Retention models in reversed-phase chromatography

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Abstract

Theoretical, empirical and semi-empirical retention models characterizing the effects of various operation parameters such as stationary phase, mobile phase composition, temperature, and programmed elution conditions on the retention in reversed-phase chromatography are reviewed. Special attention is focused on the possibilities of applications for prediction of retention, method development and optimisation. Models correlating sample structure and retention are discussed. Theoretical and practical limitations of the models and importance of accurate data acquisition in the application are emphasized. Special topics such as behaviour of ionic compounds in reversed-phase LC, selectivity adjusting using ternary mobile phase, or dual retention behaviour on a single column depending on the mobile phase are also addressed.

What and for whom?
This is an education oriented review of the various developed models for retention with the purpose of helping the academic readers with already a good basic background. The purpose of the work is to review simple models practically useful for prediction (estimate) of the effects of sample structure and of working parameters on retention, with attention to theoretical and experimental limitations. Hence, practical aspects are more emphasized than the rigorous theory. I think that the reading may be useful for advanced master and PhD students of analytical separations and possibly could be of some use as reference work for practitioners developing HPLC methods. *Pavel Jandera*.

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1. Characteristic features of reversed-phase systems

Retention in reversed-phase (RP) liquid chromatography depends on a variety of factors, including the chemistry of stationary phase chemistry and the nature of the support, the structure of sample, composition of the mobile phase and temperature. The most characteristic features of reversed-phase chromatography (RPC) are higher polarity of the mobile phase in

comparison with the stationary phase (usually a non-polar alkyl phase chemically bonded on a suitable support), increased retention for less polar samples and more polar mobile phases, opposite to the normal (straight) phase LC mode.

The retention properties of bonded alkylsilica stationary phases, most frequently used in RP HPLC, should be ideally the same as of a liquid alkane stationary phase immobilized on a solid support. However, reversed-phase behavior can be observed with various stationary phases containing more or less polar moieties chemically bonded on inorganic supports, on organic polymer stationary phases and sometimes even on naked silica gel supports, depending on the mobile phase and sample. The retention is usually lower on weakly or moderately polar stationary phases, such as phenyl or cyanopropyl, than on alkyl silica phases, requiring lower concentrations of organic solvent to elute weakly polar samples, but the selectivity of separation on such phases often differs from that on C_{18} or C_8 phases. The selectivity for non-polar compounds is generally higher on alkyl silica bonded phases.

2. Advantages and limitations of retention models.

Retention models employ theoretical concepts, which are believed to control the retention mechanism, to describe quantitatively the effects of various properties of the sample and separation system on retention. Theoretical models provide better insight into principles governing the distribution of sample compounds between the stationary and the mobile phase, on the basis of certain basic ideas on retention mechanism. Through the understanding of the retention mechanism, rational systematic method development and optimisation of separation methods can be performed, in contrast to the trial-and-error empirical approach. Retention models are usually formulated as equations describing the effects of sample structure and (or) various experimental conditions. Better understanding of the role of stationary phase provides efficient tools for development of new columns with improved characteristics for reversed-phase LC suitable to solve various separation problems. On the other hand, rational description of the effects of the mobile phase on separation allows characterization and prediction of retention and separation selectivity at changing isocratic or gradient conditions.

Rigorous physically consistent theoretical models employ a number of physicochemical constants which are difficult and time-consuming to determine. Our present knowledge of quantitative aspects of the retention mechanism on molecular level is too limited to allow calculating the retention characteristics directly from the physico-chemical parameters of solutes. Hence, sophisticated theoretical models generally are not suitable for practical prediction of retention data. Simple semi-empirical or empirical models are more suitable to provide expressions that can be directly applied for prediction and optimization of RP separations. Various methods of correlation of retention - usually expressed in the term of logarithm of the

retention factor - with some structural descriptors have been proposed for this purpose. Alternatively, the retention can be characterized in relation to a set of standard compounds used to calibrate the retention scale.

3. Retention models and experimental data - practical considerations

The retention data are measured in terms of retention times, t_R, which depend on the flow rate of the mobile phase and on the column dimensions. The retention times can be normalized to dimensionless retention factors, k, independent of the flow rate and column geometry *via* the column hold-up time (dead time), t_m, which is the time necessary to elute the whole volume of the mobile phase contained in the column: $k = (t_R - t_m)/t_m$, The retention factor is directly proportional to the distribution constant in the system, K_D, *via* the phase ratio in the column, Φ, i.e., the ratio of the volumes of the stationary phase, V_s, and of the mobile phase, V_m: $k = K_D \cdot \Phi = K_D \cdot (V_s//V_m)$. This means that for correct thermodynamic correlation of the experimental retention data, the volumes of both phases in the column should be known. Unfortunately, the stationary phase in RP is not very clearly defined under changing operation conditions (is this the volume of the bonded moiety only, should the volume of the support be included and how can we include the volume of the liquid solvating the bonded chains into the volume of the stationary phase??), so that the determination of the phase volume in the column is not straightforward. For meaningful correlations of the retention and thermodynamic data, at least the phase ratio should be constant and independent of changing operation conditions and the volume of the mobile phase in the column (V_m) (or the column hold-up time, t_m) should be accurately determined. Even the exact measurement of V_m may be problematic and hence some theoreticians put the very definition of the retention factor into question.

The determination of the hold-up time in RP LC has been studied extensively since the introduction of this separation mode [G.E. Berendsen, P.J. Schoenmakers, L. de Galan, G. Vigh, Z. Varga-Puchony, J. Inczedy, J. Liquid Chromatogr. 3 (1980) 1669]. The static method based on the calculations of V_m from the differences in weight of the packed column filled with two solvents of sufficiently different densities does not consider the solvation liquid layer in the stationary phase and provides the upper limit of the hold-up volume in the column. Another method, originating from gas chromatography, assumes a linear relationship between the logarithm of the net elution time, log ($t_R - t_m$), and the number of carbon atoms, n, in a homologous series. Based on this assumption, t_m can be calculated from the best-fit regression constants of the t_R *versus* n data plot.

Most frequently used and most convenient is the determination of t_m from the elution time of a suitable non-retained marker compound. This of course raises the question of the

correct selection of such a hold-up time marker. The most simple approach estimates the hold-up time from the first base-line disturbance in the chromatogram. This method can be very inaccurate, especially if the sample contains large or negatively charged molecules, which may be partly excluded from the pores of the column packing and provide thus underestimate hold-up times. The same applies to bromide, nitrate, or other small inorganic anions occasionally used as hold-up volume markers, which may be excluded from a large part of pores due to repulsive electrostatic interactions with partially ionized residual silanol groups in the stationary phase. Theoretically more accurate should be employing the deuterated (or non-deuterated) pure solvents used as the components of the mobile phase (water, methanol or acetonitrile), but these compound require refractometric detection, which is rarely available in laboratories nowadays. Hence, small polar molecules such as uracil or thiourea are most frequently used as the markers of the column hold-up volume. However, the elution times of these compounds, like the elution times of pure solvents depend more or less on the composition of the mobile phase, on which they often show a U-turn dependence [G.E. Berendsen, P.J. Schoenmakers, L. de Galan, G. Vigh, Z. Varga-Puchony, J. Inczedy, J. Liquid Chromatogr. 3 (1980) 1669].. It is difficult to decide whether this dependence reflects correctly the effects of the mobile phase on the hold-up volume, or whether the marker compounds may be retained in the mobile phases with a low concentration of organic modifier (RP mechanism) and (or) in the mobile phases with low concentrations of water (HILIC mechanism, see more details in the paragraph on dual retention mechanism in RP systems) - the second case seems more probable.

As for the practical use of various retention models we need to be able to measure the retention factors as correctly as possible under changing operating conditions, such as composition of the mobile phase or temperature, we need to use some convention concerning the determination of the column hold-up volume. Probably the best possibility is to use a single V_m value for a given column, regardless the changing operation conditions, such as the elution volumes of uracil or thiourea in a mobile phase containing 50 - 70% organic modifier, which usually correspond to the realistic value of the total column porosity corresponding to 65 - 70% of the void column volume for conventional C_8 and C_{18} particulate bonded phases. If necessary, the volume of the stationary phase may be calculated as the difference between the volume of the empty column tube and V_m determined in this way. The accuracy of this approach can be criticised, but at least this approach is practical and - if widely used - may provide comparable retention data measured in different laboratories as a remedy to common practice of most k published in literature, determined using various approaches, which are often non-specified. Further, the considerations of the reliability of the thermodynamically useful retention data strongly favour RP models based on a relative retention scale, as most errors in the

measurements are likely to largely compensate if the retention data are related to a reference compound.

Important practical consideration concerns the determination of the model equation parameters. The models usually are described by equations such as log k = f(x_1, x_2, ...), where x_i denote one or more operation parameters (temperature or volume fractions of one or more components of the mobile phase) and f is the mathematical function such as linear, polynomial, logarithmic, exponential or combined function). The model parameters are usually determined by linear or non-linear regression, fitting the data to the model equation. The fitting procedure is based on the least square method, minimizing the sum of the second powers of differences between the experimental data and the data calculated from the best-fit model equation: Σ (log $k_{i,exp}$ - log $k_{i,\,cal}$)^2. The application of this approach usually assumes the normal (Gaussian) error distribution, which may apply for the elution times, but generally is not the case for log k. Due to the error propagation law, small errors in t_m lead to much stronger errors in small k than in large ones. Hence, fitting experimental k (or, even worse, experimental log k) may affect the accuracy of the best-fit model parameters. This means that the data fitting should theoretically employ non-linear regression based on t_R. However, usual commercial data editor software has linear regression options only, which requires data linearization, introducing errors. Non-linear regression can be performed using special statistical software, which id commercially available, but is less common in most laboratories. Further, the optimization algorithms often may not converge if not enough data points are used in the non-linear regression, or may provide non-realistic or biased results if the data are subject to a systematic error, such as wrong value of t_m.

Reliable determination of more than three parameters of retention equations by fitting the model requires a large number of experimental data obtained over a sufficiently broad range of experimental data, which often would require impractically time-consuming measurements of very high retention factors. As a rule of thumb, increasing number of parameters in the model equations generally provide better fit to an experimental set of data, but better fit alone does not prove the physical significance of underlying theoretical model, sometimes it means better fit to a systematic experimental error in data acquisition..

Hence, the retention models should be as simple as possible to be useful in practice for method development and optimization, even though they may not be thermodynamically rigorous. More than three-parameter model equations for one operation variable condition rarely bring significant improvement in the prediction of retention for increased efforts and time in the acquisition of necessary experimental data.

4. Theoretical models of retention mechanism in reversed-phase LC

Hundreds original research papers, tens of books, chapters in monographs and special volumes in prestigious journals dealing with this topic, yet there is still no common agreement on the basic idea whether the retention in RP LC is a partition or an adsorption process, or a combination of both. In spite of widespread applications, the exact mechanism of retention in reversed-phase chromatography is controversial. A realistic retention model of reversed-phase LC should characterize the whole chromatographic system, comprising the role of the sample structure, of the column (stationary phase) of the mobile phase, temperature, etc.. However, because of complexity of the involved phenomena, various theoretical models emphasize different aspects of the retention process. Reversed-phase LC involves a plethora of interactions: between the mobile phase components and the stationary phase, between the analyte and the stationary phase and the analyte and the mobile phase. In analytical LC, the sample concentration is low enough so that the interactions between the molecules of the analytes need not be taken into account, which however does not apply to overloaded column conditions controlled by non-linear distribution isotherms, used in preparative LC. Some models are essentially transferred from gas chromatography and do not pay much attention to the role of the mobile phase in separation, some other models assume the predominant role of interactions in the mobile phase.

Thermodynamic models understand the retention process as establishing the equilibrium between the individual system components in the stationary and in the mobile phase to achieve the lowest energy in the whole system. Molecular modelling employing the quantum chemical calculations such as molecular dynamics or Monte Carlo simulations have achieved amazing progress recently [J.L. Rafferty, L. Zhang, J.I. Siepmann, M.R. Schure, Anal. Chem. 79 (2007) 6551] and simulation calculations give good idea on the distribution of molecules on the microscopic scale, but due to the complexity of calculations involved, they usually cannot take into account interactions between more than several tens or several hundreds of molecules at maximum. Such simulations are helpful to get insight into the interactions at the molecular level, but still it is difficult to transfer them in a straightforward manner to macro-scale thermodynamic description of the reversed-phase retention process, useful for method development and optimization.

The most important thermodynamic models of RP LC include those based on the partition theory [K.A. Dill, J. Phys. Chem., 91 (1987) 1980], the Hildebrand solubility parameter theory, [J.H. Hildebrand, R.L. Scott, *The Solubility of Non-Electrolytes*, Dover, New York, 1964], the regular solution theory [R. Tijssen, H.A.H. Billiet, P.J. Schoenmakers, J. Chromatogr., 122 (1976) 185], the UNIFAC theory [J.H. Park, J.E. Lee, M.D. Jang, J.J. Li, P.W.

Carr, J. Chromatogr., 586 (1991) 1], the Flory-Huggins mean field lattice theory [D.E. Martire and R.E. Boehm, J. Phys. Chem., 87 (1983) 1045] (for polymers in solutions), the concept of molecular connectivity [B. L. Karger, J.R. Gant, A. Hartkopf and P.H. Weiner, J. Chromatogr., 128 (1976) 65], the solvophobic theory [Cs. Horváth, W. Melander and I. Molnár, J. Chromatogr., 125 (1976) 129], [Cs. Horváth and W. Melander, J. Chromatogr. Sci., 15 (1977) 393], the statistical thermodynamics [R.E. Boehm, D.E. Martire, J. Phys. Chem., 84 (1980) 3620], the adsorption mechanism [R.P.W. Scott, J. Chromatogr. A, 656 (1993) 51], or combinations of adsorption and partition in partition-displacement model [M. Jaroniec, J. Chromatogr. A, 656 (1993) 37].

4.1 Partition models: Solvophobic and regular solution theories

In the early years of development, chemically bonded phases for HPLC were considered by many authors as immobilized liquid stationary phases. Hence it is not surprising that some retention models originally developed to describe the distribution of a sample between the stationary and the mobile phase in liquid-liquid chromatography by partition mechanism were transferred to reversed-phase chromatography on chemically bonded phases. The partition mechanism assumes that sample molecules are fully surrounded by the alkyl chains in the stationary phase. The partitioning process is controlled by the differences in the molecular interactions of the sample with the stationary and with the mobile phase.

A popular partition model employs the theory characterizing the molecular interactions by solubility parameters, $\delta = (-E/V_x)^{1/2}$, which are a measure of the connectivity of molecules, proportional to the square root of the cohesive energy density, $-E$, characterizing the sum of all intermolecular forces, including interactions between permanent and induced dipoles, proton-acceptor and proton-donor interactions and dispersion interactions. (V_x is the molar volume of a liquid substance) [J.H. Hildebrand, R.L. Scott, *The solubility of Non-Electrolytes*, Dover, New York, 1964]. According to this model, the energy of interactions is proportional to the product of solubility parameters of the molecules in contact in the separation system, including the sample, the mobile and the stationary phases. Hence, the distribution constant is related to the differences between the solubility parameters of the sample and of the stationary phase on one side and between the sample and the mobile phase on the other and is proportional to the molar volume of the sample.

The solubility parameters form the basis of the regular solution theory, assuming random mixing of the system components. Based on these models, relationships between the retention factor, temperature and composition of a two-component aqueous-organic mobile phase in RP chromatography were developed [P. Jandera and J. Churáček, J. Chromatogr., 91 (1974) 207],

[R. Tijssen, H.A.H. Billiet and P. Schoenmakers, J. Chromatogr., 128 (1976) 65]. Even though the attempts to calculate *a-priori* the constants of the model equations directly from the published solubility parameters were not successful, the results were in qualitative agreement with theory, using equation parameters based on experimental measurements [P. Jandera, J. Churáček and L. Svoboda, J. Chromatogr., 174 (1979) 35], [P.J. Schoenmakers, H.A.H. Billiet, R. Tijssen and L. De Galan, J. Chromatogr., 149 (1978) 519].

The regular solution theory in RP chromatography was criticized for inaccuracy when used for quantitative predictions [P.W. Carr, J. Li, A.J. Dallas, D.I. Eikens, L.C. Tan, J. Chromatogr. A, 656 (1993) 113]. One problem is the vagueness of the definition of the solubility parameter of the stationary phase, which is difficult to be measured experimentally. Further, the underlying solubility parameter equations apply only to the systems in which the excess entropy and the volume of mixing liquids are zero, which does not apply to water as a solvent [B.L. Karger, L.R. Snyder, C. Eon, Anal. Chem., 50 (1978) 2128]. The chemically bonded phases are generally considered to be monomolecular layers in which chemical bonds to the support surface decrease the chain mobility and thus also the entropy of the bonded phase, compared with the free molecules in liquid stationary phase. However, partial chain mobility permits the solute molecules to penetrate between the bonded chains to various depths, depending on the solute structure, length of the bonded alkyl, composition of the mobile phase and temperature [G.E. Berendsen, L. De Galan, J. Chromatogr. 1980, 196, 21].

The UNIFAC theory is an alternative to the regular solution theory, based on the idea that a solution can be viewed as a mixture of independent functional groups of all the components in solution and assumes that any functional group contributes to the activity coefficient of a molecule independently of the other functional groups, so that the free energy of interactions of one species with another one is the additive sum of independent functional group contributions. Structural parameters derived from the Van der Waals surface areas and volumes of the functional groups are used to calculate activity coefficients in mixed mobile phases [J.H. Park, M.D. Jang, J.J. Chae, H.C. Kim, J.K. Suh, J. Chromatogr. A, 656 (1993) 69]. The model is not accurate enough for quantitative prediction of retention, but it yieldss semi-empirical equations in agreement with the solubility parameter and regular solution theory.

Horváth et al. [Horváth, C.; Melander, W.; Molnár, I., *J. Chromatogr.*, 125 (1976) 129; Horváth, C.; Melander, W., *J. Chromatogr. Sci.*, 15, (1977) 393] developed the retention model for RP LC based on solvophobic theory [O. Sinanoglu, in: B. Pullman, ed., *Molecular associations in Biology*, Academic Press, New York, 1968, p. 427.]. Here, the mobile phase (solvophobic) interactions are understood as the main driving force of the formation of associates of the

solutes with the non-polar stationary phase. The solvophobic model distinguishes three steps contributing to the free energy change in the solvation process: creation of a cavity in the mobile phase to accommodate the sample molecule, reduction in the free volume and interactions of the molecule placed in the cavity with the surrounding solute molecules. The retention results principally from a decrease in the contact area of the solute with the mobile phase caused by its transition from the bulk mobile phase to the surface of the stationary phase. Replacement of weaker interactions between a non-polar or a moderately polar solute and polar mobile phase by mutual interactions between strongly polar molecules of the mobile phase in the space element of the mobile phase originally occupied by a solute molecule results in overall energy decrease in the phase system, which is the driving force of the retention in absence of strong (polar) interactions of the solute with the stationary phase. The model yields rather complicated expressions for the retention in terms of log *k*, including various physico-chemical parameters, which are not readily available, so that practical use of the solvophobic model for characterization and prediction of retention is difficult.

4.2. Adsorption and Partition-displacement models

According to the adsorption model, the sample molecules are adsorbed at a surface solution formed on the bonded alkyl chains in the stationary phase [J.G. Dorsey, K.A. Dill, Chem. Rev. 89 (1989) 331]. The surface solution is formed of the mobile phase components, but in different concentration ratio than in the bulk mobile phase. The adsorbed sample molecules displace the molecules of the organic solvent adsorbed at the interface layer [R.P.W. Scott, J. Chromatogr. A, 656 (1993) 51]. The combined partition-displacement model characterizes the retention as two-step process, involving the formation of an adsorbed solvent layer by a displacement mechanism, followed by the partition of the sample between the adsorbed layer and the bulk mobile phase [M. Jaroniec, D.E. Martire, J. Chromatogr., 351 (1986) 1].

5. Semi-empirical models.

Rather than attempting to provide rigorous thermodynamic description of RP separation systems, which are difficult to apply in practice because of the lack of accurate enough calculation parameters, semi-empirical models use simple equations to relate some basic thermodynamic properties to the operation variables of the separation system, to enable reasonable estimates of retention on changing operation conditions from readily accessible information. Usually, some thermodynamic meaning can be often attributed to the equation parameters, usually at a cost of significant simplifications.

5.1. Retention indices.

Retention indices have been for long years used to characterize the retention in gas chromatography (GC), where mobile phase effects are absent [Pacáková, V.; Feltl, L. *Chromatographic Retention Indices. An Aid to Identification of Organic Compounds*, E. Horwood, Chichester, 1992]. The use of retention indices in LC is less straightforward, as the retention is strongly affected by even minor changes in the mobile-phase composition; hence the LC retention indices usually can be applied only over a limited mobile phase composition range. In this approach, sample retention is related to that of a series of standards with regular structural increments. The selection of suitable standards is essential for successful use of a retention-index scale.

RPC is especially useful for separations in homologous or oligomeric series with different number of non-polar or weakly-polar structural units. Assuming that the retention follows the Martin rule of the additivity of structural contributions to the free energy of retention, which is directly proportional to the logarithms of the retention factors (log *k*) [A.J.P. Martin, *Biochem. Soc. Symp.*, 3 (1949) 4], linear relationship between the log *k* and the number of carbon atoms (i.e., the number of repeat methylene groups), n_C, (Eq. 1) applies in various homologous series - **Figure 1A** [P. Jandera, J. Chromatogr., 314 (1984), 13]:

$$\log k = \log \alpha \; n_C + \beta \qquad (1)$$

a characterizes the methylene group (hydrophobic) selectivity and *β* represents the contribution of the end group in the series to the retention.

