ELSEVIER

Journal of Chromatography A, 664 (1994) 139–147

JOURNAL OF CHROMATOGRAPHY A

# Influence of analyte stereochemistry and basicity on peak shape of basic compounds in high-performance liquid chromatography with reversed-phase columns, using pyridine and alkyl-substituted derivatives as probe compounds

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(First received November 26th, 1993; revised manuscript received December 22nd, 1993)

**Abstract**

Column efficiency, peak asymmetry and retention factor of a series of sixteen compounds comprising pyridine and some alkyl-substituted derivatives, with $pK_a$ values ranging from 5.17 to 6.74 is reported on a RP-HPLC column previously shown to be especially suitable for the analysis of basic compounds. Compound stereochemistry in close vicinity of the basic group is found to influence peak shape strongly: however, stereochemistry at more remote positions is also of some significance. The effect of $pK_a$ on peak shape is discussed in the light of measurements of the degree of protonation of the compounds in the methanolic mobile phase as well as water.

## 1. Introduction

The analysis of basic compounds by HPLC using RP columns continues to be an area of concern, and the problems inherent in such work are now well recognised [1,2]. Nevertheless, relatively little is known about the effect of compound stereochemistry and basicity on the reduction of column performance which is often experienced with these compounds. Ascah and Feibush [3] found peak asymmetry increased with increasing $pK_a$ of the analyte, while the stereochemistry in close vicinity of the basic nitrogen atom was also thought to influence peak shape. Vervoort *et al.* [4] also found that there was a strong correlation between the $pK_a$ of a compound and its asymmetry factor, with peak tailing increasing with increasing $pK_a$. The same authors postulated that the so-called "flexibility" of protonated N atoms influenced their ability to interact with underivatised silanol groups. Nevertheless, these studies have incorporated basic compounds of quite varied stereochemistry and $pK_a$, which can make attribution of peak shape to specific features of the compound difficult.

We have evaluated a series of RP columns for the analysis of basic compounds using a test based on that of Engelhardt *et al.* [5]. This procedure has allowed the identification of columns which give acceptable and reproducible peak shape for analysis of pyridine, even when the mobile phase does not contain buffering components [6]. In the present study we have investigated the analysis of sixteen compounds comprising pyridine and various alkyl-substituted derivatives. These compounds form a more closely related group than those used in previous

0021-9673/94/$07.00 
*SSDI* 0021-9673(93)E0082-6

studies, with a fairly narrow range of $pK_a$ values (5.17 to 6.74) and similar stereochemistries. The compounds include derivatives with substitution of alkyl groups of varying chain length in 2-, 3- and 4-positions relative to the basic group; furthermore some compounds have virtually identical $pK_a$ values but different stereochemical features, enabling the influence of stereochemistry to be studied in the absence of the other variable. In this way we hoped to be able to discover more about the influence of compound nature on peak shape. A few commercial companies report test results for pyridine and substituted pyridines with their columns. A related aim of the work was to assess the relative degree of difficulty of analysis of these compounds, and to identify compounds more difficult to analyse than pyridine, which could be used to assess the performance of improved RP columns which are likely to become available in the future.

