High Performance Liquid Chromatography of Triglycerides: Controlling Selectivity with Reverse Phase Columns

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ABSTRACT

Rapid separations of triglycerides by chain length and degree of unsaturation have been made by high performance liquid chromatography (HPLC) using reverse phase columns. Several different bonded columns were evaluated for use in reverse phase HPLC of triglycerides, and differences in selectivity are discussed. Selectivity was also modified by adding silver ion to the solvent, which produces marked changes in selectivity of solute triglycerides.

INTRODUCTION

High performance liquid chromatography (HPLC) is a relatively new technique for the analysis of triglycerides. Separations based on chain length and degree of unsaturation have been reported for triglycerides using reverse phase (RP) and conventional silica HPLC systems (8). Triglycerides occur naturally in complex mixtures containing many similar components, so that useful HPLC separations are difficult. The components may differ by as little as one double bond or a few carbon atoms or even differ only in the positions of the acyl groups on the glycerol molecule. An analysis of the two most important factors that control the separations, i.e., the column and the eluting solvent system, has not been reported. During the course of our work with HPLC of lipid mixtures, we have used various types of HPLC columns and solvent systems. Control of these parameters can greatly affect the separations.

The first choice that must be made in developing an HPLC separation is that of the column. Reverse phase columns are generally considered to be best for separating a homologous series of components. Fats and oils contain triglycerides made up of a complex mixture of homologous series of chain lengths and a homologous series of degrees of unsaturations, so that reverse phase HPLC would appear to be the procedure of choice. Despite the surprising fact that separations of chain length among triglycerides could be made on silica columns (8), the most efficient separations have indeed been obtained with the reverse phase (RP) columns. Because it is important that columns give reproducible results, both packing material and packing
procedures must be carefully controlled. Reproducibly packing the high-efficiency, micro-particle columns seems to be an "art form," it is probably advisable for the average user to buy prepacked columns.

Reverse phase columns are made by bonding functional groups (usually hydrophobic chains) to the silica by reaction of the appropriate chloro or alkoxy silane with a fully hydroxylated silica. Several excellent reviews discuss this reaction (9,10). For steric reasons, complete reaction of all silanol groups does not occur. The resulting silica surface, then, is a mixture of silanols and bonded functional groups. Additional silanols can be removed by following the initial bonding with reaction with trimethyl chlorosilane. Common commercial bonding materials are octyl silanes and octadecyl silanes. The silanes can be monofunctional, bifunctional, or trifunctional. When bifunctional or trifunctional silanes are used, any unreacted groups on the silane can be easily hydrolyzed to form a polymeric coating. Although this might seem desirable because of the increased amount of bonding, such polymers generally deteriorate the efficiency of the column (10). Free residual hydroxyl groups also can greatly affect the performance of the column, and capping them with methyl trichlorosilane will change the selectivity of the resulting column. The role of the chain length of the bonded phase is not completely clear. Some differences in selectivity can be attributed to chain length (11); however, the greatest change is observed in retention (12). The longer chain lengths have higher capacity, which means that more material can be put onto the column without overloading, an important consideration in preparative work. With shorter chain lengths, the mass transfer is faster and better efficiencies can be obtained (12). Unfortunately, most manufacturers of RP-LC columns do not supply details about how their columns are prepared or packed. Some manufacturers do supply the percentage of carbon loading, but these figures might be misleading because of the effects of polymerization on columns that are made from the reaction of polyfunctional silanes. Also, these figures do not indicate whether the column has been methyl capped and how this affects selectivity. The most important features in an RP column for use with triglyceride mixtures are the column's efficiency (theoretical plate count) and the column's retentiveness for triglycerides. High efficiency is important because many of the components are closely related and only slightly separated. Columns with higher plate counts can show separations that may not be achieved on the lower efficiency columns, even though both types have the same selectivity. A column with high retentiveness for triglycerides is desirable because the separation of these components occurs in solvents in which the component triglycerides are only sparingly soluble, and it has been noted that some components can be lost on the column because of poor solubility in the elution solvent (1).