Eq. (1) can be applied also to lower oligomers, where n_C denotes the number of various more or less polar repeat monomer units, up to molar masses of 2000–3000 Da [P. Jandera, J. Chromatogr., 449 (1988), 361].

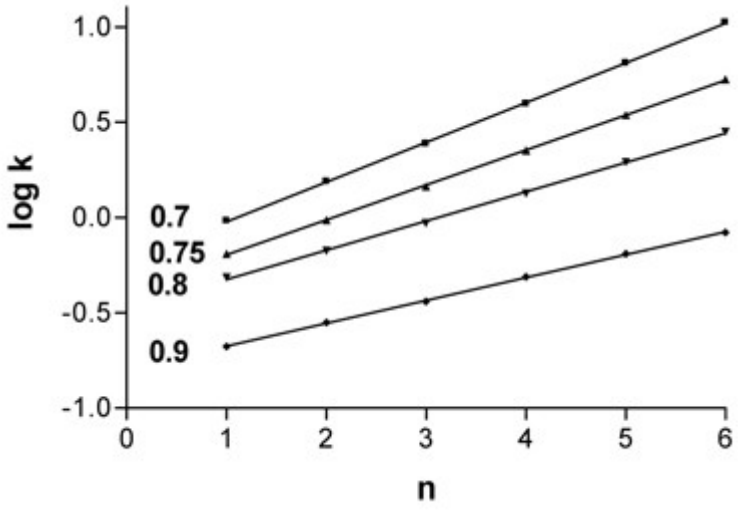

Fig. 1A. 1Retention factors, **k**, of homologous n-alkylbenzenes with **n** carbon atoms in the alkyls. Silasorb SPH C18 (7.5 μm) column

(300x4.0 mm I.D.); mobile phases containing 60 (1), 65 (2), 70 (3), 80 (4) and 90 (5) % vol. methanol in water. Points - experimental data, lines - best fit plots of Eq. (1).

Isocratic conditions usually provide separations of a limited number of oligomers only and gradient elution should be used to accomplish separation over a broader range of repeat monomer units. Unlike to homologous series, the oligomer selectivity term, log α, can be either positive or negative, depending on whether the oligomers elute in the order of increasing or decreasing size. Even though the retention in RP HPLC usually increases with increasing sample size, some oligomers with relatively polar repeat monomer units may behave opposite to this rule of thumb. This occurs if the polarity of the mobile phase is lower than the polarity of the repeat monomer group, which then shows a higher affinity to the mobile than to the stationary phase and increasing number of the repeat monomer units speeds up the elution - see example in **Figure 1B**.

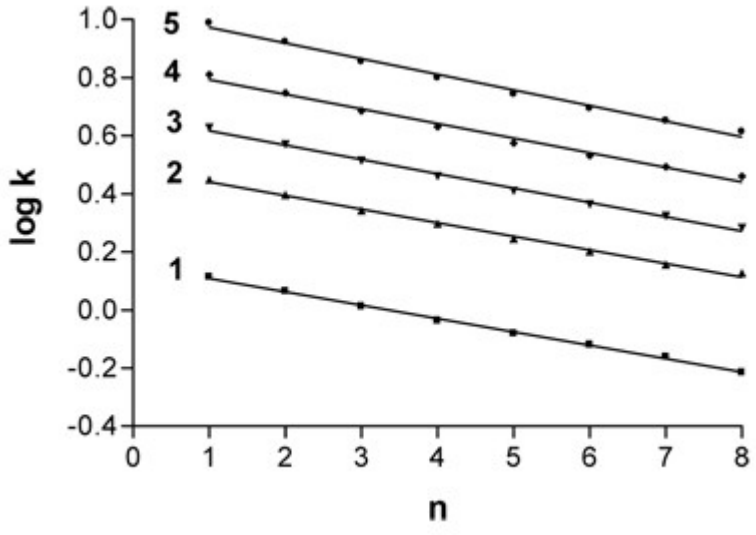

Fig. 1B. Retention factors, **k**, of oxyethylene nonylphenyl ethers with **n** oxyethylene groups. Silasorb SPH C18 (7.5 μm) column (300x4.0 mm I.D.); mobile phases containing 60 (1), 50 (2), 45 (3), 40 (4) and 35 (5) % 2-propanol in water. Points - experimental data, lines - best fit plots of Eq. (1).

Separation selectivity in a homologous or in an oligomer series usually increases with increasing molar volume and with increasing polarity and decreasing concentration of the organic solvent in the mobile phase. This may explain some unexpected and apparently strange reversal of the elution order for oligomers with the same repeat monomer group, but different end groups. For example, ethylene glycols elute in the order of increasing size in propanol - water mobile phases (α>1), but the order of elution of oxyethylene nonylphenyl ethers is reversed, as the bulky nonylphenyl end group requires a higher concentration of the organic solvent in the mobile phase to accomplish the elution in short enough time. In these mobile phases, the oligomer selectivity decreases to α <1 [P. Jandera, Chromatographia, 26 (1988) 417].

The parameters *a* and *B* of Eq. (1) determined in different RP systems can be used to compare hydrophobic properties of stationary phases in liquid chromatography and to calibrate the scale of retention indices, *I*:

$$\log k = A + BI \qquad (2)$$

The contribution of a methylene group to the retention indices is attributed a constant value, most frequently $\Delta I_{(CH2)}$ = 100. Retention indices of non-homologous compounds are determined by logarithmic interpolation between the retention factors of a series of homologous standards and provide a measure of sample hydrophobicity to retention. However, the homologous series of compounds to be widely applicable as a retention index scale in RPLC should meet several criteria [V. Pacáková, L. Feltl, *Chromatographic Retention Indices. An Aid to Identification of Organic Compounds*, E. Horwood, Chichester, 1992, p. 285] ; [R.M. Smith, J. Chromatogr., 236 (1982) 313]:

(a) Strong UV absorption at 254 nm, allowing addition of only small amounts of standards to samples;
(b) Constant retention over a broad pH range and in the presence of ion-pair reagents;
(c) availability at reasonable cost;
(d) retention time range covering the elution time interval of all sample components;
(e) good chemical stability in common liquid chromatography mobile phases;
(f) linear plots between log *k* and the number of carbon atoms or characteristic functional groups in the molecules of the calibration standards;
(g) absence of interactions with the support (silica gel);
(h) independence of the retention indices on the mobile phase composition.

These criteria rule out *N*-alkanes, used for the calibration of the retention index scale in GC, or halogenoalkanes, because of the absence of UV absorption, and many amines, phenols and weak organic acids, whose ionization changes in dependence on pH. Obviously, aromatic compounds are most suitable standards for the calibration of the retention indices scale in reversed-phase HPLC. Alkylbenzenes, benzoic acid esters, polycyclic aromatic hydrocarbons, alkylarylketones, but also aliphatic alkan-2-ones have been suggested as suitable candidates [R.M. Smith, Adv. Chromatogr., 26 (1987) 277]; [R.M. Smith, N. Finn, J. Chromatogr. A 537 (1991) 51]; [R.M. Smith, in *Retention and Selectivity in Liquid Chromatography*. R.M. Smith, Ed.; Elsevier, Amsterdam, 1995; 93–144].The retention indices scale based on alkylarylketones [R.M. Smith, J. Chromatogr., 236 (1982) 313] has been successfully applied in reversed-phase HPLC of various samples [R.M. Smith, T.G. Hurdley, R. Gill, A.C. Moffat, Chromatographia, 19 (1984) 401]; [R.M. Smith, T.G. Hurdley, R. Gill, A.C. Moffat, Chromatographia, 19 (1984) 407].

Retention indices determined by logarithmic interpolation between the retention factors of a calibration homologous series are reproducible at isocratic conditions (constant composition of the mobile phase and constant temperature) and can provide a basis for measurements of polar functional group contributions to the retention. The substituent increments of functional groups, I_S, can be determined as the differences between the retention index of a parent compound, $I_{R\text{-}H}$, and those of derivatives with functional group substituents, $I_{R\text{-}F}$:

$$I_S = I_{R\text{-}F} - I_{R\text{-}H} \quad (3)$$

The substituent increments I_S depend less on the composition of the mobile phase than the retention indices of functionalized more or less polar compounds and reflect the effects of specific interactions with different stationary phases. They can be transferred between various homologous series for first-approximation prediction of the effects of functional groups on retention. Further, I_S allow direct comparison of different columns in reversed-phase HPLC with respect to the substituent selectivity within a specific class of compounds.

5.2. The model of interaction indices

In spite of its inherent limitations, the solvophobic theory of retention is useful as the starting point in the derivation of a simplified semi-empirical description of reversed-phase systems to characterize and predict the retention and the selectivity of separation in reversed-phase chromatography in the model of interaction indices [P. Jandera, H. Colin and G. Guiochon, Anal. Chem., 52 (1982) 435], where the interactions with the non-polar stationary phase are considered less significant than the polar interactions in the mobile phase, which are the main driving force of the retention. To first approximation, the transition of a solute molecule from the bulk mobile phase to the surface of the stationary phase results from a decrease in the contact area of the solute with the mobile phase, like in the solvophobic interactions models. Replacement of weaker interactions between a moderately polar solute and a strongly polar mobile phase by mutual interactions between strongly polar molecules of the mobile phase in the space originally occupied by a solute molecule results in overall energy decrease in the system, which is the driving (solvophobic) force of the retention in absence of strong (polar) interactions of the solute with the stationary phase.

Hence, the retention decreases with increasing polar interactions, but decreases with the volume in which the interactions occur, proportional to the molar volume of the solute, V_X. The effect of V_X is distinguished from the polarity effects, measured in terms of interaction indices, I_X. To distinguish between the two structural effects, "*specific retention factors*", k^*, corrected for V_X are used, which decrease with increasing I_X of sample solutes (Eq. (4)) [P. Jandera, H. Colin and G. Guiochon, Anal. Chem., 52 (1982) 435]:

$$\log k^* = \frac{\log k - \log \phi}{V_X} = A - BI_X \qquad (4)$$

The parameters *A* and *B* depend on the column type and on temperature and decrease at increasing volume fraction of the organic solvent in the aqueous-organic mobile phase, φ. Ideally, I_X are independent of the mobile phase composition. $\Phi = V_S / V_M$ is the phase ratio, i.e., the ratio of the volume of the stationary, $V_S = (V_C - V_M)$, and the mobile, V_M, phases in the column. V_M is determined as the elution volume of a non-retained column hold-up volume marker (uracil or thiourea); V_C is the geometrical volume of the empty column. Interaction indices of some standard compounds potentially useful for calibration of the I_X scale are listed in **Table 1**.

Table 1. Retention test standards and their descriptors.
I_X – interaction index, Eq. (4), V_X – McGowan characteristic volume ($cm^3/mol.10^{-2}$), π_2^{*H} – solute dipolarity/polarizability, $\Sigma\alpha_2^H$ – hydrogen bonding basicity, $\Sigma\beta_2^H$ – hydrogen bonding acidity, Eq. (7).

Analyte No.	***Analyte***	***Symbol***	$I_{X\ (theor)}$	V_X	π_2^{*H}	$\Sigma\alpha_2^H$	$\Sigma\beta_2^H$
1	Benzene	BE	2.76	0.716	0.52	0.00	0.14
2	Toluene	MB	2.46	0.857	0.52	0.00	0.14
3	Ethylbenzene	EB	2.44	0.998	0.51	0.00	0.15
4	Propylbenzene	PB	N.A.	1.139	0.50	0.00	0.15
5	Butylbenzene	BB	1.93	1.280	0.51	0.00	0.15
6	Amylbenzene	AB	1.68	1.421	0.50	0.00	0.15
7	Hexylbenzene	HB	1.43	1.562	0.50	0.00	0.15
8	Phenol	PHE	8.55	0.775	0.89	0.60	0.30
9	Benzonitrile	BENI	5.32	0.871	1.11	0.00	0.33
10	Nitrobenzene	NB	4.49	0.891	1.11	0.00	0.28
11	Anisole	ANS	3.85	0.916	0.75	0.00	0.29
12	*p*-dichlorobenzene	DCLB	1.05	0.961	0.75	0.00	0.02
13	*3*-chlorotoluene	CLT	2.20	0.980	0.67	0.00	0.07
14	Aniline	ANL	6.62	0.816	0.96	0.26	0.50
15	Acetophenone	ACE	5.60	1.014	1.01	0.00	0.48
16	*2*-bromonitrobenzene	BRNB	3.44	1.066	1.32	0.00	0.26

The parameters *A* and *B* of Eq. (4) should be determined in each stationary/mobile phase system using a set of suitable calibration standards covering the interaction indices scale (Table 2) [H. Colin, G. Guiochon, P. Jandera, Anal. Chem. 55 (1983) 442], [H. Colin, G. Guiochon, P. Jandera, Chromatographia, 17 (1983) 83] The parameter *A* is a measure of column

hydrophobicity; the parameter *B* is the response of the retention to a change in the sample polarity (including all selective contributions) and can be used to characterize the column selectivity for polar compounds. Eq. (4) applies not only for bonded alkylsilica stationary phases, but also for non-polar organic polymer stationary phases such as polymethacrylate monolithic columns (Fig. 2).

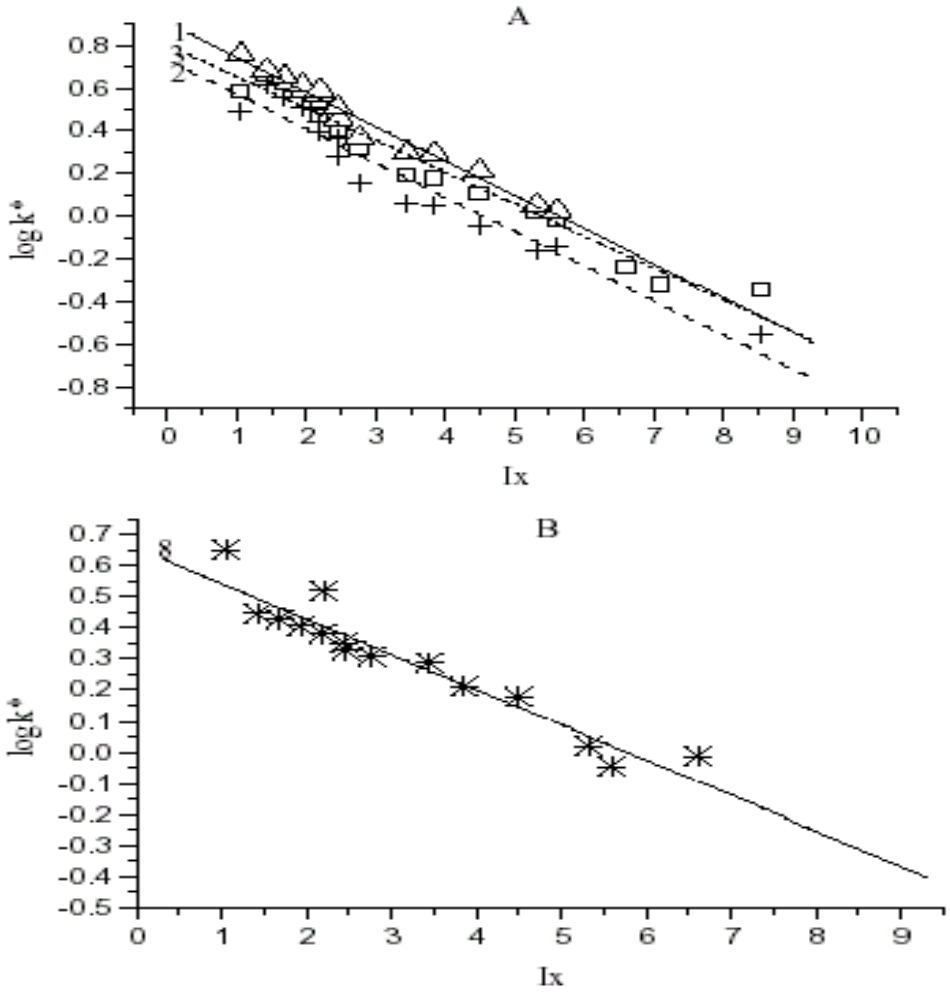

Fig. 2. Validity of the interaction indices model (Eq. (4)). Experimental data - points; lines – best fit plots. A – three alkylsilica C18 columns, B – polymethacrylate column; Mobile phase 70% acetonitrile.

Non-equilibrium between the selective (dipole-dipole, proton-donor and proton-acceptor) polar interactions with the stationary phase may cause deviations from the linearity of the log k^* versus I_X plots expected at the basis of model Eq. (4), so that the scatter (standard deviation) of the experimental data points around the linear regression calibration line is a measure of specific polar interactions in the stationary phase [P. Jandera, in. R.M. Smith, Ed:. *Retention and Selectivity in Liquid Chromatography*. Elsevier, Amsterdam, 1995, p. 135].

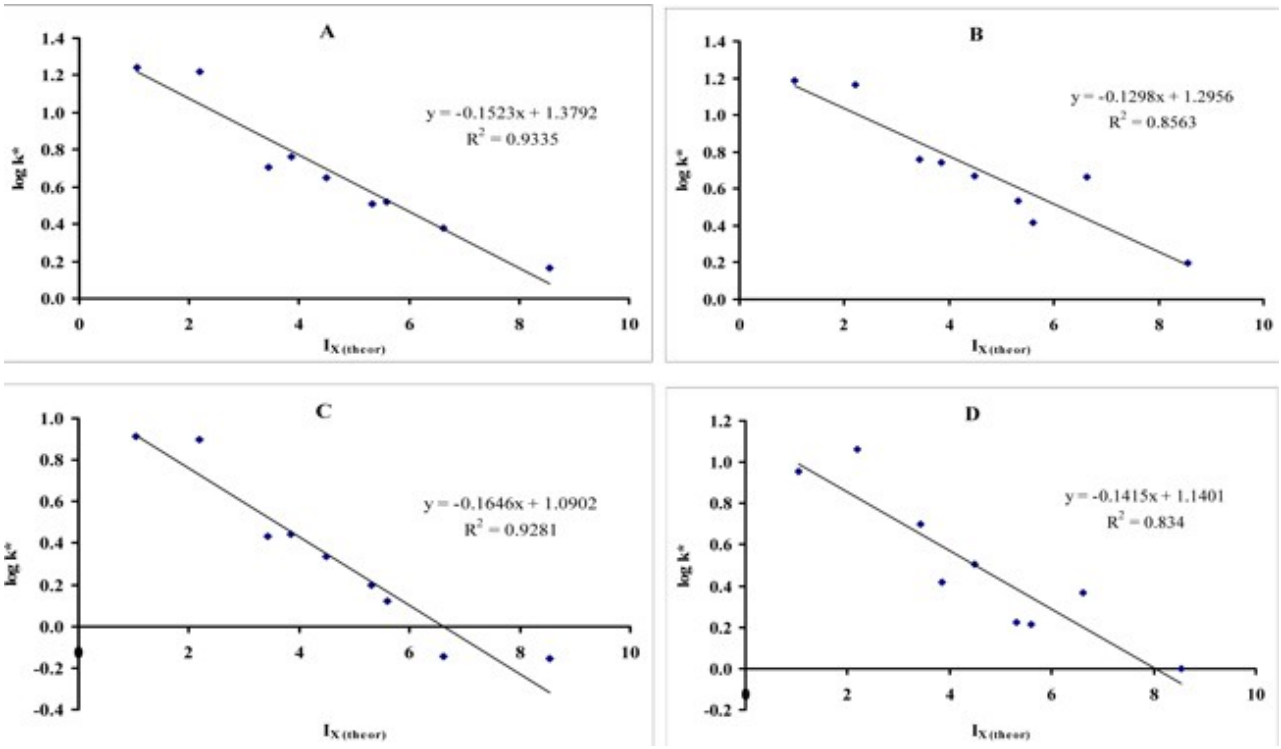

Fig. 3 Effects of the column type on the fit of the interaction indices model, Eq. (4). Columns: Chromolith Performance RP-18e (A), Atlantis dC_{18} (B), XTerra MS C18 (C) and Discovery ZR-CARBON (D) columns. Mobile phase: 50% acetonitrile in water. *(Click on figure to enlarge)*

Fig. 3 illustrates the effects of different polarities of C_{18} stationary phases bonded to various supports: a monolithic alkyl silica stationary phase, Chromolith Performance RP-18e (A), a particulate alkyl silica phase with a polar group incorporated between the silica gel surface and the alkyl group, Atlantis dC_{18} (B), a hybrid silica gel - organic polymer based stationary phase, XTerra MS C18 (C) and a stationary phase with carbon deposited on the surface of zirconium oxide support, Discovery ZR-CARBON (D). Columns B and D with polar support or bonded moieties show impaired correlations between the experimental data and k^* predicted from the interaction indices model (Eq. (4)). **Fig. 4** shows the results of PCA based on the standard deviations of the experimentally determined I_X from the regression line of the log k^* plots in 50% acetonitrile in water.

