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Dependence of reversed-phase retention of ionizable analytes on pH, concentration of organic solvent and silanol activity

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Abstract

In reversed-phase chromatography, the retention of ionizable analytes is influenced by the ionic properties of the packing caused by surface silanol groups. We have measured the ion-exchange properties of both reversed-phase bonded phases and their underlying base materials. The probe used in this part of the study was bretylium tosylate. The acquired knowledge is then used for a complete and quantitative understanding of the retention behavior of ionizable compounds as a function of the pH of the mobile phase and the solvent composition. We have studied the retention pattern of a broad range of acids, bases, and polyfunctional analytes over the pH range from 2 to 11 and from water to 80% acetonitrile. A few application examples demonstrate the relevant findings.

Keywords: Stationary phases, LC; Retention mechanism; pH effects; Silanol activity; Ion exclusion; Hybrid packings

1. Introduction

The majority of analytes in high-performance liquid chromatography are compounds that have ionizable functional groups, such as carboxylic acids, amino groups or phenol groups. The retention of such compounds in reversed-phase chromatography depends significantly on the degree of ionization of these compounds and thus on the pH of the mobile phase. The fundamental retention behavior of such analytes has been studied by several groups (e.g. Refs. [1–5]). Recent investigations [6–17], have shed new light on some of the factors influencing retention. However, due to the stability limits of silica-based columns, only a limited pH range has been covered in most studies.

Recently, hybrid packings have become available [18,19] that are stable over a wider pH range, up to pH 12, but with retention characteristics identical to classical silica-based packings. This allows for a broader applicability of reversed-phase chromatography, and permits the study of the retention of ionizable compounds over a broader pH range than was possible in the past. In order to obtain a complete picture of the retention pattern of ionizable compounds, one would like to obtain retention data over a range exceeding ± 2 pK units around the pK_a of the analyte. With classical silica-based HPLC packings, this is possible only for simple acids and very weak bases. With the use of hybrid packings, these studies can be extended to analytes with aliphatic amino groups or phenolic groups. In addi-

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tion, we have investigated the ionic properties of silica-based and hybrid packings, which influence retention beyond the range covered by previous treatments. This paper therefore expands our understanding of the pH dependence of the retention of ionizable analytes in reversed-phase chromatography.

2. Theory

An early and very comprehensive study of the retention of ionizable compounds in reversed-phase chromatography was published by Horváth et al. [1]. In the following, we will first simplify and generalize the treatment of these workers. Then we will include effects not dealt with in the early publications.

For a simple monoprotic acid or base, the retention depends on three primary factors: the retention factor of the protonated form, k_0, the retention factor of the deprotonated form, k_1 and on the degree of deprotonation of the analyte, d:

$$k = \frac{k_0 + k_1 \cdot d}{1 + d} \tag{1}$$

If the analyte is fully protonated, the retention factor k will be that of the protonated form k_0, and if the analyte is fully deprotonated, it reaches the retention factor of the deprotonated form, k_1. The degree of deprotonation in turn depends on the pH of the mobile phase and the pK_a of the analyte:

$$d = 10^{\mathrm{pH}-\mathrm{p}K_a} \tag{2}$$

For doubly charged analytes such as divalent acids or bases or zwitterions, the equation simply expands:

$$k = \frac{k_0 + k_1 \cdot d_1 + k_2 \cdot d_1 \cdot d_2}{1 + d_1 + d_1 \cdot d_2} \tag{3}$$

As above, k_0 is the retention factor of the fully protonated form, k_1 the retention factor of the form that lost one proton, and k_2 of the form that lost two protons. The factors d_1 and d_2 contain the first and second pK_a of the analyte. Eq. (3) can be expanded at will for species with higher levels of protonation. This equation is correct for a single solvent composition and in the absence of additional ionic interactions.

In order to expand the equation to the case of variable solvent composition, two steps need to be added. The first step is a description of the influence of the solvent composition on the individual retention factors. Most authors use a simple linear relationship between the logarithm of the retention factor and the volume percent organic solvent. However, such a relationship is only accurate over a limited range of solvent composition. In addition, while it may be an appropriate approximation for methanol–water mixtures, it fails for acetonitrile–water mixtures. Schoenmakers and co-workers [5,20,21] have advocated the use of a quadratic relationship between the logarithm of the retention factor and the volume percent of organic solvent. Such a relationship can also describe the retention shift with multiple solvents and ternary mixtures of water and organic solvents. A currently popular approach [16,22–24] uses linear solvation energy relationships (LSER) [25–27]. This approach significantly complicates the association between retention and solvent composition due to the multiplicity of parameters that need to be considered. In addition, the relevant parameters are only available for a limited group of compounds. Also, a simultaneous application to aqueous mixtures of multiple solvents has not yet been demonstrated.

To account for the change in the retention factor with solvent composition, we propose to use the following type of equation:

$$\log(k) = A - \frac{B \cdot r}{1 + C \cdot r} \tag{4}$$

A, B, C are empirical constants, and r is the ratio of the volume percent of organic solvent to the volume percent of water in the mobile phase. The constant A describes the logarithm of the retention factor in the aqueous buffer without organic solvent, and the constant B is a descriptor of the change in retention with solvent composition. The constant C carries the information responsible for the curvature of the relationship between solvent composition and the logarithm of the retention factor. If the relationship is linear, the constant is 1. For the commonly known relationships of retention with solvent composition, values of C exceeding 1 are expected. The value of B in turn will depend on the value of C. The approach chosen here avoids the complications of an assump-

tion of linear or quadratic relationships between retention and the volume fraction of organic modifier [28].

Such a relationship exists for every retention factor k_i in Eq. (4):

$$k_i = k_{i,w} \cdot 10^{-B_i \cdot r/(1+C_i \cdot r)} \quad (5)$$

The resulting equation describing this relationship for an ionizable species with two stages of deprotonization is shown below:

$$k = \frac{k_{0,w} \cdot 10^{-B_0 \cdot r/(1+C_0 \cdot r)}}{1 + d_1 + d_1 \cdot d_2} + \frac{k_{1,w} \cdot 10^{-B_1 \cdot r/(1+C_1 \cdot r)} \cdot d_1}{1 + d_1 + d_1 \cdot d_2} + \frac{k_{2,w} \cdot 10^{-B_2 \cdot r/(1+C_2 \cdot r)} \cdot d_1 \cdot d_2}{1 + d_1 + d_1 \cdot d_2} \quad (6)$$

Recent studies [6,7,10–17,48,49] have established that the pH and the dissociation constant also depend on the organic solvent composition. The second step is a correction of both as a function of the volumetric ratio r:

$$d_i = 10^{\mathrm{pH}(r) - \mathrm{p}K_{\mathrm{a},i}(r)} \quad (7)$$

However, it needs to be pointed out that within the framework of chromatographic retention studies it is not possible to differentiate between the shift in pH with organic solvent composition and the shift in the pK_a of the analyte with solvent composition [17]. It is even possible that the dependence of the parameter d_i on organic solvent composition disappears, if the shift in pH and the shift in the pK_a of the analyte parallel each other. A common trend of the pK_a shift for the buffer acids phosphoric acid, citric acid and acetic acid and for the buffer bases ammonia, ethanolamine and butylamine has been demonstrated [10]. It remains to be seen if similar trends can be observed for typical analytes. The current study indicates that this may be the case. It should be noted also that the purpose of the control of pH in HPLC applications is the control of retention. This is possible in a pH range of ± 1.5 units around the pK_a of the buffer, independent on how the pH is measured — as long as the pK_a and the pH are on the same scale.

Since it is not possible to differentiate between the shift in pH with solvent composition and the shift in pK_a with solvent composition without independent measurements, we simply treat the factors d_i with a joint polynomial correction, using the aqueous pH and the aqueous pK_a as a reference:

$$d_i = 10^{\mathrm{pH} - \mathrm{p}K_{\mathrm{a},j} + e_i \cdot r + f_i \cdot r^2} \quad (8)$$

As an expansion of Eq. (1), the complete equation for the dependence of the retention on solvent composition and pH is given below for a simple monovalent ionizable analyte. In analogy to the treatment in Eq. (4), this formula can be expanded without difficulty to multivalent analytes:

$$k = \frac{k_{0,w} \cdot 10^{-B_0 \cdot r/(1+C_0 \cdot r)}}{1 + 10^{\mathrm{pH} - \mathrm{p}K_{\mathrm{a}1} + e_1 \cdot r + f_1 \cdot r^2}} + \frac{k_{1,w} \cdot 10^{-B_1 \cdot r/(1+C_1 \cdot r)} \cdot 10^{\mathrm{pH} - \mathrm{p}K_{\mathrm{a}1} + e_1 \cdot r + f_1 \cdot r^2}}{1 + 10^{\mathrm{pH} - \mathrm{p}K_{\mathrm{a}1} + e_1 \cdot r + f_1 \cdot r^2}} \quad (9)$$

Up to now, the stationary phase has been treated as a passive receptor of the ligand, without ionic properties. However, an additional complication arises from the presence of surface silanols [29–31]. The ionization of these silanols depends on the pH of the mobile phase. Negatively charged silanols should increase the retention of positively charged analytes, and decrease the retention of negatively charged analytes. Sýkora et al. [7] have attempted to evaluate the influence of silanol groups on the retention of basic analytes, but without a specific theoretical treatment. They noticed however deviations of the measurements from the simple sigmoidal behavior described by Eq. (1). Other assessments of surface silanols (e.g. Refs. [32–34]) have not measured silanol activity as a function of the pH of the mobile phase. This — together with other complications — contributes to the differences in the estimates of silanol activity reported in the literature (for a review see Ref. [35]).

