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Role of π-π interactions in reversed-phase liquid chromatography

GÉRALDINE THÉVENON-EMERIC[a], ALAIN TCHAPLA[b] and MICHEL MARTIN*[,c]
École Polytechnique, Laboratoire de Chimie Analytique Physique, 91128 Palaiseau (France)

ABSTRACT

The retention of various saturated and unsaturated solutes in reversed-phase liquid chromatography (RPLC) was investigated for various saturated and unsaturated packings in mobile phase mixtures of various compositions of methanol and acetonitrile. Non-aqueous eluents containing acetonitrile have a lower solvent strength than eluents containing methanol for saturated alkanes, alcohols and triglycerides, as expected from the solvophobic theory of retention. Very peculiar retention behaviours are observed when unsaturated solutes are analysed on aromatic stationary phases and/or when mobile phase containing acetonitrile is used with either an aromatic stationary phase or with unsaturated solutes or both. All these observations can be rationalized by taking into account the effect of π-π interactions between the π-electron systems of the solute, the stationary phase and the mobile phase. The influence of the residual silanols on silica-based RPLC supports on the retention of alcoholic solutes in pure acetonitrile is demonstrated. The influence of the concentration of a charge-transfer complexing agent (silver nitrate) in the mobile phase on the retention of unsaturated triglycerides was studied in a methanolic eluent and compared with that in a mobile phase containing acetonitrile.

INTRODUCTION

The optimization of a separation in reversed-phase liquid chromatography (RPLC) is helped by an understanding of all the different interactions involved in the system [1–3]. In the past, much work has been carried out towards understanding and quantifying the effect of the mobile phase on the retention of the solutes.

The theory of the solvophobic effect [4,5] provides a good model of the retention behaviour when the stationary phase is not involved in specific interactions with either the solutes or the solvent. In this situation the elution is mostly controlled by interactions in the mobile phase and the retention is closely related to the eluent properties. Various classifications of solvents based on different parameters, which

[a] Present address: Department of Medicinal Chemistry, Pharmacy Building, Purdue University, West Lafayette, IN 47907, USA.
[b] Present address: IUT Orsay I, LETIAM, B.P. 127, 91403 Orsay Cedex, France.
[c] Present address: Ecole Supérieure de Physique et Chimie Industrielles, Laboratoire de Physique et Mécanique des Milieux Hétérogènes (URA CNRS 857), 10 Rue Vauquelin, 75231 Paris Cedex 05, France.

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may serve as guidelines for the selection of a chromatographic eluent, are available [6–8].

However, the prediction of retention can be effective only if all the interactions between the three species involved in the chromatographic process, *i.e.* the solutes, eluent and stationary phase, are considered at the same time. Tanaka and co-workers [9,10] and Colin *et al.* [11] studied the influence of the organic modifier on retention and selectivity for a large number of solutes by looking at their retention in methanol *versus* that in acetonitrile. The investigations were performed with *n*-alkyl-bonded phases. These supports only give non-specific interactions, except for undesirable hydrogen bonding as a result of the residual free silanol groups present on the surface of the silica-based packing materials. However, many supports now in use in RPLC can exhibit interactions other than non-specific, rendering the prediction of retention more complex. Studies of retention, selectivity and/or peak shape have been performed on some of these supports, such as phenyl-bonded silica-based phases [12,13] or polymeric supports such as poly(styrene–divinylbenzene) [14]. These were usually carried out in aqueous mobile phases and focused mainly on the effect of the organic modifier on the retention of the solutes.

These supports contain π-electron systems which, in presence of other π-electron systems in either the mobile phase or the solutes, may give rise to π–π interactions. These rather specific interactions, which are one form of the more general electron donor–electron acceptor type of interactions [15], are expected to influence the retention behaviour. They may also occur between the solutes and the mobile phase.

To better understand the retention mechanism in RPLC when these interactions occur in the chromatographic system, their effect has been investigated somewhat systematically. Their influence on retention was first studied when π-electron systems are present in the mobile phase and in the solutes, then in the mobile and stationary phases, then in the solutes and in the stationary phase, and then simultaneously in these three species. For each of these cases, a comparison with similar systems in which π–π interactions are absent allows the specific influence of these interactions to be isolated as much as possible. In addition, the influence of the presence of a charge-transfer complexing agent (silver ion) with π-electron systems in the mobile phase is studied.

This investigation took place in the framework of a more general study on the separation by RPLC of homogeneous triglycerides [16]. Therefore, the solutes used in this work are mainly selected from within this family of compounds. In fact, they constitute good probes for studying the influence of π–π interactions as they can contain a relatively large number of double bonds for a fixed number of carbon atoms. Furthermore, the separation of the triglycerides according to the number of carbon atoms and to the number and position of double bonds is one of the present challenges for the analysis and characterization of fats.

EXPERIMENTAL

Reagents

All solvents were of HPLC grade (Carlo Erba, Milan, Italy). All were filtered through a Millipore GF/C 1.2-μm filter, then mixed as required and briefly sonicated. All solutions were freshly prepared. All mobile phase mixtures were prepared using a 100-ml burette.

The triglycerides were purchased from Interchim (Montluçon, France). The alkanes and alcohols were from various manufacturers.

Equipment

The LC system consisted of a Beckman Model 112 pump (San Ramon, CA, USA), a Rheodyne 7125 injection valve (Cotati, CA, USA) with a 20-μl loop and a Waters R-401 refractive index detector (Milford, MA, USA). The pre-column, column and injection valve were all thermostated using a laboratory-made water jacket. The bath temperature was controlled by a Huber HS thermostat (Offenburg-Elgersweier, Germany) with a precision of 0.1°C.

Four columns were used: an Ultrasphere ODS (an octadecyl-bonded silica-based material), 5 μm, 80 Å, 15 × 0.46 cm, from Beckman; a Zorbax *n*-propylphenyl 6 μm, 80 Å, 25 × 0.46 cm, from DuPont (Wilmington, DE, USA); a Hamilton PRP-1 (styrene–divinylbenzene polymeric material), 10 μm, 80 Å, 15 × 0.46 cm (Hamilton, Reno, NV, USA); and a laboratory-made column packed with LiChrosorb RP-18 (an octadecyl-bonded silica-based material), 10 μm, 80 Å, 20 × 0.46 cm from Merck (Darmstadt, Germany).

Methods

All retention values are reported in terms of the capacity factor, k'. The calculation of this parameter requires the determination of the void volume of the column used. The weighing method was as described previously [16]. Each k' value reported results from at least three reproducible injections.

