A study of the analysis of acidic solutes by hydrophilic interaction chromatography.

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**Abstract**

The analysis of acidic solutes was compared on a cross-linked bonded amino phase and a neutral hybrid inorganic-organic amide phase, previously shown to give reasonable retention of acidic solutes. The amino column gave strong selective retention of acids, which was governed by ionic interactions that mostly increased as the solute became more negatively charged at higher pH. While the relative selectivity of the amide column towards acids, bases and neutrals was completely different to that of the amino column, the selectivity of both columns towards acidic solutes alone was surprisingly similar. It is possible that solute charge also controls retention on the amide column, through increased solute hydrophilicity and increased hydrogen bonding between the ionised form of the acid and neutral polar column groups. On both these silica-based columns there appeared to be a competitive effect between repulsion of acidic solutes from silanols, which become increasingly ionised as the pH is raised. This effect was absent when using a polymer-based amino phase which has no silanols.

**1. Introduction**

Hydrophilic interaction chromatography has become established as a complimentary LC approach that is particularly suited to the analysis of polar or charged solutes that are difficult to retain by the ubiquitous reversed-phase technique. HILIC uses a polar stationary phase in combination typically with an aqueous-ACN mobile phase containing at least 3% water. Solutes are retained principally by partitioning between an immobilised layer of water in the stationary phase and the bulk mobile phase, by adsorption on polar column groups, and by ionic interactions [1-4]. Most HILIC columns are based on silica, probably for the same reasons as in RP, which include its pressure stability and mechanical robustness, ease of attachment of different ligands, and considerable experience of its properties and use. Ionisation of residual silanol groups that remain unreacted can give rise to cation exchange properties that contribute to retention of basic solutes. However, this effect can concurrently cause low retention or even exclusion of acidic solutes when using typical mobile phases containing ammonium formate (AF) or acetate (AA) buffers in the range ww pH 3-6.

HILIC has been widely applied in the biomedical, clinical and pharmaceutical areas. both for the separation of small molecules but also to peptides and even intact proteins [5-9] . While the majority of small-molecule pharmaceuticals are basic compounds that give good retention, a sizeable minority are acids that can show limited retention on some columns. The best neutral or quasi- neutral phases for the retention of acids appear to be highly loaded polymeric bonded zwitterionic or amide columns, where silanols are masked, or inorganic-organic hybrid columns that also have low inherent concentrations of acidic silanols [10]. The use of alternative mobile phase additives such as 0.1 % trifluoroacetic acid may confer anion exchange properties on stationary phases such as bare silica or amide, resulting in preferential retention of strongly acidic solutes. The mechanism of action of TFA is not entirely clear, but appears to involve more than mere suppression of the ionisation of silanol groups; it may even involve the column surface becoming positively charged [11, 12]. However, this technique is not suitable for the analysis of weak acids, whose ionisation is suppressed at low pH, resulting in continued low retention. Amino columns give good retention of acids but may suffer from loss of stationary phase due to attack on the silica matrix from lone pairs on the nitrogen atom. Alternatively, the amine group may generate an alkaline microenvironment in the vicinity of the column surface resulting in dissolution of the matrix.

In the present study, we have investigated the performance of a cross-linked bonded amino phase, in the hope that this bonding might confer some extra stability of the material. We have compared the performance of this phase with a hybrid amide column, which is a neutral phase that does not suffer from stability problems-indeed it can be used even at alkaline pH. Silanol effects are reduced on this column, giving reasonable retention of acidic solutes [10]. Comparison of the retention and selectivity offered by these two stationary phase types should allow a contrast of the retention mechanism on each phase to be made. Comparison of selectivity should also generate useful practical information for those interested in developing separations of these solutes. The parameters investigated that could affect the separation included the influence of buffer, its pH and concentration. Previous studies have indicated a complex relationship between retention and mobile phase pH, with some acidic solutes apparently increasing in retention with increase in pH, whereas others show decreases in retention [10]. Peak shapes under the various conditions used were also investigated.

