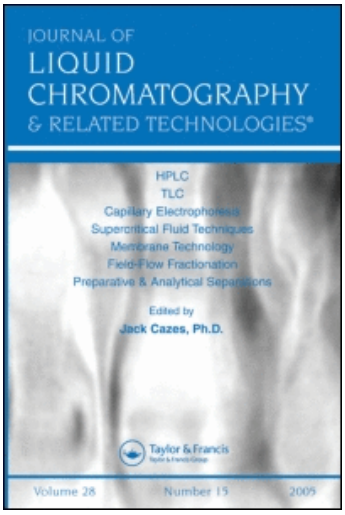

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Influence of Bonded-Phase Column Type, Mobile Phase Composition, Temperature and Flow-Rate in the Analysis of Triglycerides by Reverse-Phase High Performance Liquid Chromatography

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To cite this Article Barron, L. J. R. , Santa-María, G. and Masa, J. C. Díez(1987) 'Influence of Bonded-Phase Column Type, Mobile Phase Composition, Temperature and Flow-Rate in the Analysis of Triglycerides by Reverse-Phase High Performance Liquid Chromatography', Journal of Liquid Chromatography & Related Technologies, 10: 14, 3193 — 3212
To link to this Article: DOI: 10.1080/01483918708068307
URL: http://dx.doi.org/10.1080/01483918708068307

JOURNAL OF LIQUID CHROMATOGRAPHY, 10(14), 3193-3212 (1987)

INFLUENCE OF BONDED-PHASE COLUMN TYPE, MOBILE PHASE COMPOSITION, TEMPERATURE AND FLOW-RATE IN THE ANALYSIS OF TRIGLYCERIDES BY REVERSE-PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

Two chromatographic systems for RP-HPLC analysis of triglycerides, operating under isocratic conditions using octadecylsilane and octylsilane bonded phases, are described.

The influence of such chromatographic factors as bonded phase column type, mobile phase composition, temperature and flow rate on retention, analysis selectivity and efficiency, and separation of mixtures of homogeneous triglycerides was assessed. Linear relationships were established for the logarithm of the capacity factor and selectivity for each triglyceride in relation to temperature, the proportion of certain mobile phase components and flow rate.

The octadecylsilane bonded phase was more selective when analyzing triglycerides with a partition number below 48, while octylsilane was appropiate for separating mixtures of long chain saturated triglycerides to the detriment of the resolution of triglycerides with low partition numbers. ACN/ACE/THF (58/38/4) was a suitable mobile phase for use with the octadecylsilane bonded phase, and ACN/THF /H_2O (60/40/1) for the octylsilane bonded phase. A column temperature of 30°C and a flow rate of 1.5 mL/min resulted in acceptable resolution and analysis time in both systems.

INTRODUCTION

Reverse-phase high performance liquid chromatography (RP-HPLC) is regarded as one of the most effective methods of separating mixtures of triglycerides (TG). Despite the encouraging results that have been achieved there remain a number of obstacles to the resolution of TG mixtures, particularly when dealing with samples of natural substances.

Poor resolution of triglycerides with the same partition number (PN), separation of TG mixtures spanning a broad range of partition number and precipitation losses in the mobile phases of saturated long-chain triglycerides, are a few of the most frequently encountered difficulties.

A number of chromatographic factors, including the type of bonded phase, composition of mobile phase and column temperature, have been studied in an attempt to solve such difficulties and improve analysis results.

Octadecylsilanes (ODS) constitute the most selective bonded phases (1,2,3) as compared to other bonded phases tested, generally octylsilanes (OS). The best results obtained with ODS have been achieved using particle sizes of 5 μ (4), although particle sizes of 3 and 10 μ have also been used successfully (5,6).

The study of mobile phase composition has shown that, as the polarity of the mobile phase rises, analysis times become less feasible, and selectivity (α) increases (7,8,9). The relationship between TG capacity factors (K′) and mobile phase polarity have been calculated as the proportion of one of the mobile phase components is varied. Singleton and Pattee (7) found a polynomial relationship between K′ and the percent proportion of acetonitrile (ACN) in the mobile phase, and Pauls (8) established a linear relationship between log K′ and the percentage of the various solvents making up the mobile phase.

Parris (10) studied the influence of the proportion of tetrahidrofuran (THF) in ACN/THF mobile phases and reported that, at proportions of below 20%, resolution declined and peaks exhibited trains, whereas at proportions above 60% triglycerides with partition numbers lower than 48 were not retained. Edwards (11) suggested replacing THF with methyl tertiary butyl ether (MTBE) when using ultraviolet (UV) detectors.

Different polarity gradients have been proposed for use with complex TG mixtures in which the triglycerides span a broad range of partition numbers depending the type of detector used. Thus, Parris (12) suggested a gradient of THF in acetone (ACE) for use with infrared (IR) detectors, Robinson and Macrae (13) put forward a gradient of ethanol (ETOH) in ACN for use with UV detectors at 200-230 nm, and Herslöf and Kindmark (14) proposed gradients of from 100% ACN to 100% ETOH in an ACN/ETOH/n-hexane (H) (40/40/20, v/v) mobile phase for use with mass detectors. Myher et al. (15) suggested a gradient of n-propionitrile (PRN) in ACN when detecting triglycerides with a mass spectrometer.