## 2. Experimental

The HPLC system consisted of an SP8800 pump, a Spectra 100 variable-wavelength UV detector with time constant 0.1 s and a 9-$\mu$l flow cell (all from Spectra-Physics, San Jose, CA, USA) and a valve injector equipped with a 5-$\mu$l loop (Rheodyne, Cotati, CA, USA). We did not wish to make measurements at constant retention factor ($k'$) by varying the proportion of organic solvent in the mobile phase, since this could affect both the wetting of the stationary phase and the degree of ionisation of these analytes. However, we attempted to keep the dead volume of the system to a minimum, and used a relatively large diameter column in order to limit the influence of extra-column effects. Column efficiency values ($N$) were determined from peak widths at half height. Asymmetry factors ($A_s$) were calculated at 10% of the peak height from the ratio of the widths of the rear and front sides of the peak, using a Model 2000 data station (Trivector, Bedford, UK) in conjunction with a BASIC program. All results were the mean of at least duplicate injections of the analyte. The new column used was Inertsil ODS 5 $\mu$m, 25 × 0.46 cm I.D. (GL Sciences, Tokyo, Japan) with 14% carbon loading. All analyses were performed at 20°C. Pyridine and alkyl-substituted compounds were obtained from Sigma–Aldrich (Poole, UK). Phosphate buffer solutions (concentration 50 m$M$) were prepared by dissolving 6.803 g of $KH_2PO_4$ in 1 l of pure water, and adjusting the pH with either concentrated $H_3PO_4$ or 0.05 $M$ KOH. Unless stated otherwise, the pH of the buffer was measured before addition of the organic modifier. Uracil was used as a column void volume marker for calculation of $k'$. UV measurements were made with a Lambda-15 spectrometer (Perkin-Elmer, Beaconsfield, UK). Dilute solutions of the compounds (concentration about 10 mg $l^{-1}$) were made up in phosphate solutions with preparation and subsequent adjustment of pH as above. In order to test the UV method used to investigate the protonation of the compounds in organic solvent–water mixtures (data not available in the literature), we measured the $pK_a$ of pyridine in water (a well known value) using the same procedure. This gave a value of 5.2, in agreement with literature values [7,8].

## 3. Results and discussion

In a previous report [6] it was shown that the peak shape of pyridine in addition to that of other basic test substances, could be used as a measure of the suitability of RP columns for the analysis of basic compounds. The mobile phase consisted of methanol–water (55:45, v/v): under these conditions, the RP is totally wetted, and solute molecules can penetrate the bonded layer to interact freely with residual silanol groups. At higher water concentrations, the bonded ligands may fold up, and form a dense layer impenetrable to solutes [5,9]. If buffer solutions are not utilised, some variables connected with their composition can be eliminated from the test, and it also becomes simpler to perform. A problem with this approach is the possibility of irreproducible results for ionisable compounds which could arise due to the poor buffering capacity of the mobile phase. This could cause changes in the ionisation of analyte or column

surface groups. For this reason, we also performed experiments using a mobile phase buffered at pH 7.0, a value which should not result in excessive dissolution of the silica, which could occur at higher pH with prolonged use. Table 1 shows the reproducibility of the analysis of a solution of pyridine injected repeatedly into a column which we have found especially suitable for the analysis of basic compounds, using a simple methanol–water mixture as eluent. Many commercial columns give severe tailing for pyridine under these conditions, and furthermore we have found column performance data irreproducible. Using the Inertsil-ODS column however, the results in terms of peak shape and retention factor seem very reproducible. We performed these experiments with a new column which had not previously been used with buffered mobile phases: otherwise extensive washing may be necessary to remove the influence of these substances completely.

We investigated the effect of sample concentration by injection of pyridine solutions over a range 10 to 700 mg $l^{-1}$, corresponding to 50-ng to 3.5-$\mu$g amounts injected. Values of $N$, $A_s$, $k'$ and peak height/area were calculated for both buffered and unbuffered mobile phases. Fig. 1 shows that trends were similar for both mobile phases: in both cases the efficiency (monitored at half peak height) remains approximately constant with increasing sample concentration whereas the peak asymmetry shows a gradual worsening. Use of the Dorsey–Foley equation, or some other alternative algorithms for calculation of column efficiency, would give greater weight to the increasing asymmetry of peaks in the calculation of $N$. Nevertheless, the James–Martin procedure is precise, and the asymmetry of the peaks monitored throughout our work was not excessive [10]. Both for measurement of $N$ and $A_s$, the buffered mobile phase appears to give slightly better results for these basic compounds. Peak area and peak height measurements showed an excellent linear response with increasing concentration over the range 0–700 mg $l^{-1}$, even with the unbuffered mobile phase. For peak area (peak height) measurements, the slope of the line obtained from eight data points with methanol–water (55:45, v/v) was 7.00 (0.934), intercept 5.41 (−0.118), standard deviation of the slope 0.00851 (0.00368), standard deviation of the intercept 2.82 (1.22) standard error 5.78 (2.50), and correlation coefficient $r$ = 0.99999 (0.99995). These results indicate the absence of concentration-dependent adsorption effects. Also, the relative standard deviation of $k'$ measurements when varying the concentration of injected solutions over the range indicated in Fig. 1 was still less than 1%. These reproducible results may not be typical for more active columns. We concluded that providing the concentration of injected solutions is approximately the same, results would be sufficiently precise for meaningful comparisons of column performance for different compounds to be made, even if the mobile phase does not contain buffer components. We chose 100 mg $l^{-1}$ injections for all subsequent work because column performance data were particularly reproducible at this level.