**2. Experimental**

All experiments were performed using a 1290 ultra-high pressure liquid chromatograph (UHPLC, Agilent, Waldbronn, Germany) consisting of a binary pump, autosampler and photodiode array UV detector (0.6 L flow cell). Agilent OpenLab ChemStation software was used for data handling and instrument control. The columns were XBridge Amide (150 x 4.6 mm ID) from Waters Corp. (Milford, USA), particle size 3.5 m, pore diameter 148 Å, surface area 185 m2/g; Luna Amino (250 x 4.6mm) from Phenomenex (Torrance, USA), particle size 5.0 m, pore diameter 102 Å, surface area 374 m2/g; Asahipak Amino (250 x 4.6 mm) from Shodex (Tokyo, Japan), particle size 5.0 m, pore diameter 100 Å, surface area n/a. Columns were held at 30 oC in the column thermostat and operated at 1.0 mL/min. 5 L injections were made. Acetonitrile (gradient UV grade), formic acid, acetic acid, ammonium formate, ammonium acetate were purchased from Fisher (Loughborough, U.K.). All test solutes were obtained from Sigma-Aldrich (Poole, U.K.). Standards were prepared at concentrations of 20-50 mg/L and diluted in the exact mobile phase. Buffered mobile phases quoted usually as ww pH were prepared by adjusting the pH of its aqueous portion before addition of acetonitrile. Alternatively, the ws pH was measured directly in the final aqueous- organic phase after calibration of the electrode in conventional aqueous buffers. Calculations of solute charge, p*K*a and log D were made with the programs Marvin (ChemAxon Ltd, Budapest, Hungary), ACD I Lab (ACD, Toronto, Canada) and MedChem Designer (Simulations Plus Inc., Lancaster, USA)

**3. Results and Discussion**.

*3.1 Amino column.*

Fig. 1 shows the structures and p*K*a values of the acidic solutes used in the study. The sulfonic acids are strong acids, remaining fully negatively charged in all experiments reported. The carboxylic acids and hydroxyl substituted carboxylic acids are weak acids which range from being marginally to totally protonated under the various conditions used (see discussion below). Salicylic acid (2-OH benzoic acid) can undergo intramolecular hydrogen bonding, reducing its capacity to hydrogen bond with stationary phase groups, whereas 3- and 4-OH benzoic acids can only form intermolecular hydrogen bonds giving the possibility of increased interaction with the stationary phase.

Fig. 2a shows retention factors (*k*) for 4 neutral solutes (thiourea to uridine), 6 basic solutes/a quaternary compound (cytosine to trimethylphenylammonium chloride, TMPAC) and 9 acidic solutes (benzenesulfonic acid to 2-phenylbutyric acid) on the amino column using 5 mM ammonium formate (AF) and ammonium acetate (AA) buffers ww pH 3-6 in 85 % ACN. Note that formate has an aqueous p*K*a of 3.75, and is expected to act as a buffer in the pH range 2.75-4.75. However, the aqueous p*K*a of acetate is 4.75, so its buffer capacity is limited at pH 6.0. Nevertheless, considering the small amounts of test compounds injected, instability in the mobile phase pH is unlikely. As expected, the retention of neutral solutes was hardly affected by changing the mobile phase pH. Their retention (*k* ranged from 0.9-2.3 in all buffers) could be increased by decreasing the concentration of water in the mobile phase (results not shown). However, 85 % ACN was chosen in order to give reasonable retention of all the test solutes. Cytosine is a weak base but may behave as a quasi-neutral solute under the conditions of the experiment, as it is likely to be only slightly protonated (even using ww pH 3 buffer), considering also the influence of the high concentration of ACN in the mobile phase [13]. The other basic solutes and TMPAC showed very low retention throughout the pH range which can be attributed to repulsion between the protonated solute and similarly charged groups on the stationary phase.

