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# Comparative evaluation of bonded-silica reversed-phase columns for high-performance liquid chromatography using strongly basic compounds and alternative organic modifiers buffered at acid pH

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**Abstract**

The performance of eight silica-based RP-HPLC columns evaluated previously with relatively high-p$K_a$ bases using isoeluotropic mixtures of either methanol, acetonitrile or tetrahydrofuran in combination with phosphate buffer pH 7.0, was further examined with the same solutes and mobile phases buffered at pH 3.0. Differences in column performance dependent on choice of organic modifier were shown to be considerably reduced at acid pH, although tetrahydrofuran again gave significantly better performance than acetonitrile or methanol. While differences between columns were less apparent at pH 3.0, some columns again clearly performed better than others. Furthermore, the ranking of columns according to average asymmetry of the set of basic compounds was found to vary somewhat with pH; thus column evaluation should be performed preferentially at the pH of intended use. The dependency of column performance on choice of solute was reduced, but still evident at pH 3.0. All solutes except pyridine gave superior results at acid pH. Solutes which were the most challenging probes at pH 7.0 were not necessarily the most difficult at pH 3.0.

*Keywords:* Stationary phases, LC; Mobile phase composition; Organic modifiers; Basic compounds

## 1. Introduction

In a recent study [1] we compared the performance of eight different silica-based RP columns (as measured by column efficiency, $N$ and asymmetry factor, $A_s$) with a variety of basic test probes using mobile phases buffered at pH 7.0 with phosphate and also unbuffered solvents. The columns were selected from the newer generation of RP materials which are, according to manufacturers' data, generally prepared from very pure silicas, and are recommended for the analysis of basic compounds. It was shown that testing columns in unbuffered mobile phases must be performed with caution, and is restricted to the use of low p$K_a$ compounds, probably due to variable ionisation effects in the mobile phase. Considerable differences in column performance for higher p$K_a$ compounds were shown using buffered mobile phases dependent on the choice of organic modifier [1,2]. Tetrahydrofuran (THF) usually gave the best results, followed by methanol, with acetonitrile often giving poorest performance. However, modifier effects were somewhat dependent on the particular make of RP column. It was demonstrated that while on average, some columns clearly gave superior results to others, the performance of a given column depended significantly on the choice of the basic solute. For example, some columns showed

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relatively good results for the stronger base nortriptyline, but poor results for the weak base pyridine, whereas others showed the converse effect.

In the present study, we have compared the performance of the same eight columns using essentially the same solutes, with mobile phases containing the same three modifiers buffered instead at pH 3.0. Although some columns show exceptional stability, pH 3.0 probably represents a low limit beyond which phase stripping may occur with prolonged use [3], whereas pH 7.0 as used previously is probably the upper pH limit of long-term stability for the underlying silica, at least when using phosphate buffers and relatively low temperatures [4]. The aims of this work were:

(a) To determine whether the significant variation in column performance obtained using different organic modifiers was also apparent at acid pH.

(b) To establish whether evaluation of the columns at acid pH gave the same column ranking, based for example on the average $A_s$ of a variety of compounds, as at neutral pH. Snyder and co-workers [5] have pointed out some of the difficulties in column ranking, and further that some columns perform best at low pH and some at high pH for the same samples. However, few detailed experimental studies have been carried out in this area. If column rankings were indeed pH dependent, specific testing at the pH of intended use might be advisable. Individual solutes at the two different pH levels would give some indication whether the best column for the analysis of a compound at one pH is also likely to give the best results at a different pH.

(c) To establish whether a given column using an acidic mobile phase could give a contradictory indication of performance dependent on the solute chosen (solute dependent column ranking [5]), as found previously [1] at pH 7.0.

(d) To compare the performance of the columns with a given solute at the two pH values. Although many workers perceive that both basic and acidic compounds are best separated by reversed-phase columns at low pH where both the solutes and the silanols on the silica support are largely protonated [4,6], again there are few, systematic comparisons of performance at these "low" and "high" pH limits. It would also be possible to ascertain whether those compounds which were the most demanding at neutral pH were also the most demanding at acid pH, which should aid the selection of suitable column test probes.

