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Influence of organic solvent modifier and solvent strength on peak shape of some basic compounds in high-performance liquid chromatography using a reversed-phase column☆

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Abstract

Comparative column efficiency and peak asymmetry measurements were made for 16 pyridine derivatives using approximately isoeluotropic mixtures of methanol, acetonitrile and tetrahydrofuran (THF) in combination with phosphate buffer at pH 7.0 on a RP-HPLC column suitable for the analysis of basic compounds. On average, THF and methanol gave significantly better peak shape than acetonitrile, although considerable variations in behaviour for the different derivatives were noted. Methanolic mobile phases yielded straight line plots of $\log k'$ vs. percentage of modifier; nevertheless the increase in k' with decreasing methanol concentration is accompanied by a deterioration in peak shape which varies in extent depending on the nature of the probe compound.

1. Introduction

The difficulty inherent in the analysis of basic compounds by RP-HPLC is still an area of concern. There are important applications, for instance in pharmaceutical analysis, since many drugs contain basic groups. However, the chromatography of basic analytes is also of more general interest since such compounds can highlight the heterogeneity of the RP surface and changes in the surface which can occur with time. Differences can be revealed between apparently similar columns from different manufacturers, and even columns made from different batches of the same manufacturer's product.

Solvents of low viscosity are generally preferred in HPLC due to practical considerations, but also to the improved column efficiencies which may result. The improvement is believed to be due largely to the increased diffusion coefficients of the analytes in the mobile phase in accord with the Wilke–Chang equation [1]. Thus, acetonitrile–water mixtures may be preferred to methanol–water in RP-HPLC. However, few systematic experimental studies have been performed which detail the effect of change of the organic modifier on column efficiency (N) and asymmetry factor (A_s) if the analytes are basic compounds, which are prone to deleterious column interactions, for instance with underivatised column silanol groups. Claessens et al. [2] in a comparative study of eight columns using the probe compounds 2-hexyl- and 2-heptylpyridine, showed that large differences in A_s

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were evident for most columns when simple organic solvent–water mixtures containing either methanol, acetonitrile or THF were used as the modifier, with acetonitrile giving rise to the most asymmetric peaks. In another study, relatively little difference in A_s for pyridine was reported when using acetonitrile–water compared with methanol–water [3] ; pyridine appears to be a more severe probe of column activity under these conditions than 2-substituted derivatives of similar pK_a. In previous work [4], we investigated the performance of a range of different types of RP column using a variety of different probe compounds including pyridine (pK_a 5.2) and the considerably more basic compounds benzylamine (pK_a 9.3), quinine (pK_a 8.5) and nicotine (pK_a 8.0) [5]. It was shown that the performance for pyridine in methanol–water or methanol–phosphate buffer at pH values near the upper limit for silica-based columns, could provide some indication of column performance for the tobacco alkaloids under the same conditions.

In the present study, we have explored further the possible differences in RP column performance with different modifiers, using a set of pyridine derivatives which cover a range of pK_a values and stereochemistry. Our previous results relating compound stereochemistry and pK_a to peak shape using methanol [3] could also be compared for the different modifiers. The analysis of these compounds is of practical significance, since they are environmental pollutants originating from coal tar [6]. The study was performed on Inertsil ODS, a column which was shown to give good results for pyridine derivatives and some more basic compounds containing different structural features, detailed above, at pH 7.0. Analysis of the tobacco alkaloids (pK_a > 8) was demonstrated on the column at this relatively high, and also at low pH [4]. A column giving a reasonable peak shape for pyridine derivatives should give more reproducible data than columns producing very asymmetric peaks [7]. Similarly, we did not want to utilise probe compounds which give severe interaction with the RP surface, since we wished to study a variety of possibly non-optimum mobile phases. We performed the study with mobile phases buffered at pH 7.0. At acidic pH, pyridine derivatives show rather low retention, necessitating low concentrations of organic modifier for analysis. It was felt that differences between the various modifiers might be concealed using largely aqueous mobile phases; however, further studies are necessary to investigate this hypothesis. Furthermore, the collapse of ODS ligands onto the surface of the silica and the change in ionisation state of the surface silanols [4] might produce different results.

