for the catalyst reaction sites. More kinetic studies may further calarify the mechanism.

ACKNOWLEDGMENT

R.O. Butterfield provided the kinetic simulations.

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Optimization of Parameters for the Analysis of Triglyceride by Reverse Phase HPLC Using a UV Detector at 210 nm

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ABSTRACT

Factors affecting the quantitative analysis of triglycerides from natural oils were investigated on reverse-phase HPLC columns. The organic solvent used to dissolve the triglycerides greatly determined the extent to which the solute molecules interacted with the mobile phase and hydrocarbonaceous ligand. Chloroform-dissolved triglycerides resulted in better resolution and detection of the solute matrix of all the organic solvents used. Also, ethanol was superior to methanol as the polar organic modifier in the mobile-phase composition. Ballistic temperature programming significantly reduced analysis time and column temperature, when raised high enough, can reverse the elution of some solutes. Possible mechanisms of triglyceride solute retention on reverse-phase columns are advanced in conjunction with the various parameters used in the investigation.

INTRODUCTION

Ca. 80% of all the separations made by high performance liquid chromatography (HPLC) have been made using reverse-phase columns. The analysis of triglycerides has not been an exception. Even though reverse phase is the most popular mode of HPLC used at the present time, the mechanism of solute retention is complex and is the least understood method (1). The exact topography of the bonded hydrocarbonaceous ligand is not known, which leads to complications in attempts to interpret solute retention and solute interactions with the mobile phase and bonded support. Interaction of the mobile phase with solute molecules is generally considered the dominant force in reverse-phase chromatography (2) and the nature of this reaction is the driving force for solute distribution. In reverse-phase systems, when water is used as the primary solvent, solute retention is caused by the "hydrophobic effect" whereas, with the nonaqueous solvents, the "solvophobic theory" has been applied to solute retention (2).

Triglycerides are relatively large solute molecules that are very hydrophobic in character, and of low polarity (3). This class of lipids is soluble in such organic solvents as hexane, acetone, benzene, chloroform, methylene chloride, dioxane and tetrahydrofuran, but they have limited solubility in water. Earlier work in the HPLC analysis of oilseed triglycerides used a differential refractometer as the detector and used acetone as one of the solvents (4). The UV cutoff of this solvent negates the use of the more sensitive UV detector at 210 because of the optical windows of this solvent. Even though water has been used in the analysis of triglycerides (4), it was not considered in the present study because of the low solubility of triglycerides in water. A nonaqueous phase, consisting of solvents compatible with the UV detector at 210 nm, was used.

The objectives of this study were to investigate the various parameters necessary to optimize conditions for the analysis of triglycerides on reverse-phase columns and to provide information about triglyceride solute retention by hydrocarbonaceous ligands.

EXPERIMENTAL PROCEDURES

Triglycerides were analyzed using a Varian 5000 HPLC unit equipped with a 10 μL loop automatic valve injector. The detector was a Vari Chrom variable wavelength detector operated at 210 nm with an 8 μL cell. All analyses were performed on a MCH-10 (Varian) reverse-phase column (C-18 bonded). Methanol, ethanol and acetonitrile used in the mobile phases were HPLC-grade solvents.

Peanut oil was obtained by homogenizing the seeds 3 times with chloroform/methanol (2:1), filtering and removing the solvent by rotary evaporation. Coconut oil, which was used as a source of saturated triglycerides, was obtained commercially. The 2 triglycerides of known composition used as solute probes (glycerol-2-oleate, 1,3 dipalmitate and glycerol-2-oleate, 1,3-distearate) were obtained from

