High Performance Reversed-Phase Chromatography of Natural Triglyceride Mixtures

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ABSTRACT

High performance reversed-phase chromatography (HPRC) is an efficient and powerful tool gaining momentum in the separation of triglycerides and other lipid components. In the present study the effect of different variables in triglyceride separation has been studied. It was found that a longer hydrocarbon chain bonded to silica gel as well as the percent coverage improved the separation. Smaller particle size packing increased triglyceride resolution. A decrease in mobile phase polarity by increasing the acetone content resulted in a decrease in the retention time and resolution of triglyceride.

INTRODUCTION

The triglyceride composition of natural fats and oils is of such complexity that no one analytical technique can separate all the triglyceride components that are present (1,2).

Argentation chromatography has been used to separate natural triglycerides according to their degree of unsaturation (3-9). The separation of triglycerides according to their carbon number has been achieved by gas liquid chromatography (10-14). A combination of both techniques has been necessary for improved triglyceride separation and analysis (15-17). The use of liquid-liquid partition chromatography resulted in improved separation of triglycerides on the basis of their partition coefficient (18-20). These techniques are tedious and time consuming, and are not sufficiently reproducible for routine applications.

The introduction of high performance reversed-phase chromatography (HPRC) has revolutionalized the field of lipid separation and analysis (21-33). Recently the use of HPRC in triglyceride separation and analysis has increased (24-33). Pei, et al. (27) used Vydac reversed-phase packing to separate triglycerides using methanol:water as a mobile phase. Columns packed with μ-Bondapac C-18 have been utilized in the separation and analysis of triglycerides using different mobile phases and the refractometer as a detector (28-31). Parris (32) first separated triglycerides on a Zorbax ODS column using nonaqueous solvent mixtures of methylene chloride, tetrahydrofuran and acetonitrile as mobile phases and detected the separated components with an infrared detector.

Herslof et al. (33) recently compared the effect of mobile phases containing acetonitrile:acetone and acetone:methanol on triglyceride separation on μ-Bondapac C-18 and Spherosorb S-ODS packed columns.

As part of a broader program investigating the applications of new analytical methodology to triglyceride analysis we wish to present in this paper results comparing the effects of various column packings and mobile phase mixtures on triglyceride separation by HPRC.

EXPERIMENTAL

The HPRC instrument system employed was composed of a Tracor 995 isochromatographic pump (Tracor Instruments, Austin, TX), a Rheodyne loop injector (Model 7120), equipped with a 20-μl sample loop, and a Waters R401 differential Refractometer Detector (Waters Assoc., Milford, MA). Separations were recorded with a Hewlett Packard 3385 electronic integrator (Palo Alto, CA) at various chart speeds. Retention times were automatically printed by the recording integrator. Six commercially packed columns produced by different manufacturers were used in the present study: Partisil ODS-1 and ODS-2

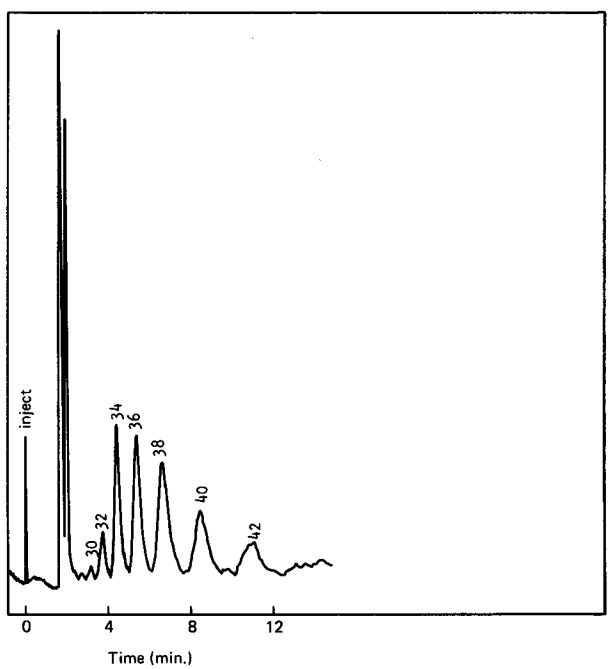

FIG. 1. The effect of acetone content on the separation of coconut oil triglycerides on a Partisil ODS-2 column. Mobile phase isopropyl alcohol:acetone:methanol:acetonitrile (1:2:3:4).