Relatively few detailed experimental studies have been performed on the effect of solvent strength (as controlled by the volume fraction of a particular organic modifier) on the retention factor and peak shape of basic analytes. Vervoort et al. [8] showed a correlation between k' and A_s for analysis of a series of basic drugs. Nevertheless, a later report by the same group [7] claimed hardly any correlation between k' and A_s. However, it is difficult to interpret these studies fully due to the lack of experimental detail given. Thus, we also performed some investigation on the effect of change of organic solvent concentration on the peak shape for the same series of basic compounds.

Despite the above justification for the experimental conditions chosen, it should be stated clearly that different combinations of sample type, pH and column can lead to different situations. Column performance as measured by A_s and N measurements may not be consistent for analytes of unrelated structure. Thus, column "A" may perform better for compound "X" while column "B" performs better for compound "Y". Similarly, comparisons of relative column performance may yield different results at different pH. Analysis of compounds with higher pK_a values (e.g. alkyl amines) could also give rise to different results. Nevertheless, the study should give additional information on the selection of optimum mobile phases for the chromatography of basic compounds.

2. Experimental

The HPLC system consisted of an SP 8800 pump, Spectra 100 UV detector with a time

constant of 0.1 s and a 9-μl flow cell (Spectra Physics, San Jose, CA, USA) and valve injector with 5 μl loop (Rheodyne, Cotati, CA, USA). We attempted to keep the dead volume of the system to a minimum, and used a relatively large diameter column in order to limit extra-column effects. N was determined from peak widths at half height ($w_{0.5}$) using the formula $n = 5.54[t_R/w_{0.5}]^2$. A_s was calculated at 10% of the peak height from the ratio of the widths of the rear and front sides of the peak; both measurements were made using a Model 2000 data station (Trivector, Bedford, UK). All results were the mean of at least duplicate injections. The columns used were Inertsil ODS 5 μm, 25 × 0.46 cm I.D. (GL Sciences, Tokyo, Japan) with 14% carbon loading. All analyses were performed at 20°C. Buffers were prepared by dissolving the appropriate quantity of KH_2PO_4 in pure water, and adjusting the pH with KOH solution of the same molar concentration, in order to maintain $[K^+]$ constant. The pH of the buffer was measured before addition of the organic modifier; this avoids difficulties inherent in the measurement of the pH of organic solvent–water mixtures, but does not allow calculation of aqueous–organic pK_a values. Uracil was used as a column void-volume marker for calculation of k'. All pyridine derivatives for HPLC analysis (from Sigma-Aldrich, Poole, UK) were made up at a concentration of 100 mg l^{-1} in the relevant mobile phase. For UV work, approximately 10 mg l^{-1} phosphate solutions of the compounds were utilised, prepared as above.

3. Results and discussion

Previously, we showed that chromatography of pyridine and some alkyl-substituted derivatives can be performed reproducibly on Inertsil ODS using methanol–water (55:45, v/v) without buffers or additives; the compounds are largely unprotonated in this mobile phase [3]. Nevertheless, unbuffered mobile phases can lead to variable ionisation due to concentration effects within the peak, leading to asymmetry [1]; indeed, we found that some derivatives gave very asymmetric peaks in unbuffered acetonitrile–water or THF–water mixtures isoeluotropic with the methanolic mobile phase. Furthermore, unbuffered mobile phases seemed to require long equilibration times and give less reproducible N and A_s values for some analyte/mobile phase combinations. Thus, we performed the study using the three solvents in combination with a pH 7.0 phosphate buffer; in any case buffers are more likely to be used in practice for the analysis of basic compounds. Table 1 shows the effect of varying the nature of the organic modifier in buffered mobile phases, on performance of the same column. Approximately isoeluotropic eluent concentrations were determined empirically; the influence of extra column band broadening should be similar for peaks of similar k'. The overall $[K^+]$ was maintained at 0.0225 M in each experiment in an attempt to maintain competetive ion-exchange interactions of the buffer constant. To ensure that no deterioration of the column took place during the study, the first results obtained were reproduced at the end of the work. Table 1 shows that stereochemical effects noted previously for the derivatives using the methanolic phase are generally reproduced for acetonitrile and THF [3]; peak asymmetry tends to decrease with increasing size and proximity of groups substituted in the region of the basic centre. Differences between column performance for the different solvents appear to be strongly dependent on the nature of the probe compound, even for this related set. Some compounds show little difference (e.g. monosubstituted 2-derivatives) whereas others show major differences (e.g. monosubstituted 4-derivatives). The difference in the results for 2-substituted derivatives from those obtained on most of the columns studied previously [2] may be due to the relative inertness of the column used in the present study. The average A_s for all 16 derivatives using either methanol, acetonitrile or THF was 1.50 (R.S.D. 9.8%), 1.81 (R.S.D. 17.1%) and 1.43 (R.S.D. 8.8%), respectively. Similarly, the average N was 14 300 (R.S.D. 8.6%), 13 900 (R.S.D. 10.2%) and 14 100 (R.S.D. 5.0%). All of the individual compounds studied showed greater A_s in the mobile phase modified with acetonitrile than in those modified with methanol or THF. Acetonitrile also gave a wider range of