Claessens *et al.* [2] found considerably greater peak asymmetry for analysis of heptylpyridine on some columns when using acetonitrile–water

Table 1
Reproducibility of analysis of pyridine on Inertsil ODS column using unbuffered mobile phase

| | $k'$ | Peak height (integrator units) | Peak area (integrator units) | $N$ (plates) | $A_s$ |
|---|---|---|---|---|---|
| Mean | 0.63 | 93.9 | 689 | 10 400 | 1.65 |
| R.S.D. (%) | 0.32 | 0.97 | 1.53 | 1.01 | 1.21 |

Mobile phase methanol–water (55:45, v/v), flow-rate 1 ml $min^{-1}$. UV detection at 254 nm. Column temperature 20°C. Results based on eight repeated injections of 100 mg $l^{-1}$ solution.

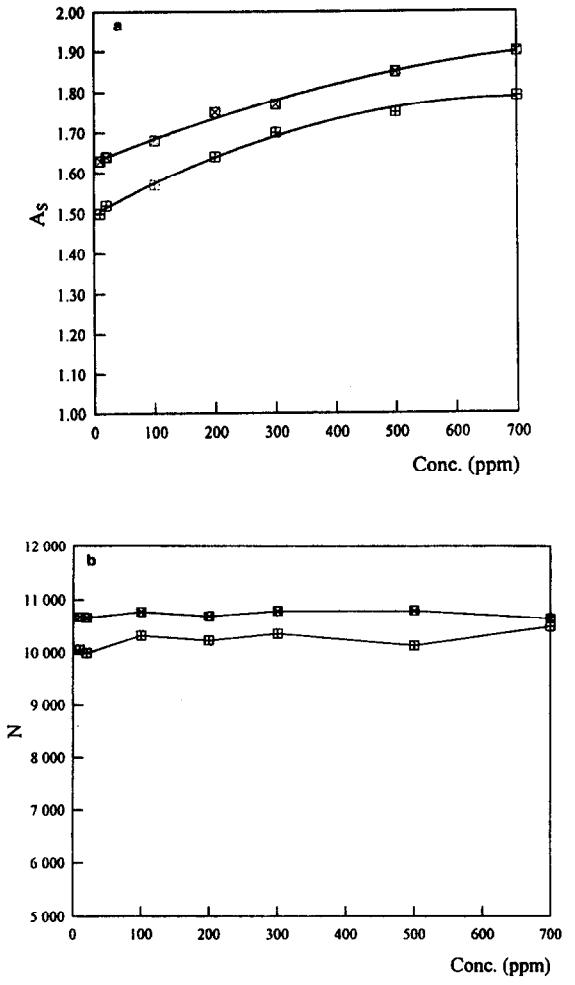


Fig. 1. (a) Plot of asymmetry factor against concentration of injected pyridine solution; mobile phase: upper curve methanol–water (55:45, v/v), lower curve methanol–phosphate buffer pH 7.0 (55:45, v/v). (b) Plot of column efficiency against concentration of injected pyridine solution; mobile phase: upper curve methanol–phosphate buffer pH 7.0 (55:45, v/v), lower curve methanol–water (55:45, v/v). Flow-rate 1 ml $min^{-1}$; column temperature 20°C; sample volume 5 $\mu$l; column Inertsil ODS; detector UV at 254 nm. ppm = mg $l^{-1}$.