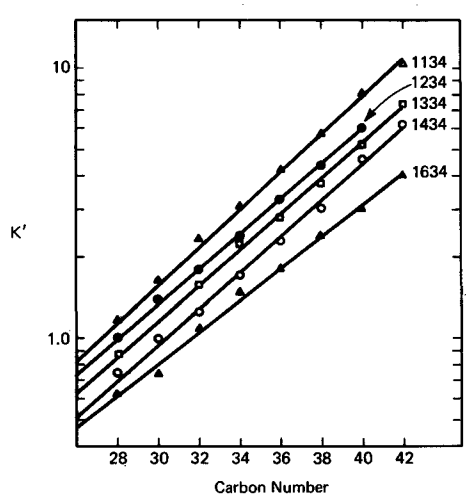

FIG. 2. The effect of decreasing mobile phase polarity on k' of triglycerides on Partisil ODS-2 using different acetone ratios on various mobile phase mixtures of isopropyl alcohol:acetone:methanol:acetonitrile.

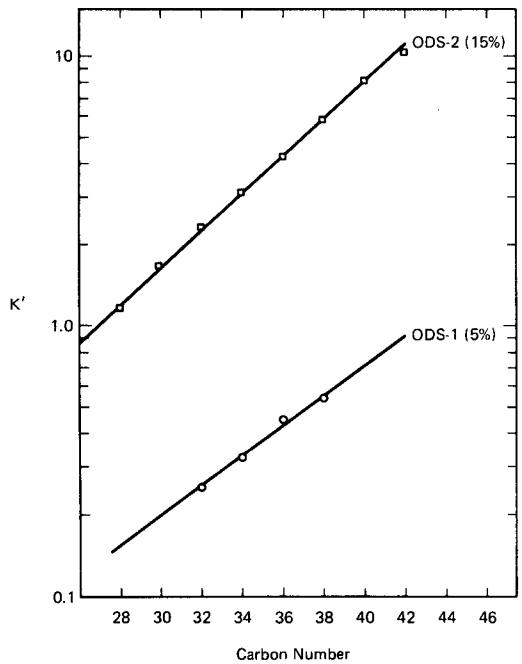

FIG. 3. The effect of bonded alkyl content on triglyceride separation.

octadecyl bonded silica of 10 μ partical size (Whatman Inc., Bridewell Place, NJ) and a Zorbax-ODS octadecyl-silica of 6-7 μ diameter (DuPont Co., Wilmington, DE). These columns were stainless steel, 250 mm in length and 4.6 mm id. In addition, three 150 mm x 4.6 mm id stainless steel columns packed with a 5 μ octyl bonded spherical silica LC-8, a 5 μ methyl bonded spherical silica LC-1 and a 5 μ octadecyl bonded spherical silica LC-18 (Supelco, Supelco Park, Bellefonte, PA) were used.

One mobile phase was employed in the present study and consisted of mixtures of methanol:acetone:isopropanol:acetonitrile ranging from 1:0:3:4 (v/v) to 1:6:3:4 (v/v). All solvents employed were of analytical reagent grade except acetonitrile, which was glass distilled. Triglycerides employed as standards were obtained from Nu-Chek-Prep (Elysian, MN) and Supelco Co. (Bellefonte, PA). Triglycerides were solubilized in either tetrahydrofuran (THF) or acetone at 100 mg/ml for each compound. Coconut oil was used as a triglyceride mixture standard because it contains a homologous series of C_{30} to C_{42} saturated triglycerides. Five to ten microliters of 20-25% coconut oil in THF were injected.

RESULTS AND DISCUSSION

Effect of Mobile Phase Polarity

Mobile phase polarity has a dramatic effect on the separation of triglycerides by HPRC. This is the result of two