RESULTS AND DISCUSSION

Relative solvent strength of methanol and acetonitrile for saturated solutes on an octadecyl-silica support

This study of the role of π–π interactions in RPLC involves the comparison of the retention of solutes in mobile phases containing acetonitrile *versus* that in eluents containing methanol. It is therefore interesting to compare first the eluotropic strengths of acetonitrile and methanol when π–π interactions are not present in the chromatographic system. There is a huge amount of data on the retention of various series of solutes in acetonitrile–water and methanol–water mixtures.

It is commonly accepted that acetonitrile is a stronger eluent than methanols, as at a given, not low, water percentage, retention in methanol–water mixtures is usually larger than in acetonitrile–water mixtures. However, it has already been observed that pure methanol or methanol–water mixtures with a low water content are stronger eluents than pure acetonitrile or acetonitrile–water mixtures with the same low water content: this was considered to be an "abnormal" behaviour of the acetonitrile–

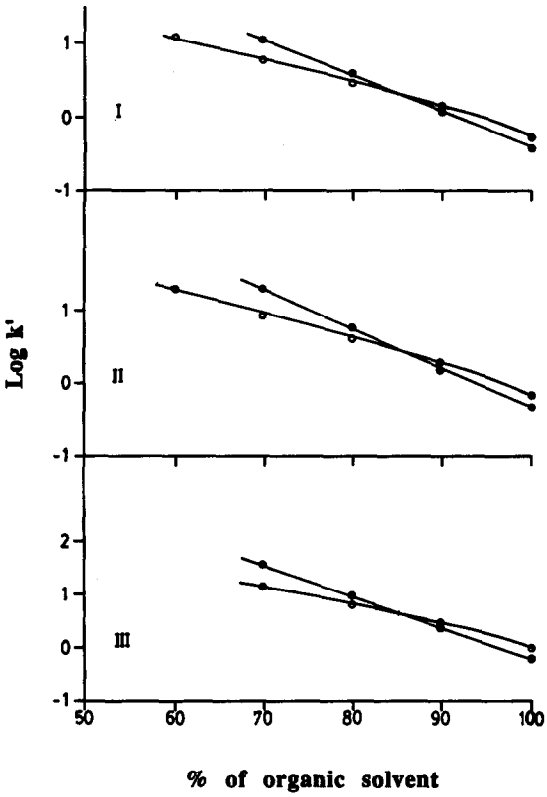

Fig. 1. Retention of *n*-hexane (I), *n*-heptane (II) and *n*-octane (III) *versus* percentage of organic modifier in water. Mobile phase: (○) water–acetonitrile; (●) water–methanol. Column: LiChrosorb RP-18. (Compilation of data from refs. 17 and 18).

water mixtures with a low water content [17]. The explanation of this contradiction can be found in the theory of the solvophobic effect. It is well documented that this effect is one of the most important phenomena governing retention in RPLC. This theory predicts that the retention increases with the surface tension of the mobile phase. The surface tension of acetonitrile (29.3 dyne/cm at 20°C) is higher than that of methanol under the same conditions (22.6 dyne/cm), whereas the surface tension of methanol–water mixtures exceeds that of acetonitrile–water mixtures when the water content is larger than about 28% (v/v). Accordingly, in the absence of water, and if no phenomena other than hydrophobic interactions are involved in the retention process, methanol must be a stronger eluent than acetonitrile. This can be verified from Fig. 1, which represents the logarithms of the capacity factors (k') of three *n*-alkanes *versus* the percentage of both methanol and acetonitrile in water, compiled from data in refs. 17 and 18. The retention of the alkane solutes, which were selected as they give only non-specific interactions, becomes lower in methanol–water than in acetonitrile–water when the amount of the organic component of the mobile phase exceeds 85% (v/v). This finding confirms the validity of the solvophobic theory for

describing the retention of non-polar solutes in RPLC. The slight difference in the water content of the cross-over points of the surface tension and retention *versus* eluent composition curves (28 and 15%, respectively) may arise from differences between the microscopic value of the surface tension of the eluent, which controls the retention, and the measured macroscopic value.

It can be noted that, although the solvophobic theory does not account for the change in retention arising from a modification of the stationary phase configuration by the mobile phase, such an influence is likely to be negligible in either pure organic eluents or those containing a low amount of water as the mechanism of penetration of the solute chains into the stationary phase, investigated previously [16], demonstrates that in these environments the alkyl chains of the bonded phase, attached to the solid surface, can move more or less freely in the mobile phase.

Influence of the presence of π-electron systems in the solutes and mobile phase with an octadecyl-silica support

In RPLC on *n*-alkyl-bonded phases, the decrease in retention of a linear solute due to the replacement of a saturated bond by an unsaturated bond in the hydrocarbon chain is well known. This phenomenon, which is observed in the presence of double or triple carbon–carbon bonds can be explained by the fact that these bonds

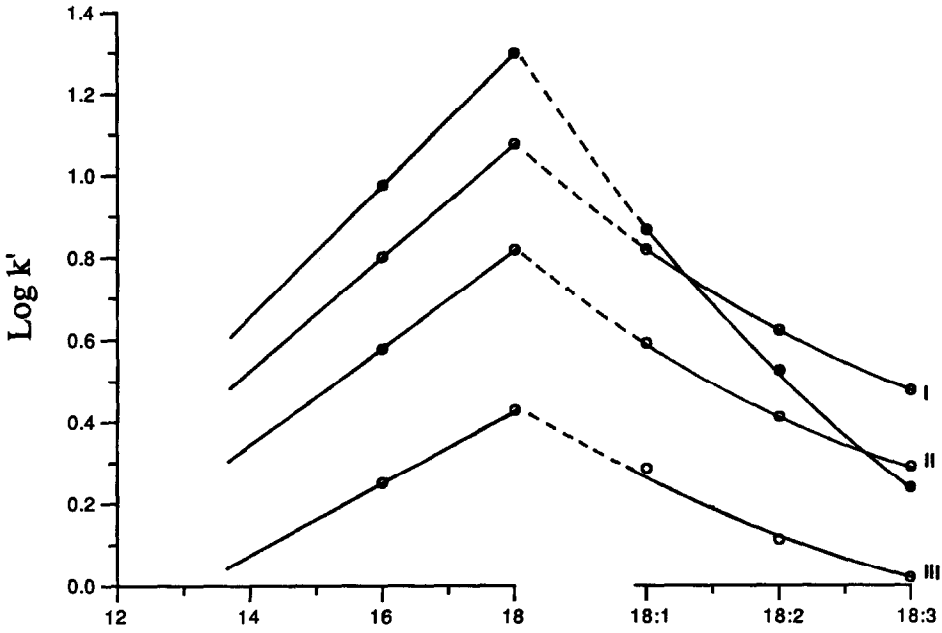

Fig. 2. Retention of homogeneous triglycerides. Left panel, retention *versus* number of carbon atoms in each chain of saturated compounds (tripalmitin and tristearin); right panel, retention *versus* number of double bonds contained in each chain with eighteen carbon atoms (18 = tristearin; 18:1 = triolein; 18:2 = trilinolein; 18:3 = trilinolenin). Column, LiChrosorb RP-18; temperature, 29°C; mobile phase: (●) acetonitrile–chloroform (75:25, v/v), (○) I = methanol–chloroform (80:20, v/v), II = methanol–chloroform (75:25, v/v), III = methanol–chloroform (65:35, v/v).