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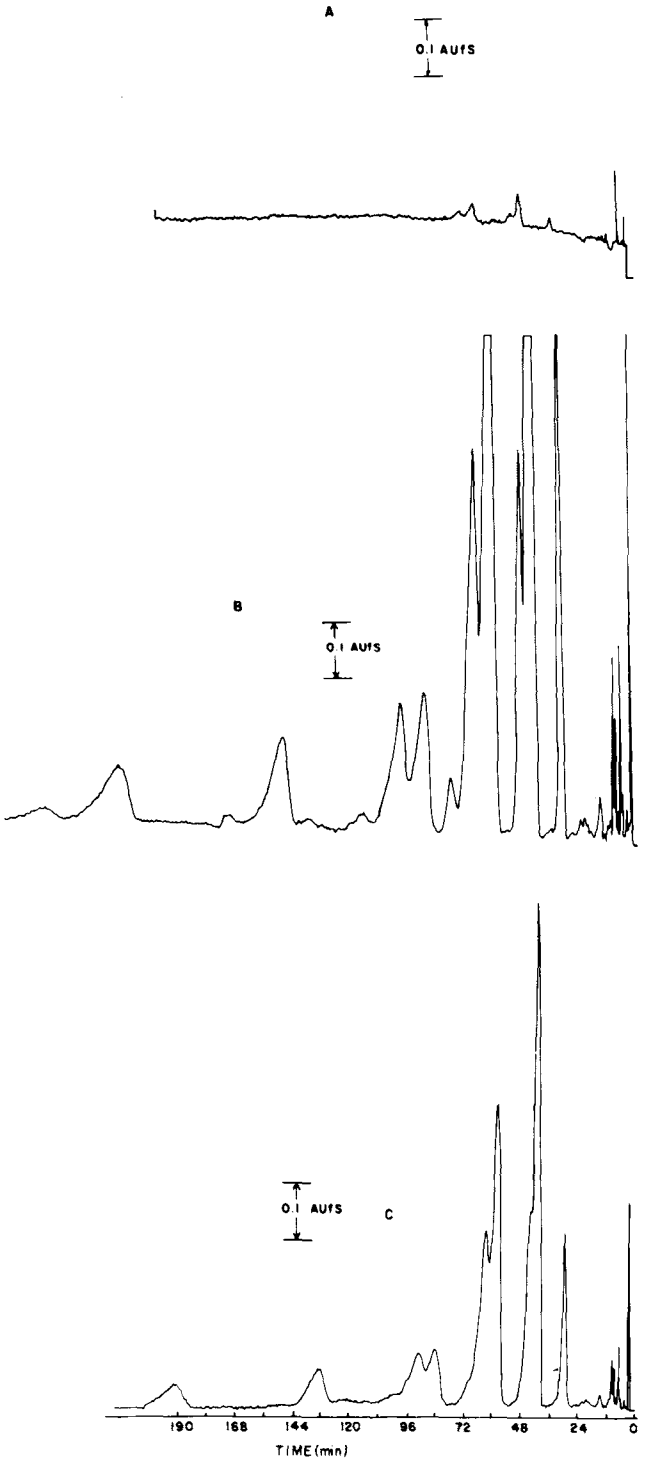

FIG. 1. Effect of sample solvent on the analysis of peanut-oil triglycerides: A. hexane; B. chloroform; C. acetone; ACN/EtOH (80/20).

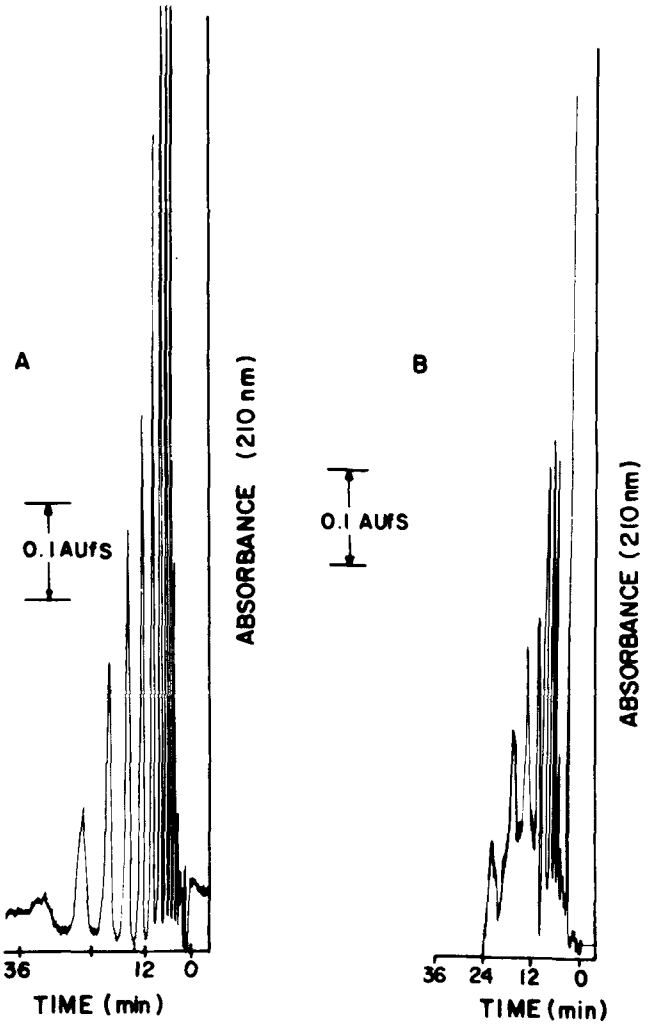

FIG. 2. Effect of sample solvent on the analysis of coconut-oil triglycerides: A. chloroform; B. acetone; ACN/MeOH (50/50).

