Journal of Chromatography, 452 (1988) 157–173
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 20 624

COMPARISON OF RETENTION MECHANISMS OF HOMOLOGOUS SERIES AND TRIGLYCERIDES IN NON-AQUEOUS REVERSED-PHASE LIQUID CHROMATOGRAPHY

MICHEL MARTIN*,*, GÉRALDINE THEVENON and ALAIN TCHAPLA
Ecole Polytechnique, Laboratoire de Chimie Analytique Physique, 91128 Palaiseau (France)

SUMMARY

The retention behaviour of saturated homogeneous triglycerides was compared to that of single chain homologous compounds in reversed-phase liquid chromatography on silica-based octadecyl bonded phases with non-aqueous binary eluents containing chloroform. For both series, in all eluents and at all investigated temperatures, a discontinuity (1–6% slope change) in log k' *vs.* the carbon atom number, n_c, of each solute hydrocarbonaceous chain was observed for a given critical number of carbon atoms, $n_{c,crit} \approx$ 12–13. Similar discontinuities were observed in ΔH° and ΔS° *vs.* n_c for the same $n_{c,crit}$ value. These and other phenomena (existence of two convergence points for log k' *vs.* n_c plotted at different eluent compositions; existence of two convergence temperatures in log k' *vs.* $1/T$ plotted for several members of a given solute series) reflect the mechanism of penetration of the solute aliphatic chains into the bonded layer. The eluotropic strength of acetonitrile–chloroform mobile phases was determined and seen to have a larger rate of variation with the solvent composition than that of some other non-aqueous binary eluents. The ratio of the slope of plots of log k' *vs.* n_c curves for triglycerides and single chain homologous series is not a whole number but has a value lying between 2 and 3. This explains why triglyceride retentions cannot be predicted from retention data for the fatty acid methyl esters. More importantly, this indicates that the triglycerides may interact with the stationary phase using various conformations, one, two or three chains penetrating into the bonded layer. These conformations, in dynamic equilibrium with each other, contribute differently to the retention. This offers, in principle, the possibility to separate unsaturated triglycerides having the same number of carbon atoms and of double bonds but differing in the distribution of the unsaturations along the chains, if the double bonds located in a non-penetrating chain can selectively interact with a mobile phase component.

INTRODUCTION

The liquid chromatographic analysis of triglycerides has been the subject of many studies which have recently been reviewed[1]. The analysis of these substances

* Present address: École Supérieure de Physique et de Chimie Industrielles, Laboratoire d'Hydrodynamique et Mécanique Physique, 10 rue Vauquelin, 75231 Paris Cedex 05, France.

0021-9673/88/$03.50 © 1988 Elsevier Science Publishers B.V.

has been a challenge to chromatographers for several reasons: the great complexity of natural triglyceride samples (vegetable or animal oils, biological samples); the absence of UV chromophores in the molecular structure of the compounds; their important hydrophobicity and, consequently, their poor solubility in aqueous solvents. The sample complexity problem is classically solved by using high efficiency columns under programmed elution conditions. In liquid chromatography, this is usually done by increasing the solvent strength of the mobile phase during the migration of the compounds through the column (gradient elution), although the relatively large temperature dependence of the triglyceride retention has made temperature programming (temperature increase) an alternative possibility[2]. Using an evaporative light scattering photometer, it has become possible to detect the triglycerides with a relatively good sensitivity and, moreover, to perform gradient elution analysis with a quite stable baseline[3–5]. Because the triglycerides all have the same polar group but differ in their apolar moieties, the separation selectivity is much larger under reversed-phase conditions than under normal-phase ones and most authors have used *n*-octadecyl bonded silica as the stationary phase. However, the insolubility of these compounds in hydroorganic solvents makes it impossible to use, as is very frequently done in reversed-phase liquid chromatography, water as a component of the mobile phase. Moreover, due to the high hydrophobicity of these solutes, it is necessary to use rather strong solvents to maintain reasonable retention. These are the reasons why the method of non-aqueous reversed-phase (NARP) liquid chromatography is generally selected for the analysis of triglycerides[6]. Although the development of the analytical instrumentation in the past few years has made feasible the liquid chromatographic analysis of this very important class of compounds, there are still problems to be solved regarding the choice of the best separation conditions and the prediction of the retention of specific triglycerides, which are usually not available as standards, in order to identify the numerous peaks observed in the chromatograms of samples of natural origin.

All previous work in this area has focused on relationships between the retention of a triglyceride and the number of carbon atoms as well as the number of double bonds in its chains. However, this work only permits the understanding of primary effects, and a fine tuning of the prediction of retention is not yet possible in many instances. Besides, some separation problems are still unsolved. For example, it has frequently been found very difficult, if not impossible, to separate triglycerides containing the same number of carbon atoms and the same total number of unsaturations, differing in the distribution of these unsaturations within the three chains. It is not possible to predict the retention of a triglyceride as a function of the retention of the three fatty acid methyl esters corresponding to the structures of each of its three chains. Indeed, it has been shown that the logarithm of the capacity factor of a mixed triglyceride must be calculated from the retention of the three homogeneous ones rather than from the retention of the three corresponding esters. So, for a mixed triglyceride ABC, where A, B, C represent three different esters, the logarithm of the capacity factor may be expressed as[7]:

$$\log k'_{ABC} = 1/3 \log k'_{AAA} + 1/3 \log k'_{BBB} + 1/3 \log k'_{CCC} \quad (1)$$

One of the purposes of the present study is to examine the causes of these phenomena, by investigation of the retention mechanism of the triglycerides on *n*-octadecyl bonded phases in non-aqueous eluents. This was done by comparing the selectivity obtained by addition of a methylene group in the triglycerides and in homologous series of single chain (*n*-alkyl) compounds under various conditions. The choice of single compounds as model reference compounds is based on the fact that a triglyceride can be considered as the sum of three linear chains (alkyl chains) attached to a head group (glyceryl group). Moreover, the retention mechanism of such linear solutes on alkyl bonded phases is relatively well documented. It has been shown that, in water–methanol and methanol–tetrahydrofuran eluents, these solutes penetrate into the layer of ligands of monomeric alkyl bonded phases[8]. As the elution of triglycerides requires binary eluents containing such solvents as chloroform or acetone, which are not classically used in reversed-phase liquid chromatography, it is first necessary to study, for later comparison purposes, the retention mechanism of linear solutes in such binary non-aqueous mobile phases. This comprises the first part of the study reported. The eluotropic strength of solvent mixtures used for the analysis of triglycerides has also been determined. The retention mechanism of the triglycerides under the same chromatographic conditions is then discussed by a comparison of the methylene selectivities of the triglycerides with those of an appropriate linear homologous series.

