Retention Behavior of Triglycerides on Reverse Phase Columns Using Pseudophase Liquid Chromatography

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ABSTRACT

The use of micellar mobile phases in decreasing the elution time of peanut triglycerides on 10μ reverse phase columns is demonstrated. Both cationic and anionic surfactants were used with "end-capped" and "non-end-capped" reverse phase columns. Surfactants added to both nonaqueous and aqueous organic mobile phases reduced the elution time of all triglyceride peaks when compared to a mobile phase without a surfactant modifier. Sodium dodecyl sulfate (SDS) as the modifier in the mobile phase was the most effective in reducing elution time for triglycerides when an "end-capped" column was used as the stationary medium. Increasing concentrations of surfactant in the mobile phase resulted in corresponding decreases in elution times. A unique feature of micellar phases is that any increase in concentration only alters the concentration of the micelles in the mobile phase. The objective of this study was to investigate the retention behavior of large, non-ionic solute molecules and to elucidate the adsorption mechanism on reverse phase columns.

INTRODUCTION

High performance liquid chromatography (HPLC) has been used in the separation and analysis of many types of compounds (1). Reverse phase HPLC is a relatively new technique for the analysis of triglycerides (2). Seed oils consist of complex mixtures of triglycerides which may differ by as little as one double bond or two carbon atoms. Although this class of compounds can be separated by chain length on silica columns (3), separations of triglycerides on bonded phase columns predominate because they are more efficient, easier to equilibrate, and retention times are more reproducible.

Retention on a bonded phase column is a very important factor in developing HPLC methodology. The length of the alkyl chain bonded to the hydroxylated silica is considered to exert the greatest influence on the retention of solute molecules. However, unreacted silanols on the bonded column material also can affect solute retention by interacting with the mobile phase. This changes the surface characteristics of the column. To prevent this from happening, commercial columns which have been "end-capped" can be used. In these columns a methyl chlorosilane reagent has been reacted with the residual silanols, thus modifying the surface of the column material. Essentially, the the bonded column becomes more hydrophobic. Therefore, valuable information can be gained about retention behavior of solute molecules by separating a mixture of compounds that have different degrees of polarity on both "end-capped" and regular reverse phase columns. The capacity factor of polar compounds on "end-capped" columns will be less when compared to capacity factors on a regular column (4). The reverse is true of less polar compounds (4).

Interactions of the mobile phase with solute molecules and with the bonded column material is another important criterion to be considered in developing HPLC methodology. Selectivity can be increased greatly by adding ionic surface active agents to the mobile phase (5). These reagents possess both hydrophobic and hydrophilic characteristics (6). Reverse phase chromatography using a mobile phase modified with surface active agents has been termed pseudophase chromatography (7). Considerable controversy exists concerning the exact adsorption mechanism in pseudophase chromatography; however, hydrophobic bonding has been widely accepted (8,9). If the capacity factor of a solute decreases with increasing concentration of surfactant added to the mobile phase, one explanation could be partitioning of the solute to surfactant aggregates in the mobile phase rather than the bulk solvent (4). Other mechanisms also may be operative.

Both anionic and cationic surface active agents were added to the mobile phase in this study. To elucidate further the effect of surfactants on the retention behavior of large non-ionic solute molecules, two types of reversed phase columns were used, "end-capped" and regular. Also, non-aqueous and aqueous organic mobile phases were investigated.

EXPERIMENTAL PROCEDURES

Peanut oil was used as the source of triglycerides and obtained by homogenizing the seeds three times with fresh chloroform:methanol (2:1), filtering, and removing the solvent by rotary evaporation. Chromatograms were obtained using a Varian 5020 liquid chromatograph. Sample components were detected at 210 nm with a variable wavelength Varichrom detector. A 30% solution of oil dissolved in chloroform was used as the sample, and injected onto the column via an automatic injection valve equipped with a 10-μl loop. A flow rate of 1.5 ml per min gave adequate response on reverse phase columns operated isocratically.

A C-18 MCH10 bonded column and "end-capped" C-18 MCH10 bonded column (Varian) were used to separate the triglycerides. Both columns were 300 mm x 4 mm.

The mobile phase consisted of acetonitrile and absolute EtOH 80:20 or a ternary mobile phase of acetonitrile, EtOH and water, 77:20:3. All were HPLC grade solvents which had been degassed by vigorously bubbling N_2 gas through the solvents for approximately 5 min.