We believe the present study alongside our previous work provides an unusual opportunity to compare the performance of a range of columns, using both high and low pH, with different organic modifiers, and with the same set of basic compounds, which cover a range of $pK_a$ and stereochemistry.

## 2. Experimental

The HPLC system consisted of P200 pump, UV 100 detector (time constant 0.05 s, 5-μl flow cell) operated at 254 or 215 nm (Thermo Separation Products, San Jose, USA) and 7725 valve injector with 2-μl loop (Rheodyne, Cotati, USA). Connections were made with minimum lengths of 0.005 in. I.D. tubing (1 in.=2.54 cm). $N$ was determined from peak widths at half height ($w_{0.5}$) using the formula $N=5.54[t_r/w_{0.5}]^2$ and also from the Dorsey–Foley equation $N_{df}=41.7[t_r/w_{0.1}]^2/[A_s+1.25]$. $A_s$ was calculated at 10% of the peak height from the ratio of the widths of the rear and front sides of the peak; all 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 (all 5-μm particle size, 25×0.46 cm I.D.) were Inertsil ODS, Inertsil ODS-2, Inertsil ODS-3 all from GL Sciences, Tokyo, Japan; Symmetry $C_{18}$ from Waters, Milford, USA; Kromasil $C_8$ and Kromasil $C_{18}$ both from Anachem, Luton, UK; Supelcosil ABZ Plus from Supelco, Bellefonte, USA and Purospher $C_{18}$ (25×0.4 cm I.D.), from Merck, Darmstadt, Germany. Surface area, carbon loading and pore diameter of these phases were recorded previously [1]. All columns were operated using a flow rate of 1.0 $cm^3$ $min^{-1}$ apart from Purospher $C_{18}$ (0.9 $cm^3$ $min^{-1}$). All analyses were performed at 30°C with the column thermostatted in a block heater (Model 7980, Jones Chromatography, Hengoed, UK). Buffers were prepared by dissolving the appropriate quantity of $KH_2PO_4$ in pure water, and adjusting the pH with concentrated phosphoric acid, in order to maintain $[K^+]$ constant. Buffer pH was measured before addition of the organic modifier. Injection of uracil using a mobile phase of acetoni-

trile–water (40:60, v/v) was used to estimate column void volume. All analytes were obtained from Sigma–Aldrich (Poole, UK). Each solute was made up at a concentration of 100 mg $l^{-1}$ in the relevant mobile phase. Solutes were injected singly, rather than in mixtures, for reasons discussed previously [1]. The loop was completely filled, resulting in a (nominal) sample mass of 200 ng being introduced; the same weight of compound was used for every one of the experiments reported below. At least 150 column volumes were purged through before use with each new mobile phase.

## 3. Results and discussion

In the present study, we have re-evaluated at pH 3.0 the exact columns previously tested with organic solvent – pH 7.0 phosphate buffers. The advantage of this approach is that both column packing variation and batch to batch variation in performance for a given stationary phase are avoided. However, the possibility of gradual deterioration in performance in a prolonged study must be considered. We evaluated the columns using the modified Engelhardt test [7] at the very beginning and end of these two studies, which were carried out over a period of about 1 year. The greatest variation was in $N$ (as measured by the half-height method), which decreased by up to 5%; $A_s$, and especially $k'$ measurements showed smaller variations. It was considered that these variations were likely to be less than could be experienced if we had substituted new columns for the second series of tests reported here. These results indicate good stability of the columns under the conditions used. However, each was probably used with a total of less than 1500 column volumes of the buffered mobile phases, and was not used for any other work. These volumes are much less than used for long-term silica stability studies by other groups [4]; furthermore these stability studies indicated that while phosphate buffer promotes silica dissolution, it occurs to a significant extent only at higher temperatures and/or pH than have been utilised in our investigations.

We used seven of the original nine basic probe compounds studied at pH 7.0. However, we rejected 2-[$N$-methyl-$N$-(2-pyridyl)amino] ethanol and procainamide for reasons stated before [1], and substituted benzylamine, a stronger base ($pK_a$=9.3) which has been used previously for column evaluation [7,8]. Due to the widely different retention characteristics of the test compounds at acid pH, we used a higher concentration of organic solvent in the mobile phases for the analysis of diphenhydramine and nortriptyline than for the other six solutes. Changing the modifier concentration can affect $N$ and $A_s$ for a given compound/column combination [2], thus results for these two compounds are not strictly comparable with those for the other probes on the same column. However, the results for each solute are comparable from column to column, and we did not wish to use gradient elution, which amongst other difficulties, would have complicated the tests due to the necessity of column re-equilibration after each analysis.