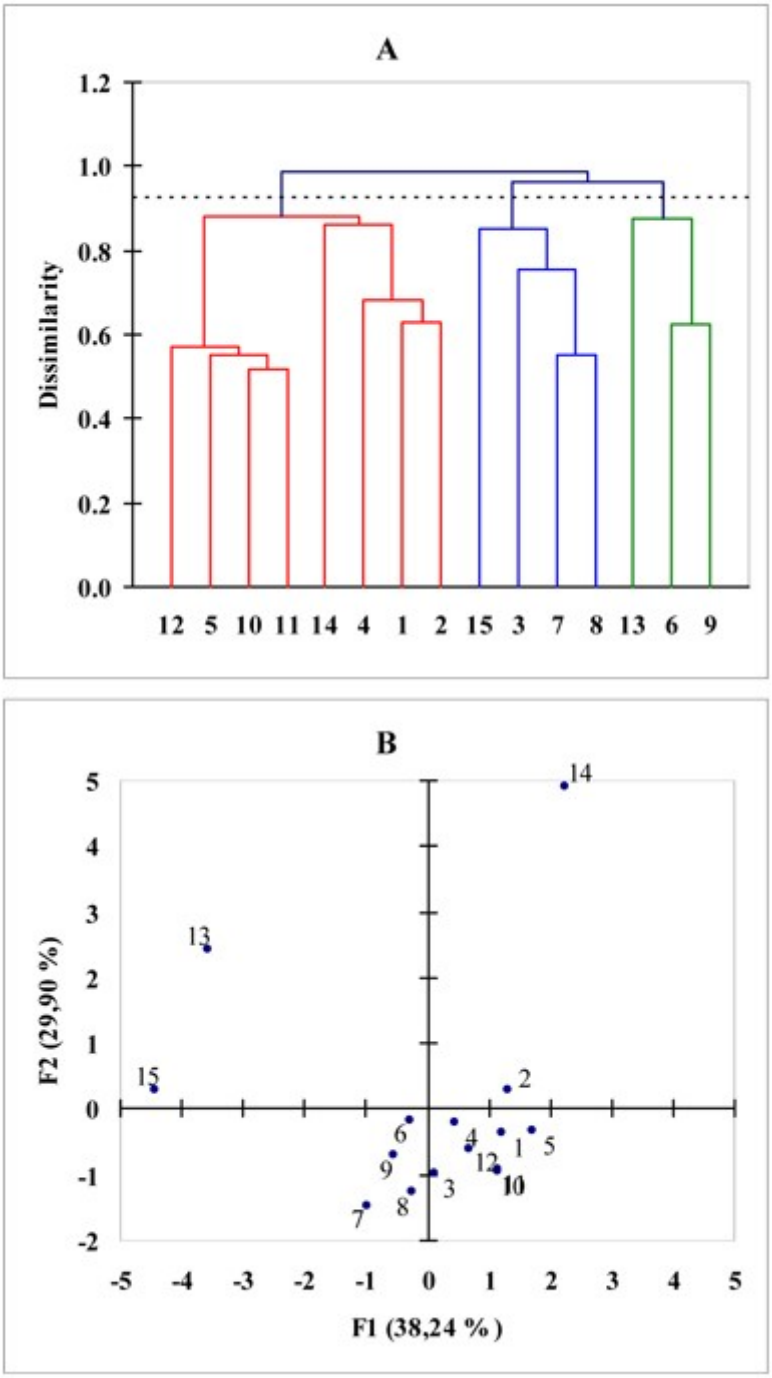

Fig. 4. The similarity dendrogram (A) and PCA score plot (B) for the columns tested with respect to the standard deviations between $I_{X\,(theor)}$ and $I_{X\,(exp)}$ of the analytes tested in methanol-water mobile phase. Columns as Table 2

Because the interaction indices model neglects the effects of the stationary phase, it can be applied only for relative predictions of retention and a suitable set of standard reference compounds is necessary to calibrate the retention (or the selectivity) scale, like in the retention indices approach.. The correct selection of the reference standard compounds for calibration of the log k^* scale is very important for the predictive power of the interaction indices approach. To first approximation, the specific interactions between the solutes and the components of the

mobile phase are neglected. The relative importance of these interactions may depend on the composition of the mobile phase and consequently, more or less significant deviations from the linear log k^* - I_x plots may be observed with some solutes and chromatographic systems. Such compounds should be avoided as reference standards as they would yield incorrect coefficients *A* and *B* of the calibration straight lines fitted by regression analysis to the data in various reversed-phase systems and the predicted capacity factors would be less accurate. Further, the time necessary to perform the calibration experiments makes it impractical to use more than five to six reference standards. These compounds should be selected to cover a rather broad range of mobile phase composition, to be stable, readily available and to absorb in the UV region for convenient detection.

Benzene (I_x = 2.76), toluene (I_x = 2.46), nitrobenzene (I_x = 4.49), acetophenone (I_x = 5.60), and anisole (I_x = 3.85), were initially selected as the calibration standards. Later, various groups of solutes were tested for least deviations from the log k^* *versus* I_x regression lines in different mobile phases. Based on these experiments, a final series of calibration standards was selected, including additional standards: 1,4-dichlorobenzene (I_x = 1.05), 3-chlorotoluene (I_x = 2.20) 3-bromonitrobenzene (I_x = 3.44), nitrobenzene (I_x = 4.49) and benzonitrile (I_x = 5.32) - **Table 1** [H. Colin, G. Guiochon, P. Jandera, Chromatographia, 17 (1983) 83]. Minor differences between the I_x of the standards were found with acetonitrile, methanol, or tetrahydrofuran used as the components of the mobile phases, reflecting different selective polarities of the solvents. However, the relative standard deviations for the interaction indices of the calibration standards in mobile phases with varying concentrations of a given organic solvent were between 4.2 and 6.8%.

6. Models based on correlations between sample structure and retention.

To characterize different types of polar selectivity, the retention scale can be based on non-homologous standard series. For this purpose, a set of column test compounds with different polarities (toluene, nitrobenzene, *p*-cresol, 2-phenylethanol and *N*- methylaniline) can be used as the aromatic equivalents of the standards introduced by Rohrschneider [L. Rohrschneider, J. Chromatogr., 17 (1965) 1] and McReynolds [W.O. McReynolds, J. Chromatogr. Sci. 8 (1970) 685] for characterization of stationary phases in gas chromatography.

6.1. Quantitative structure-retention relationships (QSRR).

Quantitative Structure-Retention Relationships (QSRRs) are statistically derived relationships between chromatographic retention parameters and the quantities characterizing molecular structure (molecular descriptors) of the test analytes [R. Kaliszan, *Structure and Retention in Chromatography. A Chemometric Approach,* Harwood Academic Publishers,

Chichester, 1997]. The concept departs from the correlations between chemical reactivity or biological activity of a substance and its partition coefficient (log *P*) in a two-phase system consisting of n-octanol and water, which has been used for long years to evaluate physicochemical properties of analytes and to predict relative biological activities within a set of drugs [A. Leo, C. Hansch, D. Elkins, Chem. Rev., 71 (1971) 525]. The log *P* values, considered as a standard measure of hydrophobicity, were conventionally determined using time-consuming static "shake-flask" technique. RPLC is attractive method for determination of log *P* on the basis of linear correlations with chromatographic retention data. On one hand, the correlation of log *P* with log k_w extrapolated to 100% water (Eqs. (12) and (13)) was found not to be significantly influenced by the nature of the RP stationary phase within a class of structurally similar compounds [T. Braumann, J. Chromatogr., 373 (1986) 191], on the other hand Abraham et al. [M.H. Abraham, H.S. Chadha, R.A.E. Leitao, R.C. Mitchell, W.J. Lambert, R. Kaliszan, A. Nasal, P. Haber, J. Chromatogr. A, 766 (1997) 35] observed significant influence of the stationary phase on log *P* of a large set of non-congeneric classes of compounds, attributed to the differences in the carbon load, density and length of the bonded alkyls, residual silanols and other properties of the columns for RPLC.

Based on these considerations, QSRR has been used not only for the selection of the most informative structural descriptors and improving the knowledge of the molecular mechanisms of separation, but also was applied for characterization and prediction of retention in RPLC [R. Kaliszan, Anal. Chem., 64 (1992) 619A]. QSRR treats the chromatographic retention as a linear function of a number of different solute-column-mobile phase interactions, described by a set of quantum chemical indices and molecular descriptors provided by calculation chemistry. A variety of descriptors have been tested to characterize the RPLC retention, to some of which it is often difficult to assign any physical sense [R.M. Smith, C.M. Burr, J. Chromatogr., 485 (1989) 325]. A set of molecular descriptors is subject to regression against retention data to obtain the parameters of regression equations, which can be used for estimation of the retention on the basis of the structure of analytes [R. Kaliszan, *Quantitative Structure - Chromatographic Retention Relationship*. Wiley, New York, 1987], [R. Kaliszan, in: P. Cheremisinoff (Ed), *Handbook of Advanced Materials Testing, Quantitative Structure-Chromatographic Retention Relationship*. Marcel Dekker, New York, 1995, p. 87]. The parameters characterize the influence of the chromatographic system (the stationary and the mobile phase) on the retention. This approach is incorporated in a commercial software for prediction of retention from the structure of analytes.

The simplest QSSR approach consists in linear correlation between the logarithms of the retention factors determined under isocratic conditions or the retention times in gradient LC of

selected test analytes and the theoretically calculated logarithms of octanol/water partition coefficients, *c*log *P*, which may be found in the literature [R. Kaliszan, M.A. van Straten, M. Markuszewski, C.A. Cramers, H.A. Claessens, J. Chromatogr. A, 855 (1999) 455]:

$$t_R = k_A + k_B \, c\log P \qquad (5)$$

The parameters k_A and k_B can distinguish the stationary phases with different chemically bonded ligands and/or supports.

6.2. Molecular modelling in QSRR.

The general QSRR equation employs the following analyte descriptors: 1) the total dipole moment, μ, accounting for the dipole-dipole and the dipole-induced dipole interactions of the analyte with the stationary and the mobile phases, 2) the electron excess charge of the most negatively charged atom, δ_{Min}, as reflecting its local analyte polarity and ability to participate in dipole-dipole, charge-transfer and hydrogen-bonding interactions on sub-molecular level and 3) water-accessible molecular surface area, A_{WAS}, describing the dispersive (London type) interactions of the analyte [R. Kaliszan, M.A. van Straten, M. Markuszewski, C.A. Cramers, H.A. Claessens, J. Chromatogr. A, 855 (1999) 455]:

$$t_R = k_1 + k_2\mu + k_3\delta_{Min} + k_4 A_{WAS} \qquad (6)$$

The regression coefficients k_1-k_4 in Eq. (6) characterize the properties of the separation system (i.e., the combination of the stationary and the mobile phase) complementary to the sample properties. If the mobile phase is the same for all columns tested, the coefficients´ k_2-k_4 reflect differences in the properties of stationary phases and enable comparative analysis of columns for RPLC [T. Baczek, R. Kaliszan, K. Novotná, P. Jandera, J. Chromatogr. A, 1075 (2005) 109].

6.3. Linear Free Energy Relationship (LFER) model.

Linear Free Energy Relationships (LFERs) are widely used to characterize chemical and biochemical processes and were successfully applied to liquid chromatography. The correlation between the retention and the structural parameters is based on the equation introduced by Abraham's group [M.H. Abraham, J.C. McGowan, Chromatographia 23 (1987) 243].

$$\log k = c + rR_2 + s\pi_2^{*\mathrm{H}} + a\Sigma\alpha_2^H + b\Sigma\beta_2^H + vV_X \qquad (7)$$

Here, *c* is the intercept, R_2 is the excess molar refraction, π_2^{*H} is a measure of the solute dipolarity and polarizability, Σa_2^H is the solute overall or effective hydrogen bonding acidity,

and ΣB_2^H is the solute overall or effective hydrogen bonding basicity, and V_X is the McGowan solute characteristic volume (**Table 1**). The coefficients *c*, *r*, *s*, *a*, *b* and *v* characterize the phase system, i.e., the particular combination of an RP-HPLC column and mobile phase. With the same mobile phase, the coefficients of Eq. (7) characterize the contribution of the stationary phases to the individual molecular interactions and can be used to characterize and compare various RP columns, like the parameters of Eq. (6) [L.C. Tan, P.W. Carr, M.H. Abraham, J. Chromatogr. A, 752 (1996) 1]. For example, **Fig. 5** shows the results of PCA based on the parameters of Eq. (7) for 15 columns.

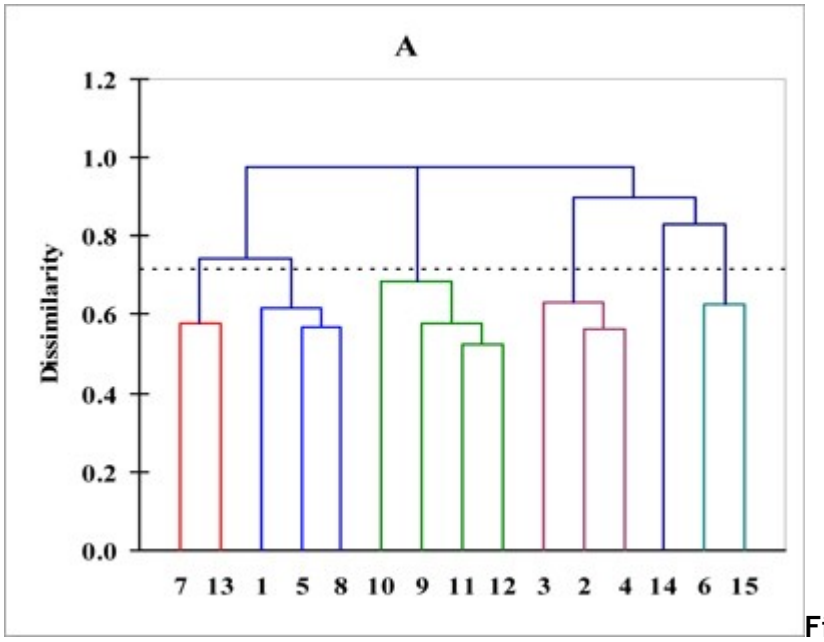

Fig. 5A

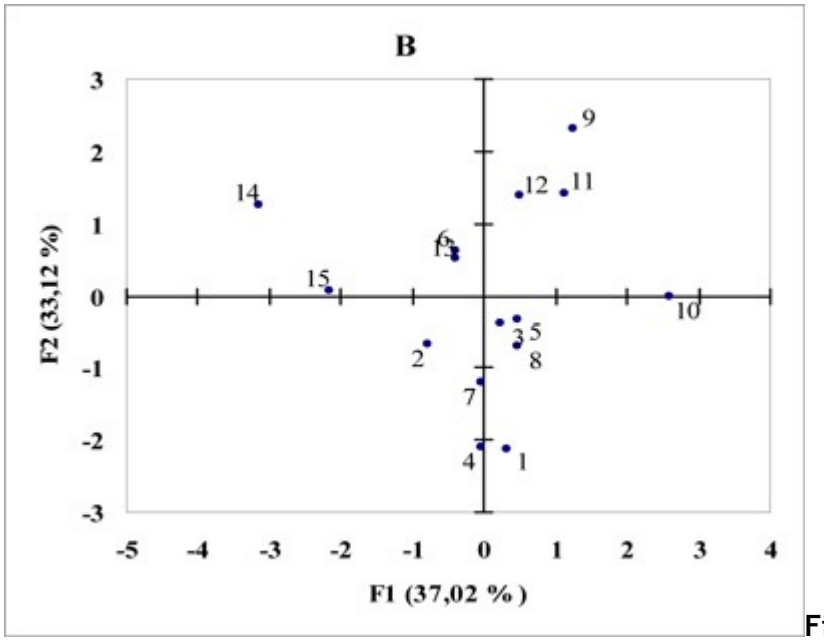

Fig. 5B. Similarity dendrogram (A) and PCA score plot (B) for 15 selected columns with respect to the parameters of LFER equation for the test analytes in **Table 1** in 50% acetonitrile-water mobile phase. Columns as in **Table 2**.

6.4. Linear Solvation Energy Relationships (LSERs).

A QSRR approach based on the Linear Solvation Energy Relationships (LSERs) is a variation of the LFER approach and is based on Eq. (8) using multiple correlations between the retention and so-called solvatochromic parameters, which take into account solubility and solvation of the solute and the stationary phase [L.C. Tan, P.W. Carr, M.H. Abraham, J. Chromatogr. A, 752 (1996) 1], [P.W. Carr, Microchem. J., 48 (1993) 4]:

$$\log k = (\log k)_0 + m_1 V_X / 100 + s_1 \pi_2^* + a_1 \alpha_2 + b_1 \beta_2 \qquad (8)$$

The parameters of Eq. (8) characterize various properties influencing the solubility of analytes: the volume of solvated solute, V_X, polarity, π_2*, hydrogen bonding basicity, B_2, and hydrogen bonding acidity, α_2. Eq. (8) is similar to Eq. (7) in that it allows to correlate the retention of different solutes on the same column and in the same mobile phase with solute properties. The regression coefficients m_1, s_1, a_1 and b_1 in Eq. (8) depend on the stationary and mobile phase properties, so that Eq. (8) can be used for comparison of the contributions of stationary phases to various molecular interactions, and consequently to the retention and selectivity in RPLC, like Eq. (7).

The LSER model was criticized as some of the solute descriptors are not clearly defined and are difficult to reproduce, so that the accuracy of the predicted retention values is not always satisfactory. The V_X term does not account completely for both cohesive and dispersion interactions and the model does not take into account ion-exchange, Lewis acid-base interactions, and shape recognition interactions [L. Rohrschneider, J. High Resol. Chromatogr., 22 (1999) 454], [L. Rohrschneider, J. Sep. Sci., 24 (2001) 3].

6.5. Hydrophobic Subtraction Model.

The Hydrophobic Subtraction Model (HSM) derived on the basis of the LFER model was successfully used for the characterization and comparison of the selectivity of different RP stationary phases. In this model, the hydrophobic contribution to the retention in RPLC is subtracted to better see the contributions of polar interactions to the retention [L.R. Snyder, J.W. Dolan, P.W. Carr, J. Chromatogr. A, 1060 (2004) 77]. The HSM approach resulted in general equation describing the selectivity (relative retention), a, in RPLC:

$$\log \alpha \equiv \log\left(\frac{k}{k_{EB}}\right) = \eta' H - \sigma' S^* + \beta' A + \alpha' B + \kappa' C \qquad (9)$$

Here, k is the retention factor of a solute, k_{EB} the value of k for a non-polar reference solute (such as ethylbenzene, *EB*) measured on the same column under the same conditions.

According to the model, the selectivity-related symbols on the right-hand side of Eq. (9) represent the eluent- and temperature-dependent properties of the solute (η' – hydrophobicity, σ' – molecular size, β' – hydrogen-bonding basicity, α' – hydrogen-bonding acidity′, κ' – partial charge, positive or negative), and the eluent- and temperature-independent properties of the column (H is the hydrophobicity, S^* the steric resistance to penetration of the analyte molecule into the stationary phase, A the column hydrogen bonding acidity, attributed to residual non-ionized silanols, B the column hydrogen bonding basicity due to the water adsorbed in the stationary phase, and C – the column cation-exchange activity due to ionized silanols).

Systematic applications of this model to the retention data of various solutes and columns have provided new insights into the nature of different solute-column interactions and their relative importance in affecting sample retention and separation. The stationary phase characteristics (parameters) of Eq. (9) measured for more than 300 different columns, including silica gel supports with bonded alkyl-, cyanopropyl-, phenylalkyl- and fluoro-substituted stationary phases and columns with embedded or end-capping polar groups, have shown that the HSM represents efficient tool for characterization and comparison of the selectivity of columns for RPLC.

7. Models describing the effects of the composition of mobile phase on retention

For a successful HPLC separation, the components of a binary, ternary or even more complex mobile phase should be adequately selected and their concentration ratio should be adjusted to provide the best separation of the sample mixture, preferably in as short run time as possible. The type of organic solvent in the mobile phase controls the selective polarity properties of the mixed mobile phase, as each solvent shows different preferences for selective dipole-dipole, proton-donor and proton-acceptor polar interactions. Most frequently used organic solvents are acetonitrile with predominating dipole-dipole properties, tetrahydrofuran with proton-acceptor and methanol with both proton-donor and proton-acceptor selectivity. An increase in the concentration of the stronger eluting component in a binary aqueous-organic mobile phase enhances the elution strength and decreases the retention factors of sample solutes.

The composition of the mobile phase controls the interactions of the solvents with analytes, but may also affect the solvation of the stationary phase and modify its properties [H.E. Slaats, W. Markowski, J. Fekete, H. Poppe, J. Chromatogr. 207 (1981) 299], [R.M. McCormik and B.L. Karger, Anal. Chem., 52 (1980) 2249], because of preferential adsorption of the organic solvents from mixed mobile phases. If a relatively small entropic contribution to the retention is neglected, theoretical considerations based either on the model of interaction indices [P. Jandera, H. Colin and G. Guiochon, Anal. Chem., 52 (1982) 435], on the solubility

parameter approach [P. Jandera and J. Churáček, J. Chromatogr., 91 (1974) 207], [R. Tijssen, H.A.H. Billiet and P. Schoenmakers, J. Chromatogr., 128 (1976) 65], or on the molecular statistical theory [D.E. Martire and R.E. Boehm, J. Phys. Chem., 87 (1983) 1062], lead to the derivation of a quadratic equation describing the effects of the volume fraction, φ, of the organic solvent(s) in the mobile phase on the logarithm of the retention factor of a solute, *k*, in a binary aqueous - organic mobile phase :

$$\log k = \log k_W - m\varphi + d\varphi^2 \qquad (10)$$

The constants *a, m, d,* depend on the type of the organic solvent in the mobile phase and of the solute. The quadratic term $d\varphi^2$ controls the extent ofcurvature of the log *k versus* φ plots. The parameter *d* increases with decreasing polarity of the organic solvent. Consequently, the log *k versus* φ plots are often linear in aqueous solutions of methanol (**Fig. 6A, top**), slightly nonlinear in acetonitrile - water mixtures and significantly curved in mobile phases containing tetrahydrofuran in water (**Fig. 6B, bottom**) [P. Jandera, Chromatographia, 19 (1984) 101].

