Study of retention and peak shape in hydrophilic interaction chromatography over a wide pH range.

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Abstract

Retention factor and column efficiency measurements were made for 14 test compounds comprising acids, bases and neutrals on two pairs of amide and bare silica HILIC columns, each pair obtained from a different manufacturer. The columns were tested with up to 6 different mobile phases with acetonitrile-water containing formic (FA), trifluoracetic (TFA), heptafluorobutyric acids (HFBA) and ammonium salt buffers at ${}_{w}^{w}$pH 3, 6 and 9. Measurements of mobile phase pH in water (${}_{w}^{w}$pH) and in the aqueous-organic mixture (${}_{w}^{s}$pH) were performed, and calculations of ionic strength made, in order to aid interpretation of the chromatographic results. Stronger acids like TFA produced very different selectivity compared with ammonium formate buffers at similar aqueous pH. On a given column using TFA as additive, the retention of strongly acidic solutes was considerably increased relative to that of bases. Some bases even showed exclusion on both amide, and the hybrid silica columns. Conversely, in ammonium formate buffers of similar aqueous pH, bases had increased retention compared with acids, particularly on the bare silica columns. This result can be attributed to the higher pH of the salt buffers when measured in the aqueous-organic phase and interaction with negatively charged silanols. It is possible that the silica surface becomes positively charged at the low pH of TFA, leading to anion exchange properties that become competitive with the cation exchange properties normally attributed to silanol dissociation, although other explanations of these results are possible. Very marked selectivity differences were obtained by use of TFA in the mobile phase. Useful selectivity differences may also be obtained with salt buffers at different pH if the use of TFA is not desired due to its relatively unfavourable properties in mass spectrometry.

1. Introduction

Hydrophilic interaction chromatography (HILIC) has over the last ten years become accepted as a complimentary LC separation mechanism to reversed-phase (RP), especially for the analysis of polar and ionised solutes that may be poorly retained by the latter technique. Many compounds of biomedical and clinical significance are amenable to analysis by HILIC [1-4]. The mechanism of the separation is complex, but recent studies have thrown more light on its detailed nature. Retention is thought to occur principally through partition of the solute between a water layer held on the surface of the polar stationary phase and the bulk mobile phase, which contains typically a high concentration of acetonitrile (ACN). However, ionic, adsorptive and even RP interactions can also occur, which are dependent on the nature of the solute, stationary and mobile phases [5-6]. The relative contributions of partition and adsorption to retention is likely to depend on the thickness of the water layer on the column surface, with adsorption contributing more when the water layer is limited. Using the Karl Fischer (KF) procedure, direct measurements of the water concentration inside the pores of different HILIC stationary phases have been made [7]. Similar investigations have been performed by using frontal analysis in combination with the KF method [8]. These studies have shown that water absorption is much greater on zwitterionic or amide-based phases, which can be attributed partially to the polymeric bonding of popular commercial phases of these types. In contrast, the water layer is much more limited on bare silica phases. Molecular dynamics studies have pointed to the existence of a tightly bound water layer close to the silica surface, followed by a more diffuse layer which gradually attains the composition of the bulk mobile phase with increasing distance from the surface [9]. Recent studies have confirmed that the mechanism of retention may be solute dependent. For example, the reduced b coefficient in the van Deemter equation determined by peak parking was appreciably smaller for cytosine compared with nortriptyline, the former solute being considerably more hydrophilic [10]. This result was caused by slower effective diffusion relative to bulk diffusion for cytosine, suggesting that it is held in the (more viscous) water layer whereas nortriptyline is held in the adjacent (less viscous) diffuse water layer containing a higher acetonitrile concentration. Broadly similar results were obtained in another study [11], suggesting nortriptyline might be retained more by a partition-like and cytosine by a more adsorption-like mechanism, at least on the bridged ethyl hybrid silica (BEH) used in that study. The complexity of the mechanism, however, is illustrated by the finding that the retention of nortriptyline at least on conventional bare silica (rather than BEH silica) is dominated by ionic interactions between the protonated base and ionised silanol groups on the stationary phase [5].

The effect of mobile phase composition in HILIC has also been investigated in more detail recently. Aqueous-acetonitrile solvents containing soluble buffer salts such as ammonium formate (AF) or ammonium acetate (AA) are recommended over solutions containing formic (FA) or acetic acids, as they produce better peak shapes, especially for ionogenic solutes [12]. Improved results may be due to the higher ionic strength of solutions containing salts compared with simple formic or acetic acid solutions and the competing effect of buffer cations with solute ions for ionised silanols on the stationary phase. Nevertheless, it was shown previously that stronger acids such as trifluoroacetic acid (TFA) could give acceptable peak shapes for acidic and basic compounds, which could be attributed to the higher ionic strength of such solutions (at least compared with FA), and/or suppression of the ionisation of silanol groups at the lower pH attained [1]. For a bare silica column, changes in selectivity were observed using TFA. With ammonium formate buffers ($_w^w$pH 3.0) in acetonitrile, long retention times of bases were observed, whereas acids eluted near the void volume of the column. Conversely, in 0.1 % TFA the order of elution was reversed, with strong acids having much longer retention times than the bases. These results might partially be attributed to the greatly decreased ionic interactions of protonated bases with ionised silanols at the lower pH of TFA. However, use of stronger acids like TFA has hardly been studied in HILIC, apart from some work in the analysis of peptides [13]. The previous study was confined to a single column, and no detailed rationalisation of the results was attempted. In this study, we first investigated in more detail the acidic properties of a number of potential HILIC mobile phases containing TFA, heptafluorobutyric acid (HFBA), phosphoric acid (PA) and formic acid (FA) in order to assist with interpreting the different results they produce. We then investigated retention and peak shape in a wide variety of mobile phases at different pH, using two pairs of silica and amide-bonded HILIC phases, with each pair coming from a different manufacturer. Only a few papers have discussed HILIC at high pH, when applied to the separation of peptides [14,15]. A selection of neutral, acidic and basic solutes was used as probes for the present study.