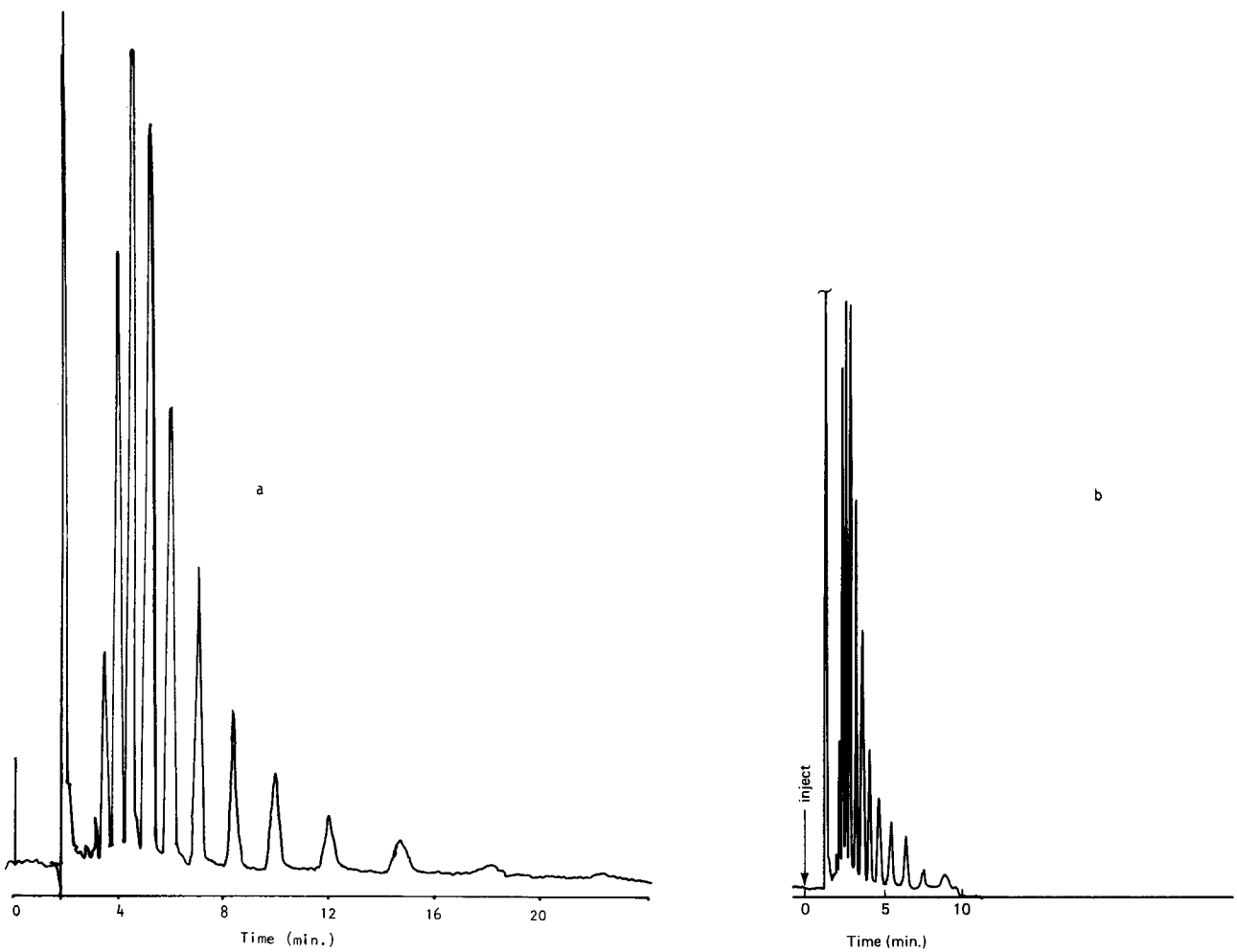

FIG. 4. The effect of acetone content on the separation of coconut oil triglycerides on a Supelco LC-8 column. Mobile phase, isopropyl alcohol: acetone: methanol:acetonitrile—(a) 1:0:3:4, (b) 1:2:3:4.

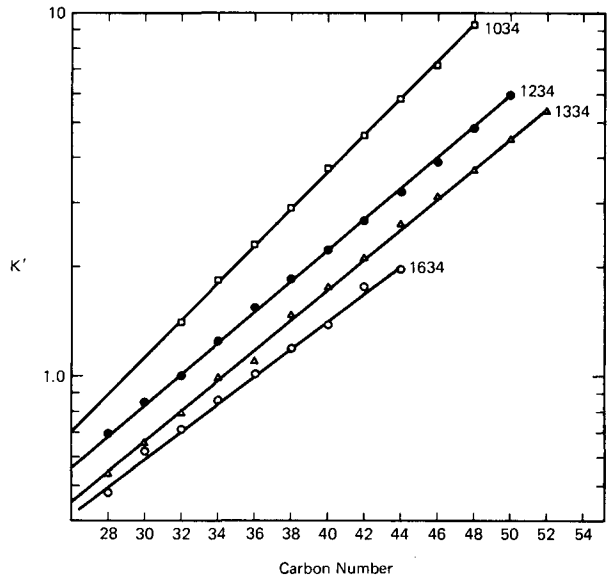

FIG. 5. The effect of decreasing mobile phase polarity on triglyceride separation using the octyl bonded phase column with a mixture of isopropyl alcohol:acetone:methanol:acetonitrile.

main factors: (a) competition between the mobile phase and the triglycerides for the stationary phase and (b) the increase or decrease in triglyceride solubility in the mobile phase which can enhance or retard their separation.