Another consideration in column testing procedures is the state of wetting of the stationary phase. Engelhardt maintained that most reversed phases are totally wetted when the mobile phase contains less than 60% water [9]. More recent studies [10,11] have indicated that ODS ligands remain wetted above about 7% (v/v) methanol but that the degree of solvation of $C_{18}$ chains is gradually reduced at concentrations below 30% methanol. Work at low pH is complicated by the reduced retention of basic compounds, requiring lower concentrations of modifier to elute them with sufficient $k'$. Thus the possibility of reduced solvation, and even phase collapse, is increased. Furthermore, different columns can show different wetting characteristics, dependent on, for example surface coverage and length of bonded chain [10]. In the present study, we have attempted to keep modifier concentration as high as possible consistent with reasonable retention, and have not used less than 30% (v/v) methanol. Studies with other modifiers seem less detailed, but it appears that wetting is improved in acetonitrile and especially THF. It seems important to use *downward equilibration* of phases [10] when using low modifier concentrations. Here, the column is first equilibrated with mobile phases containing high concentrations of modifier sufficient to fully solvate the bonded phase, followed by reduction of the modifier concentration to the desired value. We observed this precaution strictly in the present work. Alternatively, if the

phase is conditioned previously with water or eluents containing very low concentrations of modifier, the phase may collapse. It may not then be possible to restore the collapsed phase completely unless very high modifier concentrations are again used. This may be the reason for the difference in the figure obtained by Engelhardt, since this result was based on adding organic solvent to column packing suspended in water. Despite the conceptual difficulties of work at low pH, a large fraction of applications for basic compounds reported use low pH [4], so our study is justified for practical reasons. From the generally high column efficiencies reported below, it seems unlikely that wetting problems have seriously influenced the present study.

Table 1 shows $k'$, $A_s$ and efficiency measurements on the eight columns using approximately isoeluotropic eluents containing three different modifiers in combination with pH 3.0 phosphate buffer. Column efficiency is reported using both the half-height method and the Dorsey–Foley equation. Serious errors can occur in half-height column efficiency measurements if peaks are not close to Gaussian in shape; the Dorsey–Foley equation incorporates peak asymmetry in the algorithm for plate number calculation, and has been shown to give a reasonable estimation of true column efficiency [12]. The difference in value for $N$ calculated by the two methods is considerable. For example, Kromasil $C_8$ using acetonitrile–buffer gives $N=9190$ but $N_{df}=1190$ for pyridine ($A_s=4.36$). Substantial differences could occur even when smaller peak asymmetry was recorded; thus Purospher $C_{18}$ using THF–buffer gave $N=13700$ and $N_{df}=7600$ for pyridine ($A_s=1.58$). It is interesting to consider the question of how large a value of the asymmetry factor should be accepted? Although serious peak tailing leads to a number of difficulties, for instance in determining the end of a peak for quantitation, the loss of column efficiency for peaks with more moderate tailing illustrated by these results suggests that this might be used as a basis for determining "acceptable" peak asymmetry. However, to make judgements on such a basis would require a direct correlation of $A_s$ and column efficiency. While it is evident from Table 1 that a close correlation between these values does indeed exist, especially and perhaps unsurprisingly when efficiency is measured using the Dorsey–Foley procedure, the asymmetry factor does not invariably predict the column efficiency. Thus, Inertsil ODS-2 and Purospher both give $A_s=1.3$ for diphenhydramine using THF, although the former gives $N_{df}$ over twice the latter. Our previous studies [1] showed the efficiency for benzene obtained on these two columns was very similar (over 20 000 plates). Thus, we believe column efficiency values should always be reported alongside asymmetry factors. A rule of thumb for asymmetry measurements would therefore seem dangerous, although it does appear from consulting Table 1 that true column efficiencies are seriously compromised in most cases when $A_s$ exceeds about 1.5. A more important observation from the figures, however, is the generally serious effect of peak tailing on the true column efficiency, consequently on resolution, and this observation stresses the importance of reducing peak tailing to a minimum either by column or mobile phase optimisation.