Pauls (8) suggested that the effect of variations in the composition of the mobile phase on resolution might reflect the effect of polarity, chiefly a result of the number of double bonds in the TG molecule, as well as the effect of hydrophobicity due to the number of carbon atoms in the fatty acids making up the triglycerides.

Several authors have studied the effect of column temperature (T). Increases in T have been observed to bring about a decrease in TG retention time (RT) and K', as well as in α, particularly for triglycerides with the same partition number (16,17,18). The number of theoretical plates for the column for the different TG species has, moreover, been found to increase with T, improving resolution power (17).

Relationships between K' and T have also been established. Jensen (19) found a linear relationship for the log of K' and the inverse of T, and he advised a working temperature of 14.5ºC, since below that T tristearin, with a PN of 54, turned insoluble, while at higher temperatures α decreased. Frede (16) found a linear relationship based on Vant' Hoff's equation for log of K' and 1000/T(1/K') for each individual triglyceride species.

Temperature gradients have been put forward as a way to improve analysis of complex TG mixtures, and good separations have been obtained in the first part of chromatograms at temperatures of 18°C and above, while minor triglycerides have been eluted and detected in the last part of chromatograms at temperatures above 35°C. At these latter temperatures analysis times are considerably reduced, though other problems arise. On the one hand, working with refractive index (RI) detectors limits the use of multicomponent mobile phases, because T affects the chemical equilibrium each of the components in the mobile phase and the bonded phase, thereby causing variations in their proportions

(20). Furthermore, at temperatures of 35 to 50°C, α decreases considerably in the last part of chromatograms (16). Frede (16) working with RI detectors, suggested the use of unary mobile phases, specifically PRN or perhaps binary mobile phases made of mixtures of PRN/ethyl ether (EE).

In a study of flow rates, Lozano (9) reported that increasing the solvent flow rate could improve resolution in some cases.

Nonetheless, there is as yet no single method that satisfactorily solves all the problems involved in RP-HPLC analysis of triglicerides, although the separating power of the chromatographic system depends in large measure on the TG composition of the mixture being analyzed (21). The object of the present study was, firstly, to complete previous partial studies on the influence of the type of bonded-phase composition, column temperature and flow rate in the RP-HPLC separation of triglycerides, and secondly, to determine chromatographic conditions suitable for the use of OS bonded phases in the analysis of triglycerides with partition numbers higher than 48, primarily long-chain triglycerides with a low level of unsaturation.

MATERIALS AND METHODS

The chromatographic system employed consisted of a Waters model 510 pump, a Waters model U6K injector and a Waters model differential refractometric detector connected to a BBC Goerz Metrawatt SE 120 recorder. Both the columns tested and the injector loop were equipped with water bath thermostats.

Injection comprised 5 μL of pure samples of homogeneous triglyceride mixtures at a concentration of 10 mg/mL. The homogeneous triglycerides were tributirin (BuBuBu), tricaproin (CoCoCo), tricaprylin (ClClCl), tricaprin (CaCaCa), trilinolenin (LnLnLn), trilaurin (LaLaLa), trilinolein (LLL), tripalmitolein (PaPaPa), trimyristin (MMM), triolein (OOO), tripalmitin (PPP), tristearin (SSS), triarachidin (AAA) and tribehenin (BBB). All were Sigma Grade and approximately 99% pure.

The triglycerides were dissolved in chloroform (CHL)/ACE (1/1, v/v), except in the case of PPP and SSS, which were dissolved in CHL/ACE (3/1, v/v), and AAA and BBB, dissolved in CHL.

Three column were types tested, as set out below:
Waters NOVAPAK ODS 5 μ 15-cm column;
Beckman ULTRASPHERE ION PAIR ODS 5 μ 15-cm column;
Beckman ULTRASPHERE OS 5 μ 25-cm column.

The proportion of THF in the ACN/ACE/THF mobile phase (62 - X/38/X, v/v/v) was varied from 2 to 12% by volume for all three column types while holding constant the proportion of ACE. A further two solvent systems were tested in the OS column:

ACN/THF in proportions of 10, 20, 30, 40 and 50% THF by volume;

ACN/THF/H_2O, holding constant the ACN/THF ratio of 60/40 and varying the proportion of water from 1 to 6, with intermediate proportions of 1.2, 1.4, 1.6, 1.8, 2 and 4.

The range of temperatures tested ran from 25 to 40°C in 5-°C steps. Flow rates were 0.5, 1.0, 1.7 and 2.2 mL/min tested in a 15 cm × 4.6 mm i.d. stainless steel column filled in our laboratory with 5 μ SPHERISORB ODS-2 (lot 22/209). The mobile phase was ACN/ACE/THF (38/48/16 v/v/v) and the column temperature was 30°C. The number of theoretical plates (N) for the column was calculated by the 1/2 Peak Height method: $N = 5.54 \times (v/w)^2$, where v was the volume retained and w was 1/2 peak height width.

The dead volume of the columns was calculated by injecting a substance (ACN) that was not retained. All the organic solvents employed either to dissolve the triglycerides or in the mobile phases were Ferosa HPLC grade. The water used was Mili Q grade. Moreover, the solvents used in the mobile phases were filtered (0.2 μ) and degassed in an ultrasonic bath.