Hexadecyltrimethylammonium bromide (CTAB) and sodium dodecyl sulfate (SDS) were used as the cationic and anionic surfactants, respectively. In the binary organic mobile phase the appropriate concentration of SDS (0.5 to 5.0×10^{-3} M) was added to the absolute EtOH and stirred with a magnetic stirrer until all the surfactant was dissolved. When the ternary mobile phase was used, the surfactant was added to the water. Critical micelle concentrations (CMC) were determined at 25 C by measuring the conductivity of each mobile phase used and plotting the log of the concentration against the conductivity. The concentration at which the micelles appear corresponds to a change in the slope of the curve; however, this change occurs over a narrow concentration range rather than a precise point. This produces a curvilinear plot with essentially two linear portions. These linear portions of the curves were extended to intersect, and a perpendicular line was dropped from the intersecting point to the x-axis. The intersection of the perpendicular line with the x-axis gives the CMC of the mobile phase. The CMC of SDS in the binary mobile phase was found to be ca. 2.5×10^{-3}, and the CMC's in the ternary phases containing CTAB and SDS were 6.0×10^{-4} M and 6.7×10^{-3} M, respectively. Columns were always

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equilibrated with the mobile phase prior to the injection of the sample. Regeneration of the reverse phase columns from a pseudophase to an organic phase can be accomplished simply by flushing the column with water. Capacity factors (K′) and changes in the capacity factors were determined in the following manner:

$$K' = \frac{V_r - V_o}{V_o} \quad [1]$$

where V_r is the retention volume of a given solute and V_o is the interstitial volume of an unretained component. Percent change in the capacity factor was calculated according to:

$$\% K' = \frac{K'_s - K'_i}{K'_{i'}} \times 100 \quad [2]$$

where K'_i is the capacity factor of a given solute at a given mobile phase composition without surfactant, and K'_s is the capacity factor of the same solute with the same mobile phase composition except a surfactant was added.

RESULTS AND DISCUSSION

Figure 1 shows the affect of SDS on the analysis of triglycerides in peanut oil on an "end-capped" column (aqueous solvent system). Both chromatograms were obtained using the same parameters, except in Figure 1A the mobile phase was modified with SDS (8.1×10^{-3}M). The most obvious effect from the addition of SDS to the mobile phase was the large reduction in retention time of the triglycerides (peaks 1-10). However, adequate resolution was maintained. The triglyceride peaks with the longer elution time (peaks 6-10) contain a larger percentage of saturated and long chain fatty acids than peaks 1-5. These triglyceride components would be more hydrophobic than early eluting peaks which contain a high degree of unsaturation. When a pseudophase was used as the mobile phase (Fig. 1A), capacity factors (K′) of the more hydrophobic triglyceride groups showed the largest decrease. This would indicate that the surfactant (SDS) had bonded (hydrophobic bonding) to the C-18 alkyl chains on the stationary matrix, thus occupying available sites for solute molecules. Peaks 6-10 were affected to a greater extent than peaks 1-5.

Triglycerides separate on reverse phase columns according to the degree of unsaturation. Resolution of critical pair triglycerides can be enhanced by altering the stationary phase or the mobile phase. "Non-end-capped" columns have residual hydrated silanols that can react with the polar mobile phase, forming a multilayer of solvent molcules around the silanol sites (10). A solute molecule can associate with a solvent molecule in much the same way as a second layer of polar solvent is formed on the residual silanols. In this case polar solvent molecules are not displaced but become part of the stationary phase. Therefore, the stationary phase has greater interaction with the unsaturated linkage in the triglyceride groups and thus enhances resolution of closely related triglyceride pairs. "End-capped" columns have residual silanols reacted with a silanol reagent, and therefore little interaction occurs with these groups and the polar solvent. Figure 2 shows the separation of peanut oil triglycerides on a "non-end-capped" column (Fig. 2A) and an "end-capped" column (Fig. 2B). Peaks 2-3 and 4-5 are partially resolved on the "non-end-capped" column, whereas these peaks are not resolved on the "end-capped" column. This would indicate an association has occurred between the solute molecules and the polar solvent molecules adsorbed to the residual silanols. Because the stationary phase of "end-capped" columns contains more hydrophobic groups, the capacity factors of all the triglycerides increase as shown in Figure 2A.