First, the differences in performance dependent on modifier choice were considered; however, Table 1 shows little variation in $A_s$ between methanol and acetonitrile. The average $A_s$ for all eight columns and all eight solutes using methanol–buffer was 1.92, compared with 1.84 for acetonitrile–buffer. Neither does this average conceal significant differences for any individual column. Whereas at pH 7.0, Inertsil ODS gave mean column $A_s=2.33$ for methanol–buffer and 4.03 for acetonitrile–buffer [1], the comparable figures for this column at pH 3.0 are 1.48 and 1.40, respectively. Considering the mean values for a given solute averaged over all eight columns, the variation in $A_s$ between methanol–buffer and acetonitrile buffer only exceeds $\pm 0.2$ for pyridine, which gives values of 3.24 and 2.52, respectively. Even a detailed examination of the figures for individual analyte/column combinations reveals few instances where interchange of these two modifiers yields worthwhile effects, except possibly for pyridine. It is difficult to discern any trend in the pyridine results – whereas six columns gave somewhat improved $A_s$ using acetonitrile, two gave improved results using methanol. Overall, acetonitrile gave the expected increase in average column efficiency, which can be attributed to increased analyte diffusivity in the lower viscosity mobile phase [13]. However, in general agreement with pH

Table 1
Column performance data for eight basic solutes on eight different columns

