ELSEVIER

Journal of Chromatography A, 758 (1997) 37–51

JOURNAL OF CHROMATOGRAPHY A

Interactions of basic compounds in reversed-phase high-performance liquid chromatography
Influence of sorbent character, mobile phase composition, and pH on retention of basic compounds

David Sýkora*, Eva Tesařová, Milan Popl

Institute of Chemical Technology, Department of Analytical Chemistry, Technická 5, 166 28 Prague, Czech Republic

Received 20 May 1996; revised 6 August 1996; accepted 6 August 1996

Abstract

Differences in a degree of base deactivation of selected reversed-phase sorbents were evaluated. Five silica-based sorbents (SGX C_{18}, LiChrosorb RP-18, LiChrospher RP-select B, Purospher RP-18, Symmetry C_{18}), a polybutadiene-coated alumina (Aluspher RP-select B) and 2-hydroxyethylmethacrylate-based stationary phase (HEMA-BIO 1000 C_{18}) were compared. The best results were obtained for Symmetry C_{18} and HEMA-BIO 1000 C_{18}. For testing purposes a set of nineteen basic compounds differing in pK_a constants and hydrophobicity was used. The bases possessing high pK_a values (pK_a>9.0) and/or low hydrophobicity proved to be the best indicators of sorbent surface deactivation. Dependencies of capacity factor on pH of the mobile phase were measured. Shape deviations between the sigmoidal theoretically predicted and experimentally obtained curves evidence for a complex retention mechanism in which not only hydrophobic but also other interactions, for example, ion-exchange, participate. The effect of methanol content in the mobile phase on the dissociation of buffer and a basic analyte used was studied. The characteristic shifts of k versus pH of the mobile phase dependencies caused by the addition of methanol were found to be in a good agreement with theory.

Keywords: Mobile phase composition; Sorbent character; pH effects; Retention; Aminopyridine; 2-Amino-4-picoline; Picoline; 2,6-Lutidine; 2,4,6-Collidine; N-Ethylaniline; N,N-Dimethylaniline; N-Benzylmethylamine; 2-Phenylethylamine; Quinoline; 2-Methylquinoline; Dimethylquinoline; Aniline; Pyridine

1. Introduction

Reversed-phase (RP) chromatography continues to dominate applications of high-performance liquid chromatography (HPLC) [1–3].

Alkyl-bonded reversed phases with silica backbone are still very popular. Modified silica has good chromatographic properties and neutral compounds can be successfully analyzed with high efficiency and reproducibility. Separations of basic compounds often cause much more difficulties [4,5]. The majority of silica reversed-phase column packings are produced by reacting porous silica particles with an appropriate silane. Silanol groups (−SiOH) on the silica-gel surface are consumed in this reaction but steric effects prevent the reaction of more than 30–40% of all the silanols [6]. Further reaction with a short silane (endcapping) eliminates the most access-

*Corresponding author.

0021-9673/97/$17.00
PII S0021-9673(96)00691-7

ible silanols remaining from the initial bonding but typically does not substantially alter the total concentration of unreacted silanols [7]. These residual silanols can then interact with basic analytes, frequently leading to inferior separations. The bulk of available evidence suggests that the interaction of basic solute B with acidic site on the RP packing occurs by an ion-exchange process [6]:

$$BH^+ + SiO^-Na^+ \rightleftharpoons Na^+ + SiO^-BH^+ \quad (1)$$

Hereby it is assumed that the strongly acidic site (−SiOH) is in the sodium form and the basic solute B is protonated. There are many possible ways of suppressing this interaction and enhancing the efficiency of separation. The reduction of ionisation of acidic sites by employing the mobile phase of low pH (pH<4) or, in contrast, the decrease of ionisation of the basic sample by increasing the pH of the mobile phase are the easiest methods [8–10]. Other approaches take advantage of the addition of 'silanol blockers', e.g. triethylamine, to the mobile phase [6,9]. To elaborate a reliable chromatographic method is often a very time-consuming process.

A great deal of work has been done on the characterisation and comparison of different reversed-phase silica surfaces in the last two decades. A wide range of analytical methods has been employed for those studies [2,11]. On the basis of the knowledge gained new silica-based RPs possessing lower silanol activity have been synthesised and modified [12].

Recently, many manufacturers have developed new technologies for the preparation of 'deactivated' silica and have improved bonding procedures for preparation of RPs. As a result, at present, base deactivated RPs are on the market. Special manufacturing procedures deliver a high-purity silica with silanol groups homogeneously spread on the surface. As a consequence, the addition of triethylamine or other blocking agents can be diminished, or, in some cases, avoided. Columns packed with those sorbents are mainly intended for separations of biological and pharmaceutical samples [10,11,13–17]. The rapidly increasing number of newly introduced base-deactivated RP sorbents of different brands enable us to compare rather excessive sets of packings by means of chemometric methods [16,17].

Problems with residual silanols can be also circumvented by using polymeric sorbents. Generally, they have wider pH operating ranges (often from 1 to 14) and provide longer lifetimes, but they display somewhat lower efficiency than silica-based reversed-phase sorbents of the same particle size [3,18].

The use of high-purity silica or alumina as a support for polymer coating is another alternative for the preparation of a new reversed-phase sorbent [19–21]. Polymer-bonded or coated phases are intermediates between bonded silica and polymeric packings. They combine the excellent mechanical properties of silica beads with high chemical inertness of the polymeric layer. Polymer coating should shield silanols and render the modified silica or alumina surface more suitable for the separation of basic compounds.

Very promising results have been achieved in the development of new phases convenient for the separation of basic compounds. However, some difficulties are outlasting and there is no doubt that research in this area has to continue.

In this study seven different reversed-phase sorbents have been compared from the point of view of their potential for the separation of basic compounds. The degree of base deactivation of particular sorbents has been estimated on the basis of relations among the solute hydrophobicity, the pK_a value and its retention characteristics. The influence of the methanol content in the mobile phase on the dissociation of basic compounds has been studied in detail.

2. Theoretical

2.1. Establishment of pH* operational scale

The definition and determination of pH is a key question when using aqueous solvent with an organic modifier. For a quantitative evaluation of the effects which influence the ionisation of bases in aqueous–organic solutions a theory for pH measurement elaborated by de Ligny et al. [22] and Bates at al. [23] should be employed. Operationally, the pH* value (pH in an aqueous–organic system) of a tested mixed solution, designated by pH^*_x, is determined in

the normal manner by comparing the measured e.m.f. value, E_x^*, with that obtained for a standard buffer solution, E_{st}^*, of known pH_{st}^* in the identical solvent composition:

$$pH_x^* = pH_{st}^* + \frac{(E_x^* - E_{st}^*)F}{2.3RT} \quad (2)$$

F is the Faraday constant, R the universal gas constant, T is the temperature.

Because the calibration of a pH-meter with standards of known pH_{st}^* values has been inconvenient, another method for determination of pH_x^* has been proposed. It has allowed the use of aqueous standard buffers instead of mixed standard buffers:

$$pH_x^{app} = pH_{st} + \frac{(E_x^* - E_{st})F}{2.3RT} \quad (3)$$

where pH_x^{app} is the measured pH value of a tested sample in a mixed aqueous–organic solution referred to an aqueous standard buffer, pH_{st} is the known value of an aqueous standard buffer and E_{st} is the measured e.m.f. of that buffer. Due to the additional correction for the liquid junction and ignoring the difference in the standard potentials of the glass electrode between the mixed solvent and the pure aqueous media, as this term has been shown to be small [24], the following equation should be considered [25,26]:

$$pH_x^* = pH_x^{app} - \delta \quad (4)$$

where δ is a correction factor. Values of δ have been published for most of the methanol–aqueous mixed solvents [23,27,28].

2.2. Dissociation of basic compounds in aqueous–organic solutions

For a buffer solution in an aqueous–organic mixture the Henderson–Hasselbach equation can be applied [29]:

$$pH^* = pK_{a(A)}^* + \log([A^z]/[AH^{z+1}]) \quad (5)$$

where $pK_{a(A)}^* = -\log K_{a(A)}^*$ and $K_{a(A)}^*$ is the appropriate dissociation constant of a buffer in an aqueous–organic solvent, $[A^z]$ and $[AH^{z+1}]$ are molar concentrations of single respective forms of the buffer in an aqueous solution, z is the charge, and activity coefficients have been neglected.

