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Effect of the eluent pH and acidic modifiers in high-performance liquid chromatography retention of basic analytes

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Abstract

The retention of ionogenic bases in liquid chromatography is strongly dependent upon the pH of the mobile phase. Chromatographic behavior of a series of substituted aniline and pyridine basic compounds has been studied on C_{18} bonded silica using acetonitrile–water (10:90) as the eluent with different pHs and at various concentrations of the acidic modifier counter anions. The effect of different acidic modifiers on solute retention over a pH range from 1.3 to 8.6 was studied. Ionized basic compounds showed increased retention with a decrease of the mobile phase pH. This increase in retention was attributed to the interaction with counter anions of the acidic modifiers. The increase in retention is dependent on the nature of the counter anion and its concentration in the mobile phase. It was shown that altering the concentration of counter anion of the acidic modifier allows the optimization of the selectivity between basic compounds as well as for neutral and acidic compounds.

Keywords: pH effects; Mobile phase composition; Retention behaviour; Anilines; Pyridines

1. Introduction

When developing HPLC methods for pharmaceutical compounds, it is often necessary to control the retention of analytes by changing and optimizing experimental variables such as: pH of mobile phase, types and concentration of mobile phase additives, buffers, and eluent composition. For the separation of ionic and ionogenic (ionizable) solutes such as basic compounds, the variations in the mobile phase pH can lead to the extreme changes in the selectivity. The degree of ionization of these basic solutes and mobile phase additives is affected by the eluent pH. By definition the acidity constant of basic analyte B is

$$K_{a(BH^+)} = \frac{[H^+][B]}{[BH^+]} \quad (1)$$

The HPLC retention of basic compounds as a function of pH shows sigmoidal dependence as described in Fig. 1. The following equation (2) (derived by Horváth et al. [1]) is used to describe the effect of ionization of basic solutes:

$$k = \frac{k_0 + k_1 \cdot \dfrac{[H^+]}{K_a(BH^+)}}{1 + \dfrac{[H^+]}{K_a(BH^+)}} \quad (2)$$

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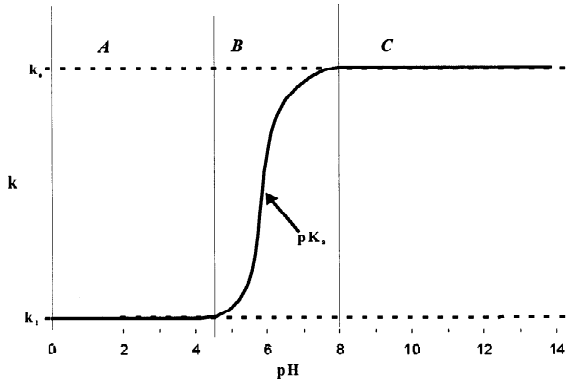

Fig. 1. Theoretical curve showing basic analyte retention on a reversed-phase column as a function of pH. Region A is where the basic analyte is in its fully protonated form, (BH^+); region B is where both unionized and ionized forms, ($B^+H^+ \Leftrightarrow BH^+$), are present and the pH at the inflection point denotes the pK_a of the compound; region C is where the compound is in its unionized form, (B).

where k_0 and k_1 are the retention factors of the neutral and ionized solute, respectively, BH^+ is the protonated basic analyte, $K_a(BH^+)$ is the acid dissociation constant for the base and $[H^+]$ is the proton concentration.

The greatest retention is at pH above the analyte pK_a, as shown in section C of Fig. 1. The pK_a of a compound corresponds to the pH at the inflection point of the curve. At high pH, basic analytes are in an unionized form, so they show greater hydrophobic interaction with the C_{18} stationary phase. Significant changes in retention occur for pH values within ± 2 units of the pK_a. In this pH range, analytes may show irregular peak shape, such as fronting or tailing. Lower pH leads to ionization of the basic analyte and a resulting decrease in retention. Further lowering of the pH should not have any effect upon basic analyte retention since the analyte is in its fully ionized form as shown in section A of Fig. 1.

1.1. pK_a shift

The pH of the mobile phase is usually taken to be the same as that of the aqueous fraction. However, the pK_a value of the acid used to prepare the buffer changes with the solvent composition [2–4] and so does the pH of the buffer [5,6]. This will affect the ionization of bases in an organic–aqueous mixture. Therefore, the chromatographic pK_a cannot be directly correlated to the potentiometrically determined pK_a. Also, it has been shown that the pK_a of basic compounds is shifted to lower values and acidic compounds to higher values with increasing organic composition [7,8]. Barbosa et al. found from potentiometric titrimetry that pK_a of typical acidic buffers shifts to higher values by approximately by 0.3 pH units per each 10% organic phase added. Therefore the working pH of an aqueous–organic eluent should be considered when studying the ionization of basic compounds.

1.2. Ion interaction

Oppositely charged ions in the liquid phase have a tendency to attract one another. The strength of this attraction may depend on the dielectric constant of the mobile phase and on the solvation of individual ions. It has been shown that the retention factors of protonated nitrogen containing species were altered when different acidic modifiers were employed in the eluent [9–22]. The concentration [9,10] and hydrophobic nature of the counter anions of the modifier [11–20] plays an integral part of this effect. This is usually attributed to ion-pair formation with hydrophobic counter anions [21].

Analyte structure [14,22] and the degree of solvation, including any secondary interactions may also affect the basic analyte retention. An increase in retention for aminoindanol was obtained with mobile phase pH change from 3 to 1 using perchloric, trifluoroacetic, nitric and phosphoric acid on a silica-based crown ether column [23]. The authors attributed this effect to the type and acid counter anion concentration which affected the analyte solvation. The retention of propranolol (basic racemate) on a CHIRALCEL OD-R column [24] was shown to increase when counter anions such as perchlorate and nitrate were used as mobile phase additives. Other primary, secondary and tertiary amines showed similar retention behaviour [24]. An increase in retention of basic ophthalmic compounds on a C_{18}

column with a decrease of pH was obtained with phosphate, trifluoroacetate, and perchlorate as counter anions [25].

All these effects are attributed to the ionic interaction of protonated analyte with oppositely charged species which results in either the formation of stable ion pairs or the disruption of the analyte solvation. Ion interaction will be used as a general term to denote the effects related to the analyte ionic interactions.

1.3. Comparison of retention models

An increase of the protonated analyte retention is observed with decrease in pH or with increase of acidic modifier concentration. Several models for the description of the effect of these anionic reagents upon retention of the protonated species are proposed.

The formation of an ion-pair with the solute in the mobile phase with consequent retention of the neutral complex on the reversed-phase column has been suggested as a model [10,26–30]. Ion-pair formation in the mobile phase and adsorption of the noncomplexed free ionic analyte, such as a protonated basic analyte has also been suggested as a model [31]. Another model suggests the modification of the hydrophobic character of the stationary phase where the counter anion of the acidic modifier is first sorbed onto the column and then a dynamic ion-exchange process occurs where the solute molecules interact with the sorbed counter anion [9,32–35] The last model is usually employed when a surfactant-like acidic modifier is used and when this modifier contains a hydrophobic moiety of significant size (like octylsulfonic acid) [9,35,36].