EXPERIMENTAL

Reagents

Methanol, acetonitrile and chloroform were HPLC grade (Carlo Erba, Milan, Italy). All solvents were filtered through a Millipore GF/C 1.2-μm filter. The solutions were then degassed by sonication.

Alkanes, fatty acid methyl esters, phenylalkanes, chloroalkanes and *n*-alkyl benzoates were from different sources. Triglycerides and diglycerides were from Interchim (Montluçon, France).

Equipment

The liquid chromatographic system comprised a Model 112 pump (Beckman, San Ramon, CA, U.S.A.), a Model 7125 injection valve with a 20-μl loop (Rheodyne, Cotati, CA, U.S.A.) and a Model R 401 refractive index detector (Waters, Milford, MA, U.S.A.). Two columns were used. The Ultrasphere 5-μm ODS column, 15 cm $\times$ 4.6 mm, was a gift from Beckman, and a μBondapak 10-μm C_{18} column, 30 cm $\times$ 3.9 mm, was obtained from Waters. For all experiments, the temperature of the precolumn, of the injection valve and of the column was controlled using a laboratory made water jacket by means of a Model HS 40 thermostat (Huber, Offenburg-Elgersweier, F.R.G.) with a precision of 0.1°C. The eluent flow-rate was 0.8 ml/min.

Methods

Samples were always dissolved in the solvent or solvent mixture used as the mobile phase in order to avoid the precipitation of the solutes on the column, a change in the solute peak shape[9] or the modification of the retention time due to the presence of an excess of strong solvent in the injected sample[10].

All retention values are reported in terms of the capacity factor, k'. The calculation of this factor requires the measurement of the void volume of the column (column hold-up volume). It has been shown that, in order to determine this volume, a convention must be adopted regarding the adsorption equilibrium[11] and that the various possible conventions lead to void volumes which may differ by as much as 20%[12]. In the present study, the recommended[11] weighing method[13] has been used because it provides a value which does not depend on the mobile phase composition and is linked to the total column porosity. It was shown that it closely corresponds to the convention that no liquid phase component is adsorbed in terms of volume[12]. In order to minimize the possible error in the capacity factor and although we are in the following concerned only with relative, not absolute, k' values, only capacity factors greater than 0.4 were considered. Each value of k' is the result of at least three reproducible injections.

RESULTS AND DISCUSSION

Retention mechanism of single chain compounds

Martin's equation[14] predicts a linear relationship between the free enthalpy of transfer of a single chain compound from the mobile phase to the stationary phase and the number of carbon atoms of the chain. As this free enthalpy is linearly related to the logarithm of the capacity factor, a linear relationship is expected between this logarithm and the number, n_c, of carbon atoms of the solute. Such a linearity has often been observed[15,16] and has even served as a basis for the determination of the hold-up volume[17]. However, a slight discontinuity in the plot of log k' *vs.* n_c has recently been observed when working with monomeric alkyl bonded phases[8,18]. Because this effect is small and plotting the retention in logarithmic coordinates tends to level off small variations, the relationship between the retention and chain length of a solute is more appropriately investigated in terms of the quadratic methylene selectivity, α, defined as

$$\alpha(n_c) = (k'_{n+1}/k'_{n-1})^{1/2} \qquad (2)$$

where k'_{n+1} and k'_{n-1} represent the capacity factors of homologous single chain compounds with $n + 1$ and $n - 1$ carbon atoms, respectively. This expression has the advantage of revealing details that do not appear on a logarithmic scale. Furthermore, the calculation of a ratio minimizes the effects of experimental errors. The plot of α *vs.* n_c is nevertheless closely related to that of log k' *vs.* n_c. A linear variation of log k' should correspond to a constant α value when n_c is changing. In fact, the plot of α *vs.* n_c reveals a discontinuity, dependent on the chain length of the ligand and corresponding to the point where a break appears in the plot of log k' *vs.* n_c. This phenomenon, observed with numerous homologous series and with various ligand chain lengths, has been attributed to the penetration of the solutes into the bonded ligands. Evidence in support of this mechanism is provided by the fact that the break in the plot of log k' *vs.* n_c is not observed for methyl bonded phases as well as for bonded phases synthesized using a trifunctional alkyl silane leading to so-called polymeric bonded phases. In the first case the methyl chain is obviously too short for the solute to penetrate into the ligand layer, while in the second case the sup-

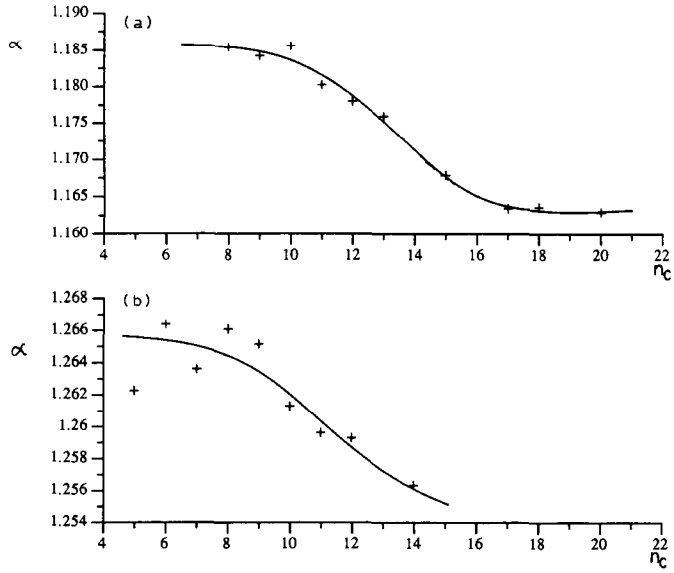

Fig. 1. Quadratic selectivity, α, *vs.* the number of carbon atoms for different homologous series on an Ultrasphere ODS column. Temperature: 20°C. (a) Solutes, *n*-alkanes; mobile phase, acetonitrile–chloroform (70:30, v/v). (b) Solutes, phenylalkanes; mobile phase, acetonitrile–chloroform (90:10, v/v).