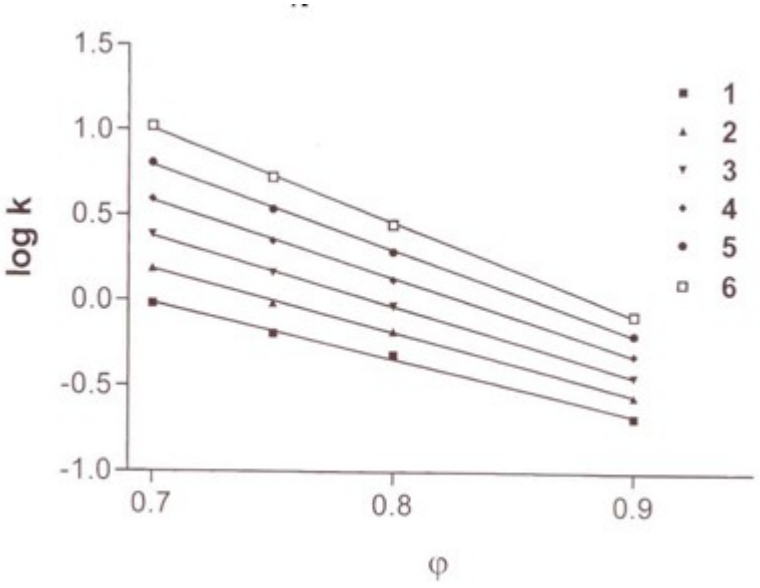

Fig. 6A

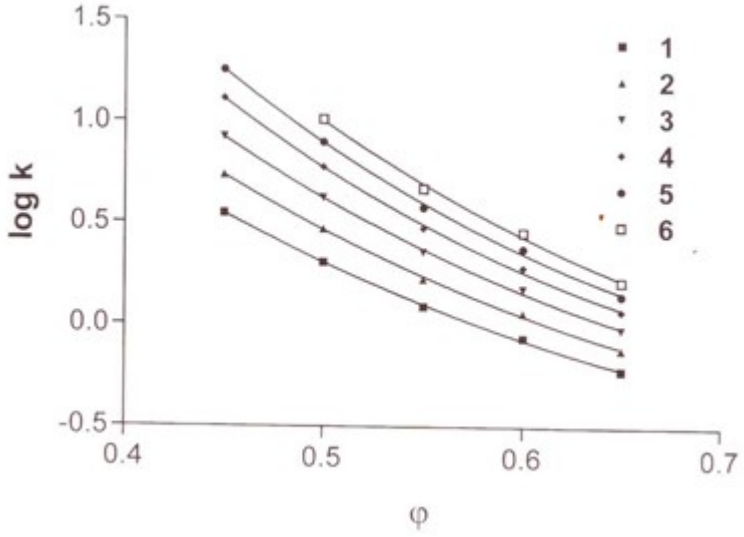

Fig. 6B Effects of the volume fraction of organic modifier on the retention factors, *k*, of *n*-alkylbenzenes (methyl to hexyl) on a C_{18} column. A - methanol; B – tetrahydrofuran.

The parameter *d* and the curvature of the log *k versus* φ plots increase with the size of the solute molecules, but it often can be neglected to first approximation in methanol - water and in acetonitrile - water mobile phases, so that Eq. (10) is reduced to the well-known and widely used linear solvent strength (LSS) equation, Eq. (11). [L.R. Snyder, J.W. Dolan, J.R. Gant, J. Chromatogr. 165 (1979) 3], [P. Jandera and J. Churáček, J. Chromatogr., 91 (1974) 207], [P. Jandera, J. Churáček and L. Svoboda, J. Chromatogr., 174 (1979) 35]:

$$\log k = \log k_W - m\varphi = a - m\varphi \qquad (11)$$

The constants *a* in Eq. (10) and Eq. (11) increase as the polarity of the solute decreases and as its size increases. *a* theoretically stands for the logarithm of the retention factor in pure water as the mobile phase, k_w, but the values of log k extrapolated to pure water from experimental plots using either linear or quadratic regression analysis do not give accurate description of the solute retention in water [P. Jandera, J. Kubát, J. Chromatogr., 500 (1990) 281] and may be up to one order of magnitude lower than the experimental retention factors in pure water (which are too large to be readily determined on a conventional column, but can be aqquired using very short columns). **Fig. 7** illustrates the errors originating from extrapolation of the retention to 100% water from the "common conditions" composition range of the mobile phase (limited by dashed lines).

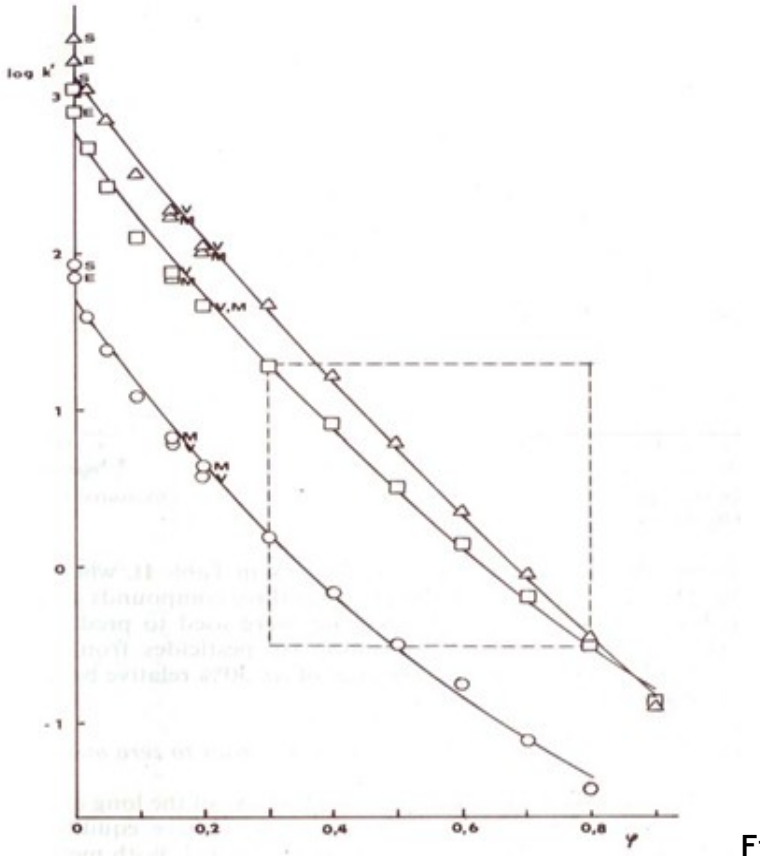

Fig. 7. Effects of the volume fraction of methanol on the retention of fluometuron (triangles), monuron (squares) and methomyl (circles) on a C_{18} stationary phase over a broad composition range of aqueous-organic mobile phase. V - conventional column, 300x3.6 mm i.d.; M - microcolumn, 30x1mm i.d. Dashed lines limit the retention data available in mobile phases containing 30 - 80% methanol.

Even the quadratic Eq. (10) fitted to the experimental data in the concentration range 2 - 80% methanol yields underestimated extrapolated k_w. In addition to the theoretical deviations from linearity, expected on the basis of Eq. (10), preferential sorption of the organic solvent in the stationary phase may be an additional reason of these discrepancies [R.M. McCormik, B.L. Karger, Anal. Chem., 52 (1980) 2249]. Generally, the parameter *m* characterizes the solvent strength effect on the retention and increases with decreasing polarity of the organic solvent and with increasing size of the sample molecule.

Other models based on either adsorption or partition mechanism in RPC or on their combination result in more complex equations for the retention factors, which usually involve three or more parameters [L. Peichang, L. Xiaoming, , J. Chromatogr., 292 (1984) 169, M. Jaroniec, *J. Chromatogr. A*, 656 (1993) 37] To describe the retention in reversed-phase chromatography over the full concentration range, extension of Eq. (10) by an additional third-power [P. Nikitas, A. Pappa-Louisi, P. Agrafiotou, J. Chromatogr. A, 946 (2002) 9, 33] or square root [P.J. Schoenmakers, H.A.H. Billiet, L. de Galan, J. Chromatogr., 185 (1979) 179] terms was

suggested:

$$\log k = a - m\varphi + d\varphi^2 + e\varphi^3 \qquad (12)$$

$$\log k = a - m\varphi + d\varphi^2 + e\sqrt{\varphi} \qquad (13)$$

Another retention equation was suggested by Scott [R.P.W. Scott, J. Chromatogr., 158 (1978) 183] to describe the retention for adsorption model of RP LC, where one solvent molecule displaces one solute molecule:

$$\frac{1}{k} = a + b\varphi \qquad (14)$$

Formally equal retention equation results from another derivation presented later [K. Kaczmarski, W. Prus, T. Kowalska, J. Chromatogr. A 869 (2000) 57]. Eq. (20) represents a simplified version of the earlier introduced Eq. (15) [M. McCann, J.H. Purnell, C.A. Wellington, Faraday Soc. Symp. Ser., 15 (1980) 82]:

$$\frac{1}{k} = a + b\varphi + b\varphi^2 \qquad (15)$$

Theoretical derivation of Eq. (15) was presented later, assuming adsorption mechanism with strong interactions between the solute and organic modifier in the mobile phase only [P. Nikitas, A. Pappa-Louisi, P. Agrafiotou, J. Chromatogr. A, 946 (2002) 9, 33.]. Based on statistical thermodynamic treatment, Peichang and Xiaoming introduced another retention equation [L. Peichang, L. Xiaoming, , J. Chromatogr., 292 (1984) 169]:

$$\log k = a + d\varphi + e\log\left(1 + b\varphi\right) \qquad (16)$$

Eq. (15) is the enlarged form of the retention equation presented by Antia and Horvath [F. Antia, Cs. Horvath, J. Chromatogr. 550 (1991) 411], Eq. (17), which can be formally re-arranged into the Eq. (14).

$$\log k = a - \log\left(1 + b\varphi\right) \qquad (17)$$

Another four-parameter retention equation for adsorption model of reversed-phase mechanism was presented by Nikitas et al. who compared limiting conditions for theoretical validity of various reversed-phase retention models described by Eqs. (12) –(18) [P. Nikitas, A. Pappa-Louisi, P. Agrafiotou, J. Chromatogr. A, 946 (2002) 9, 33.]:

$$\log k = a + d\varphi - \log\left(1 + b\varphi\right) - \frac{c\varphi}{1 + b\varphi} \qquad (18)$$

Eq. (18) can be reduced to a four-parameter equation by deleting the logarithmic term on the right-hand side [P. Nikitas, A. Pappa-Louisi, J. Chromatogr. A, 1068 (2005) 279.].

These models however have one common drawback, which limits their practical usefulness for prediction of retention. Reliable determination of more than three parameters of retention equations by fitting the model requires a large number of experimental data obtained over a sufficiently broad range of mobile phase composition, where the plots of log *k versus* φ are significantly non-linear, which usually would require impractically time-consuming measurements of very high retention factors. Even best-fit parameters of three-parameter equation cannot be attributed much physical meaning, but they may be in some cases useful to improve the prediction of retention data in comparison to the linear solvent strength retention equation, Eq. (11). Finally, all the models are based on the unrealistic assumption that the molar volumes of the solvents in the mobile phase are independent of the composition of the mobile phase and neglect the volume contractions during mixing of water with organic modifiers. Hence, some differences in retention may be observed depending on whether pre-mixed mobile phases are delivered by a single pump, or are mixed from the components directly in the instrument using one pump (or channel) for each component. The volume fractions φ in the above equations relate to the volume fractions before mixing rather than to the real concentrations in the mixed mobile phase, which can affect the equation model constants, in addition to neglecting the activity coefficients.

7.1. Ternary mobile phases

If the selectivity of separation in binary aqueous-organic mobile phases is not satisfactory, mobile phases containing two or three organic solvents may provide better balance of the selective polarity contributions resulting in improved selectivity of separation. The so-called selectivity triangle (**Fig. 8A**) is useful for rapid qualitative orientation in the selective polar contributions to the polarity of ternary or quaternary aqueous-organic mobile phases, containing a mix of organic solvents (methanol, acetonitrile and (or) tetrahydrofuran) [L.R. Snyder and J.L. Glajch, J. Chromatogr., 214 (1981) 1].

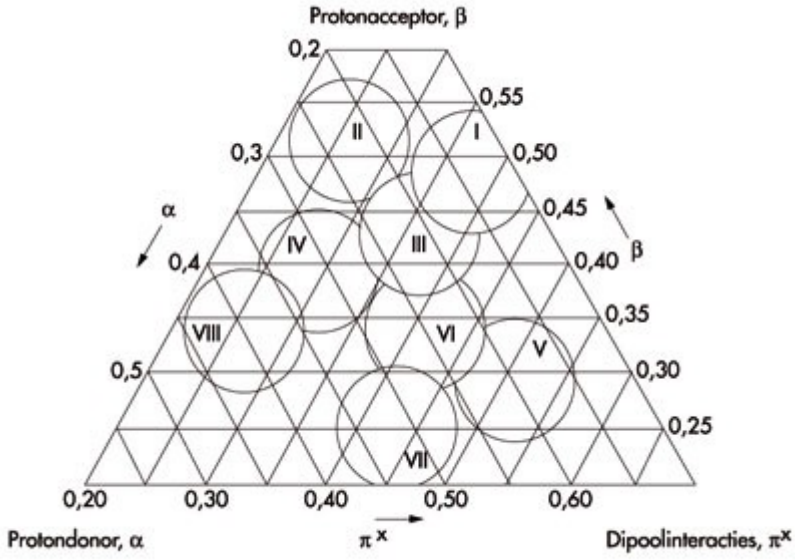

Fig. 8A. Solvent selectivity triangle.

The original triangle classified common organic solvents into 8 groups, according to predominating selectivity polar interactions, proton donor (x_d), proton acceptor (x_e) a dipole-dipole (x_n).

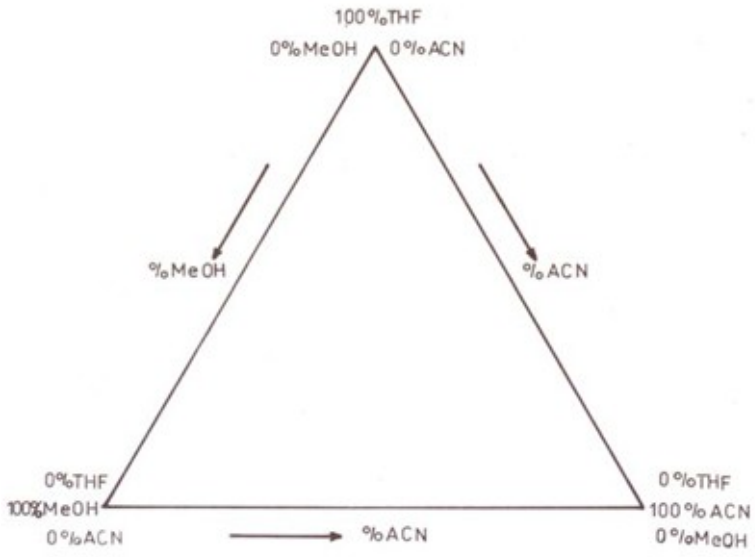

Fig. 8B. Solvent selectivity triangle.

Fig. 8B shows the reversed-phase solvent selectivity triangle cut out and transformed from the original one, with the apices corresponding to pure organic solvents - methanol, acetonitrile and tetrahydrofuran - (or to their iso-eluotropic mixtures with approximately equal elution strengths adjusted by appropriate diluting organic solvents with water). For any quaternary mobile phase, the distances of a point in the triangle from the apices represent the proportions of the individual selective contributions to the polarity corresponding to the concentration ratios of the three strong solvents. A point on a side of the triangle corresponds to the proportion of the selective polar interactions in a ternary mobile phase [J.L. Glajch, J.J. Kirkland and L.R. Snyder, J. Chromatogr., 238 (1982) 269].

For a ternary mobile phase containing organic solvents X and Y in water, Eqn. (19) was introduced to describe the effects of their concentrations, φ_x, φ_y, on the retention [1H. Colin, G. Guiochon and P. Jandera, Anal. Chem., 55 (1983) 442]:

$$\log k = a - m_x\varphi_x - m_y\varphi_y + d_x\varphi_x^2 + d_y\varphi_y^2 + 2\sqrt{d_x d_y}\,\varphi_x\varphi_y \qquad (19)$$

where a, m_x, m_y, d_x, d_y are the constants of Eq. (10), related to the binary mobile phases comprised of water and solvent X, and of water and solvent Y, respectively.

The contributions of the terms with d_x, d_y to log k often can be neglected over a more or less limited range of the concentrations φ_x, φ_y and then the retention can be calculated from the following simple equation, Eq. (20) [1H. Colin, G. Guiochon and P. Jandera, Anal. Chem., 55 (1983) 442.]:

$$\log k = a - m_x\varphi_x - m_y\varphi_y \qquad (20)$$

The values of the constants a, m_x, m_y and, if necessary, d_x and d_y can be determined in binary mobile phases, which theoretically enable prediction of the retention in ternary mobile phases X - Y - water from the retention data in binary mobile phases X - water and Y - water, using Eq. (19) or Eq. (20). When applying this approach, one often meets difficulties originating in significant differences in the values of the constant a determined by regression of the log k *versus*. φ plots for the individual binary mobile phases containing different organic solvents X and Y, i.e., a_x differs from a_y. A simple but efficient empirical remedy is to use the balanced mean value of the two experimental constants a_x and a_y for different compositions of ternary mobile phases [1P. Jandera, J. Churáček and H. Colin, J. Chromatogr., 214 (1981) 35., 1P. Jandera, H. Colin and G. Guioichon, Chromatographia, 16 (1982) 132.]:

$$a = \frac{a_x\varphi_x + a_y\varphi_y}{\varphi_x + \varphi_y} \qquad (21)$$

The errors of the k predicted from the data in binary mobile phases on a given column using Eqs. (20) and (21) are usually 5% or less [P. Jandera, J. Churáček and H. Colin, J. Chromatogr., 214 (1981) 35., 1P. Jandera, H. Colin and G. Guioichon, Chromatographia, 16 (1982) 132.].

8. Models describing simultaneous structural and mobile phase effects on retention

8.1. Homologous and oligomer series

The parameters of Eq. (1) describing the retention in homologous or oligomer series depend on the composition of the mobile phase, to first approximation as:

$$\alpha = a_1 - m_1\varphi \ , \ \beta = a_0 - m_0\varphi \qquad (22a, 22b)$$

Using Eqs. (22a) and (22b) and assuming validity of Eq. (11), i.e., of the LSS model, Eq. (1) can be adapted to describe the effects of varying organic solvent concentration on the retention in a homologous series (or an oligomeric series) in dependence on the number of the repeat structural units, n_C [P. Jandera, J. Chromatogr., 314 (1984) 13]:

$$\log k = a_0 + a_1 n_C - (m_0 + m_1 n_C)\varphi = (a_0 + a_1 n_C)(1 - p\varphi) - q\varphi \qquad (23)$$

Generally, the retention increases with increasing number of repeat units, n_C, and both the retention and selectivity of separation increase with decreasing concentration of the organic solvent in the mobile phase (**Fig. 1A**), but some oligomers with a moderately polar repeat unit are occasionally eluted in order of decreasing n_C, such as with oxyethylene nonylphenyl ethers (**Fig. 1B**) [P. Jandera, M. Holčapek, L. Kolářová, Int. J. Polym. Anal. Charact., 6 (2001) 261].

The constants of Eq. (23) characterize the homologous (or oligomer) selectivity of separation for one repeat unit (such as methylene selectivity), a_1, and the "end- group" selectivity (e.g., phenyl selectivity in alkylbenzene series), a_0, extrapolated to pure water and the effects of changing concentration of the organic solvents in binary mobile phases on the repeat group (m_1) and end-group selectivity (m_0). a_0, a_1, m_0, m_1, p and q depend on the number and type of the repeat (methylene or monomer) and the end groups in the series, on the column and on the type of the organic solvent in the mobile phase. Hence, the constants determined by multi-linear regression of log k of methyl- to hexylbenzene on the volume fraction of methanol or acetonitrile in aqueous-organic mobile phases can be used to characterize reversed-phase methylene selectivity in binary aqueous-organic mobile phases with varying composition [P. Jandera, J. Chromatogr. A, 656 (1993) 437].

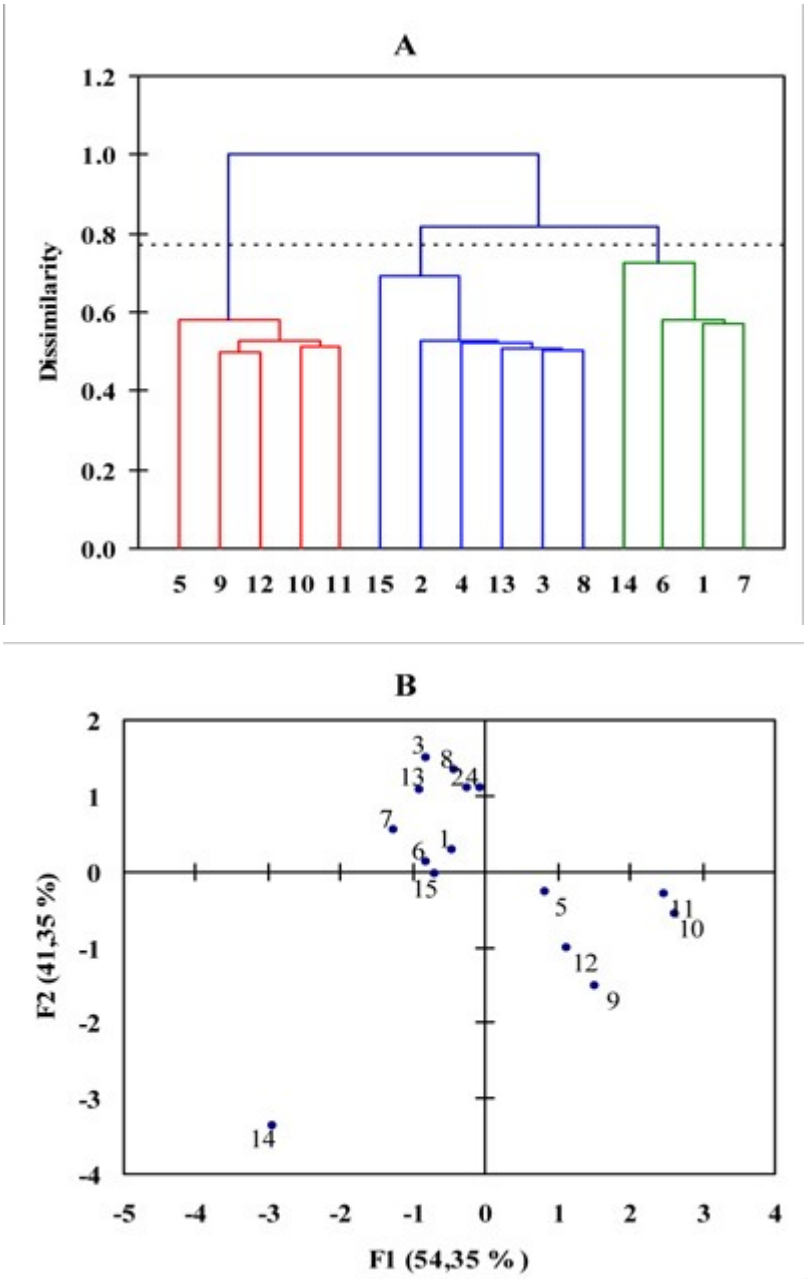

Fig. 9. Similarity dendrogram (A) and PCAscore plot (B) for the columns in Table 2 usingo the parameters a0, a1, m0, m1 for n-alkylbenzenes (C1–C6) in mobile phases containing 50–90% methanol.