mixtures rather than methanol–water mixtures. They attributed this result to the ability of methanol to hydrogen bond with silanol groups, and thus give a deactivation effect in comparison with acetonitrile. We found that substitution of an isoeluotropic acetonitrile–water mixture (approximately 45:55) for the methanol–water mixture gave an increase of only about 10% in $A_s$ for pyridine, for injections of the same amount of analyte. The small difference we found may be due to the relative inertness of the stationary phase employed in our work. Most of the RP-columns investigated in the other study [2] were well established materials not especially suited for the analysis of basic compounds.

The seventeen compounds selected for study in Table 2 allow investigation of the effect on peak shape of a number of different features of the analyte. These include steric effects around the basic pyridine nitrogen atom, whole molecule steric effects, and ionisation effects which are related to the various indicated $pK_a$ values of the derivatives. Values of $N$, $A_s$ and $k'$ are reported in Table 2 for each substance. All compounds gave reduced performance in comparison with benzene, a neutral substance included for comparison purposes. Duplicate injections were performed to give mean results reported for the unbuffered mobile phase; the same procedure was repeated for the buffered mobile phase. For each mobile phase, the individual results contributing to the mean value showed close agreement, which was predicted by the more detailed reproducibility study for pyridine shown in Table 1. Fig. 2 shows a representative chromatogram of six of the compounds using the methanol–water mobile phase. As with pyridine, all compounds showed slightly better performance with a mobile phase buffered at pH 7.0 rather than water in admixture with methanol, although differences were rather small. Some clear trends are visible in the results. Peak asymmetry increases and column efficiency drops along the series 2-methylpyridine ($A_s = 1.41$ in methanol–water), 3-methylpyridine ($A_s = 1.56$) and 4-methylpyridine (1.83), with a corresponding drop in $N$. Similar results are seen for 2-ethylpyridine ($A_s$ = 1.36 in methanol–water), 3-ethylpyridine ($A_s$ = 1.52) and 4-ethylpyridine ($A_s = 1.70$). The same trends are seen for these two sets of compounds when considering performance using the buffered mobile phase. Evidently, 2-substitution of the pyridine ring reduces the access of the basic nitrogen atom to column active sites, with the

Table 2
Column performance data for pyridine and alkyl-substituted pyridines

| Compound | p$K_a$ (water at 25°C)[a] | $k'$ | $N$ (plates) | $A_s$ |
|---|---|---|---|---|
| Pyridine | 5.17 | 0.63 | 10 400 | 1.65 |
| | | *0.60* | *10 600* | *1.57* |
| 2-Methylpyridine | 5.96 | 1.15 | 12 500 | 1.41 |
| | | *1.10* | *12 800* | *1.35* |
| 3-Methylpyridine | 5.68 | 1.34 | 11 700 | 1.56 |
| | | *1.29* | *12 100* | *1.47* |
| 4-Methylpyridine | 6.00 | 1.33 | 10 600 | 1.83 |
| | | *1.27* | *11 400* | *1.64* |
| 2-Ethylpyridine | 5.89 | 2.08 | 13 300 | 1.36 |
| | | *2.03* | *14 000* | *1.24* |
| 3-Ethylpyridine | 5.80 | 2.58 | 12 500 | 1.52 |
| | | *2.51* | *12 800* | *1.39* |
| 4-Ethylpyridine | 5.87 | 2.67 | 11 800 | 1.70 |
| | | *2.68* | *13 100* | *1.47* |
| 2,3-DMP | 6.57 | 2.30 | 12 000 | 1.43 |
| | | *2.19* | *12 700* | *1.26* |
| 2,4-DMP | 6.74 | 2.47 | 12 200 | 1.58 |
| | | *2.37* | *13 000* | *1.36* |
| 2,6-DMP | 6.71 | 2.07 | 12 600 | 1.49 |
| | | *1.98* | *13 500* | *1.29* |
| 3,4-DMP | 6.47 | 2.49 | 11 000 | 1.85 |
| | | *2.39* | *12 200* | *1.58* |
| 3,5-DMP | 6.09 | 2.86 | 12 500 | 1.57 |
| | | *2.78* | *13 100* | *1.46* |
| 2-Propylpyridine | 6.30 | 4.09 | 14 300 | 1.27 |
| | | *4.02* | *14 000* | *1.20* |
| 4-Isopropylpyridine | 6.02 | 4.93 | 13 100 | 1.53 |
| | | *4.99* | *13 100* | *1.38* |
| 3-Butylpyridine | [b] | 11.4 | 14 900 | 1.31 |
| | | *11.7* | *16 300* | *1.25* |
| 4-*tert.*-Butylpyridine | 5.99 | 7.94 | 13 600 | 1.47 |
| | | *8.30* | *13 600* | *1.34* |
| Benzene | – | 4.99 | 19 200 | 1.12 |
| | | *5.00* | *19 100* | *1.13* |