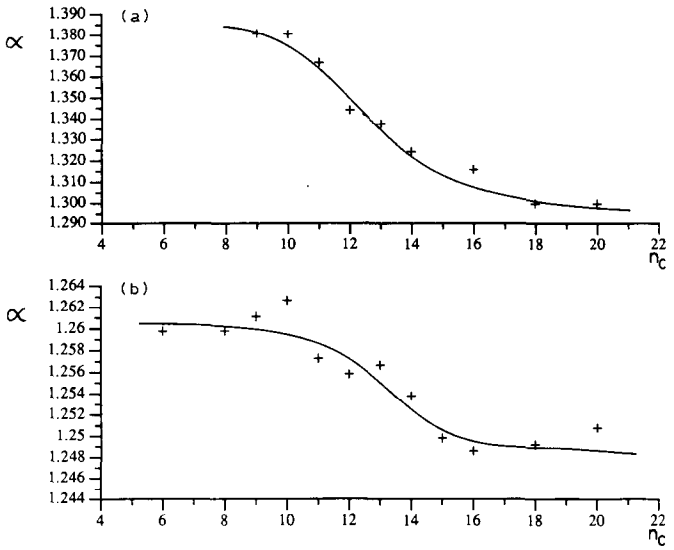

Fig. 2. Quadratic selectivity, α, *vs.* the number of carbon atoms for different homologous series on an Ultrasphere ODS column. (a) Solutes, fatty acid methyl esters; mobile phase, acetonitrile; temperature, 17°C. (b) Solutes, alkyl benzoates; mobile phase, acetonitrile–chloroform (90:10, v/v); temperature, 20°C.

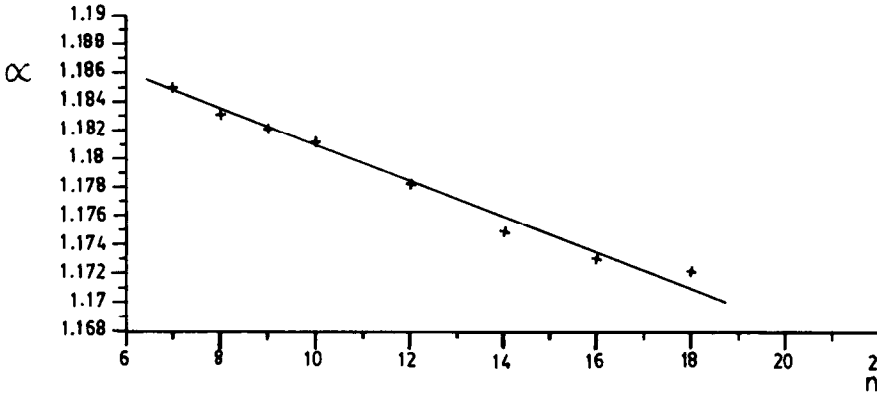

Fig. 3. Quadratic selectivity, α, *vs.* the number of carbon atoms for the series of chloroalkanes on a μBondapak column with methanol as the mobile phase, at 20°C.

posedly more erratic structure of the polymeric phases either does not allow the solute insertion or allows it to various extents in such a way that there is no reason to observe a break in α *vs.* n_c at any particular n_c value.

We have investigated the retention behaviour of different homologous series in binary non-aqueous mobile phases typically used for the analysis of triglyceride mixtures. Figs. 1 and 2 show some examples of plots of α *vs.* n_c for different homologous series, with different eluents and temperatures, for the Ultrasphere column. Fig. 3 shows the same type of plot for the μBondapak column. The curves in Figs. 1 and 2 for the monomeric Ultrasphere phase are quite similar. The methylene quadratic selectivity is roughly constant for small solute carbon numbers, then a discontinuity (decrease) occurs before the selectivity again becomes approximately constant as n_c increases. In contrast, the curves for μBondapak do not exhibit any discontinuity but regularly decrease with increasing n_c. The results presented in Figs. 1–3 and similar results obtained with different homologous series and different non-aqueous mobile phases appear to be in close agreement with those reported previously principally for aqueous mobile phases[8]. They are interpreted in terms of a mechanism of penetration of the solute alkyl chain into the octadecyl bonded phase. Under the non-aqueous eluent conditions used here or in the low water content of the hydroorganic eluents previously used, the ligands of the monomeric stationary phase can be reasonably pictured as extending with a relatively high mobility into the mobile phase as confirmed by NMR conformational studies[19,20]. Because of the solvophobic effect[21], the alkyl chains of the solutes tend to penetrate into the bonded layer. This penetration can occur relatively freely up to some solute carbon number, the alkyl chain mainly adopting a regular, commonly called all-*trans* or zigzag conformation which favours a close dispersion interaction with the bonded phase. Above this critical carbon number, which was shown to be correlated with the bonded chain length[8], the solute chain must slightly change its conformation in order fully to penetrate into the bonded layer and provide optimum interaction with it. This is supposed to result in a looser contact with the bonded phase, reflected by a slight decrease in the interaction energy associated with the addition of a methylene group to the solute and, consequently, in the quadratic methylene selectivity.

The magnitude of this decrease depends on the experimental conditions and from Figs. 1 and 2 is seen to vary from 1 to 6%. In spite of the relatively small value of this variation, the discontinuity in α is clear and unambiguous in Figs. 1 and 2. A detailed statistical study of the error associated with the α determination has been carried out for experimental conditions very similar to the present ones[22]. It shows that the relative error (standard deviation) for the magnitude of the discontinuity in α is equal to 0.25%. This is several times smaller than the magnitude of the discontinuity and indicates clearly that the latter cannot be accounted for by experimental errors.

Further support to the hypothesis of a penetration mechanism is the fact that the discontinuity in α appears in the same range of n_c values, for monomeric octadecyl bonded phase, whatever the nature of the homologous series and the mobile phase composition. This range lies between about 10 and 14 with an average at about 12. This confirms previous observations that this critical carbon number increases less rapidly than the chain length of the alkyl bonded phase[8]. This possibly reveals that long ligand alkyl chains, such as octadecyl chains, do not adopt the all-*trans* conformation (straight chain) but show a gauche conformation so that they are bent to some extent, as observed in Fourier transform infrared spectroscopic studies[23]. It should be noted that we do not observe in Figs. 1 and 2 a different behaviour of the phenylalkanes and benzoates from the other series. The critical carbon number is the same for all series, in contrast with a previous report[8]. A detailed study of the particular behaviour of the phenylalkanes and benzoates will be published elsewhere.

Fig. 3 confirms the previous results about the μBondapak phase, for which there is no discontinuity in the plots of α *vs.* n_c, but a regular decrease of the methylene selectivity. Similar curves have been obtained with acetonitrile–chloroform eluents of various compositions. Clearly this bonded phase is not a monomeric one.