The extremely high selectivity of the amine column for acidic solutes compared with bases and neutrals is immediately indicated in Fig. 2a. High *k* values were generally obtained for all acids in 85% ACN, particularly for 3-and 4-OH benzoic acids at ww pH 6, which both gave *k* > 50. Guo and Gaiki [14] found that the retention of acids showed a drastic decrease as the salt concentration increased using an amino phase, in direct contrast with other phases studied which included a zwitterionic, amide and silica phase. They interpreted this result on the basis of electrostatic interactions between the positively charged amino phase and negatively charged solutes. An estimate of the contribution of ionic retention to these high *k* values would be useful in understanding the overall mechanism. Cox and Stout showed that a plot of *k* against the reciprocal of buffer concentration should yield a straight line indicative of ionic retention [15, 16]. Fig. 3 shows such plots for the 9 acidic solutes over the range 5-20 mM AF, with the pH maintained at ww pH 4.4, in the middle of the data range of Fig. 2a. The plots show excellent linearity with R2 values ranging from 0.989 to 0.999. The plots can be extrapolated to infinite buffer concentration (1/[M+] = 0) to reveal the percentage contribution to retention of the non-ionic (“hydrophilic”) mechanism, which could include adsorption and partition. Table 1 shows these values were 7-19 % at 5 mM AF concentration, which means that for every acidic solute, over 80% of the retention is due to ionic effects. The calculation was repeated at 20 mM buffer concentration (Table 1) where the percentage contribution of hydrophilic processes increased to the range 21-48%, with reduced ionic retention. Clearly the buffer anion competes with the acidic solutes for retentive sites on the stationary phase and can be used to suppress the contribution of ionic processes to overall retention. As a control for these experiments, the effect of buffer concentration on the retention of basic and neutral solutes was also investigated, as this result will affect the overall selectivity of the system. Fig. 4 shows a small increase in retention for neutrals with increasing salt concentration, which can be interpreted as an increase in the thickness of the stationary phase water layer due to attractive forces imparted by the salt [17]. A somewhat greater increase in retention was shown by the basic compounds and the quaternary (TMPAC) than for the neutrals, probably due to screening of the repulsive forces between the solute and ionised column groups of the same charge as buffer concentration was increased. Fig. 5 indicates the peak shape of some acids, bases and neutrals on the amino column using 85% ACN at 5 mM buffer concentration ww pH 4.4. The average plate count for these 8 solutes was 22,300 in the 25cm column, indicating a reduced plate height h = 2.2. Some tailing was noted on the peak of the base procainamide (USP tailing factor = 1.4) and some slight fronting (0.9) on the peaks of the strong acids p-XSA and 2-NSA. Nevertheless, this good performance suggests it is unnecessary to use higher buffer concentrations in an attempt to improve peak shape. Higher buffer concentrations will both tend to reduce the preferential selectivity of the amino column for acidic compounds and reduce the sensitivity of electrospray mass spectrometry detection [18].

As the retention of the acidic solutes at constant 5mM buffer concentration appears to be governed largely by ionic retention processes, the mean solute charge and its variation with pH should be an important factor to consider. Table 2 shows both the solute charge and log D value for the acidic solutes over the pH range 3 to 7, shown as the average generated by three different calculation packages. The average value was taken as there can be variations in these estimated values dependent on the particular algorithms used in these programs [19]. Further care is needed in the use of such data in that calculated charge is based on p*K*a values in water, rather than those in the aqueous organic mobile phase. As the p*K*a of buffer components is also dependent on the organic concentration in the mobile phase, the pH measured in the aqueous component will differ [20]. Indeed, Table 3 shows marked differences in the experimentally measured ww pH and ws pH of the mobile phase which are particularly large when the ACN concentration is highest. For example, the ws pH of AF buffer ww pH 3.0 was measured at 6.1. In general, the p*K*a of the (conjugate acid of) basic solutes is lower in organic rich solvents, whereas the p*K*a of acids is raised. In other words, the acidity of acids, and the basicity of bases is moderated in these solvents. These difficulties can present a barrier for rigorous modelling systems which attempt to predict retention in HILIC [21, 22] . Some data is available concerning the p*K*a of formic acid buffers in mobile phases containing up to 90 % ACN [20, 23]. However, considering the high aqueous content of the solvent composition in the vicinity of the stationary phase surface [24, 25], it is difficult to justify the exclusive use of aqueous-organic values, which in any case are only scarcely available, at least for the solutes in the present study. Thus interpretations will be mostly based on the solute charges based on the aqueous values given in Table 2.