EXPERIMENTAL PROCEDURES

Chromatograms were obtained with a Waters Associates ALC-201 liquid chromatograph. Samples were injected as 20% solutions in CHCl₃ by means of a U6K septumless injector. Components were detected with either a Waters differential refractometer (RI) or a DuPont infrared (IR) detector set at 5.75 μ. A 1-mm CaF₂ cell was used with the IR detector. The sensitivity of the IR detector at 0.1 AUFS using this cell was approximately equal to that of the RI detector at the 8X setting. Sample sizes of 0.1-2 mg injected onto 25-30 cm x 3.8 mm columns gave adequate response on both detectors. The best isocratic chromatograms were obtained when solvents were adjusted so that all components were eluted within 20 column volumes (ca. 60 ml). A flow rate of 1 ml/min seemed to give optimal resolution with all 30 cm x 3.8 mm and 25 cm x 4.6 mm columns. The 50 cm x 9.4 mm and 30 cm x 7.8 mm semipreparative columns had optimal flow rates of 2-4 ml/min. The following columns were used: (a) 30 cm x 3.8 mm and (30 cm x 7.8 mm) μ-Bondapak C-18 (Waters Associates); (b) a 30 cm x 3.8 mm μ-Bondapak-phenyl (Waters Associates); (c) a 30 cm x 7.8 mm Triglyceride Analysis column (Waters Associates); (d) a 25 cm x 4.6 mm Partisil-10 ODS column (Whatman); (e) a 25 cm x 4.6 mm and a 50 cm x 9.4 mm Partisil-10 ODS-2 column (Whatman); (f) a 25 cm x 4.6 mm Zorbax ODS column (DuPont); and (g) a 25 cm x 4.6 mm Zorbax C-8 column (DuPont).

GLC analysis after collection identified some of the components. The procedures used for GLC were previously reported (1). Theoretical plate counts were determined for all columns, using the equation:

\[ N = \frac{V_R}{W} \]

where \( V_R \) is the retention time from injection and \( W \) is the peak-width as determined from tangents to the sides of the peak. Acenaphtylene was used as a reference compound. The elution solvent was adjusted for a \( R' \) of 2-5 for the acenaphthylene on all columns. Calculated plate counts were always within 10% of the manufacturer's claim for that column.

RESULTS AND DISCUSSION

Four types of columns with 18 carbon bonded alkyl chains were examined. They were μ-Bondapak C-18 (Waters Associates), Zorbax ODS (DuPont), and Partisil 10 ODS and ODS-2 (Whatman). Differences in retentiveness and selectivity were noted for all four columns. Three other RP columns, the proprietary Waters Triglyceride Analysis column, the μ-Bondapak alkyl phenyl column (Waters), and the Zorbax C-8 (DuPont) column were also tested.

The bulk of the previously published data (1-3) on HPLC of triglycerides was obtained from the μ-Bondapak C-18 columns. This packing is a micro-particle, fully porous, irregular-shaped silica (10 μ particle size) to which a C₁₈ group (not defined whether monofunctional or difunctional) has been chemically bonded at the 10% level by weight. The prepacked columns (3.9 mm id x 30 cm) are specified to have a minimum of >3,000 plates/column (>4,000 plates/meter). The typical value for eight columns was 3,700 plates. The μ-Bondapak C-18 column has moderate retentiveness for triglycerides. Some difficulties are observed for fully saturated triglycerides (e.g., triglycerides) because they are nearly insoluble in the elution solvents. The selectivity found for the μ-Bondapak C-18 column has been reported previously (1,2). Not much difference is observed between the selectivity for palmitoyl acyl groups and oleoyl acyl groups, although the oleoyl-containing triglycerides elute slightly faster. Figure 1 shows the HPLC chromatogram obtained for soybean oil using the μ-Bondapak C-18 column. The peaks resulting from separation (by number of double bonds) of triglycerides with eight to two double bonds/molecule have been described in detail (1,2). The Waters Triglyceride Analysis column, though not identical to the μ-Bondapak C-18 column, gave separations that were very similar in selectivity and column efficiency. The Waters column contains a proprietary packing and very little information is given about it. It is a gel column of

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some type, and the manufacturer says it is therefore fragile and susceptible to solvent swelling and channeling with alcohol or water solvent systems. Although the Waters column was somewhat less efficient than the µ-Bondapak C-18 columns, its larger size (7.8 mm × 30 cm) compensated for this deficiency; overall resolution of components with this column and µ-Bondapak C-18 was about the same.