RESULTS AND DISCUSSION

The molecular variables affecting TG retention in bonded phase silica columns were the carbon number (CN) and the number of double bonds (ND) of the fatty acids making up the triglycerides. The partition number, defined by Wada et al. (22) as PN = CN - 2 × ND, was used to establish the molecular identity of each triglyceride. The chromatographic variables capacity factor (K′) and selectivity (α) were used to assess retention of triglycerides and system affinity for a TG pair.

Selectivity was calculated with respect to LaLaLa, which was it was present in a region of the chromatogram in which retention time (RT) did not undergo any substantial variation with the differing chromatographic factors applied (bonded phase,

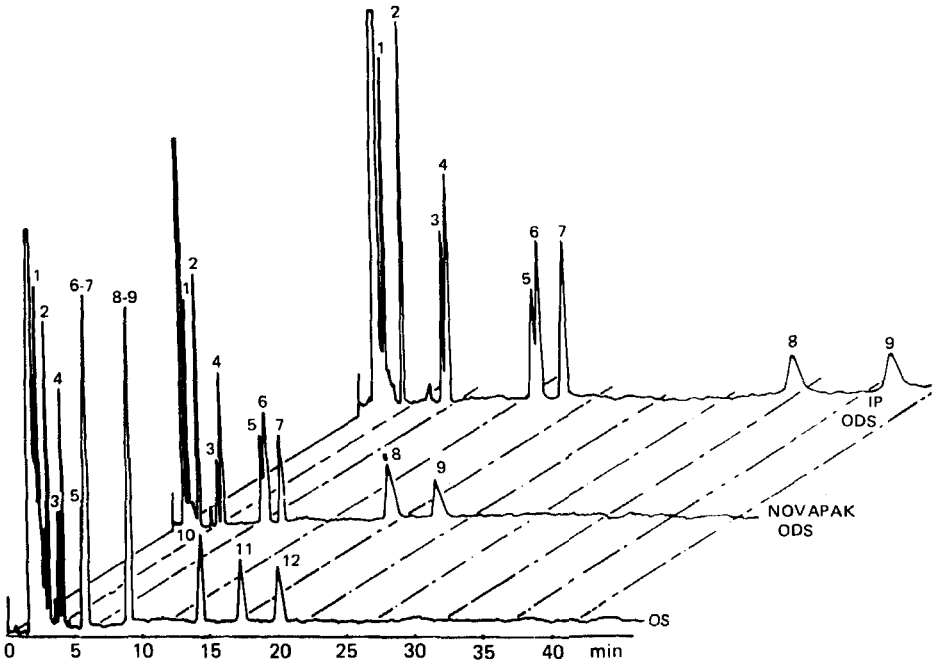

Figure 1: Chromatograms for the ION PAIR ODS (IP), NOVAPAK ODS and OS column types under identical operating conditions: ACN/ACE/THF (58/38/4) mobile phase, column temperature (T) = 30ºC and flow rate (F) = 1.5 mL/min. Numering of peaks: 1 = ClClCl; 2 = CaCaCa; 3 = LnLnLn; 4 = LaLaLa; 5 = LLL; 6 = PaPaPa; 7 = MMM; 8 = OOO; 9 = PPP; 10 = SSS; 11 = AAA; 12 = BBB. TG abbreviations as in Table 1.

proportion of THF and temperature). Selectivity was also calculated for TG pairs with the same PN, i.e., LnLnLn-LaLaLa (PN = 36), LLL-PaPaPa and PaPaPa-MMM (PN = 42), and OOO-PPP (PN = 48).

Under the chromatographic conditions used, the triglycerides eluted in ascending order of PN, and, in the case of triglycerides with the same PN, those with more unsaturations eluted first (Figure 1).

Type of Bonded Phase

Table 1 summarizes the values of RT and α for the homogeneous triglycerides in the three column types tested under

Table 4: Slope (m) and y-intercept (a) values, coefficient of correlation (r), and standard error ($S_{y.x}$) for log α/THF lines for TG pairs with the same PN (PN = 36, 42 and 48) in the NOVAPAK ODS column with an ACN/ACE/THF (62 - X/38/X), T = 30°C and F = 1.5 mL/min. Abbreviations for α for TG pairs with the same PN as in Table 2.

log α/THF	PN	a	m	r	$S_{y.x}$
α_{LnLnLn}	36	-0.03	-0.002	-0.650	0.439
α_{LLL}	42	-0.01	-0.002	-0.620	0.453
α_{PaPaPa}	42	-0.07	-0.000	-0.074	0.576
α_{OOO}	48	-0.07	0.000	-0.018	0.577

numbers ranging from 24 to 48. At this proportion, decreases in RT, K′ and α for the triglycerides were moderate, analysis time acceptable and resolution good for triglycerides with the same partition number (PN = 36, 42 and 48).

OS bonded phase

An identical study of the mobile phase as described above for the ODS columns was carried out for the OS column, and Figure 4 presents identical results to those obtained with the ODS columns. It should be noted that none of the ACN/ ACE/THF mobile phases tested separated the pair OOO-PPP (line 8-9) and that separation of triglycerides with a PN of 42 (lines 5, 6 and 7), though quite incomplete, commenced an 8% THF. Triglycerides with a PN higher than 48, SSS (line 10), AAA (line 11) and BBB (line 12), exhibited very reasonable elution times (14.4, 17.4 and 20.8 minutes, respectively) at 4% THF.