The acidic solutes can be divided into 3 groups according to their retention behaviour with change in pH on the amino column (Fig. 2a). The first group (BSA, 2-NSA, p-XSA) shows a continual decrease in retention with increasing pH; for instance BSA shows a decrease from *k* = 14.8 at pH 3.0 to 9.8 at pH 6.0. These solutes are strong acids which are fully charged (-1) throughout the range ww pH 3-7 (Table 2). Retention decrease with increasing pH may be due to some reduction in the number of protonated amino groups on the stationary phase as the pH is raised. Alternatively, the increasing ionisation of underlying stationary phase silanol groups on the base silica, providing (repulsive) cationic exchange sites in opposition to the anion exchange sites furnished by the amino groups, could cause this decrease in retention. Note that this silanol effect, which generally causes increases in retention of bases on neutral stationary phases, has been demonstrated on many silica-based HILIC columns [16] .The second group of acidic solutes (3- and 4-OH benzoic, 2-phenylbutyric acids) shows continual strong increases in retention with increasing pH. At ww pH 3.0, these solutes bear very small negative charges (-0.11, -0.03 and -0.03 respectively, Table 2) while at ww pH 6, they are all (almost) fully charged (-0.99, -0.97 and -0.97 respectively) and thus are able to interact with protonated column amino groups. Clearly, this increase in solute ionisation must have a more significant effect on their retention than the effect of any changes in the ionisation of the stationary phase, and correlates well with the increase in retention of the various solutes. There is a particularly strong change between retention at ww pH 3.0 and 4.4. The third group of solutes (salicylic, acetylsalicylic and 4-aminosalicylic acids) show increases in retention between ww pH 3.0 and 4.4, but decreased retention at pH 6.0. These solutes are already mostly charged at pH 4 (-0.93, -0.78, -0.76 respectively). It seems likely that any small additional increase in solute charge at pH 6.0 is outweighed by the decrease in charge of the stationary phase and/or the increased repulsion due to increased ionisation of silanol groups.

If increasing silanol ionisation as pH is raised explains the reduction of retention of strong acids and even some weak acids (by opposing the effect of increased solute charge which otherwise augments interaction with the stationary phase), then this effect should be moderated at higher buffer concentration due to the competitive effect of buffer cations. Indeed, increasing the pH of the mobile phase from ww pH 4.4 to 6.0 at 20 mM buffer concentration instead of 5 mM on the Luna column (Fig 2d) demonstrated exactly the same pattern of retention effects of the acids but in moderated form. Differences in the performance of the silica-based Luna column compared with a polymer-based amine column with no silanols should reveal their contribution to retention. Fig. 2c shows retention data over the same pH range (4.4 to 6.0) for the polymer based Asahipak amino column again with 20 mM buffer.The overall retention profile of this column was similar to that of the silica amino phase, although higher retention was shown for *all* acidic solutes at pH 6.0 rather than pH 4.4. No decreases in retention between ww pH 4.4 to 6.0 were observed for the stronglyacidic solutes on the Asahipak column, in contrast to the behaviour of the silica-based Luna amino column (Fig. 2d). This observation lends weight to the argument that increasing silanol ionisation at higher pH can explain the decrease in retention of completely ionised strong acids at higher pH on silica-based stationary phases. It was not possible to compare the performance of silica and polymer columns at 5 mM buffer concentration (where silanol effects in the former are more significant) due to excess retention of acidic solutes on the polymer column. Increasing the aqueous content of the mobile phase was considered, but not performed as it would decrease the comparability of the data.