2. Experimental.

Experiments with 4.6 mm ID columns were performed with a 1100 binary solvent mixing system optimised for low extra column volume, equipped with a UV detector (1 μL flow cell), and with a 1290 binary high pressure mixing instrument with photodiode array detector (0.6 μL flow cell) for 2.1 mm columns (Agilent, Waldbronn, Germany). 5 μL injections were used for the former, and 1 μL injections for the latter system. The columns used were Atlantis silica (5 μm particle size, pore Size 100 Å, surface area 360 m^2/g), 25 cm x 0.46 cm ID ;

XBridge BEH Amide (3.5 μm particle size, pore size 140 Å, surface area 190 m^2/g), 15 cm x 0.46 cm ID; XBridge HILIC, (3.5 μm particle size, pore size 136 Å, surface area 183 m^2/g), 15 cm x 0.46 cm ID, all from Waters (Milford, USA) ; Poroshell HILIC (2.7 μm particle size, pore size 120 Å, surface area 130 m^2/g) and AdvanceBio Glycan Map-an amide phase (2.7 μm particle size, pore size and surface area unavailable) both 10 cm x 0.21 cm ID (Agilent). Flow rates were 1.0 mL/min. for the 0.46 cm ID and 0.25 mL/min for the 0.21 cm ID columns. A higher linear flow velocity than the geometrically scaled value was used in the narrow bore columns as the optimum flow velocity increases with decrease in particle size. Temperature was maintained at 30 ^{o} C using the Agilent column compartments. Acetonitrile (far UV grade), ammonium formate (AF), ammonium acetate (AA), trifluoroacetic (TFA) and orthophosphoric acid were obtained from Fisher (Loughborough U.K.). AF and AA buffers were prepared by adjusting 5 mM aqueous solutions of the salt to pH 3.0 and pH 6.0 with formic (FA) and acetic (AA) acids respectively. Ammonium bicarbonate (AB) buffer was prepared by adjusting the 5 mM aqueous solution to pH 9.0 with ammonia. Note that preparation of the buffers in this way results in a different concentration of acid anion at different pH. For example, 5mM AF $_{w}^{w}$ pH3 requires >30 mM/L of added FA to reach the desired pH, whereas 5 mM AA $_{w}^{w}$ pH 6 requires < 0.3 mM/L of added acetic acid. Nevertheless, according to the results (see below) the added formic acid is largely undissociated in high concentrations of ACN, and thus less likely to give major effects on the retention of ionised solutes. The overall concentration of ammonium ions potentially involved in ion exchange with dissociated silanol groups remains constant at 5 mM, and the ionic strength at least 5 mM due to the salt. This would not be the case if alternatively, the buffers had been made up using the same starting concentration of acid (rather than salt) and adjusting the pH with ammonia solution. Standards were prepared at a concentration of 20 mg/L and made up in the exact mobile phase. The pH values of the mobile phase quoted are those either in the aqueous portion of the buffer ($_{w}^{w}$pH), as measured in the organic-aqueous combination with the electrode calibrated in aqueous buffers ($_{w}^{s}$pH) or as the true thermodynamic pH, equivalent to that measured in the organic-aqueous solution with the electrode calibrated in organic-aqueous buffers ($_{s}^{s}$pH). pH was measured using a Metrohm 827 meter equipped with Unitrode electrode. All test solutes were obtained from Sigma-Aldrich (Poole, U.K.). Log D and log P values were calculated as the average from 3 different programs: ACD version 12.0 (ACD labs, Toronto, Canada), Marvin (ChemAxon, Budapest, Hungary) and MedChem Designer (Simulations Plus, Lancaster, California, USA). Column efficiency was measured from the first (M_1) and second (M_2) statistical moments according to the relationship:

$N = M_1^2/M_2$ (1)

3. Results and discussion

3.1 Measurement of ${}_w^spH$, ${}_w^wpH$ and ionic strength of aqueous-organic mobile phases.

Fig. 1 (a) shows the variation in ${}_s^spH$ with ACN concentration for TFA, FA and HFBA at 13.1 mM concentrations, which is the molar concentration equivalent to 0.1 % v/v TFA often used in LC work. Also included are measurements for 0.1 % v/v formic acid (26.5 mM) and 0.1 % (v/v) of concentrated (85 %) phosphoric acid (14.7 mM), which are commonly used in practice. (Note that phosphoric acid is completely soluble at acetonitrile concentrations up to at least 90 % v/v in water at this concentration. It was used as a chromatographic mobile phase without evidence of precipitation in a previous study [12]). The true thermodynamic ${}_s^s$ pH can be derived from the experimentally measured ${}_w^s$ pH using published delta values according to the equation [16]

${}_s^spH = {}_w^spH - \delta$ (2)

where δ is a term that incorporates both the Gibbs free energy for transference of 1 mole of protons from the standard state in water to the standard state in the hydroorganic solvent at a given temperature, and the residual liquid junction potential (the difference between the liquid junction potential established during calibration in aqueous solutions, and that in the hydroorganic mixture). Delta was calculated from the empirical equation:

$\delta = X(a+bT)/(1+cX)$ (3)

where T is the temperature on the Celsius scale, X is the ACN concentration and a,b,c are the fitting parameters given in [16] appropriate to the concentration scale (% v/v in the present case, equation validated over the range 0-90 % ACN, v/v). It is notable that whereas the ${}_s^spH$ of the various acids in 0% ACN (equivalent to the ${}_w^wpH$) covers a relatively small range (1.9-2.8), the range of ${}_s^spH$ at 90 % ACN is considerably greater (2.5-5.2). Fig. 1 (b) shows the ionic strength of these solutions calculated from the ${}_s^spH$. The very low ionic strength of phosphoric and formic acids in high concentrations of ACN is readily apparent. In contrast, the ionic strength of TFA and HFBA remains above 3 mM/L even in 90% ACN. Table 1 shows the ${}_w^wpH$ and ${}_w^spH$ of the salt buffer mobile phases used in this study. It is not possible to calculate ${}_s^spH$ in 95 % ACN as delta values are not available [13]. The ${}_w^spH$

values are clearly high, suggesting that ionisation of silanols on silica-based columns is likely to occur. While the detailed effect of the organic solvent on the pK_a of silanols is unknown, they may tend to become less acidic, somewhat moderating their ionisation. Ionic interactions of basic solutes may be reduced by competition with salt buffer cations. The salt concentration maintains the buffer strength at least to 5 mM, which is greater than any of the acidic solutions shown in Fig. 1. As shown previously, the pH and ionic strength in the aqueous-organic phase are important in the detailed interpretation of retention and peak shape data [12].

3.2 *Comparison of retention of acids, bases and neutrals in 95 % ACN containing different additives*.