Let us now consider changes in the dissociation of a basic compound in a buffered aqueous–organic solution. The dissociation can be again described by the Henderson–Hasselbach equation applied to both the basic solute and the buffer as follows [30]:

$$\log([B]/[BH^+]) = pK_{a(A)}^* - pK_{a(B)}^* + \log([A^z]/[AH^{z+1}]) \quad (6)$$

where $[B]$ and $[BH^+]$ are the molar concentrations of non-ionised and ionised form of the basic solute, $pK_{a(B)}^* = -\log K_{a(B)}^*$ and $K_{a(B)}^*$ is the dissociation constant of the basic solute in the aqueous–organic solvent, and activity coefficients have been again neglected.

The ratio $[A^z]/[AH^{z+1}]$ can be expressed using the pH of the aqueous buffer and its dissociation constant (stated as $pK_{a(A)}$) in water and Eq. (6) may be rewritten:

$$\log([B]/[BH^+]) = pH - pK_{a(A)} + pK_{a(A)}^* - pK_{a(B)}^* \quad (7)$$

and then transformed into:

$$\log([B]/[BH^+]) = pH - pK_{a(B)} + \Delta pK_{a(A)} - \Delta pK_{a(B)} \quad (8)$$

where $\Delta pK_{a(A)} = pK_{a(A)}^* - pK_{a(A)}$ and $\Delta pK_{a(B)} = pK_{a(B)}^* - pK_{a(B)}$. Eq. (8) is valid for buffered solutions within the pH range near the appropriate $pK_{a(A)}$. The dependence of a solute dissociation in a mixed solution on the pH of an appropriate aqueous buffer is shifted with respect to the same dependence in an aqueous buffer. The magnitude of the shift is given by the difference $\Delta pK_{a(AB)} = \Delta pK_{a(A)} - \Delta pK_{a(B)}$. Apparently, the total shift corresponds to a difference of the two terms, $\Delta pK_{a(A)}$ and $\Delta pK_{a(B)}$, where the first one represents the influence of an organic modifier on the dissociation constant of the buffer and the other one the same influence on the dissociation constant of the solute. The direction and magnitude of the individual shifts depend on the buffer quality and the kind and the amount of the organic modifier in the mobile phase.

2.3. Chromatographic aspects of the theory

Theoretical models describing a sigmoidal dependence of the distribution coefficient (K_D) and/or the capacity factor (k) on the pH of the mobile phase for acidic and basic compounds on reversed-phase sorbents have been derived and partly experimentally verified [31–38]. The equation for the capacity factor of a monoprotic base has been established as follows:

$$k = \frac{k_0 + k_1 \frac{[H^+]}{K_{a(B)}}}{1 + \frac{[H^+]}{K_{a(B)}}} \quad (9)$$

k_0 and k_1 are the capacity factors of a neutral and completely ionised base, respectively, $K_{a(B)}$ is the dissociation constant of a protonated basic compound (its conjugated acid) in an aqueous mobile phase and $[H^+]$ is the concentration of the hydrated proton.

Eq. (9) was originally elaborated for aqueous solutions only; however, it is of general validity. For aqueous–organic solvents $[H^+]$ and $K_{a(B)}$ should be substituted by $[H^+]^*$ and $K^*_{a(B)}$, respectively, where $[H^+]^*$ is the concentration of the solvated proton in a mixed aqueous–organic solvent and $K^*_{a(B)}$ is the dissociation constant of a protonated basic compound in the same solvent. If more convenient $[H^+]$ (pH measured before the addition of organic modifier) and $K_{a(B)}$ values are applied in Eq. (9) with mixed solvents, a specific shift of the experimentally obtained k versus pH dependence in comparison to the same dependence in aqueous solvents has to be expected.

Eq. (9) has been proposed for cases where the interaction between a sorbent and a solute is exclusively controlled by their hydrophobicity, usually expressed as log P (P is the partition coefficient of a solute between n-octanol and water). It is not applicable if more interactions of different origin are involved in the separation process.

Thus, it can be concluded that shifts of sigmoidal curves of k versus pH of the mobile phase are related to the influence of an organic modifier on the dissociation of both the basic solute and the buffer. On the other hand, eventual deformations of the sigmoidal curves give evidence of the presence of non-hydrophobic interactions on a tested reversed-phase sorbent.

3. Experimental

3.1. Chemicals

The bases tested are listed in Table 1. 2-Aminopyridine, 3-aminopyridine, 2-amino-4-picoline, N-benzylmethylamine, 2-phenylethylamine were obtained from Aldrich (Milwaukee, WI, USA), the other bases from Lachema (Brno, Czech Republic). Water was redistilled, methanol, orthophosphoric acid (85%) and sodium hydroxide were purchased from Lachema. All chemicals were analytical-reagent grade.

3.2. Liquid chromatographic system

A modular HPLC system was used, consisting of a Model LCP 3001 HPLC pump (Ecom, Prague, Czech Republic), a Model 7125 Rheodyne sample valve (Cotati, CA, USA) with a 20-μl sample loop

Table 1
Basic compounds used in the study

No.	Compound name	pK_a [a]	pK_a [b]	log P [c]	log P [d]
1	2-Aminopyridine	6.71	6.84	–	0.50
2	3-Aminopyridine	6.03	5.25	0.11	0.20
3	4-Aminopyridine	9.18	8.61	0.28	0.26
4	2-Amino-4-picoline	7.48	7.67	1.02	1.02
5	2-Picoline	5.94	6.02	–	1.17
6	3-Picoline	5.66	5.60	1.20	1.17
7	4-Picoline	6.03	6.08	1.22	1.17
8	2,6-Lutidine	6.60	6.78	–	1.69
9	2,4,6-Collidine	7.43	7.61	–	2.21
10	N-Ethylaniline	5.12	5.50	2.26	2.13
11	N,N-Dimethylaniline	5.10	5.06	2.31	2.18
12	N-Benzylmethylamine	9.54	9.52	–	1.24
13	2-Phenylethylamine	9.81	9.81	1.41	1.45
14	Quinoline	4.80	4.64	2.06	2.05
15	2-Methylquinoline	5.42	5.66	–	2.57
16	2,4-Dimethylquinoline	–	6.64	–	3.09
17	2,6-Dimethylquinoline	–	6.22	–	3.09
18	Aniline	4.62	4.58	0.98	0.99
19	Pyridine	5.20	5.25	0.64	0.64

[a] pK_a constants in aqueous solution at 25°C, ion strength $I \approx 0.01$ [45].

[b] pK_a constants in aqueous solution at 25°C, computed with software package PALLAS for prediction of pK_a constants [46].

[c] Partition coefficients in n-octanol–water taken from [47].

[d] Partition coefficients in n-octanol–water computed with software package PALLAS for prediction of log P values; CDR fragment database was used in this case [48].

and a Model UV Monitor 1205-1280 (Waters, Framingham, MA, USA) operating at 254 nm. Signal acquisition and data handling were performed with a CSW Data station, v.1.6 (Data Apex, Prague, Czech Republic).

For sorbents and columns see Table 2.

Columns were thermostatted at 30°C in the water bath. Mobile phases were preheated in a water bath immersed capillary (2 m length, 0.75 mm I.D.) inserted between the HPLC pump and the injection valve. The injection volume of all the basic solutes was 5 μl at sample concentrations of 10 mmol/l. For the determination of column void volumes deuterated water was used according to Knox and Kaliszan [39].

3.3. Mobile phases composition

All experiments were carried out with mixtures of methanol–sodium phosphate buffer, where the pH value was measured before the addition of methanol in all instances. Mixed buffers were prepared as follows: 25 mmol orthophosphoric acid was diluted to 700 ml redistilled water, adjusted to the required pH with 0.5 mol/l NaOH and diluted with water to 1 l. Aqueous-organic buffers were prepared by mixing methanol with the aqueous buffers (v/v). For experiments on silica-based reversed phases the methanol–buffer ratio was 6:4, for Tessek Separon HEMA-BIO 1000 C_{18} sorbent the ratio 2:8 was applied and Aluspher RP-select B sorbent was tested in aqueous buffers. Silica-based sorbents were measured in the pH range from 2.5 to 7.5, the other sorbents in the range from 2.5 to 10.5.