Eluotropic strength in non-aqueous reversed-phase liquid chromatography

The average methylene selectivity value varies from one curve to another in Figs. 1 and 2. We have observed that, for a given mobile phase composition, this average value does not depend on the homologous series selected, at least to a first approximation. This is clearly seen in Figs. 1b and 2b plotted at the same temperature and with the same mobile phase composition for two different series (phenylalkanes and alkylbenzoates). The range of α values scanned is nearly the same for these two series. However, the methylene selectivity does significantly depend on the mobile phase composition. As it is a monotonous function of the eluting power of the mobile phase, the average α value gives an indication of this solvent eluting power. In Fig. 4a, the logarithm of the average methylene selectivity, obtained from the slope of a least mean square linear regresssion of log k' *vs.* n_c for *n*-alkanes (from *n*-octane to *n*-docosane), is plotted as a function of the volume fraction of chloroform in acetonitrile. (The variation of the methylene selectivity associated with the penetration mechanism is nearly negligible compared to the influence of the mobile phase composition on this selectivity. Therefore we have used data for all members of the homologous series for solvent strength determinations without taking into account the effect of the penetration mechanism.) The continuous decrease in log α with increasing volume fraction of chloroform clearly indicates, as expected, that chloroform is a stronger eluent than acetonitrile on octadecyl bonded phases.

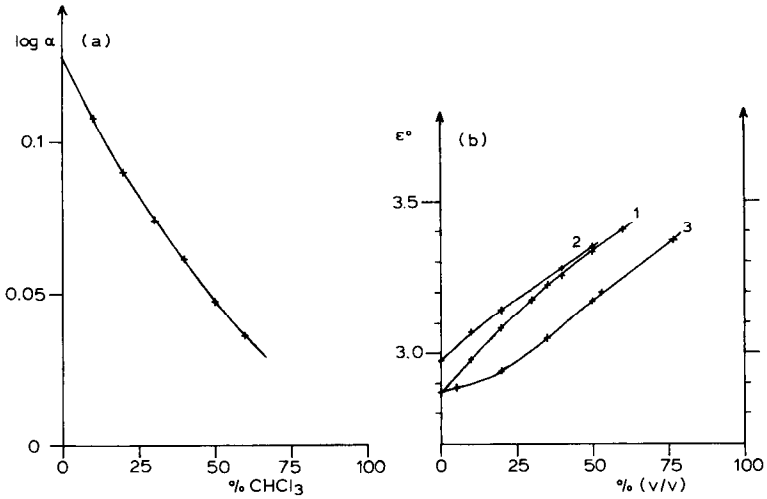

Fig. 4. (a) Logarithm of the average methylene selectivity (slope of log k' *vs.* n_c) *vs.* the volume fraction of chloroform in acetonitrile on the Ultrasphere ODS column. (b) Eluotropic strength of the acetonitrile–chloroform mixture *vs.* the volume fraction of chloroform (curve 1). For comparison, previously reported values[25] of the eluotropic strength of methanol–tetrahydrofuran and acetonitrile–ethyl acetate mixtures are also plotted *vs.* the volume fractions of tetrahydrofuran (curve 2) and ethyl acetate (curve 3), respectively.

Furthermore, as the solvophobic effect is the main retention mechanism in reversed-phase liquid chromatography[21], the average α value provides a very useful means to determine the eluotropic strength of a mobile phase. Following the reasoning of Snyder[24] for the definition of the eluotropic strength, ε°, in normal-phase liquid chromatography, the ε° value in reversed-phase liquid chromatography has been shown to be easily derived from log α

$$\varepsilon^\circ = (\log \alpha_{H_2O} - \log \alpha)/v_0 \tag{3}$$

where α_{H_2O} is the methylene selectivity with pure water as the eluent and v_0 is the volume of the methylene group, which was estimated to be 0.168 in units of 100 ml[25]. In this equation, the methylene group is used for measuring the change in retention when changing the eluent, and water serves as the reference solvent for which ε° is by definition equal to zero. Although not experimentally determined using the *n*-alkane series in the present study, the log α value in pure acetonitrile can be estimated by extrapolation of the curve in Fig. 4 with a relatively good precision. It is found to be equal to 0.126. It is remarkable that this extrapolation gives an α value equal to 1.34 which is very close to the average value obtained in pure acetonitrile for the fatty acid methyl ester series, as seen in Fig. 2a. Again this reflects the fact that the methylene selectivity does not depend on the series investigated. The corresponding log α value is somewhat larger than the value (log α = 0.1101) previously measured by Colin *et al.*[25] on a different octadecyl bonded phase column. They determined the ε° value of acetonitrile to be 2.87. Using this value together with our extrapolated value of log α, one finds that log α_{H_2O} in our system is equal to 0.608 [log α_{H_2O} = log α(acetonitrile) + $v_0\varepsilon^\circ$(acetonitrile)], which is remarkably close to the two previously

determined values of 0.592[25] and, especially, 0.60[26]. These small differences probably reflect the slightly different properties of different brands of octadecyl bonded phases. The variations of the eluotropic strength of the acetonitrile–chloroform system are plotted as a function of the mobile phase composition in Fig. 4b. For comparison, the curves previously determined in the same ε° range for the acetonitrile–ethyl acetate and methanol–tetrahydrofuran binary mixtures[25] are also plotted in this Figure. To be rigorously comparable, all curves should be plotted at the same temperature. In fact, the previous curves were obtained at 25°C while our work was performed at 17°C. Although we have observed, for acetonitrile–chloroform (60:40, v/v), a relative log α variation of -0.5% per °C, the temperature effect on ε° should be sufficiently small for the curves in Fig. 4b to be correctly compared because the temperature variations of log α_{H_2O} and $-\log \alpha$ most likely compensate each other.

Curves like those of Fig. 4b are interesting as they provide a basis for the optimization of the mobile phase composition, when one attempts to separate compounds differing by their apolar moieties, such as triglycerides, in non-aqueous reversed-phase liquid chromatography. Indeed, the weakest eluents found in these systems are acetonitrile and methanol. It appears that, among the three solvent mixtures compared in Fig. 4b, the acetonitrile–chloroform system gives the largest rate of increase of ε° with the volume fraction of the strong solvent when this fraction is lower than 50%. It is interesting to compare this system with the acetonitrile–acetone mixture as this system has been used relatively frequently for the analysis of triglyceride samples[1]. Although the eluotropic strength of acetonitrile–acetone mixtures has not been reported, it should lie between 2.87 and 3.19, which are the ε° values of pure acetonitrile and pure acetone, respectively[25]. Therefore, according to Fig. 4b, larger ε° values can be obtained by adding chloroform rather than acetone to acetonitrile. Indeed, acetonitrile–chloroform (70:30, v/v) has about the same eluting power as pure acetone. Therefore, when performing a gradient elution analysis, one should be able to scan a wider range of triglyceride chain lengths using chloroform rather than acetone as the strong solvent. Alternatively, curves like those of Fig. 4b can be used to select two isoeluotropic solvent mixtures for the optimization of the composition of ternary mobile phase systems.