Results for mobile phase methanol–water (55:45, v/v) shown in normal characters; for mobile phase methanol–phosphate buffer pH 7.0 (55:45, v/v) shown in italics. All other conditions as in Table 1.
[a] All p$K_a$ values from ref. 7 except 2,3-DMP, ref. 8.
[b] Value not available.

effect decreasing successively for 3- and 4-substitution. Peak shapes for the dimethylpyridines (DMPs) are almost entirely predictable by a summation of the position-dependent steric effects of the individual methyl groups. For example, peak asymmetry increases along the series 2,3-DMP ($A_s = 1.43$ in methanol–water), 2,4-DMP ($A_s = 1.58$) and 3,4-DMP ($A_s = 1.85$). Increasing the size of the substituent at the 2-position seems to reduce peak asymmetery: a decrease is observed for the series 2-methylpyridine ($A_s = 1.41$ in methanol–water), 2-ethylpyridine ($A_s = 1.36$) and 2-propylpyridine ($A_s = 1.27$), accompanied by an increase in $N$. Despite the apparent importance of stereochemistry in close proximity to the basic group however, steric effects still appear to influence results when alkyl groups are present at more remote

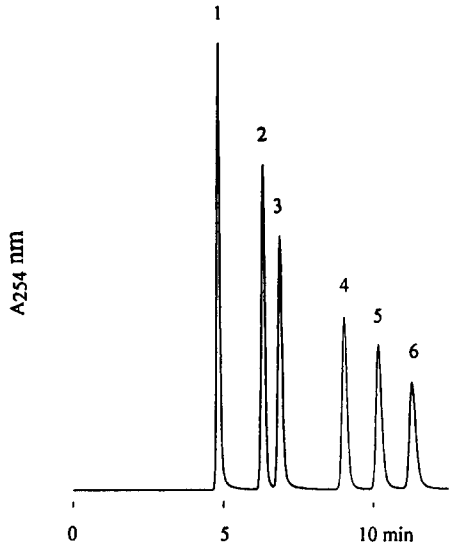


Fig. 2. Separation of pyridine and some derivatives using Inertsil ODS column. Mobile phase methanol–water (55:45, v/v). Peaks: 1 = pyridine; 2 = 2-methylpyridine; 3 = 3-methylpyridine; 4 = 2,6-DMP; 5 = 2,4-DMP; 6 = 3,5-DMP. Other conditions as in Fig. 1.

sites. Thus $N$ increases and peak asymmetry decreases for the series 4-methylpyridine ($A_s$ = 1.83 in methanol–water), 4-ethylpyridine ($A_s$ = 1.70), 4-isopropylpyridine ($A_s$ = 1.53) and 4-*tert.*-butylpyridine (1.47). It would appear for these compounds that the overall size of the molecule may also influence its ability to penetrate the bonded ligands and interact with the column surface. The relative effect of substituents in positions adjacent to, and remote from the basic centre can be judged by inspection of results for 2-methylpyridine and 4-*tert.*-butylpyridine which have virtually identical p$K_a$ values. Thus, a methyl group in the 2-position seems to give a similar steric effect to the *tert.*-butyl group in the 4-position. In summary it appears that steric effects around the basic nitrogen atom are of major importance, while steric effects for the whole molecule have a significant but lesser effect.