destroy the conformational similarity between the ligands (the bonded octadecyl moieties) and the linear chain of the solute. This results in a weaker interaction between the hydrocarbon chains of the solute and the ligand and in a greater surface area of contact of the solute–ligand complex with the eluent and, hence, in a weaker retention according to the solvophobic theory. However, the consideration of interactions between the solute and the stationary phase alone provides only a partial explanation of the retention phenomenon. Interactions between all species present in the chromatographic system must be taken into account. The decrease in retention associated with the presence of unsaturated bonds also arises from interactions between the solute and the eluent. Thus the greater polarizability of the unsaturated solutes leads to a greater interaction with polar solvents and contributes to lowering the retention.

This phenomenon is illustrated in Fig. 2, which shows the retention of homogeneous saturated triglycerides (tripalmitin and tristearin) and of unsaturated triglycerides containing eighteen carbon atoms in each of their ester chains (triolein, trilinolein and trilinolenin) on an *n*-octadecyl-bonded phase on silica (ODS). On the left-hand part of the figure, it is seen that, whatever the nature of the organic modifier, log k' of the saturated solutes increases with the number of carbon atoms of the alkyl chains. It has been previously observed that this increase is linear [16].

It is also noted that, in mixtures with 25% chloroform, methanol is a stronger eluent than acetonitrile for saturated solutes on an ODS stationary phase. This confirms the observations made in pure methanol and acetonitrile mobile phases as well as in acetonitrile–water and methanol–water eluents with a low water content and indicates that, in the chloroform-containing eluents, the solvophobic effect remains the dominating factor controlling the retention, in spite of the varying degree of hydrogen bonding interactions of chloroform with acetonitrile and methanol.

On the right-hand part of Fig. 2 are reported, for binary eluents of a different nature and composition, the retention of saturated and unsaturated homogeneous triglycerides with eighteen carbon atoms in each chain. In all instances a decrease is observed in the retention factor when one or more double bonds are inserted into the carbonaceous skeleton of the solute. Several conclusions can be deduced from these observations.

(1) Whatever the nature of the solvents in the binary eluents or the composition of the mobile phase, the retention drop increases when the number of double bonds in each chain is increased.

(2) The amount of chloroform in the mobile phase does not significantly affect the magnitude of the decrease in retention due to the unsaturated bonds. This is clear from Fig. 1 where the right-hand parts of the curves for three different compositions of the methanol–chloroform mixture are nearly parallel.

(3) The magnitude of the decrease in retention is much larger in an acetonitrile–chloroform mixture than in a methanol–chloroform mobile phase. This can be interpreted by specific interactions occurring between the π-electrons of the solutes and those of the mobile phase. Indeed, acetonitrile, with its cyano group, possesses a site rich in electrons, as do unsaturated compounds. The interaction of these sites results in a high affinity of the unsaturated solutes for the mobile phase. Consequently, in the presence of such interactions, the solute retention is reduced.

(4) The decrease of log k' and, hence, of the standard free enthalpy (Gibbs

function) of transfer of the solute from the mobile phase to the stationary phase, *versus* the number of double bonds is not linear. This probably results, according to the solvophobic theory, from the non-linearity of the variation between the number of double bonds of the solute and reduction of the surface area of contact of the solute and the ligand with the eluent when forming the solute–ligand complex. In addition, the polarizability of the solutes may not be a linear function of the number of double bonds. Accordingly, Fig. 2 shows that for triglycerides possessing eighteen carbons in each of their three chains, the presence of one, two or three ethylenic bonds has the same effect as the withdrawal of 2.6, 4.8 or 6.5 methylene groups, respectively, from each chain of the solute in acetonitrile–chloroform eluents or the withdrawal of 1.9, 3.3 or 4.2 methylene groups from each solute chain in methanol–chloroform mixtures. These results show that parameters such as the partition number (NP), or the equivalent carbon number (ECN) must always be given with well defined chromatographic conditions. The NP number has been defined by Goiffon *et al.* [19] to relate the retention of triglycerides to their number of carbon atoms and double bonds. The ECN has been introduced by Podlaha and Toregard [20] and represents the number of carbon atoms of a hypothetical saturated triglyceride which would elute at the same time as the unsaturated triglyceride in question.

Another illustration of the influence of the nature of the mobile phase mixture

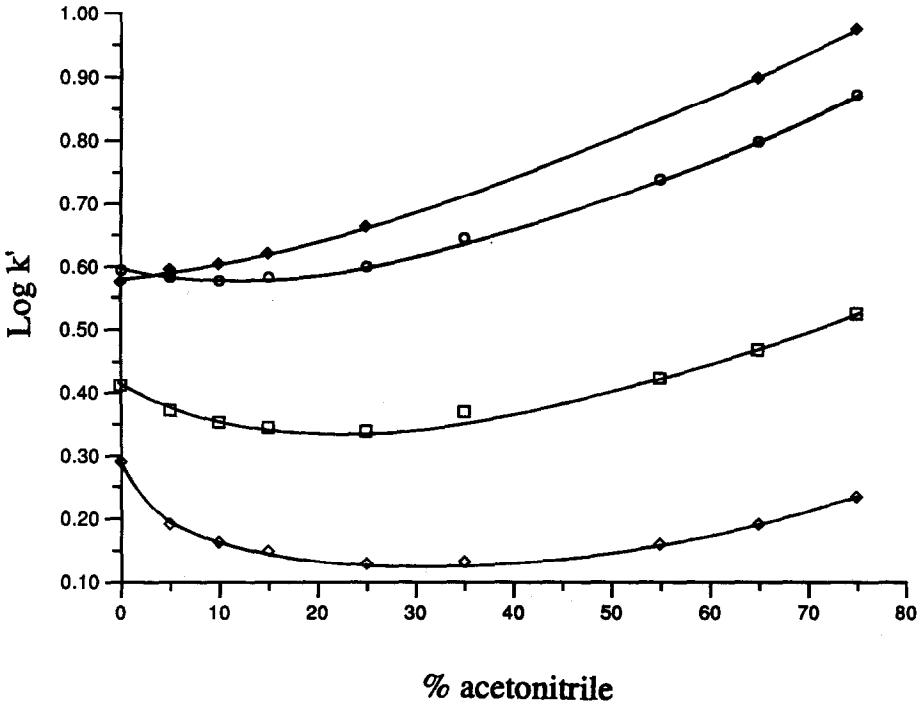

Fig. 3. Retention of homogeneous triglycerides *versus* amount (x) of acetonitrile in the mobile phase. Column, LiChrosorb RP-18; temperature, 29°C; mobile phase, methanol–acetonitrile–chloroform ($75-x$:x:25, v/v/v). Triglycerides: (◆) tripalmitin; (○) triolein; (□) trilinolein; (◇) trilinolenin.