Retention mechanism of triglycerides

Evidence of the penetration of the triglycerides. If it is easy to visualize the penetration of a single chain solute into the layer of ligands of a bonded phase, the conformational structure of more complex molecules, such as triglycerides, in interaction with a bonded phase is not so trivial. In order to get some insight into this conformation and better to understand the retention behaviour of the triglycerides in non-aqueous mobile phases, we have investigated the retention of saturated homogeneous triglycerides on the monomeric Ultrasphere bonded phase. The results are reported on Fig. 5 for different temperatures and in Fig. 6 for different mobile phases in terms of log k' *vs.* the number of carbon atoms (including the carbon of the ester bond) of each chain of the triglyceride molecules. A discontinuity in the slope of these curves is clearly visible for a number of carbon atoms equal to 13 in each chain. Such a break in the curves is equivalent to discontinuity in the corresponding plots of α *vs.* n_c. As in the above study of homologous series, it can be interpreted as reflecting a penetration mechanism of the triglyceride molecules into the bonded

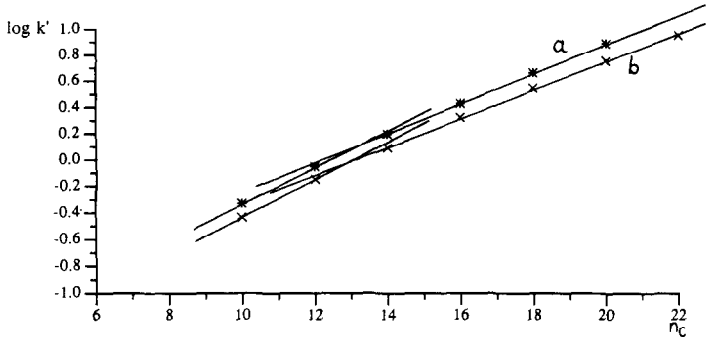

Fig. 5. Plots of log k' *vs.* the number of carbon atoms for homogeneous saturated triglycerides on the Ultrasphere ODS column. Mobile phase: acetonitrile–chloroform (60:40, v/v). Temperatures: (a) 35°C; (b) 40°C.

layer. Furthermore, the break appears for the same number of carbon atoms as for the homologous series. This reveals that the triglycerides do not penetrate into the ligand layer using their glyceryl head. Indeed, in this case, the break should have appeared for a lower n_c value due to the steric hindrance (equivalent to about two carbon atoms) of the glyceryl moiety.

By plotting curves like those of Fig. 6 for seven different compositions of acetonitrile–chloroform mobile phases at a given temperature (17°C), it appears that the curves all converge to a given point, as previously observed for single chain homologous series in aqueous mobile phases[27]. However, because of the slight discontinuity in the plots of log k' *vs* n_c, separate extrapolation of the straight lines before and after the break point at the critical number of carbon atoms, reveals not one, but two convergence points; the coordinates of these points are: log $k' = -1.60$ and $n_c = 0.6$, log $k' = -1.50$ and $n_c = -0.4$, for the lines extrapolated before the break and after the break, respectively. Such a behaviour for the triglycerides is entirely similar to the behaviour of single chain homologous series[8].

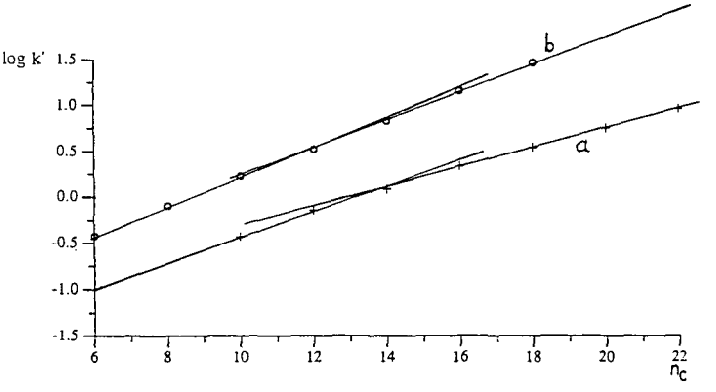

Fig. 6. Plots of log k' *vs.* the number of carbon atoms for homogeneous saturated triglycerides on the Ultrasphere ODS column at 20°C. Mobile phases: (a) acetonitrile–chloroform (50:50, v/v); (b) methanol–chloroform (75:25, v/v).

From the variations of the retention with the temperature, one can obtain access to the thermodynamic variables associated with the transfer of a solute from the mobile phase to the stationary phase. The capacity factor of a soluble is related to the standard Gibbs free energy of transfer, $\Delta G°$

$$\log k' = C - \Delta G°/2.3\ RT \tag{4}$$

where C is a constant taking account of the amounts of mobile and stationary phases. The standard enthalpy of transfer, $\Delta H°$, can be determined from the slope of the linear plot of $\log k'$ *vs.* $1/T$.

$$\log k' = C - (\Delta H°/2.3\ RT) + (\Delta S°/2.3\ R) \tag{5}$$

where $\Delta S°$ represents the entropy of transfer of the solute. Plots of $\log k'$ *vs.* $1/T$ have been made for the alkane and triglyceride series at eight different temperatures ranging from 17 to 55°C, *i.e.*, at temperatures above that corresponding to the end of the phase transition noted for octadecyl bonded phases[18], for acetonitrile–chloroform (60:40, v/v) as the mobile phase. These plots are effectively linear (correlation coefficient of the least mean square linear regression larger than 0.999 for all solutes, but the least retained for which this coefficient is 0.996). By extrapolating these lines to low $1/T$ values, two different intersection points or, more precisely, two different intersection domains are found. The curves for solutes with numbers of carbon atoms lower than the critical carbon atom number, $n_{c,crit}$, converge to a temperature which is about 100 K larger than the convergence temperature of curves for n_c larger than $n_{c,crit}$. These convergence temperatures (490 and 590 K) are approximately the same for the *n*-alkane and triglyceride series. Again, such a behaviour is quite similar to that observed for the *n*-alkane series in methanol–water or pure methanol eluents[22]. It was shown[22] that the meaning of the convergence temperature for homologous series is identical to the concept of compensation temperature associated with the enthalpy–entropy compensation[28]. It is interesting that the invariance of the two convergence temperatures observed under largely different operating conditions (aqueous or non-aqueous mobile phases, simple or more complex solute series) indicates that the interaction mechanism of the solutes with the stationary phase is the same for all conditions examined. The existence of a penetration mechanism of the solute apolar chains into the bonded layer is reinforced by the fact that the plots of $\Delta H°$ *vs.* n_c and $(\Delta S° + 2.3\ RC)$ *vs.* n_c are not linear over all the n_c but present a discontinuity for a n_c value close to $n_{c,crit}$, again as was observed for simple series in aqueous mobile phases[22].