In order to improve as much as possible TG separation in the OS column less polar mobile phases consisting of mixtures of ACN/THF were tested, since THF seemed to have an important effect on α for TG with the same PN. Because the main problem area was non-separation of triglycerides with a PN of 42 and 48, a mixture of equal proportions of these triglycerides was injected.

Figure 5 presents changes in log K′/THF. A sharp decrease in RT can be observed. Separation of TG with a PN of TG with PN of 42 (lines 5, 6 and 7) began to be appreciable starting at 40% THF, whereas at this proportion separation of the TG pair with a PN of 48 (lines 8 and 9) was partial.

Table 2: Selectivity (α) for triglycerides with the same PN (PN = 36, 42 and 48) in the three colum types with an ACN/ACE/THF (58/38/4) mobile phase, T = 30°C and F = 1.5 mL/min. α_{LnLnLn} = α for LnLnLn vs. LaLaLa; α_{LLL} = α for LLL vs. PaPaPa; α_{PaPaPa} = α for PaPaPa vs. MMM; α_{OOO} = α for OOO vs. PPP. TG abbreviations as in Table 1.

α	NOVAPAK ODS	ULTRASPHERE IP ODS	ULTRASPHERE OS
α_{LnLnLn}	0.86	0.93	0.95
α_{LLL}	0.93	0.96	0.97
α_{PaPaPa}	0.86	0.87	1.00
α_{OOO}	0.82	0.81	1.00

These results indicate that the ODS bonded phases were better suited for analysis of triglycerides with a PN of between approximately 24 and 48.

The OS bonded phase may be suitable for rapid separation of long-chain triglycerides with a low level of unsaturation and a PN higher than 48, to the detriment of resolution, particularly of triglycerides with the same PN. These results substantiate the findings reported by other authors concerning the application of different bonded phases in RP-HPLC analysis of triglycerides (1,3).

Composition of the Mobile Phase

ODS bonded phase

Figure 2 presents the chromatograms for differing proportions of THF in the mobile phase. As the proportion of THF in the mobile phase increases, RT can be observed to decrease, this decrease being much more pronounced for triglycerides with higher partition numbers. At higher proportions of THF (8 and 12%) the RT was acceptable (27 and 23 minutes, respectively). Chromatographic analysis of this triglyceride was unacceptably long at lower proportions of THF in the mobile phase. Chromatographic analysis of AAA and BBB was unfeasible at all proportions, as the elution times required were excessive.

The differences in retention of triglycerides observed in function of their PN and the proportion of THF are explicable by the model put forward by Geng and Regnier (23) for interpreting non-polar solute retention in reverse-phase chromatography. The

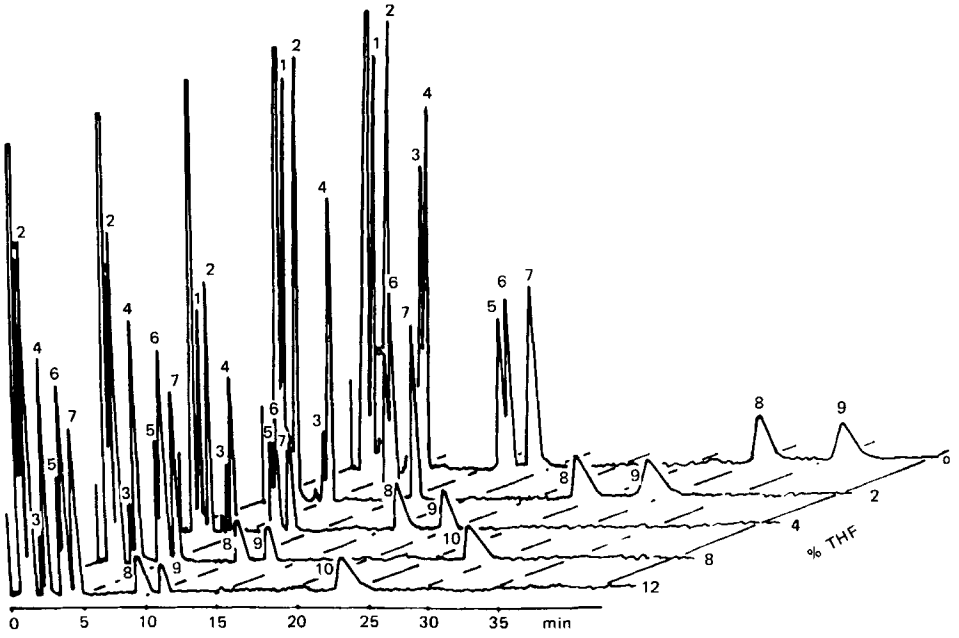

Figure 2: Chromatograms for the NOVAPAK ODS column at varying percentages of THF in an ACN/ACE/THF (62 - X/38/X) mobile phase, T = 30ºC and F = 1.5 mL/min. Peak numbers as in Figure 1.

model predicts that the displacement of solute molecules adsorbed by the bonded phase is accompanied by adsorption of a stoichiometric number of solvent molecules (Z). Solute retention is directly related to its molecular structure. In the case of triglycerides, their structure is dependent upon both the CN and the ND, which explains why RT was dependent upon PN, which relates CN and ND.