The separation of coconut oil triglycerides on a Partisil ODS-2 column is shown in Figure 1, where the effect of an increase in the acetone content is clearly evident. The separation shown in Figure 1 was achieved using 20% acetone and was satisfactory with baseline resolution. Increasing the acetone content to 33.3% decreased not only the separation time but also the resolution, resulting in clustering of these triglycerides, preventing baseline separation.

Further increasing the acetone content impairs resolution. The effect of a change in the acetone content on the capacity factor is shown in Figure 2. The ratio of the mobile phase mixture of 1:2:3:4 was shown as the optimum mixture. A decrease in the acetone content results in band broadening and increased k′ value to the extent that possible detection is impaired. The effect is more drastic on Partisil ODS-1 where the carbon coverage is 15% compared to a k′ value of more than 6.0 on Partisil ODS-2 (Fig. 3), thus illustrating the effect of bonded phase content on the partitioning effect of such columns and the retention of triglycerides.

A separation of coconut oil on the 150-mm Supelcosil LC-8 column with an octyl bonded phase using the same mixture of the multi-component mobile phase but changing the acetone content is shown in Figure 4. Using a mobile phase without addition of acetone gave a satisfactory baseline resolution (Fig. 4), which, however, required more than 16 min for the complete separation. The addition of 20% acetone resulted in decreasing the separation time with a satisfactory resolution (Fig. 4) and sharper and more symmetrical peaks. The addition of more acetone, however, decreased the elution time but impaired the resolution of triglycerides. This interrelationship is shown in Figure 5 where the capacity factor vs the carbon number is plotted. Varying the acetone content in the mobile phase mixture of isopropyl alcohol:acetone:methanol:acetontrile resulted in the noticeable change in the capacity factor of the triglycerides. It can be seen that the higher the triglyceride carbon content, the greater the change in k′ values. From the data shown in Figure 5, the optimum mobile phase composition appeared to be 1:2:3:4, which resulted in k′ values between 0.3 and 6.0 for triglycerides with a carbon content from 28 to 48, which makes it possible to separate triglycerides having a carbon content of more than 48 carbons in a reasonable time with an acceptable resolution.

The separation of coconut oil triglycerides on a Zorbax ODS column using 20% acetone in the mobile phase mixture is shown in Figure 6a. While excellent separation was