Further insight into the penetration mechanism can be gained from the comparison of the slopes of the plots of $\log k'$ *vs.* n_c for the triglycerides with those of single chain homologous series. Although such a comparison should naturally be done with the series of fatty acid methyl esters, which are structurally similar, the retention values of these compounds in the eluents used for triglyceride analysis are too small to provide an acceptable precision. However, as we noted above that the α values do not depend, to a first approximation, on the series selected, we used, for this purpose, the series of *n*-alkanes which, being less polar than the esters, have a greater retention.

Procedure for determination of the number of penetrating chains. According to eqn. 4, the slope of log k' *vs.* n_c, which was seen to be equal to log α, is directly proportional to the difference in the ΔG° values of two consecutive members of the series investigated, *i.e.*, for an homologous series, to the free enthalpy of transfer, $\Delta G^\circ_{CH_2}$, associated with the addition of a methylene group to a given compound. The following expression is therefore obtained:

$$\log \alpha = -\Delta G^\circ_{CH_2}/2.3\ RT \tag{6}$$

For triglycerides, the slope of log k' *vs.* n_c corresponds to the decrease in the free enthalpy, $(\Delta G^\circ_{CH_2})_{tri}$, associated with the addition of one methylene group in each chain of the molecule. Interesting information from the comparison of the slopes can be gained by calculating either the ratio or the difference of the log α values. The former, R_α, reflects the ratio of the $\Delta G^\circ_{CH_2}$ values for the triglycerides and for the *n*-alkanes

$$R_\alpha = (\log \alpha)_{tri}/(\log \alpha)_{alk} = (\Delta G^\circ_{CH_2})_{tri}/(\Delta G^\circ_{CH_2})_{alk} \tag{7}$$

where the suffixes tri and alk refer to the homogeneous saturated triglyceride series and *n*-alkane series, respectively, while the latter (logarithm of the α ratio) measures their difference. The relevant comparison basis which is to be selected depends on the objective pursued. Our purpose, in the present study, is to get some information on the interaction of the triglyceride chains with the bonded layer and, specifically, to evaluate the number of chains which penetrate into the layer. This can be done by calculating the ratio R_α.

Indeed, in reversed-phase liquid chromatography, it is accepted that the solvophobic effect is the dominating mechanism for retention, especially when considering the retention change associated with the addition of methylene groups to the solute molecules. The retention theory based on this effect predicts that the free enthalpy of transfer of a solute is proportional to the reduction in the overall molecular surface area of species (solute and ligand) immersed in the eluent resulting from the association of the solute with a ligand[21]. Accordingly, the change in log k' of consecutive members of the triglyceride series, $(\log \alpha)_{tri}$, is directly related to the variation of the contact areas between the solutes with the ligands when adding a methylene group to each chain. The variation of contact area due to the interaction of one methylene group with the ligand is measured by $(\log \alpha)_{alk}$. Therefore, the ratio R_α provides a scale for measuring this variation for the triglyceride series in units of the contact area of a methylene group. It should be noted that such a scaling procedure can be applied to any series of compounds differing in the number of structural units of a given kind *e.g.*, aromatic groups. However, in the specific case of molecular compounds with multiple chains, such as the triglycerides, R_α directly measures the number of methylene groups interacting with the stationary phase when one methylene is added to each chain and, consequently, the number of chains penetrating into the bonded layer.

Number of penetrating chains for the triglycerides. Because we have seen above that, for both the *n*-alkane and triglyceride series, the value of log α depends slightly on the number of carbon atoms penetrating into the bonded layer, the comparison

TABLE I

RATIO OF THE LOG α VALUES OF THE TRIGLYCERIDES AND n-ALKANES

Column: Ultrasphere ODS. A = acetonitrile; B = methanol; C = chloroform.

Mobile phase	*T (°C)*	*log α n-alkanes*	*Correlation coefficient*	*log α triglycerides*	*Correlation coefficient*	R_α
A–C (60:40, v/v)	20	0.065	0.9999	0.152	0.9999	2.34
	25	0.061	0.9998	0.148	0.9997	2.42
	35	0.058	0.9996	0.139	0.9995	2.39
	45	0.057	0.9987	0.136	0.9985	2.39
A–C (70:30, v/v)	20	0.077	0.9998	0.182	1	2.35
B–C (90:10, v/v)	20	0.086	0.9999	0.206	0.9999	2.41
A–C (70–30, v/v)	17	0.081	0.9998	0.194	0.9999	2.39
A–C (55–45, v/v)	17	0.061	0.9997	0.144	0.9994	2.37
A–C (50–50, v/v)	17	0.058	0.9991	0.135	0.9995	2.34

of log α must be done either before or after the appearance of the break in log k' *vs.* n_c (or, which is equivalent, of the discontinuity in α *vs.* n_c) using the same range of carbon numbers. The results of such comparisons are shown in Table I for different temperatures and mobile phase compositions. The log α values reported in this table correspond to the slopes of the least mean square linear regressions of the log k' *vs.* n_c data for alkanes and triglycerides with 10, 12 and 14 carbon atoms in the aliphatic chains. Under all conditions tested, the R_α value is almost the same, close to 2.4.

This value confirms the conclusion, derived above from the position of the break in log k' *vs.* n_c, that the glyceryl moiety does not penetrate into the bonded layer. Indeed, such a penetration would have necessarily induced the penetration of the three alkyl chains and led to an R_α value equal to three.

However, it is somewhat surprising that this ratio is not a whole number. A value higher than 2 eliminates the possibility of penetration of only one or two chains of the triglycerides into the bonded ligands. Therefore, this suggests that the penetration of the three chains must be considered and that at least one chain can undergo only a partial penetration. However, because of the way R_α is defined, this cannot be due to the fact that a length of a chain cannot penetrate entirely into the bonded layer but, instead, reveals that there is a dynamic conformational equilibrium such that either one, two or three chains penetrate into the stationary phase. The value of R_α therefore reflects the statistical time average of the different conformations at the interface between the mobile and stationary phases.