Two types of Partisil-10 octadeyl silane (ODS) columns were evaluated. Partisil-10 ODS is a 10-µ porous silica with 400 m²/g surface area bonded with a C₁₈ group (not defined whether mono- or difunctional). The “carbon load” (carbon load refers to %C vs %Si) of this column is somewhat lower than most of the other commercial RP C₁₈ packings. Partisil-10 ODS-2 has higher carbon load, which is said to be achieved through a different bonding process followed by “methyl capping” of the residual silanol groups. The plate counts specified for these columns are >18,000 plates/meter (or ca. 6,000 plates/25 cm column); the 25-cm columns examined had plate counts of 6,600 (ODS) and 7,800 (ODS-2), respectively. A semipreparative 50 cm × 9.4 mm Partisil M-9 ODS-2 column had a plate count of 13,500. The Partisil-10 ODS column did not have sufficient retentiveness for successful chromatography of triglycerides. The Partisil-10 ODS-2 column had slightly higher retention for triglycerides than the µ-Bondapak C-18 columns; to obtain the equivalent retention values (K') for a given triglyceride, a slightly stronger solvent was needed. The higher efficiencies of the Partisil-10 ODS-2 yielded sharper peaks with markedly different selectivity than that obtained with C₁₈ µ-Bondapak columns. For example, complete baseline separation of tripalmitin and triolein was possible at a K' value of ca. 5, whereas only partial separation of these two triglycerides could be achieved at K' = 20 on µ-Bondapak C₁₈. Experiments with alteration of the solvent system using mixtures of methanol, ethanol, acetonitrile, acetone, methylene chloride and THF tended to show that changes in selectivity for triglycerides seemed to depend more on column packing factors and that selectivity was only slightly altered by eluting solvents. This was quite unexpected, because a major change in selectivity for the fatty esters, methyl palmitate and methyl oleate occurred when the eluting solvent was changed from water/methanol to water/acetonitrile. The elution order of the methyl ester peaks was reversed on both Partisil-10 ODS-2 and µ-Bondapak C-18. A possible reason for the inability of solvent to markedly influence selectivity in triglycerides could be that the large size of these molecules makes the difference in double bond number less important to solubility properties than in the much smaller fatty esters.

Figure 2 is the HPLC chromatogram for soybean oil from the Partisil-10 ODS-2 column (50 cm × 9.4 mm). The solvent used to elute the triglycerides (total elution in ca. 6 column volumes) from the Partisil ODS-2 column was 3:1 acetone/acetonitrile, a substantially stronger solvent than...
the 1:1 acetone/acetonitrile solvent that produces similar retention with \( \mu \)-Bondapak C-18. The greater retentiveness of Partisil-10 ODS-2 for triglycerides means that stronger solvents were used to elute components and, therefore, separations are less prone to the loading and solubility problems previously reported (1) using \( \mu \)-Bondapak C-18 columns.

Considerable fine structure is now visible instead of the broad peaks observed with the \( \mu \)-Bondapak C-18 column. For example, peaks 3 and 4 of Figure 1 are now partially resolved into two peaks each. Peak 3 of Figure 1 has an equivalent carbon number (ECN) (1) of 42 and contains C\(_{52}\) triglycerides with five double bonds (primarily PLLn) and C\(_{54}\) triglycerides with six double bonds (primarily LLLn and others). In Figure 2, separation is observed between the C\(_{52}\) triglycerides (smaller peak) and the C\(_{54}\) triglycerides (larger peak). Some indication of further separation is present on the back side of the larger peak. Peak 4 of Figure 1 has an ECN of 44, containing about equal amounts of C\(_{54}\) triglycerides with five double bonds (primarily LOL and LnOO) and C\(_{52}\) triglycerides with four double bonds (primarily PLLL). These are resolved clearly into two peaks in Figure 2. Similarly, three major peaks are observed in Figure 2 in the vicinity of peak 5 of Figure 1. These triglycerides are composed primarily of mixtures of palmitoyl and oleoyl acyl groups with an ECN of 48. Figure 2 was generated using the semipreparative (50-cm) column which had 13,500 plate count. With the analytical (25 cm \( \times \) 4.6 mm) column (7,800 plates), the separations achieved were not quite as sharp. The differences could be attributed to the column efficiency because the selectivity and retentiveness of the two columns were identical.