Surfactants can be used with a non-aqueous mobile phase (11) as well as an aqueous-organic mobile phase. Table I shows the reduction in capacity factors of triglycerides from peanut oil using three different concentrations of SDS in a binary organic mobile phase of ACN/EtOH on a "non-end-capped" reverse phase column. Capacity factors decreased with increasing concentration of SDS added to

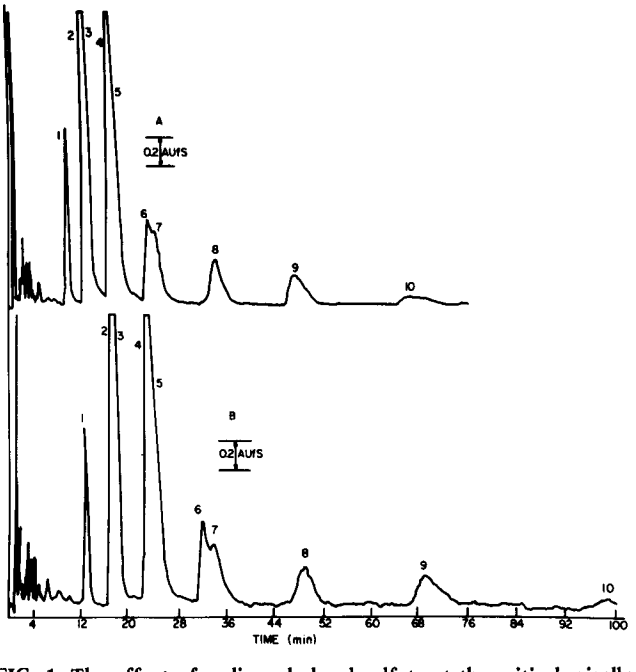

FIG. 1. The effect of sodium dodecyl sulfate at the critical micelle concentration on the analysis of peanut oil triglycerides using an "end-capped" reverse phase column using an aqueous mobile phase (ACN/EtOH/H_2O, 77/20/3). (A) Sodium dodecyl sulfate at the critical micelle concentration added to the mobile phase. (B) No sodium dodecyl sulfate added.

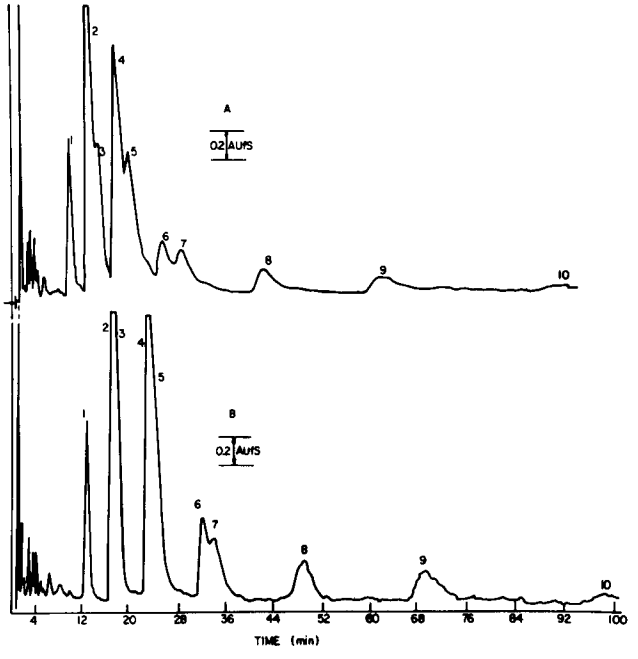

FIG. 2. The effect of unreacted silanols on the separation of peanut oil triglycerides using an aqueous mobile phase (ACN/EtOH/H_2O, 77/20/3). (A) "Non-end-capped" columns. (B) An "end-capped" column.

TABLE I

Percent Changes in Capacity Factors (% K′) of Peanut Triglycerides as a Function of SDS Concentration on a "Non-End-Capped" Reverse Phase Column Using a Non-Aqueous Mobile Phase[a]

	% K′		
HPLC peak no.	0.5×10^{-3} M	2.0×10^{-3} M[b]	5.0×10^{-3} M
1	−5.45	−9.09	−10.00
2	−6.25	−6.25	−9.6
3	−5.68	−6.82	−10.00
4	−7.08	−8.85	−9.71
5	−7.81	−8.59	−14.29
6	−6.13	−9.20	−12.41
7	−7.03	−10.81	−13.50
8	−13.88	−17.71	−21.00
9	−8.20	−11.54	−12.39
10	−11.30	−13.04	−11.65

[a]Mobile Phase - ACN/EtOH; 80/20.
[b]Average of 3 observations.

TABLE III

Percent Changes in Capacity Factors in Peanut Triglycerides as a Function of CTAB on an "End-Capped" Reverse Phase Column[a]

	% K′[b]	
HPLC peak no.	5.0×10^{-4} M	9.2×10^{-4} M
1	−10.34	−12.09
2	−7.52	−11.25
3	−7.52	−11.25
4	−5.46	−13.64
5	−5.46	−13.64
6	−7.83	−9.74
7	−7.90	−10.35
8	−8.22	−11.77
9	−7.99	−11.24
10	−9.26	−12.56

[a]Mobile Phase - ACN/EtOH/H_2O 77/20/3.
[b]Average of 3 observations.