Applied Science Inc., State College, PA. HPLC parameters are listed in the figure legends.

The parameters tested in this study were: (a) the effect of sample solvent on triglyceride analysis; (b) the effect of mobile phase composition on the sorption behavior of triglycerides; (c) the effect of sample load level on triglyceride analysis; and (d) temperature effects on triglyceride analysis.

RESULTS AND DISCUSSION

Effect of Sample Diluent

Optimization of parameters in the analysis of triglycerides on reverse-phase columns can be enhanced by understanding the theory of thermodynamic equilibrium of solute retention. Sample diluent, an important factor in the kinetics of equilibration, affects solute dispersion. Figure 1 shows the effects of 3 diluents on the chromatographic behavior of peanut-oil triglycerides on reverse-phase columns using acetonitrile (ACN) and ethanol (EtOH) as the mobile phase. Peanut triglycerides dissolved in hexane provided only a minute chromatographic trace, whereas dissolution in chloroform yielded excellent detection and resolution (Fig. 1A and 1B). These results can best be explained by invoking the solvophobic theory of Horvath and Melander (2). According to this theory, a solute molecule can be brought into solution by making an appropriate hole in the solvent followed by a reduction in free volume caused by van der Waals forces and electrostatic interactions. The energy required to make such a cavity in the solvent is calculated from the surface area of the solute molecule and the surface tension of the solvent. The physical properties of chloroform (solute diluent) lowers the energy level required to make a cavity in the mobile phase (ACH/EtOH), which allows the solute molecules to interact

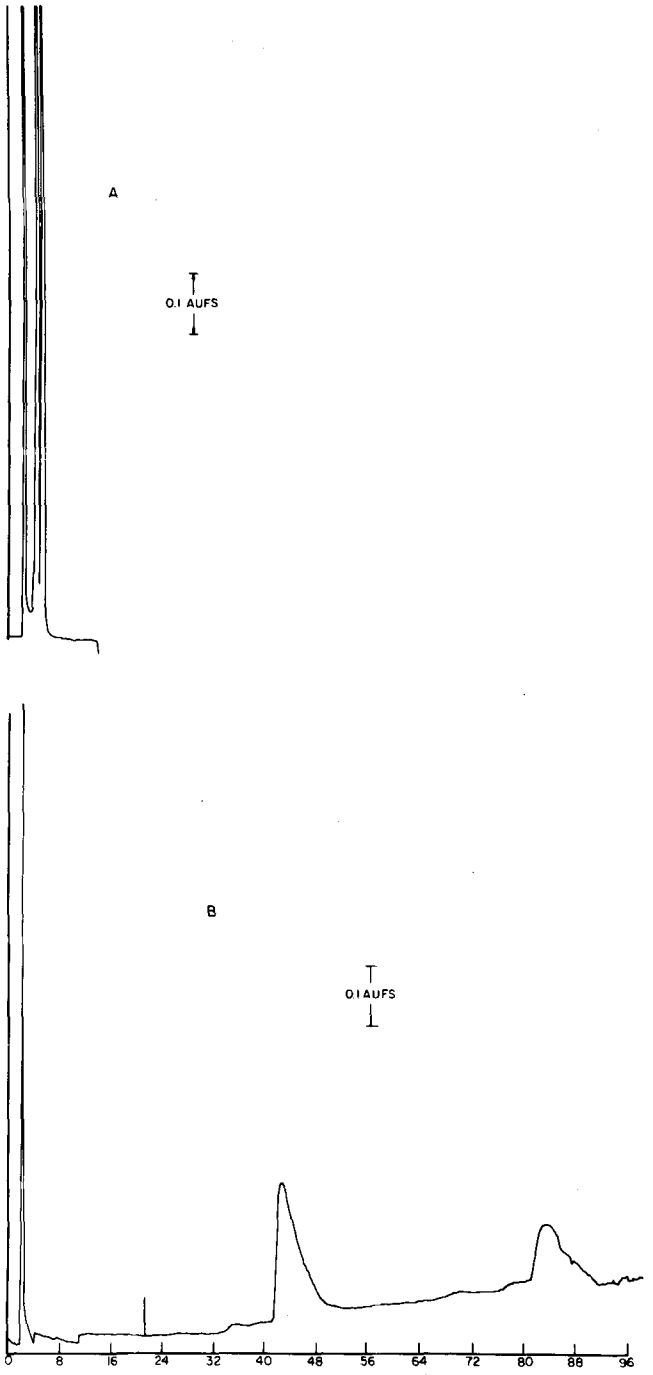

FIG. 3. Effect of mobile phase on the resolution of 2 known solute pairs—glycerol-2-oleate 1,3-dipalmitate and glycerol-2-oleate 1,3-distearate: A. ACN/EtOH (20/80); B. ACN/MeOH (20/80).