Table 1
Effect of organic modifier on column performance data for pyridine and alkyl substituted pyridines

Compound	pK_a (water at 25°C)[a]	k'	N	A_s
Pyridine	5.17	0.59[b]	11 400	1.69
		0.45[c]	11 900	1.78
		0.40[d]	13 200	1.55
2-Methylpyridine	5.96	1.09	14 000	1.45
		0.73	14 000	1.54
		0.74	13 800	1.46
3-Methylpyridine	5.68	1.26	13 100	1.57
		0.96	12 900	1.82
		0.97	13 800	1.61
4-Methylpyridine	6.00	1.23	12 700	1.73
		0.93	11 600	2.12
		0.86	13 400	1.60
2-Ethylpyridine	5.89	2.02	15 300	1.32
		1.69	15 900	1.44
		1.89	15 400	1.32
3-Ethylpyridine	5.80	2.42	14 400	1.53
		1.93	14 100	1.78
		2.30	14 900	1.45
4-Ethylpyridine	5.87	2.53	14 000	1.65
		2.06	12 900	2.31
		2.15	14 500	1.45
2,3-Dimethylpyridine	6.57	2.10	14 700	1.36
		1.35	14 700	1.55
		1.48	14 000	1.46
2,4-Dimethylpyridine	6.74	2.29	14 500	1.46
		1.37	13 900	1.70
		1.53	13 300	1.49
2,6-Dimethylpyridine	6.71	1.98	14 800	1.37
		1.10	14 700	1.55
		1.31	13 600	1.46
3,4-Dimethylpyridine	6.47	2.28	13 300	1.72
		1.60	12 300	2.23
		1.60	13 500	1.51
3,5-Dimethylpyridine	6.09	2.70	14 200	1.55
		1.83	13 900	1.86
		2.21	13 800	1.49
2-Propylpyridine	6.30	3.98	16 200	1.26
		2.87	16 700	1.34
		4.57	15 200	1.25

Table 1. *Continued.*

Compound	pK_a (water at 25°C)[a]	k'	N	A_s
4-Isopropylpyridine	6.02	4.66	14 800	1.50
		3.61	13 400	2.14
		4.59	15 000	1.30
3-Butylpyridine	–[e]	10.75	15 900	1.35
		7.86	15 500	1.60
		13.71	14 200	1.19
4-*tert.*-Butyl pyridine	5.99	7.83	15 300	1.45
		5.80	14 500	2.23
		8.85	14 500	1.27

[a] All pK_a values from Ref. [9] except 2,3-DMP, Ref. [5].
[b] Methanol–phosphate buffer (0.05 *M*) pH 7.0 (55:45, v/v).
[c] Acetonitrile–phosphate buffer (0.0375 *M*) pH 7.0 (40:60, v/v).
[d] THF–phosphate buffer (0.03 *M*) pH 7.0 (25:75, v/v).
[e] Value not available. For comparison purposes, benzene gave $N = 17\,000$ with $A_s = 1.22$ with this column using methanol–water (55:45, v/v).
Flow-rate 1 cm^3 min^{-1}; column Inertsil ODS batch "a"; detector UV at 254 nm. For other conditions, see Experimental.

values for the compounds (shown by the increased R.S.D.). Overall, methanol and THF gave much more similar results.