4. Results and discussion

The basic solutes have been chosen to cover a wide range of hydrophobicity and pK_a constants (Table 1). The compounds have low molecular weights and are well-characterised species. All of them can be sorted into small consistent groups of structurally related compounds (often isomers), where some members of the individual group have similar pK_a constants and the other ones possess essentially different pK_a values at comparable hydrophobicity of all the compounds within the group. The individual groups of bases are at the same time mutually diverse in their hydrophobicity.

Selected stationary phases (Table 2) include five silica-based reversed-phase sorbents with different

Table 2
Columns and sorbents used in the study

Column		Sorbent	Manufacturer, Notes
1	CGC 150×3 mm (Compact Glass Cartridge)	Separon SGX C_{18} 7 μm	Tessek, Prague, Czech Republic Conventional silica-based RP for general purpose
2	CGC 150×3 mm	LiChrosorb RP-18 7 μm	Merck, Darmstadt, Germany Conventional silica-based RP for general purpose
3	CGC 150×3 mm	LiChrospher RP-select B 5 μm	Merck, Darmstadt, Germany Silica-based RP Compatibility for basic compounds
4	LiChroCART 125×4 mm (Stainless steel cartridge)	Purospher RP-18 5 μm	Merck, Darmstadt, Germany Base-deactivated silica RP Latest generation of silica-based RPs
5	Symmetry 150×3.9 mm (Stainless steel cartridge)	Symmetry C_{18} 5 μm	Waters, Milford, MA, USA Base-deactivated silica RP Latest generation of silica-based RPs
6	CGC 150×3 mm	Separon HEMA-BIO 1000 C_{18} 10 μm	Tessek, Prague, Czech Republic Hydroxyethylene methacrylate-based RP Specially developed for bioapplications
7	CGC 150×3 mm	Aluspher RP-select B 5 μm	Merck, Darmstadt, Germany Alumina coated with polybutadiene Developed for separation of basic compounds, especially suitable for high pH of the mobile phase (up to 12)

degrees of surface deactivation. One polymeric sorbent and a polymer-coated aluminium oxide have been added to the set of columns to compare silica-based sorbents with stationary phases of different nature. The sorbent HEMA-BIO 1000 C_{18} is known to have an extremely low concentration of ionic sites on its surface. Aluspher RP-select B is claimed to be a suitable sorbent for the separation of basic compounds in alkaline and/or non-buffered mobile phases.

Mixed methanol–sodium phosphate buffers have been applied as mobile phases in the experiments because of their widespread use in RP-HPLC. The ratio of methanol–buffer has been established for individual columns with the aim of obtaining k preferentially between 0.5 and 20 for most of the bases.

The above-mentioned inputs created prerequisites for the study of several different effects within a rather limited number of experiments.

In the first part of this work differences among the sorbents were studied. The main effort was focused on the estimation of the extent of the eventual non-hydrophobic interactions on individual sorbents. For this purpose capacity factors of all the tested bases on all the columns in the buffered methanolic mobile phases of different pH were measured extensively.

Figs. 1–7 show the dependencies of the measured capacity factors on the pH of the mobile phases for four of the tested bases on all seven sorbents. These selected bases were chosen with the aim of differing considerably in their sensitivity to the presence of ion-exchange centres on the sorbent surface.

As mentioned previously (see Section 2), sigmoidal curves would be obtained only if analyte retention depends solely on hydrophobic interaction. If the retention mechanism includes also other interactions, the k versus pH dependence can show completely different shapes or at least some deviation from the ideal sigmoidal shape. The difference between the obtained curve and the theoretically predicted one is closely related to the degree of the sorbent surface deactivation, physico-chemical characteristics of the solute (mainly pK_a value and hydrophobicity) and the composition of the mobile phase.

It has been found that the higher the pK_a value of

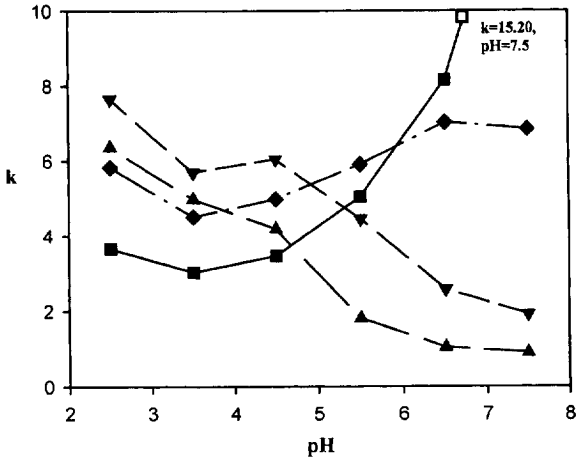

Fig. 1. Dependence of capacity factors on pH. Sorbent: SGX C_{18}. Mobile phase: methanol, 25 mmol/l sodium phosphate buffer (6:4). ▲=2-aminopyridine, ▼=2-amino-4-picoline, ♦=4-aminopyridine, ■=2-phenylethylamine.

the solute, the higher its ability to reveal undesirable interaction with the sorbent. This result is in accord with observations of other authors [6,9]. 2-Aminopyridine with a rather low pK_a value ($pK_a=6.84$) was the least sensitive probe of the shown bases in Figs. 1–7. It gave nearly ideal sigmoidal dependencies on five of the seven sorbents. The properties of SGX C_{18} and Aluspher RP-select B made a great

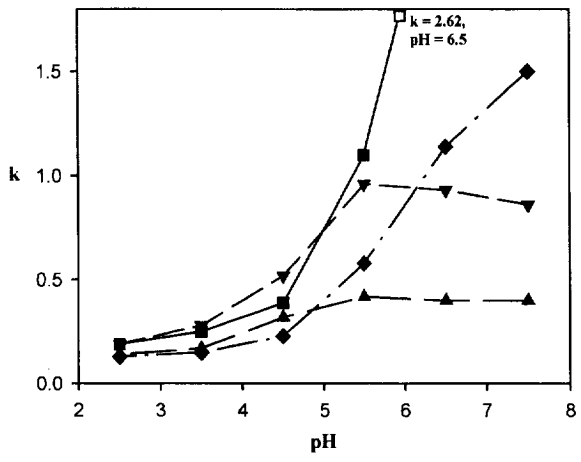

Fig. 2. Dependence of capacity factors on pH. Sorbent: LiChrosorb RP-18. Mobile phase and symbols as in Fig. 1.

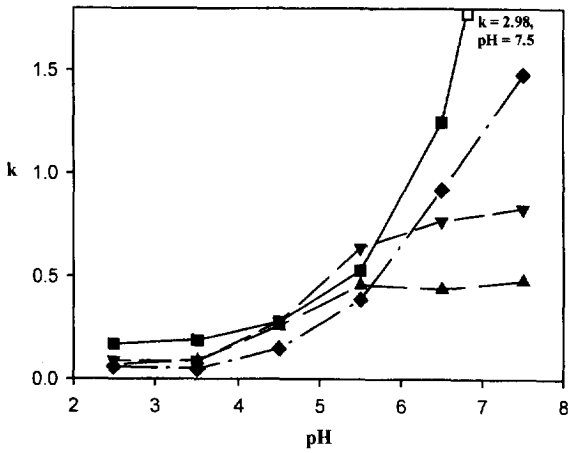

Fig. 3. Dependence of capacity factors on pH. Sorbent: LiChrosorb RP-select B. Mobile phase and symbols as in Fig. 1.