with the mobile phase. On the other hand, hexane (solute diluent), because of its physical properties, cannot lower the energy enough to bring about dissolution of the solute molecules into the mobile phase (ACH/EtOH). Therefore, the hexane (solute diluent) encapsulated triglycerides were effectively screened from interacting with the mobile phase and the bonded alkyl stationary phase.

Acetone (Fig. 1C), the most polar of triglyceride diluents, resulted in reduced detection and resolution compared with the chloroform dissolved triglycerides (Fig. 1B). The high dielectric constant of acetone apparently resulted in less reduction of the solute cavity size and therefore less

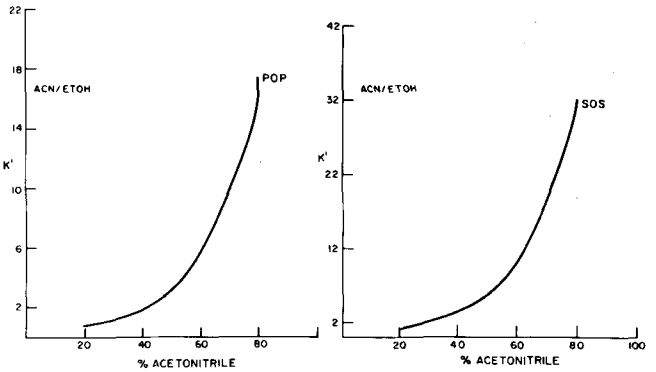

FIG. 4. Changes in the capacity factor with decreased mobile-phase polarity—glycerol-2-oleate 1,3 dipalmitate (POP) and glycerol-2-oleate, 1,3-distearate (SOS).

solvent-solute interactions. Chloroform, because of its physical and chromatographic properties, interacted to a greater extent with the mobile phase and hydrocarbonaceous ligand. Other organic lipid solvents were tested, such as acetone, carbon tetrachloride, benzene and methyl sulfoxide, but gave inferior results when compared with chloroform.

Coconut oil, a well-defined natural oil containing a homologous series of saturated triglycerides, was also used to determine sample diluent effects on fast emerging peaks (Fig. 2). Apparently chloroform (Fig. 2A) allowed more interaction of the solute matrix with the mobile phase and the bonded hydrocarbonaceous ligand, resulting in a clearly defined chromatogram, whereas acetone (Fig. 2B) produced a very rapid localized transient gradient as the solvent passed over the solute matrix, resulting in poor resolution and decreased detector response.

Effect of Mobile Phase Composition on the Sorption Behavior of Triglycerides

Two solute triglyceride probes of known composition, glycerol-2-oleate 1,3 dipalmitate and glycerol-2-oleate 1,3 distearate, as well as naturally occurring oils such as peanut and coconut, were used to determine the sorption behavior of triglycerides on reverse-phase columns. The effects of using ACN/EtOH (80/20) and ACN/MeOH (80/20) on the reverse-phase chromatography of glycerol-2-oleate 1,3 dipalmitate and glycerol-2-oleate 1,3 distearate is shown in Figure 3. A very rapid analysis resulted, with excellent peak shape and adequate resolution, when EtOH was used as the secondary solvent. However, substituting an equal amount of MeOH for EtOH resulted in increased solute retention, poor detector response and asymmetric peaks. MeOH forms a monomolecular layer on octadecyl-derived silica (5), which may explain the increase in solute retention caused by MeOH. Therefore, it may compete with solute molecules for adsorption sites on the bonded phase. Also, use of MeOH would increase the hydrophobicity of the mobile phase, thus decreasing solute-stationary phase interactions.