In the case of inorganic acids, such as H_3PO_4, $HClO_4$ or trifluoroacetic acid (TFA), the formation of ionic pairs in the mobile phase is also used [37]. This effect is dependent on the charge and size of the ions and the solvent's dielectric constant. The formation of stable ionic pairs is most favorable for smaller ions with high charges in solvents of low dielectric constant. Therefore, the formation of stable ionic pairs in water will usually occur only to a small extent [38].

The solvation of the analyte with polar solvent suppresses the coulombic attraction between opposite ions. The ion interaction of the protonated basic analyte with the counter anion of the acid is affecting the solvation of the analyte. The higher the disruption of the analyte solvation shell the greater the analyte retention since the virtual polarity (hydrophilicity of the solvated cluster) of the analyte is decreased. Counter anions that have been shown to increase the disorder of water are called chaotropic counter anions [39]. The increase in retention is related to the chaotropic nature of the counter anion.

The majority of the studies of the acidic modifier effects have been performed with analytes of zwitterionic nature such as peptides and proteins. There is a lack of experimental work on the effects of the type and concentration of inorganic mobile phase additives on the retention of small basic analytes.

In this paper the effects of the type and concentration of several acid counter anions on the retention of primary and secondary nitrogen containing compounds on a typical reversed-phase C_{18} column are investigated in detail. The compounds used in this study were a series of substituted aniline and pyridine compounds.

2. Experimental

2.1. Apparatus

The chromatographic system used was a model 1100 HPLC from Hewlett-Packard (HP; Little Falls, DE, USA). The chromatograms were processed using HP software. The column used was Zorbax Eclipse XDB-C_{18}, (Hewlett-Packard) 150×4.6 mm I.D., particle diameter 5 μm, bonding density 3.4 $\mu mol/m^2$. The Eclipse XDB-C_{18} column has a nominal surface area of 180 m^2/g, and a pore size of 80 Å.

The column temperature was controlled by a circulating water-bath Brinkman Model RC6 Lauda (Lauda-Konigshofen, Germany). pH measurements were performed with a Fisher Accumet pH meter 15 on the aqueous eluent component before the addition of the organic modifier. The electrode was calibrated with pH 1.0, 2.0, 4.0, 7.0, and 10.0 standard solutions.

2.2. Chemicals

Orthophosphoric acid (analytical grade), perchloric acid (redistilled) and trifluoroacetic acid (spectrophotometeric grade), water (HPLC grade), and acetonitrile (HPLC grade) were obtained from Sigma (Milwaukee, WI, USA). Disodium hydrogenphosphate heptahydrate was purchased from Fisher Scientific (Fairlawn, NJ, USA). All aqueous mobile phases were filtered with Nylon 66 membrane filter (Fisherlane). The following compounds were used: Aniline (Baker), *N*-methylaniline (Eastman, TN, USA), pyridine, 2-ethylpyridine, 3-ethylpyridine, 4-ethylpyridine, 2,4-dimethylpyridine, 2,6-dimethylpyridine, 3,4-dimethylpyridine, 3,5-dimethylpyridine, phenol, *p*-toluenesulfonic acid, benzenesulfonic acid, labetolol, and metoprolol (Aldrich), 2-*n*-propylpyridine, 4-*n*-propylpyridine, benzylamine, 2-methylbenzylamine, 3-methylbenzylamine, 4-metylbenzylamine (Lancaster Labs., Lancaster, PA).

2.3. Chromatographic conditions

The retention data was recorded at 25°C using isocratic conditions with a flow-rate of 1 ml/min for the Zorbax Eclipse XDB-C_{18}. UV detection was at 254 nm for the entire study. The aqueous portion of the mobile phase was a 10 m*M* sodium dihydrogenphosphate buffer (pH 8.96) adjusted with the different acidic modifiers (perchloric, trifluoroacetic acid, or orthophosphoric acid) over the pH range of 1.3 to 8.6. The organic modifier used was acetonitrile and the eluent composition was buffer–organic (90:10). For the studies without the phosphate buffer, the pH of the water was adjusted with perchloric or trifluoroacetic acids. The trifluoroacetate and perchlorate counter anion concentrations are 5–68 m*M* and 6–60 m*M*, respectively.

All analyte solutions except for the benzylamines were prepared by dissolution in the eluent to give a concentration of 0.1–0.2 mg/ml. Benzylamines were dissolved in water–acetonitrile (70:30) to give a concentration of 0.2 mg/ml. Injections of 1–5 μl of these solutions were made.

The $t_0$value obtained for the Zorbax XDB-C_{18} column with minor disturbance method, deuterated components and pyknometry [40,41] at 25°C) was 1.46 min with less than a 2% RSD from three different methods and six different experiments. Since the void volume is the total volume of the liquid phase in the column it will not change with the mobile phase composition as shown by determination of the void volume with deuterated components. The retention factors calculated were the average of triplicate injections. Also, a test mixture of aniline, phenol and pyridine was used as a system suitability check before and after each experiment to determine the stability of the column and system performance.

3. Results and discussion

3.1. Comparison of the effect of acidic modifiers on retention using 10 mM sodium phosphate buffer

3.1.1. Phosphoric acid

The pH of a 10 m*M* sodium phosphate buffer (original pH 8.96) was adjusted with phosphoric acid and the retention factors were obtained at each pH. The pH plotted is the pH of the buffer before the addition of organic eluent. Plots of the retention factors for several basic compounds versus pH are shown in Figs. 2 and 3. For a series of mono and dialkylsubstituted pyridines the retention factors level off at a pH of 2 units below the titrmetric pK_a

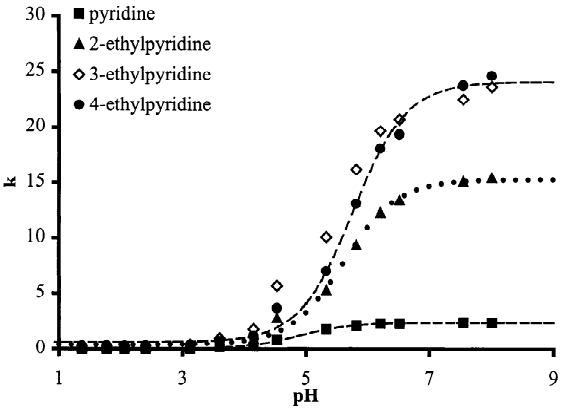

Fig. 2. Retention factor versus pH for a series of pyridines of increasing hydrophobicity. Chromatographic conditions: Column 150×4.6 mm Zorbax XDB-C_{18} mobile phase: acetonitrile–10 m*M* sodium phosphate buffer adjusted with phosphoric acid (10:90) flow-rate, 1.0 ml/min; 25°C, UV, 254 nm; sample: 1 μl injection.