The differences in column performance for the individual solvents could be due to the different hydrogen bonding abilities of the modifier as noted by Claessens et al. [2]; however, this explanation does not fully account for our results. We believed that different degrees of protonation of the analytes in the different mobile phases could be a contributing factor; protonated analytes can participate in ion-exchange interactions with ionised surface silanols. We monitored the protonation of 2,4-dimethylpyridine (2,4-DMP) and pyridine (highest and lowest pK_a) in acetonitrile–buffer and THF–buffer using a UV method employed previously [3], which is based on the increased absorptivity of the protonated species (see Fig. 1). The approximate pH of the aqueous buffer (measured prior to mixing with organic solvent), which when combined with modifier leads to half protonation of pyridine was 3.2, 3.9 and 4.1 for buffered isoeluotropic methanol, acetonitrile and THF mixtures, respectively; methanol results are calculated from previous data [3]. The corresponding approximate values for 2,4-DMP are 4.6, 5.0 and 5.5, respectively. It should be noted that these are not aqueous–organic pK_a values (see Experimental). The variation in values for the different modifiers may be at least partially due to their appreciably different concentrations in the isoeluotropic mixtures and differing influence on the ionisation of the buffer used. At aqueous pH of 7.0 prior to combination with THF, 2,4-DMP appears to be very slightly protonated, although not measurably protonated in the other two mobile phases. Furthermore, the highest pK_a derivatives 2,4- and 2,6-DMP give slightly increased tailing using THF instead of methanol, in contrast with the average results reported above, although the increases seem hardly significant. We substituted a pH 6.0 buffer for the pH 7.0 buffer in the THF mobile phase. Fig. 1 shows 2,4-DMP to be substantially protonated at this lower pH in combination with THF and the derivative shows greater protonation than in isoeluotropic methanol or acetonitrile mixtures. The k' values for 2,4-and 2,6-DMP fell by ca. 25%, presumably due to decreased interaction with the ODS ligands, whereas low pK_a compounds (e.g. pyridine) showed little change in k'. However, we were unable to detect significant changes in A_s for any of the analytes. A