Finally, the influence of p$K_a$ of these compounds on peak shape must be considered. From the results in Table 2 it appears that using this inert ODS phase, p$K_a$ has a lesser influence within this group of compounds than stereochemical effects, although the range of p$K_a$ values within the group is not very large. Pyridine with the smallest p$K_a$ in the set (5.17) gives more asymmetric peaks than 2,4-DMP with the largest (6.74). In fact only 4-methylpyridine and 3,4-DMP, which according to the above arguments should give relatively small stereochemical effects, give peaks which are substantially more asymmetric than pyridine:these results could be attributed to their significantly higher p$K_a$. This result is also evident from the data reported by Ascah and Feibush [3]; however, these authors worked with a much more limited set of pyridine derivatives and used an electrostatically shielded RP column, which is not directly comparable with the more conventional column used in our work. Furthermore the mobile phase used [3] contained only 2% organic modifier which would not lead to full wetting of the bonded ligands. Nevertheless, it does appear that 4-methylpyridine and 3,4-DMP can be used as more severe tests of a column for activity towards basic compounds.

In order to investigate more fully the effect of compound p$K_a$ on peak asymmetry we monitored the variation of the degree of protonation with pH, of the compound with highest and lowest p$K_a$. We used a UV spectrophotometric method based on the increased absorptivity of the pyridium cations over that of the unprotonated species. Fig. 3 shows the absorbance spectra of 2,4-DMP in methanol–buffer solutions, at pH 1.6 and 9.0 (55:45, v/v). In a

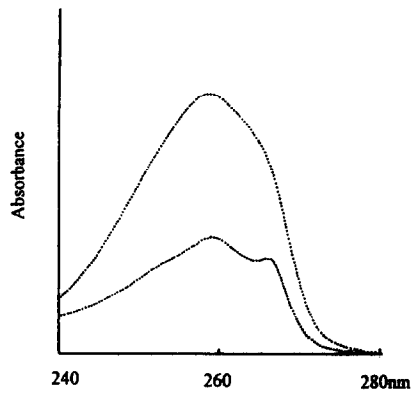


Fig. 3. Absorbance spectra of same concentrations 2,4-DMP in methanol–phosphate buffer pH 1.6 (55:45, v/v) (upper curve) and methanol–phosphate buffer pH 9.0 (55:45, v/v) (lower curve).

standard procedure, the absorbance of the compound is measured at a wavelength where the largest difference in absorptivity of the species occurs [11]; we chose 255 nm for pyridine and 259 nm for 2,4-DMP. Fig. 4a and c show plots of absorbance at the selected wavelength *versus* pH of the buffer for both compounds studied. It can be seen that even 2,4-DMP appears to be unprotonated in methanol–phosphate buffer pH 7.0 (55:45, v/v), which is unexpected by casual consideration of its p$K_a$ in water. There has been much debate about the measurement of pH in mobile phases containing significant proportions of organic solvents [12], although such measurements are not necessary to establish the above result. (Fig. 4a and c). Nevertheless, measurement of pH prior to addition of the organic solvent could not reveal any resultant effect on pH, or possible contamination by acids or bases. Therefore, we also measured the pH of the solutions after addition of the methanol. Bates *et al.* [13] noted that pH values obtained with glass electrodes standardised with aqueous buffers in alcohol–water mixtures, are subject to no simple interpretation. However, it was shown that for methanol–water mixtures over the range 8:92 to 68:32, the liquid junction potential of the electrode was sufficiently constant with pH to estab-