The ratio R_α is a measure of a $\Delta G°$ ratio for methylene groups. It is interesting to compare the other thermodynamic variables associated with the transfer of a methylene group from the mobile phase to the stationary phase. It has been noted above that plots of $\Delta H°$ *vs.* n_c and ($\Delta S°$ + 2.3 RC) *vs.* n_c for the triglyceride series are made of two linear parts with a discontinuity for $n_c = n_{c,crit}$. There is also a discontinuity at the same $n_{c,crit}$ value for the n-alkane series in the same eluent. The slopes of these plots correspond to the enthalpy and entropy of transfer of a methylene group, $\Delta H°_{CH_2}$ and $\Delta S°_{CH_2}$, respectively. One can compare the corresponding values for tri-

glyceride and alkane series for a range of n_c values which are either lower or larger than $n_{c,crit}$. In acetonitrile–chloroform (60:40, v/v) as the mobile phase, one obtains the following results:

$$(\Delta H^\circ_{CH_2})_{tri}/(\Delta H^\circ_{CH_2})_{alk} = 2.7 \qquad \text{for } n_c \leqslant n_{c,crit}$$

$$(\Delta H^\circ_{CH_2})_{tri}/(\Delta H^\circ_{CH_2})_{alk} = 2.2 \qquad \text{for } n_c > n_{c,crit}$$

$$(\Delta S^\circ_{CH_2})_{tri}/(\Delta S^\circ_{CH_2})_{alk} = 2.2 \qquad \text{for } n_c \leqslant n_{c,crit}$$

It is noticeable that these enthalpy and entropy ratios, corresponding to the addition of a methylene group to the hydrocarbonaceous chains, are similar to R_α. The difference between these ratios and R_α, which amounts to about $\pm 10\%$, is similar to that observed for the series of *n*-alkyl *o*-phthalates in aqueous eluents[22] and is most likely due to the precision of the experimental determinations. The fact that these ratios are not whole numbers again indicates that there are several conformations, in dynamic equilibrium with each other, for the triglycerides interacting with the stationary phase.

Possible conformations of diglycerides and triglycerides at the interface with the bonded layer. Numerous studies have been carried out on the conformation of glycerol-based molecules in different states. X-ray and NMR spectroscopic studies performed on the crystalline structures of the triglycerides have shown that, in the solid state, the conformation of these molecules is such that the two extreme chains are oriented in the same direction while the central chain is oriented in the opposite direction[29,30]. Such a configuration may lead to the penetration of either one or two chains. Under liquid chromatographic conditions, however, different conformations may be encountered due to the free rotation of the bonds. It was shown that gauche conformations of the glyceryl moiety exist in aqueous solutions[31]. Furthermore, one can expect that the solvophobic effect will tend to force all aliphatic chains to interact with the ligands. This was shown in a conformational study of triglyceride films on water[32].

We have performed similar retention studies on homogeneous saturated 1,2- and 1,3-diglycerides in acetonitrile–methanol (90:10, v/v). A slight (11%) retention difference is found between isomers. The secondary alcohols (1,3-diglycerides) are less retained than the primary alcohols (1,2-diglycerides), as observed for *n*-alcohols[15]. The number of available diglycerides is not enough for a break to be detected in log k' *vs.* the number of carbon atoms in each chain of the diglyceride molecules. Nevertheless, log α values can be calculated from retention data for 1,2- and 1,3-diglycerides with 12 and 14 carbon atoms in each aliphatic chain and compared to the corresponding value (log $\alpha = 0.116$) for *n*-alkanes in the same mobile phase. The resulting R_α values obtained for the 1,2- and 1,3-diglycerides are both equal to 1.8. This value again is not a whole number and corresponds to a time average of molecular conformation in which either one or the two chains interact with the stationary phase. One can easily calculate that the configurations corresponding to the two interacting chains are four times more probable than the configurations in which only one chain is interacting. If one now assumes that this probability ratio is kept

unchanged for triglycerides, it becomes possible to solve the system of equations giving an R_α value equal to 2.4. One then finds that the probabilities, P_1, P_2 and P_3, for configurations corresponding to one, two and three interacting chains are equal to 10, 40 and 50%, respectively. Because of the uncertainty about the above assumption and, to a lesser extent, of the somewhat limited precision of the R_α determination, these probability values for the occurrence of the different conformations must be regarded with caution. Nevertheless, the relatively large value of P_3 is quite remarkable. Indeed, in the free mobile phase, the probability of finding the three aliphatic chains of a diluted triglyceride oriented in the same direction is expected to be quite low. Therefore, the relatively large value of P_3 for three chains penetrating into the bonded layer certainly reflects the strong influence, and the cooperative contribution, of the solvophobic effect on the conformation of the molecules at the interface between the two phases.

CONCLUSIONS

Several conclusions can be drawn from the results presented above.

(1) This study confirms that the previously observed mechanism of penetration of the hydrocarbonaceous solute chains into the layer of bonded ligands prevails under widely varying reversed-phase liquid chromatography conditions, for aqueous as well as non-aqueous mobile phases and for simple as well as more complex solute molecular structures, such as the triglycerides. Various indicators can be used to illustrate this mechanism (break in log k' *vs.* n_c or discontinuity in α *vs.* n_c; existence of two intersection points or domains for log k' *vs.* n_c under different mobile phase compositions; existence of two different convergence temperatures for log k' *vs.* $1/T$; invariability of these temperatures when changing the mobile phase composition; break in ΔH° and ΔS° *vs.* n_c). The positive response of all these indicators and their sensitivity to a constant $n_{c,crit}$ value considerably reinforces the support for the penetration mechanism and gives a coherent image of the retention mechanism.

(2) A procedure has been established to determine the number of penetrating chains for complex solute molecules with multiple hydrocarbonaceous chains. The concept on which this procedure is based, *i.e.*, measurement of the incremental contact area with the stationary phase for structurally related compounds in terms of the contact area of a reference structural unit (here, a methylene group), can be extended to other types of structural units (for instance, aromatic rings).

(3) The application of this procedure to triglycerides has revealed that these molecules can interact with the bonded stationary phase using various conformations in dynamic equilibrium with each other. When interacting with the ligand layer, a triglyceride may have one, two or more frequently, three chains penetrating into the layer. Of course, each of these conformations does not lead to the same energy of interaction. The overall retention of a triglyceride in the column is the result of a statistical time averaging of these various free energies. It is most likely that such conformational equilibria influencing significantly the retention exist for other types of compounds having several more or less flexible hydrocarbonaceous chains, such as, for instance, branched polymers or hydrocarbons present in petroleum fractions. The diglycerides have a similar behaviour, one of their chains or, most frequently, the two chains penetrating into the stationary phase.

(4) The eluotropic strength, ε°, of acetonitrile–chloroform mixtures in reversed-phase liquid chromatography has been determined and compared to that of other non-aqueous elements. In the present case, the use of the methylene group as the reference solute for ε° determinations is justified by the fact that triglycerides must first be separated according to their numbers of methylene groups. It is shown that the rate of variation of ε° with the volume fraction of the strongest solvent component is larger for acetonitrile–chloroform mixtures than for solvent mixtures for which ε° determinations have been carried out. Consequently, during an acetonitrile–chloroform gradient elution, one can scan a larger range of triglyceride hydrophobicities, *i.e.*, analyse triglycerides with longer chains than by carrying out a gradient elution with other mixtures. Furthermore, the comparison of the relative influences of temperature and mobile phase variations on retention indicates that an acetonitrile–chloroform gradient elution analysis is much more efficient than a temperature gradient (in the practical range 15–55°C) for scanning a large range of triglyceride chain lengths.