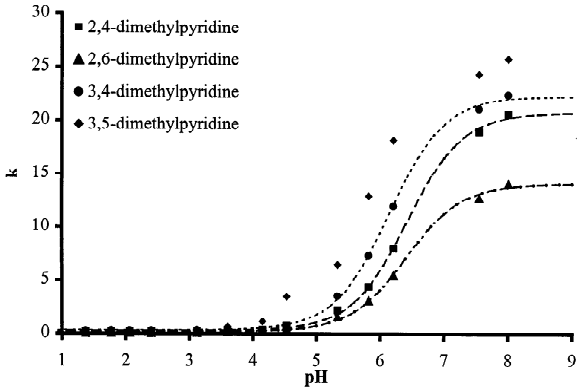

Fig. 3. Retention factor versus pH for an isomeric series of dimethylpyridines. Chromatographic conditions identical to Fig. 2.

values of the analytes. Inflection point of the analyte retention dependence on the mobile phase pH was used as chromatographic pK_a. These values were determined for all compounds that had expected pK_a values within the pH range studied (Table 1).

3.2. Eluent composition influence on the pK_a

The retention factors of the basic compounds were plotted versus the pH of the aqueous portion of the mobile phase before the addition of the organic and the inflection point which correlates to the chromatographic pK_a was calculated for each plot using Eq. (1). The analyte chromatographic pK_a values determined in eluent systems modified with the three acidic modifiers and the analyte literature pK_a values are compared in Table 1. The chromatographic pK_a values determined for the basic compounds in the aqueous–acetonitile (90:10) eluents modified with the three different acidic modifiers do not correspond to the literature values which were determined by potentiometry in aqueous solutions. The chromatographic pK_a determined in aqueous/organic mixtures is lower than those measured in an aqueous solution and this effect has been observed by others [42–45]. This attributed to a pH shift of the aqueous phase upon the addition of the organic and/or the suppression of the pK_a of the basic compound [46]. The pH shift of the mobile phase occurs since the pK_a values of the particular acids employed increase upon the addition of organic. Barbosa and Sanz-Nebot [5,6] found from potentiometric titrimetry that the pK_a of various acidic buffered systems shifts approximately 0.3 pH units per each 10% organic phase added. Bosch and Roses [4] also had shown the variation in the pK_a values of polyprotic acids is dependent not only on the solvent composition but also on the type of acid. They attributed this to the different solute properties of the acids: Charge, volume, polarity, and hydrogen acceptor and bonding capabilities. These

Table 1
Comparison of pK_a values measured using reversed-phase HPLC with literature values (titrimetry)[a,b]

Compound	Lit.	Chromatographic experiments			Average
		H_3PO_4	TFA	$HClO_4$	
Pyridine	5.17	4.85	4.75	5.03	4.88
2-Ethylpyridine	5.89	5.62	5.56	5.62	5.60
3-Ethylpyridine	5.80 (20°C)	5.42	5.3	5.43	5.38
4-Ethylpyridine	5.87	5.8	5.66	5.8	5.75
2,4-Dimethylpyridine	6.74	6.42	6.27	6.31	6.33
2,6-Dimethylpyridine	6.71	6.41	6.27	6.27	6.32
3,4-Dimethylpyridine	6.47	6.2	6.02	6.08	6.10
3,5-Dimethylpyridine	6.09	5.82	5.72	5.76	5.77
Aniline	4.6	4.08	4.13	4.2	4.14
N-Methylaniline	4.85	4.4	4.44	4.64	4.49

[a] All literature pK_a values were determined at 25°C in water unless otherwise noted [50].

[b] All chromatographic pK_a values were determined in a 90% aqueous buffer containing 10 m*M* sodium phosphate buffer adjusted with perchloric, trifluoroacetic or phosphoric acid and 10% acetonitrile at 25°C. The best fits of the theoretical curves to the experimental data were found by using a nonlinear least squares curve fitting software, Mathcad 8.

may affect the pH of an organic/aqueous mixture and the solvation of the solutes in the mixed solvent which will both have an influence on the pK_a determination of acid and basic solutes. Phosphoric, trifluoroacetic and perchloric acid were used to adjust the pH of the aqueous portion of the mobile phase. The chromatographic pK_a values determined for the basic compounds are shown in Table 1 when the different acidic modifiers employed. Minor differences between the pK_a values for the same compound were obtained with the three different acidic modifiers. This is attributed to the suppression of the acidic modifier ionization upon addition of organic to different degrees.

The chromatographic pK_a values for the basic compounds studied were about 0.3–0.5 pH units less than their literature values. Even if the pK_a is corrected for the pH shift of the mobile phase upon the addition of organic solvent, the chromatographic pK_a values are still lower than their literature values. This suppression in the pK_a may be attributed to specific solvation effects, including preferential solvation of the organic eluent components on the analyte. The environment in the vicinity of the solute is different than that of the bulk eluent environment. Therefore, the ionization of the base at the same pH in aqueous medium, as opposed to organic–aqueous medium, may not be equivalent. The ionization of different classes of basic compounds is suppressed to different degrees in the organic–aqueous medium. This suppression in the pK_a values can be related to basic analyte solvation by the eluent components. The basic solute in the eluent will interact with solvents in the eluent and is preferentially solvated by them. This explains why there is not a consistent change in pK_a at certain percentages of organic modifier between different classes of basic compounds such as the aniline and pyridine species shown in Table 1.

3.2.1. Trifluoroacetic acid and perchloric acid

The adjustment of pH of a 10 m*M* sodium dihydrogenphosphate buffer (pH 8.96) with perchloric or trifluoroacetic acid instead of phosphoric acid lead to the same dependence of analyte retention on pH, until pH 3. As can be seen in Figs. 4 and 5, the decrease of mobile phase pH below 3 leads to a slight increase of the analyte retention. At pH above

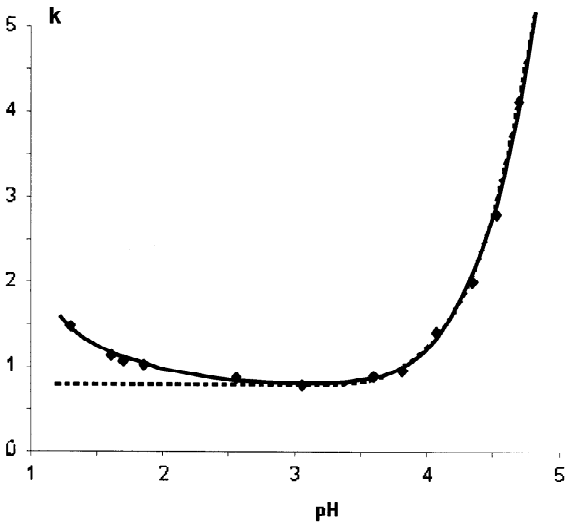

Fig. 4. pH dependence of the retention of 4-ethylpyridine. Column 150×4.6 mm Zorbax XDB-C_{18} Mobile phase: acetonitrile–10 m*M* sodium phosphate buffer adjusted with trifluoroacetic acid (10:90); flow-rate, 1.0 ml/min; 25°C, UV, 254 nm; sample: 1 μl injection.