The ion-exchange retention of positively charged analytes K^{n+} is due to the following equilibrium with negatively charged silanols sites SiO^-:

$$K^{n+} + n \cdot SiO^- \leftrightarrow K^{n+}SiO_n^- \quad (10)$$

n is the valency of the analyte. From this equilibrium, one can write down the ion-exchange retention factor for the species K^+:

$$k_{ex} = \phi \frac{[K^{n+}SiO_n^-]}{[K^{n+}]} = \phi \cdot K_{ex} \cdot S^n \quad (11)$$

ϕ is the phase ratio, and K_{ex} is the equilibrium constant for the ion-exchange process of Eq. (12). The concentration of negatively charged silanols S is a function of the pH of the mobile phase. The ionization of these surface silanols depends on one hand on the pH of the mobile phase [36,37]. On the other hand, the overall process of silanol ionization is likely to be a specific property of a specific packing material, being different for a silica, a silica-based bonded phase or an organic–inorganic silica hybrid.

We can combine the retention effects of cation exchange and reversed-phase for a positively charged analyte, by summing up the energies of both retention mechanisms:

$$\ln(k) = \ln(\phi) + \frac{\Delta G_{CE}}{RT} + \frac{\Delta G_{RP}}{RT} \quad (12)$$

ϕ is the phase ratio, ΔG_{CE} and ΔG_{RP} are the Gibbs free energies of the cation exchange and the reversed-phase processes. Consequently, the retention of an ionic and hydrophobic species will arise from the combination of three independent factors: the purely hydrophobic retention k_{rp} of the ion and the purely ionic retention, which in turn depends on the equilibrium for the ion-exchange process K_{ex} and the ionization of the surface silanols S:

$$k = k_{rp} \cdot K_{ex} \cdot S \quad (13)$$

An example analyte that follows this equation would be a hydrophobic quaternary amine. The retention pattern of such an analyte can be used to measure the influence of the ionization of silanols on retention.

We can now also modify the retention equation for ionizable positively charged analytes that undergo both ionic retention on surface silanols and hydrophobic retention on the bonded phase as a function of their degree of ionization:

$$k = \frac{k_{0,rp} \cdot K_{ex} \cdot S + k_1 \cdot d}{1 + d} \quad (14)$$

This can be viewed simply as a modification of the retention factor of the protonated base with the silanol activity of the packing. It accounts for the observed shift in retention of a fully protonated base outside the pH range covered by the protonation/deprotonation equilibrium of the analyte proper.

For negatively charged analytes, the negative charge of the surface silanols leads to exclusion of the analytes from the pores of the packing and its surface. In order to assess the impact of this phenomenon, we need to express the retention factor k_i relative to the number of molecules in the interstitial volume N_i:

$$k_i = \frac{N_s + N_p}{N_i} = \frac{N_p}{N_i} \cdot \left(\frac{N_s}{N_p} + 1 \right) \quad (15)$$

N_s and N_p are the number of molecules or ions in the stationary phase and the pores of the particle. We need to consider separate equilibria for the interaction of the analyte with the stationary phase and the penetration of the negatively charged analyte into pores that carry a negative charge due to the ionization of the surface silanols. This effect is well known in ion-exchange chromatography as the Donnan exclusion mechanism (e.g. Ref. [38]).

Rewriting the equation with the concentrations in the various phases, we obtain:

$$k_i = \frac{V_p}{V_i} \cdot \frac{C_p}{C_i} \cdot \left(\frac{V_s}{V_p} \cdot \frac{C_s}{C_p} + 1 \right) \quad (16)$$

V_s, V_p and V_i are the volume of the stationary phase, the pore volume and the interstitial volume. C_i is the concentration of the analyte in the interstitial volume, C_p is its concentration in the pores, and C_s is its concentration in the stationary phase or on the surface of the packing. The pore penetration can be examined by studying the first ratio on the right-hand side of the equation, and the interaction of the analyte with the stationary phase is expressed in the parentheses. Expressed with the Donnan equilibrium coefficient K_D and the reversed-phase partitioning coefficient K, we obtain:

$$k_i = \frac{V_p}{V_i} \cdot K_D \cdot \left(\frac{V_s}{V_p} \cdot K + 1 \right) \quad (17)$$

For a 1:1 electrolyte, Werner [38] expressed the Donnan equilibrium coefficient as follows:

$$k_i = \frac{V_p}{V_i} \cdot \left(\sqrt{1 + \frac{1}{4} \cdot \left(\frac{S}{C_i} \right)^2} - \frac{1}{2} \cdot \frac{S}{C_i} \right) \cdot \left(\frac{V_s}{V_p} \cdot K + 1 \right) \quad (18)$$

S is the concentration of negatively charged silanols on the surface of the packing, and C_i is the concentration of mobile phase anions in the interstitial volume. We can see that the retention of negatively charged analytes depends on both the buffer concentration and the concentration of negatively charged surface silanols. At a sufficiently high concentration of the mobile phase anions compared to the concentration of ionized silanols, the effect of the parameters in the first parentheses is small.

The retention factor of a weak acid, such as benzoic acid, is a simultaneous function of the ionization of the acid, the ionization of the silanols, and the concentration of the buffer ions in the mobile phase:

$$k_i = \frac{\varepsilon_0}{\varepsilon_i} \cdot \frac{k_0 \cdot \left(\frac{\varepsilon_i}{\varepsilon_p} + 1 \right) + 1 + f(S) \cdot (k_1 + 1) \cdot d}{1 + d} \quad (19)$$

We will use this form of the equation for the curve fitting of our results obtained with acidic analytes.

3. Results

3.1. Experimental

The instrument used in the pH-retention studies comprised a Waters 2690 Solvent Manager and a Waters 2487 multiwavelength UV detector equipped with a microbore cell. Data management was performed using Waters Millenium 32, version 3.2. The columns used were 3.9 mm×20 mm XTerra MS C_{18}, XTerra RP_{18}, Symmetry C_{18} or Resolve C_{18} cartridge columns operated in the Sentry Universal Guard Holder. In addition, special cartridge columns of the same dimensions were prepared for the study of Symmetry and Resolve silica or underivatized XTerra particles. The cartridge columns and the column holder were immersed into a Neslab recirculating water bath set at 23°C. A new column was used for each pH study. With such an approach it is possible to obtain large amounts of retention data up to retention factors approaching 300 in a short period of time.

Buffers were prepared for pH 2.0, 3.0, 6.0, 7.0, 8.0 and 11.0 using phosphate, for pH 4.0 and 5.0 using acetate, for pH 9.0 and 10.0 using bicarbonate. The sodium concentration was kept constant at 30 m*M* for the entire study. The buffers were either prepared starting off with a 30 m*M* sodium hydroxide solution and adding the appropriate acid at 1 N concentration, or, in the case of the bicarbonate buffers, a 30 m*M* solution of $NaHCO_3$ was used to adjust the pH to the desired values. The pH measurements were performed in the aqueous solution using an Orion Model 720A pH meter. The acetonitrile concentration was varied in 10% increments from 0 to 80%. The samples were prepared in concentrations of 1 mg/ml. Samples used in this study were benzoic acid ($pK_a = 4.2$), ketoprofen ($pK_a = 3.49$), suprofen ($pK_a = 3.98$), indoprofen ($pK_a = 3.92$), ethyl paraben ($pK_a = 8.4$), vanillin ($pK_a = 7.4$), 3-ethylaniline ($pK_a = 4.6$), amitriptyline ($pK_a = 9.1$), nortriptyline ($pK_a = 9.98$), benzocaine ($pK_a = 2.8$), hydroxyzine ($pK_a = 7.98$, 11.98), sulfadiazine ($pK_a = 6.5$), and cetirizine ($pK_a = 3.32$, 7.58, 11), fexofenadine ($pK_a = 4.32$, 9.50), and metaproterenol ($pK_a = 8.8$). Bretylium tosylate was the test compound for the assessment of the silanol activity as a function of pH. Doxepin, ketoconazole and astemizole were used in application examples. All compound structures are shown in Fig. 1.