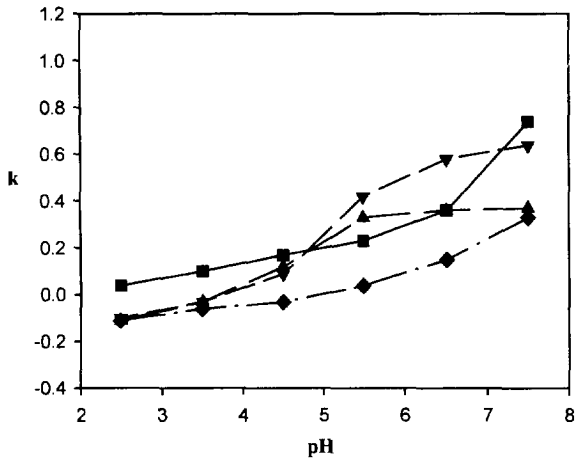

Fig. 5. Dependence of capacity factors on pH. Sorbent: Symmetry C_{18}. Mobile phase and symbols as in Fig. 1.

difference from the other sorbents, so that it was possible to distinguish them even with such a non-sensitive base. 2-Amino-4-picoline ($pK_a = 7.67$) was a better indicator of ion-exchange sites on the sorbent surface. This compound possesses the capability of disclosing not only strong ion-exchange interactions on SGX C_{18} and Aluspher RP-select B but it also reveals a slight difference in the degree of the surface deactivation between LiChrosorb RP-18 (Fig. 2) and LiChrospher RP-select B (Fig. 3) where the latter has proved to be a better base-deactivated sorbent. Both 4-aminopyridine ($pK_a = 8.61$) and 2-phenylethylamine ($pK_a = 9.81$) were found to be extremely sensitive to the presence of ion-exchange centres on the sorbent surface. The only pair of sorbents which could not be distinguished by means of 4-aminopyridine was Purospher RP-18 (Fig. 4) and Symmetry C_{18} (Fig. 5). However, the most basic compound within the studied set, 2-phenylethylamine, showed stronger retention on Purospher RP-18 than on Symmetry C_{18}, and thus gave evidence of

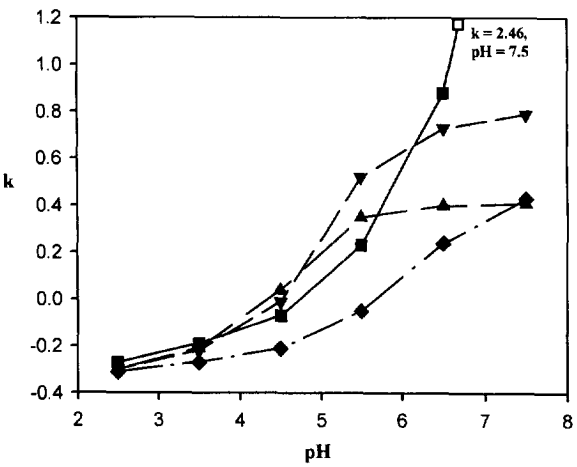

Fig. 4. Dependence of capacity factors on pH. Sorbent: Purospher RP-18. Mobile phase and symbols as in Fig. 1.

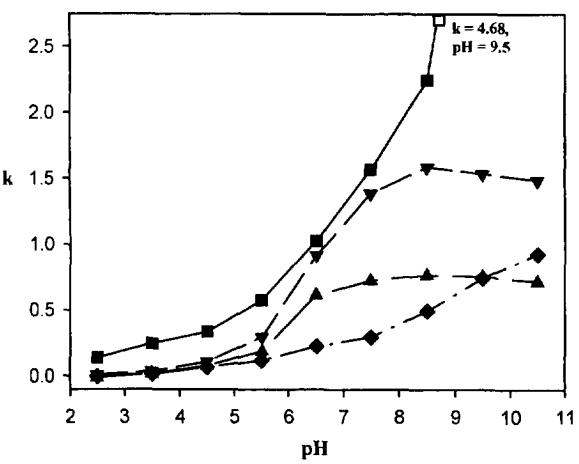

Fig. 6. Dependence on capacity factors on pH. Sorbent: HEMA-BIO 1000 C_{18}. Mobile phase: methanol, 25 mmol/l sodium phosphate buffer (2:8). Symbols as in Fig. 1.

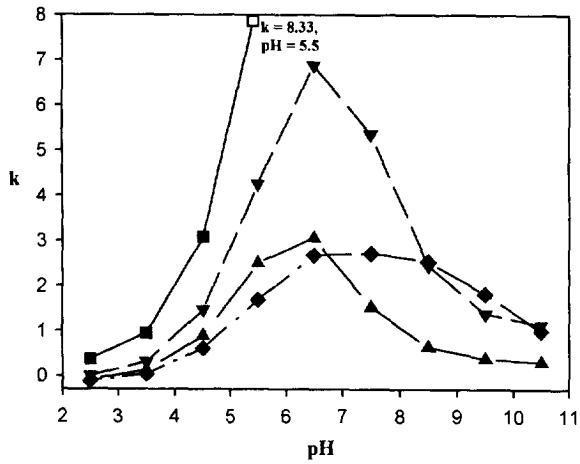

Fig. 7. Dependence of capacity factors on pH. Sorbent: Aluspher RP-select B. Mobile phase: 25 mmol/l sodium phosphate buffer. Symbols as in Fig. 1.

the best base deactivation of Symmetry C_{18}. Along with N-benzylmethylamine (results not shown) 2-phenylethylamine (both having $pK_a > 9.0$) proved to be the most sensitive testing solutes.

The analysis of all the obtained data with SGX C_{18} (Fig. 1) shows its divergence from the other tested silica-based sorbents. No tested base gave a reasonable sigmoidal curve on SGX C_{18}. This sorbent provided enormously strong interaction with basic compounds even at pH 2.5. This phenomenon might be explained by the presence of metallic impurities in the silica matrix and/or the presence of a portion of the residual silanols possessing very low pK_a values in the SGX C_{18} matrix. These unexpected high capacity factors of the bases at pH 2.5 were recently reported by Wan et al. [37] on Hypersil ODS (conventional non-base-deactivated packing).

LiChrosorb RP-18 proved to be a much better deactivated sorbent (Fig. 2). On LiChrosorb RP-18 certain departures of sigmoidal curves from the ideal shape were observed only for bases with medium and high pK_a constants. Herewith, the enhanced retention of 2-amino-4-picoline at pH 5.5 in comparison to pH 6.5 and 7.5 was caused by ion-exchange interactions which influence the retention process at that pH. On the contrary, 2-aminopyridine with a low pK_a value gave a very good sigmoidal curve. For the bases of high hydrophobicity (log $P > 1.1$) and low pK_a constant ($pK_a < 7.0$), a good agreement of the theoretically predicted and the measured data was found. The sorbent LiChrospher RP-select B (Fig. 3) displayed even lower ion-exchange interaction than LiChrosorb RP-18. Nevertheless, high retention of 4-aminopyridine gave an unquestioned proof of non-hydrophobic interaction of the solute on this sorbent, at least at pH above 5. Low retention of 4-aminopyridine was obtained on Purospher RP-18 (Fig. 4) that showed ideal behaviour with regard to nearly all selected bases. The low capacity factor of 4-aminopyridine even at pH 7.5 was in accordance with the rather low value of log P of the analyte (Table 1) and indicated a highly base-deactivated surface of Purospher RP-18. However, the retention of 2-phenylethylamine was unsatisfactory high at pH 7.5 (see the relative difference of the curves for 2-phenylethylamine on Purospher RP-18 (Fig. 4) and on Symmetry C_{18} (Fig. 5) in comparison to the other three bases). The only silica-based sorbent which passed successfully all the tests was Symmetry C_{18} (Fig. 5). The data measured on Purospher RP-18 and Symmetry C_{18} revealed subtle differences between these two well-base-deactivated sorbents. The retention of 2-phenylethylamine on Symmetry C_{18} was considerably lower than on Purospher RP-18, and this low capacity factor was in good agreement with the log P value for this compound (Table 1).

The polymeric sorbent HEMA-BIO 1000 C_{18} (Fig. 6) showed exclusively hydrophobic interaction with the bases and sigmoidal curves were obtained for all the tested basic compounds, independent of their hydrophobicity.