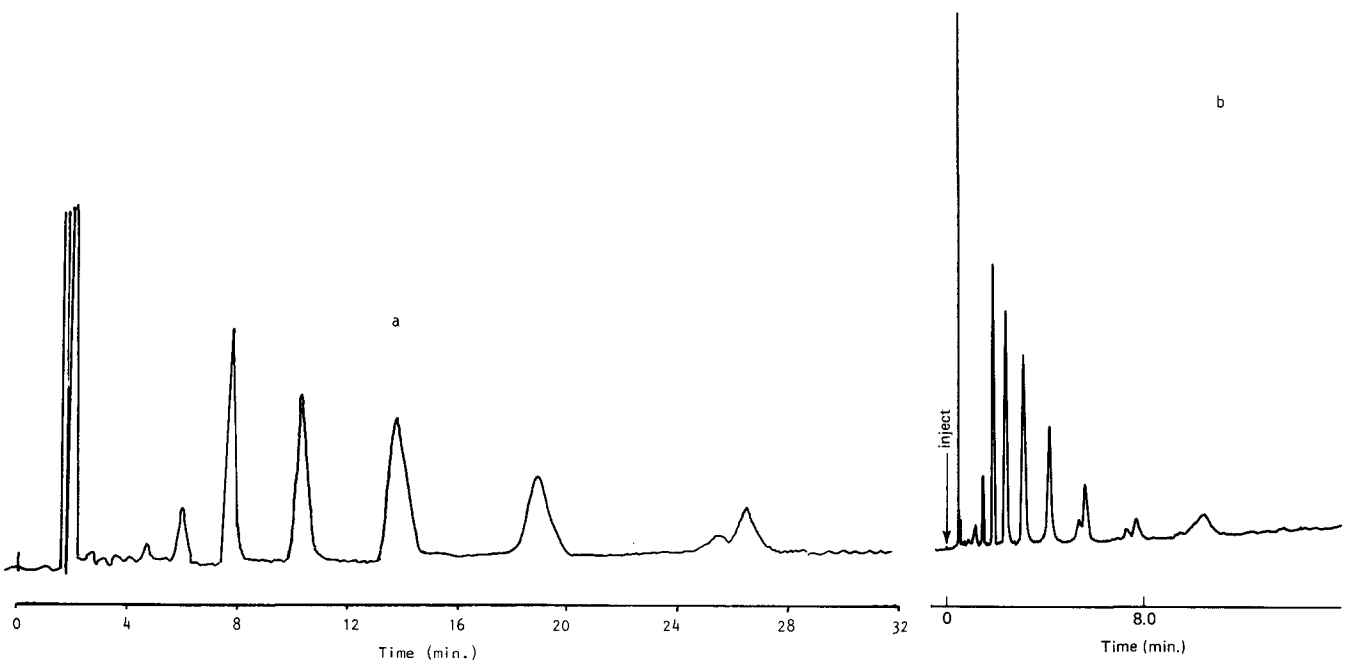

FIG. 6. The effect of changing acetone content on the separation of coconut triglycerides using 6 μm-250 × 4.6 Zorbax ODS (DuPont) mobile phase, isopropyl alcohol:acetone:methanol:acetonitrile—(a) 1:2:3:4, (b) 1:5:3:4.

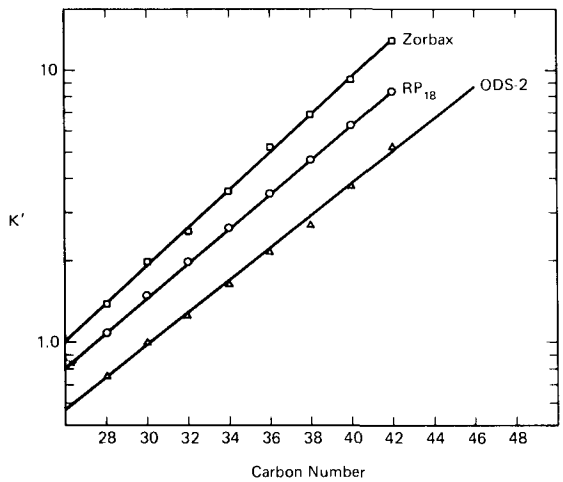

FIG. 7. The effect of particle size on triglyceride separation.

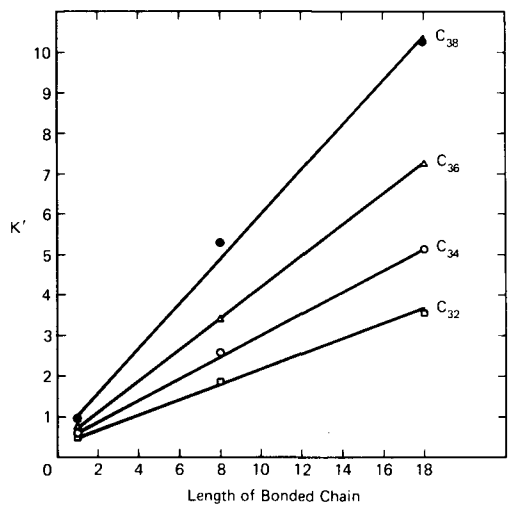

FIG. 8. The effect of bonded hydrocarbon chain length on the k′ value of triglycerides using a mobile phase mixture of isopropyl alcohol:acetone:methanol:acetonitrile.

obtained, the peaks were asymetric and the k′ values very large. However, the incorporation of 38.5% acetone in the mobile phase resulted in decreasing the separation time from more than 32 min to less than 12 min (Figure 6b). The bands were sharper and more symmetrical.

A comparison of the effect of the difference in particle size on triglyceride separation is shown in Figure 7. The results indicated that the 5 μ Supelcosil LC-18 column was the more efficient of the columns evaluated. Because of the smaller particle size of this packing, it was possible to use a smaller sample and improved resolution with decreased retention times compared with the columns with larger particle size.