Plots of the capacity factors of glycerol-2-oleate 1,3 dipalmitate and glycerol-2-oleate 1,3 distearate vs mobile phase composition are shown in Figure 4. The instructive feature of these plots is that 2 types of solute retention mechanisms may be operative for triglyceride solute retention on reverse-phase columns. At high EtOH concentrations, the curvature suggests that a retention regime may be operative where the thermodynamic distribution coefficient and the phase ratios are changing simultaneously, resulting in nearly incremental changes in the capacity factor. As the

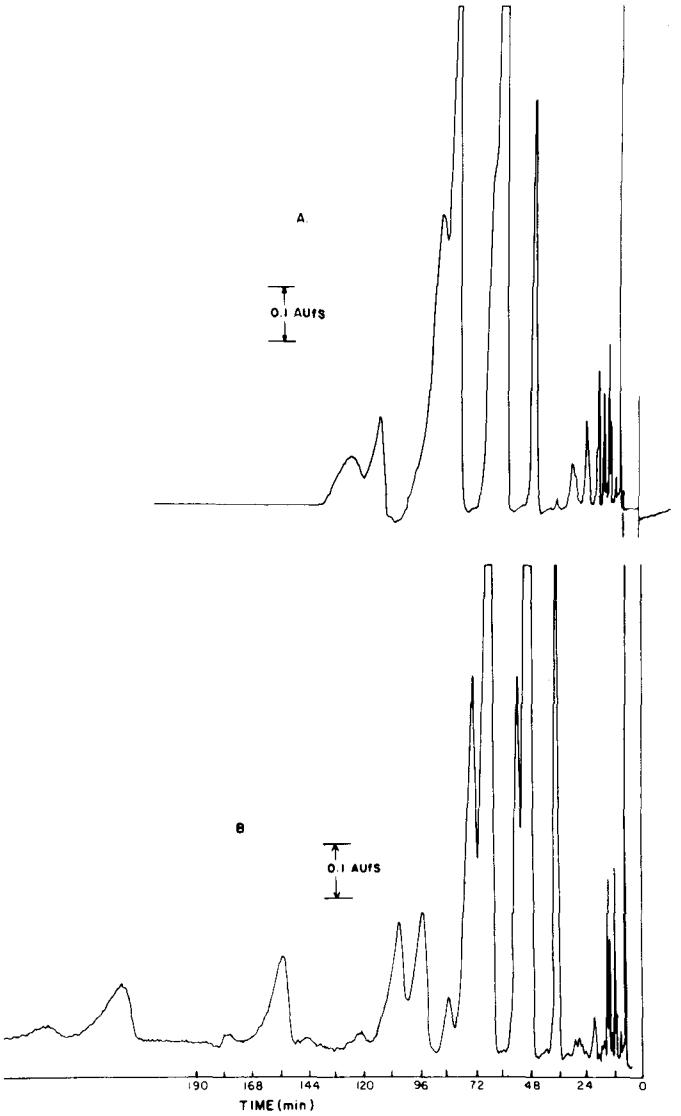

FIG. 5. Effect of mobile phase on the resolution of peanut-oil triglycerides: A. ACN/MeOH (50/50); B. ACN/EtOH (80/20).

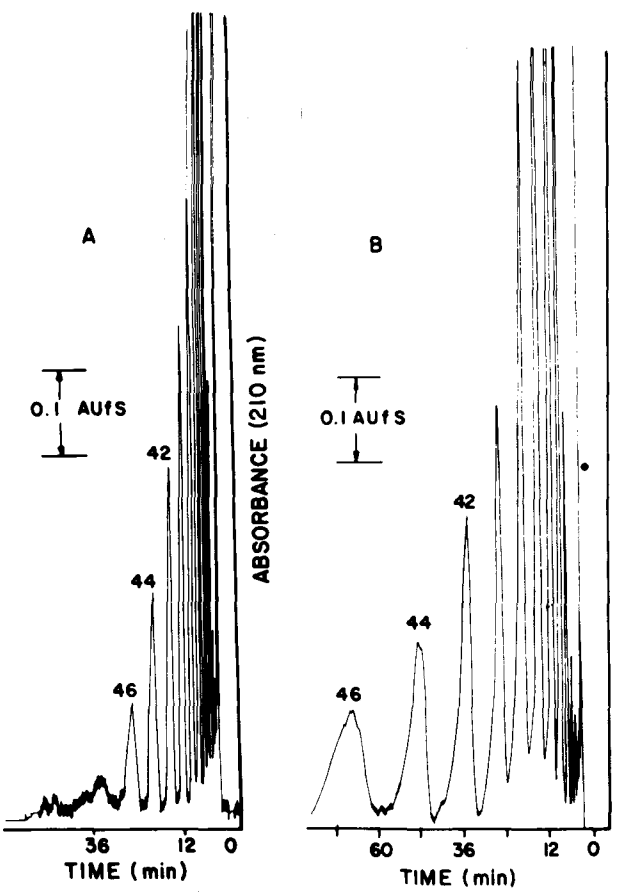

FIG. 6. Effect of temperature on the analysis of coconut-oil triglycerides: A. 60 C; B. ambient; ACN/MeOH (50/50).