Figure 9 shows the results of the PCA classification of the columns of **Table 2** based on the parameters a_0, a_1, m_0, m_1 of Eq. (23).

Table 2. Some columns for RP HPLC and their properties.
L – column length (mm), *i.d.* – inner diameter (mm), V_m – hold-up volume (average values for acetonitrile-water and methanol-water) (mL), *%C* – carbon load, *S* – specific surface area ($m^2.g^{-1}$), F_{as} – factor of asymmetry (aniline, 50% acetonitrile-water).

Column No.	***Trade name, dimensions, (L x I.D.), particle size***	***Manufacturer***	V_m	***%C***	***S***	F_{as}
1	Chromolith Performance RP-18e, 100 x 4.6	Merck, Darmstadt, Germany	1.41	N.A.	300	1.2
2	Atlantis dC_{18}, 150 x 3.9, 3µm	Waters, Milford, MA, USA	1.16	12.1	323	1.2
3	XTerra MS C18, 30 x 4.6, 5µm	Waters, Milford, MA, USA	0.31	15.0	175	2.0
4	Nova-Pak C18, 50 x 3.9, 4µm	Waters, Milford, MA, USA	0.34	7.3	120	1.0
5	Aqua 3µ C18 125A, 30 x 2, 3µm	Phenomenex, Torrance, CA, USA	0.08	15.0	320	1.2
6	Zorbax SB-Aq, 50 x 4.6, 3.5µm	Agilent, Palo Alto, CA, USA	0.50	N.A.	180	1.0
7	Zorbax 300Extend-C18, 150 x 4.6, 5µm	Agilent, Palo Alto, CA, USA	1.51	4.0	45	1.0
8	Hypersil ODS, 60 x 4.6, 3µm	Agilent, Palo Alto, CA, USA	0.62	10.0	170	1.2
9	Ace 3 Phenyl, 10 x 2.1, 3µm	ACT, Aberdeen, Scotland	0.02	9.5	300	1.1
10	Ace 3 C18, 10 x 2.1, 3µm	ACT, Aberdeen, Scotland	0.02	15.5	300	1.1
11	Ace 3 C8, 10 x 2.1, 3µm	ACT, Aberdeen, Scotland	0.02	9.0	300	1.1
12	Ace 3 C4, 10 x 2.1, 3µm	ACT, Aberdeen, Scotland	0.02	5.5	300	1.2
13	Discovery ZR-CARBON C18, 150 x 4.6, 5µm	Supelco Park, Bellefonte, PA, USA	1.58	3.0	30	1.2
14	Discovery HS PEG, 150 x 4.6, 5µm	Supelco Park, Bellefonte, PA, USA	1.82	12.0	300	1.2
15	Discovery HS F5, 150 x 4.6, 5µm	Supelco Park, Bellefonte, PA, USA	1.62	12.0	300	1.2

Valkó and Slégel [K. Valkó, R. Slégel, J. Chromatogr., 631 (1993) 49] and other authors [G. Rippel, E. Alattyani, L. Szepesy, J. Chromatogr. A, 668 (1994) 301] characterize the hydrophobicity of stationary phases by the parameter, φ_0, characterizing the volume fraction of the organic solvent necessary to achieve standardized sample retention, k = 1, which can be calculated combining the parameters of Eq. (11):

$$\varphi_0 = -\log k_W / m \qquad (24)$$

Assuming validity of Eq. (17) in a homologous or oligomeric series, a common intersection point of the fan-like plots of log k_w vs. m for homologues with different alkyl lengths can be expected [P. Jandera, in R.M. Smith (Ed.) *Retention and Selectivity in Liquid Chromatography*, Elsevier, Amsterdam 1995, p.260], with coordinates given by Eqs. (25) and (26):

$$\varphi_C = 1/p \qquad (25)$$

and

$$\log k_C = -q/p \qquad (26)$$

Fan-like plots with a common convergence point have been observed experimentally with numerous series and chromatographic systems [P. Jandera, J. Chromatogr. A, 656 (l993) 437], [P.J. Schoenmakers, H.A.H. Billiet, L. de Galan, *J. Chromatogr.,* 185 (1979) 179]. The coordinates of the convergence point corresponding to co-elution conditions in a homologous or oligomer series depend on the type of the stationary phase and may be used for column characterization. The "co-elution" volume fraction of the organic solvent", φ_c and n_c depend not only on the type of the series, but also on the organic solvent in the mobile phase. φ_c = 1.08 - 1.16 for methanol - water, 0.95 for dioxane - water and 0.86 for tetrahydrofuran - water mobile phases [P. Jandera, in R.M. Smith (Ed.) *Retention and Selectivity in Liquid Chromatography*, Elsevier, Amsterdam 1995, p.260]. Of course, φ_c >1 and (or) the negative values of n_c lack the physical meaning and indicate that the co-elution cannot be observed for this homologous series within the real mobile phase composition range.

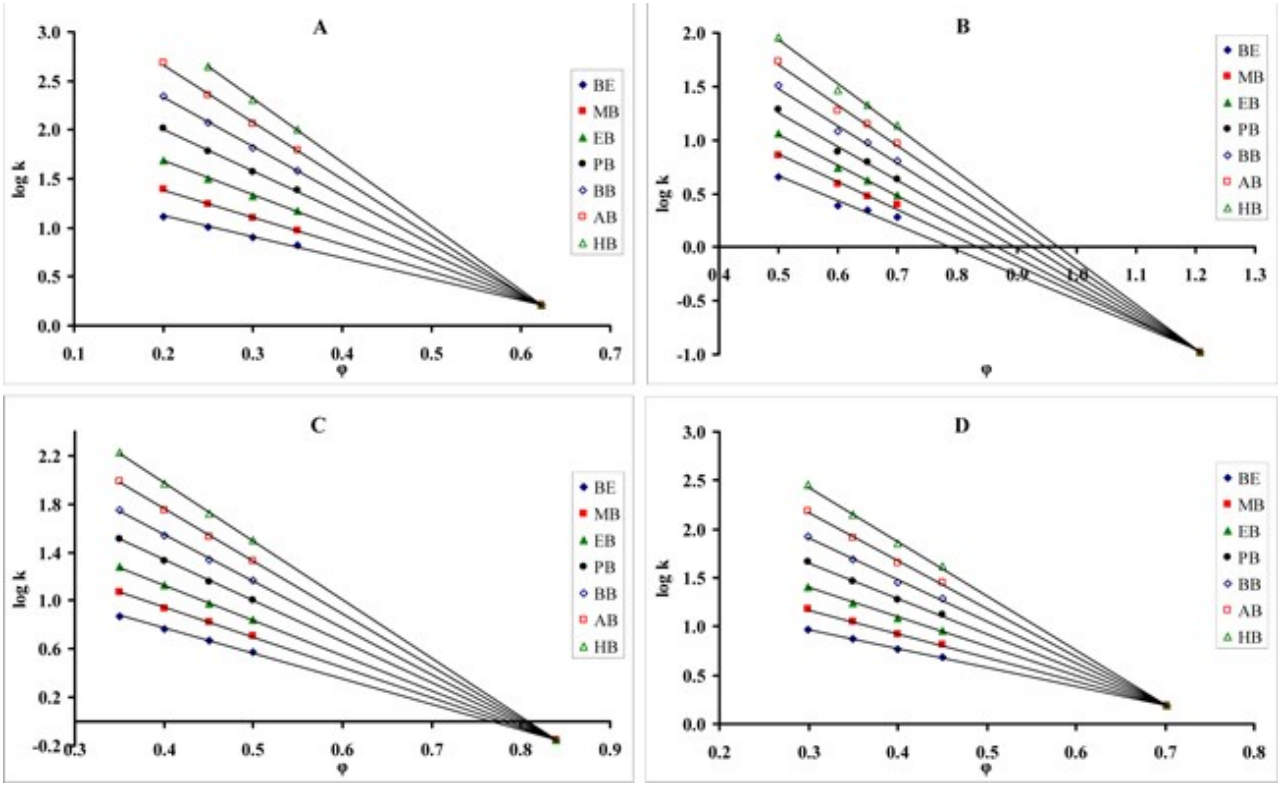

Fig. 10. Co-elution volume fraction of acetonitrile, φ, for *n*-alkylbenzenes (C_0–C_6) on ACE 3 Ph (A), ACE 3 C18 (B), ACE 3 C4 (C) columns. *(Click on figure to enlarge)*

The effects of the stationary phase on the convergence coordinates are illustrated in **Fig. 10,** showing that even if co-elution of a sample does not occur in the real composition range of the mobile phase for a column with longer bonded alkyl chains (C18) (**Fig. 10 B**), it may be shifted into a real organic solvent concentration and higher retention factors region for stationary phases with shorter bonded alkyls (**Fig. 10 C and 10 D**) or with more polar (phenyl, **Fig. 10 A**) bonded moieties.

Estimation of the co-elution conditions is practically important for the development of two-dimensional LCxLC separations of oligomers, where the separation selectivity according to one type of the structural distribution (such as for methylene or other repeat monomer units) should be suppressed in one dimension to enhance the separation according to another type of structural unit distribution (such as of polar functional end groups) [P. Jandera, M. Halama, L. Kolářová, J. Fischer, K. Novotná, J. Chromatogr. A, 1087 (2005) 112].

8.2. Non-homologous compounds with various functional groups.

Some general sample structural effects were observed in RP systems comprised of various combinations of stationary and mobile phases. The retention increases for compounds with greater hydrophobic surface area and decreases for compounds containing polar functions.

The polarity effect is sometimes very well defined. For example, a double bond in a hydrocarbon chain of long fatty acids and their esters decreases the retention almost regularly to that of a saturated alkyl shorter by two methylene groups, almost independently of the stationary and the mobile phases [P. Jandera, K. Novotná, L. Kolářová, J. Fischer, Chromatographia, 60 (2004) S27].

Compounds with branched alkyls are usually less retained than the compounds with straight chains containing the same number of carbon atoms. Further, alkyl silica gel bonded phases show preferential retention of rigid, planar solutes over non-planar, bulky ones. This feature is even more apparent with alkyl aryl bonded phases.

To characterize the most characteristic properties of stationary phases, simple tests were suggested, most often based on the relative retention of some selected sample compounds. Hydrophobic selectivity can be characterized by relative retention of benzene and various alkylbenzenes, or of polyaromatic hydrocarbons. Silanol activity tests are based on relative retention of various more or less polarstandards, such as the relative retention of *N*,*N*-diethyltoluamide (DETA) and anthracene in acetonitrile, the relative retention of theophyline and caffeine in 40% methanol or in buffered aqueous methanol and acetonitrile, the relative retention of naphthalene and nitronaphthalene in 60% methanol in water containing 5% sodium acetate, the relative retention of aniline and phenol in 55% methanol, the relative retention of aniline and phenol in 60% methanol; Lesellier et al. [E. Lesellier, C. West, A. Tchapla, J. Chromatogr. A, 1111 (2006) 62] used sub-critical fluid chromatography of cis- and trans-carotenoid compounds to characterize hydrophobicity, hydrogen bond interactions and steric selectivity to distinguish polar-endcapped, amide, carbamate and ether stationary phases, just to mention a few examples. A plethora of such methods were described in the literature, various methods were compared in several reviews, e.g., [S.D. Rogers, J.G. Dorsey, J. Chromatogr., 892 (2000) 57–65.**]**, [H.A. Claessens, M.A. van Straten, C.A. Cramers, M. Jezierska, B. Buszewski, J. Chromatogr. A, 826 (1998)135], [H. A. Claessens, TrAC Trends in Analytical Chemistry, 20 (2001) 563] [K. Krupczyńska, B. Buszewski, P. Jandera, Anal. Chem. 76 (2004) 226A]. These tests usually employ a single composition of the mobile phase and the results may significantly change in other mobile phases, as illustrates **Fig. 11** for the relative retention of phenol and aniline to benzene in mobile phases containing 60% acetonitrile or 60% methanol.

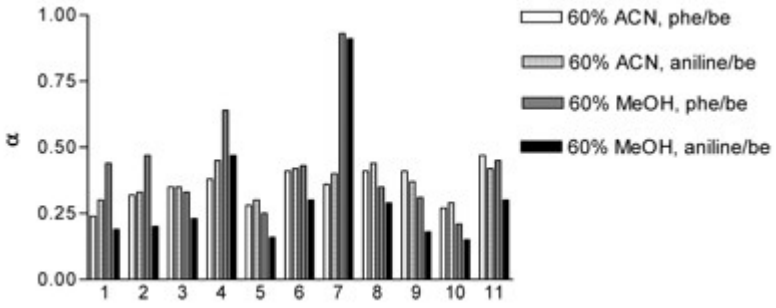

Fig. 11

On the x-axis: Columns: 1. Zorbax 300 SB-C18, **2.** Zorbax Eclipse XDB-C18, **3.** LUNA C8(2), **4.** LUNA Phenylhexyl, **5.** LUNA C18(2), **6.** LUNA C18, **7.** Astec Polymer C18, **8.** LiChrospher 60 RP select B, **9.** XTerra RP8, **10.** Symmetry C18, **11.** Symmetry Shield RP8

8.3. Solvatochromic parameters

The solvatochromic parameters assess solvent properties such as polarity and hydrogen bonding donor and acceptor capabilities, which can be measured from the UV-VIS spectral shifts of the absorption band maximum of an indicator probe, depending on the energy of solvation. To characterize the polar solvation of both the stationary and the mobile phases, the polarity parameter $E_T(30)$ scale was introduced [B.P. Johnson, M.G. Khaledi, J.G. Dorsey, Anal. Chem., 58 (1986) 2354]. Like the dependence of log *k* on the volume fraction of the organic solvent described by Eq. (11), the plots of log *k versus* the normalized parameter of the mobile phase, E_{Tm}^N, [C. Reichardt, *Solvents and Solvent Effects in Organic Chemistry*, VCH, Weinheim, 2nd ed., 1988] are linear in the commonly used composition range of acetonitrile-water and methanol-water mobile phases [M. Rosés, E. Bosch, Anal. Chim. Acta, 274 (1993) 147]:

$$\log k = q + pE_{Tm}^N \qquad (27)$$

Similar equation can be written for the stationary phase and Eq. (26) can be re-written as:

$$\log k = \log k_0 + p(E_{Tm}^N - E_{Ts}^N) \qquad (28)$$

where E_{Ts}^N refers to the stationary phase. Eq. (28) is similar to the linear solvation energy relationships developed by Kamlet and co-workers [M.J. Kamlet, M.H. Abraham, P.W. Carr, R.M. Doherty, R.W. Taft, J. Chem. Soc., Perkin Trans. 2 (1988) 2087], [M.J. Kamlet, J.L.M. Abboud, M.H. Abraham, R.W. Taft, J. Org. Chem., 48 (1983) 2877] to describe the distribution of a solute between two liquid phases. Several hundreds of the solute parameters *p* are compiled in reference data potentially useful for prediction of retention at changing separation systems (columns, organic modifier) using semi-empirical correlation equations.

According to the LSER formalism, log *k* can be calculated as the sum of a constant term (log k_0 in Eq. (28)) and solute-solvent interaction terms, which are described by the product of the solute characteristic parameter *p*, and the difference between the solvent parameters of

the mobile and the stationary phases, ($E_{Tm}^N - E_{Ts}^N$). E_{Ts}^N is assumed to be independent of the mobile phase to first approximation, which is not fully true, as the stationary phase is solvated by the mobile phase, and therefore its properties change with the mobile phase composition to some extent.

8.4. The model of lipophilic and polar indices.

Methods considering the structural effects and simultaneously the effects of the mobile phases are based on a set of reference compounds used for calibration of the retention scale. An example is the method characterizing samples by lipophilic and polar indices. The basic idea is similar to that of the single retention index approach, but the approach distinguishes between the non-polar (hydrophobic) and the polar contribution to RP retention; each contribution being characterized by an independent index. A sample compound can be considered as an equivalent to a member of a calibration homologous series, for which we do not know *a priori* the values of n_C. Using the experimental log k_w and m of the sample determined by linear regression of the log k vs. φ plots (Eq. (11)) and the parameters a_0 , a_1 of the calibration homologous series (Eq. (23)), we can calculate the hypothetical equivalents to the constant q: q_i, and to the number of methylene units n: n_{ce}, for each sample solute, using Eqs. (29) and (30) [P. Jandera, J. Chromatogr. A, 656 (1993) 437].

$$n_{ce} = \frac{a - a_0}{a_1} \tag{29}$$

$$q_i = m - p(a_0 + a_1 n_{ce}) \tag{30}$$

The lipophilic index n_{ce} is a measure of the non-polar contribution to the retention of the solute, whereas the polar contribution to retention can be characterized by the polar index, q_i [P. Jandera, Chromatographia, 19 (1984) 101]. The parameters q_i, and n_{ce} determined on various columns can be compared to evaluate the lipophility and the polarity of the stationary phases, as illustrated by the principal component analysis for 15 columns (**Table 2**) in **Fig. 12** and **Fig.13**.

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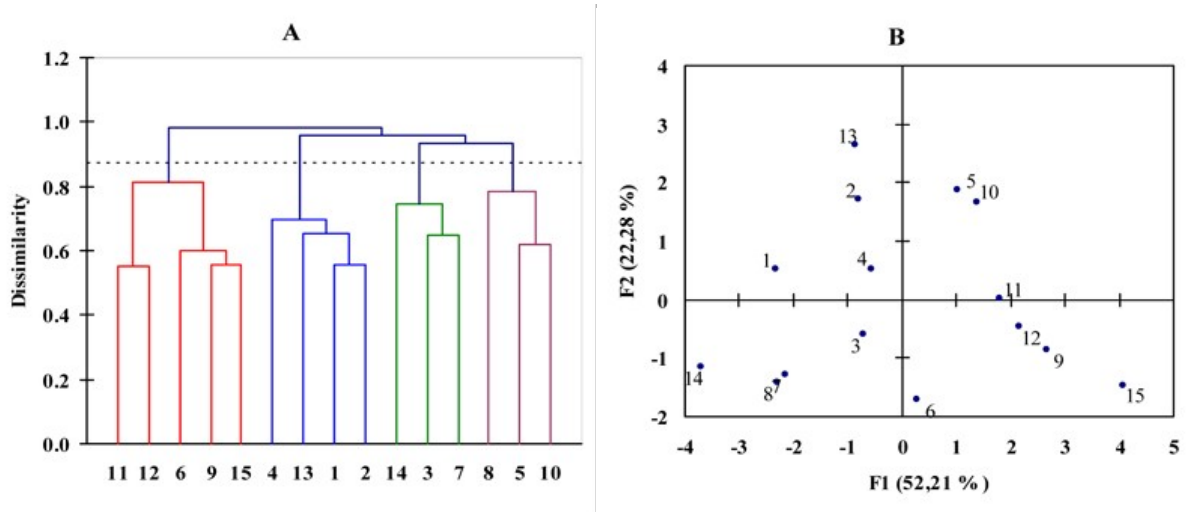

Fig. 12

Fig. 12: Similarity dendrogram (A) and PCA score plot (B) for the columns in **Table 2** based on lipophilic indices, n_{ce}, of the analytes in **Table 1** in 50–90% methanol mobile phases

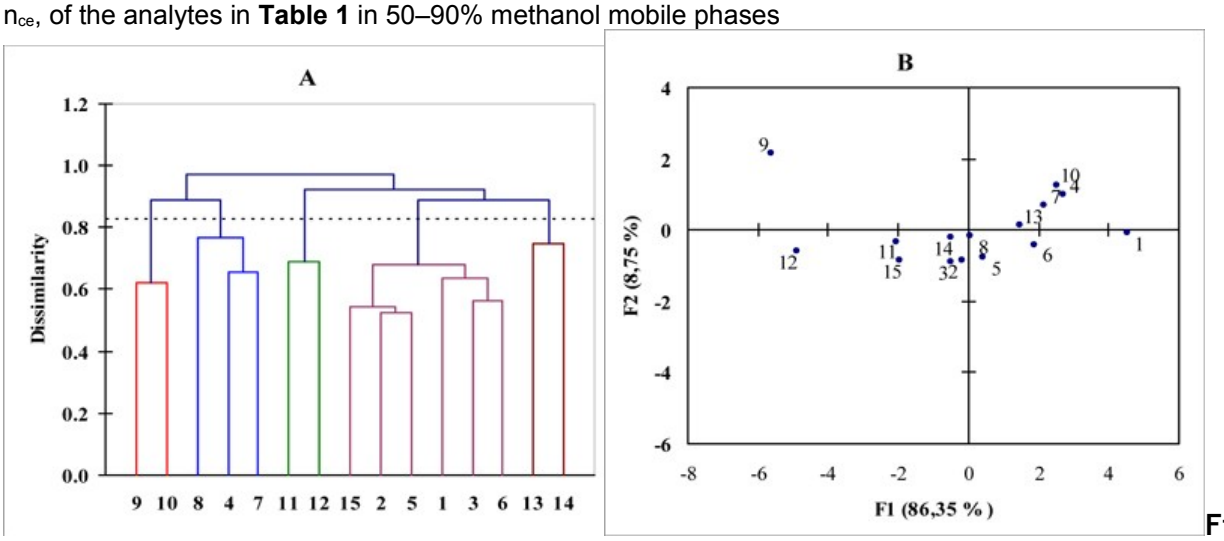

Fig. 13

Fig. 13: Similarity dendrogram (A) and PCA score plot (B) for the columns in **Table 2** based on polar indices, q_i, of the analytes in **Table 1** in 50–90% methanol mobile phases.