and of the presence of alkene bonds in the solutes on retention is shown in Fig. 3. This represents the variation of log k' of homogeneous saturated and unsaturated triglycerides with the composition of a ternary methanol–acetonitrile–chloroform mobile phase. The amount of chloroform is fixed (25%, v/v) and the percentage composition of the acetonitrile–methanol mixture varies from 0:75 to 75:0. For the saturated solute, the only effect of the addition of increasing amounts of acetonitrile is the decrease of the eluotropic strength of the mobile phase, resulting in an increase in solute retention, as observed for tripalmitin. Another parameter affects the retention of unsaturated solutes. Indeed, the addition of acetonitrile favours the interaction between the solute and solvent due to the presence of the π-electron systems. Consequently, two opposite phenomena compete: the increased solvophobicity of the eluent due to the addition of acetonitrile, which results in an increase in the retention, and a stronger interaction between the solutes and the solvent, which results in a decrease in the retention. Classically, such a competition between two effects leads to an optimum in the curves. In this case, a minimum is observed for the retention of unsaturated compounds *versus* the amount of acetonitrile. The addition of the first aliquots of acetonitrile results in a drop in the retention because the solutes interact with the mobile phase. A further increase in the amount of acetonitrile causes a continuous decrease in retention up to the point where the π–π interaction cannot compensate for the higher solvophobicity of this solvent compared to methanol. This then results in an increase in retention. That a minimum rather than a maximum is observed in Fig. 3 can be qualitatively understood from the variation of the magnitude of the two effects with the amount of acetonitrile added to the mobile phase. The variation of the solvophobicity of the eluent with composition is rather monotonous, as is the variation of the eluent surface tension according to the solvophobic theory of retention in RPLC [4]. In contradiction to this, the variation in the effect of π–π interactions with the eluent composition is expected to be largest for the smallest percentages of acetonitrile in the eluent as once the amount of acetonitrile has reached the point where each solute molecule is surrounded by one or two acetonitrile molecules, the intensity of the π–π interactions will not change significantly with a further addition of acetonitrile in the mobile phase. Consequently, the solvophobic effect is predominant for large percentages of acetonitrile whereas the π–π interaction effect dominates at low percentages. The strength of the interaction between acetonitrile and the solute depends on the number of unsaturated bonds in the solute chains. Therefore, the amount of acetonitrile for which the solvophobic effect becomes the predominant phenomenon is also dependent on the number of unsaturated bonds in the solutes. This is why the decrease in the retention and the percentage of acetonitrile corresponding to the minimum of the curves increase from triolein to trilinolein to trilinolenin.

It is interesting to note in Fig. 3 that the acetonitrile–chloroform (75:25) binary solvent is a stronger eluent for trilinolenin than the methanol–chloroform (75:25) mobile phase, whereas the opposite is true for tripalmitin, triolein and trilinolein. This reflects the fact that the intensity of the π–π interactions between acetonitrile and trilinolenin, which is the most strongly unsaturated of these four triglycerides, overcomes the greater solvophobicity of acetonitrile compared to methanol. Similarly, it can be seen from an analysis of the retention data of Stalcup *et al.* [21] that the four polyaromatic hydrocarbons investigated are retained more in pure methanol than in pure acetonitrile, except, perhaps, the phenanthrophenanthrene.

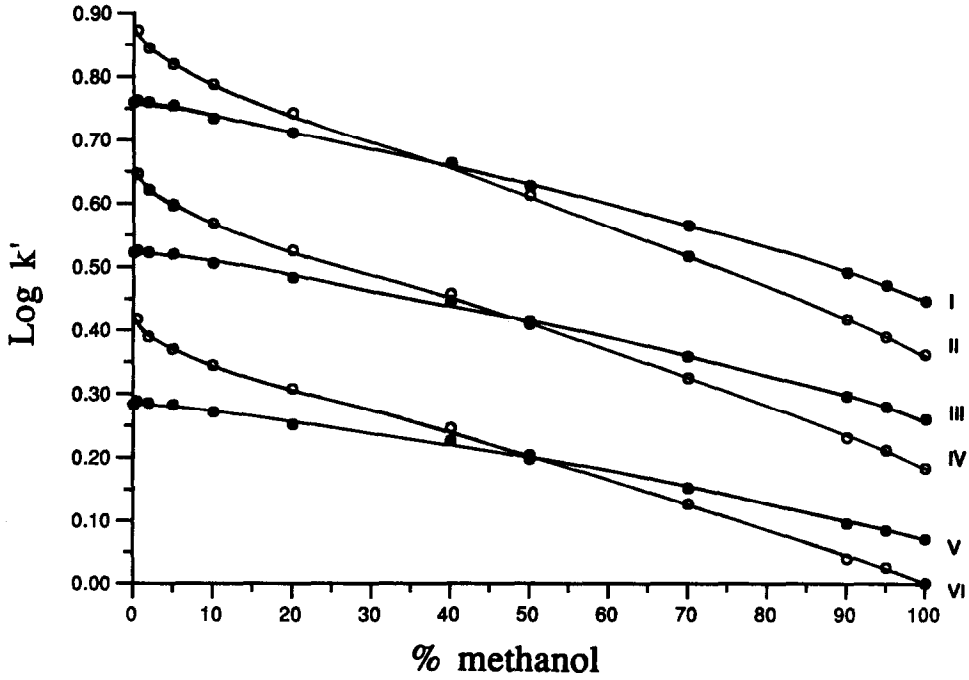

Fig. 4. Retention of *n*-alkanes (●) and *n*-alcohols (○) *versus* the amount of methanol (v/v) in the acetonitrile–methanol mobile phase. Column, Ultrasphere ODS; temperature, 25°C. Solutes: (I) *n*-dodecane, (III) *n*-decane, (V) *n*-octane, (II) *n*-octadecanol, (IV) *n*-hexadecanol, (VI) *n*-tetradecanol.