4, the curve shows the typical sigmoidal behavior seen in Figs. 2 and 3. This increase of retention time with a decrease of pH was found for all basic analytes studied using both perchloric (Table 2) and trifluoroacetic acid (Table 3).

The possible ion-exchange interactions between a basic analyte and dissociated silanol groups on the column surface should not be prevalent at these low pH values. The pK_a of normal silanols on a silica surface has been estimated to have a value of 7, but

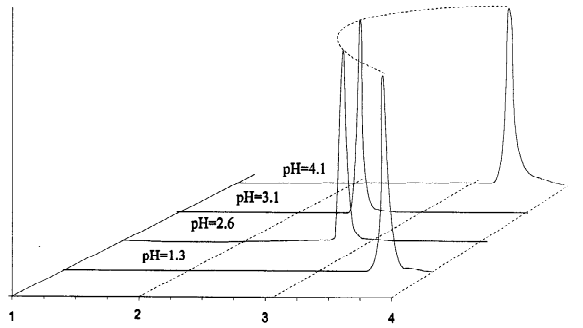

Fig. 5. The effect of TFA (pH adjustment) on retention of 4-ethylpyridine. Column and chromatographic conditions same as in Fig. 6. Retention time at pH 4.1 is 3.5, at pH 3.1 is 2.6 at pH 12.6 is 2.7 and at pH 1.3 is 3.6 min.

Table 2
Retention factor at each pH adjustment with trifluoroacetic acid[a]

pH	Aniline	*N*-Methyl-aniline	Pyridine	2-Ethyl-pyridine	3-Ethyl-pyridine	4-Ethyl-pyridine	2,4-Dimethyl-pyridine	2,6-Dimethyl-pyridine	3,4-Dimethyl-pyridine	3,5-Dimethyl-pyridine
4.08	2.50	5.79	0.46	0.98	1.88	1.40	0.68	0.47	0.91	1.24
3.82	1.64	2.49	0.30	0.67	1.18	0.95	0.57	0.41	0.73	0.89
3.6	1.26	2.54	0.22	0.58	0.99	0.89	0.54	0.39	0.68	0.77
3.06	0.78	1.47	0.14	0.50	0.78	0.79	0.53	0.39	0.68	0.70
2.56	0.74	1.34	0.15	0.55	0.83	0.87	0.61	0.43	0.75	0.76
1.86	0.82	1.38	0.17	0.64	0.94	1.02	0.72	0.52	0.90	0.91
1.7	0.88	1.49	0.19	0.67	0.99	1.06	0.75	0.54	0.92	0.94
1.61	0.91	1.60	0.20	0.72	1.06	1.14	0.81	0.57	1.00	1.00
1.3	1.04	1.93	0.23	0.92	1.38	1.48	1.05	0.73	1.28	1.31

[a] Column 150×4.6 mm Zorbax XDB-C_{18}; mobile phase: Acetonitrile–10 m*M* sodium phosphate buffer adjusted with trifluoroacetic acid, pH 1.3–8.6 (10:90); flow-rate, 1.0 μl/min; 25°C; UV, 254 nm; sample: 1 μl injection.

this value also depends on the environment and metal impurities of the silica matrix [47–49]. The effect of pH on silanol retention activity should resemble a titration curve. At low pH, accessible residual silanols will be protonated and should show minimal electrostatic interactions, any further decrease of the pH should not have an effect on the basic analyte retention.

The chromatograms for aniline at pH from 1.3 to 7.1 are shown in Fig. 6. As the pH approaches the pK_a of aniline the peak shape becomes broad and severe fronting is observed. In this pH region, peak asymmetry is attributed to secondary equilibria effects. Upon further decrease of the pH the retention of aniline decreases until pH 2.6, then retention starts to increase as the pH is lowered further. Even for higher pK_a bases such as the benzyl amines, the increases in retention are observed at pH values less than 3.

Our experiments have shown that differences in analyte retention is affected by different types and concentrations of acidic modifier. These effects are observed on the same column. Increased retention was obtained with both trifluoroacetic and perchloric acid modifiers but not with the phosphoric acid. This suggests that the observed effect could be attributed to the influence of the acidic mobile phase modifier and not to any specific pH or pH influence on the stationary phase properties. The actual concentration of the counter anion of the acidic modifier actually plays a role on the basic analyte retention.

3.3. Comparison of modifiers

Fig. 7 shows an overlay of the retention factors for 4-ethylpyridine as a function of pH using three different acid modifiers. At pH values above 7, the retention factors of 4-ethylpyridine (neutral at these

Table 3
Retention factor at each pH adjustment with perchloric acid[a]

pH	Aniline	*N*-methyl-aniline	Pyridine	2-Ethyl-pyridine	3-Ethyl-pyridine	4-Ethyl-pyridine	2,4-Dimethyl-pyridine	2,6-Dimethyl-pyridine	3,4-Dimethyl-pyridine	3,5-Dimethyl-pyridine	2-Methyl-benzylamine	3-Methyl-benzylamine	4-Methyl-benzylamine
4.04	2.53	5.60	0.34	1.10	2.05	1.63	0.70	0.50	–	1.44	3.06	3.99	3.97
3.78	1.69	3.47	–	0.77	1.31	1.11	0.64	0.47	0.82	0.98	2.99	3.87	3.86
3.51	1.18	2.29	0.26	0.58	0.95	0.89	0.56	0.41	0.71	0.78	2.83	3.71	3.67
3.02	0.79	1.47	0.17	0.56	0.86	0.88	0.62	0.45	0.77	0.78	3.24	4.20	4.16
2.55	0.70	1.26	0.17	0.56	0.84	0.90	0.64	0.47	0.79	0.80	3.39	4.43	4.40
1.82	0.83	1.47	0.22	0.71	1.05	1.14	0.82	0.59	1.01	1.02	4.38	–	–
1.65	0.85	1.50	0.22	0.74	1.09	1.20	0.84	0.61	0.03	1.03	–	5.64	5.58

[a] Column 150×4.6 mm Zorbax XDB-C_{18}; mobile phase: Acetonitrile–10 m*M* sodium phosphate buffer adjusted with perchloric acid, pH=1.65–8.6 (10:90); flow-rate, 1.0 ml/min; 25°C; UV, 254 nm; sample: 1 μl injection.