The retention scale based on lipophilic (n_{ce}) and polar (q_i) indices is suitable also for characterization of the retention of azodyes with complex structures in ion-pair HPLC. The indices provide useful information on the structure of sulfonated azodyes with broad range of molecular weights containing 1 – 5 sulfonic acid groups in mobile phases with various dialkyl- or trialkylammonium ion-pairing reagents, compatible with HPLC/MS analysis. Mobile phases with 0.0025 mol/l triethylammonium acetate or tributylammonium acetate acetate provide sufficient retention and separation selectivity even for complex dyes with molecular weights over 1000 and can effectively substitute tetraalkylammonium ion-pairing reagents in HPLC/MS. The polar indices are proportional to the number of sulfonic acid groups and the lipophilic indices to the molecular weight of dyes and increase with the number of carbon atoms and the lengths of the alkyl chains in the molecules of ion-pairing reagent, **Fig. 14**

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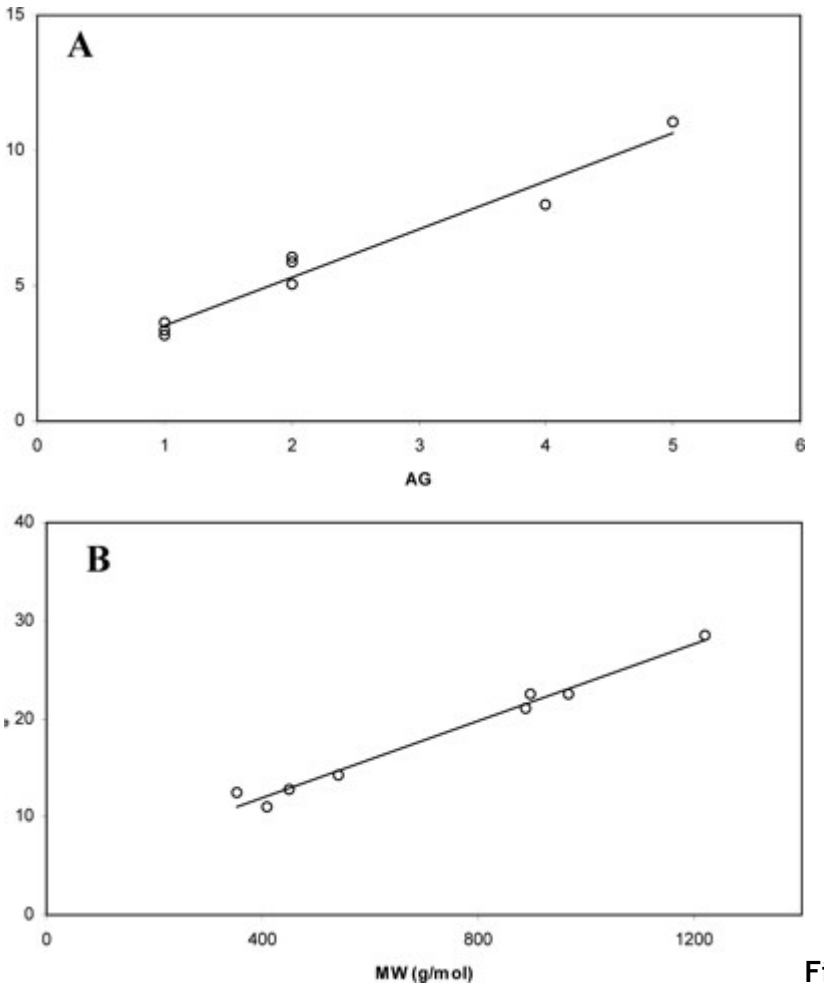


Fig. 14. Structural dependences of the polar indices q_i (A) and of the lipophilic indices n_{ce} (B) of complex (poly)sulfonated azo dyes on the number of sulfonic acid groups in the dye molecules, *AG*, and on the molecular weights, *MW*, in methanol-water mobile phases with 0.0025 mol/l triethylammonium acetate.

The retention data can be estimated from the model using the molecular weight and the number of sulfonic acid groups as structural parameters of dyes and used as supporting material in the structure elucidation by the mass spectra [D. Vaněrková, P. Jandera, J. Hrabica,. J. Chromatogr. A, 1143 (2007) 112].

Like with the constants α, ß of homologous or oligomer series, also the parameters *A*, *B* of the models of retention indices, Eq. (2) and interaction indices, Eq. (4) are affected by the composition of the mobile phase and this dependence can be - to first approximation - described by semi-logarthmic equations similar to Eq. (22a) and Eq. (22b):

$$A = A'_0 - A'_1 \varphi \ , \quad B = B'_0 - B'_1 \varphi \qquad \text{(31a), (31b)}$$

Similar equations were suggested also to describe the effects of the mobile phase on the constants of the QSRR models.

9. Ionic compounds

9.1. pH effects

Samples containing ionized or ionizable organic compounds - strong or weak acids or bases - usually are difficult to separate by RPC in pure aqueous - organic mobile phases. However, successful RPC separations of ionic samples are often possible with ionic additives to the mobile phase. Weak acids or bases are partially ionized in aqueous-organic mobile phases used in RP LC, depending on pH: weak bases are completely ionized at pH < pK_A - 1.5 and weak acids at pH > pK_A + 1.5. (pK_A = - log K_A). K_A is the acidity (i.e. the dissociation) constant, which characterizes the degree of ionization of a weak acid or base. If both the non-ionized and ionized forms of a sample are present in the mobile phase, the experimental retention factor is assumed to represent an average of the retention factors of the two forms, such as k_{HA} for the non-ionized form and k_A for the ionized form of a weak acid. The ionized species is usually much less retained under RP conditions than the non-dissociated one, $k_{HA} >> k_A$. Hence the retention of a monoprotic weak acid depends strongly on pH of the mobile phase:

$$k = \frac{k_{HA} + k_a K_A 10^{pH}}{1 + K_A 10^{pH}} \qquad (32)$$

Similar considerations apply also for the retention of weak bases or poly-protic acids and bases. Addition of a buffer to the mobile phase can be used to suppress the ionization of weak acids at lower pH and of weak bases at higher pH and to eliminate undesirable chromatographic behavior of ionic species. However, suppressing the ionization of basic compounds is limited with silica-based columns, due to their poor chemical stability in the alkaline pH range.

The control of mobile-phase pH by adding a buffer can be used not only to suppress the ionization of weak acids or bases, but also to control the selectivity of separation by working at a suitably adjusted pH in the range within ±1.5 units around the pK_A of weak acids of bases, which may be ionized to different degrees, i.e., the concentration ratios of the ionized and neutral species differ for the individual sample components. Weak acids are eluted in order of decreasing and weak bases in order of increasing. Hence, the choice of a suitable buffer additive to the mobile phase is dictated by the pK_A values of sample compounds. A buffer is usually composed of a salt and a corresponding acid or base, with pH adjusted by the concentration ratios of the two components. Each type of buffer can be used only within certain pH limits, where it has adequate buffer capacity. At low concentrations, the buffer capacity may be insufficient for reproducible separations, but 10 to 50 mM buffers have usually adequate buffer capacities for most HPLC separations (at higher concentrations, problems with

buffer solubility in mobile phase or with corrosion of stainless-steel parts of the HPLC instrument may occur). Buffers useful in various pH ranges can be found in general chemical tables. Phosphate buffers (pH 2.1 - 3.1 and 6.2 - 8.2) and acetate buffers (pH 3.8 - 5.8) are most often used in HPLC.

It should be noted that the presence of organic solvent in the mobile phase causes changes in the dissociation of both the sample and the buffer, which causes a change in the pH of the mobile phases. Their combined effects leads to shifts of the retention factors from the behavior expected on the basis of Eq. (32) with pH and K_A measured in purely aqueous media [D. Sýkora, E. Tesařová, M. Popl, J. Chromatogr. A, 758 (1997) 37]. To obtain the real pH of the mobile phase, the pH measured in aqueous-organic mobile phases using a pH-meter calibrated with the standard buffers dissolved in water should be corrected using an empirical parameter, depending on the concentration of the organic modifier in the mobile phase [M. Roses, X. Subirats, E. Bosch, J. Chromatogr. A, 1216 (2008) 1756].

Basic compounds can interact with residual silanols of alkyl silica bonded phases, which are ionized to anionic SiO^- groups at pH > 6. These interactions are often irreproducible from one column to another and often result in strong and variable retention and tailing peaks of analytes. The retention can be decreased by increasing the concentration of a buffer added to the mobile phase. The addition of an alkylamine to the mobile phase often can improve peak shapes as the basic amine is preferentially attracted to ionized silanol groups by ion-exchange process, blocks them and suppress their harmful effect on the separation of basic samples.

Acid anions often elute as strongly deformed peaks close to the column hold-up volume or may be even excluded from the pores of the packing particles. The residual silanol groups on the silica surface are partially ionized to $\equiv SiO^-$ groups in pure water or in aqueous salt solutions. Even a very small excess of negative charges caused by the residual SiO^- or other groups in the bonded phase is sufficient to create negative surface (zeta) potential and an electric double-layer in the solution adjacent to the negatively charged surface of the bonded stationary phases, from which sample anions are more or less strongly repulsed, so that they cannot access a part of the pore volume, much like macromolecules in steric-exclusion chromatography [P. Jandera, S. Bocian, M. Molíková, B. Buszewski, J. Chromatogr. A, 1216 (2009) 237]. Addition of a neutral salt such as sodium sulfate to the mobile phase can suppress these repulsive interactions and induce salting out of the acids from the mobile into the stationary phase, so that even very strong naphthalene tri- and tetra-sulfonic acids can be separated as sharp symmetrical peaks, but the selectivity of separation depends strongly on the type of the stationary phase (**Fig. 15**) [P. Jandera, S. Bunčeková, M. Halama, K. Novotná, M. Nepraš, J. Chromatogr. A, 1059 (2004) 61]. However, this approach is useful only for acids containing a

bulky hydrophobic part in their molecules.

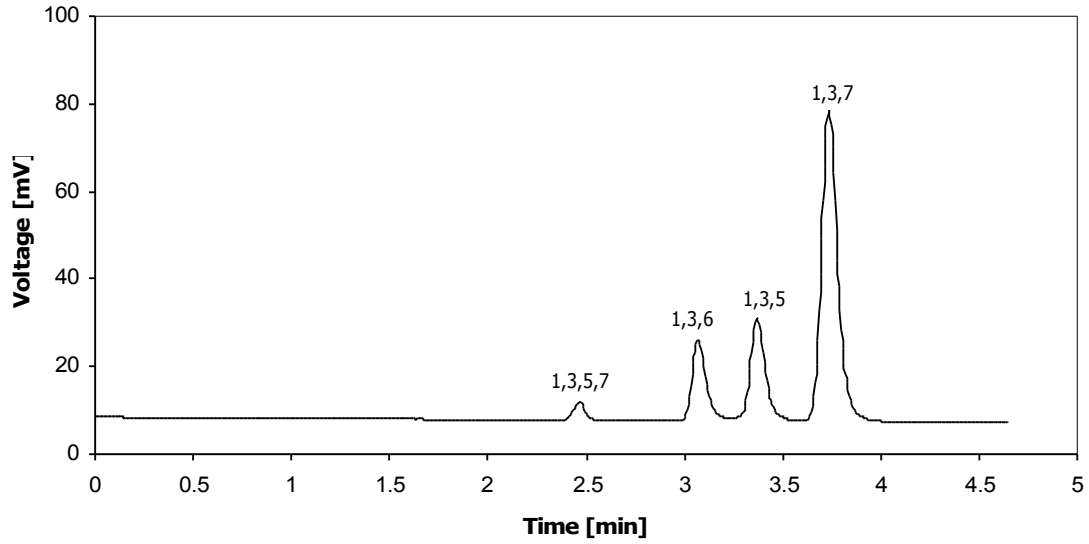


Fig.15.
Separation of 1,3,5,7-naphthalene tetrasulphonic acid, 1,3,6-, 1,3,5- and 1,3,7-naphtalene trisulphonic acids on a Zorbax SB Aq column. Mobile phase: 0.4 M Na_2SO_4, flow rate: 1 ml min^{-1}, temperature 40°C.

9.2. Ion-pair chromatography

Strong acids and strong bases are completely ionized over the whole pH range of the mobile phases useful for chromatography on chemically bonded alkylsilica columns and their chromatographic behavior is not affected by the pH of the mobile phase. Such compounds can be separated using ion-pair chromatography (IPC) in reversed-phase systems, where an ion-pairing reagent with surface-active properties is added to aqueous - organic mobile phases containing usually methanol, acetonitrile or tetrahydrofuran. The molecules of suitable ion-pairing reagents contain a completely ionized strongly acidic or strongly basic group and a bulky hydrocarbon part in their molecules. Basic substances can usually be separated by using salts of C_6 - C_8 alkanesulphonic acids, and acidic substances can be separated with tetrabutylammonium or cetyl trimethylammonium salts in the mobile phase. Ion-pair additives greatly increase the retention and improve the peak symmetry, either through formation of neutral ionic associates with increased affinity to a non-polar stationary phase, or by modification of the surface properties of a non-polar stationary phase, which then acts towards the ionized sample much like a liquid ion exchanger coated on a solid support.

The addition of an ion-pairing reagent into the mobile phase slightly decreases the retention of non-ionized molecules, but increases the retention of ions carrying opposite charges. Hence, aqueous-organic mobile phases with the ion-pair reagent usually contain also a

buffer to adjust the pH enhancing the ionization of weak acids (pH > 7) or bases (pH < 7) and formation of ion-pair associates. Adequate ion-pairing reagent concentrations in IPC are in between 10^{-4} - 10^{-2} mol/l, depending on the sample, column and other components of the mobile phase.

An advantage of IPC with respect to RPC without ion-pair additives consists in suppressed silanol effects by stronger interactions either between the ion-pair reagent and analytes or between the reagent and the ionized silanol groups. The effects of the volume fraction of the organic solvent in the mobile phase and the structural effects on the retention are similar as in RP chromatography of nonionic compounds, but the ion-pairing reagent contributes to the hydrophobic retention by the bulky non-polar parts in their molecules. The model of polar and hydrophobic indices was applied to ion-pair chromatography of synthetic acid dyes [[D. Vaněrková, P. Jandera, J. Hrabica,. J. Chromatogr. A, 1143 (2007) 112]].

10. Stationary phase effects

10.1. Chemically bonded silica-based phases

Stationary phases for RP LC based on silica gel are prepared by chemical modification of the silica surface using reactions of the surface silanol (Si-OH) groups with organosilanes (halogeno- or alkoxy-) to obtain stationary phases with Si-O-Si-R bonds (R are most often C_8 or C_{18} alkyls). Monofunctional reagents such as alkyldimethylmonochloro silanes yield well defined monomeric packings, as one silanol group reacts with one silane molecule, resulting in flexible "fur-" or "brush-" like structure of the alkyl chains on the silica surface. The retention generally increases with increasing content of carbon atoms in the chemically bonded phase and with increasing length of the bonded alkyl chains, but only up to a certain "critical" length of the bonded alkyls [K. Karch, I. Sebastian and I. Halász, J. Chromatogr., 122 (1976) 3], [G.E. Berendsen and L. De Galan, J. Chromatogr., 196 (1980) 21

The chemistry of the chemically bonded ligands and of the support, the degree of surface coverage of bare silica gel or other support material and the homogeneity of the arrangement of chemically bonded phase on the surface all more or less affect the retention by specific polar and non-specific interactions between analyte, stationary phase and mobile phase. The alkyl groups are not evenly distributed across the silica surface, but are aggregated to a degree that depends on the average bonding density and the nature of the surrounding liquid phase, which affects the orientation of the chains at the surface.

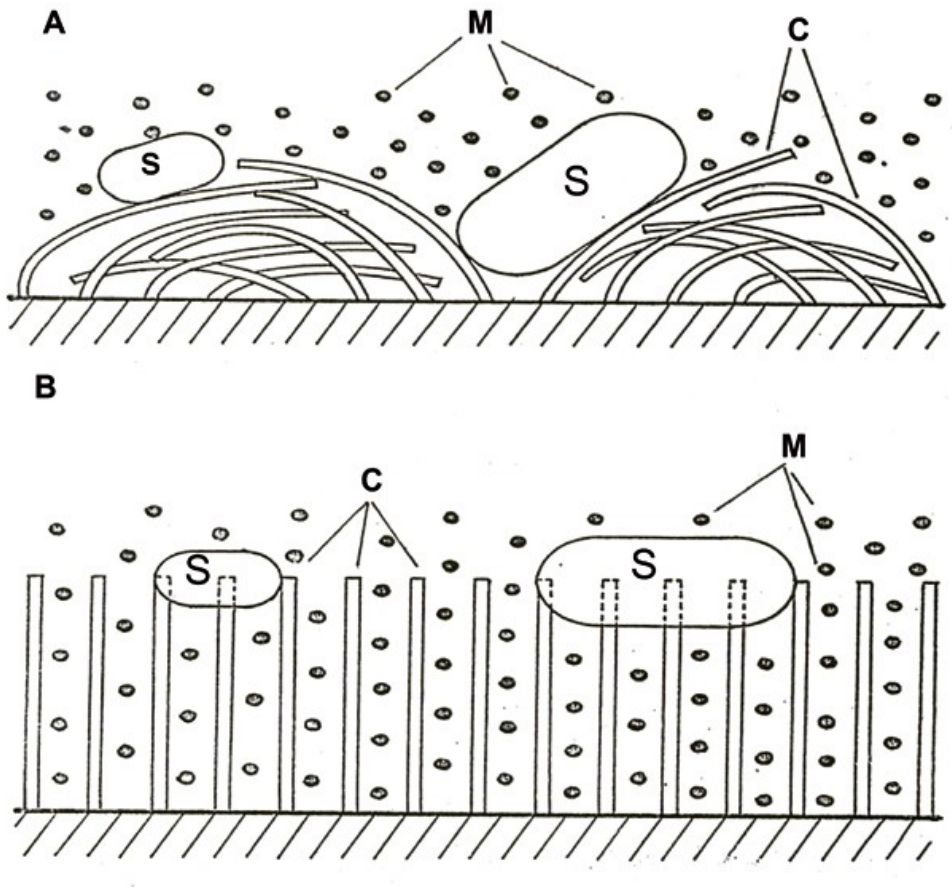


Fig. 16 (A and B)

Schematic representation of the behavior of monomeric chemically bonded alkyl silica phases in highly aqueous (A) and in organic-rich (B) mobile phases.

In organic rich mobile phases, the alkyl chains are solvated by the organic component of the mobile phase and are extended away from the surface (**Fig. 16 B: Bottom**) and show some features of liquid-liquid phase distribution, comparable to octanol-water Hansch methods. On the other hand, water-rich mobile phases have poor solvating quality for the bonded alkyl chains, which tend to "collapse", i.e., to form a phase separated from the bulk mobile phase with a thin interphase (**Fig. 16 A: Top**). Here, the specific affinity of the sample to adhere to the bare solid surface appears more favourable than that of the solvent. The regular solution theory was combined with the "self-consistent theory of adsorption" to account for these effects [R. Tijssen, P.J. Schoenmakers, M.R. Boehmer, L.K. Koopal, H.A.H. Billiet, J. Chromatogr. A, 656 (1993) 135].

For separations of hydrophilic and other compounds requiring highly aqueous mobile phases, so-called "aqua" bonded phases are designed, with improved solvation of the bonded material preventing the "collapse" of hydrophobic alkyl chains. For this purpose, a polar (amide, carbamate, etc.) group may be incorporated between the alkyl chain and the surface of the

silica gel support, or the non-reacted residual silanol groups are end-capped with polar hydrophilic ligands instead of hydrophobic short alkyl groups.

Most common complications of the RP behavior originate in specific polar interactions between sample and residual groups on the adsorbent support. Steric reasons do not allow all the silanol groups to react with rather bulky silanization reagents and from 7-8 μmol of silanol groups per m^2 of surface area, only approximately 2 - 4 μmol/m^2 of silane can be chemically modified. Hence, at least 50% of the original silanol groups remain non-reacted on the support after chemical modification and the residual silanols may show unwanted interactions with solutes. Some polar and especially basic solutes can be strongly retained by the residual silanol groups, which results in their poor and irreproducible separation, band tailing or distorted shape.