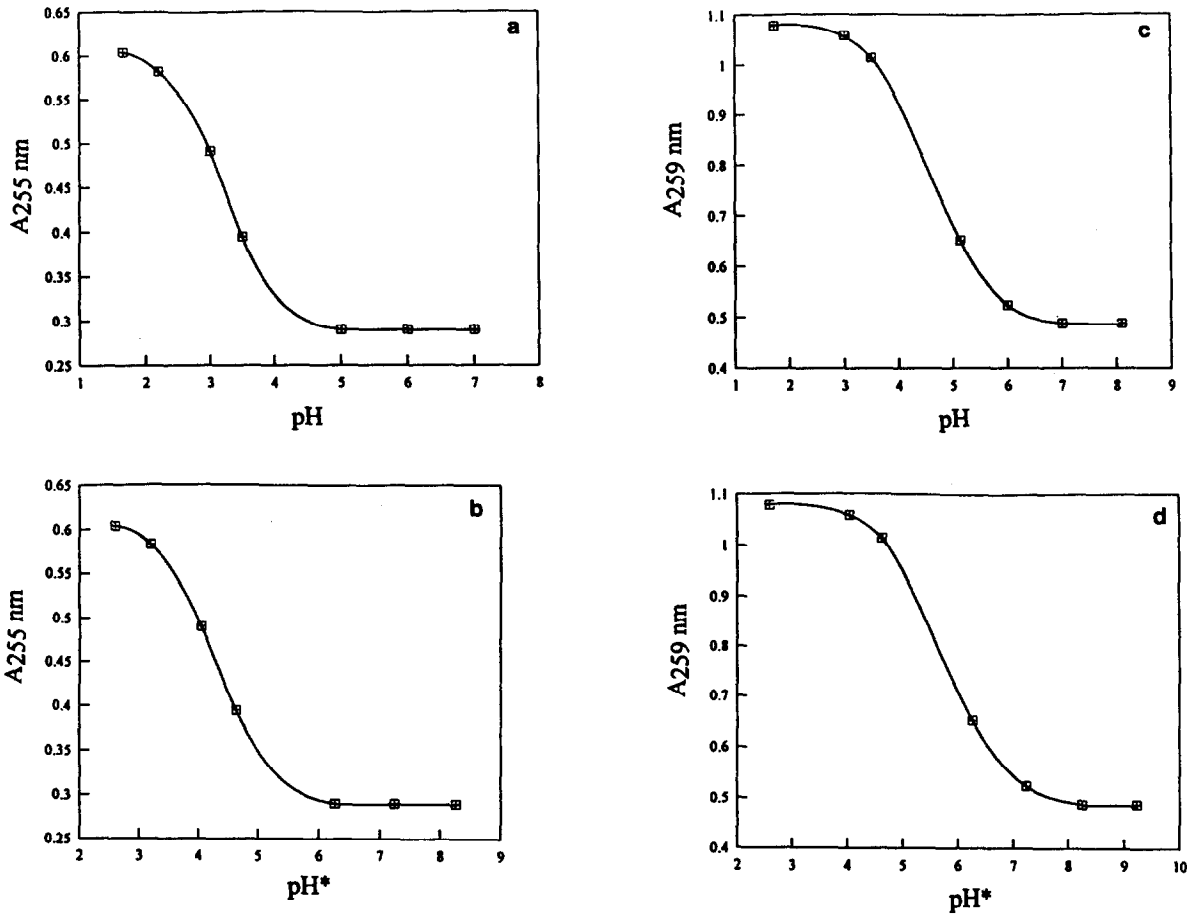


Fig. 4. Plot of UV absorbance against pH. (a) Pyridine in methanol–phosphate buffer (55:45, v/v); pH measured *before* organic solvent addition. (b) Pyridine (same concentration) in methanol–phosphate buffer (55:45, v/v); pH* measured *after* organic solvent addition. (c) 2,4-DMP in methanol–phosphate buffer (55:45, v/v); pH measured *before* organic solvent addition. (d) 2,4-DMP (same concentration) in methanol–phosphate buffer (55:45 v/v); pH* measured *after* organic solvent addition.