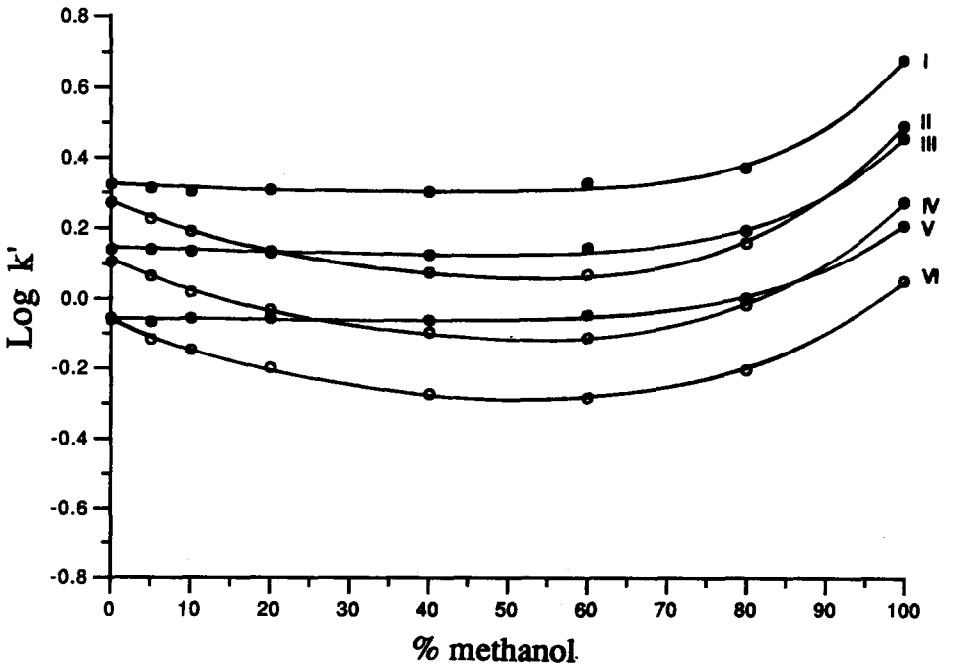

Fig. 5. Retention of *n*-alkanes (●) and *n*-alcohols (○) *versus* the amount of methanol, (v/v) in the acetonitrile–methanol mobile phase. Column, Hamilton PRP-1; temperature, 20°C. Solutes: (I) *n*-decane, (III) *n*-octane, (V) *n*-hexane, (II) *n*-hexadecanol, (IV) *n*-tetradecanol, (VI) *n*-dodecanol.

Influence of the presence of π-electron systems in the mobile and stationary phases with saturated solutes

The effect of the presence of ethylenic bonds in the solute on the retention on the ODS support in various eluents has revealed the essential influence of the π–π interactions between the solute and the eluent. It is interesting to examine these interactions when only the mobile and stationary phases are involved. To do this, a study of the retention of saturated small molecules as a function of the relative amount of methanol and acetonitrile was considered. Owing to its larger retention characteristics for these solutes, a poly(styrene–divinylbenzene) support was preferred to a bonded phenylpropyl silica for this purpose.

To increase the understanding of the results, similar experiments were also performed on the ODS support. The purpose of this comparative study was to unravel the individual effects of π–π interactions and the difference in solvophobicity of the methanol and acetonitrile eluents. The results are reported, in terms of log k' *versus* percentage methanol, in Fig. 4 for the ODS phase and in Fig. 5 for the poly(styrene–divinylbenzene) support. The curves on Fig. 4 for three *n*-alkanes and three *n*-alcohols confirm the results presented in the first part of the Results and Discussion section: the retention of these solutes decreases when the proportion of methanol increases. Pure acetonitrile is more solvophobic than pure methanol and is thus a weaker eluent when solvophobic interactions dominate the retention.

It is interesting to note the rapid decrease in retention for the alcohols when the first aliquots of methanol are added to acetonitrile. This rapid decrease is not observed for the *n*-alkanes. It can be explained by the hydrogen bond interactions occurring in the mobile phase between the alcoholic solutes and the methanol. In addition, the significant peak tailing which is observed with acetonitrile as the eluent for the alcohols, but not for the alkanes, disappears when the amount of methanol in the mobile phase exceeds 0.5% (data not shown). This phenomenon is most likely to be related to competition between the alcoholic solutes and methanol for the interactions, through hydrogen bonds, with the free silanol sites present on the surface of the support. The competition is displaced in favour of the eluent with increasing amounts of methanol. It is important to be aware of this phenomenon when working with non-aqueous mobile phases. It indicates that, although acetonitrile can form hydrogen bonds through its free electron pair on the nitrogen atom [22,23], this bonding is not strong enough to hinder the interaction of the alcoholic solutes with the silanol groups. Obviously, these effects (hydrogen bond interactions in the mobile phase and silanol interactions) contribute simultaneously to the rapid decrease in retention of the alcohols noted at low percentages of methanol in the eluent.

The results obtained on poly(styrene–divinylbenzene) are reported in Fig. 5. Surprisingly, the retention of the *n*-alkanes remains nearly constant when acetonitrile is replaced by methanol. It would have been expected that, as a result of the higher solvophobicity of acetonitrile, the retention of these compounds would decrease when the amount of methanol increased, as was observed on the ODS support. However, another effect has to be taken into consideration in this instance. Acetonitrile can interact by means of π–π interactions with the aromatic rings of the poly(styrene–divinylbenzene) support, whereas methanol does not. The addition of methanol to the mobile phase weakens the interaction of the eluent with the support. Consequently, interactions of the solute with the stationary phase are favoured when methanol is

added. This should result in an increase in the retention of the solutes. The two phenomena, lower solvophobicity and lower eluent interaction with the stationary phase with increasing methanol amount in the mobile phase, have antagonistic effects on the retention. The direction of the retention variation will depend on which phenomenon is the stronger. For the alkanes, these effects are nearly equivalent. The retention is approximately constant over a wide range of methanol–acetonitrile mixtures. It is noticeable that the point is never reached at which the higher eluent strength of methanol is predominant. The decrease in retention is never observed for the alkanes. Conversely, when the amount of methanol is greater than 60%, an increase in retention is observed. This indicates that when the amount of acetonitrile is high enough, a solvent layer is formed at the surface of the stationary phase, rendering the interaction of the solute with this phase more difficult. When the percentage of acetonitrile is low enough, the poly(styrene–divinylbenzene) becomes more accessible to the solutes and their retention increases. For the alcohols, the same phenomenon occurs, except that the retention decreases with the addition of the first aliquots of methanol. This is attributed to the interaction between these solutes and methanol, through hydrogen bonds between their polar heads, which is strong enough to compensate for the increased accessibility of the surface of the stationary phase.