To reduce the number of residual silanol groups, a small-molecule silane such as trimethylchlorosilane or hexamethyldisilazane is often used for modification of the bonded phase in the second-step "end-capping." reaction. The "end-capping." procedure improves the properties of the stationary phase, but cannot completely remove all silanols and prevent the bonded phase from interactions with basic solutes.

Stationary phases chemically bonded on silica gel are generally not stable enough outside the pH range from 2 to 8. At higher pH, the silica gel support gets gradually dissolved in the mobile phase, which causes continuous decrease in retention and eventually the collapse of the column bed.

Longer-chain alkyl-bonded phases usually are more stable against hydrolysis than short-chain bonded phases. The stability of a silica-based chemically bonded phase at lower pH can be improved by using silane reagents with more bulky sterically protecting groups, such as diisopropyl- or diisobutyl- instead of dimethyl-alkylchlorosilanes, which shield the Si-O-Si bond and minimize its hydrolysis. Another approach relies on bi-dentate stationary phases, prepared by modification of the silica gel surface with bidentate silanes containing C_{18} or C_8 alkyls and two reactive groups, separated by a -CH_2-CH_2- or a -CH_2-CH_2-CH_2- "bridge". Such "bridged" bonded phase is attached *via* two siloxane groups to two silanol groups on the surface of the silica gel support and efficiently shields a part of non-reacted silanol groups on the silica gel support from the direct contact with mobile phase, improving thus the long-term column stability, both at low and at high pH [J.J. Kirkland, J.B. Adams, M.A. van Straten and H.A. Claessens, Anal. Chem., 70 (1998) 4344]. **Figure 17** illustrates schematically some approaches used to suppress the residual silanol activity of stationary phases used in RP LC.

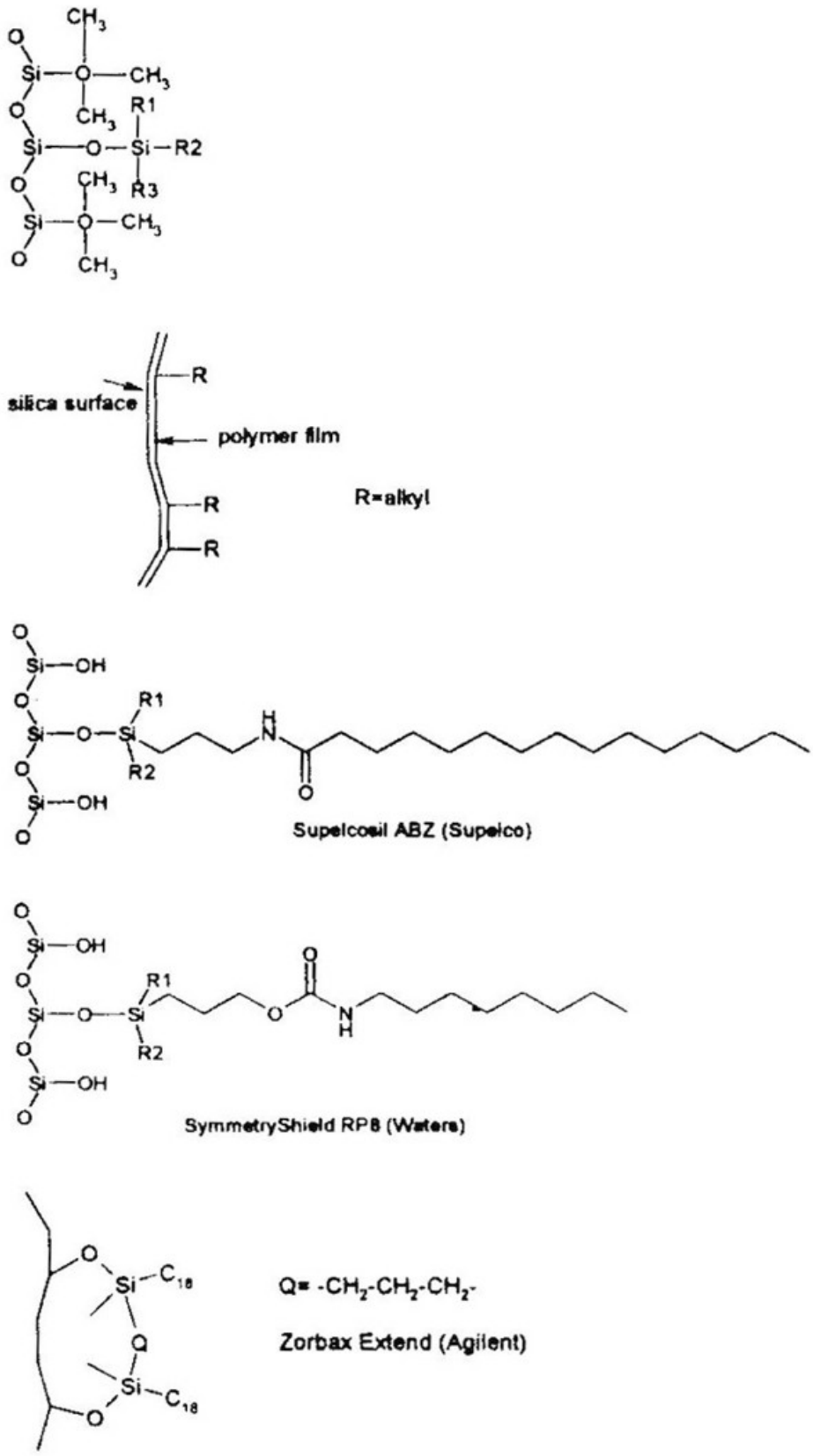


Fig. 17.

Examples of various approaches to suppressing the silanol activity and improving compatibility with water-rich mobile phases: shielding of silanol groups by endcapping, polymer encapsulation, amide or carbamate spacing groups between the silica support and alkyl chains, bidentate alkyl attachment.

Non-polar or moderately polar stationary phases with other chemically bonded moieties, such as branched hydrocarbons, perfluoroalkanes, cholesterol or alkylaryl groups show different separation selectivities which can be useful for specific separations, but they are far less frequently used than bonded C_{18} or C_8 phases. For example, the presence of an aromatic ring in the chemically bonded substituents results in preferential retention of aromatic compounds and increased shape selectivity - rigid-rod-like molecules are retained more strongly than plate-like molecules, and these are retained more strongly than molecules with flexible chains [C.H. Lochműller, M.L. Hunnicutt and J.F. Mullaney, J. Phys. Chem., 89 (1985) 5770].

Reversed-phase columns differ also in the properties of the silica gel support. High purity of the silica gel support is very important as its possible contamination with metals such as Fe, Al, Zn, etc., may result in formation of metal chelates with some polar solutes, which are then either completely retained, or eluted as tailing bands. To meet these requirements, silica gel type B support is made by aggregating ultra-pure silica sols (sol-gel particles), whereas the silica gel type C (silica hydride) support has major part of the original silanol (Si-OH) groups replaced with silane (Si-H) groups *via* a silanization / hydrosilation procedure alumina [J.J. Pesek, J.E. Sandoval and M. Su, J. Chromatogr., 630 (1993) 95]. The original silica gel type A prepared by gelation of soluble silicates become dissolved at pH > 8, but the type B silica gel made by aggregation of silica sols is stable up to pH 9 and some fully reacted end-capped alkyl bonded phases with these supports can be used up to pH 11.

Hybrid stationary phases containing mixed silica gel - organic backbone represent recent improvement in technology of materials for reversed-phase HPLC. These materials show increased thermal and chemical stability with respect to pure silica gel supports due to lower population of surface silanol groups.

10.2. Non-silica stationary phases for RP HPLC

Aluminium, titanium and zirconium oxide particles exhibit hardness and mass transfer properties comparable to silica, but are much more stable over a broad pH range, from pH = 0 to 12 - 14. This property makes these materials attractive as bonded phase substrates, but bonding organic moieties to their surface is much more difficult than chemical modification of silanol groups. However, the surfaces of these oxides are highly active and can interact with some analytes by ligand-exchange interactions, which deteriorate the separation and make the retention process irreproducible.

These difficulties are overcome by alternative surface coating procedure by which oligomers or polymers are deposited on the support surface and then fixed by cross-linking to

form a polymeric layer (e.g., polybutadiene or alkylated polymethylsiloxane) around the support core [G. Schomburg, A. Deege, J. Kőhler and U. Bien-Vogelsang, J. Chromatogr., 282 (1983) 27], [H. Figge, A. Deege, J. Kőhler and G. Schomburg, J. Chromatogr., 351 (1986) 393]. The inorganic surface encapsulated by a non-polar stationary phase does not come into contact with the mobile phase or with the analyte, so that these materials can be used in the pH range 1 - 14 to take the advantage of full suppression of the ionization of strongly basic compounds for their efficient separation [J. Yu and Z.-E. Rassi, J. Chromatogr., 631 (1993) 91]. Main disadvantages of these packings is lower efficiency compared to chemically bonded phases because of hindered mass transfer in the relatively thick coating layer.

Chemical stability of carbon over the entire pH range has led to considerable interest in the development of carbon-based stationary phases for RPC [J.H. Knox, B. Kaur and G.R. Millward, J. Chromatogr., 352 (1986) 3]. The retention and selectivity behavior of carbon phases significantly differs from that of chemically bonded phases for RPC. Carbon adsorbents have greater affinity for aromatic and polar substances so that compounds can be separated that are too hydrophilic for adequate retention on a C_{18} column. Fixed adsorption sites make these materials more selective for the separation of geometric isomers [J.H. Knox, K.K. Unger and H. Mueller, J. Liquid Chromatogr., 6 (1983), Suppl. 1, 1].

10.3. Dual retention mechanism dependent on the mobile phase

Stationary phase effects on the retention and separation selectivity are usually less significant in mobile phases containing 20 - 80% organic solvent in water than in water rich mobile phases used for separations of strongly polar samples or, on the other hand, in mobile phases with low concentrations of water, or in non-aqueous mobile phases. Non-aqueous reversed-phase chromatography is often used for separations of very lipophilic compounds such as fats or oils. There, the residual silanol groups or other polar moieties in the stationary phase contribute to the retention by polar interactions with polar substituents on the bulky hydrocarbon backbone, such as hydroxy, carbonyl ester or other groups. The relative polarity of the mobile phase with respect to the stationary phase is distinguishing mark for the classification of normal-phase and reversed-phase systems; the importance of polar interactions increases in less polar mobile phases [J. Fischer, P. Jandera, J. Chromatogr. A, 684 (1994) 77]. Under these conditions, the retention behavior is controlled by dual mechanism involving solvophobic interactions and polar interactions with the residual silanol or other polar groups. Eq. (33) can describe the retention in aqueous-organic mobile phases resulting from the combination of the non-polar (RP) and the polar (NP) mechanisms:

$$k = \frac{\Psi_1}{K_1} + \frac{\Psi_2}{K_2} = k_1 + k_2 \qquad (33)$$

Ψ_1 and Ψ_2 are the phase ratios of the hydrocarbon moieties and of the polar groups in the stationary phase, respectively, K_1 is the equilibrium constant for the solvophobic (reversed-phase) retention and K_2 that for the polar (normal-phase) contributions to the retention. The retention factor, k, is comprised of the solvophobic, k_1, and of the polar, k_2, contributions, which depend on the polarity of the solute, on the chemistry of the stationary phase and on the composition of the mobile phase.

Dual retention mechanism, where the NP and the RP effects contribute simultaneously to the retention, is rather common phenomenon in chromatography of polar compounds on various chemically bonded phases. Nahum and Horváth [A. 1Nahum, Cs. Horváth, J. Chromatogr, 203 (1981) 53] noted that the dependences of the retention factors, k, of some crown ethers and peptides on the composition of methanol-water binary mobile phases with alkylsilica bonded stationary phases show minima. Even on "bare" silica, siloxane bonds may show some RP interactions. In reversed-phase systems with binary mobile phases (e.g., methanol - water or acetonitrile - water), the retention decreases as the concentration of the less polar solvent (methanol) increases, whereas in normal-phase chromatography the retention decreases with increasing concentration of the more polar solvent (water).

1Aqueous-organic mobile phases with low concentrations of water are employed in “hydrophilic interaction chromatography” (HILIC) [A.J. Alpert, J. Chromatogr. 449 (1990) 177] on bonded phases containing more or less polar groups, such as silica gel with low surface silanol groups population, diol, aminopropyl, or specially designed "HILIC" (e.g., polyhydroxyethyl aspartamide. Like in non-aqueous normal-phase chromatography, the retention increases as the sample polarity increases and as the concentration of water in the mobile phase decreases, opposite to RPC. HILIC provides excellent separation selectivity for some strongly polar samples, such as peptides and proteins, carbohydrates, oligonucleotides, etc., which are often difficult to separate in HPLC, as they are very weakly retained in RPC or very strongly retained in non-aqueous NPC [P. Hemström, K. Irgum, J. Sep. Sci., 29 (2006) 1784]. **Figure 18** shows an example of separation of phenolic acids on a bonded polyethylene glycol column in water-rich (reversed-phase) and acetonitrile-rich (HILIC) aqueous-organic mobile phases.

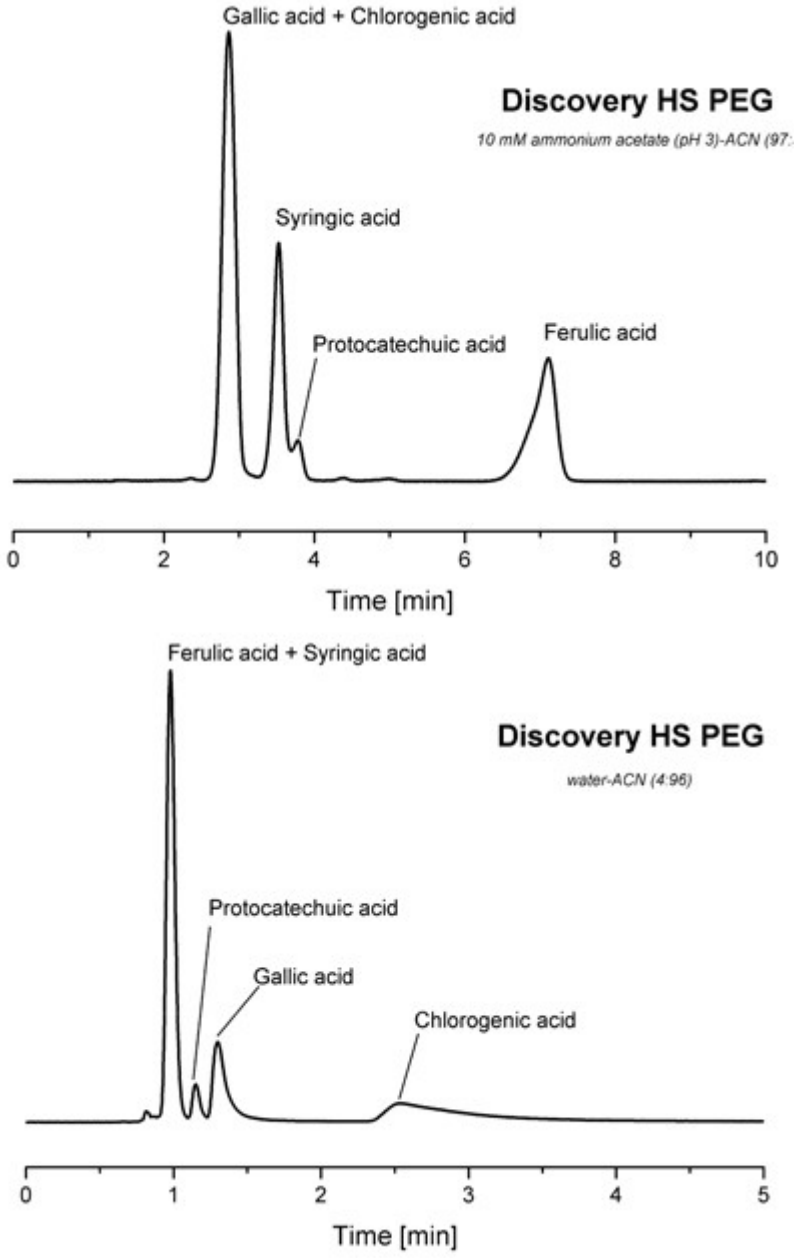


Fig. 18. Separation of phenolic acids on a bonded polyethylene glycol column by RP HPLC (3% acetonitrile and by NP HPLC (96% acetonitrile) conditions, illustrating dual retention mechanism.

Most "HILIC" columns and some RP columns with residual polar groups may show "U-turn" dependence of retention on the volume fraction of water in the mobile phase, φ. Increasing concentration of water decreases *k* in the low water concentration range (HILIC behavior), but increases *k* in the high water concentration range (RP behavior). The minimum retention at the "U-turn" composition of the mobile phase corresponds to the transition from the RP to the NP (HILIC) mechanism - see example in **Fig. 19**.

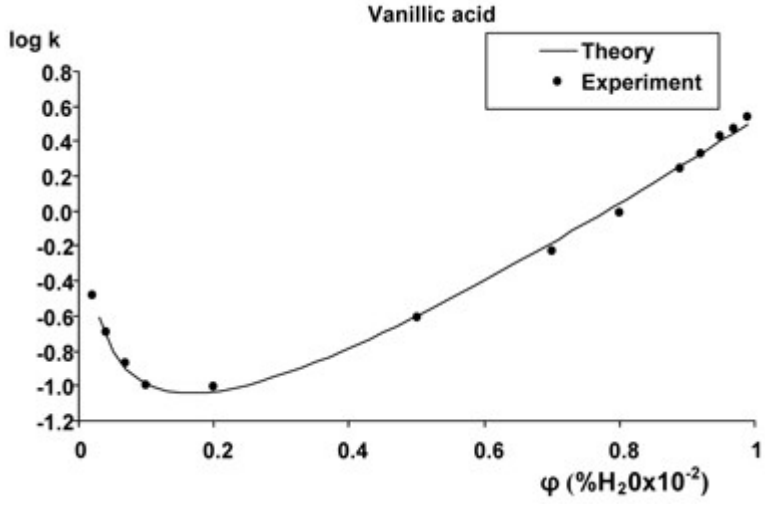


Fig. 19. Dual retention mechanism on a discovery HS PEG column controlled by the volume fraction of water, φ, in the mobile phase. Theory - best fit of Eq. (32).

These effects can be described by Eq. (34) with the parameters m_{RP} and m_{HILIC} characterizing the RP and HILIC contributions to the retention, respectively [G. Jin, Z. Guo, F. Zhang, X. Xue, Y. Jin, X. Liang, Talanta, 76 (2008) 522]:

$$\log k = a + m_{RP} \cdot \varphi - m_{HILIC} \cdot \log \varphi \qquad (34)$$

According to Eq. (34), the "U-turn" mobile phase composition at the minimum of retention can be expected at $\varphi_{min} = m_{HILIC} / 2.301 m_{RP}$ and generally depends on the stationary phase and solute; consequently with various stationary phases different mobile phase "U-turn" composition can be expected. For many compounds (but not all) the retention at the "U-turn" point is very low.

11. Model of gradient reversed-phase chromatography

In the gradient elution mode, the composition of the aqueous-organic mobile phase changes in time, *t*, most often according to a pre-set linear gradient program:

$$\varphi = A + B' F_m t = A + BV = A + \frac{\Delta \varphi}{V_G} V \qquad (35)$$

V_G is the gradient volume corresponding to the change in the volume fraction, φ, of the organic solvent changing from $\varphi = A$ at the start of the gradient to the final gradient concentration $\varphi_G = A + \Delta\varphi$ at the end of the gradient; Δφ is the gradient range and *B* or *B'* are the steepness (slope) of the gradient, characterizing the increase in φ in the time unit, or in the volume unit of the mobile phase, respectively; F_m is the flow-rate of the mobile phase.

The retention decreases continuously in course of gradient elution to speed up the

elution of strongly retained compounds, and - unlike to the isocratic mode - can be assumed constant only within a very small (differential) time interval *dt*, in which the sample zone migrates along an infinitesimally small distance in the column, corresponding to a differential part of the column hold-up volume V_m, dV_m,

$$d\left(t'_R\right) = k_i\, d\left(t_m\right) \quad ; \quad d\left(V'_R\right) = k_i\, d\left(V_m\right) \qquad (36a,\ 36b)$$

The differential equations (36a) or (36b) can be integrated after introducing the actual dependence of *k* on the time, *t* (or on the volume of the mobile phase, *V*, which has passed through the column) from the start of the gradient until the elution of the band maximum. Assuming that the column hold-up volume does not change significantly with changing mobile phase in the gradient range, the change in $d(t_R')$ during a gradient run is independent of the change in $d(t_m)$. Considering the change in the variables during gradient elution in the limits from 0 to t_R' for $d(t_R')$and from 0 to t_m for $d(t_m)$, Eq. (36a) can be integrated within these limits [P. Jandera, J. Churáček, *Gradient Elution in Column Liquid Chromatography*, Elsevier, Amsterdam 1985.]:

$$\int_0^{t_R - t_m} \frac{d\left(t'_R\right)}{k_i} = \int_0^{t_m} d(t_m) = t_m \qquad (37)$$

The LSS retention equation, Eq. (11), combined with linear gradient profile Eq. (35) can be introduced into the fundamental gradient equation (Eq. 37), whose integration yields the solution for the elution volumes, V_R, for so-called "linear solvent strength" (LSS) gradients [L.R. Snyder, J.W. Dolan. *High-performance Gradient Elution. The Practical Application of the Linear-solvent-strength Model*. Hoboken, N.J.: Wiley-Interscience, 2007]. Taking into account the contribution of the instrumental dwell volume, V_D, to the retention, the elution volumes in gradient reversed-phase chromatography can be calculated from Eq. (38) [P. Jandera, *Adv. Chromatogr*. 43, 1, 2005]:

$$V_R = \frac{1}{m\,B}\log\left\{2.31\,m\,B\left[V_m\,10^{(a\,-\,m\,A)} - V_D\right] + 1\right\} + V_m + V_D \qquad (38)$$

Eq. (38) was found to describe adequately the retention for a variety of gradient methods (see, e.g., Refs. [M.A. Quarry, R.L. Grob, L.R. Snyder, *J. Chromatogr.*, 285 (1984) 1] and [P. Jandera, *J. Chromatogr. A* 1126, 195, 2006]). For reversed-phase systems where the retention is controlled by the quadratic Eq. (10), the integration of Eq. (37) requires numerical solution [P.J. Schoenmakers, H.A.H. Billiet, R. Tijssen, and L. De Galan, *J. Chromatogr.*, 149: 9 (1978), P.J. Schoenmakers, H.A.H. Billiet, L. de Galan, *J. Chromatogr.*, 185 (1979) 179]. However, linear approximation usually provides good agreement between the calculated and the experimental gradient retention data.