lish an operational scale of pH* measurements, where pH* = (observed pH $-\delta$) and $\delta$ is a correction term [13]. Fig 4b and d shows absorbance for the compounds plotted against pH*. The previous results are confirmed in that pH* of the methanol–phosphate buffer pH 7.0 (55:45, v/v) was approximately 8.25, indicating 2,4-DMP was virtually unprotonated in this system. The approximate $pK_a$ values for pyridine and 2,4-DMP in methanol–water (55:45, v/v) were calculated as 4.3 and 5.8, respectively. The depression of the $pK_a$ of bases in alcohol–water mixtures compared with the values in aqueous solution is well known [11]. More accurate calculation of these values would require measurements in buffer solutions over a much narrower pH range around the $pK_a$ values. Nevertheless, our results for the diminution of $pK_a$ for these compounds in this solvent are broadly comparable with those found by Bacarella *et al.* [14] for aniline, N-methylaniline and N,N-dimethylaniline in methanol–water mixtures of similar concentration.

The lesser influence of the $pK_a$ of the compounds within the pyridine group on chromatographic peak shape could be attributed to the probability that they are all largely unprotonated under the conditions of study, since it is likely that the protonation of all these compounds in methanol–water follows a similar pattern. Larger effects of $pK_a$ within a group might occur when some compounds are partially protonated, some fully protonated, and some not protonated, at the prevailing pH of the mobile phase. Thus, these compounds seem to give rise to asymmetric peaks when present as the neutral compounds, probably by hydrogen bonding with silanol groups. Nevertheless, the possible contribution of ion-exchange interactions between a basic analyte (B) and dissociated silanol groups on the column surface cannot be ruled out. Such interactions are governed by two equilibria:

solute ionisation: $B + H^+ \rightleftharpoons BH^+$

ion exchange: $BH^+ + K^+SiO^- \rightleftharpoons K^+ + BH^+SiO^-$

Depending on the values of $pK_a$ (in the mobile phase) and of the ion-exchange constant, it is possible to obtain substantial retention of $BH^+$ in this way, even if there is relatively little ionisation in the mobile phase. Thus, ionic effects may still contribute significantly to peak asymmetry for 2,4-DMP even at pH 7, in view of the much stronger ion-exchange attraction between protonated bases and silanols. Furthermore, on more active ODS phases, compounds with high $pK_a$ could show greater peak asymmetry, due to increased influence of ion exchange. Ion-exchange effects probably account for the differences in performance results for buffered compared with unbuffered mobile phases shown in Table 2. Finally, the existence of interactions with impurities in the silica such as metals cannot be ignored even though these compounds could not possibly undergo the very strong interactions experienced by analytes with chelating properties [15].

## 4. Conclusions

Measurements of column performance for pyridine and alkyl-substituted derivatives were performed reproducibly using a stationary phase especially suitable for the analysis of basic compounds. In comparative studies, the concentration of injected solutions should be kept reasonably constant, since peak asymmetry shows a gradual increase with the amount of compound injected. Steric factors have an important influence on peak shape. Alkyl groups in close proximity to the basic site have the most pronounced effect, apparently hindering interactions with the column surface. Nevertheless, steric effects are still demonstrable at more remote sites. On the inert ODS phase studied here, the effect of $pK_a$ was less important. Furthermore, for this set of compounds, UV measurements showed that the analytes were largely unprotonated under the conditions of the study. A scale of the degree of difficulty of analysis of these compounds was established: only 4-methylpyridine and 3,4-DMP appear to be significantly more severe tests of column activity towards basic compounds than pyridine itself.

## 5. Acknowledgements

The author thanks M. Norman, University of the West of England, for assistance with the preparation of the figures in this work and GL Sciences for the generous gift of HPLC columns.

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