Retention factors (*k*) of the 14 test compounds including neutrals uracil to 2-deoxyuridine), bases/quaternary compound (cytosine to trimethylphenylammonium) and acids (4-OH benzoic acid to p-xylenesulfonic acid) on the Waters BEH amide phase in 95 % ACN using a variety of acid additives and buffers (TFA, HFBA, FA at 13.1 mM concentration; AF $_w^w$ pH 3, AA $_w^w$ pH 6 , AB $_w^w$ pH 9) are shown in Fig. 2 (a). Besides the influence of high concentrations of ACN on mobile phase pH, pK_a shifts for the solutes will also occur, as well as changes in the pK_a of silanols noted above. The pK_a of acids is increased while that of the conjugate acids of basic compounds is reduced in high concentrations of ACN i.e. they become weaker acids and bases [16]. These pK_a shifts may change the degree of ionisation of the weaker acidic and basic solutes 4-OH benzoic acid, cytosine and pyridine. Most of our discussion below relates to the stronger acidic and basic solutes, which will remain fully ionised in acidic solutions (pH measured in water) despite these shifts in their pK_a. However at higher pH (e.g $_w^w$ pH 9) the ionisation of even the stronger bases may be reduced by these pK_a shifts, as discussed below. Solute abbreviations are defined in Table 2. *k* values on the Atlantis bare silica phase (originating from the same manufacturer) are shown in Fig. 2 (b); AB buffer at $_w^w$pH 9 was not used with the Atlantis column due to the possibility of dissolving the stationary phase under these conditions. Nevertheless, the solubility of silica at high pH in 95 % ACN is likely to be considerably less than in water, and merits further experimental investigation.

As expected, the neutral compounds gave greater retention on the amide phase than with bare silica, which may be attributed to a thicker water layer on the former [7]. On both stationary phases, while retention of the neutral compounds was reasonably independent of the buffer composition, use of salt buffers gave small increases in retention compared with the simple acid mobile phases. This observation may be connected with enhancements in the thickness of the water layer on the surface that occur in the presence of salt [17].

Retention of the bases and the quaternary compound in the salt buffers (AF, AA) was generally higher on the silica column than that of the neutrals, attributable to the interaction of the protonated solute with the larger number of ionised silanols that exist on this phase [18]. Retention of the bases in these buffers was less on the hybrid amide phase, which possesses a smaller number of acidic silanols [19]. On the silica column, retention of the stronger bases and TMPAC increased in AF $_{w}^{w}$ pH 6 compared with AF $_{w}^{w}$ pH 3, presumably as the proportion of ionised silanols increased. This increase in retention at $_{w}^{w}$pH 6 was much less marked on the amide column, again consistent with lower silanol acidity [19]. Using the amide phase however, procainamide (along with the bases diphenhydramine, nortriptyline and the quaternary TMPAC) showed *negative k* in TFA and HFBA (Fig. 2). It might be argued that ionisation of the silanol groups is suppressed at the low pH of these mobile phases (see Fig. 1), leading to reduced ionic retention. However, procainamide (log D_{pH2} –2.7) is considerably more hydrophilic than uracil (log D_{pH2} -0.78, see Table 2) and more hydrophilic than uridine (log D_{pH2} -2.1) and should arguably show greater retention in TFA or HFBA even in the absence of attractive ionic interactions. Uracil and uridine (k ~1.8 and k > 5) are well retained under the same conditions (Fig. 2). Fig. 3 shows chromatograms of a reduced set of test compounds on the Atlantis silica column. While the basic solutes do not show negative k with TFA/HFBA on this column, the retention of the basic solutes (peaks 5,6,8) relative to neutral (peaks 3,4) and acidic compounds (peaks 1,2) is drastically reduced compared with use of AF pH 3. An alternative explanation of the reduced retention of bases is that ion pairing of cationic solutes with TFA and HFBA reduces their hydrophilicity. Indeed, it is possible that the bases nortriptyline and procainamide (Fig. 3, peaks 5 and 6) show reduced retention in HFBA compared with TFA due to ion pairing effects which should be greater for HFBA. Nevertheless, ion pairing cannot explain the negative k values using the amide phase.

Retention factors of the acidic compounds on both columns in the salt buffers showed a complimentary pattern. k for benzenesulfonic acid (BSA), naphthalene-2-sulfonic acid (2-NSA) and p-xylene sulfonic acid (p-XSA) was low or even negative, especially in AF $_{w}^{w}$pH 6 and AB $_{w}^{w}$pH 9 (see Figs. 2,3). This observation is consistent with repulsion of the solute from ionised silanols on the columns, which increases as the pH is raised. Exceptionally, the weak acid 4-OH benzoic acid showed increased retention (k >5 on both columns) at $_{w}^{w}$ pH 6 compared with $_{w}^{w}$pH 3 which might be attributable to somewhat increased ionisation and thus greater hydrophilicity at the higher pH (see Fig. 2). Overall retention of the acids was somewhat greater on the amide compared with the silica phase, consistent with reduced repulsion from the (fewer) ionised silanols on this phase. The stronger acids were only excluded using the $_{w}^{w}$pH 9 buffer with the amide column (Fig. 2). In marked contrast, TFA and HFBA additives gave dramatic increases in retention of the strong

acids, especially on the amide column, giving *k* for BSA, 2-NSA and p-XSA of 53, 32, 36 respectively in TFA, (Fig. 2). The increase in retention of these strong acids was less marked on the silica phase in TFA, but p-XSA (peak 1) and 2-NSA (peak 2) were still the longest retained of the probes (see Fig. 3) in this mobile phase. An explanation of the results again might be that the lower pH of these mobile phases merely suppresses the ionisation of silanols, facilitating hydrophilic interactions and thus retention of the strong acids. However, the exceptional increase in the retention of the strong acids on the BEH amide column in TFA and HFBA cannot be explained on the basis of hydrophilic interactions alone; log D_{pH2} values for BSA, 2-NSA and p-XSA are relatively modest (-1.5, -0.5, -0.8 respectively).

The experiments were repeated using columns from a different manufacturer (Agilent Glycan amide and bare silica columns) in order to ascertain whether these unusual results were in any way exclusive to the Waters columns. For example, residues of basic catalysts used in some manufacturers' ligand bonding processes can exist on the stationary phase surface, giving rise to positive charges in acidic mobile phases [20]. The Agilent columns (superficially porous, 2.7 μm particles) also have the advantage that they are made from the same base silica, allowing a better comparison of performance to be made. Very similar results were obtained. Fig. 4 shows illustrative chromatograms of a subset of 8 test compounds on the Agilent silica column using TFA and AF $_w^w$ pH 3. The pattern of elution of the peaks is very similar to that on the Atlantis column (Fig. 3) showing the same drastic reduction in retention of the bases (peaks 5,6,8) in TFA compared with AF, and increase in retention of the acids (peaks 1 and 2). Similarly the results for the Agilent Glycan amide (Fig. 5) compare extremely well with that of the Waters amide column in Fig. 2. Nortriptyline and procainamide (peaks 5 and 6) were again excluded on the Agilent amide column, while the acids p-XSA and 2-NSA (peaks 1 and 2) had the longest retention times using TFA. In contrast, the use of AF pH 3 shows much longer retention times of the bases with much shorter retention times of the acids.