Table 3 presents the slope and y-intercept values for the linear relationship between logarithm of K´ and the proportion of THF in the mobile phase (log K´/ THF). The slope of the log K /THF lines was different for each TG and increased with PN. This change in slope can also be explained using the previously mentioned model of Geng and Regnier (23), because Z can be expected to be larger the greater the surface area of the TG for contact with the bonded phase, and such surface area increases as a triglyceride's PN increases. Table 3 also shows that the slope of the log K´/THF

Table 3: Slope (m) and y-intercept (a) values for log K′/THF lines for the different triglycerides at different temperatures (T) in the NOVAPAK ODS column, using an ACN/ACE/THF (62 - X/38/X) mobile phase and F = 1.5 mL/min. TG abbreviations as in Table 1.

T°C		ClClCl	CaCaCa	LnLnLn	LaLaLa	LLL	PaPaPa	MMM	OOO	PPP
25	m	-0.025	-0.034	-0.045	-0.042	-0.050	-0.051	-0.051	-0.060	-0.061
	a	-0.14	0.36	0.79	0.82	1.20	1.21	1.28	1.65	1.76
30	m	-0.024	-0.034	-0.045	-0.042	-0.051	-0.051	-0.051	-0.060	-0.061
	a	-0.20	0.27	0.70	0.73	1.09	1.11	1.16	1.53	1.61
35	m	-0.024	-0.033	-0.044	-0.042	-0.051	-0.051	-0.050	-0.061	-0.060
	a	-0.26	0.19	0.60	0.65	0.99	1.00	1.05	1.41	1.46
40	m	-0.024	-0.033	-0.044	-0.042	-0.050	-0.050	-0.050	-0.061	-0.060
	a	-0.32	0.11	0.51	0.56	0.88	0.89	0.94	1.29	1.33

lines did not vary appreciably with column temperature (T) for each individual TG, which indicates that the effect of THF on retention predominates over the possible effect of changes in T.

Figure 3 depicts variations in the log of α with changes in proportion of THF(log α/THF). It shows that as in the case of log K′/THF, the change was linear and distinct for each triglyceride. As the proportion of THF increases, the value of log α approached zero, indicating a general loss of resolution in the chromatogram. Separation of TG pairs with the same PN (PN = 36, 42 and 48) was unaffected by the proportion of THF, as can be seen from the slope values given in Table 4, all less than |0.003|. Furthermore, the correlation coefficient for changes in log α/THF (Table 4) were in no case statistically significant for p minor than 0.05.

The results described above for the NOVAPAK column were likewise obtained using ION PAIR ODS column.

These results explain Lozano's (9) finding that the presence of 4% THF in the ACN/ACE mobile phase was advisable for the analysis of vegetable oil triglycerides. Thus, as the proportion of THF in the mobile phase increased, α was enhanced for TG pairs with the same PN, accompanied by a general decrease in resolution in the chromatogram (Figure 3). As a result, a proportion of 4% THF appears to be advisable for analyzing TG pairs with partition

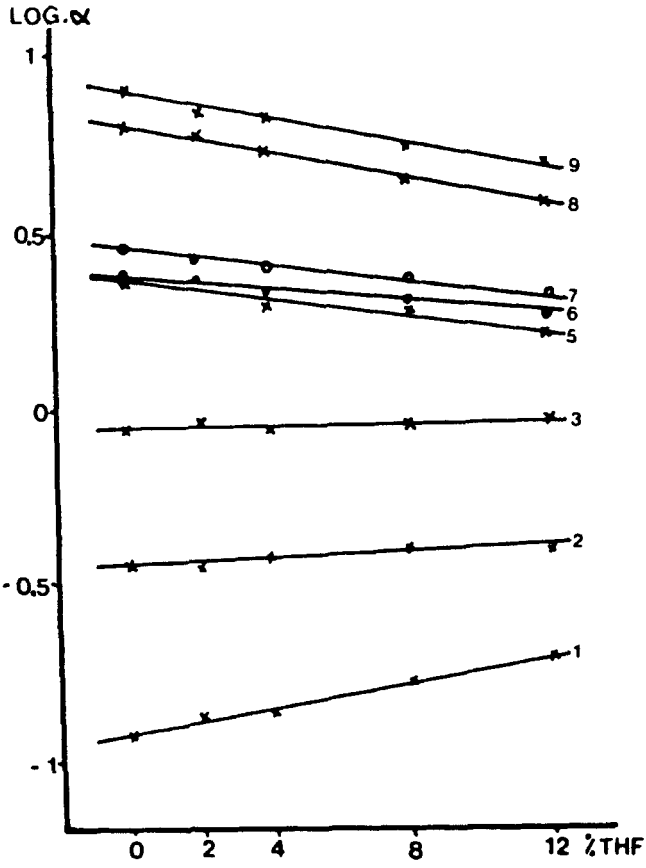

Figure 3: Changes in log α/THF for the NOVAPAK ODS column with an ACN/ACE/THF (62 - X/38/X) mobile phase, T = 30ºC and F = 1.5 mL/min. Line numbers correspond to the peak numbers in Figure 1.