Gradient elution is especially useful for separation of high-molecular compounds, for

which it is often difficult to find isocratic mobile phase providing separation over a wider range of molecular weights. The conventional model of gradient elution can principally explain gradient separations of large molecules, considering the effect of increasing size of molecules on the values of the constants of Eq. (11). Eq. (38) was found to describe adequately the retention of oligomers and lower homopolymers and copolymers up to the molar masses 10 000 – 30 000 Da [P. Jandera, *J. Chromatogr.*, 449 (1988) 361, L. Kolářová, P. Jandera, E.C. Vonk, H.A. Claessens, *Chromatographia,* 59 (2004) 579, F. Fitzpatrick, R. Edam, P. Schoenmakers, *J. Chromatogr. A*:, 988 (2003) 53]; for higher polymers the accuracy of the determination of retention model parameters is too low. The differences between the retention behaviour of small and large molecules can be explained by additive contribution of various structural elements in the molecule to the free energy of the retention, in terms of logarithms of the retention factors (Martin rule) [A.J.P. Martin, *Biochem. Soc. Symp.*, 3 (1949) 4] The retention of homo-polymers and homo-oligomers increases with regularly increasing number of a single type of repeat monomer units, *n*, and the constants *m*, *a* in Eqs. (11) and (38) are directly proportional to *n* [P. Jandera, *J. Chromatogr.*, 314 (1984) 13, P. Jandera, *J. Chromatogr.*, 449 (1988) 361]:

$$m = m_0' + m_1' n \qquad (39a) \quad ; \quad a = \log k_0 = a_0' + a_1' n \qquad (39b)$$

For reversed-phase LC of peptides and proteins with irregular structural units, Stadalius et al. [M.A. Stadalius, H.S. Gold, L.R. Snyder, *J. Chromatogr.*, 296 (1984) 31] suggested the following approximate dependence of the parameter *m* of Eq. (11) on the molecular mass, M_r :

$$m = 0.48\, M_r^{0.44} \qquad (40)$$

Because log *k* varies linearly with the product *m*.φ in reversed-phase systems - see Eq. (11), even a small change in the volume fraction of the organic solvent, φ, causes a much more significant change in the retention (*k*) of large molecules than in the retention of small molecules and often increasing the concentration of the organic solvent by even a few tenths of per cent may cause transition from “full retention” to “no retention – full elution” behaviour, in formal agreement with both “on-off” and “critical elution” models. Hence, only a narrow composition range of the mobile phase is available for the elution of large molecules, so that isocratic separation of high-molecular polymers is virtually impossible and gradient elution is a “must” in interaction chromatography of polymers. Further, the accuracy of the determination of the parameters *a* and *m* strongly decreases for high-molecular-weight polymers and there is a limiting molecular weight where the parameter *m* is very large, so that separation of polymers with molecular weights higher than this limit is not possible by gradient reversed-phase chromatography.

Ternary gradients may improve the resolution when the selectivity of binary gradients is not sufficient for the separation of a particular sample, especially when different selectivities

are desired for separation of the early-eluting and of the late-eluting compounds. In ternary solvent gradients, the concentrations of two organic solvents (such as acetonitrile and methanol) in water change simultaneously, most often in a linear manner, so that the gradient of each of the two strong solvents is described by Eq. (35) with different parameters *A* and *B*. Using the parameters of Eqs. (19) - (21) determined under isocratic conditions, it is possible to predict elution volumes in the "elution strength" (iso-selective) ternary gradients the concentration ratio of the two strong solvents is kept constant to maintain constant selectivity, but the sum of the concentrations linearly increases, whereas in "selectivity" ternary gradients the sum of the concentrations of the two strong solvents is constant, but their concentration ratio changes [P. Jandera, *J. Chromatogr.*, 485 (1989) 113]. **Fig. 20** shows an example of the effects of ternary gradient on reversed-phase separation of phenols [P. Jandera, J. Churáček, H. Colin, *J. Chromatogr.*, 214 (1981) 35].

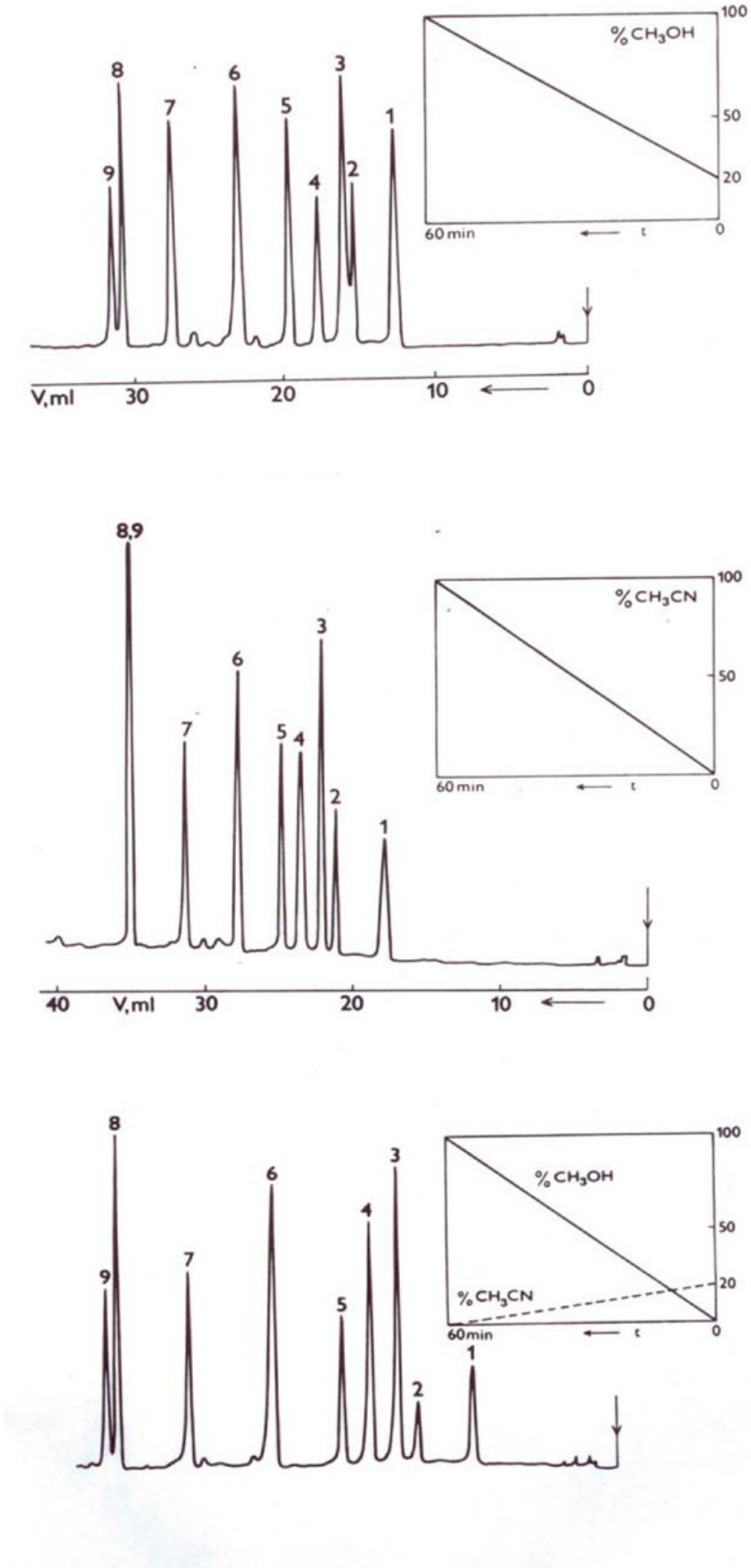


Fig. 20. Selectivity optimization using ternary gradients water- methanol - acetonitrile.

Whereas early eluting compounds 2 and 3 are poorly separated using a binary gradient of methanol in water and strongly retained compounds 8 and 9 co-elute with a gradient of acetonitrile in water, the optimized ternary gradient of simultaneously increasing concentration of methanol and decreasing concentration of acetonitrile significantly improves the resolution of both the early- and the late-eluting compounds.

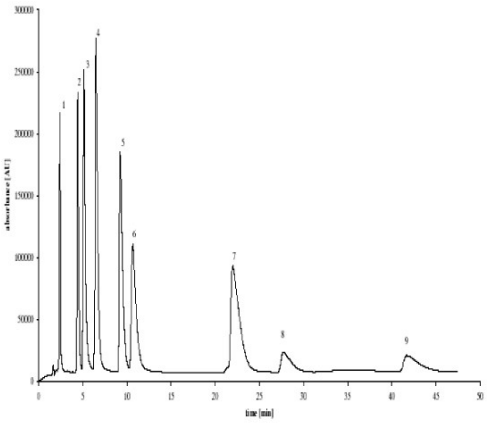
Ternary gradients with simultaneously changing pH and volume fraction of the organic solvent in a binary aqueous-organic mobile phase, φ, run on an alkylsilica column were employed for simultaneous determination of the characteristics of drug candidates in a mixture, the acidity (pK_A) and the lipophilicity (φ_0, calculated from Eq. (24), which is directly proportional to the partitioning coefficient between water and *n*-octanol, log *P*, a conventional measure of lipophilicity). The determination is based on fitting a theoretical model, assuming linear change of pK_A with the concentration of organic modifier, to the experimental gradient retention times [P. Wiczling, P. Kawczak, A. Nasal, R. Kaliszan, *Anal. Chem.* 76 (2006) 239].

11. Temperature effects - enthalpy and entropy of retention.

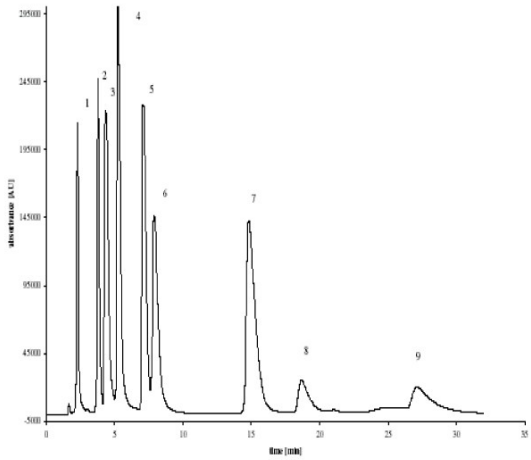
Adjusting the temperature has not been so far frequently used for controlling the retention than the elution strength of the mobile phase, as in reversed-phase chromatography, a 4 - 5°C increase in temperature causes a decrease in retention corresponding approximately to a 1% increase in concentration of methanol or acetonitrile [T. Andersen, P. Molander, R. Trones, D.R. Hegna, T. Greibrokk, *J. Chromatogr. A*, 918 (2001) 221]. Adjusting of temperature is usually less effective in improving the quality of HPLC separations than varying the composition of the mobile phase.

The main reason for relatively rare use of temperature as the operation parameter in HPLC is poor temperature stability of most silica-based columns, whose applications are usually limited to the temperatures < 60°C, especially in aqueous mobile phases at a pH below 3 or above 6. Further, using some solvents with lower boiling temperatures is restricted at higher temperatures. Only recently bidentate bonded or zirconia-based stationary phases have been introduced, allowing operation at as high temperature as 100 –200°C [T. Greibrokk, T. Andersen, J. Chromatogr. A 1000 (2003) 743], [J. Li, P.W. Carr, Anal. Chem. 69 (1997) 2202]. On the other hand, separation at elevated temperatures offers some undeniable advantages, such as improvement in the peak shape and efficiency. Increased temperature usually affects favorably the separation selectivity of ionic compounds. The regulation of temperature is very convenient and simple, as it requires only a column thermostat, which can be often connected to the HPLC system controller to enable automated optimization of temperature. Adjusting the temperature may be very useful for fine tuning of separation, especially if used in combination with the control of the mobile phase composition or of the gradient profile [J.W. Dolan, L.R. Snyder, N.M. Djordjevic, D.W. Hill, T.J. Waeghe, *J. Chromatogr. A*, 857 (1999) 1., P.L. Zhu, L.R.

Snyder, J.W. Dolan, N.M. Djordjevic, D.W. Hill, L.C. Sander and T.J. Waeghe, J. Chromatogr. A, 756 (1996) 21].



Isothermal conditions, 40 ºC



Isothermal conditions, 70 ºC

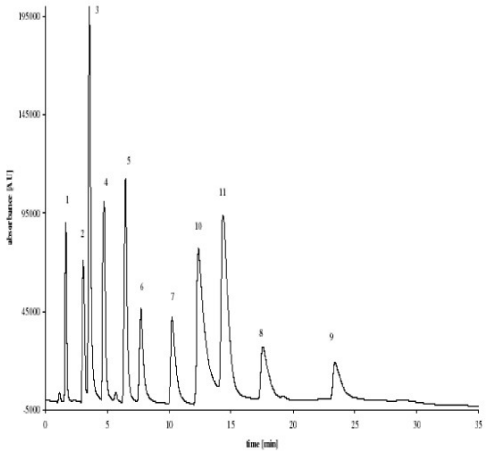


Fig. 21. Separation of 11 phenolic acids on a Discovery ZR-Carbon 150 x 4.6 mm, 5 µm column in 100 % MeOH + 0.05 M H_3PO_4 under isothermal and programmed temperature conditions.

Figure 21 illustrates decrease in the time of separation of phenolic acids at increased temperature and increased number of separated compounds at programmed temperature in comparison to isothermal conditions.

The temperature effects on chromatographic retention are explained on the basis of van ´t Hoff model, according to which the distribution coefficient K_D is proportional to the change of the Gibbs energy of retention, ΔG^0, and is related to the corresponding changes of entropy, ΔS^0, and enthalpy, ΔH^0, connected with the transfer from the mobile to the stationary phase:

$$\Delta G^0 = \Delta H^0 - T\Delta S^0 = -RT \ln K_D \qquad (41)$$

The effect of temperature on the retention factor, k, is then described by Eq. (42):

$$\ln k = \ln K_D + \ln \frac{V_S}{V_M} = -\frac{\Delta G^0}{RT} = -\frac{\Delta H^0}{RT} + \frac{\Delta S^0}{R} + \ln \frac{V_S}{V_M} = A_i + \left(\frac{B_i}{T}\right) \qquad (42)$$

The parameter A_i is related to the standard partial molar enthalpy of transfer of solute i from the mobile phase to the stationary phase and the parameter B_i to the standard partial molar entropy of transfer of solute i from the mobile phase to the stationary phase and to the phase ratio (the ratio of the volumes of the stationary, V_S, and of the mobile, V_M, phases in the chromatographic system. R is the gas constant, and T is the thermodynamic temperature in Kelvins [C.F. Poole, *The Essence of Chromatography*, Elsevier, Amsterdam, 2003], [T.L. Chester, J.W. Coym, J. Chromatogr. A, 1003 (2003) 101].

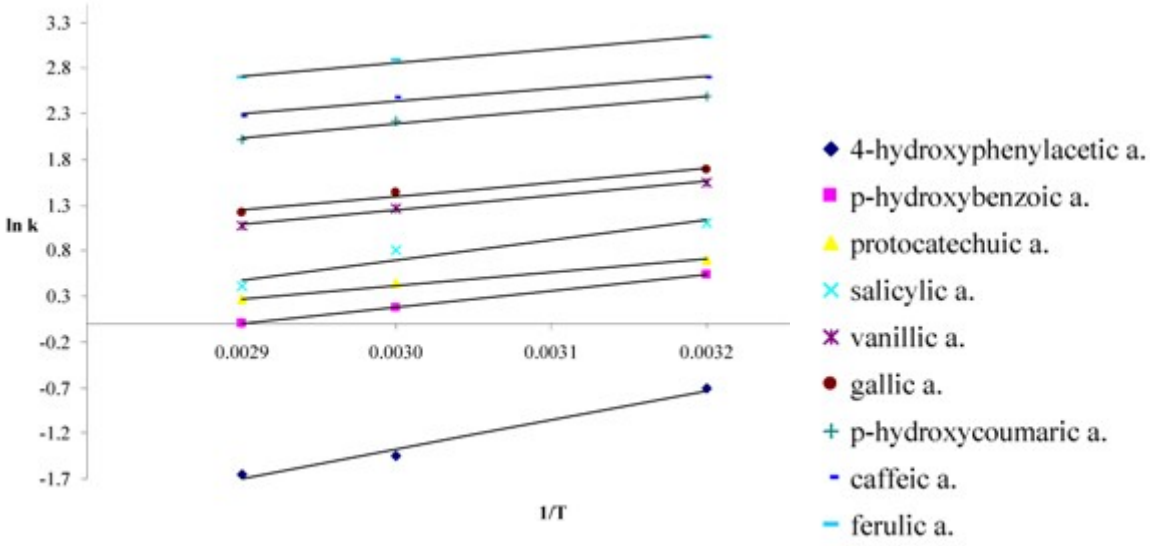


Fig 22.

Van't Hoff temperature effects on retention factors of phenolic acids.

Discovery ZR-Carbon column, 150 x 4.6 mm, 5 µm, 100 % MeOH + 0.05 M H_3PO_4

Figure 22 illustrates validity of van't Hoff plots for phenolic acids on a zirconia carbon

column suitable for high-temperature operation. Plotting ln *k versus* the reciprocal values of absolute temperature 1/*T* (Eq. 41) over a sufficiently broad temperature range, one may calculate ΔH^0 from the slope and ΔS^0 from the intercept of the plot. Van´t Hoff plots may provide information on whether or not the retention mechanism(s) may change over the studied temperature range. The enthalpy and the entropy contributions to chromatographic retention and selectivity at various temperatures can be calculated, which reveal information about the nature of the chromatographic retention and selectivity [R.P.J. Ranatunga, P.W. Carr, Anal. Chem., 72 (2000) 5679].

From adapted Eq. (42), so-called compensation temperature can be calculated at which the enthalpic and the entropic contributions to the retention are at equilibrium. Selectivity changes at varying temperature can be caused by either enthalpic or entropic effects. The latter are usually attributed to shape related changes in conformation of the solutes adsorbed in the stationary phase. In this case, temperature regulation can be used for optimizing the resolution.

Non-linear enthalpy changes at increasing temperature can appear as the result of non-linearly increasing adsorption enthalpy with increasing molar masses of analytes, or of temperature-related changes of the solvation of molecules in the mobile or stationary phases [D. Bolliet, C.F. Poole, Analyst, 123 (1998) 295], [D.V. McCalley, J. Chromatogr. A, 902 (2000) 311]. Deviations from the validity of Eq. (33) indicate either mixed retention mechanism, secondary chemical equilibria [J.W. Li, Anal. Chim. Acta, 369 (1998) 21], or entropic changes in the solute (or stationary phase) conformation in the stationary phase [L.C. Sander, S.A. Wise, J. Sep. Sci., 24 (2001) 910], [Y.X. Chen, C.T. Mant, R.S. Hodges, J. Chromatogr. A, 1010 (2003) 45].

If a single mechanism controls the retention (such as hydrophobic interactions in a homologous series), decrease in the retention and separation selectivity (in terms of relative retention) is usually observed at increasing temperature [D. Bolliet, C.F. Poole, Analyst, 123 (1998) 295]. Increased retention and/or selectivity may be occasionally observed at elevated temperatures, which are attributed to secondary interactions [D.V. McCalley, J. Chromatogr. A, 902 (2000) 311], reduced ionization or reduced solubility in the mobile phase at higher temperatures and for systems with a temperature-dependent phase ratio [T.L. Chester, J.W. Coym, J. Chromatogr. A, 1003 (2003) 101].

Empirical linear or polynomial models of relationships between the retention and temperature are occasionally used in prediction and optimization of separation conditions:

$$\log k = a_0 - a_1 T \qquad (43)$$

The best- fit constants of Eq. (43), a_0, a_1, etc. do not have any physical meaning. T in Eq. (43) is often expressed in degrees Celsius for convenience [P.L. Zhu, J.W. Dolan, L.R. Snyder, D.W. Hill, L. Van Heukelen, T.J. Waeghe, J. Chromatogr. A, 766 (1996) 51].

Temperature gradients are simple to use, do not need complex gradient pumps and column equilibration after the end of the experiment requires less time than in gradient elution [P. Jandera, L.G. Blomberg, E. Lundanes, *J. Sep. Sci.*, 27 (2004)_1402]. However, temperature programming usually provides a limited change in retention compared to mobile phase composition gradients, so that a large rise in temperature during the run is necessary to reduce significantly the retention of strongly retained compounds. The exception is separation of polymers, as large molecules are much more sensitive to a change in temperature or in mobile phase composition than the small ones [B. Ooms, *LC-GC*, 14(4) (1996) 306], [M.H. Chen, M.H.; Cs. Horváth J. Chromatogr. 788 (1997) 51], and programmed temperature represents there useful alternative to solvent gradients.