(5) This study permits a clear understanding of why the retention of a triglyceride cannot be correctly predicted from the retention of the three corresponding fatty acid methyl esters. Indeed, the aliphatic chain of these single chain esters does penetrate into the stationary phase. By combining the retention data of the three esters, one implicitly assumes that the three chains interact with the stationary phase, while, in fact, only 2.4 chains have been shown statistically to penetrate into the bonded layer.

(6) The identification of triglycerides in complex samples is frequently done by calculating the capacity factor of a mixed triglyceride from those of homogeneous ones according to eqn. 1 using diagrams where linear interpolations of log k' *vs.* the number of carbon atoms are performed[7]. However, because it is shown that there is a break in such curves, much care must be used not to interpolate curves on both sides of the critical carbon atom number.

(7) It is frequently thought that unsaturated triglyceride isomers having the same number of carbon atoms and of unsaturations, but differing in the positions of the double bonds along the three chains, cannot be separated. This can be understood since, when the chains penetrate into the stationary layer, there is no interaction specificity according to the position of the double bonds. However, the fact that the triglycerides may have different conformations when interacting with the ligands and that one, or more rarely, two chains may not penetrate into the bonded layer but stay entirely surrounded by solvent molecules, can give a selectivity basis according to specific interactions of the mobile phase with the unsaturations remaining outside the bonded layer. Admittedly, the corresponding selectivity values are likely to be small since these "outside chain" conformations are statistically not very favourable and since there might be several different conformations giving one outside chain (this might be the central chain or a lateral chain) which tends to average and somewhat cancel out these different interactions. Nevertheless, in principle, the separation of such isomers can occur provided that the efficiency of the chromatographic system is sufficient, as seems to have been recently observed[33]. It is expected that the separations will be improved by using a mobile phase which gives strong specific interactions with unsaturated compounds and which simultaneously has an appropriate eluotropic strength to provide an adequate retention.

ACKNOWLEDGEMENT

We are grateful to MM. Anselme and Azemar (Beckman, Gagny, France) for the gift of the monomeric Ultrasphere column.

REFERENCES

1 L. J. R. Barrón and G. Santa-Maria, *Chromatographia*, 23 (1987) 209.
2 E. Frede, *Chromatographia*, 21 (1986) 29.
3 A. Stolyhwo, H. Colin, M. Martin and G. Guiochon, *J. Chromatogr.*, 288 (1984) 253.
4 J. L. Robinson and R. Macrae, *J. Chromatogr.*, 303 (1984) 386.
5 A. Stolyhwo, H. Colin and G. Guiochon, *Anal. Chem.*, 57 (1985) 1342.
6 N. A. Parris, *J. Chromatogr.*, 149 (1978) 615.
7 J. P. Goiffon, C. Reminiac and D. Furon, *Rev. Fr. Corps Gras*, 4 (1981) 199.
8 A. Tchapla, H. Colin and G. Guiochon, *Anal. Chem.*, 56 (1984) 621.
9 P. K. Tseng and L. B. Rogers, *J. Chromatogr. Sci.*, 16 (1978) 436.
10 M. Tsimidou and R. Macrae, *J. Chromatogr. Sci.*, 23 (1985) 155.
11 F. Riedo and E. Sz. Kováts, *J. Chromatogr.*, 239 (1982) 1.
12 E. sz. Kováts, in F. Bruner (Editor), *The Science of Chromatography*, (Journal of Chromatography Library, Vol. 32), Elsevier, Amsterdam, 1985, p. 205.
13 E. H. Slaats, J. C. Kraak, W. J. T. Brugman and H. Poppe, *J. Chromatogr.*, 149 (1978) 255.
14 A. J. P. Martin, *Biochem. Soc. Symp.*, 3 (1949) 4.
15 H. Colin and G. Guiochon, *J. Chromatogr. Sci.*, 18 (1980) 54.
16 P. Jandera, *J. Chromatogr.*, 314 (1984) 13.
17 G. E. Berendsen, P. J. Schoenmakers, L. De Galan, G. Vigh, Z. Varga-Puchony and J. Inczedy, *J. Liq. Chromatogr.*, 3 (1980) 1669.
18 D. Morel, J. Serpinet, J. M. Letoffe and P. Claudy, *Chromatographia*, 22 (1986) 103.
19 R. K. Gilpin and M. E. Gangoda, *J. Chromatogr. Sci.*, 21 (1983) 352.
20 E. Bayer, A. Paulus, B. Peters, G. Laupp, J. Reiners and K. Albert, *J. Chromatogr.*, 364 (1986) 25.
21 Cs. Horváth, W. Melander and I. Molnár, *J. Chromatogr.*, 125 (1976) 129.
22 A. Tchapla, S. Héron, H. Colin and G. Guiochon, *Anal. Chem.*, (1988) in press.
23 L. C. Sander, J. B. Callis and L. R. Field, *Anal. Chem.*, 55 (1983) 1068.
24 L. R. Snyder, *J. Chromatogr.*, 6 (1961) 22.
25 H. Colin, G. Guiochon, Z. Yun, J. C. Diez-Masa and P. Jandera, *J. Chromatogr. Sci.*, 21 (1983) 179.
26 B. L. Karger, J. R. Gant, A. Hartkopf and P. H. Weiner, *J. Chromatogr.*, 128 (1976) 65.
27 H. Colin, A. M. Krstulovic, M.-F. Gonnord, G. Guiochon, Z. Yun and P. Jandera, *Chromatographia*, 17 (1983) 9.
28 W. Melander, D. E. Campbell and Cs. Horváth, *J. Chromatogr.*, 158 (1978) 215.
29 L. H. Jensen and A. J. Mabis, *Acta Crystallogr.*, 21 (1966) 770.
30 D. L. Dorset, W. A. Pangborn, A. J. Hancock, T. C. Van Soest and S. M. Greenwald, *Z. Naturforsch., Teil C*, 33 (1978) 50.
31 G. Fourche, C. Clément, B. Lemaire and C. Lussan, *Nouv. J. Chim.*, 5 (1981) 91.
32 T. Bursh, K. Larsson and M. Lundquist, *Chem. Phys. Lipids*, 2 (1968) 102.
33 G. Semporé and J. Bézard, *J. Chromatogr.*, 366 (1986) 261.