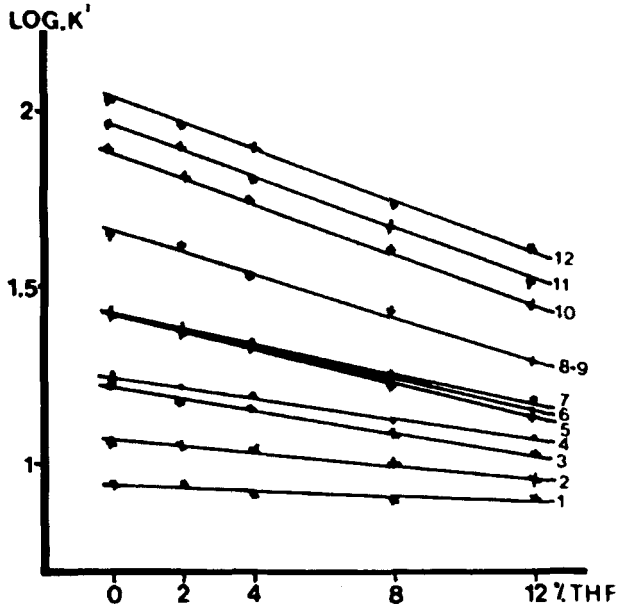

Figure 4: Changes in log K'/THF for the OS column with an ACN/ACE/THF (62 - X/38/X) mobile phase, T = 30ºC and F = 1.5 mL/min. Line numbers as in Figure 3.

In view of these results, a mobile phase consisting of ACN/THF (60/40 v/v) was selected as the starting point for improving resolution on the OS column, since with such a mixture triglycerides with a PN of 42 were separable and triglycerides with a PN of 48 began to be separable, with no adverse effect on overall resolution in the chromatogram, as occurs at higher proportions of THF (Figure 6).

Varying proportions of water added to the ACN/THF (60/40, v/v) mobile phase, in an attempt to enhance retention and TG separation. Figure 7 presents the chromatograms for proportions of 0, 1, 2, 4 and 6 parts water. RT increased with the proportion of water. Furthermore, as compared to the mobile phase with no water, separation of triglycerides with a PN of 42 (peaks 5, 6 and 7) was slightly enhanced by using one part water, while no appreciable

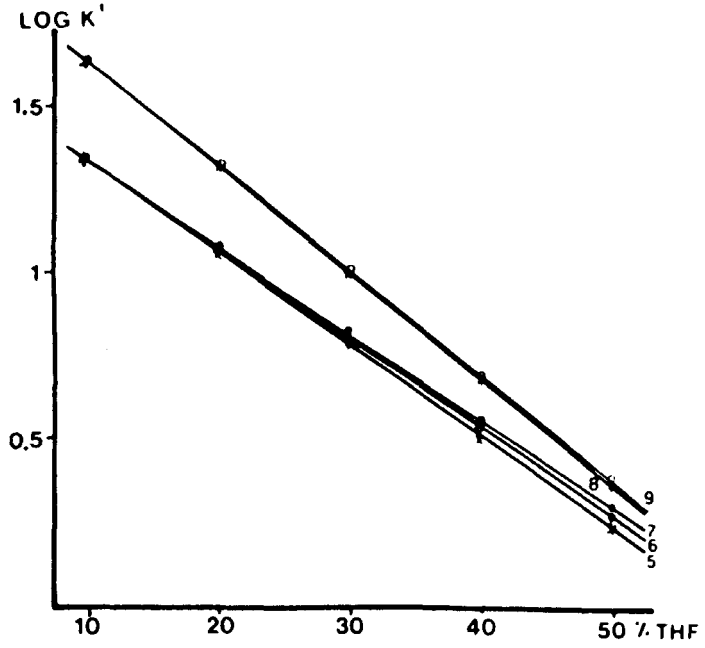

Figure 5: Changes in log K'/THF for triglycerides with partition numbers of 42 and 48 in the OS column with an ACN/THF (100 - X/X) mobile phase, T = 25ºC and F = 1.5 mL/min. Line numbers as in Figure 3.

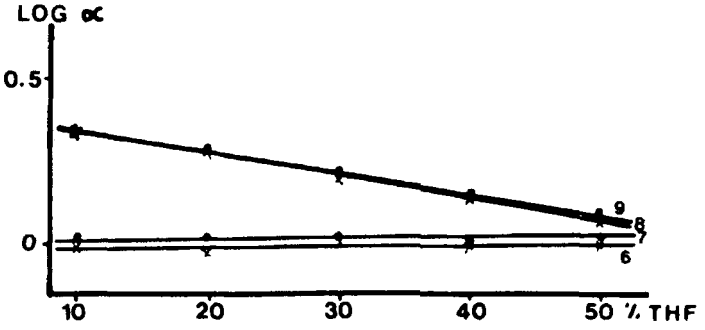

Figure 6: Changes in log α/THF for triglycerides with partition numbers of 42 and 48 in the OS column with an ACN/THF (100 - X/X) mobile phase, T = 25ºC and F = 1.5 mL/min. Line numbers as in Figure 3.