| | Pyridine | | | | Nicotine | | | | Amphetamine | | | | Codeine | | | | Quinine | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $k'$ | $N$ | $N_{df}$ | $A_s$ | $k'$ | $N$ | $N_{df}$ | $A_s$ | $k'$ | $N$ | $N_{df}$ | $A_s$ | $k'$ | $N$ | $N_{df}$ | $A_s$ | $k'$ | $N$ | $N_{df}$ | $A_s$ |
| Inertsil ODS | 0.25[a] | 10200 | 2650 | 2.27 | 0.25 | 12000 | 6160 | 1.73 | 2.60 | 10700 | 7790 | 1.21 | 0.89 | 10200 | 7830 | 1.18 | 12.30 | 8800 | 7820 | 1.07 |
| | 0.05[b] | 17300 | 11300 | 1.38 | 0.05 | 13200 | 7810 | 1.57 | 2.27 | 14800 | 10200 | 1.36 | 0.68 | 14600 | 12100 | 1.12 | 4.67 | 10800 | 8620 | 1.17 |
| | 0.13[c] | 7340 | 5060 | 1.55 | 0.19 | 15300 | 7230 | 1.81 | 2.54 | 15500 | 9100 | 1.51 | 1.56 | 14600 | 13100 | 1.01 | 7.34 | 14200 | 12800 | 1.03 |
| Inertsil ODS-2 | 0.19 | 6930 | 2820 | 2.11 | 0.12 | 8370 | 4600 | 1.82 | 1.69 | 14400 | 9770 | 1.48 | 0.47 | 10100 | 7280 | 1.42 | 5.22 | 13300 | 11200 | 1.27 |
| | 0.02 | 12200 | 7080 | 1.74 | 0.02 | 9350 | 4310 | 2.00 | 1.63 | 15300 | 11300 | 1.46 | 0.45 | 11800 | 9170 | 1.34 | 2.20 | 12100 | 9670 | 1.36 |
| | 0.07 | 10900 | 6650 | 1.31 | 0.08 | 11800 | 6020 | 1.91 | 1.56 | 16300 | 10400 | 1.62 | 0.85 | 14900 | 12500 | 1.27 | 3.62 | 16700 | 13800 | 1.34 |
| Inertsil ODS-3 | 0.26 | 7420 | 2320 | 2.53 | 0.24 | 9460 | 4880 | 1.91 | 2.82 | 11200 | 7150 | 1.64 | 0.95 | 8500 | 6990 | 1.28 | 10.57 | 10400 | 7810 | 1.51 |
| | 0.02 | 10200 | 6680 | 1.53 | 0.01 | 7230 | 4620 | 1.76 | 1.79 | 13000 | 10700 | 1.31 | 0.52 | 9420 | 7850 | 1.22 | 3.03 | 9150 | 7450 | 1.30 |
| | 0.11 | 13500 | 6700 | 1.70 | 0.16 | 10400 | 5470 | 1.81 | 2.50 | 14900 | 9480 | 1.67 | 1.61 | 13500 | 12200 | 1.17 | 7.04 | 13800 | 11.400 | 1.37 |
| Kromasil $C_{18}$ | 0.30 | 2980 | 293 | 5.35 | 0.22 | 13900 | 5330 | 2.12 | 2.76 | 10200 | 4380 | 2.03 | 0.82 | 9540 | 6840 | 1.37 | 8.08 | 10300 | 7410 | 1.44 |
| | 0.04 | 14900 | 7560 | 1.64 | 0.03 | 11900 | 7870 | 1.43 | 1.96 | 13500 | 9830 | 1.36 | 0.53 | 11600 | 9820 | 1.14 | 2.78 | 10500 | 8760 | 1.19 |
| | 0.15 | 7450 | 928 | 4.39 | 0.16 | 13300 | 3980 | 2.33 | 2.44 | 11600 | 3680 | 2.60 | 1.39 | 13200 | 10900 | 1.20 | 5.60 | 14200 | 10300 | 1.44 |