While solute charge seems to be a major factor in explaining the retention of acidic solutes, the generally higher retention of the carboxylic compared with the sulfonic acids at pH 6 where all solutes are fully charged (Table 2) remains problematic to rationalise. Particularly 3- and 4-OH benzoic acids have much higher *k* than the sulfonic acids while having less negative log D values. It is feasible that the greater possibility of hydrogen bonding for these benzoic acids can explain the results [21, 22]. Hydrogen bonding will be considered in more detail below.

Table S1 shows selectivity changes which can be obtained on the Luna amino column by variation of the ww pH over the range 3.0 to 6.0. The correlation coefficient R for all 19 solutes (acids, bases and neutrals) for 5 mM AF buffer pH 3 compared with buffer pH 6 was only 0.226, indicating almost no correlation. Similarly, the correlation coefficient between *k* at these two pH values for the 9 acidic solutes alone was only -0.424. These indicate that changing the pH of the mobile phase has a considerable effect on the selectivity of the amino phase. Changing the pH over the range 4.4 to 6.0 seems to have a much smaller effect on the correlations for the whole group of solutes, and for the acidic solutes alone (R = 0.877 and 0.784 respectively). This smaller change can be interpreted in terms of the smaller change in the ionisation of the solutes that occurs over this pH range.

*3.2 Amide column*. *Comparisons with the* *amino column.*

As the bonded ligand groups on the amide column remain uncharged over the pH range of this investigation, its study can allow further elucidation of the mechanism of retention of acidic solutes, and the relative contribution of individual processes to retention. An advantage of the bridged ethyl hybrid (BEH) amide column is its robustness and stability at high pH. Retention was first studied in the same mobile phases (85 % ACN containing 5 mM ammonium salt buffers ww pH 3.0, 4.4 and 6.0) as for the amine column. However, due to the generally lower retention, of acidic solutes, the ACN concentration was increased to 95 % which increased retention with little change in selectivity (results not shown). Fig. 2b shows that incontrast to the behaviour of the amine stationary phase, the retention of neutral, basic and acidic solutes was much more similar to one another on the amide column, in accord with the lack of ionic repulsion effects for basic and attractive effects for acidic solutes that had occurred on the amine column. The contrasting retention properties of the amine and amide columns can be considered using correlation analysis. Table S2 shows that the correlation of *k* at a given pH on the amide column (95% ACN) with that of the amine column (85 % ACN) was indeed very poor when considering all solutes at pH 3.0 (R = -0.0133) and at pH 4.4 (R = 0.221). Somewhat surprisingly when considering the different properties of these two columns, the correlations of *k* for the 9 acidic solutes alone in these mobile phases were considerably better (R = 0.861, 0.948 and 0.965 at pH 3.0, 4.4 and 6.0 respectively). This reasonable correlation is visibly evident merely from inspection of the bar graphs for the acids in Figs 2a and 2b. A possible consideration is that solute charge governs retention both on the amine phase through ionic interactions, and on the amide column through increased hydrophilic interactions.

Fig. 6 shows chromatograms for the amide column over the range ww pH 3.0 to 9.0 for a test mixture of acidic, basic and neutral solutes and for exclusively acidic solutes in Fig. 7. As with the amine column, the neutral solutes thiourea (peak 3) and uracil (peak 4) show little variation in *k* over the entire pH range. The bases nortriptyline and procainamide (peaks 5 and 6) increase in retention as the pH increases from ww pH 3.0 to 6.0. It seems that increased silanol ionisation at higher pH, producing increased ionic interaction of bases, is a feature even of this relatively inert hybrid stationary phase. At pH 9, retention of these bases drops, probably due to suppression of solute ionisation and reduction of ionic interaction effects.