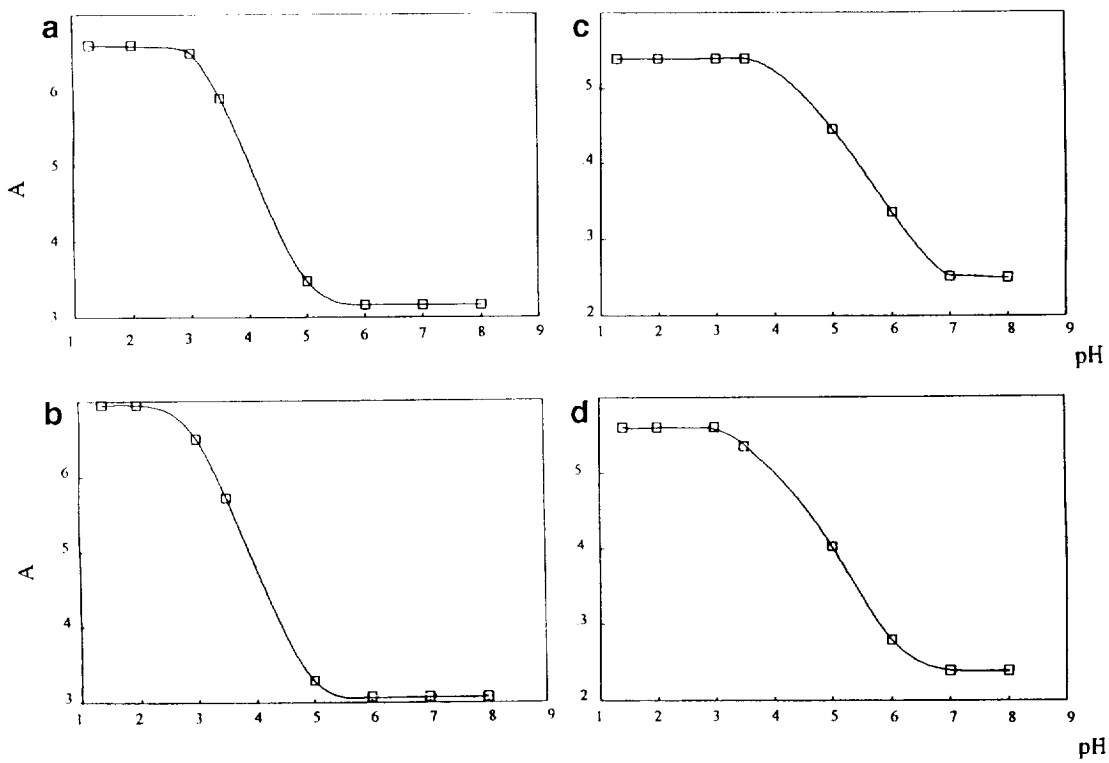

Fig. 1. Plot of UV absorbance at 255 nm (a, b), and 259 nm (c, d) against pH. (a) Pyridine in THF–phosphate buffer (25:75, v/v); (b) pyridine in acetonitrile–phosphate buffer (40:60, v/v); (c) 2,4-dimethylpyridine in THF–phosphate buffer (25:75, v/v); (d) 2,4-dimethylpyridine in acetonitrile–phosphate buffer (40:60, v/v).

difficulty in such investigations is the concurrent change in ionisation of silanols, especially since the pH is in the region of their average pK_a. Furthermore, the competitive effect of buffer K^+ ions may reduce ion-exchange effects. This same factor may also reduce the influence of the relatively small pK_a differences in these analytes on peak tailing, emphasising stereochemical effects. The relative influence of compound stereochemistry and pK_a could vary from column to column, leading to changes in the relative asymmetry of the analytes. Other factors may contribute to the differences in A_s shown for individual compounds with the different modifiers. For instance, spectroscopic studies show distinctly different RP solvation characteristics of methanol–water compared with acetonitrile–water. Increased solvation of the bonded ligands in the latter mobile phase may allow greater penetration of components to the silanols [10].

Finally, we investigated the effect of organic solvent strength on peak shape for these compounds. Methanol was used due to its apparent suitability for chromatography of these compounds and the greater solubility of phosphate buffer in higher concentrations of this solvent. The overall phosphate buffer concentration of the mixture was maintained at 0.0225 *M* throughout the experiments which utilised 25–65% methanol and produced variations in k' of approximately an order of magnitude. For these experiments, we had to replace the column due to some deterioration after prolonged use. The second column, packed with a different batch of the same ODS material, showed improved N and A_s in absolute terms, however, values relative to pyridine are similar to those for the first column, and differences of similar magnitude are also shown in peak shape for the neutral compound benzene. Fig. 2 shows a plot of log k' vs. volume

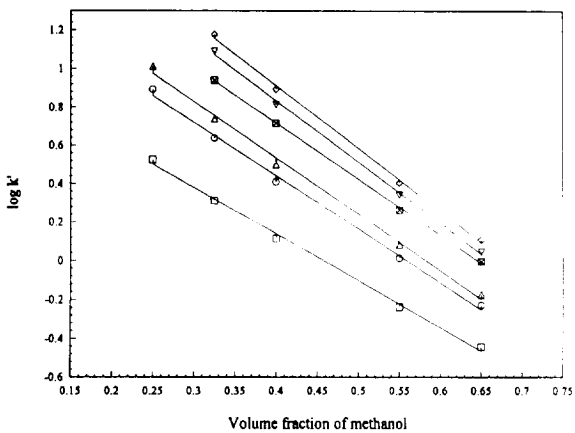

Fig. 2. Plot of log k' against volume fraction of methanol. Column Inertsil ODS batch "b"; detector UV at 254 nm. (□) pyridine, (○) 2-methylpyridine, (△) 3-methylpyridine, (⊠) 2,6-dimethylpyridine, (▽) 3,4-dimethylpyridine, (◇) 3,5-dimethyl pyridine. For composition of mobile phases, see Table 2. For other conditions see Table 1.