In most studies, XTerra MS C_{18} or XTerra RP_{18} packings were used. XTerra is a new generation packing based on a silicon organic–inorganic hybrid. The packing is generated by the copolymerization of tetraethoxy silane with methyl triethoxy silane. This process generates a material with methyl groups incorporated into its matrix. These methyl groups increase the pH stability of the packing over classical silica-based packings [18]. This feature is an important aspect of the current study, since pH values to 11 and higher were needed. The silicon–oxygen matrix on the other hand provides the packing with a hardness comparable to silica. At the same time, the surface of the packing contains silanol groups, which allow a surface modification of the packing with classical silanization techniques. The XTerra MS C_{18} packing is surface-derivatized with a trifunctional

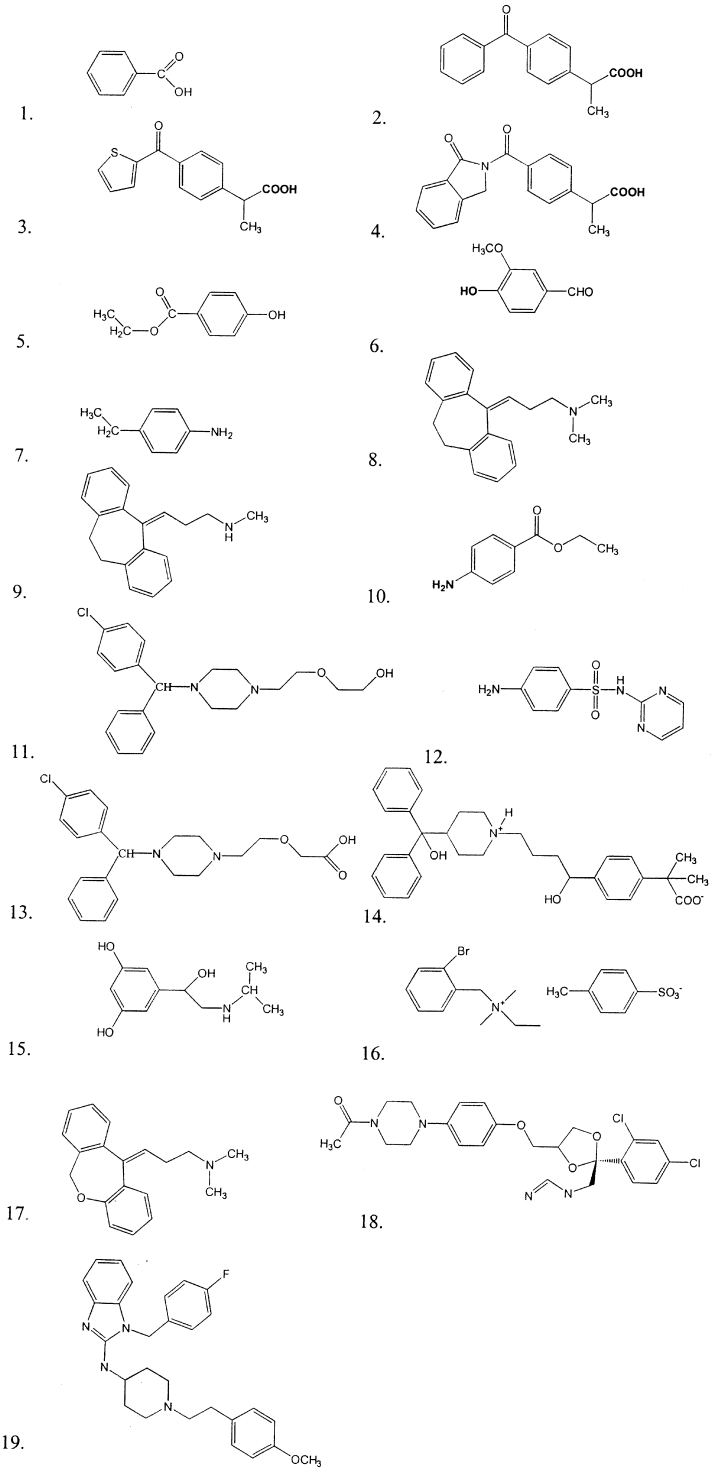

Fig. 1. Analytes used in this study. (1) Benzoic acid, (2) ketoprofen, (3) suprofen, (4) indoprofen, (5) ethyl paraben, (6) vanillin, (7) 3-ethylaniline, (8) amitriptyline, (9) nortriptyline, (10) benzocaine, (11) hydroxyzine, (12) sulfadiazine, (13) cetirizine, (14) fexofenadine, (15) metaproterenol, (16) bretylium tosylate, (17) doxepin, (18) ketoconazole, (19) astemizole.

C_{18} silane. The XTerra RP_{18} packing is based on a monofunctional silane with embedded polar group [40,41]. The polar group is a carbamate group. The surface coverage with the functional groups is about 2.2 to 2.3 $\mu mol/m^2$ for both packings. The surface coverage is limited by the presence of a large amount of methyl groups on the surface of the packing before bonding. This concentration of methylsilicon groups on the surface is estimated to be around 2.5 $\mu mol/m^2$ [42]. Due to the presence of both the bonded functional groups on the surface and the presence of methylsilicon groups, the silanol activity of the XTerra packings is much reduced compared to classical silica-based packings. As will be shown in the next section, the acidity of the surface silanols is affected as well.

The curve fitting procedures were carried out using ProStat Version 2.0a for Windows from Polysoftware International, Sandy, USA. This software allows for a non-linear curvefit to custom equations using the Levenburg algorithm refined by Marquardt and gives extensive feedback on the quality of the parameter estimation. We initially tested the procedure for a complete data set without any predetermined parameters. The procedures functioned well, based on the fact that the known aqueous pK_a values were predicted with little error. We then proceeded to use fixed pK_a values for the final curve fitting procedures reported here.

3.2. Measurement of silanols

Since the beginning of bonded-phase chromatography, it has been understood that surface silanols on a bonded phase participate in the retention process, especially for positively charged analytes [35]. Conversely, one can use the retention properties of such analytes to assess the activity of surface silanols [39,43] and to compare different packings [32,34]. It is possible to achieve a relative performance rating of different packings. For example, Sykora [8] concluded that Symmetry bonded phases exhibited the best deactivation at the time of the study. However, the methods of different authors yielded different results for different packings [44]. It should be noted that the analytes used for the assessment of the silanol activities of a packing were organic amines. One of the possible explanations of the discrepancies among the different measurements is the fact that the ionization of simple amines at neutral pH in the presence of a large volume fraction of an organic solvent is only partial [39]. Thus retention is due to a mixed retention mechanism involving the hydrophobic properties of a packing, the silanophilic properties of the packing, and the degree of ionization of the analytes.

In this study, we were interested in the interaction between hydrophobic retention and silanophilic retention as a function of the pH of the mobile phase. Since the silanophilic interaction is expected to shift with the pH of the mobile phase, we needed an analyte whose ionization does not change with pH. For this reason, we selected the quaternary amine bretylium.

For bonded phases, the retention of the bretylium ion is expected to depend on both the ion-exchange interaction with residual silanols and the hydrophobic interaction with the reversed-phase ligand. The effect of silanol interaction on the retention of bretylium is best studied using underivatized phases, such as silicas or the underivatized XTerra hybrid packing. We selected Resolve silica as a representative of an older, low purity silica and Symmetry silica as a representative of a newer high purity silica together with the hybrid packing. In addition, the organic concentration of the mobile phase was varied. Fig. 2a and b shows the retention of the bretylium ion as a function of the pH for Resolve silica, Symmetry silica and the XTerra hybrid for 50% acetonitrile and 20% acetonitrile. The retention pattern for both silicas is similar: retention is low at pH 2 and increases slowly to pH 5. For pH values higher than 5, the retention increases significantly, to retention factors in the range of 20 at 50% acetonitrile, pH 8 or above 50 at 20% acetonitrile, pH 7. It is interesting to note that the data for the low purity silica and the high purity silica parallel each other. The general ionization pattern of silanols does not change significantly between a high-purity and a low-purity silica. Considering the differences in the performance of bonded phases based on these different silicas, this is a surprising finding and merits additional more detailed investigations.

The steep rise of retention with pH at pH values larger than 6 indicates a general silanol pK_a around 7 for silicas, in agreement with some of the reports in

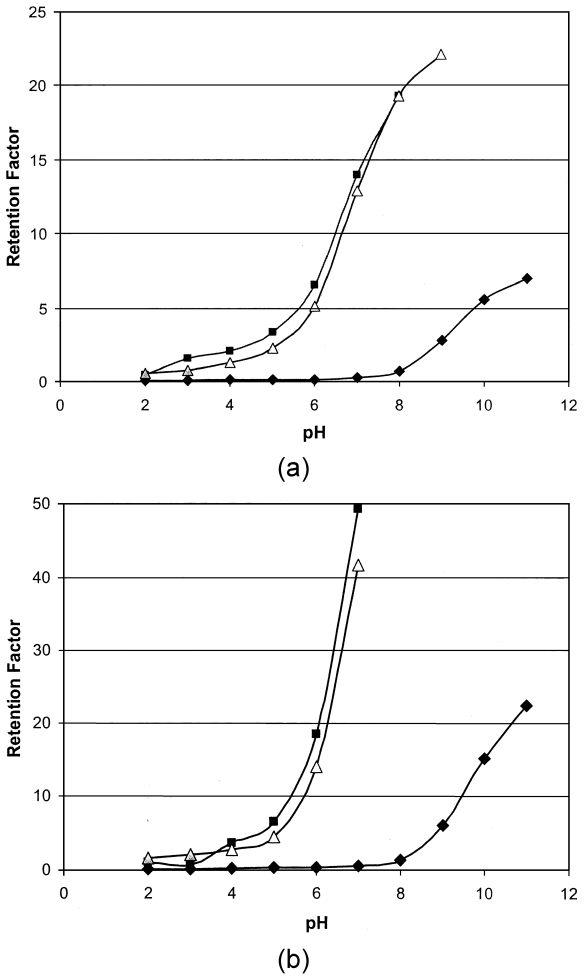

Fig. 2. Bretylium retention as a function of the pH of the mobile phase on underivatized stationary phases. (a) 50% acetonitrile, (b) 20% acetonitrile. Symbols: {triangle} Resolve silica, {square} Symmetry silica, {diamond} inorganic–organic hybrid.

the literature [31,45]. We used a curvefit to a single-step ionization of the silanols to estimate a p*K* of the silanols on the various packings. While this is clearly an oversimplification, it allows us to compare the apparent p*K* values of different packings to each other. As shown in Table 1, we obtained a p*K* of 7.1 for both the Resolve silica and the Symmetry silica in 50% acetonitrile. In 20% acetonitrile, it was not possible to obtain reliable data for silica at pH values of 8 or higher, and thus it was not feasible to determine a p*K* value. For the hybrid packing, the improved stability of the packing allowed us to estimate the p*K* values for both 50% and 20% acetonitrile. The values were significantly higher than those obtained for the silicas, around 10 in both mobile phases. The shift in the p*K* relative to silica correlates well with the significantly reduced tailing of basic analytes on hybrid-based bonded phases compared to silica-based bonded phases.