The retention behaviour of the acidic solutes on the amide column (Figs. 2b, 6, 7) surprisingly seems to fall into the same three groups as previously shown on the amine column. The first group of solutes, the sulfonic acids (BSA, 2-NSA and p-XSA) again show continual decrease in retention as the pH is increased, followed by exclusion at pH 9. This behaviour is attributable in this case solely to repulsion of these fully charged solutes from increasingly ionised column silanols. Although their retention on the amide phase is considerably less, behaviour with pH increase generally mirrors that of the amine phase (see above). The second group of acids (3- and 4-hydroxy benzoic, 2-phenyl butyric acids) show increased retention with increasing pH up to ww pH 6. These increases are considerable for 3- and 4-OH benzoic acids (peaks 13 and 7 respectively, Fig. 7). The third solute group of acidic solutes (salicylic, acetylsalicylic and 4 amino salicylic acids) again shows similar behaviour to that on the amine phase. These solutes show only minor increases in retention as pH increases, mostly between ww pH 3.0-4.4 followed by reduced retention at higher pH. This behaviour can be explained again by the substantial charge already on these solutes at ww pH 4, (-0.93,-0.78.-0.76 respectively) leading to increased repulsion from the column surface at higher pH. Further increase to ww pH 9 for all acidic solutes reduces the retention. The retention behaviour of all acidic solutes as the mobile phase pH is increased appears to be a balance of increased retention as solute ionisation increases counteracted by mounting repulsion from increasingly ionised silanols.

This pattern of retention of acids is exactly the same (albeit at lower overall *k*) as on the amine phase. This result is initially surprising as the amide column should have no positively charged sites for ion exchange. However the increasing negative charge on these weakly acidic solutes as the pH is raised my have two effects.

i) It increases the solute hydrophilicity reducing the log D values until they become negative at ww pH 6 (Table 2), such that increased partitioning into the water layer occurs. For example, 3-OH and 4-OH benzoic acids have log D 1.40 and 1.51 respectively at pH 3 and -0.63 and - 0.15 respectively at pH 6. However, these log D values are modestly negative at pH 6 in comparison with those for the sulfonic acids at the same pH (-2.59, -1.83 and -2.13 for BSA, 2-NSA and p-XSA respectively). At pH 6, the charge on all the acids is close to one, thus similar repulsion effects from ionised silanols should occur in either case. On the basis of log D values at pH 6, the sulfonic acids should have much greater retention than the benzoic acids (as was argued also for the case of the amino column above), but Fig. 2b, Fig, 6 and Fig. 7 clearly indicate that the reverse is true. These data are therefore difficult to explain on the basis of hydrophilicity alone.

Ii) Increased hydrogen bonding between the solute and stationary phase may be important, as shown by the increased retention of 3-and 4-OH benzoic acids, which are capable of intermolecular bonds through either the hydroxyl or carboxyl groups on the molecules. In contrast, intramolecular hydrogen bonding may be of greater significance for salicylic acid, reducing any interactions with the stationary phase. The importance of H-bonding has for these solutes has previously been noted by Schuster and Lindner [21]. These authors also noted major increases in the retention of 4-OH benzoic acids over the range ww pH 3 to 5 compared with little or no change for salicylic acid. They offered the explanation of intramolecular hydrogen bonding with the stationary phase for 4-OH benzoic acid, which is prevented by intramolecular hydrogen bonding of the salicylic acid. However, this argument does not explain fully the large increases in *k* with increasing pH for the 4-OH acid. A possible rationale is that hydrogen bond strength increases between a neutral silanol and negatively charged acid, so retention follows the solute charge as indicated in Table 2 (see arguments for amine phase above).

The retention increase for weak acids that occurs with increasing mobile phase pH has been observed previously on a variety of stationary phases [10] including bare silica, sulfobetaine, zwitterionic, BEH amide (neutral stationary phase), TSK amide (neutral-from a different manufacturer) and diol (neutral). Thus, the increase does not seem attributable to some unusual property of the (Waters) BEH amide phase, that could have resulted from some unique proprietary method of ligand bonding [26].