The extreme selectivity differences using either AF pH 3 or TFA were illustrated by the correlation coefficients of retention factor of the 14 test compounds on the same column with these two mobile phases. These were -0.12 and 0.00 for the Waters amide and bare silica columns respectively. For the Agilent amide and bare silica columns, the correlations were -0.15 and 0.31. Thus there is almost no correlation between the retention factors for the same column in these pairs of mobile phases. The results indicate an opportunity for use of TFA or HFBA to manipulate the selectivity of a HILIC separation. Note that in a previous study, the low retention or apparent exclusion of bases together with enhanced retention of acids observed with TFA/HFBA was not shown on any of the columns investigated (zwitterionic, bare silica, amide or silica-hydride) using 0.1 % v/v phosphoric acid in the

mobile phase [18], which suggests that the lower pH of TFA and HFBA (Fig. 1) is critical in order to observe this phenomenon.

3.3 Comparison of selectivity in salt buffers at different pH on pH stable amide column.

The potential differences in selectivity of a given separation mechanism is a topic of much current interest in RPLC, for instance in two dimensional separations, where so-called "orthogonal" selectivity is desired. Although not without problems, 2D separations using the same basic mechanism (e.g. RP x RP) with a pH change are attractive in that similar organic solvent modifier concentrations could be used in each dimension, avoiding complications of interfacing more unrelated techniques [21]. The similarity of buffer nature and concentration between first and second dimension also improves the compatibility of the technique. Furthermore, some workers prefer not to use TFA or HFBA with LC-MS, due to the suppression of the detector signal that results [22]. For these reasons, we investigated changes in selectivity using 5 mM ammonium salt buffers at $_{w}^{w}$ pH 3.0-9.0. The Waters hybrid amide column was used as it is claimed to be stable over the pH range of 1-12. An additional advantage of salt buffers is that they maintain ionic strength (see above) and generally give improved peak shape with ionisable compounds (see below). Fig. 6 shows the separation of the 8 compound subset using 95 % ACN containing AF $_{w}^{w}$ pH 3, AA $_{w}^{w}$ pH 6 and AB $_{w}^{w}$ pH 9. Visual comparison of the chromatograms indicates a considerable difference in selectivity between pH 3 and 6. The correlation coefficient R was 0.73 when considering all 14 test compounds. Clearly there appears to be a much closer correlation for this set of compounds when considering the correlation of retention at pH 6 and pH 9. However, the correlation coefficient R was poorer (0.47), when considering all 14 test compounds. This result was partially due to the very high retention of TMPAC at $_{w}^{w}$ pH 9 (k = 44) compared with that at $_{w}^{w}$ pH 6; see Fig.2. The ionisation of silanols at $_{w}^{w}$ pH 9 and subsequent ionic interaction with the fully protonated quaternary compound explains its high retention. In contrast, the ionisation of even the stronger bases like diphenhydramine, nortriptyline and procainamide ($_{w}^{w}$ pKa ~9-10) is suppressed at this high pH leading to lower retention and reduced hydrophilicity (see Fig. 2), especially considering that the pK_a of the conjugate acid of these bases is shifted to lower values, due to the effect of the organic solvent (see above). The retention of more strongly basic compounds (e.g. nortriptyline and procainamide) increases modestly from $_{w}^{w}$ pH 3 to 6, presumably due to somewhat increased ionic interactions with ionised silanols even on this hybrid support material. In agreement with these arguments, retention of strongly acidic compounds (BSA, 2-NSA, p-XSA) gradually decreased as the pH is raised, indicating increased repulsion from ionised silanol groups. Indeed, all acids except 4-OH benzoic acid (noted above) show negative k at

$_w^w$ pH 9. Clearly, the effect of changes in pH will depend on the changes in the degree of ionisation of solutes that this causes, which in turn will depend on the nature of the solute and its pK_a.

These results confirm that changing the mobile phase pH using salt buffers is an effective method to alter the selectivity in a HILIC separation, found previously to be second only to changing the nature of the stationary phase [18].

3.4 *Comparison of column efficiencies with different mobile phases*.

Fig. 7 shows the efficiencies (calculated by the statistical moments procedure) for the BEH amide (6 different mobile phases with 95 % ACN) and Atlantis silica column respectively (5 different mobile phases). In general, efficiencies for the neutral solutes showed much less variation with change in the mobile phase than for the ionogenic solutes. However, lower efficiencies were obtained for uridine and deoxyuridine when using mobile phases other than AF $_w^w$ pH 3, especially with the amide column. It is possible that these differences are related to differences in the water layer formed on the stationary phase in these different buffers. Peak shapes on both columns when using FA were extremely poor for acidic and basic compounds (see Fig. 7). This result seems attributable to the low ionic strength of this mobile phase (see Fig. 1, [12]). Efficiencies for bases on the silica column were very good in all other buffers, although they clearly have low retention in HFBA and TFA. As noted above (Fig. 1), the ionic strength of these solutions of stronger acids in high concentrations of ACN is greater than that of formic acid. Ionic interactions of protonated bases with ionised silanols are suppressed using the stronger acids. Efficiencies for most bases on the amide column in TFA/HFBA (Fig. 7) are of little interest due to the apparent exclusion of these solutes. However, in the salt buffers, efficiencies for the bases on the amide column were lower than on the silica column. The average reduced plate height for the bases in AF $_w^w$ pH 3 and AA $_w^w$ pH 6 was 4.8 and 7.6 respectively for the amide column and 2.6 and 3.1, respectively for the silica column. The better performance of the silica column is initially surprising in view of the strong contribution of ionic retention for bases in comparison to that for the amide column [18]. Other authors have found better performance for bare silica in comparison with an amide column for the analysis of mixtures that consist largely of basic compounds [23]. It may be that the balance of ionic and hydrophilic interactions is crucial in determining peak shape. The acidic solutes gave mostly poor efficiency on both pairs of columns when using TFA/HBA, with peaks suffering from strong tailing while giving high retention (see Figs. 3,4,5,7). For example, 2-NSA and p-XSA gave < 5000 plates on the Agilent amide column, and < 3000 plates on the Agilent silica column (Fig. 5 and 4); similarly poor results were shown on the Waters columns (Fig. 7). In this case the peak shape might be again an

unfavourable combination of hydrophilic and positively charged *anionic* retention sites on the stationary phase surface (see below). Peak shapes for acidic solutes were apparently improved in salt buffers, but the usefulness of this result is questionable due to the low retention times, especially using the silica column. Clearly, the efficiency of a column is not a constant, and is dependent on the retention factor through the B and C terms of the van Deemter equation, and due to the greater proportional effect of extra-column bandspreading for early peaks. However, we believe these variations are small in comparison with those observed here due to changes in the mobile phase.