The effect of the alkyl chain bonded to silica on the separation of saturated triglycerides using a solvent mixture of IPA:Ac:M:Cn at a ratio of 1:1:3:4 (v/v) idealized for the octadecyl bonded columns is shown in Figure 8. It can be seen that using only one carbon atom (CH_3) as a stationary phase bonded to silica did not result in any appreciable separation of the different triglycerides with very close k′ values. Bonding an octyl group resulted in increased separation with greater k′ values ranging 1.5 to 5.0. The bonding of a longer chain length (C-18) resulted in more separation of the triglycerides than both the LC-1 and LC-8 columns. This indicated that increasing the bonded chain length allowed more partitioning of the triglycerides within the stationary phase and in addition allowed the solubility of the triglyceride in the mobile phase to play an important part in the overall separation achieved.

A comparison of the effect of increasing the acetone content on the triglyceride separation on the different columns investigated is shown in Figure 9. A change in acetone content from 11.1% to 45% in the mobile phase mixture resulted in a decrease of the k′ values on the Zorbax ODS column from 10 to 4.5. The Supelcosil LC-18 column showed a more gradual change, from 7 to 3; the decrease of k′ values on the Partisil ODS-2 column was from 4.5 to 2 and from 3 to 1 on the Supelcosil LC-8. The effect of acetone on the k′ values is smaller on Supelcosil LC-8, which has an octyl chain bonded to silica, followed by Partisil ODS-2, where the particle size is larger and the bonded alkyl chain is longer. The effect of acetone on k′ values of triglycerides on Supelcosil LC-18 is more gradual than on Zorbax ODS. This is prob-

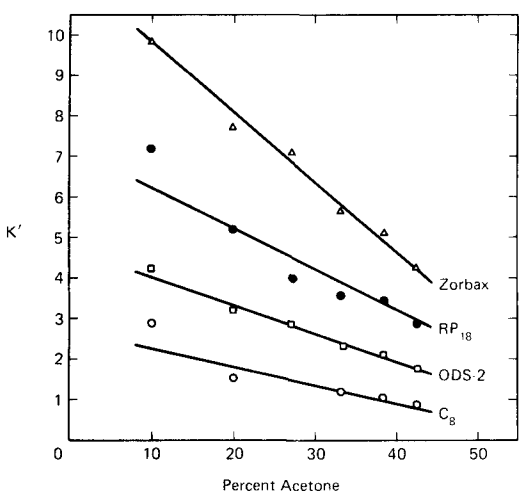

FIG. 9. The effect of decreasing mobile phase polarity on triglyceride separation on the various columns tested.

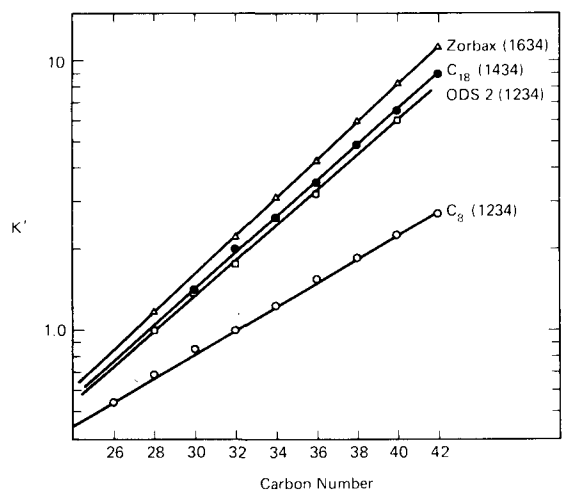

FIG. 10. K′ vs triglyceride carbon number obtained with the optimum mobile phase found for each column evaluated.

ably due to the difference in column length and particle size. These data show clearly that decreasing the solvent polarity or triglyceride solubility by addition of more acetone resulted in a decrease in the elution time of triglycerides, which in turn was due to the combination of both solubility and partition effects, as has been shown in Figures 1, 4 and 6.

A series of experiments were carried out to determine the optimum solvent combination for each column under study. A summary of this data for comparison purposes is presented in Figure 10. From the data shown in Figure 9 it can be seen that the optimum mobile phase is different for each column packing depending on the particle size, the alkyl bonded chain, the surface coverage, etc. It is shown in Figure 8 that there is a compatability between a longer chain bonded to a larger particle size and a shorter chain bonded to a smaller particle size (Partisil ODS-2 vs Supelcosil LC-8). Impressed separation of saturated triglycerides was obtained with Supelcosil LC-8 in a shorter time than with Partisil ODS-2 using the same mobile phase mixture. This was especially true for triglycerides with carbon numbers higher than C38.

ACKNOWLEDGMENT

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