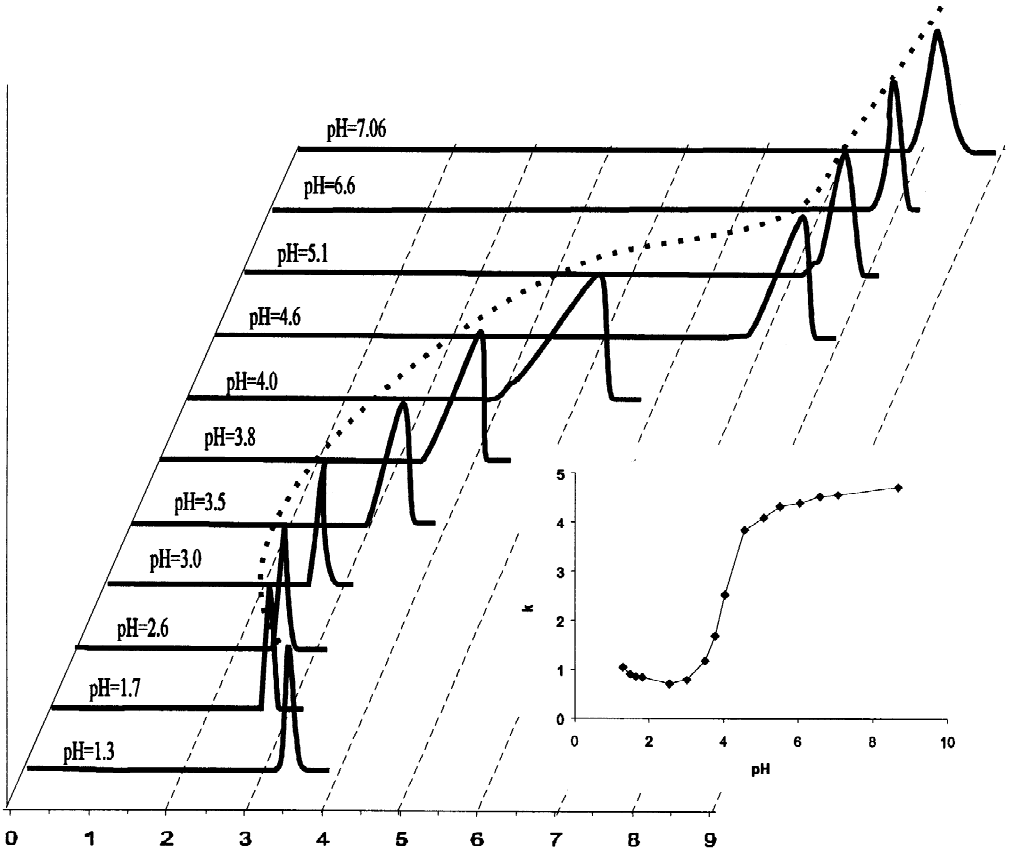

Fig. 6. Effect on retention of aniline when perchloric acid is used as the acidic modifier throughout pH region 1.3–7.1. Column 150×4.6 mm Zorbax XDB-C_{18} mobile phase: acetonitrie–10 m*M* disodium hydrogenphosphate buffer adjusted with perchloric acid, pH 1.3–7.1 (10:90): flow-rate, 1.0 ml/min; 25°C; UV, 254 nm; sample: 1 μl injection.

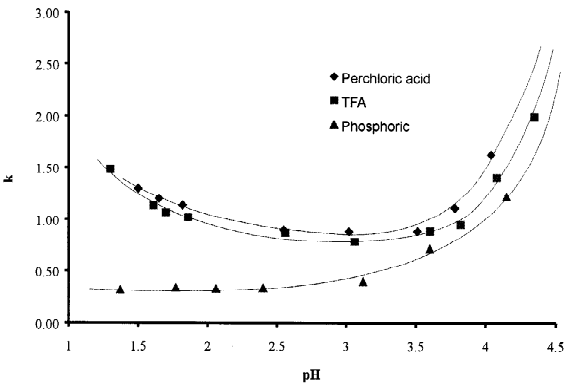

Fig. 7. Comparison of retention factors of 4-ethylpyridine as a function of pH with three different acidic modifiers. Same chromatographic conditions as Fig. 6 except for mobile phase modifiers.

pH values), with all three acidic modifiers shows a plateau effect. The chromatographic pK_a values obtained for 4-ethylpyridine are similar with the three different modifiers (see Table 1). The sigmodial curves obtained are very similar with all three acidic modifiers in the pK_a region. Upon further decrease of the pH, the retention factors obtained are similar until pH 4. Below pH 4, 4-ethylpyridine shows different retention factors and this change is specific to the type of acidic modifier used. The decrease of pH with addition of trifluoroacetate and perchlorate causes an increase in retention whereas with the dihydrogen phosphate counteranion a ''plateau-like'' effect is observed.

The decrease in mobile phase pH is achieved by an increase in concentration of perchloric and trifluoroacetic acid. This increase in concentration of

the counter anion of the acid led to an increase in retention for all the basic analytes. In the proposed explanation, both the type and concentration of the counter anion play a role on the increased retention of the basic analytes.

3.4. Chaotropic effect

Different acids affect the retention of basic analytes to different degrees in the low pH region (Fig. 7). The counter anion of the acid interacts with the positively charged basic analyte and may form an ion-associated complex. This interaction alters the retention of the basic analytes due to the changes in charge density, polarizability and solvation. Counter-anions that have a less localized charge, high polarizability and lower degree of hydration usually show a significant effect on the retention of basic analytes and are known as chaotropic ions [24]. These chaotropic ions are known to change the structure (hydrogen bonding) of water in the direction of greater disorder [39]. Therefore, the hydrogen bonding of the solvation shell of the basic analytes is disrupted due to ion interaction with the chaotropic anions. The counter anions of the acids employed in our studies (perchlorate, trifluoroacetate and dihydrogenphosphate) fall into this class of chaotropic anions.

As the concentration of the counter anion increases the solvation of the protonated basic analyte decreases. The primary sheath of water molecules around the basic analytes is disrupted and this decreases the solvation of the basic analyte. The decrease in the analyte solvation increases the analyte hydrophobicity and leads to increased interaction with the hydrophobic stationary phase and increased retention for the basic analytes.

Mobile phase pH not only effects the protonation of basic analytes but also the ionization of the acidic modifier. The chaotropic effect, discussed above is dependent on the concentration of the free counter anion and the concentration of the protons in solution. This suggests that the increases in retention of the protonated basic analytes may be observed with an increase in concentration of the counter anion by the addition of a salt at a constant pH. The increase in analyte retention was similar for both perchloric and trifluoroacetic acids systems that contained 10 m*M* of phosphate buffer salt (Tables 2 and 3). The concentration of the perchloric acid and the trifluoroacetic though were not the same at similar pH values so the chaotropic effect may be hidden within the plot of k versus pH. In the following studies the retention of the basic compound is plotted versus concentration of the counter anion in the mobile phase. The pH is solely needed for the protonation of the basic analyte and any further decrease in the pH leads to an increased concentration of the counter anion of the acidic modifier.