| Kromasil $C_8$ | 0.28 | 2890 | 229 | 6.46 | 0.21 | 16800 | 6500 | 1.78 | 2.57 | 13800 | 5910 | 2.08 | 0.71 | 12600 | 8340 | 1.58 | 8.43 | 11900 | 8630 | 1.48 |
| | 0.04 | 18400 | 11400 | 1.48 | 0.03 | 15800 | 11500 | 1.31 | 2.07 | 18300 | 14200 | 1.32 | 0.54 | 16500 | 13600 | 1.15 | 2.67 | 13600 | 11300 | 1.18 |
| | 0.13 | 9190 | 1190 | 4.36 | 0.15 | 16800 | 7860 | 1.83 | 2.48 | 16300 | 8650 | 1.85 | 1.32 | 18100 | 15500 | 1.17 | 5.48 | 16300 | 12600 | 1.41 |
| Symmetry $C_{18}$ | 0.54 | 4160 | 944 | 3.13 | 0.45 | 4170 | 490 | 5.61 | 2.38 | 11300 | 6380 | 1.59 | 0.87 | 9690 | 6790 | 1.38 | 6.60 | 9000 | 5250 | 1.77 |
| | 0.31 | 12400 | 5470 | 2.01 | 0.33 | 3930 | 543 | 5.58 | 2.37 | 12900 | 9430 | 1.37 | 0.84 | 11000 | 8380 | 1.28 | 3.28 | 8000 | 3290 | 2.49 |
| | 0.37 | 9370 | 3400 | 1.92 | 0.39 | 5880 | 648 | 5.50 | 2.32 | 13500 | 7240 | 1.72 | 1.43 | 13100 | 10500 | 1.23 | 5.17 | 11100 | 5130 | 2.26 |
| Superco ABZ+ | 0.03 | 6890 | 3660 | 1.96 | 0.00 | 13800 | 6020 | 2.03 | 0.67 | 11700 | 7000 | 1.65 | 0.19 | 10700 | 7250 | 1.49 | 1.83 | 7610 | 5560 | 1.36 |
| | 0.00 | 16600 | 10100 | 1.65 | 0.00 | 14300 | 7870 | 1.70 | 0.66 | 14200 | 9600 | 1.51 | 0.15 | 12700 | 9040 | 1.42 | 0.90 | 10100 | 7520 | 1.30 |
| | 0.00 | 18100 | 7530 | 2.16 | 0.00 | 16100 | 6780 | 2.03 | 0.62 | 13800 | 7060 | 1.85 | 0.34 | 18000 | 11700 | 1.67 | 1.40 | 10500 | 7780 | 1.35 |
| Purospher | 0.04 | 6960 | 2780 | 2.08 | 0.00 | 8260 | 3450 | 2.05 | 0.29 | 7160 | 2880 | 2.08 | 0.07 | 5300 | 3070 | 1.66 | 2.17 | 2540 | 941 | 2.27 |
| | 0.00 | 13700 | 7600 | 1.58 | 0.00 | 11000 | 6140 | 1.70 | 0.19 | 11000 | 7400 | 1.44 | 0.00 | 8230 | 5100 | 1.48 | 0.48 | 5590 | 4140 | 1.38 |
| | 0.00 | 8990 | 2440 | 2.77 | 0.00 | 10700 | 3930 | 2.20 | 0.20 | 9700 | 4600 | 1.80 | 0.11 | 9100 | 5810 | 1.49 | 0.92 | 4200 | 2200 | 2.01 |
| Mean solute | | 6054 | 1962 | 3.24 | | 10845 | 4679 | 2.38 | | 11308 | 6408 | 1.72 | | 9579 | 6799 | 1.42 | | 9231 | 6828 | 1.52 |
| | | 14463 | 8399 | 1.63 | | 10839 | 6333 | 2.13 | | 14125 | 10333 | 1.39 | | 11981 | 9383 | 1.27 | | 9980 | 7594 | 1.42 |
| | | 10605 | 4237 | 2.52 | | 12535 | 5240 | 2.43 | | 13950 | 7526 | 1.83 | | 14313 | 11526 | 1.28 | | 12625 | 9504 | 1.53 |