The higher retention factors for bretylium in 20% acetonitrile compared to 50% acetonitrile demonstrates that the interaction of the analyte with the silica surface is not exclusively due to ion-exchange. A second mechanism can be attributed to hydrophobic interaction, most likely due to siloxane bridges on the silicas or a combination of siloxane bridges and methyl groups on the surface of the hybrid packing.

The retention of bretylium on the three bonded phases Resolve C_{18}, Symmetry C_{18} and XTerra MS C_{18} with 50% acetonitrile (Fig. 3a) shows large differences between the unendcapped Resolve C_{18} and the two newer phases. Resolve silica is a classical, lower purity silica. In addition, Resolve C_{18} is not endcapped. It therefore has a large silanol

Table 1
Estimated p*K* values of underivatized phases in 50% and 20% acetonitrile, derived from the curvefit to the retention of the bretylium ion

	p*K*	Bretylium retention factor		
		Acidic pH	Basic pH	Correlation coeff.
Symmetry silica	p*K*=7.11	$k_0 = 0.667$	$k_1 = 27.22$	$r^2 = 0.9973$
Resolve silica	p*K*=7.14	$k_0 = 0$	$k_1 = 26.22$	$r^2 = 0.9967$
XTerra hybrid 50%	p*K*=9.82	$k_0 = -0.1$	$k_1 = 9.5$	$r^2 = 0.9947$
XTerra hybrid 20%	p*K*=10.45	$k_0 = -0.2$	$k_1 = 36.1$	$r^2 = 0.9961$

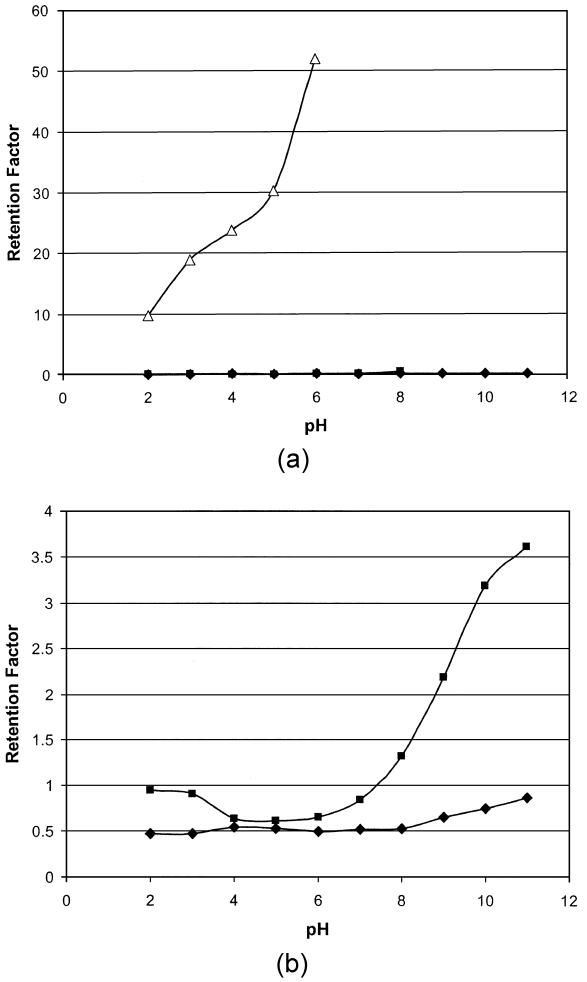

Fig. 3. Bretylium retention as a function of the pH of the mobile phase on bonded phases. (a) 50% acetonitrile, (b) 20% acetonitrile. Symbols: {triangle} Resolve C_{18}, {square} Symmetry C_{18}, {diamond} XTerra MS C_{18}.

activity. In 20% acetonitrile, no retention data for bretylium could be obtained with Resolve C_{18} due to the fact that the retention factors were larger than about 100. The very large retention factors for bretylium on Resolve C_{18} demonstrate that the combined ionic and hydrophobic interaction increases retention quite significantly, as shown in Eqs. (14) and (15). The increase in retention far away from the pK of the silanols obtained with the underivatized silica demonstrates that surface silanols are not uniform in nature and comprise multiple species [31,36,48]. On the other hand, good deactivation of the silica due to endcapping and the use of a high-purity silica reduces the retention of bretylium to less than 1 in 50% acetonitrile and less than 3.5 in 20% acetonitrile. The bonding to the surface also causes a shift in the pK of the residual surface silanols for the fully endcapped Symmetry C_{18} packing to a value of 9.2 ($r^2 = 0.9983$) in 20% acetonitrile and 9.0 ($r^2 = 0.9923$) in 50% acetonitrile. A dependence of the small ionization of the XTerra MS C_{18} surface on pH in a 100% aqueous mobile phase was complicated in our study due to the adsorption of acetate on the surface of the packing when an acetic acid buffer was used. This adsorption increased the retention of bretylium measurably. Using only inorganic buffers, the retention of bretylium increased generally only by roughly a factor of 2 at alkaline pH compared to acidic pH (Table 2a). This increase is much smaller than the retention shift observed for Symmetry C_{18} (Fig. 3b) and agrees with the expectation based on the lower silanol content of the XTerra matrix.

The pK_a values of the silanols are around 7 for underivatized silica. For XTerra, we measured values around 10. For the bonded phase Symmetry C_{18}, we obtained values around 9. The shift in the pK_a values of the silanols due to the presence of bonded phase or methyl groups incorporated into the matrix of the packing may originate in the same mechanism that shifts the pK_a's of acids and bases in the presence of organic solvents [6,7,10–17].

A curvefit to the data in Table 2a results in the overall expression for the XTerra MS C_{18} packing:

$$k = 39.2 \cdot 10^{-20.81r/(1+6.58r)} \cdot \left(1 + 10^{0.154 \cdot \mathrm{pH} - 1.678}\right) \quad (20)$$

with a correlation coefficient of 0.9989. In agreement with Eq. (15) in the Theory section, we interpret the first part of this equation as arising from the hydrophobic interaction. The second part — in the parentheses on the right — expresses the influence of the residual surface ionization of the XTerra MS C_{18} bonded phase. Note that this treatment ignores the theoretically possible shift in silanol activity as a function of the organic solvent composition. The analysis of this data set did not provide any evidence for such a shift. This could be due to the broad spacing of the pH values used in this study.

Table 2
Bretylium retention on XTerra MS C_{18} (a) and XTerra RP_{18} (b) as a function of pH and vol.% acetonitrile

Buffer	pH	50%	40%	30%	20%	10%	0%
(a) XTerra MS C_{18}							
Phosphate	2	0.046	0.105	0.186	0.473	2.609	43.248
Phosphate	3	0.043	0.091	0.176	0.469	2.08	40.518
Phosphate	6	0.063	0.105	0.184	0.497	2.191	42.378
Phosphate	7	0.058	0.116	0.198	0.515	2.11	50.58
Phosphate	8	0.067	0.113	0.156	0.525	2.344	52.76
Bicarbonate	9	0.104	0.186	0.314	0.652	2.787	62.537
Bicarbonate	10	0.136	0.202	0.331	0.745	3.026	66.66
Phosphate	11	0.16	0.272	0.392	0.869	3.474	80.35
(b) XTerra RP_{18}							
Phosphate	2	0.064	0.133	0.203	0.453	1.363	5.014
Phosphate	3	0.058	0.119	0.233	0.46	1.366	5.002
Phosphate	6	0.054	0.116	0.204	0.464	1.343	4.885
Phosphate	7	0.043	0.115	0.2	0.455	1.339	4.875
Phosphate	8	0.05	0.108	0.202	0.484	1.353	4.856
Bicarbonate	9	0.1	0.181	0.306	0.618	1.604	5.456
Bicarbonate	10	0.168	0.26	0.377	0.729	1.867	6.466
Phosphate	11	0.2	0.32	0.464	0.881	2.22	7.871

For the data for the XTerra RP_{18} packing in Table 2b we obtain:

$$k = 4.82 \cdot 10^{-6.44r/(1+2.60r)} \cdot \left(1 + 10^{0.336 \cdot \mathrm{pH} - 3.881}\right) \quad (21)$$

with a correlation coefficient of 0.9992. The hydrophobic retention is lower on the phase with the incorporated polar group, in agreement with our general experience with phases with an incorporated polar group. The ionic retention pattern reflected in the terms in the parentheses is similar to the pattern on the underivatized hybrid packing: a measurable increase in retention does not occur until pH values 9 and higher. The trifunctionally bonded XTerra MS C_{18} packing on the other hand showed already an increase in ionic retention at lower pH values. This difference can be attributed to the influence of silanols from the trifunctional ligand rather than silanols in the matrix of the packing.