We considered that if adsorption was a significant contributor to the retention mechanism for the amide column, at least under conditions of low water content of the mobile phase, then it might be possible to reduce the retention of solutes by increasing the buffer concentration giving rise to competitive displacement of solutes. Fig. 8 shows *k* values for all solutes using either 5 mM or 10 mM AA pH 6.0 in 95% ACN. We used 95% ACN as the mechanism is more likely to be influenced by adsorption when the water content of the mobile phase is small. 10 mM of the buffer salt is the maximum concentration in such a mobile phase due to solubility considerations. Neutral solutes showed increases in retention at 10 mM concentration, consistent with the salt enhancing the thickness of the water layer. As expected, basic solutes show reduced retention at the higher concentration, consistent with competition of the buffer cation for retentive interaction with ionised silanols. However, both strong acids (BSA, 2-NSA, p-XSA) and weak acids show enhanced retention at higher buffer concentration. It appears that the dominant effect is screening of acids from repulsive interactions with ionised silanols; no evidence was obtained for competitive hydrogen bonding with the stationary phase ligands between solute and buffer anion, although such an effect might be swamped by the former process.

Table S3 indicates that important selectivity changes are again produced by changing the ww pH over the range pH 3.0 to 6.0 for the BEH amide column. A particularly large selectivity difference is shown for the acidic solutes alone when changing the pH from 3.0 to 6.0, where the correlation coefficient of *k* values is -0.03, indicating virtually no correlation. This lack of correlation is demonstrated visually in Fig. 7 where the order of elution of the peaks at pH 3.0 is 10, 7, 12 ,9, 1,13,11 compared with 1, 9,10,11,12, 7,13 at pH 6.0.

The peak shapes of acidic, basic and neutral solutes were generally very good on the amide column as shown in Fig. 6. Using 5 mM AF pH 4.4, the average plate count for the solutes shown was17800 plates for the 15 cm column, indicating a reduced plate height h = 2.4. All compounds showed good peak symmetry (USP tailing factor 1.0 to 1.15) except for the base procainamide which gave a tailing peak (1.5) and a poorer plate count (10,000). The average plate count for the 9 acidic solutes under the same conditions was 17900, with h = 2.4. These results indicate good performance for all types of solute. However, consideration of Fig. 6 and 7 using pH 9 indicates some increased tailing of peak 6 ( procainamide) and fronting of some of the acid solutes such as peaks 7 and 13 (4-OH and 3-OH benzoic acids) at high pH. Measurements of performance at lower pH were repeated after the pH 9.0 experiments. Closely similar results to those performed before exposure to these high pH conditions were obtained. Thus, the poorer performance at pH 9.0 was not caused by column deterioration at this high pH.

Finally, we considered the possibility that selectivity differences could arise from using a different buffer salt (AF or AA) at the same ww pH. Lindner considered that changing from formate to acetate may influence the thickness of the water layer [21]. The higher molecular volume of acetate could lead to swelling of the water layer compared with formate. It was reported that elevated retention occurs when formate is changed to acetate [27]. Alternatively AF and AA might have different ion pair properties with solute cations, affecting their hydrophilicity and retention. A different consideration (Table 3) also reveals that the ws pH of 5 mM AA buffer and AF buffers in 95% ACN (both ww pH 4.4 before addition of the organic solvent) is significantly different (7.7 and 7.2 respectively). The higher ws pH may be a reflection of the stronger acidity of formic acid relative to acetic acid. Changes in selectivity can be visualised for both the acids/bases/neutral mix (Fig. 9a) and for the acid mixture (Fig. 9b). When the ws pH differs (pH 7.7 for AA and 7.2 for AF, top two chromatograms in each Figure), some small selectivity differences are notable. Adjusting the ws pH of the AA buffer from 7.7 to that of the AF buffer (7.2) produced much more similar selectivity in the chromatograms. Thus, it may simply be that the difference in ws pH between these two buffers at the same ww pH accounts for most of the differences in their selectivity.

No significant changes in retention or peak shape were noted for either column during the course of this study.