Alumina coated with polybutadiene (Fig. 7) showed strong interaction with the tested solutes. The surface of this sorbent is not sufficiently shielded with the polymeric layer and non-hydrophobic interactions participating in the retention process. A better agreement of the theoretical and the obtained data was found for hydrophobic compounds with sufficiently high log P (log $P > 1.6$) and $pK_a <$ 9.0 at the same time. The capacity factors of only eight of nineteen compounds fit Eq. (9).

Another view of the results is demonstrated in Figs. 8–12. There depict the dependencies of log P (Table 1) on log k for five sorbents. Values of log k are limiting values which were obtained from Eq. (9) by means of the non-linear least-square method

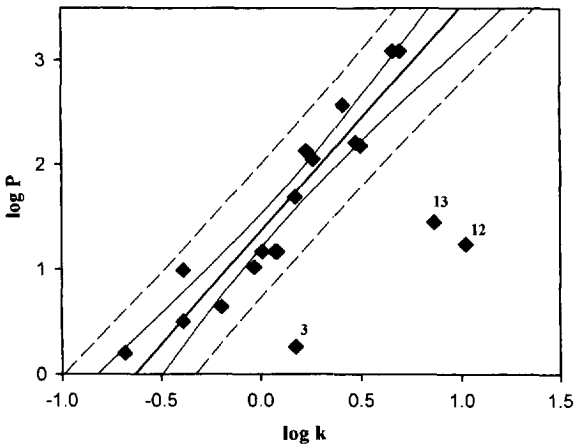

Fig. 8. Dependence of calculated log P ([48]) on calculated limiting values of log k for all the studied bases on LiChrosorb RP-18. For numbering of bases see Table 1. Central thick line depicts calculated regression, two thin lines specify 95% confidence limits for the regression and two thin dashed lines circumscribe the 95% confidence interval for the population.

(n.l.s.m.); they represent logarithms of capacity factors of the non-ionised form of the bases (for the detailed description of the calculation procedure see caption to Fig. 16).

Due to strong non-hydrophobic interaction of the basic compounds on SGX C_{18} and Aluspher RP-select B these two sorbents had to be excluded from the evaluation.

Generally, the partition coefficient P of a solute is known to be related to its capacity factor [11]. Therefore, if only hydrophobic forces are responsible for the retention of the analyte, the retention must depend solely on its hydrophobicity. It means that the higher the hydrophobicity the higher the capacity factor of a given solute. The dependence of log P on log k should be linear. Of course, this assumption is justified only on the condition that measured data are obtained at pH values where the tested compounds are ionised exactly to the same extent. Because of the wide range of pK_a constants of the tested bases,

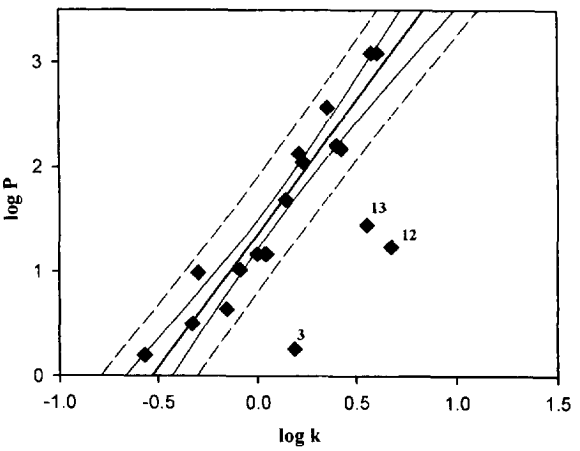

Fig. 9. Dependence of calculated log P ([48]) on calculated limiting values of log k for all the studied bases on LiChrosorb RP-select B. For explanation of symbols see Fig. 8.

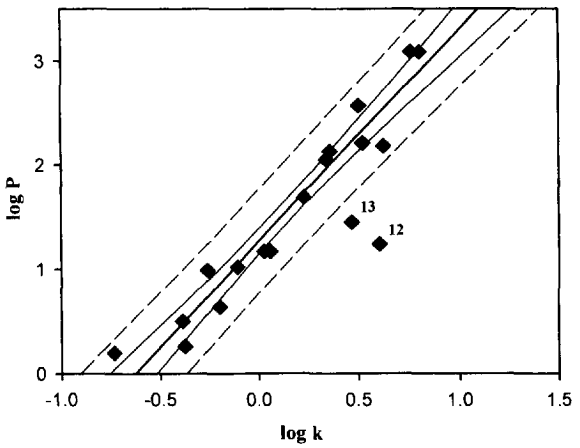

Fig. 10. Dependence of calculated log P ([48]) on calculated limiting values of log k for all the studied bases on Purospher RP-18. For explanation of symbols see Fig. 8.

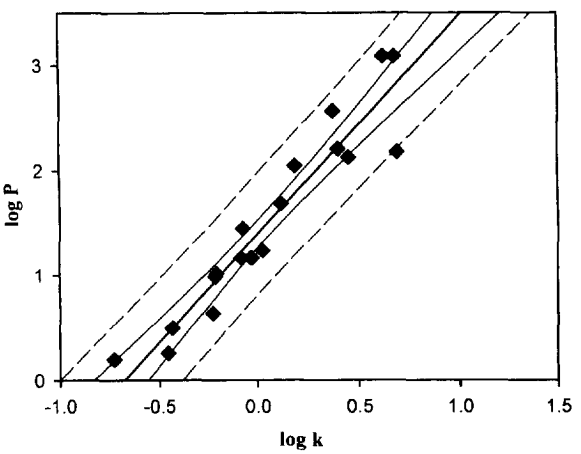

Fig. 11. Dependence of calculated log P ([48]) on calculated limiting values of log k for all the studied bases on Symmetry C_{18}. For explanation of symbols see Fig. 8.

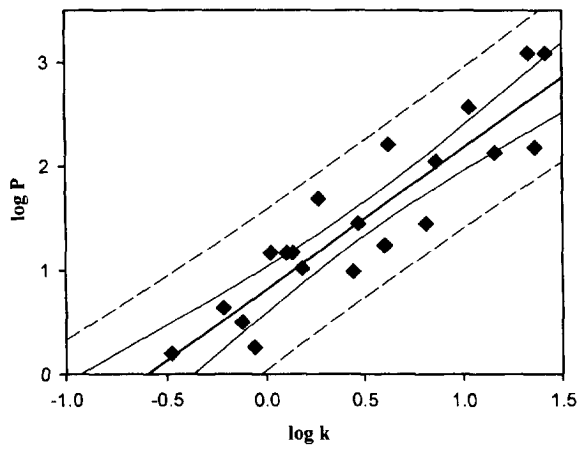

Fig. 12. Dependence of calculated log P ([48]) on calculated limiting values of log k for all the studied bases on HEMA-BIO 1000 C_{18}. For explanation of symbols see Fig. 8.

log k of their non-protonated forms, expressed as limiting log k values, were correlated with the corresponding log P values.

Fig. 8 shows the dependence of log P on log k for LiChrosorb RP-18. Three compounds are perspicuously outside the linear dependence. The capacity factors which did not match the linear dependence give evidence of the presence of non-hydrophobic interactions between those solutes and the stationary phase. Generally, the number of bases and their distance from the line is strongly related to the level of surface deactivation. The most basic solutes, 4-aminopyridine, 2-phenylethylamine and N-benzylmethylamine, as mentioned above, have been excellent indicators of ion-exchange centres on the sorbent surface. This is the reason why their values of log k are high. The exclusion of the data for these three solutes yielded a satisfactory regression coefficient ($r=0.9648$). Under those circumstances LiChrospher RP-select B (Fig. 9) gave an even better regression coefficient ($r=0.9609$). On Purospher RP-18 (Fig. 10) only two basic compounds were found to be outliers. 4-Aminopyridine fell into the confidence interval for the population, only N-benzylmethylamine and 2-phenylethylamine were excluded from the regression ($r=0.9695$). Fig. 11 demonstrates the extraordinary properties of Symmetry C_{18}, when no base within the tested set has been eliminated from the regression, and even the strongly basic compounds fit well the linear dependence ($r=0.9514$). Quite low values of regression coefficients calculated for silica-based RPs can be explained by significant diversity of the bases studied; it is well known that better regression coefficients are usually obtained for homologous series.