TABLE I

Effect of Temperature on Column Parameters in the Analysis of Coconut-Oil Triglycerides (C-44, C-46)

Temperature	α	K′	N	H	R
Ambient	1.42	12.67	578	0.52	1.65
60 C	1.40	7.50	1589	0.19	2.50

α—column selectivity.
K′—capacity factor.
N—theoretical plates.
H—height equivalent to theoretical plates.
R—resolution.

polarity of the mobile phase decreased, the curves began to flatten, indicating a partitioning mechanism. Lochmuller (1) observed similar results with bonded phases of different chain lengths, relatively small solutes and the same mobile phase. In this study, the chain length of the bonded phase was kept constant, the mobile phase composition was varied and large solute moleules were used. The capacity factor is a product of 2 terms: the thermodynamic distribution coefficient and the phase ratio. Two distinct regions in the plot of capacity factor vs mobile phase polarity were identified. Information obtained using known solute probes to understand the theory of solute retention can have a practical application. For instance, the flat region of the capacity-factor plot vs mobile-phase composition corresponded to the optimum phase composition for analysis of the complex seed-oil triglycerides.

In another set of experiments, various solvent combinations were tried with peanut-oil triglycerides to determine optimum solvent pairs and composition. When ACN/MeOH was used as the mobile phase, the concentration of MeOH had to be increased to 50% before the triglycerides could be eluted in a reasonable length of time. When EtOH was used as the polar organic modifier (Fig. 5B) with ACN, resolution and detection were improved, and only 20% EtOH was required. An explanation for the poor resolution observed when MeOH was used in the mobile phase may be that MeOH, the more hydrophobic of the 2 alcohols, forms a monomolecular layer on the bonded phase, hindering the solute adsorption process.

Effect of Load Level on Triglyceride Analysis

The most effective way to have optimal kinetic parameters in HPLC analysis is to reduce effectively the absolute plate height, H (6). Theoretically, for the best column efficiency, the absolute plate height should be as small as possible. In the present study, as the sample load level (1-12 mg) was increased, H increased gradually until, at ca. 12 mg, an abrupt change occurred in the curve when solute concentration was plotted against H (mm). This change could be

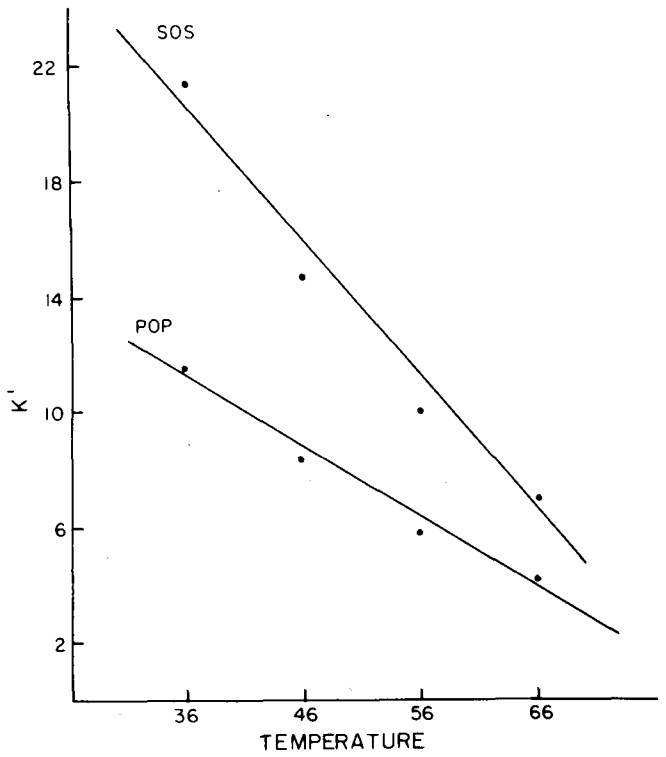

FIG. 7. Effect of temperature on capacity factors—glycerol-2-oleate 1,3-dipalmitate (POP) and glycerol-2-oleate 1,3-distearate.

attributed to errors in plate H measurements, caused by a lack of stationary phase for solute adsorption, and increased load levels, which cause band spreading to become more prevalent.