3.5 Interpretation of retention results at low pH.

The strong retention of acids at the low $_s^s$ pH of TFA and HFBA, in combination with the low retention or even exclusion of protonated basic compounds point to the possible existence of *anionic* retention sites on the columns under these conditions. This behaviour was not observed with either phosphoric acid (used in a previous study, [12]) or formic acid, and thus these sites are only apparent at very low pH. The magnitude of the effect was greater with amide columns than bare silica columns. This observation can be related to the co-existence of cationic retention sites (negatively ionised silanols) as well as anionic sites. The former are gradually suppressed as the mobile phase pH is lowered. It appears for the amide columns that the influence of the anionic sites outweighs the cationic sites in TFA and HFBA mobile phases, whereas the numbers of sites may be more evenly balanced for the two silica columns. This observation seems reasonable in that conventional bare silica columns show greater cationic retention at higher pH than amide columns [18]. Cationic retention may be further suppressed on columns based on hybrid silica, as is the case for the Waters amide column [19,24], which may explain the greater ease of the dominance of anionic retention sites at low pH. Table 2 shows retention data on a *hybrid* bare silica column which shows exclusion of the bases nortriptyline and diphenhydramine with strong retention of the acidic probes. This result demonstrates the possibility of greater anionic retention on the hybrid bare silica than is the case for the conventional bare silica columns. Again, the very low retention of TMPAC and procainamide (k = 0.11 and 0.06 respectively) seems incompatible with their high negative log D_{pH2} values (-2.2 and -2.7 respectively), which indicate a greater hydrophilicity than any of the strongly retained acidic probes.

The existence of the anionic retention sites could be explained by

a) Protonation of stationary phase ligands (at least for the bonded phases).
b) Presence of residues of basic catalyst (used in the bonding process) on the surface, or residues of an amine intermediate used to prepare the phase.

c) Hydrolysis of bonded ligands at low pH to yield positively-charged groups.
d) The presence of metal ions on the column surface.
e) Further protonation of the stationary phase silanols, or incorporation of hydroxonium ions into the tightly bonded layer of water close to the surface.

The protonation of stationary phase ligands seems unlikely to be a major contributor. The pK_a of the amide group is too low for this to take place. In addition, bare silica, which has no bonded ligands, clearly shows similar behaviour to the bonded amide phases, especially for the hybrid bare silica column, which has a reduced competing effect from negatively charged silanols. Residues of a weakly basic catalyst have been identified as the source of protonated sites on some kinds of RP columns [19,20]. However, such a catalyst would also be expected to be protonated in phosphoric acid solutions for which no evidence of this unusual retention behaviour was previously shown [12]. While the catalyst effect seems to occur with some Waters columns (amongst others), we obtained broadly similar results on the Agilent stationary phases. Furthermore, the bare silica columns should employ no bonded ligand catalyst. Some older reversed-phases with embedded polar amide groups were prepared using a two-step reaction in which a free amine was first bound to silica, but this approach was generally discontinued due to the influence of unbonded amino groups on chromatographic properties. It seems unlikely that HILIC amide phases would be prepared using this method [25]. Hydrolysis of bonded amide ligands at low pH to give cationic sites could occur but is difficult to assess the possibility due to the proprietary nature of the bonding process. Neither of these effects could occur with bare silica phases. Metal ions that originate from the column hardware, especially the frits, could be responsible for the presence of positive charges on the surface [26]. Experiments in which the metal complexing agents acetylacetone or EDTA were incorporated into the mobile phase however, did not indicate any appreciable effects, although EDTA is rather insoluble in HILIC mobile phases and had to be used in concentrations < 1mM. This leaves further protonation of the silanol groups, or an association of quasi-immobilised hydroxonium ions within the water layer in close contact with the column surface, as the remaining possibility. For silica, many different values of the point of zero charge (pzc) have been determined, ranging from values less than 2 to greater than 3 [27-29]. It appears that the pzc can be considerably affected by the presence of different anions and cations. The electrokinetic behaviour of silica is thus likely to be quite different in the presence of different acids. For example, the interfacial region is depleted of anions at pH > 2.5 due to electrostatic repulsion from the surface, and their effects thus become insignificant. Conversely, anion effects can be considerable at pH < 2.5 as electrostatic repulsion is minimised. It appears that some ions can be specifically adsorbed, inducing considerable shifts in pzc, whereas other ions are not specifically

adsorbed (so-called “inert” electrolytes) and are adsorbed by electrostatic forces. The values of pzc in these reports [26-29] are consistent with the possibility that the silica matrix has an overall positive charge in TFA/HFBA mobile phases, at least in the BEH amide and bare silica columns studied in this work.

Finally, we noted some drift of strong acids to shorter retention and strong bases to longer retention with time when using TFA as a mobile phase. This finding might be related to hydrolysis of siloxane bonds creating additiona silanols at low pH [30].

4. Conclusions

The selectivity of amide and bare silica columns obtained from different manufacturers was studied in HILIC separations, using different buffers and additives over a wide pH range and a selection of acidic, basic and neutral test compounds. The pH of solutions of the acids formic, phosphoric, TFA and HFBA used covered a relatively narrow range when measured in water ($_w^w$pH 1.9-2.8), but a much wider range in 90 % ACN when the true thermodynamic pH was considered ($_s^s$pH 2.4-5.2). These differences can explain the considerable changes in selectivity produced by use of these additives. Furthermore, the $_s^s$pH can be used to demonstrate the very low ionic strength of formic acid solutions in high concentrations of ACN, which can explain the poor peak shapes of ionogenic compounds. TFA and HFBA give somewhat greater ionic strength as they are stronger acids; ammonium salt buffers alternatively maintain much higher ionic strength and give better peak shape.

Surprising and useful differences in selectivity were obtained on a given column by the use of stronger acid additives such as TFA or HFBA compared with ammonium salt buffers. Pronounced retention of strongly acidic solutes, and reduced retention or even exclusion of basic solutes, was noted under these conditions. It is possible that at the lower pH of these additives, the ionisation of silanols is almost entirely suppressed, leading to enhanced retention of acid solutes (due to the absence of repulsive effects). Furthermore, ionic retention of bases might be reduced by ion pair formation that could also reduce the hydrophilicity of these solutes. However, exclusion of basic solutes on two amide phases and also a hybrid bare silica phase is difficult to explain. According to log D values, these bases should be more hydrophilic and thus more retained than any of the acid probes used. It is possible that some sites on the silica surface can become protonated at the low pH of the mobile phase, or that hydroxonium ions become incorporated into the tightly bound layer of water close to the surface, leading to additional retention of anions. Especially for conventional bare silica phases, silanol dissociation to produce cationic sites is a process that acts in the opposite direction. However, trends of anionic retention were also obtained on bare silica columns, especially a hybrid silica that is known to have low concentrations of

acidic silanols that lead to this competitive cationic retention effect. Peak shape measurements in the various mobile phases were generally supportive of this hypothesis. Nevertheless, other explanations of these unusual retention effects, such as the possible influence of metals, and artefacts involved in column preparation, or in use at low pH are considered. Some of these alternatives would only apply to bonded phases, and not to bare silica. However, it is possible that more than one mechanism is responsible for the apparent anionic retention properties, especially for amide phases. In future studies, we intend to make zeta-potential measurements to compare surface charge with different materials in combination with various eluents.