fraction of organic solvent in the mobile phase for some of the compounds. The plots are generally linear over the range 25–65% methanol which is fairly typical for RP systems, although not usual for basic compounds, whose plots often give severe curvature [11]. Presumably, the column used was sufficiently inert towards these compounds to give this behaviour without the use of amine additives such as triethylamine. Measurements of k' and corresponding N and A_s are shown in Table 2. Some compounds (e.g. 4-methylpyridine) gave considerable tailing at high k' values. This lends weight to our decision not to use probe compounds giving greater detrimental interaction, which could have prevented observation of any trends by giving extreme tailing under these non-optimum conditions. For all compounds, A_s increases as k' increases. However, A_s of some compounds hardly varies with k' over the ranges studied (e.g. 2-propylpyridine); others show considerable variation (e.g. 4-methylpyridine). These results may merely be a reflection of the relative degree of detrimental interaction of the compound with the column, as influenced by stereochemical and pK_a effects. Fig. 3 shows selected plots of A_s vs. k'; they are approximately linear but have very different slope. N values reflect the trends in A_s values, although some peaks are not sufficiently asymmetric to affect N calculated using the half-height method. The effects of instrumental dead volume are noticeable in reduced N for some peaks with low k'. The variation of A_s with k' can be attributed to overloading of the silanols by the retained basic solutes as discussed by Snyder and co-workers [1,12]. At very low sample concentrations, these sites may preferentially interact with sample molecules but are not overloaded, whereas for more practical sample concentrations, they become saturated. At high k', a greater fraction of the sample band is present in the stationary phase to overload these sites. Our previous monitoring of A_s using injection of 0.05–3.5 μg pyridine with the same column with methanol–phosphate buffer pH 7.0 (55:45, v/v) gives direct evidence of such overloading effects [3]. Nevertheless, the increase in asymmetry with sample amount was somewhat less than that with increase in k' shown in Table 2. It is possible that other factors contribute, such as the decreased ionisation of both sample molecules and silanol groups in mobile phases containing high concentrations of modifier.

Our previous comparisons of A_s for pyridine derivatives were largely between compounds of very similar k' [3]. 4-Substitution with alkyl groups of increasing chain length gives substantial increase in k', and thus should give increased tailing, although the reverse is found for THF and methanolic mobile phases. It is possible that whole molecule stereochemical effects can explain the data [3]; these may be partially obscured by differences in k', since the factors have opposing influence. Use of Table 2 to compare A_s of compounds giving similar k' in different solvents poses conceptual difficulties, because changes in solvent composition lead to other changes (e.g. ligand solvation effects and ionisation effects) noted above.

4. Conclusions

Significant differences in peak shape were obtained for pyridine and alkyl-substituted de-

Table 2
Effect of solvent strength on column performance data for pyridine derivatives

Compound	Mobile phase composition													
	65:35			55:45			40:60			32.5:67.5			25:75		
	k'	N	A_s	k'	N	A_s	k'	N	A_s	k'	N	A_s	k'	N	A_s
Pyridine	0.36	12 600	1.37	0.58	12 500	1.47	1.30	11 700	1.62	2.05	10 900	1.90	3.35	10 800	2.11
2-Methylpyridine	0.59	13 900	1.24	1.03	14 500	1.24	2.57	14 600	1.28	4.33	14 600	1.47	7.79	14 800	1.77
3-Methylpyridine	0.67	13 400	1.35	1.21	13 400	1.42	3.16	12 900	1.53	5.48	12 400	1.87	10.3	12 200	2.34
4-Methylpyridine	0.66	12 600	1.40	1.19	12 700	1.60	3.07	11 900	1.90	5.30	11 200	2.42	9.95	10 900	3.26
2-Ethylpyridine	0.97	14 500	1.15	1.80	15 200	1.14	5.29	15 800	1.16	[a]	[a]				
3-Ethylpyridine	1.15	14 300	1.26	2.20	14 000	1.37	6.99	14 200	1.49	[a]	[a]				
4-Ethylpyridine	1.18	13 800	1.31	2.27	13 900	1.49	7.16	13 600	1.73	[a]	[a]				
2,3-Dimethylpyr.	1.05	16 400	1.19	1.93	14 100	1.19	5.72	13 700	1.31	[a]	[a]				
2,4-Dimethylpyr.	1.13	14 100	1.20	2.17	14 400	1.31	6.25	14 500	1.47	11.4	14 200	1.84	[a]		
2,6-Dimethylpyr.	0.99	14 600	1.15	1.83	14 800	1.19	5.19	15 400	1.23	8.70	15 500	1.42	[a]		
3,4-Dimethylpyr.	1.11	13 200	1.41	2.20	13 700	1.67	6.51	12 700	2.07	12.3	11 900	2.77	[a]		
3,5-Dimethylpyr.	1.28	14 100	1.32	2.54	14 000	1.49	7.82	14 400	1.75	15.0	14 000	2.19	[a]		
2-Propylpyr.	1.67	14 900	1.09	3.47	15 300	1.11	12.1	15 800	1.14	[a]	[a]				
4-Isopropylpyr.	1.92	14 000	1.25	4.10	14 400	1.37	15.3	14 600	1.59	[a]	[a]				
3-Butylpyr.	3.76	15 000	1.12	9.26	15 900	1.23	[a]	[a]	[a]						
4-*tert.*-Butylpyr.	2.91	13 700	1.20	6.81	14 500	1.34	[a]	[a]	[a]						