3.5. Comparison of trifluoroacetic acid, perchloric acid and phosphoric acid without addition of buffer

The influence of trifluoroacetate, perchlorate and dihydrogenphosphate anions on the retention of the basic compounds is compared in the absence of buffer. The concentration of the trifluoroacetate anion in the studied region was varied between 5 and 68 m*M*. The concentrations for the perchlorate anion are between 6 and 60 m*M*. The concentrations for the dihydrogenphosphate counter anion are between 4.5 and 91 m*M*. An accurate representation of the influence of the counter anion on the basic analyte retention is evident when the retention factors for 3,4-dimethylpyridine and 2-propylpyridine are plotted versus the concentration of free counter anions of perchlorate, dihyrodgenphosphate, and trifluoroacetate. These are shown in Fig. 8a and b.

A greater increase in retention of the basic compounds is observed when perchlorate is employed. At every concentration studied, perchlorate shows a greater effect on retention than trifluoroacetate and phosphate. This is attributed to the greater chaotropic nature of the perchlorate ion. Similar results are obtained with other basic analytes studied.

However, in the phosphate buffer system the effect of trifluoroacetate and perchlorate anions upon analyte retention are similar as shown in Tables 2 and 3. The difference in strength of the chaotropic anions is hidden when the results were plotted versus the pH and not the counter anion concentration. Also, a greater amount of both acids is needed to reach the desired pH in the low pH range due to the buffering capacity of the phosphate buffer. Therefore, the concentration of counter anions is different

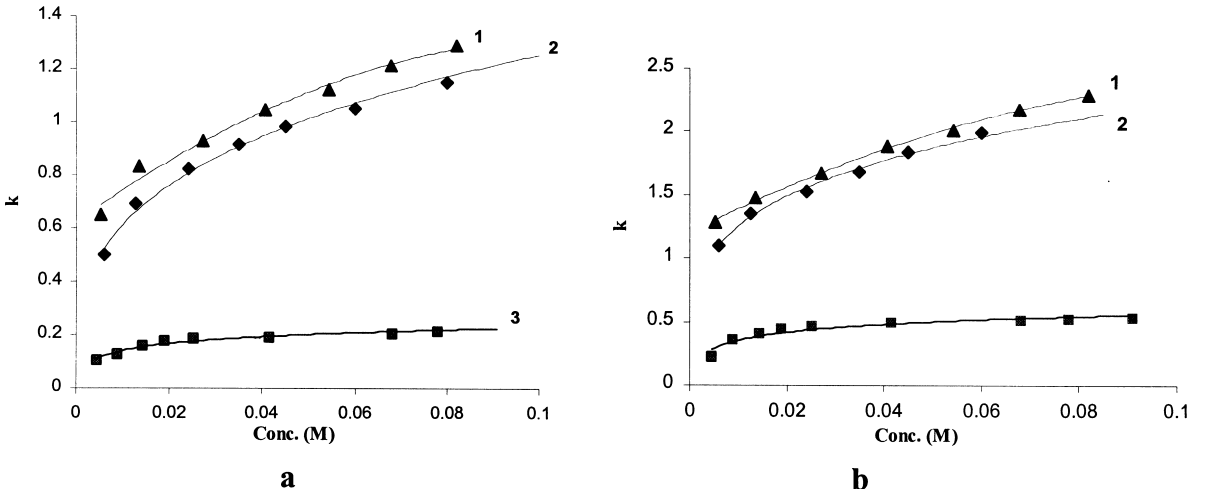

Fig. 8. Comparison of the chaotropic effect on basic analytes caused by trifluoroacetate, dihydrogenphosphate and perchlorate counter anions plotted against counter anion concentration curves: (1) perchloric acid, (2) trifluoroacetic acid and (3) is for phosphoric acid. (a) 3,4-Dimethylpyridine, (b) 2-propylpyridine. Column 150×4.6 mm Zorbax XDB-C_{18}; mobile phase: acetonitrile–HPLC-grade water adjusted with the following acids (10:90); sodium phosphate buffer 4.3–73 m*M* adjusted with phosphoric acid and/or solely phosphoric acid, pH 1.8–2.3, trifluoroacetic acid and perchloric acid, pH 1.3–1.9: flow-rate, 1.0 ml/min; ambient temperature, UV detection at 254 nm; sample: 1 μl injection.

in the same pH range with the buffered and unbuffered mobile phases. There also are competing effects on the retention due to the presence of the dihydrogenphosphate anions from the phosphate buffer. The effects of the trifluoroacetate and perchlorate anions on the solvation of the basic analytes may be suppressed to different degrees.

3.6. Counter anion of salt versus acid

In order to demonstrate that the increase in basic analyte retention is associated with an increase of the counter anion concentration experiments were performed at variable pH and a constant pH. In one experiment, the concentration is changed solely by adjusting the concentration of perchloric acid with simultaneous change in pH. In several other experiments the perchlorate concentration in the mobile phase is increased at a constant pH with the addition of sodium perchlorate. A plot of the concentration of perchlorate anion from various experiments (solely perchloric acid adjustment and perchloric acid + $NaClO_4$) versus retention factor are shown Fig. 9. For example, at pH 1.91 the mobile phase was divided into three portions and sodium perchlorate was added at different concentrations. A pH 2.0 mobile phase was treated in the same fashion. The mobile phases in which sodium perchlorate was added are denoted as runs 5, 9, 11 (pH 1.91) and runs 10, 12, 13, 14 (pH 2.0) in Table 4.

There are two ways in which the concentration of the counter anion may be increased:

1. Increase the amount of acid added.
2. The addition of a salt that contains the same counter anion as the acid.

In the first approach not only does the concentration change but so does the pH. Therefore, with an increase in concentration of acid there is a decrease in pH.