HEMA-BIO 1000 C_{18} (Fig. 12) was confirmed to be a sorbent without ion-exchange centres. The dependence log P versus log k for all the tested bases is linear ($r=0.9056$). The explanation for an even lower correlation coefficient in comparison with the silica-based RPs has not been found yet. However, the completely different character of this RP sorbent within the tested set might be responsible for the result.

On the basis of all the stated measurements the order of degree of the surface deactivation for the silica-based reversed-phase sorbents can be established, in ascending order, as follows: SGX C_{18} < LiChrosorb RP-18 < LiChrospher RP-select B < Purospher RP-18 < Symmetry C_{18}. Sorbent HEMA-BIO 1000 C_{18} is comparable to Symmetry C_{18} from the point of view of base deactivation but by no means with respect to the efficiency. Aluspher RP-select B showed very strong non-specific interactions for ionised basic compounds (for more detailed discussion see below).

Besides the strong influence of the pK_a values of the bases on their behaviour on the reversed-phase sorbents, the relation between hydrophobicity and the charge of the solutes and their retention on the sorbents was studied. The hydrophobicity of an analyte is a very important parameter to be taken into account, as has already been mentioned. Practically it means that two bases possessing similar pK_a values and differing in their hydrophobicity were found to be distant in their abilities to describe undesirable ion-exchange interactions on the sorbent surfaces. In this respect the hydrophilic solutes proved to be significantly more sensitive probes of residual ion-exchange centres than the hydrophobic ones. This effect was rather subtle (considering capacity factors only but by no means with respect to the efficiency and the asymmetry) on silica-based RP sorbents (Fig. 13). The sorbent Aluspher RP-select B turned out to be a more descriptive example of this generally valid phenomenon; therefore, it was chosen to illustrate the above-mentioned statement.

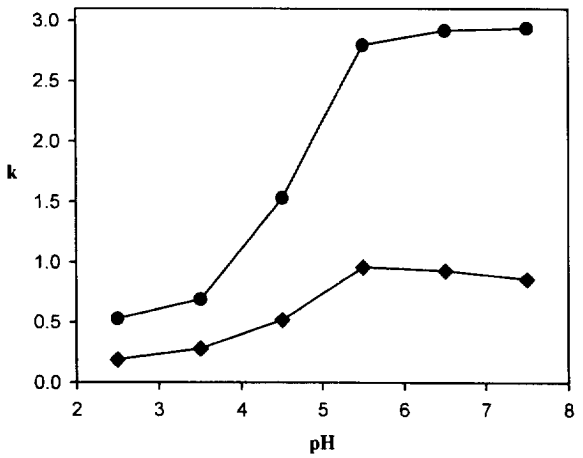

Fig. 13. Influence of hydrophobicity of two selected basic compounds on their retention under altering pH. Sorbent: LiChrosorb RP-18. Mobile phase: methanol, 25 mmol/l sodium phosphate buffer (6:4). ♦=2-amino-4-picoline (pK_a=7.67), ●=2,4,6-collidine (pK_a=7.61).

Owing to its amphoteric character, aluminium oxide (without polymeric coating) can be used depending on pH as a cation or an anion exchanger. The transition between these two mechanisms takes place at a certain pH value where the net charge of the surface is zero (zero point of charge, ZPC) [40]. In the case of polymer-coated alumina, if the shielding of the surface is not complete the matrix of the sorbent can influence the retention behaviour of basic compounds and the proportion of hydrophobic to cation or anion interactions is significantly dependent on the pH of the mobile phase.

The influence of the hydrophobicity of the selected pairs of bases possessing similar dissociation constants but different hydrophobicities on the retention behaviour on Aluspher RP-select B in relation to the pH of the mobile phase is depicted in Figs. 14 and 15. Different shapes of the curves for 3-picoline versus N-ethylaniline and 2-amino-4-picoline versus 2,4,6-collidine are caused by differences in the hydrophobicities of these compounds exclusively (because the pK_a values are very similar within each pair). When the mobile phase has a high pH value (pH 10.5) all the bases are retained only by hydrophobic interaction as they are non-ionised. Decrease of the pH of the mobile phase is followed by an increase of the protonisation of the bases and a decrease of the number of negatively charged centres on the sorbent surface at the same time. The more hydrophobic compounds, N-ethylaniline and 2,4,6-collidine, despite the fact that they are dissociated to the same extent as their counterparts within the pair, are influenced significantly less by ion-exchange interaction, and the obtained dependencies of k versus pH are sigmoidal without marked signs of deformation. More hydrophilic compounds, 3-picoline and 2-amino-4-picoline, are retained preferentially by ion-exchange interaction in the pH

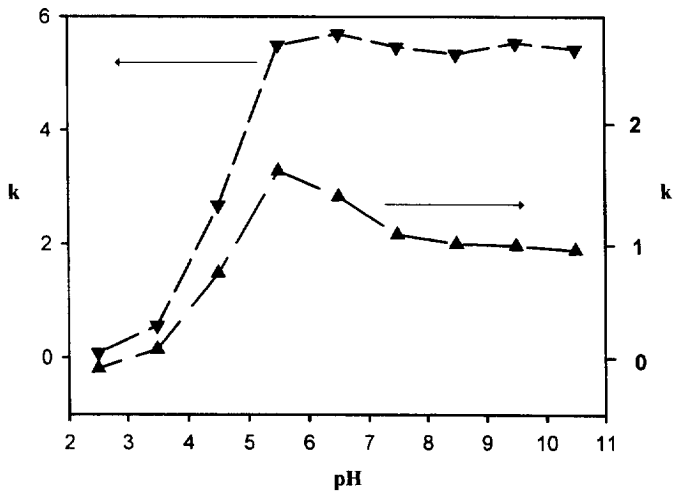

Fig. 14. Influence of hydrophobicity of two selected basic compounds on their retention under altering pH. Sorbent: Aluspher RP-select B. Mobile phase: 25 mmol/l sodium phosphate buffer. ▲=3-picoline (pK_a=5.60), ▼=N-ethylaniline (pK_a=5.50).

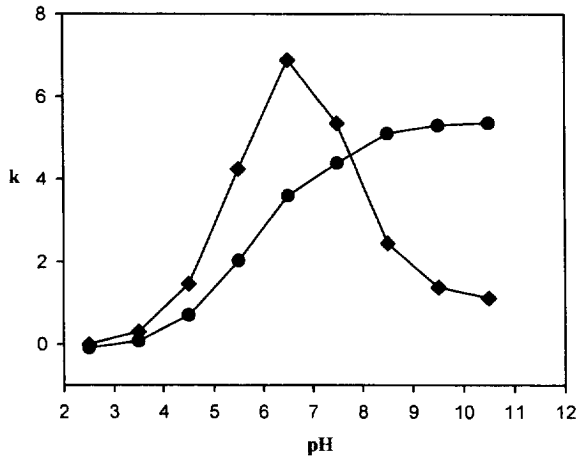

Fig. 15. Influence of hydrophobicity of two selected basic compounds on their retention under altering pH. Sorbent: Aluspher RP-select B. Mobile phase: 25 mmol/l sodium phosphate buffer. ♦=2-amino-4-picoline (pK_a=7.67), ●=2,4,6-collidine (pK_a=7.61).