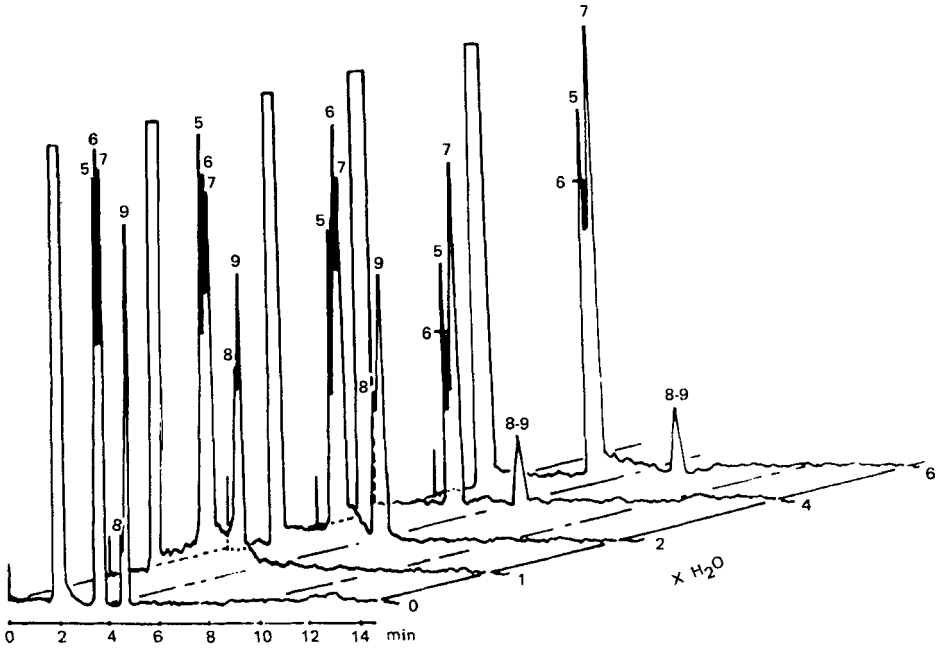

Figure 7: Chromatograms for TG mixtures with partition numbers of 42 and 48 in the OS column at differing proportions of H_2O in an ACN/THF/H_2O (60/40/X) mobile phase, T = 25ºC and F = 1.5 mL/min. Peak numbers as in Figure 1.

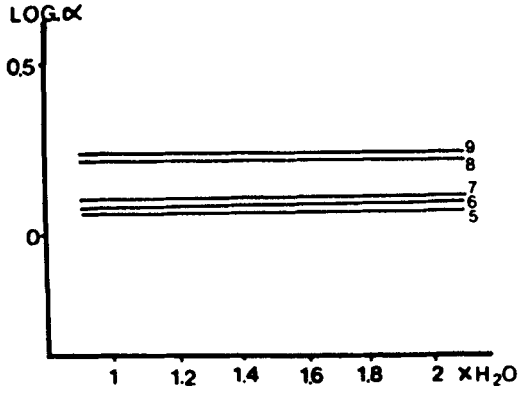

Figure 8: Changes in log α/H_2O for triglycerides with partition numbers of 42 and 48 in the OS column with an ACN/THF/H_2O (60/40/X) mobile phase, T = 25ºC and F = 1.5 mL/min. Line numbers as in Figure 1.

variation was observed in the resolution of triglycerides with a PN of 48 (peaks 8 and 9).

The separations were not improved by increasing the proportion of water to 6 parts, and even exhibited a tendency to worsen at proportions above two parts water. The difficulty in separating triglycerides encountered at proportions higher than two parts water was due to the low solubility of triglycerides in the mobile phase, which could result in precipitation losses.

Additionally, Figure 8 shows that selectivity remained practically constant for each TG at all the different proportions of water. It also shows that separation of the pair OOO-PPP, though very incomplete, occurred at a proportion of one part water, and that the separation of this pair was not improved by raising the proportion of water to two parts.

As in the analysis of the composition of the mobile phase carried out for the ODS columns, the effect of variations in the proportion either of THF or water in the composition of the mobile phase in the OS column predominated in all cases over the effect of temperature.

On the basis of the results set out above, a mobile phase of ACN/THF/H_2O (60/40/1, v/v) would seem most appropiate for analyzing mixtures of triglycerides with a PN greater than 24, provided, as pointed out in the considerations made concerning the bonded phase, the use of OS phases is at all times directed at analyzing long-chain triglycerides with a low level unsaturation, which corresponds to partition numbers higher than 48. In any event, studies using mobile phases made from these same components would seem to be in order, but with a higher proportion of THF, which appears to determine the α for separations of triglycerides with the same PN (Figure 6), as well as with a higher proportion of water, in order to prevent the sharp drop in RT brought about by proportions of THF above 40% (Figure 5).