Useful selectivity differences by use of ammonium salt buffers at different pH can also be obtained when change of the nature of the buffer (e.g. for on-line 2-dimensional applications) or use of TFA (e.g. in some mass spectrometry applications) is not desired.

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5. References

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6. Legend to Figures

Fig. 1 (a) ${}_{s}^{s}$ pH of solutions of different acid additives in acetonitrile as a function of organic solvent concentration; (b) Ionic strength of solutions of different acid additives in acetonitrile as a function of organic solvent concentration.

Fig. 2 Retention factor (*k*) for neutral (uracil to 2-deoxyuridine), basic (cytosine to TMPAC) and acidic (4-OH benzoic acid to p-XSA) solutes in 95 % ACN (v/v) containing 0.1 % (v/v) trifluoroacetic acid (13.1 mM), heptafluorobutyric acid (13.1 mM), formic acid (13.1 mM), 5mM ammonium formate ${}_{w}^{w}$ pH 3.0, 5mM ammonium acetate ${}_{w}^{w}$ pH 6.0 and 5mM ammonium bicarbonate ${}_{w}^{w}$ pH 9.0 on (a) Waters BEH amide column. (b) Waters Atlantis silica column. For other details, see experimental section.

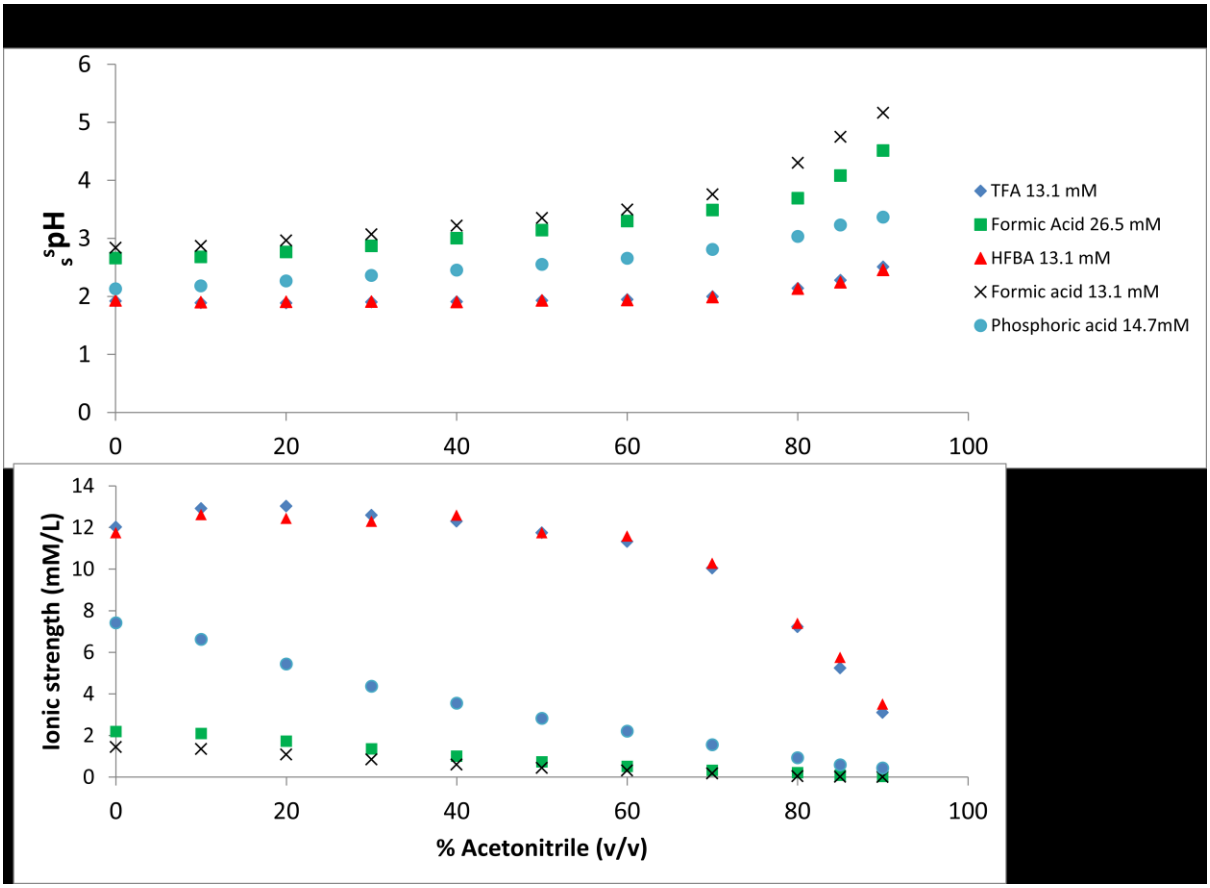
Fig. 3 Chromatograms of a subset of the test compounds on Atlantis silica using 95 % acetonitrile containing 13.1 mM heptafluorobutyric , trifluoroacetic, formic acids and 5 mM ammonium formate ${}_{w}^{w}$ pH 3.0. Peak identities: 1 = p-xylenesulfonic acid (p-XSA);2= 2 naphthalenesulfonic acid (2-NSA); 3 = thiourea; 4 = uracil; 5 = nortriptyline; 6 = procainamide; 7 = 4-hydroxybenzoic acid; 8 = cytosine. Flow rate 1.0 mls/min. For other conditions see Fig.2.

Fig. 4 Chromatograms of a subset of the test compounds on Agilent bare silica (shell column) in 95% acetonitrile containing TFA or ammonium formate ${}_{w}^{w}$ pH 3.0. Flow rate 0.25 mls/min. For peak identities see Fig. 3.

Fig. 5 Chromatograms of a subset of test compounds on Agilent glycan-amide (shell column) in 95% acetonitrile containing TFA or ammonium formate $_{w}^{w}$ pH 3.0. For peak identities see Fig. 3.

Fig. 6 Chromatograms of test compounds on BEH amide in 95% acetonitrile containing 5mM ammonium formate $_{w}^{w}$ pH 3.0, 5mM ammonium acetate $_{w}^{w}$ pH 6.0 and 5mM ammonium bicarbonate $_{w}^{w}$ pH 9.0. For peak identities see Fig. 3.