Similarly, the analysis of the retention data of bretylium on Symmetry C_{18} could be accommodated by the same model:

$$k = 25.3 \cdot 10^{-12.85r/(1+4.99r)} \cdot \left(1 + 10^{0.137 \cdot \mathrm{pH} - 1.384}\right) \quad (22)$$

with a correlation coefficient of 0.9981. Therefore, the contribution to retention from silanols is generally larger on Symmetry C_{18} than on XTerra MS C_{18}, but it is not substantially different.

3.3. Ion-exclusion effects

The presence of anionic silanol sites on the surface of a packing also creates ion-exclusion effects for anionic analytes. Therefore, reduced retention of anions is expected at high pH for both underivatized packings and for reversed-phase bonded phases. We employed the tosylate ion as a probe with nearly pH-independent charge to assess the ion exclusion effects. As outlined in the Theory section, we use k_i, the retention factor relative to the retention of a species that is both unretained and excluded from the pores of the packing for this study. The retention of such an analyte was assumed to occur at an interstitial fraction of 0.40.

Fig. 4 shows the retention factor k_i as a function of the pH of the mobile phase for Resolve silica and the underivatized hybrid packing. The tosylate ion was used as a probe. The organic solvent concentration was varied from 50% acetonitrile to 20%

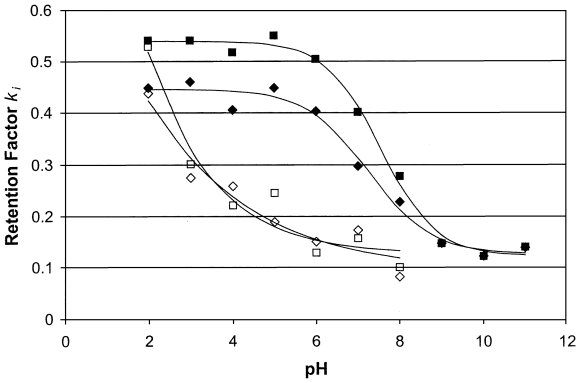

Fig. 4. Tosylate retention as a function of the pH of the mobile phase on the inorganic/organic hybrid packing and Resolve silica. {diamond} 50% acetonitrile, {square} 20% acetonitrile; full symbols: inorganic/organic hybrid packing; open symbols: Resolve silica.

acetonitrile to assess the impact of hydrophobic retention.

The primary observation of Fig. 4 is the lack of ion-exclusion effects for the hybrid silica below pH 6. On the other hand, ion-exclusion effects are already observed above pH 2 for the Resolve and the Symmetry (not shown) silicas. This confirms the findings of the last section which discussed the retention of bretylium as a function of the pH. However, the ion-exclusion phenomenon is different from the ion-exchange phenomenon, since it depends on the ratio of the silanol ion concentration on the surface of the packing to the ion concentration in the interstitial mobile phase (Eq. (18)). An analysis of the ion-exclusion Eq. (18) shows that the half-point of the ion exclusion curve is reached when the concentration of negatively charged surface silanols in the pores reaches around 1.5× the ionic concentration in the interstitial space. At the standard mobile phase concentration of 30 m*M*, this translates to an ionized silanol concentration of around 0.18 $\mu mol/m^2$ for both packings. This value is reached around pH 7 for the underivatized hybrid packing.

In the absence of ion-exclusion effects, i.e. below pH 6 for the hybrid packing and at pH 2 for Resolve silica, a slight increase in retention is observed for 20% acetonitrile compared to 50% acetonitrile. This shift in retention is once again due to hydrophobic interaction with the underivatized packings, including the influence of siloxane bridges on the Resolve silica. This finding parallels the results obtained with the bretylium ion. The retention data for the tosylate ion on Symmetry C_{18}, XTerra MS C_{18} and XTerra RP_{18} are shown in Fig. 5a–c. For Symmetry C_{18}, the retention drops significantly, by about a factor of 2 to 3, between pH 2 and pH 8. The shift in retention is smaller for XTerra MS C_{18}: between pH 2 and pH 11, the shift is less than 40% in 100% water, and it drops to under 10% in 30% acetonitrile. The reduction in the retention is similar for the bonded phase with incorporated polar group, XTerra RP_{18} in Fig. 5c. In agreement with the bretylium ion-exchange data, the ionic effect on the retention of an anionic species is stronger with a silica-based packing than with a bonded phase based on the hybrid packing.

In addition, the ion-exclusion effect depends on the charge and the concentration of the negatively charged buffer ion: it is weaker for the singly charged phosphate ion at pH 2 than for the doubly charged phosphate ion at pH 7 or the triply charged phosphate ion at pH 12. At the same time, the experiment was designed with a constant concentration of the Na^+ counterion. Consequently, the concentration of the buffer ion was higher for the singly charged buffer ion at low pH than for the multiply charged buffer ion at high pH. This compensates partially for the change in charge of the phosphate ion. Fig. 5 includes the results obtained with phosphate buffers at pH 2, 3, 7, 8, 9, and 11. A higher reduction in retention is observed for the buffers based on multiply charged phosphate ions.

Since we will exploit the ion-exclusion effect to correct the retention pattern of acidic analytes for the XTerra packings, we used a polynomial curvefit to quantitate its influence. We employed the following equation for this purpose:

$$k_i = k_0^{1+P(\mathrm{pH}-2)} \cdot 10^{-B\cdot r/(1+C\cdot r)} \qquad (23)$$

The function $P(\mathrm{pH}-2)$ is simply an empirical polynomial function with $(\mathrm{pH}-2)$ as the independent variable. A fifth-power polynomial was found to be adequate. Table 3 contains the coefficients for the retention pattern of the tosylate ion on the XTerra packings. The difference in the polynomial pH correction between XTerra MS C_{18} and XTerra RP_{18} is small and statistically insignificant.

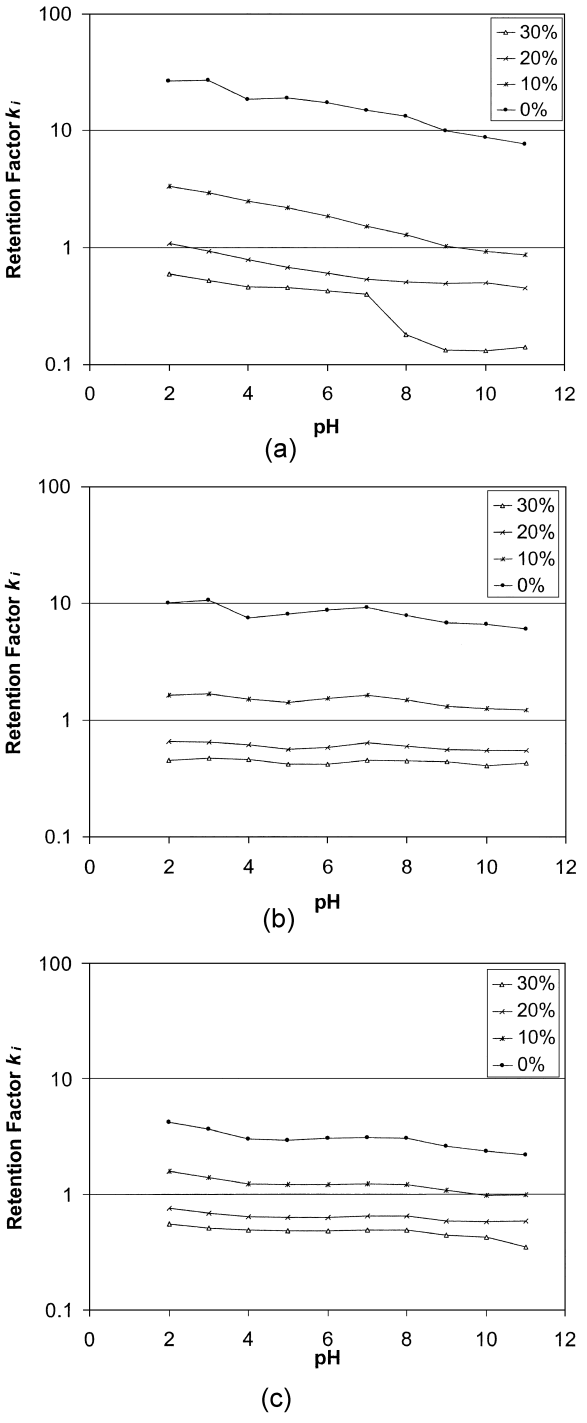

Fig. 5. Tosylate retention on bonded phases as a function of the pH of the mobile phase. (a) Symmetry C_{18}, (b) XTerra MS C_{18}, (c) XTerra RP_{18}. The retention is shown at 0%, 10%, 20% and 30% acetonitrile.