Column temperature

Figure 9 shows the changes in log K′/T. Retention times decreased with T, and the linear variation in log K′/T differed for each TG, such that as the PN increased, the slope of log K′/T lines also increases. This was because at higher temperatures TG solubility in the mobile phase was greater, and this effect was more pronounced for the least soluble triglycerides, i.e., long-chain triglycerides with a low level of unsaturation,

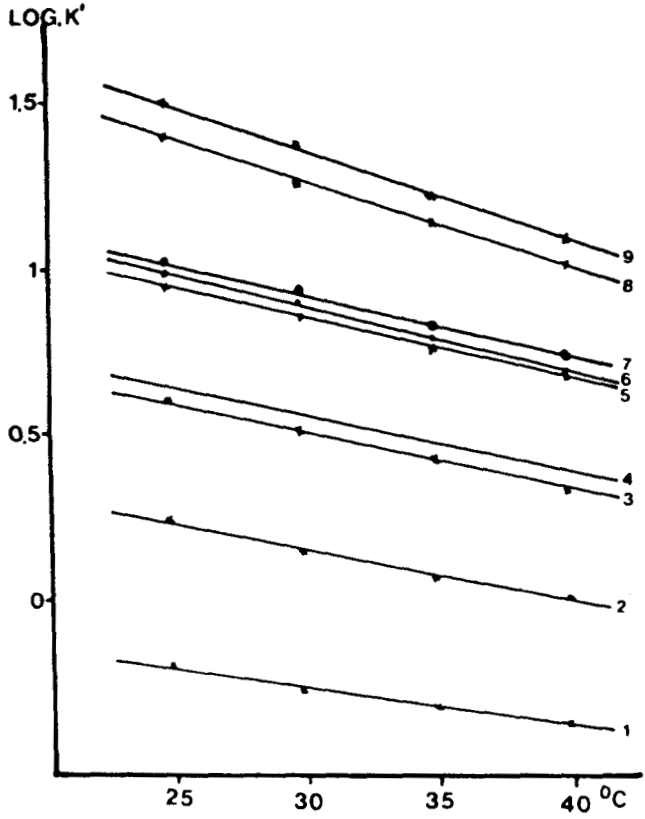

Figure 9: Changes in log K'/T in the NOVAPAK ODS column with an ACN/ACE/THF (58 / 38 /4) mobile phase and F = 1.5 mL /min. Line numbers as in Figure 3.

corresponding to those with higher partition numbers. At a 40°C, the elution time for SSS was acceptable (about 23.5 minutes), impossible to achieve at lower temperatures.

On the other hand, increases in T resulted in lowered selectivity, although separation of pairs with the same PN (PN = 36, 42 and 48,) was relatively unaffected by changes in T (Table 5). In all cases the slope of log α/T lines was less than 0.003

Table 5: Slope (m) and y-intercept (a) values, coefficent of correlation (r) and standard error (S_r) for log α/T lines for TG pairs with the same PN (PN = 36, 42 and 48) in the NOVAPAK ODS column with an ACN/ACE/THF (58/38/4) mobile phase and F = 1.5 mL/min. Abbreviations for α for TG pairs with the same PN as in Table 2.

log α/T	PN	a	m	r	S_r
α_{LnLnLn}	36	-0.08	0.001	0.673	0.523
α_{LLL}	42	-0.03	0.000	0.258	0.683
α_{PoPoPo}	42	-0.11	0.002	0.924	0.270
α_{OOO}	48	-0.17	0.003	0.983	0.131

and the correlation coefficient was not statistically significant for p minor than 0.05, except for the pair OOO-PPP (Table 5).

Similar results were obtained when working with the ION PAIR ODS and OS columns with selected mobile phases for each, as well as for the rest of the mobile phases tested for each of the thrfâ column types.

A T of 30°C seemed to be best suited for TG analysis using the systems described above, because it resulted in moderate decreases in RT, K′ and α, and did not bring about any significant reduction in the resolution of TG pairs with the same PN, while ensuring a good level of TG solubility in the mobile phase, particularly for higher partition numbers. Although, when working with the OS column, a T of 25°C yielded better separation of triglycerides than did other temperatures, precipitation of triglycerides with partition numbers higher than 54 (SSS, AAA and BBB) from the mobile phase occurred.

Flow rate

As the flow rate (F) increased, retention times for the triglycerides decreased and the column slowly lost its efficiency, as shown in Table 6 by the number of theoretical plates (N) for each TG at the various flow rates.

The results obtained demonstrated that it was possible to use relatively high flow rates with minimal loss of column efficiency. For example, for the chromatographic system used and for the flow rates studied, the flow rate of 2.2 mL/min was the most useful as it required the least analysis time, yet still provided adequate peak separation, with a working pressure of 1500 PSI.

Table 6: Number of theoretical plates (N) in the SPHERISORB ODS-2 column with an ACN/ACE/THF (38/46/16) mobile phase and T = 30°C for each flow rate (mL/min) studied. $\bar{X}$ = mean; CV = coeff. of variation (%). TG abbreviations as in Table 1.

TG	0.5		1.0		1.7		2.2	
	$\bar{X}$	CV	$\bar{X}$	CV	$\bar{X}$	CV	$\bar{X}$	CV
LnLnLn	3647	5.0	2677	6.0	2552	4.0	2576	2.7
LaLaLa	5550	4.1	4361	7.0	3706	6.4	3305	10.4
LLL	5916	2.3	4405	5.0	3418	6.4	3331	3.6
MMM	5781	1.3	4627	2.0	4420	4.5	3462	5.9
OOO	6236	3.4	5152	5.0	4721	3.2	3814	1.8
PPP	7164	1.9	5676	6.0	4590	5.9	4101	5.1

ACKNOWLEGEMENTS

The authors express their gratitude to D. Durant de Marevil and J. Koumoutsos for their help on this work.

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