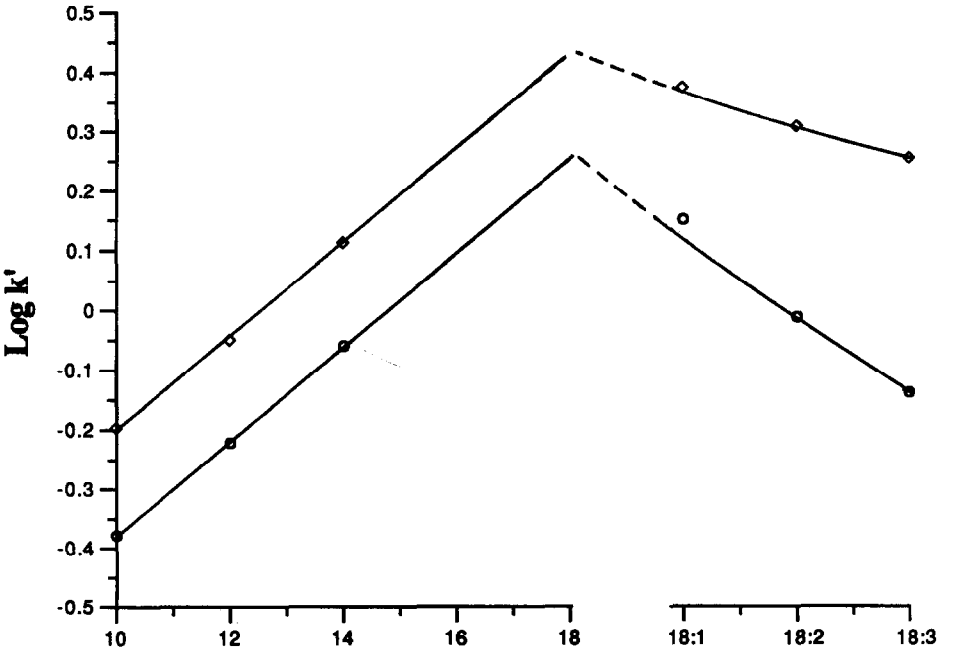

Fig. 6. Retention of homogeneous triglycerides. Left panel, retention *versus* number of carbon atoms in each chain of saturated compounds (tricaprin, trilaurin and trimyristin); right panel, retention *versus* number of double bonds contained in each chain with eighteen carbon atoms (18:1 = triolein; 18:2 = trilinolein; 18:3 = trilinolenin. Column, Zorbax *n*-propylphenyl; temperature, 20°C; mobile phase, (◇) methanol, (○) acetonitrile.

Influence of the presence of π-electron systems in the solutes, stationary phase and mobile phase

The π–π interactions discussed above were taking place between the mobile phase and the solute or the stationary phase. However, these interactions can simultaneously involve these three species. Fig. 6 shows the retention results obtained for saturated and unsaturated triglycerides with a silica support bonded with a propylphenyl phase, which can lead to π–π interactions through the π-electrons of the phenyl groups. This figure can be described in exactly the same way as Fig. 2 for an ODS phase: on the left-hand part of the figure, the logarithms of the capacity factors of the saturated solutes increase linearly with the number of carbon atoms of the solute chains. This phenomenon does not depend on the nature of the organic modifier. As for the ODS support, the right-hand part of the figure shows that the presence of unsaturated bonds in the molecular structure of the solute decreases the retention. The higher the number of unsaturated bonds, the more important is the decrease in retention. Again, the decrease of log k' is not proportional to the number of double bonds.

When comparing the two curves corresponding to the phenyl phase in Fig. 6, it is noted that, as in the case of the ODS support, the magnitude of the decrease in retention due to an unsaturated bond is larger with acetonitrile than with methanol as

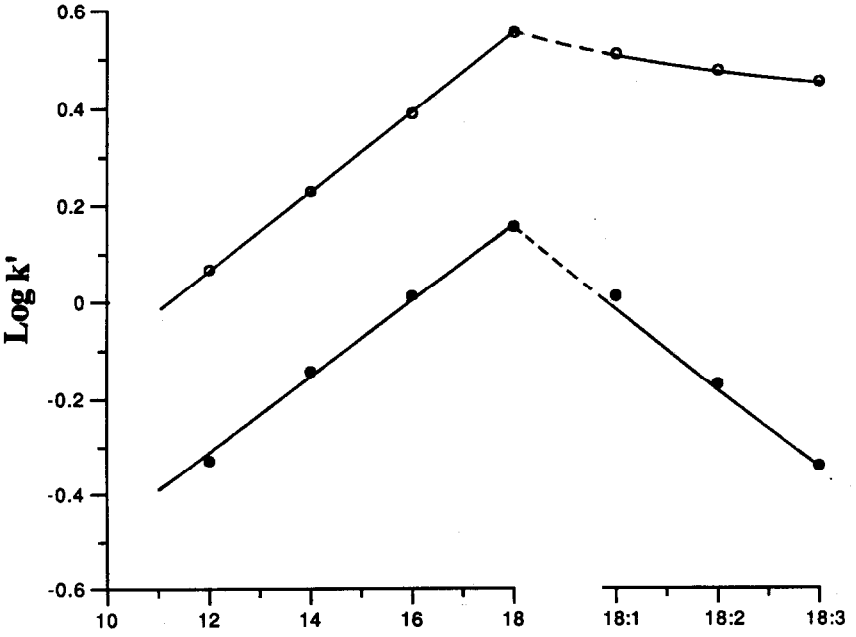

Fig. 7. Retention of homogeneous triglycerides. Left panel, retention *versus* number of carbon atoms in each chain of saturated compounds (trilaurin, trimyristin, tripalmitin and tristearin); right panel, retention *versus* number of double bonds contained in each chain with eighteen carbon atoms (18 = tristearin; 18:1 = triolein; 18:2 = trilinolein; 18:3 = trilinolenin). Column, Hamilton PRP-1. (○) Mobile phase, methanol–chloroform (70:30, v/v); temperature, 20°C. (●) Mobile phase, acetonitrile–chloroform (70:30, v/v); temperature, 15°C.

the mobile phase. This, as with the ODS support, can be explained by the fact that acetonitrile interacts with the π-electrons of the solutes, which decreases their retention.