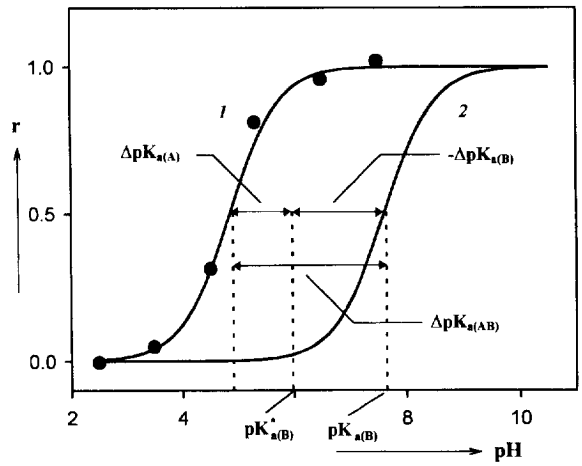

Fig. 16. Influence of methanol on the sift of r versus pH dependence. Sorbent: Symmetry C_{18}. Mobile phase: methanol, 25 mmol/l sodium phosphate buffer (6:4), $\Delta pK_{a(A)} = -1.100$ ([41]). Analyte: ●=2,4,6-collidine. Curves 1 and 2 were constructed as follows: *Curve 1*: Experimentally obtained capacity factors for the given analyte in the methanol–buffer solution were fitted through Eq. (9). Known values of $[H^+]$ (pH measured before addition of methanol to buffer) was inserted into Eq. (9) and k_0, k_1 and the location of the inflection point of Curve 1 were computed by the non-linear least square method (n.l.s.m.). Then k versus pH dependencies were substituted by r versus pH dependencies, where $r=([B]/([BH^+]+[B]))$. *Curve 2*: Theoretical dependence of k versus pH for the given analyte in the aqueous solution was described by Eq. (9), where $K_{a(B)}$ and $[H^+]$ are known values for the given basic compound and appropriate k_0, k_1 were obtained as described for *Curve 1*. Again, dependencies of k versus pH were substituted by r versus pH dependiences. The meaning of the other symbols is explained in Section 4.

range 5–8 and the shapes of the k versus pH curves are strongly distorted. As the pH continuously decreases the positive charge on the alumina surface gradually rises and a decrease of the retention of all the solutes is observed.

The results in Figs. 13–15 demonstrate the crucial role of the proper selection of particular basic compounds for testing of RP sorbents.

Another aspect studied in the framework of this study was the influence of methanol content in the mobile phase on the retention characteristics of basic solutes. This can be derived from the theory mentioned in Section 2 that for a specified basic compound the higher the content of methanol in a buffer, the more pronounced the shift of the experimentally obtained sigmoidal curve, with respect to the same dependence measured in the aqueous buffer, as expected. It is important to stress that the shift should not be dependent on the kind of RP (RP backbone, brand, batch, etc.) but exclusively on the methanol content in the mobile phase if the interaction of the solutes is based solely on hydrophobic interactions. Figs. 16 and 17 depict examples of the dependencies of r, where r is the ratio of the concentration of non-protonated base to the total concentration of base, versus pH experimentally obtained and theoretically predicted on Symmetry C_{18} at the ratio of methanol–buffer 6:4 and on HEMA-BIO 1000 C_{18} at the ratio 2:8. (The quantity r is more suitable than the capacity factor for the comparison of the sigmoidal dependencies.) These two sorbents were chosen because of their high degree of base deactivation, hence exhibiting the ideal sigmoidal curves. The distance between the inflection points of the experimental curve 1 and the theoretical curve 2 (calculated for aqueous buffer) is labelled $\Delta pH_{a(AB)}$, where index (AB) means that the resulting shift is a combination of two individual shifts caused by the change of dissociation of the phosphate buffer $\Delta pK_{a(A)}$ and the change of dissociation of the basic compound $\Delta pK_{a(B)}$ in the methanol–buffer solution. The values of $\Delta pK_{a(A)}$ are known for methanol–phosphate buffers [41], pK_{a2} and pK^*_{a2}

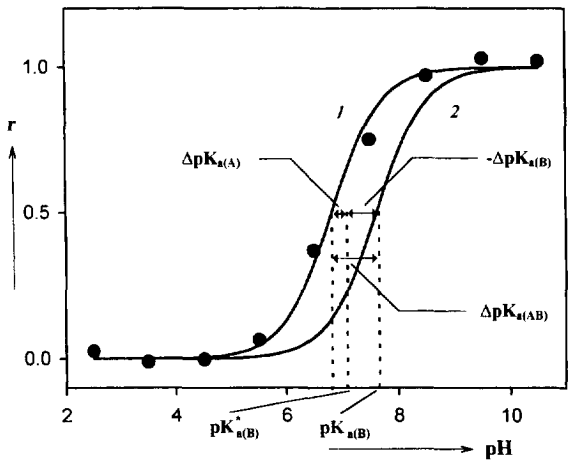

Fig. 17. Influence of methanol on the shift of r versus pH dependence. Sorbent: HEMA-BIO 1000 C_{18}. Mobile phase: methanol, 25 mmol/l sodium phosphate buffer (2:8), $\Delta pK_{a(A)} = -0.185$ ([41]). Analyte: ● = 2,4,6-collidine. Curves 1 and 2 were obtained as described for Fig. 16. The meaning of the other symbols is explained in Section 4.

constants (derived from the second dissociation constant) of the phosphate buffer were used in this case. As a consequence, the appropriate $\Delta pK_{a(B)}$ can be easily determined. The procedure of the determination of $\Delta pK_{a(B)}$ for 2,4,6-collidine on Symmetry C_{18} and HEMA-BIO 1000 C_{18} is demonstrated in Figs. 16 and 17. The simplification (exclusive application of pK_{a2} and pK^*_{a2}) is acceptable in most instances with regard to the chosen mobile phases, pH values and the dissociation constants of the tested basic compounds. Because $\Delta pK_{a(A)}$ values are always positive for a phosphate buffer and at the same time, $\Delta pK_{a(B)}$ values in methanolic solutions are negative (for methanol content not exceeding approx. 80%), both effects work in the same direction for bases dissolved in a methanol–phosphate buffer mixture. As a result, for instance to achieve the same dissociation of the basic compound in the aqueous-organic solution as in the aqueous one, the methanolic buffer must be prepared by mixing methanol with the aqueous phosphate buffer of pH about 2.5 units lower, for the final methanol content approx. 60%.

The comparison of the differences of shifts in Figs. 16 and 17 shows a strong dependence on the methanol content in the mobile phase. The distance of the theoretical (for aqueous solvent) and the experimentally obtained curves with Symmetry C_{18} is bigger than with HEMA-BIO 1000 C_{18} for the same base (2,4,6-collidine) as a consequence of the higher content of methanol applied with the former sorbent. These findings are in agreement with the theoretical assumption.

4.1. Practical aspects of the study

As follows from Eq. (8) and from Figs. 16 and 17 the addition of methanol to the aqueous phosphate buffer of a given pH value leads to a considerable shift of the dissociation of a basic solute in a given buffer, represented by $\Delta pK_{a(AB)}$. On the contrary, the same change of this mobile phase has relatively little effect on the dissociation of an acidic solute because of partial compensation of the shifts of the dissociation constants of the phosphate buffer and the acidic compound.

There are many different approaches for plotting k or r values versus acidity of the mixed aqueous-organic mobile phase. Some authors do not take into account changes of the dissociation of both buffers and solutes after the addition of organic solvent to the mobile phase e.g., [34,35,37]. The results of this study demonstrate that such a simplification is hardly justifiable even at low concentrations of the organic modificator in the mobile phase (Fig. 17). As a consequence, provided that no correction is made for the change of buffer dissociation (see Eq. (8) and Figs. 16 and 17), the pK^*_a values of solutes obtained in this way are not in good agreement with data measured by potentiometric titration. Other researchers employ Eqs. (3) and (4), e.g. [25,42], eventually Eq. (2), e.g. [36], and plot the capacity factors against pH*. The latter two approaches owing to the known pH* values of the mobile phase enable the calculation of the pK^*_a values of solutes directly from chromatographic data. However, in the present work, pH of the mobile phases was measured before the addition of methanol exclusively and k and/or r values were plotted against this pH in all cases. There are two advantages connected with such a graphical description: (1) The plots directly show the changes of the degree of dissociation of the solute due to the addition of methanol to the mobile phase. It means that this representation displays a sum of

the shifts caused by changes of dissociation of both the solute and the buffer. This value is often more important (e.g., for optimisation of the separation) than the exact knowledge of pK_a^* of the solute. (2) The measurement of pH is very convenient and the application of Eq. (8) leads to approximate values of pK_a^* of the bases. Of course, for more precise determination of their pK_a^* values the method of direct pH* measurements with a calibration of pH meter in the aqueous-organic solvent should be preferred. (Moreover, a constant ionic strength of the mobile phase within changes of pH should be maintained [25,38]).