Temperature Effects on Triglyceride Analysis

Temperature is another parameter that can be used for optimal analysis on reverse-phase columns. Overall resolution of a particular solute matrix can be improved and analysis time reduced because temperature affects every term in the resolution equation (7). $R = \frac{1}{4}(k'/1+k')(\alpha-1)\sqrt{N}$ where k' = capacity factors, α = column selectivity and N = theoretical plates.

In the analysis of coconut-oil triglycerides, both band spreading and analysis time were less at 60 C than at ambient temperature when ACN/EtOH was used as the mobile phase (Fig. 6). Applying the resolution equation to 2 of the peaks in the chromatograms (Fig. 6) made the effect more apparent (Table I). Increasing the temperature from ambient to 60 C reduced the capacity factor and dramatically increased the number of theoretically available plates. It also considerably reduced absolute H so that column efficiency increased (Table I). Even though resolution was adequate at both temperatures, it was improved at 60 C. Elevated temperatures produced narrower peaks, which contributed to overall improved resolution. Also, viscosity of the mobile phase and the solute matrix was reduced, thus permitting more interaction of the solute molecules with the bonded phase.

One of the most effective ways to visualize temperature effects on solute retention is to plot capacity factor vs temperature (7). Figure 7 shows the effect of temperature on 2 known solute triglyceride probes. Elevated temperature will affect the larger solute molecules to a greater extent. Theoretically, the temperature can be elevated to a point where no resolution occurs, then continued temperature elevation will reverse the elution order. However, in the present study, reversal of elution order did not occur because of column temperature limitations. The reversal phenomenon may be best explained by the solvophobic theory. Alkyl hydrocarbon chains bound to a silica base are somewhat rigid in nature, yet enough mobility is available to solvate each other according to the patch model of