Fairly similar results are observed on the poly(styrene–divinylbenzene) support, as seen in Fig. 7. The main difference between Figs. 6 and 7 is that, to obtain moderate retention levels with the polymeric support, stronger eluents have to be used. For this reason acetonitrile and methanol are mixed with 30% chloroform. However, as was observed in Fig. 2, the only effect of chloroform is to control the overall solvophobicity of the mobile phase as curves with various percentages of chloroform are parallel for both saturated and unsaturated solutes.

It is interesting to compare the curves obtained in methanolic mobile phase on the three supports shown in Figs. 2, 6 and 7. The decrease in retention due to the replacement of an alkane bond by an alkene bond in the solute chains is larger on the ODS support than on the phenylic phases. Indeed, on the *n*-alkyl-bonded phase, the presence of one, two or three ethylenic bonds in each chain of a triglyceride gives the same retention as that which would be obtained by withdrawing, respectively, 1.9, 3.3 and 4.2 methylene groups in each chain. On the aromatic supports, the corresponding numbers of methylene units to be withdrawn are much lower and are equal to 0.5, 1.6 and 2.2 for the propylphenyl phase and 0.45, 0.9 and 1.2 for the polymeric support, respectively. It can be seen in Fig. 2 that the presence of chloroform in the mobile phase does not affect these numbers. Clearly, these large differences between the ODS phase and the aromatic supports result from π–π interactions between the aromatic sites of the support and the ethylenic bonds of the solute. Indeed, when adding a double bond to a solute, its retention is favoured because of the π–π interaction of the double bond with the support, but the solvophobic effect overcomes this effect so that the retention decreases. However, it decreases less strongly on the aromatic stationary phase than on the ODS support. Consequently, the relative retention difference between triolein, trilinolein and trilinolenin is lower on the aromatic support than it is on the ODS support when methanol or a methanol–chlorofom mixture is used as the eluent.

In contradiction with what is found on the ODS support, in Figs. 5–7, acetonitrile appears to be a stronger eluent than methanol on the two aromatic supports [propylphenyl phase bonded on silica and poly(styrene–divinylbenzene)]. This can be explained by the interaction occurring between the π-systems of the acetonitrile and of the support. Such an interaction lowers that of the solute with the stationary phase. Indeed, the solute has to displace the solvent molecules to interact with the stationary phase. The π–π interaction between the support and the acetonitrile renders this displacement more difficult. Consequently, the solutes interact less strongly with the support, making their retention lower in such an eluent. This effect is fairly significant as it largely overcomes the intrinsically lower solvent strength of the acetonitrile, for both saturated and unsaturated solutes.

It is interesting to determine the effect of acetonitrile on the retention of unsaturated solutes with the stationary phases containing π-electrons, in terms of the numbers of methylene units to be withdrawn to an unsaturated solute to obtain the same retention as a saturated compound of the same series. For homogeneous triglycerides with one, two or three double bonds in each chain, these numbers per chain are 1.3, 3.3 and 5.0 for the propylphenyl silica, 1.8, 4.2 and 6.4 for the polymeric PRP-1

support and equal to 2.6, 4.8 and 6.5 for the ODS support, respectively. These numbers are larger with acetonitrile than with methanol, which is explained by the π–π interactions between the solutes and the eluent. However, the relative differences in these numbers between the ODS support and the aromatic supports is much lower in acetonitrile than in methanol, especially for the polymeric support, which probably reflects the effect of the π–π interactions between the acetonitrile and the aromatic stationary phases.

The data shown in Figs. 1–6 clearly reflect the competitive interactions occurring among the three species engaged in the chromatographic retention process: the stationary phase, mobile phase and the solute. When at least two of these are involved in π–π interactions, the relative strength of these interactions, compared to the other interactions associated with the solvophilic or solvophobic eluent properties which affect the retention in RPLC, can drastically change the selectivities of the separations. If the elution order of the saturated triglycerides is never affected, whatever the nature of the support and the eluent used, the relative retention of the saturated and unsaturated triglycerides is strongly affected by the nature of these phases. Accordingly, great care must be taken, when using partition numbers or equivalent carbon numbers for triglycerides, to specify all the parameters pertaining to the nature of the mobile and stationary phases.

Influence of the addition of a π-complexing agent in the mobile phase on the retention of saturated and unsaturated solutes

The charge-transfer interactions of silver salts with unsaturated compounds is well known [24,25]. This type of interaction is, like the π–π interactions discussed herein, one form of the more general electron donor–electron acceptor group of interactions [26]. It was used to separate unsaturated triglycerides according to their number of double bonds by liquid chromatography with the silica stationary phase loaded with silver nitrate [27]. Separations of unsaturated compounds by RPLC were also performed by adding silver nitrate to the mobile phase [28].

The influence of the addition of silver nitrate to the mobile phase on the retention of saturated and unsaturated triglycerides in RPLC with an ODS support was studied and the results are given in Fig. 8 as a function of the concentration of silver nitrate in methanol–chloroform (75:25) and acetonitrile–chloroform (75:25) eluent mixtures. For both the methanol- and acetonitrile-containing mobile phases, the retention of the saturated compounds slightly increases with an increasing concentration of the salt. This behaviour can be understood from the solvophobic theory as the addition of a salt to a solvent increases its surface tension and thus contributes to an increase in the retention.

In contrast, the retention of the unsaturated triglycerides decreases when the amount of silver ion in the methanolic eluent is increased. This results from the complexation of the ethylenic solutes by the silver salt which, as it occurs in the mobile phase, contributes to the decrease in the retention. This effect largely overcomes the solvophobic effect and is larger for polyunsaturated than for monounsaturated compounds.