The evaluation of the sorbents and the study of different parameters influencing the separation of basic compounds were based mainly on utilisation of measured capacity factors of the given solutes. Capacity factors showed better reproducibility than measurements of peak asymmetry and efficiency. However, in the course of this study, efficiencies and asymmetries were also measured. Thus, the present results are in fact supported also by other chromatographic data. However, as McCalley has indicated, the relationships among capacity factors, peak asymmetry, efficiency and the stereochemical effects of a solute are very complex and difficult to interpret [43]. These additional aspects will be a subject of a detailed evaluation elsewhere [44].

In the course of experiments on Purospher RP-18 unusually high retention of aldehydes and non-dissociated carboxylic acids has been found. Elemental analysis confirmed a small quantity of nitrogen on the sorbent (0.3% m/m). This sorbent cannot be used universally; acidic compounds and aldehydes might cause difficulties.

5. Conclusion

Considerable differences among some of the studied reversed-phase sorbents with regard to basic compounds have been found. Of the set of silica-based sorbents SGX C_{18} has shown very strong non-hydrophobic interactions. Symmetry C_{18} has exhibited very good properties and HEMA-BIO 1000 C_{18} has proved to be another stationary phase of choice for the separation of bases. The lower efficiency of HEMA-BIO 1000 C_{18}, however, makes Symmetry C_{18} the preferable stationary phase (of the reversed-phase packings studied) for the separation of complex mixtures of basic compounds. The alumina matrix of Aluspher RP-select B is not fully shielded with the polymeric layer and as a result strong undesirable interactions of basic compounds have been found with this sorbent.

The ability of a basic solute to reveal non-hydrophobic interactions on the surface of a sorbent depends mainly on its dissociation constant. The strongly basic compounds are the most sensitive probes. The hydrophobicity of solutes was shown to be another important factor for evaluation of the degree of surface deactivation. In this respect hydrophilic compounds were found to be better indicators of undesirable ion-exchange centres.

The presence of methanol in the mobile phase has a strong influence on the dissociation of both a buffer and a basic solute. The observed changes of dissociation have been successfully quantified and a good agreement with the theory has been obtained.

References

[1] J.G. Dorsey, W.T. Cooper, J.F. Wheeler, H.G. Barth and J.P. Foley, Anal. Chem., 66 (1994) 500.
[2] J.G. Dorsey, W.T. Cooper, B.A. Siles, J.P. Foley and H.G. Barth, Anal. Chem., 68 (1996) 515.
[3] R.E. Majors, LC·GC Int., 9 (1996) 214.
[4] E. Bayer and A. Paulus, J. Chromatogr., 400 (1987) 1.
[5] B. Buszewski, J. Schmid, K. Albert and E. Bayer, J. Chromatogr., 552 (1991) 415.
[6] M.A. Stadalius, J.S. Berus and L.R. Snyder, LC·GC Int., 6 (1988) 494.
[7] G.B. Cox, J. Chromatogr., 656 (1993) 353.
[8] J.L. Glajch and J.J. Kirkland, LC·GC Int., 3 (1990) 50.
[9] R.J.M. Vervoort, F.A. Maris and H. Hindriks, J. Chromatogr., 623 (1992) 207.
[10] I.M. Mutton, J. Chromatogr. A, 697 (1995) 191.
[11] T.L. Ascah and B. Feibush, J. Chromatogr., 506 (1990) 357.
[12] J.J. Kirkland, C.H. Dilks Jr. and J.E. Henderson, LC·GC Int., 6 (1993) 436.
[13] J. Paesen, P. Claeys, E. Roets and J. Hoogmartens, J. Chromatogr., 630 (1993) 117.
[14] D.V. McCalley, J. Chromatogr., 636 (1993) 213.
[15] J. Verne-Mismer, M. Lamard and J. Wagner, J. Chromatogr., 645 (1993) 251.
[16] R.J.M. Vervoort, M.W.J. Derksen and F.A. Maris, J. Chromatogr. A, 678 (1994) 1.
[17] B.A. Olsen and G.R. Sullivan, J. Chromatogr. A, 692 (1995) 147.

[18] B. Gawdzik, J. Chromatogr., 600 (1992) 115.
[19] M. Petro and D. Berek, Chromatographia, 37 (1993) 549.
[20] M. Hanson, A. Kurganov, K.K. Unger and V.A. Davankov, J. Chromatogr. A, 656 (1993) 369.
[21] A. Kurganov, V. Davankov, T. Isajeva, K. Unger and F. Eisenbeiss, J. Chromatogr. A, 660 (1994) 97.
[22] C.L. de Ligny, P.F.M. Luykx, M. Rehbach and A.A. Weinecke, Recl. Trav. Chim. Pays-Bas, 79 (1960) 699.
[23] R.G. Bates, M. Paabo and R.A. Robinson, J. Chem. Phys., 67 (1963) 1833.
[24] W.J. Gelsema, C.L. de Ligny and H.A. Blijleven, Recl. Trav. Chim. Pays-Bas, 86 (1967) 852.
[25] J.L.M. van de Venne, J.L.H.M. Hendrikx and R.S. Deelder, J. Chromatogr., 167 (1978) 1.
[26] B.L. Karger, J.N. LePage and N. Tanaka, in C. Horvath (Editor), HPLC, Advances and Perspectives, Academic Press, New York, NY, 1980, Ch. 3, p. 113.
[27] C.L. de Ligny and M. Rehbach, Rec. Trav. Chim. Pays-Bas, 79 (1960) 713.
[28] W.J. Gelsema, C.L. de Ligny, A.G. Remijnse and H.A. Blijleven, Recl. Trav. Chim. Pays-Bas, 85 (1966) 647.
[29] H.A. Laitinen and W.E. Harris, Chemical Analysis, Chimija, Moscow, 1979.
[30] M. Popl, J. Fähnrich and V. Tatar, in J. Cazek (Editor), Chromatographic Analysis of Alkaloids, Marcel Dekker, New York, NY, 1990, Ch. 2, p. 25.
[31] C. Horváth, W. Melander and I. Molnár, Anal. Chem., 49 (1977) 142.
[32] D.J. Pietrzyk, E.P. Kroeff and T.D. Rotsch, Anal. Chem., 50 (1978) 497.
[33] P.J. Schoenmakers, S. van Molle, C.M.G. Hayes and L.G.M. Uunk, Anal. Chim. Acta, 250 (1991) 1.
[34] J.A. Lewis, D.C. Lommen, W.D. Raddatz, J.W. Dolan, L.R. Snyder and I. Molnar, J. Chromatogr., 592 (1992) 183.
[35] J.A. Lewis, J.W. Dolan, L.R. Snyder and I. Molnar, J. Chromatogr., 592 (1992) 197.
[36] J. Barbosa, V. Sanz-Nebot and I. Toro, J. Chromatog. A, 725 (1996) 249.
[37] Q. Wan, M.C. Davies, P.N. Shaw and D.A. Barrett, Anal. Chem., 68 (1996) 437.
[38] P.J. Schoenmakers and R. Tijssen, J. Chromatogr. A, 656 (1993) 577.
[39] J.H. Knox and R. Kaliszan, J. Chromatogr., 349 (1985) 211.
[40] C.J.C.M. Laurent, H.A.H. Billiet and L.D. Galan, J. Chromatogr., 285 (1984) 161.
[41] A. Leitold and G. Vigh, J. Chromatogr., 257 (1983) 384.
[42] B. Rittich and M. Pirochtova, J. Chromatogr., 523 (1990) 227.
[43] D.V. McCalley, J. Chromatogr. A, 708 (1995) 185.
[44] D. Sýkora, Ph.D. Thesis, Institute of Chemical Technology, Prague, 1996.
[45] D.D. Perrin, Dissociation Constants of Organic Bases in Aqueous Solution, Butterworth, London, 1965.
[46] Software package PALLAS for Windows v.1.2 — pK_a Prediction Module, pKalc 3.1, CompuDrug Chemistry, Budapest, Hungary, 1995.
[47] A. Leo, C. Hansch and D. Elkins, Chem. Rev., 71 (1971) 525.
[48] Software package PALLAS for Windows v.1.2 — log P Prediction Module, Prolog P 5.1, CompuDrug Chemistry, Budapest, Hungary, 1995.