| | Benzylamine | | | | Diphenyhydramine | | | | Nortriptyline | | | | Mean column | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $k'$ | $N$ | $N_{df}$ | $A_s$ | $k'$ | $N$ | $N_{df}$ | $A_s$ | $k'$ | $N$ | $N_{df}$ | $A_s$ | $N$ | $N_{df}$ | $A_s$ |
| Inertsil ODS | 0.79 | 15900 | 7370 | 1.54 | 2.64 | 9280 | 5030 | 1.71 | 8.62 | 9660 | 8410 | 1.16 | 10843 | 6633 | 1.48 |
| | 0.53 | 14800 | 8260 | 1.57 | 3.11 | 10600 | 8860 | 1.15 | 12.39 | 9220 | 8490 | 1.07 | 13165 | 9455 | 1.30 |
| | 0.67 | 15900 | 8100 | 1.63 | 5.73 | 14400 | 10500 | 1.39 | 14.62 | 12100 | 9890 | 1.30 | 13668 | 9473 | 1.40 |
| Inertsil ODS-2 | 0.46 | 12000 | 6550 | 1.79 | 1.72 | 12100 | 8960 | 1.39 | 5.02 | 13900 | 10900 | 1.39 | 11388 | 7760 | 1.58 |
| | 0.39 | 12100 | 6700 | 1.79 | 3.08 | 15000 | 12800 | 1.32 | 12.02 | 17700 | 13900 | 1.51 | 13194 | 9366 | 1.57 |
| | 0.41 | 12000 | 8680 | 1.50 | 4.18 | 20200 | 15100 | 1.51 | 10.80 | 19400 | 13600 | 1.66 | 15275 | 10844 | 1.52 |
| Inertsil ODS-3 | 0.80 | 10200 | 5930 | 1.74 | 2.73 | 9810 | 6660 | 1.60 | 9.10 | 11600 | 7770 | 1.65 | 9824 | 6189 | 1.73 |
| | 0.42 | 11900 | 8570 | 1.44 | 2.90 | 10600 | 8910 | 1.28 | 12.01 | 13000 | 11000 | 1.32 | 10563 | 8223 | 1.40 |
| | 0.65 | 12800 | 6210 | 1.92 | 6.08 | 17700 | 13200 | 1.54 | 15.50 | 17500 | 12900 | 1.62 | 14263 | 9695 | 1.60 |
| Kromasil $C_{18}$ | 0.77 | 11200 | 4560 | 2.06 | 2.75 | 7070 | 2270 | 2.64 | 9.25 | 7710 | 3460 | 2.17 | 9113 | 4318 | 2.40 |
| | 0.46 | 14300 | 9150 | 1.44 | 2.89 | 9710 | 7800 | 1.28 | 11.61 | 10800 | 8820 | 1.31 | 12151 | 8701 | 1.35 |
| | 0.64 | 14100 | 5370 | 2.11 | 6.23 | 11800 | 3870 | 2.98 | 15.81 | 10800 | 3430 | 3.09 | 12056 | 5307 | 2.52 |
| Kromasil $C_8$ | 0.74 | 14300 | 6000 | 2.03 | 2.27 | 13100 | 7800 | 1.79 | 6.67 | 13500 | 9640 | 1.50 | 12361 | 6631 | 2.34 |
| | 0.51 | 19100 | 14600 | 1.30 | 3.96 | 14400 | 11300 | 1.28 | 15.15 | 13800 | 12000 | 1.27 | 16238 | 12488 | 1.29 |
| | 0.65 | 19100 | 8820 | 1.90 | 6.26 | 18500 | 12600 | 1.65 | 14.57 | 16600 | 12400 | 1.52 | 16361 | 9953 | 1.96 |
| Symmetry $C_{18}$ | 0.87 | 10300 | 3690 | 2.13 | 2.54 | 10000 | 7060 | 1.39 | 7.90 | 11000 | 8530 | 1.28 | 8703 | 4892 | 2.29 |
| | 0.77 | 13700 | 9500 | 1.37 | 4.90 | 10900 | 7430 | 1.60 | 19.72 | 12100 | 8420 | 1.64 | 10616 | 6558 | 2.17 |
| | 0.77 | 12900 | 5290 | 1.97 | 5.50 | 15600 | 10900 | 1.53 | 13.81 | 15700 | 11600 | 1.54 | 12144 | 6839 | 2.21 |
| Supelco ABZ+ | 0.18 | 11300 | 5320 | 1.97 | 0.55 | 10500 | 7130 | 1.49 | 1.80 | 9680 | 6980 | 1.41 | 10273 | 6115 | 1.67 |
| | 0.11 | 15000 | 9400 | 1.57 | 1.36 | 11900 | 8900 | 1.37 | 5.60 | 12000 | 9630 | 1.32 | 13350 | 9008 | 1.48 |
| | 0.12 | 13600 | 6700 | 1.82 | 1.27 | 14600 | 10400 | 1.49 | 3.41 | 14000 | 10400 | 1.49 | 14838 | 8544 | 1.73 |
| Purospher | 0.00 | 4570 | 2060 | 1.67 | 0.35 | 6630 | 3990 | 1.57 | 1.45 | 5140 | 3270 | 1.56 | 5820 | 2805 | 1.87 |
| | 0.00 | 4290 | 3280 | 1.29 | 0.66 | 8440 | 6320 | 1.31 | 3.08 | 9160 | 7350 | 1.25 | 8926 | 5916 | 1.43 |
| | 0.00 | 5370 | 2470 | 1.42 | 0.75 | 11800 | 8950 | 1.30 | 2.22 | 10200 | 7830 | 1.32 | 8758 | 4781 | 1.79 |
| Mean solute | | 11221 | 5185 | 1.87 | | 9811 | 6113 | 1.70 | | 10300 | 7370 | 1.52 | 9794 | 5668 | 1.92 |
| | | 13149 | 8683 | 1.47 | | 11444 | 9040 | 1.32 | | 12200 | 9951 | 1.34 | 12273 | 8714 | 1.50 |
| | | 13221 | 6455 | 1.78 | | 15575 | 10690 | 1.67 | | 14500 | 10256 | 1.69 | 13415 | 8179 | 1.84 |