It is shown in Fig. 9 that as the concentration of the perchlorate anion increases the retention of the basic analytes increases at constant pH and at variable pH. As a result of addition of acid there is a consequent increase in the concentration of the counter anion of the acid and a simultaneous decrease in pH. pH only affects the protonation of the analyte. Only, the protonated analyte species can undergo ionic association with the counter anion of the acid that is employed. Hence, when the protonated base interacts with the counter anion this leads

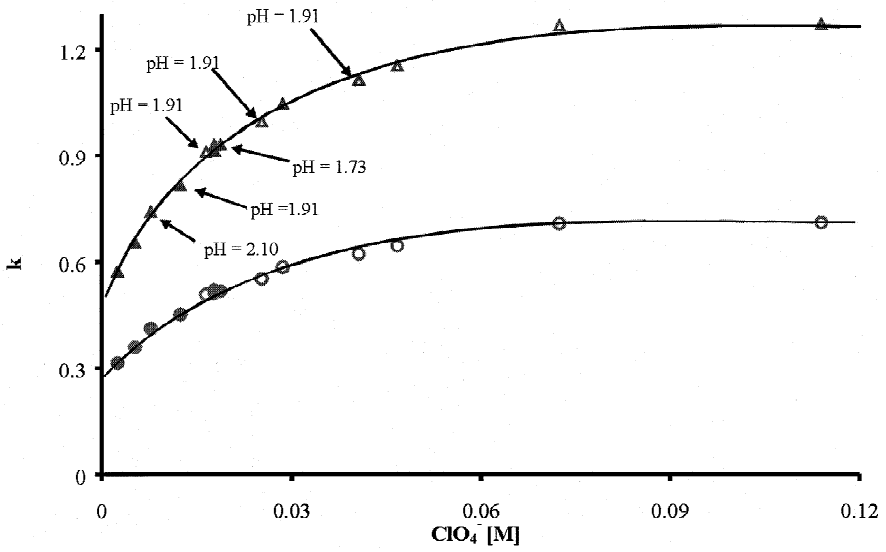

Fig. 9. Retention factor of 2-ethylpyridine and 4-ethylpyridine versus concentration of perchlorate anion. Upper dependence (closed and open triangles) is for 4-ethylpyridine and the bottom dependence (open and closed circles) is for 2-ethylpyridine. Closed triangles and circles represent the retention factors obtained with a buffer whose concentration was modified solely with the addition of perchloric acid (variable pH). Open triangles and circles represent the retention factors obtained with a buffer whose concentration was modified with the addition of perchloric acid and sodium perchlorate at a constant pH. Conditions: Zorbax Eclipse XDB-C_{18}, 90% aqueous adjusted with sodium perchlorate and/or perchloric acid and 10% acetonitrile; temperature: 25°C; wavelength: 254 nm.

to changes in its solvation and an increase in its hydrophobicity. The higher the counter anion concentration in the mobile phase, the greater the desolvation of basic analyte. This causes an increase in analyte retention.

The addition of sodium perchlorate causes an increase of total ionic strength to a greater extent than addition of perchloric acid. However, it is shown that the increase in retention is independent on other ionic species in the mobile phase and the retention increase can be solely attributed to interaction with the perchlorate anion. Further studies of this chaotropic effect at controlled ionic strength with NaCl needs to be performed which is a subject of a future study. Fig. 10 also shows the increase in the retention of several basic compounds with the increase in the perchlorate concentration from sodium perchlorate 2.5–114 m*M*.

3.7. Selectivity

If a basic analyte is not fully protonated in the pH region studied the chaotropic effects may not be observed. On the other hand, the effect of the chaotropic agent upon the solvation and retention of fully protonated basic analyte is significant. This is demonstrated in Fig. 11, for phenylethylamine and *o*-chloroaniline.

The pK_a of *o*-chloroaniline is 2.64 and for phenylethylamine is 9.83 and the working pH was from 1.6 to 1.8. The concentration of perchlorate anion in this pH range was 10 and 25 m*M*, respectively. In this pH region the *o*-chloroaniline is not fully protonated while phenylethylamine is. A decrease in retention was observed for *o*-chloroaniline and this was due to the change in its ionization. An increase in concentration of the perchlorate anion from 10 m*M* to 25 m*M* leads to an increase in retention for phenylethylamine due to the chaotropic effect on the retention. A dramatic reversal of elution order is obtained.

The separation of neutral, acidic and basic compounds at a constant pH with increasing perchlorate concentration is shown in Fig. 12. The retention of acidic and neutral components is virtually unaffected due to the lack of interaction with the negatively

Table 4
Retention factors of basic compounds obtained at increasing perchlorate concentrations[a]

	Run													
	1: $HClO_4$, pH 2.59, 2.5 m*M* ClO_4^- (0.0025 *M* ClO_4^-)	2: $HClO_4$, pH 2.25, 5.2 m*M* ClO_4^- (0.0052 *M* ClO_4^-)	3: $HClO_4$, pH 2.1, 7.7 m*M* ClO_4^- (0.0077 *M* ClO_4^-)	4: $HClO_4$, pH 1.91, 12.4 m*M* ClO_4^- (0.0124 *M* ClO_4^-)	5: $HClO_4$ + $NaClO_4$, pH 1.91, 16.4 m*M* ClO_4^- (0.0164 *M* ClO_4^-)	6: $HClO_4$, pH 1.78, 17.71 m*M* ClO_4^- (0.01771 *M* ClO_4^-)	7: $HClO_4$, pH 1.78, 17.71 m*M* ClO_4^- (0.01771 *M* ClO_4^-)	8: $HClO_4$, pH 2, 18.7 m*M* ClO_4^- (0.0187 *M* ClO_4^-)	9: $HClO_4$ + $NaClO_4$, pH 1.91, 25.2 m*M* ClO_4^- (0.0252 *M* ClO_4^-)	10: $HClO_4$ + $NaClO_4$, pH 2, 28.2 m*M* ClO_4^- (0.0285 *M* ClO_4^-)	11: $HClO_4$ + $NaClO_4$, pH 1.91, 40.6 m*M* ClO_4^- (0.0406 *M* ClO_4^-)	12: $HClO_4$ + $NaClO_4$, pH 2, 46.7 m*M* ClO_4^- (0.0467 *M* ClO_4^-)	13: $HClO_4$ + $NaClO_4$, pH 2, 72.4 m*M* ClO_4^- (0.0724 *M* ClO_4^-)	14: $HClO_4$ + $NaClO_4$, pH 2.01, 113.9 m*M* ClO_4^- (0.1139 *M* ClO_4^-)
Aniline	0.50	0.55	0.61	0.66	0.75	0.76	0.74	0.76	0.80	0.84	0.89	0.91	0.99	0.99
pyridine	0.03	0.04	0.06	0.07	0.09	0.10	0.09	0.09	0.10	0.11	0.12	0.12	0.14	0.14
N-Methylaniline	0.94	0.99	1.08	1.17	1.33	1.32	1.30	1.34	1.42	1.50	1.58	1.64	1.78	1.78
N,*N*-Dimethylaniline	1.17	1.22	1.31	1.40	1.61	1.58	1.54	1.62	1.72	1.81	1.91	1.98	2.18	2.19
2,4-Dimethylaniline	0.34	0.41	0.47	0.52	0.59	0.60	0.58	0.59	0.63	0.67	0.71	0.74	0.81	0.82
3,4-Dimethylaniline	0.46	0.53	0.61	0.67	0.76	0.77	0.76	0.77	0.82	0.87	0.93	0.96	1.05	1.06
2-Ethylpyridine	0.31	0.36	0.41	0.45	0.51	0.52	0.51	0.52	0.55	0.58	0.62	0.64	0.71	0.71
4-Ethylpyridine	0.57	0.65	0.74	0.81	0.91	0.93	0.91	0.93	0.99	1.05	1.11	1.15	1.27	1.27
2-*n*-Propylpyridine	0.98	1.08	1.21	1.31	1.46	–	1.46	1.50	1.59	1.67	1.78	1.85	2.02	2.04
4-*n*-Propylpyridine	1.92	2.16	2.41	2.65	2.97	–	2.97	3.06	3.25	3.42	3.63	3.76	4.12	4.14

[a] The makeup of the aqueous portion of the mobile phase is indicated. $HClO_4$ + $NaClO_4$ indicates that sodium perchlorate was added to the solution to increase the concentration of perchlorate anion without changing the pH. $HClO_4$ indicates that perchloric acid was added to the solution to increase the concentration of the perchlorate anion.