TABLE II

Percent Changes in Capacity Factors of Peanut Triglycerides as a Function of SDS Concentration on a "Non-End-Capped" and an "End-Capped" Reverse Phase Column[a]

	% K′					
	2.0×10^{-3} M		5.0×10^{-3} M		8.1×10^{-3} M	
HPLC peak no.	"Non-end-capped"	"End-capped"	"Non-end-capped"	"End-capped"	"Non-end-capped"	"End-capped"
1	−2.94	−10.34	−4.41	−19.03	−8.82	−24.20
2	−2.11	−8.70	−2.11	−18.75	−7.37	−22.51
3	−1.87	−8.70	−3.74	−18.75	−8.41	−22.51
4	−1.48	−8.18	−1.48	−18.17	−11.11	−24.75
5	−2.63	−8.18	−2.36	−18.17	−9.87	−24.75
6	−1.52	−8.45	−3.03	−21.43	−10.10	−25.98
7	+0.45	−9.15	−2.25	−21.95	−9.00	−26.82
8	0.0	−10.08	−3.82	−24.38	−8.82	−29.42
9	−2.79	−10.35	−2.99	−25.44	−7.97	−30.77
10	−0.80	−10.70	−1.99	−26.75	−13.01	−32.10

[a]Mobile Phase - ACN/EtOH/H_2O 77/20/3.
[b]Average of 3 observations.

the mobile phase. Peak 10 was the least affected by increased concentration of SDS. This triglyceride group would be the most hydrophobic. Therefore, it may be a stronger competitor with a surface active agent for the hydrophobic surface. Since "non-end-capped" columns have residual hydrated silanols and the degree of unsaturation and hydrophobicity varies from triglyceride to triglyceride, each triglyceride would be affected differently by the presence of SDS.

To determine if an interaction occurred between the residual silanols and surfactant added to the mobile phase when chromatographing triglycerides on reverse phase columns, two types of columns were used, a "non-end-capped" and an "end-capped" column. In this experiment a ternary aqueous-organic mobile phase was used. Table II shows a comparison of capacity factor reductions (% K′) for peanut oil triglycerides separated on both column types using three different concentrations of SDS. For the lowest concentration of surfactant used in the mobile phase (2.0×10^{-3} M), capacity factor reduction (% K′) was small for most of the triglyceride peaks on a "non-end-capped" column. On the other hand, capacity factor reductions (% K′) are much more pronounced on the "end-capped" column at the same concentration of surfactant. This difference indicates that some interaction occurred between the hydrated silanols and the polar solvent, which affected the retention of the triglycerides on the "non-end-capped" column. If no interaction had occurred, capacity factor reductions would have been similar on both types of column. Increased concentration of surfactant in the mobile phase (5.0×10^{-3} M and 8.1×10^{-3} M) produced a more pronounced reduction in triglyceride capacity factors. However, the "end-capped" column showed the largest reduction (% K′) in capacity factors for triglycerides, and this can be attributed to the more hydrophobic nature of the stationary matrix of the "end-capped" column, which would allow more hydrophobic bonding with the surfactant.

Cationic surfactants also can be used to create a pseudophase for liquid chromatography. Table III shows the affect of CTAB on triglyceride capacity factor reductions on an "end-capped" column. The reductions were not of the same magnitude as those produced by SDS (Table II) on a similar type column. This may be due to the bulky character of the CTAB molecules and the more compact charge layer of CTAB vs that of SDS molecules.

Results of this study show that triglyceride analysis time can be effectively reduced on reverse phase columns using pseudophase liquid chromatography without sacrificing resolution (12). Hydrophobic bonding of the surfactant to the octadecyl chains appears to have the greatest effect on capacity factor reductions. However, at high concentrations of surfactant in the mobile phase, saturation of the column would occur and some partitioning of the solute molecules

to the micelles is likely. Also, the presence of hydrated silanols on "non-end-capped" columns reduces the effectiveness of added surfactants to the mobile phase, primarily due to interactions of these groups with the polar solvent. Other advantages of using micellar mobile phases in the analysis of triglycerides on reverse phase columns over traditional mobile phases include improved peak shape, which permits better integration and the use of less solvent. The use of a pseudo mobile phase in reverse phase chromatography provides an alternative in the analysis of hydrophobic solutes.