Table 3
Curve-fit results of the retention of the tosylate ion on XTerra MS C_{18} and XTerra RP_{18} packings

Packing	XTerra MS C_{18}	XTerra RP_{18}
Correlation coefficient	0.9973	0.9976
K_0	9.01	3.82
B	12.54	5.51
C	7.45	4.46
Polynomial		
d	4.83×10^{-2}	-8.21×10^{-2}
e	-7.64×10^{-2}	-2.44×10^{-2}
f	2.74×10^{-2}	1.90×10^{-2}
g	-3.74×10^{-3}	-3.23×10^{-3}
h	1.73×10^{-4}	1.64×10^{-4}

3.4. Retention patterns of acids, bases and zwitterions at a single mobile-phase composition

Our ultimate goal is the understanding of the retention pattern of acids, bases and neutral analytes as a function of the pH and the solvent composition. It is convenient to start with an examination of the retention pattern at a single solvent composition. In Fig. 6, the logarithm of the retention factor is plotted as a function of the pH for an acidic analyte, ibuprofen and a basic analyte, doxepin. For the acidic analyte, we observe high retention at acidic pH, where the analyte is not ionized, and low retention at pH values above 8. In the transition

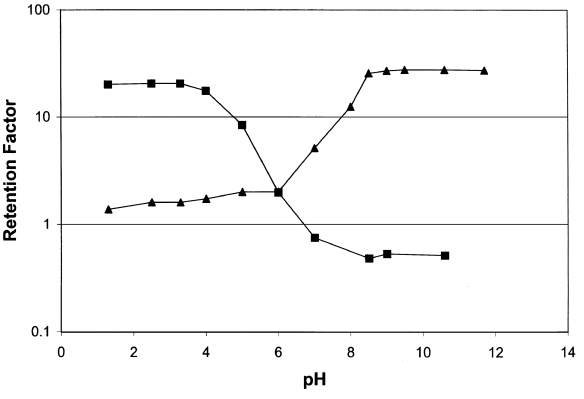

Fig. 6. Plot of the logarithm of the retention factors of an acidic analyte, ibuprofen, and a basic analyte, doxepin, as a function of the pH of the mobile phase. Column: 5 μm XTerra RP_{18}, 3.9 mm×20 mm; mobile phase: 35% acetonitrile, 65% 20 m*M* buffer. {triangle} Ibuprofen, {square} doxepin.

region, roughly from pH 4 to pH 8, one notices a gradual transition from one retention pattern to another, described well by Eq. (1) in the Theory section. For the basic analyte, we observe the opposite pattern: low retention at acidic pH and high retention at basic pH, with a transition region in between. In addition, a careful examination of the retention pattern also shows a slow increase in retention for the protonated base in the pH range from 2 to 6. As discussed in the previous section this increase is due to the pH-dependent ionization of the surface silanols and ion-exchange with these silanols.

For a zwitterionic analyte, fexofenadine in Fig. 7, the change in the elution pattern with pH is more complex. The reason for this more complicated pattern can be found in Eq. (4): for every stage in the ionization process, a different retention of the analyte is observed, and the steps are due to the change in the ionization. On the diagram, we have also marked the aqueous pK_a's of the analyte. As one can see, there is a significant difference in the measured transition pattern relative to the transition range expected from the aqueous pK_a of 9.5. The reason for this departure is discussed in the next section.

3.5. Combining pH change with change in organic composition

For a complete understanding of the retention pattern of charged analytes in reversed-phase chromatography, several components need to be combined with each other. One needs to study the effect of the solvent composition on the retention of the differently charged forms of the analyte. This is commonly a curved exponential relationship between the retention factor and the acetonitrile concentration in the mobile phase. Furthermore, one needs to combine the retention pattern of the differently charged forms of the analyte at every single solvent composition. The pivotal points around which this relationship revolves are the pK_a values of the analytes in the buffers used. As has been pointed out in the literature (e.g. Refs. [6,16,17,13,48]), the pK_a values of the buffers are in turn a function of the solvent composition. In addition, one can expect that the pK_a values of the analytes may very well depend on the concentration of the organic solvent as well (e.g. Ref. [48]). Lastly, the ionization of the surface silanols will affect positively charged analytes through additional ion-exchange retention and negatively charged analytes through ion-exclusion. In the following, we will examine the combination of these phenomena.

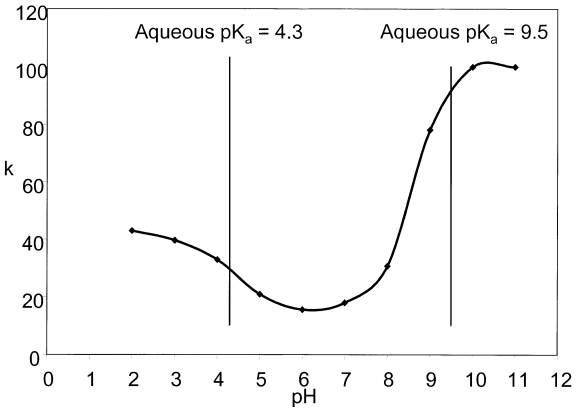

Fig. 7. Retention of a zwitterionic analyte, fexofenadine, as a function of the pH of the mobile phase. The vertical lines mark the pK_a values of the analyte measured in water. Column: 5 μm XTerra RP_{18}, 3.9 mm×20 mm; 20% acetonitrile, 80% 30 m*M* buffer.

Knowledge of the influence of silanols on the retention of ionic compounds can be combined quantitatively with our knowledge of the hydrophobic retention. A combination of Eq. (11) with the influence of silanols determined for the different packings in Eqs. (20)–(22) results in the complete pattern for simple analytes with a single basic functional group

$$k = \frac{k_{0,w} \cdot 10^{-B_0 \cdot r/(1+C_0 \cdot r)} \cdot \left(1 + 10^{S_0 \cdot \mathrm{pH} - S_1}\right)}{1 + 10^{\mathrm{pH} - \mathrm{p}K_{a1} + e_1 \cdot r + f_1 \cdot r^2}} + \frac{k_{1,w} \cdot 10^{-B_1 \cdot r/(1+C_1 \cdot r)} \cdot 10^{\mathrm{pH} - \mathrm{p}K_{a1} + e_1 \cdot r + f_1 \cdot r^2}}{1 + 10^{\mathrm{pH} - \mathrm{p}K_{a1} + e_1 \cdot r + f_1 \cdot r^2}} \quad (24)$$

S_0 and S_1 are the constants describing the silanol activity of a packing as a function of the pH and therefore the silanophilic ionic interaction with the basic analytes. This interaction happens only for the fraction of the analyte that is positively charged. Therefore, the relevant modification affects only the first term in the numerator of Eq. (24). The second

term, which describes the retention of the uncharged form of the analyte, is not altered.

We have investigated the retention behaviour of four basic analytes with the capability of carrying a single positive charge: amitriptyline, nortriptyline, 3-ethylaniline and benzocaine. The pK_a values of the analytes in water were given in the literature. In order to reduce the number of constants to be determined in Eq. (24), we adopted the literature values for the pK_a. Based on preliminary studies, we also assumed that the constants C_0 and C_1 were identical, i.e. that the curvature of the retention factors with solvent composition (but not the slopes) is the same for the ionized and the unionized form of the analytes. This still leaves seven undetermined constants in Eq. (24). The results are shown in Table 4 for both XTerra MS C_{18} and XTerra RP_{18}. On the upper part of the table, we see the constants obtained from the curvefit to Eq. (24) as described in the last paragraph. The correlation coefficients were generally quite excellent, approaching or exceeding 0.99.

The best correlation was obtained for amitriptyline on XTerra MS C_{18} with a correlation coefficient of 0.99981. An examination of the coefficients e and f used for correcting for the pH shift in the presence of the organic solvent and the corresponding pK_a-shift reveals that the pattern of the curve is similar for the different analytes. In addition, since these are solution properties, no difference is expected between the two XTerra packings. Therefore an attempt was made to use the coefficients obtained for amitriptyline on XTerra MS C_{18} as general coefficients for all analytes. The results are shown in the lower part of Table 4. The change in the correlation coefficients is negligible, which means that one can assume within the accuracy of this study that the same correction for a shift in the pK_a applies for all four basic analytes, despite the significant differences in their basicity.