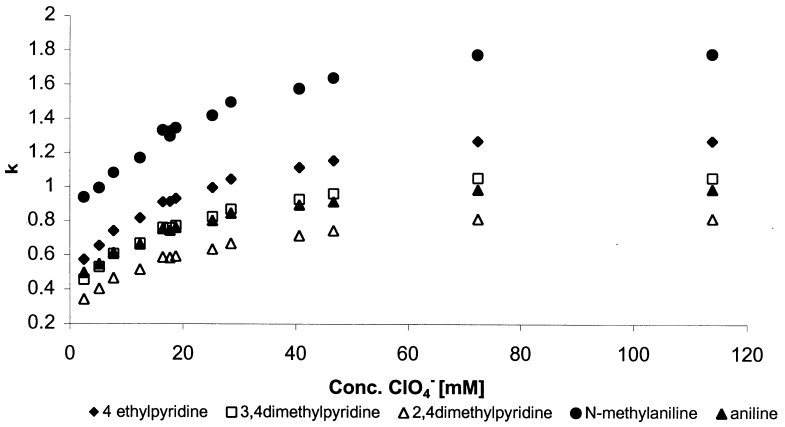

Fig. 10. Retention factor versus perchlorate concentration at aqueous–acetonitrile (90:10). Concentration region 2.5–114 m*M* of perchlorate anion. Column: 15×0.4 cm Zorbax XDB-C_{18} mobile phase: acetonitrile–aqueous perchloric acid and $NaClO_4$. (10:90). Aqueous portion of mobile phase made as according to Table 4; flow-rate, 1.0 ml/min; Temperature 25°C; UV, 254 nm; sample: 1 μl injection.

charged perchlorate anion. This work proposes interesting possibilities for improved resolution and selectivity of both acids and bases in a mixture at a constant pH.

4. Conclusion

The dependencies of the retention of basic analytes on the mobile phase pH are measured. Chromatographic pK_a values of basic analytes determined from these dependencies show a noticeable shift compared to the literature pK_a values.

At low mobile phase pH, in a region where basic analyte is fully protonated, any further pH decrease leads to an increase in analyte retention. This effect is attributed to the influence of the acidic modifier counter anions on the analyte solvation. Change of the counter anion concentration by simple salt addition produces the same effect on the analyte retention, although mobile phase pH remains constant. The chaotropic effect has been found to be dependent on the nature of the anionic species and independent of the source of the anion (acid or salt), with the propensity for analyte desolvation following the order: ClO_4^-, CF_3COO^-, $H_2PO_4^-$.

Highest effect on the retention of basic analyte is observed at low counter anion concentration. A further increase of the counter anion concentration causes a gradual leveling off of the retention of basic analyte. This effect is consistent with the suggested model on the influence of the counter anions on the analyte solvation. At certain counter anion concentrations the disruption of the basic analyte solvation is completed and any further concentration increase will not show a further effect. Counter anion concentration does not show a noticeable effect on the retention of neutral and acidic compounds.

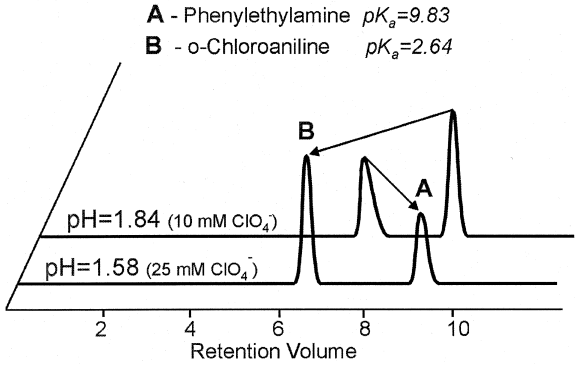

Fig. 11. Decrease in retention of *o*-chloroaniline is governed by ionization while increase in retention of phenylethylamine is governed by chaotropicity. Column 15×0.46 cm Zorbax XDB-C_{18}; mobile phase: Acetonitrile–HPLC grade water adjusted with perchloric acid, pH 1.58 and pH 1.84 (10:90); flow-rate, 1.0 ml/min; 25°C; UV, 254 nm; sample: 1 μl injection..

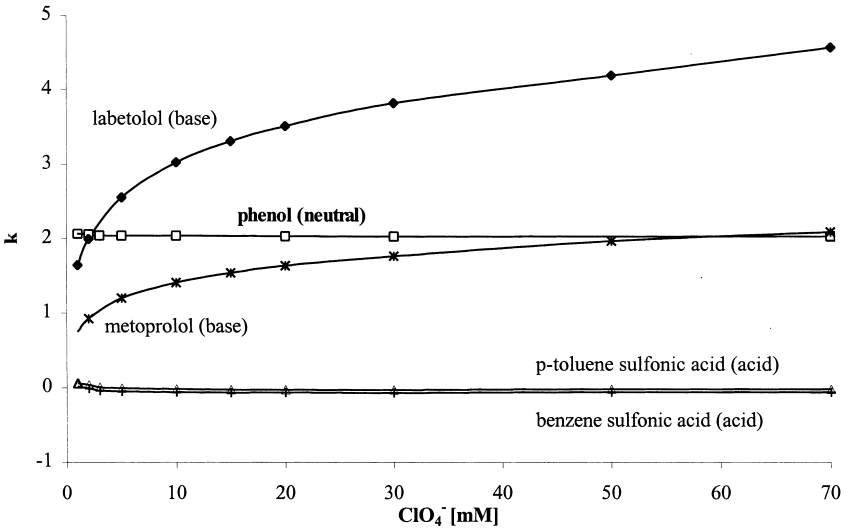

Fig. 12. Retention factor versus perchlorate concentration at aqueous–acetonitrile (70:30). Concentration region 1–70 m*M* of perchlorate anion. Column: 15×0.46 cm Zorbax XDB-C_{18}; mobile phase: acetonitrile–aqueous perchloric acid and $NaClO_4$ (30:70), pH 2.9 for 1–20 m*M* and pH 2.6 for 30–70 m*M*; flow-rate, 1.0 ml/min; tempreature, 25°C, UV at 220 and 254 nm; sample: 1 μl injection.

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