**4. Conclusions**.

The silica-based amino phase gave pronounced and selective retention of acidic solutes compared with bases and neutrals. In a mobile phase with 5 mM buffer concentration and ww pH 4.4 in 85 % ACN, ~80-95% of its retention of a variety of acidic solutes was attributable to ionic processes. The overall selectivity for acidic, basic and neutral solutes of a neutral amide phase was completely different to that of the amino phase, showing similar retention for each group of compounds. However, the selectivity for acidic solutes alone was surprisingly similar on both columns. This result may be because retention on the amide phase also depends on solute charge. For the amine column, the driving force of retention is the ionic attraction between protonated groups on the column surface and oppositely charged acidic solutes. As pH is raised, weak acids become increasingly charged, increasing retention, but this process is counteracted by increased ionisation of silanol groups which gives rise to repulsive effects. This hypothesis was given weight by comparison with a polymer–based amino phase, which in the absence of silanols demonstrated no analogous repulsion effects. On both silica-based amine and amide columns, increased solute ionisation as the mobile phase pH is raised increases the solute hydrophilicity (reduces log D values) and can contribute to increased retention. For the amide column, it is possible that hydrogen bonding strength increases between ionised solute and neutral column groups as the pH is raised, thus explaining increased retention. Again, these retentive forces can be counteracted at higher pH by increasing silanol ionisation, even on the relatively inert hybrid stationary phase used in the study. Thus for some acidic solutes, retention decreases at the highest pH.

Although their stability was not investigated rigorously, no deterioration in performance of the amino columns was noted throughout the course of this study over a period of several months of use.

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**6. Legend to Figures**

Fig. 1 Structure of the test solutes used. Numbers in parantheses are the aqueous p*K*a values of the strongest acid group, calculated from the average of values generated by Marvin and ACD labs programs.

Fig. 2 *k* values for neutral (thiourea to uridine) basic (cytosine to TMPAC) acidic (BSA to 2 – phenylbutyric) solutes using 5 mM ww pH 3-6 buffers. Flow 1 mL/min; column temperature 30 o C; UV detection;~ 20ng injected on column. a) Luna amino column, 85% ACN- buffers. b) BEH amide column 95 % ACN-buffers. c) Asahipak amino column 85% ACN-20 mM buffers, d) Luna amino column 85% ACN-20 mM buffers.

Fig. 3 Plots of *k* vs the inverse of buffer cation concentration at ww pH 4.4 and 85% ACN for 9 acidic solutes on Luna amino column. Other conditions as Fig. 2.

Fig. 4 Effect of buffer concentration for Luna amino column on *k* of neutral and basic solutes. Mobile phase 5-20 mM AF ww pH 4.4 in 85 % ACN. Other conditions as Fig. 2.

Fig. 5 Chromatogram of a mixture of acidic, basic and neutral solutes on Luna amino column using 5mM AF ww pH 4.4 in 85% ACN. Peak identities: 1 p-xylenesulfonic acid; 2= naphthalene-2-sulfonic acid; 3= thiourea; 4= uracil; 5= nortriptyline; 6 = procainamide; 7= 4-hydroxybenzoic acid; 8 = cytosine. Other conditions as Fig. 2.

Fig. 6 Chromatograms of a mixture of acidic, basic and neutral test compounds on an BEH amide column using 95% ACN containing 5mM ammonium formate buffers pH 3.0, 4.4; ammonium acetate buffer pH 6.0; ammonium bicarbonate buffer pH 9.0. Other conditions as Fig. 2.

Fig. 7 Chromatogram of mixture of acids on BEH amide column. Conditions and mobile phases as Fig. 6. Peak identities: 9= salicylic acid; 10 = 2-phenylbuturic acid; 11 = 4-aminosalicylic acid; 12 = acetylsalicylic acid; 13 = 3-hydroxybenzoic acid. Other conditions as Fig. 2.

Fig.8 *k* values for neutrals, bases and acids on BEH amide column. Mobile phase 5 or 10 mM AA ww pH 6.0 in 95% ACN. Other conditions as Fig.2.

Fig. 9 a) Chromatograms of neutral, basic and acidic solutes and b) Acidic solutes on BEH amide column using 5mM AA and AF ww pH 4.4 in 95% ACN and in 5mM AA ws pH 7.2 in 95 % ACN. Peak identities as Fig. 5 .

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