Variation of k', N and A_s with solvent strength using Inertsil ODS column. 65:35 = methanol–0.064 *M* phosphate buffer pH 7.0 (65:35, v/v); 55:45 = methanol–0.05 *M* phosphate buffer pH 7.0 (55:45, v/v); 40:60 = methanol–0.0375 *M* phosphate buffer pH 7.0 (40:60, v/v); 32.5:67.5 = methanol–0.033 *M* phosphate buffer pH 7.0 (32.5:67.5, v/v); 25:75 = methanol–0.030 *M* phosphate buffer pH 7.0 (25:75, v/v). For comparison purposes, benzene gave $N = 19\,200$ with $A_s = 1.03$ using methanol–water (55:45, v/v). Column Inertsil ODS batch "b". Other conditions as in Table 1.

[a] Data not obtained due to long retention time.

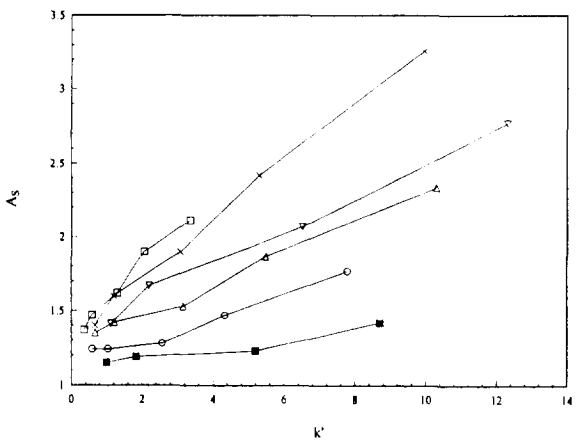

Fig. 3. Plot of A_s against k'. (×) 4-Methyl pyridine; for other symbols see Fig 2. Column Inertsil ODS batch "b". For other conditions see Table 1.

rivatives when using a silica-based RP column, depending on the choice of organic modifier in mobile phases buffered at a pH near the upper limit of such columns. However, the magnitude of these differences varies considerably with the analyte, even within the related set of compounds studied. Differences in protonation of the individual compounds in the different mobile phases were studied as a possible influential factor in explaining the differences in peak shape. However, it appears that the situation is complex, with several possible contributing effects. For the Inertsil ODS column used, mobile phases modified with acetonitrile gave significantly worse results than those modified with methanol or THF; this finding was in general agreement to that in a previous study [2]. Nevertheless, our work in progress indicates that the optimum choice of modifier may depend both on the nature of the analyte and the particular ODS phase utilised. All of these results indicate that the modifier is a neglected but important factor in the optimisation of the chromatography of basic compounds.

Steric hindrance around the basic nitrogen atom of the analyte reduces the influence of silanol effects [12]; this effect can explain why 2-substituted pyridines gave less tailing than 3- or 4-substituted derivatives of similar pK_a with each of the modifiers used. Tailing appears to decrease with increasing size and proximity of groups substituted near the basic centre.

Peak tailing increases for a particular analyte as k' increases with decreasing methanol concentration. The magnitude of this increase depends on the analyte. Steric effects may again explain why 2-derivatives, especially when substituted with larger alkyl groups, show little tailing even at high k' on the relatively inert column utilised in this study. Increased tailing of other analytes may be explained partially in terms of the increased overloading of silanol groups as k' increases; however, it is likely that other factors contribute to this effect.

It should be emphasised that these results were obtained using a single column with a rather narrow range of solutes. More work is necessary using different columns and solutes of entirely different structure, at high and low pH before deciding if any more general conclusions can be drawn.

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