The other constants obtained from the curve-fit merit a few comments as well. The ratio of the B-values to the C-values obtained from the curvefit are measures of the change in hydrophobic retention with solvent composition. These corrected slopes depend of course on the hydrophobic area of the molecule. However, one expects a similarity of both values for the ionized form of the molecule and the non-ionized form. Indeed, a good correlation is

Table 4
Results of the curvefitting procedure for singly charged basic analytes to Eq. (24)

	XTerra MS C_{18}				XTerra RP_{18}			
	Amitriptyline	Nortriptyline	3-Ethyl aniline	Benzocaine	Amitriptyline	Nortriptyline	3-Ethyl aniline	Benzocaine
pK	9.1	10.0	4.6	2.8	9.1	10.0	4.6	2.8
log ($k_{0,w}$)	2.83	4.45	0.52	0.77	2.27	2.43	0.19	0.71
log ($k_{1,w}$)	4.18	6.66	1.00	0.92	3.33	3.33	0.71	0.86
B_0	22.0	62.3	3.41	2.47	14.8	17.6	1.22	2.41
B_1	27.7	85.6	3.40	2.84	17.8	20.7	1.63	2.85
C	6.53	12.7	2.56	1.92	5.07	5.81	1.44	2.28
e	2.84	2.11	2.87	3.60	2.62	2.70	2.95	3.65
f	−0.97	−0.56	−0.74	−7.34	−0.62	−0.73	−0.73	−1.56
r^2	0.9998	0.9943	0.9976	0.9945	0.9896	0.9901	0.9957	0.9998
pK	9.1	10	4.6	2.8	9.1	9.98	4.6	2.8
log ($k_{0,w}$)	2.83	5.39	0.48	0.79	3.07	2.85	0.16	0.71
log ($k_{1,w}$)	4.18	7.51	1.06	0.92	4.47	3.85	0.72	0.86
B_0	22.0	94.1	3.17	2.73	29.6	25.3	0.88	2.31
B_1	27.7	121.9	4.21	2.88	37.6	29.9	1.72	2.85
C	6.53	16.1	3.15	1.99	8.22	7.45	1.55	2.28
r^2	0.9998	0.9927	0.9938	0.9943	0.9824	0.9868	0.9943	0.9998
B_0/C	3.37	5.84	1.01	1.37	3.60	3.40	0.57	1.01
B_1/C	4.24	7.57	1.34	1.45	4.57	4.01	1.11	1.25

obtained between both values shown at the bottom of Table 4, independent of the nature of the analyte. In addition, there is a high correlation ($r^2 = 0.9944$) between the logarithm of the extrapolated retention factor in the purely aqueous mobile phase and the slope of the curves for both the ionic forms and the non-ionic forms of all analytes. In addition, the slope of this correlation is close to 1 (0.953). The high correlation and the slope of unity indicate that the underlying factor is independent of the nature of the analyte or its ionic form. The thought is intriguing that the fundamental parameter is identical and is a descriptor of the hydrophobic effect [46]. However, one has to remain aware of artifacts in curvefitting procedures [47].

For acidic analytes with a negatively charged ionized form, one needs to include ion-exclusion effects. The measurement of the ion-exclusion effect itself has been described above and the results are given in Table 3. The combination of the ion-exclusion effect with the reversed-phase retention of acidic analytes results in the following equation:

$$k_i = \frac{\varepsilon_p}{\varepsilon_i} \cdot \left[\frac{\left(\frac{\varepsilon_i}{\varepsilon_p} + 1\right) \cdot k_{0,w} \cdot 10^{-B_0 \cdot r/(1+C_0 \cdot r)} + 1}{1 + 10^{pH - pK_{a1} + e_1 \cdot r + f_1 \cdot r^2}} + \frac{\left(k_{1,w} \cdot 10^{-B_1 \cdot r/(1+C_1 \cdot r)} + 1\right) \cdot 10^{f(pH)} \cdot 10^{pH - pK_{a1} + e_1 \cdot r + f_1 \cdot r^2}}{1 + 10^{pH - pK_{a1} + e_1 \cdot r + f_1 \cdot r^2}} \right] \quad (25)$$

As outlined in the Theory section, it is more appropriate for the study of negatively charged and therefore partially excluded analytes to use the retention factor k_i based on an excluded peak. Ion-exclusion influences only the negatively charged form of the analyte and therefore affects only the second term in the denominator of Eq. (25). We fitted the data for the singly charged acidic analytes benzoic acid, ketoprofen, suprofen, indoprofen, ethylparaben and vanillin against this equation. An examination of the results indicated that the correction of the pK_a with solvent composition was insignificant for these acidic analytes. This can be explained by the fact that all our buffers were prepared from acids. The shift in the pK_a of the analytes and the buffers with solvent composition follows a similar pattern [48]. Within the framework of our experiments, a more detailed examination of this pattern was not possible. Therefore, the coefficients correcting the pK_a with organic composition were set to 0. In addition, a simple linear correction was sufficient for the description of the influence of the silanol groups on the retention of the charged form of the analytes:

$$f(\text{pH}) = 0.0109 - 0.0135 \cdot \text{pH} \quad (26)$$

The results of the curvefit to Eq. (25) for the singly charged acidic analytes are given in Table 5 for both the XTerra MS C_{18} and the XTerra RP_{18} packing. Good correlation coefficients were obtained throughout. The table also contains the corrected slopes B/C. As described above for the basic analytes, general relationships were obtained between both the intercepts and the corrected slopes for the ionized form and the unionized form of the sample acids on both columns. Furthermore, both relationships had the same slope and the same intercept, so they could be combined with each other to yield:

$$Y = 0.94 \times X - 0.75 \quad r^2 = 0.9363 \quad (27)$$

where X stands for the value of the non-ionized form and Y for the same value of the ionized form of the analyte. Such a general relationship can be used to predict the retention pattern of different acidic analytes from a limited data set. In addition, for all acidic analytes, their non-ionized and their ionized form and for both columns, a single relationship was found that correlates with a high precision the intercepts with the corrected slopes of the curves given in Table 5:

$$Y = 0.95 \times X + 1.39 \quad r^2 = 0.9757 \quad (28)$$

From this, one can conclude that the relationship between retention, pH and solvent composition follows rather simple rules at least for simple monovalent acids. This should encourage us to continue to examine such relationships with the ultimate goal of an ab initio prediction of retention.

3.6. Retention patterns for more complex analytes

Finally, we will examine the retention pattern for more complex analytes. The first example is sulfadiazine (Fig. 8). Sulfadiazine is a conjugated sulfon-

Table 5
Results of the curvefitting procedure for singly charged acidic analytes to Eq. (25)

	Benzoic acid	Ketoprofen	Suprofen	Indoprofen	Ethylparaben	Vanillin
XTerra MS C_{18}						
pK	4.2	3.49	3.98	3.92	8.4	7.4
log ($k_{0,w}$)	1.906	6.070	4.893	6.485	2.692	2.292
log ($k_{1,w}$)	0.573	4.486	4.336	5.398	1.704	1.394
B_0	8.64	35.9	26.5	50.0	11.7	13.8
B_1	7.42	39.4	45.1	70.8	16.6	16.5
C_0	2.49	5.05	4.31	6.44	2.92	4.33
C_1	3.14	6.96	8.17	10.84	6.00	7.01
R^2	0.9944	0.9892	0.9978	0.9957	0.9986	0.9886
B_0/C_0	3.47	7.11	6.15	7.76	4.01	3.19
B_1/C_1	2.36	5.66	5.52	6.53	2.77	2.35
XTerra RP_{18}						
pK	4.2	3.49	3.98	3.92	8.4	7.4
log ($k_{0,w}$)	1.369	5.310	3.224	4.665	2.190	1.497
log ($k_{1,w}$)	0.228	3.345	3.252	3.866	1.057	0.898
B_0	4.07	28.5	10.1	25.2	6.94	5.56
B_1	3.87	23.3	25.4	38.0	10.96	8.85
C_0	1.32	4.57	2.15	4.27	1.99	2.16
C_1	1.76	5.10	5.59	7.54	5.99	4.63
R^2	0.9903	0.9864	0.9960	0.9943	0.9913	0.9924
B_0/C_0	3.08	6.24	4.70	5.90	3.49	2.57
B_1/C_1	2.20	4.57	4.54	5.04	1.83	1.91

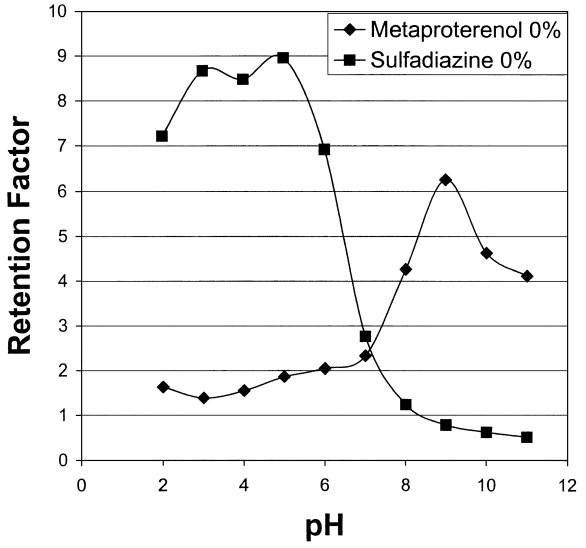

Fig. 8. Retention pattern of more complex analytes I: sulfadiazine and metaproterenol. Column: 5 μm XTerra RP_{18}, 3.9 mm×20 mm; mobile phase: 100% aqueous buffer.

amide with a pyrimidine ring. The retention increases first from pH 2 to pH 3, then remains roughly constant until pH 5. At higher pH values, the retention decreases until it becomes stable again at pH values above 10. The retention shift at acidic pH values corresponds to a deprotonation of the pyrimidine group, while around neutral pH the sulfonamide group becomes negatively charged. This is in agreement with the reported pK_a of 6.5 for sulfadiazine.

Metaproterenol, a bronchodilator, contains a secondary amine and two phenolic groups. At acidic pH, the amino group is protonated, resulting in lowered retention (Fig. 8). Retention increases with pH from pH 6 to pH 9, then decreases again towards higher pH. The increase in retention is due to the loss of charge on the molecule due to the deprotonation of the amino group. At the highest pH values, the phenolic groups become negatively charged, which once again results in a decrease in retention.