The other C-18 column evaluated was a Zorbax ODS column. Zorbax ODS is a 5-\( \mu \)-diameter porous silica microsphere which is bonded with a linear aliphatic octadecyl hydrocarbon monomolecular layer (monofunctional silane). Maximal surface coverage is said to be achieved with the bonding process (25% bonded phase by weight). The manufacturer does not note whether the silica has been methyl capped. The Zorbax ODS columns showed the highest retentiveness of all RP columns evaluated. They also yielded the highest plate counts. The manufacturer specifies that plate counts will exceed 8,000 for a 25 cm \( \times \) 4.6 mm column, and the columns tested ranged from 12,000 to 15,000 plates/column—substantially higher than the minimum specification. The separations achieved with Zorbax ODS columns were the most complete of any from the columns evaluated (Fig. 3). Using acetone to elute the triglycerides yielded similar \( K' \) values to using acetonitrile/acetonitrile (1:1) with Partisil 10 ODS-2 columns. The selectivity of the Zorbax ODS and Partisil ODS-2 columns was virtually identical, but higher plate count and higher retentiveness of the Zorbax column for triglycerides led to slightly superior separations.

The Zorbax C-8 column was also evaluated with triglyceride mixtures. Zorbax C-8 is formed by bonding a monomolecular layer of octyl silanes to the Zorbax microparticulate support. The retentiveness of the Zorbax C-8 column was similar to that of \( \mu \)-Bondapak C-18 with the same \( K' \) values, but the selectivity was somewhat different. The 25 cm \( \times \) 4.6 mm column was quite efficient, showing a plate count of \( \sim 10,000 \) and symmetrical peaks were observed for soybean oil (Fig. 4). No separation was achieved between tripalmitin and triolein.

The last column evaluated for HPLC of triglycerides was the \( \mu \)-Bondapak-phenyl column (bonded phenyl group at 16% by weight), but sufficient retention to elute triglycerides in a usable manner was not achieved.

Useful separations were achieved with \( \mu \)-Bondapak C-18, Partisil ODS-2, Zorbax ODS and Zorbax C-8 columns. The selectivities and retentiveness of all four types of columns were different; Zorbax ODS columns were the most retentive. Separations were sufficient to do quantitative semipreparative work when components were collected and analyzed by GLC with an internal standard (1-3). The selectivity of the separation could not be significantly altered by using changes in eluting solvent.

In RP chromatography, adding silver ion to the mobile phase has been shown to act in a similar manner as adding silver ion to the silica in conventional analysis (13). Silver ion is quite soluble in acetonitrile, a primary solvent for the analyses.
HPLC of triglycerides, so the effect of silver ion on triglyceride HPLC was easily studied. The difference in selectivity of one double bond vs two carbon atoms was considerably changed; the elution of the more unsaturated components decreased relative to the saturated analogs. Silver ion concentrations studied were 0 to 0.2 N AgNO₃. Vonach and Schomburg (13) have shown that the relationship of silver ion concentration and log K' is linear for a wide range of chain lengths of alkanes, alkenes, alkanols and alkyl benzenes. The best separation we obtained for triglycerides was at 0.15-0.2 N AgNO₃ in the mobile phase. Figure 5 shows the separation of tripalmitin, triolein, palmitodiolein, and oleoyl dipalmitin on a μ-Bondapak C-18 column, with no silver ion and 0.2 N silver ion in the mobile phase. Adding silver ion increases the retention of all components slightly, but the influence was less for unsaturated components. With the Zorbax ODS column, the separation of these four triglycerides is almost complete and no silver ion is present (Fig. 6). This resolution can be completed by the addition of 0.2 N AgNO₃ to the solvent system. Figure 7, the HPLC chromatogram of soybean oil, was typical of the improved separations that were obtained for a natural triglyceride mixture by adding silver ion to the mobile phase. Vonach and Schomburg (13) show similar improvements in HPLC of olive oil with the addition of silver ion. This alteration did not appear to affect efficiency or life time. Because of the sensitivity of silver ion to light, the system was always thoroughly purged after use, and silver-containing solutions were not left in prolonged contact with the cells of the detector.

Conceivably, the proper column and mobile phase (with silver ion added) will control the selectivity of reverse phase HPLC to the extent that useful separations can be made with nearly any triglyceride mixture. Identification of the components is still somewhat tenuous, and identification and quantitation must depend on a secondary analysis technique such as GLC.

REFERENCES


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