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Chlorinated Long-Chain Fatty Acids. Their Properties and Reactions. XII. The Dechlorination Pathways of Sodium 9(10)-Chloro-10(9)-Oxooctadecanoates in Aqueous Sodium Hydroxide Solution

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ABSTRACT

The dechlorination pathways of the equal mixture of 9-chloro-10-oxo- and 10-chloro-9-oxooctadecanoic acids in aqueous sodium hydroxide solution were investigated. The reaction product mixture of these acids, isolated after dechlorination, was found to contain α-hydroxyoxo and long-chain alkanedioic acids at a weight ratio of 15 to 1. The most abundant compounds formed were 9-hydroxy-10-oxo and 10-hydroxy-9-oxooctadecanoic acids. The minor reaction products consisted of Favorskii rearrangement products, 2-heptyl-1,11-undecanedioic, 2-octyl-1,10-decanedioic and 2-nonyl-1,9-nonanedioic acids. On the other hand, the expected α,β-unsaturated oxoacids could not be detected in the reaction product mixture.

INTRODUCTION

We previously have described the alkaline dechlorination of an equal mixture of 9-chloro-10-oxo- (*1a*) and 10-chloro-9-oxooctadecanoic acids (*1b*), which was found to occur easily and at a rate comparable to those of the corresponding chlorohydrins, i.e. *threo-* and *erythro*-9(10)-chloro-10(9)-hydroxyoctadecanoic acids under similar conditions (1,2). In general, the dehalogenation of α-haloketones by alkoxide bases may yield various reaction products, such as Favorskii esters (carboxylic acid derivatives), α-hydroxy ketals, α-hydroxy ketones, α-alkoxy ketones and α,β-unsaturated ketones (3). In our continuing studies on the reactions of chlorinated long-chain fatty acids, the present paper deals with dechlorination pathways of an equal mixture of *1a* and *1b* in aqueous sodium hydroxide solution as checked by product analysis using chromatography and spectroscopy.

EXPERIMENTAL

Model compounds.

Equal amounts of *1a* and *1b* were prepared by chromic acid oxidation of an equal mixture of *threo*-9-chloro-10-hydroxy- and *threo*-10-chloro-9-hydroxyoctadecanoic acids (15 g) in glacial acetic acid according to Corin et al. (4). The crude product (11 g) was recrystallized three times from petroleum ether (bp 40-60 C) at −17 C to give 5.8 g of *1* (39%): mp (uncorrected) 32.5-36.5 C; ^{1}H NMR(CCl_4) δ 0.90 (t, 3H, terminal $-CH_3$), 1.32 (m, chain $-CH_2$, 2.31 (t, 2H, $-CH_2CO_2H$), 2.63 (t, 2H, $-CH_2CO-$), and 4.10 ppm (broad s, 1H, $-CHCl-$); IR(KBr) 1725 (C=0), 1710 (COOH), and 680 and 600 cm^{-1} (C-Cl). MS of methyl ester mixture (*1a, 1b*), m/z (% rel. intensity): 349(0.2), 347(0.5), 317(1.3), 315(3.8), 310(1), 279(1.3), 250(1), 248(2.7), 185(100), 157(3.4), 157(7), 141(81), 125(31), 57(45), 55(62).

Spectroscopy

IR spectra were obtained with a Perkin Elmer 180 spectrophotometer in KBr. The viscous samples were run as thin films on KBr disks. ^{1}H NMR spectra were recorded on a Jeol PMK 60 spectrometer in CCl_4 with tetramethylsilane as internal reference. Mass spectra were taken on an LKB 9000 GC/MS instrument under electron impact at 70 eV.

Gas-liquid chromatography

A Hewlett Packard 5700A gas chromatograph was equipped with a flame ionization detector (FID) and a 2 m x 3 mm ID. stainless steel column packed with 3% Silar 10C on Chromosorb Q (80/100 mesh). The temperature was programmed from 220 to 260 C, 4 C/min. The GC/MS analyses were performed with a 2.4 m x 3 mm ID. glass column packed with 1% XE-60 on Gas-Chrom Q (100/120 mesh) by programming from 150 to 220 C, 5 C/min. The acids were methylated with diazomethane in diethyl ether containing methanol (9:1, v/v). The TMSi ethers of hydroxy ketonic acid methyl esters were prepared with BSTFA-IMCS (bis(trimethylsilyl)trifluoroacetamide-trimethylchlorosilane, 3:1, v/v) and heated at 75 C for 15 min.

Thin-layer chromatography

Analytical TLC was done on glass plates (20 x 20 cm) with