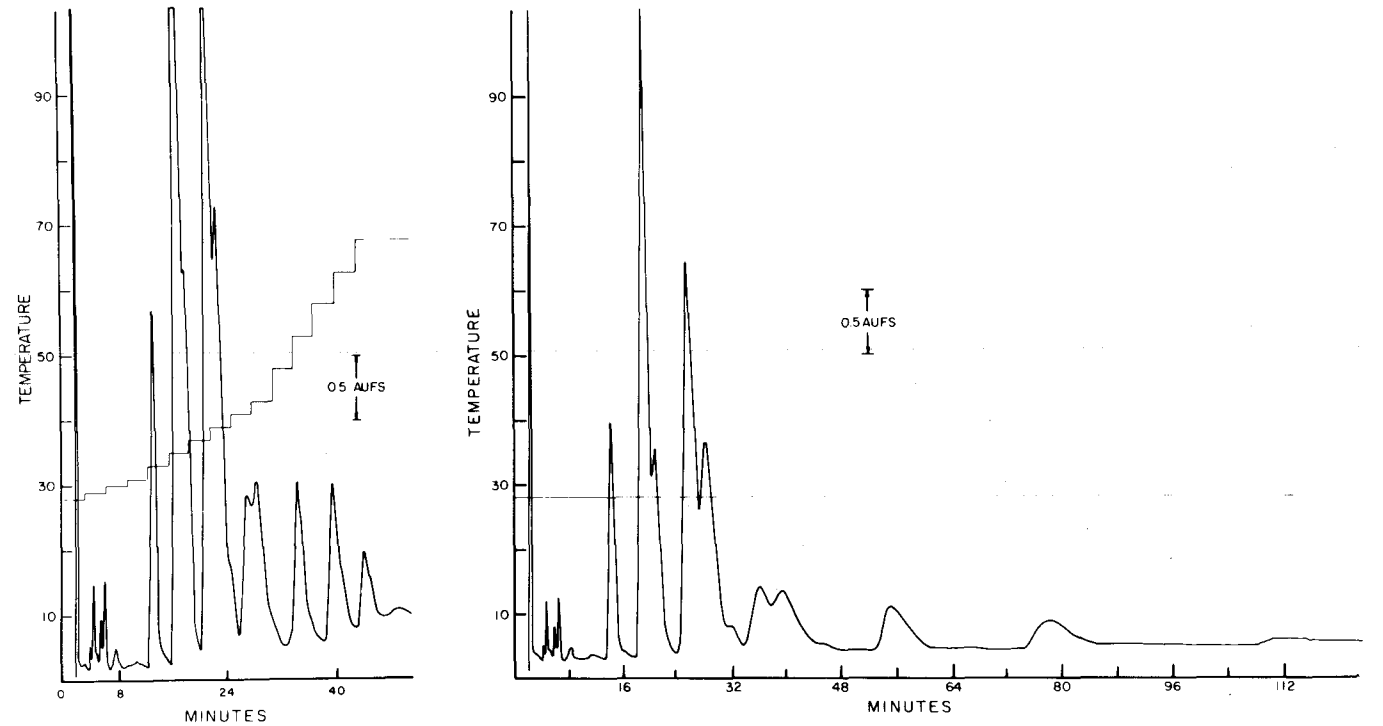

FIG. 8. Effect of temperature programming on peanut-oil triglyceride separation—ACN/EtOH (80/20).

Lochmuller (1). As the temperature rises, the alkyl chains apparently become vertical to the silica base, affording increased adsorption sites for the larger solutes and reducing solute retention. As indicated in Figure 7, as the temperature was elevated, the larger solute probe was affected to a greater extent. Regeneration of the column returns the bonded alkyl chains to their original geometry (7).

Another technique that can be used effectively for chromatographing a complex solute matrix on reverse-phase columns is temperature programming. Figure 8 compares temperature programming and an isocratic run of a natural oil triglyceride sample. The temperature program was performed ballistically, in increments, as shown in Figure 8. Temperature programming resulted in very little loss in resolution, but significantly reduced analysis time. Even though this technique has not been widely explored, it has been used with some interesting effects (7,8).

The analysis of triglycerides on reverse-phase columns in this study shows that $CHCl_3$ was superior to other lipid solvents when used as the sample solvent. A mobile phase using absolute ethanol as the polar modifier resulted in better resolution of triglycerides compared with methanol. A ballistic temperature program was used effectively to reduce analysis time of triglycerides. The above parameters resulted in adequate resolution for triglyceride groups of natural oils on reverse-phase columns.

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A Novel Method for Spectrophotometric Determination of Triglycerides

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ABSTRACT

A new method for the spectrophotometric determination of triglycerides at microgram levels has been developed. The method relies on the quantitative degradation of *O*-acyl lipids with hydrazine and the subsequent conversion of the fatty acid hydrazides to the corresponding *N'*-isopropylidenealkanohydrazides by reaction with acetone. Results are presented for triolein and some oils of plant origin.

INTRODUCTION

Triglycerides are determined most frequently by analyzing the glycerol generated by their saponification or transesterification. Although a number of methods, including an enzymatic one, have been used for the determination of glycerol, the preferred method involves oxidation of the glycerol with periodic acid to yield formaldehyde, which can be assayed spectrophotometrically after condensation with chromotropic acid to give a violet compound (1,2).

Recently, we have described the quantitative degradation of *O*-acyl lipids with hydrazine to their constituent fatty acid hydrazides (3). These hydrazides, on reaction with acetone, are converted into their corresponding fatty acid *N'*-isopropylidenealkanohydrazides, R—CO—NH—N = $C(CH_3)_2$, which strongly absorb ultraviolet light at 229 nm. Because these derivatives can be resolved by gas chromatography (GC) (3) and by high performance liquid chromatography (HPLC) (4), the hydrazinolysis-acetonization method can be used for fatty acid analyses of acylglycerols.

In this communication, we have modified the method of hydrazinolysis, followed by acetonization, for the spectrophotometric determination of triglycerides. The procedure has been designed to eliminate interference caused by incomplete removal of reactants. It has been tested with a number of triglycerides of plant origin.

EXPERIMENTAL PROCEDURES

All chemicals were purchased from E. Merck (Darmstadt, West Germany), except 98% hydrazine hydrate, which was from Fluka AG, Buchs, Switzerland. Triolein was obtained from Sigma Chemical Co., St. Louis, MO.

Preparation of Palmitic Acid Isopropylidene Hydrazide

Ca. 1 g of methyl palmitate was refluxed with 20 mL of 50% hydrazine hydrate in ethanol for 3 hr. To the reaction mixture, 50 mL of water was added and the resulting precipitate was filtered, washed with water and crystallized from 90% ethanol to yield pure palmitic acid hydrazide (mp 112-113 C). About 100 mg of the hydrazide was refluxed with 30 mL of acetone for 2 hr. Acetone was removed under reduced pressure and the *N'*-isopropylidenealkanohydrazide was crystallized from acetone (mp 71-72 C).

Preparation of Acetonized Hydrazinolysates of Triglycerides

Triglyceride (20-120 μg) was heated at 60 C with 0.1 mL

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