However, in the acetonitrile–chloroform eluent, this effect is not observed and the retention of the unsaturated compounds is seen to increase slightly, with an increasing concentration of the salt in the eluent. The behaviour is then fairly similar

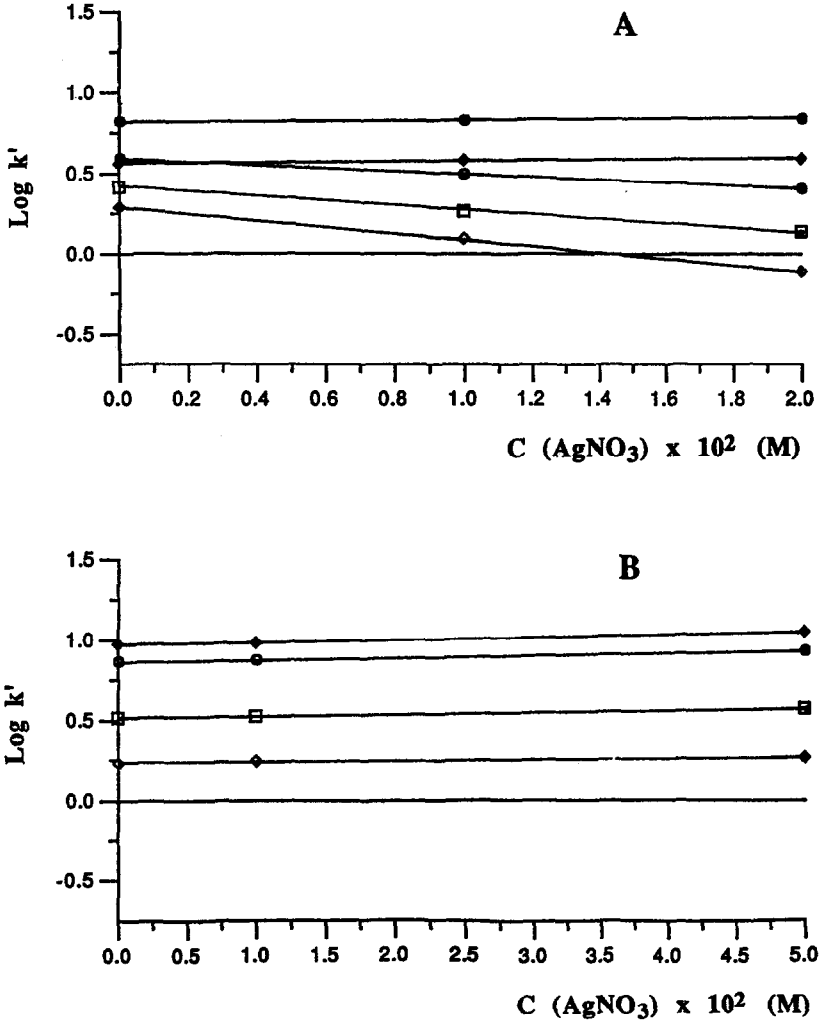

Fig. 8. Retention of homogeneous triglycerides *versus* molar concentration of silver nitrate in the mobile phase. Column, LiChrosorb RP-18; temperature, 29°C. Mobile phase: (A) methanol–chloroform (75:25, v/v); (B) acetonitrile–chloroform (75:25, v/v). Solutes: closed symbols, saturated triglycerides (●, tristearin; ◆, tripalmitin); open symbols, unsaturated triglycerides (○, triolein; □, trilinolein; ◇, trilinolenin).

to that of saturated compounds. Thus, acetonitrile hinders the complexation of the silver ion with the double bonds of the triglycerides, which can be explained by the fact that a charge-transfer complex is formed between the silver ion and the electron-rich cyano group of the acetonitrile. As the amount of acetonitrile in the mobile phase is much larger than that of the triglycerides and largely exceeds that concentration required to complex the silver ions, these ions cannot interact with the double bonds of the triglycerides.

CONCLUSIONS

This investigation of the retention of various saturated and unsaturated solutes in RPLC has shown evidence of the following effects.

(1) In non-aqueous mobile phases, eluents containing acetonitrile have a lower eluotropic strength for saturated compounds than eluents containing methanol. This is in agreement with the solvophobic retention theory of RPLC, but in contradiction with the common belief based on extrapolation of the retention behaviour in water–organic eluents with relatively large amounts of water.

(2) The solvophobic effect is not the major retention-controlling mechanism in RPLC when relatively strong specific interactions occur in the chromatographic system. In some instances, this results in the inversion of the eluting power of eluents containing methanol and acetonitrile, as shown for the highly unsaturated trilolenin.

(3) The study of the relative retention of saturated and unsaturated homogeneous triglycerides on an ODS based material with acetonitrile–methanol mixtures has revealed the significant influence on retention of the π–π interactions occurring between the double bonds of the solutes and the cyano group of acetonitrile.

(4) A similar study on small molecules such as *n*-alcohols revealed the inability of acetonitrile to hinder the formation of hydrogen bonds between the solute and the free silanol groups remaining on the surface of the support. The addition of a very small amount of methanol to the non-aqueous mobile phase is enough to prevent significant peak tailing.

(5) Experiments performed with stationary phases possessing unsaturated sites, such as the poly(styrene–divinylbenzene) or phenylpropyl-bonded silica-based supports, have clearly demonstrated the large influence on retention of π–π interactions when only the stationary phase and the mobile phase are involved. The presence of unsaturated bonds in the support tremendously influences the rate of change of retention when replacing acetonitrile by methanol in the mobile phase.

(6) A comparison of the effect of the presence of double bonds in the solutes on their retention on an ODS support and on aromatic supports has shown that π–π interactions can exist simultaneously among the three species of the system, solute–stationary phase–mobile phase, and that their interplay tremendously influences the selectivity of the separation.

(7) π–π interactions occurring between π-electron-containing molecular systems are one kind of the more general electron donor–electron acceptor group of interactions, as are charge-transfer interactions. Is is shown that such interactions between unsaturated solutes and silver ions present in the mobile phase can be removed by the complexation of the ions with acetonitrile.

This investigation was mainly concerned with the effects of π–π interactions on the retention of saturated and unsaturated solutes. These effects are rather complex as π–π interactions can occur between the components of the chromatographic system, *i.e.* the solute, mobile phase and stationary phase. Although the above discussion is only qualitative, this study shows that taking these π–π interactions into account when at least two of these components possess π-electron bonds allows a coherent description of the various retention effects observed.

This study has particularly illustrated the difference in eluting power between methanol and acetonitrile when double bonds are present either in the solute molec-

ular structure or in the stationary phase. Much work has been devoted over the past few years and is still devoted to the problem of finding the mobile phase composition which gives the best separation of a given sample mixture by RPLC. Usually, two binary aqueous mobile phases (acetonitrile–water and methanol–water) with the same eluotropic strength, *i.e.* providing the same k' range, are selected for the elution of the sample components. Optimization procedures are then used to find the composition of the mixture of these two binary solvents which provides the best separation of the mixture. Sometimes quaternary solvents, generally including tetrahydrofuran as a mobile phase component, are used instead of ternary eluents for this purpose. These results suggest that one of the major effects of modifying the relative content of acetonitrile–methanol in the mobile phase is to affect the relative retentions of the various pairs of sample components due to the interplay of π–π interactions between acetonitrile and the solutes. A proper quantitative treatment of these interactions is expected to greatly facilitate the search for the optimum mobile phase composition.

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