Fig. 7 Column efficiencies (N, moments method) for 14 test compounds on (a) BEH amide column. (b) Atlantis silica column. For other conditions see Fig. 2.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TFA 13.1 mM
Formic Acid 26.5 mM
HFBA 13.1 mM
Formic acid 13.1 mM
Phosphoric acid 14.7mM
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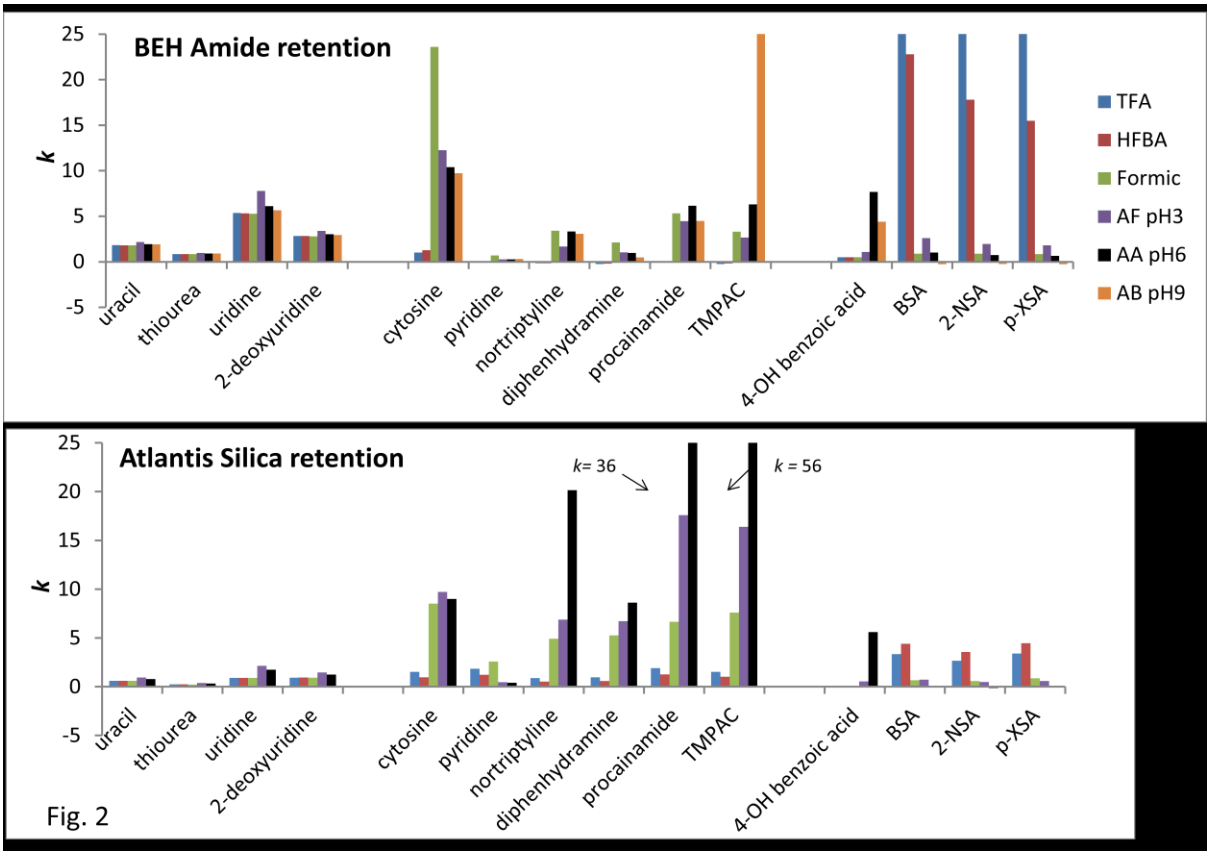
100
% Acetonitrile (v/v)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uracil
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2-deoxyuridine
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pyridine
nortriptyline
diphenhydramine
procainamide
TMPAC
4-OH benzoic acid
BSA
2-NSA
p-XSA
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HFBA
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AA pH6
AB pH9
Atlantis Silica retention
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procainamide
TMPAC
4-OH benzoic acid
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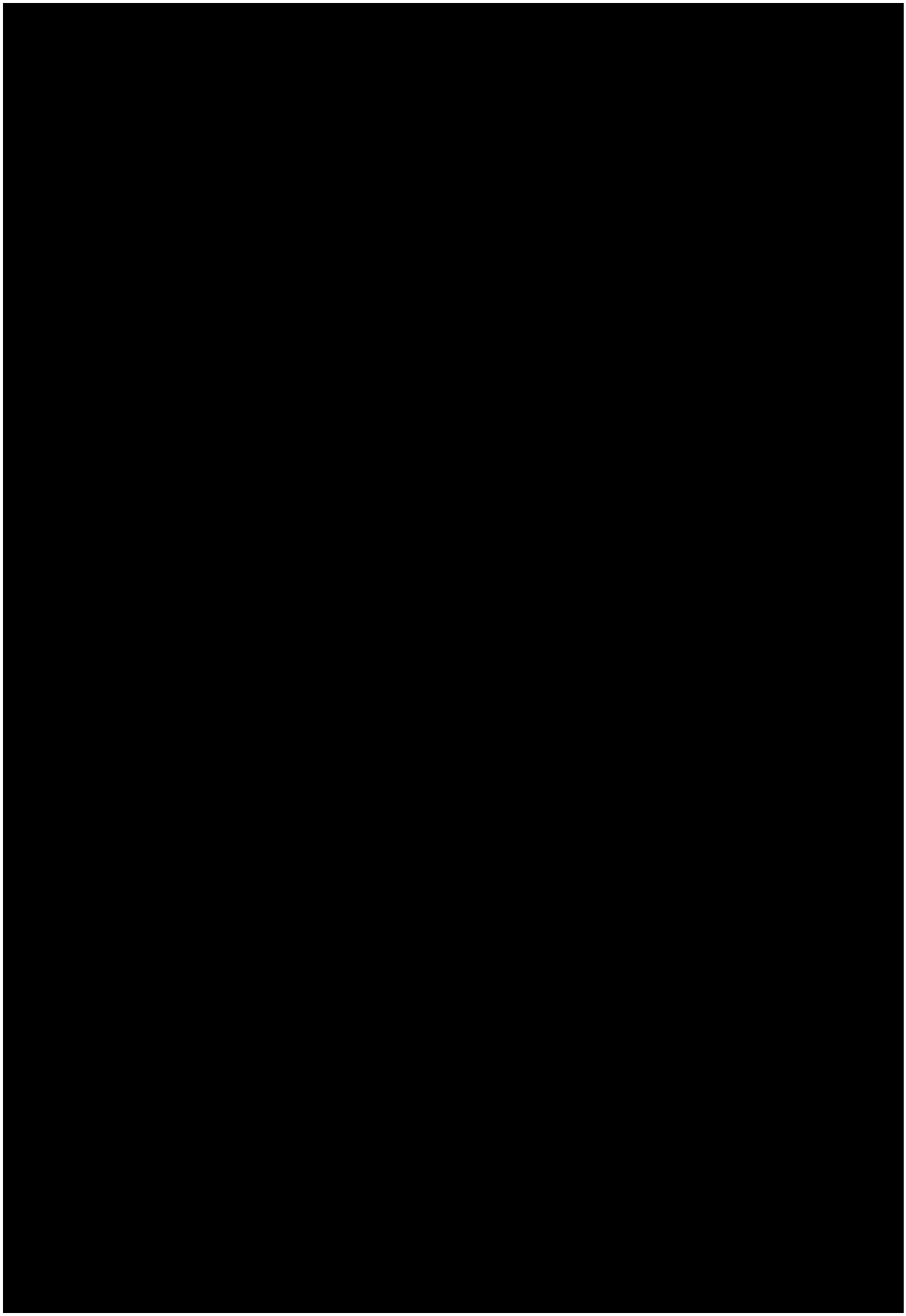
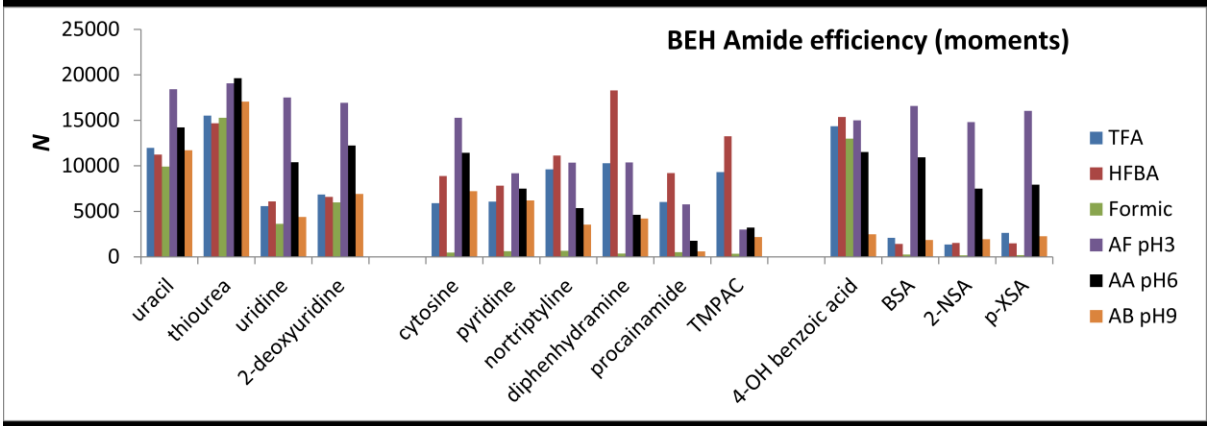
2-NSA
p-XSA