Hydroxyzine and cetirizine (Fig. 9) are two closely related compounds used as antihistamines. Hydroxyzine has two tertiary amines as basic functional groups. Cetirizine adds a carboxylic acid group to

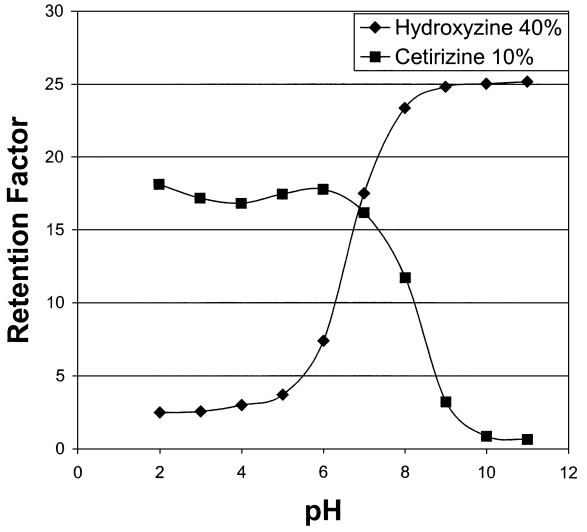

Fig. 9. Retention pattern of more complex analytes II: hydroxyzine and cetirizine. Column: 5 μm XTerra RP_{18}, 3.9 mm×20 mm; mobile phase: hydroxyzine: 40% acetonitrile; cetirizine: 10% acetonitrile.

this. Hydroxyzine shows low retention at acidic pH. Between pH 4 and pH 8 retention increases roughly 10-fold, to remain unchanged until pH 11. Thus, only one ionization step is found within the examined pH range. Cetirizine shows stable and high retention at acidic pH, but the retention is significantly lowered compared to hydroxyzine. The expected pattern is high retention at pH values below 3, followed by a drop in retention around pH 4 due to the ionization of the carboxylic acid group. Then, at pH 7, an increase in retention is expected due to the loss of ionization of one of the two tertiary amino groups. However, only a slight drop in retention is found around pH 4, where one expects an increase in the total ionization of the analyte. Furthermore and contrary to expectation, the retention drops about 30-fold between pH 7 and pH 11. This must be interpreted as an increase in polarity. The most logical explanation of the observed pattern is a structural change of the analyte in solution or on the surface of the packing that accompanies the ionization of the carboxylic acid group around pH 4. The opposite charges induce a folding of the molecule that exposes a larger hydrophobic area to the reversed-phase packing. This is followed by another structural change as one of the tertiary amino groups is deionized around pH 8. The resulting unfolded structure with only two ionic groups is more polar than the internally folded structure containing three ionic groups. To our knowledge, this is the first time that conformational changes have been related to the retention pattern of a small molecule.

3.7. Applications

The retention change with pH at a single solvent composition is significant. As shown in Fig. 10 for a few singly charged acidic and basic analytes, retention decreases by a factor of 10 to 30 from the non-ionized to the ionized form of the analyte. The retention for the neutral reference compound toluamide does of course not change with pH. For ionizable compounds, on the other hand, changes of selectivity and retention occur in the intermediate pH range. The chart shows selectivity shifts for the selected singly charged basic analytes as an example. At acidic pH, the elution order of the tricyclic antidepressants is doxepin, imipramine and nortriptyline, with close elution of the latter two compounds. At alkaline pH, the elution order is nortriptyline, doxepin and imipramine, with near coelution of nortriptyline and doxepin. In the intermediate pH range, the resolution of all three compounds is possible, but the pH of the mobile phase needs to be tightly controlled to maintain consistent retention. On the other hand, when the analytes are either not ionized or fully ionized, the retention and the res-

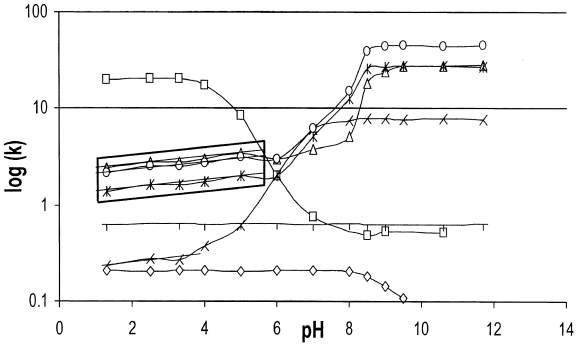

Fig. 10. Change in retention with pH for neutral, acidic and basic analytes. {plus} Toluamide, {square} ibuprofen, {star} doxepin, {circle} imipramine, {triangle} nortriptyline, {diamond} acetaminophen, {cross} lidocaine. Column: 5 μm XTerra RP_{18}, 4.6 mm× 150 mm; mobile phase: 35% acetonitrile, 65% 20 m*M* buffer.

olution are only weakly dependent on pH or completely independent. The weak dependence of retention on pH for the positively charged analytes is due to the ion-exchange interaction with the surface silanols, outlined in detail in the previous paragraphs. The parallel lines drawn through the retention data for basic analytes in the charged form emphasize the common relationship with the silanol activity. For the two other analytes, the change in retention is unremarkable. The acidic analyte ibuprofen changes from high retention to low retention as the pH increases. Acetaminophen is a phenol. Retention decreases in the alkaline pH range.

Due to the overall large change in retention with pH, it is necessary in practical applications development to compensate for the ionization of the analytes with a change in solvent composition. For the monovalent analytes in this study we found that in general a decrease in acetonitrile concentration by about 20% compensates for the ionization of the analytes. An example of this is shown in Fig. 11. In these chromatograms, the retention pattern of ketoconazole and astemizole are compared at pH 2 and pH 10. Note that the acetonitrile concentration was changed from 30% acetonitrile at pH 2 to 50% acetonitrile at pH 10. The resulting retention times

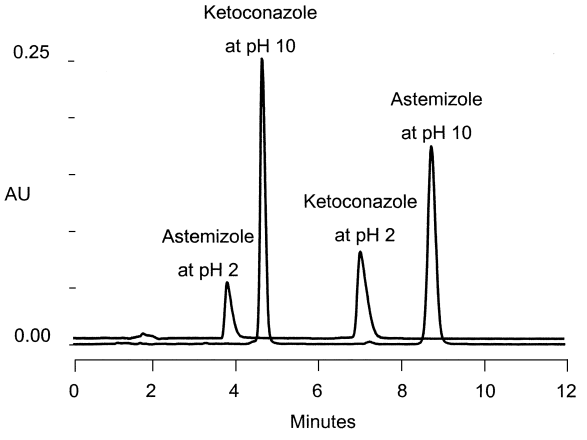

Fig. 11. Comparison of the chromatograms at pH 2 and 30% acetonitrile and pH 10 at 50% acetonitrile. The retention time is similar, but the elution order has changed. Column: XTerra® RP18, 4.6×150 mm, 3.5 μm; mobile phase A: 30% acetonitrile, 60% water, 10% 100 m*M* sodium phosphate buffer, pH 2.0; mobile phase B: 50% acetonitrile, 40% water, 10% 100 m*M* CAPSO, pH 10.

are similar, but the elution order of both analytes has changed. In such a simple case, the analysis can be carried out at both acidic pH and basic pH, but the change in selectivity is noteworthy.

4. Summary and conclusions

In this paper, we have revisited the retention patterns of ionizable analytes. The influence of the ionization of surface silanols on the retention of ionic and ionizable analytes has been established. We have used bretylium tosylate to measure the ion-exchange and ion-exclusion properties of silicas, underivatized hybrid packings and the corresponding C_{18}-bonded phases. The ion-exchange properties of a high-purity silica and a classical silica are very similar. However, the properties of the hybrid packing are substantially different. The ionization of the hybrid surface is shifted to significantly higher pH values compared to silica. This could explain the good peak shapes obtained for ionizable analytes when hybrid packings are employed. While the retention of the bretylium ion was used for the measurement of the ion-exchange properties of a packing, the retention of the tosylate ion could be used to assess the ion-exclusion properties.

Knowledge of the ionization of the surface silanols can be used to correctly establish the retention shifts of ionizable analytes outside their p*K* range. Positively charged analytes interact with negatively charged surface silanols via an ion-exchange mechanism. This results in an amplified retention. Conversely, negatively charged analytes are subject to ion-exclusion effects. For basic analytes, we have confirmed the reports in the literature that the pK_a of ionizable analytes and/or the buffers changes with the organic composition of the mobile phase. For acidic analytes, it is expected that the pK_a of the analytes and the pK_a of acidic buffers follow a similar pattern with solvent composition. Therefore, a shift with solvent composition could not be observed, at least not with the large pH steps used in this study.

From the standpoint of the practitioner of HPLC, pH changes are a powerful tool for manipulating the retention and the selectivity pattern of a separation. Ten- to 30-fold changes in retention time have been

observed with the change in the ionization of the analytes. As a rule of thumb, the change in ionization can be compensated by a roughly 20% change in organic composition to obtain the same retention window. Changes in pH are accompanied by changes in the selectivity of a separation. As a salient example, the complete reversal of the elution order with pH has been demonstrated.

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