Fig. 2

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pyridine
nortriptyline
diphenhydramine
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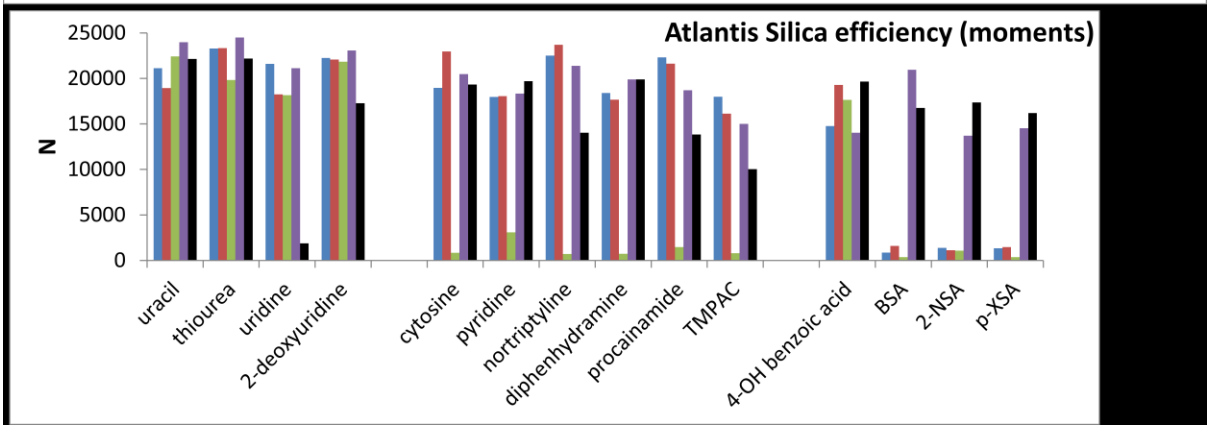
AB pH9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cytosine
pyridine
nortriptyline
diphenhydramine
procainamide
TMPAC
4-OH benzoic acid
BSA
2-NSA
p-XSA

Table 1 $_w^w$ and $_w^s$ pH (95 % ACN) of salt buffers.

Buffer	pH in aqueous solution ($_w^w$ pH)	pH in 95% ACN ($_w^s$ pH)
5 mM ammonium formate	3.0	5.9
5 mM ammonium acetate	6.0	7.0
5 mM ammonium bicarbonate	9.0	9.0

Table 2. Log D values at pH 2 and pH 6 compared with retention factor of probes on BEH silica. Mobile phase 95% ACN (v/v) containing 0.1 % TFA (v/v). For other details, see Experimental Section.

Solute	**Log D (pH2)**	**Log D (pH6)**	***k* BEH silica TFA**
Uracil	-0.78	-0.78	0.41
Thiourea	-1.03	-0.90	0.19
Uridine	-2.10	-2.10	0.60
2-deoxyuridine	-1.59	-1.59	0.63
Cytosine	-2.70	-2.25	0.13
Pyridine	-1.46	-1.05	0.16
Nortriptyline	0.94	0.94	-0.05
Diphenhydramine	0.32	0.32	-0.05
Procainamide	-2.68	-2.29	0.11
Trimethylphenylamm. Chl. (TMPAC)	-2.15	-2.15	0.06
4-OH benzoic acid	1.53	-0.15	0.05
Benzenesulfonic acid (BSA)	-1.46	-2.59	8.0
Naphthalene-2-sulfonic acid (2-NSA)	-0.48	-1.83	6.6
p-xylenesulfonic acid (p-XSA)	-0.76	-2.13	7.7

Log D values are the average of 3 estimation programs (see Experimental section).