[a] Mobile phase methanol–0.0321 *M* phosphate buffer pH 3.0 (30:70, v/v). [b] Mobile phase THF–0.0243 *M* phosphate buffer pH 3.0 (7.5:92.5, v/v). [c] Mobile phase acetonitrile–0.0265 *M* phosphate buffer pH 3.0 (15:85, v/v)

For nortriptyline and diphenhydramine, the corresponding mobile phases were: [a] Methanol–0.0321 *M* phosphate pH 3.0 (55:45, v/v). [b] THF–0.0243 *M* phosphate buffer pH 3.0 (20:80, v/v). [c] Acetonitrile–0.0265 *M* phosphate buffer pH 3.0 (28:72, v/v).

Note for convenience of buffer preparation, the phosphate concentration was not adjusted to reproduce the overall concentration in the corresponding mobile phases above. For other conditions, see Section 2.

7.0 results, THF gave improvement in $A_s$ for most columns (average for eight solutes) and each individual solute (average for eight columns). The overall average asymmetry for THF ($A_s$=1.50 for all eight columns and eight solutes) is significantly better than for methanol or acetonitrile, although this result is amplified by the exceptional improvement in performance of both Kromasil columns when used with THF, which was also found at pH 7.0. Furthermore, the overall average column efficiency is highest with THF, at least when the measurement is made by the Dorsey–Foley method ($N_{df}$=8714). However, we have already commented on the practical drawbacks and hazards associated with use of THF [1].

Secondly, column rankings based on overall mean $A_s$ for the solutes with the three mobile phases were considered at pH 3.0, and compared with results at pH 7.0. As discussed previously [1], we believe that column assessment should be based on as wide a range of compounds as possible, due to significant variations which can occur for a given column with different solutes. However, differences in $k'$ (and thus retention/peak volume) for a given solute could give rise to variations in the effect of extra-column dispersion on the system. Thus, we used a low dispersion instrument with small injector and detector volumes, in conjunction with relatively large diameter columns to minimise these effects. The reduction in column efficiency produced by the equipment was calculated as less than 10% in the worst case [14]. Furthermore, $k'$ for a given solute appeared reasonably constant from column to column (Table 1), suggesting a similar influence of any extra column effects. However, both the Supelco and Purospher columns gave similar but lower $k'$; for the former, high efficiency was maintained despite low $k'$, although the latter gave relatively low efficiency. These results confirm the rather low efficiency shown by the Purospher column is not due to extra column dispersion. For the Supelco column, low $k'$ is attributable (as with its results at pH 7.0) to electrostatic shielding of the phase. Purospher gave similar $k'$ to other columns at pH 7.0; it is possible that its lower $k'$ and $N$ (despite median values for $A_s$) at pH 3.0 may be partially attributable to reduced phase wetting. Nevertheless, there is a further problem (other than that of extra-column effects) with low $k'$ values which must be considered. According to the work of Snyder's group [15], the strong retention of protonated bases on ionised silanols, with subsequent overloading of the relatively small number of these sites, is a major contributor to band broadening and tailing. The sample capacity of the strong sites was estimated to be as little as 1% of the capacity of the weak sites. If the overloaded silanol model is applicable, then as $k'$ decreases, a much larger sample weight could be injected before tailing occurs. Further, the model would suggest that below $k'$=1.0, this permissible sample weight increases steeply; thus introducing a variable into the data. To overcome this problem, it is possible to evaluate columns by varying the percentage of organic modifier to give the same value of $k'$ for each column and a given solute. However, a difficulty with this alternative approach is that varying the modifier concentration can affect the degree of solvation of the bonded phase and thus the penetration of sample components to the column surface, also the ionisation of the buffer and analyte, introducing other variables. It should be noted that the mass of sample injected in the present study was low (200 ng in every case). Our preliminary calculations, based on an estimate of the apparent column saturation capacity $w_s$, would indicate that this mass of sample is too small to give rise to significant variation in $N$ dependent on the somewhat different $k'$ values for a particular solute shown by the different columns. Furthermore, the general similarity of $k'$ between different columns shown for a given solute/mobile phase combination again means that the situation is quite similar for each column. We believe these considerations are complex, and warrant a separate detailed investigation. We do not wish to present a formal ranking of the columns based, for example, on mean asymmetry factors; besides the fact that rankings can change dependent on test compounds used, manufacturers may improve products, sometimes indicating this by name changes. For example, an end-capped version of the Purospher product is now available (Purospher-E) which according to manufacturers' data, also has a lower metal content of the silica than the column we tested. Rankings should also take into account column efficiency, and it could be argued that efficiency as calculated by the Dorsey–Foley equation might be a better evaluation parameter; rankings could change dependent on the assessment criteria. Furthermore, despite much tight-

er quality control limits achieved by manufacturers, batch to batch variations still occur so some columns may be unrepresentative of performance with strongly basic compounds; however, our tests with the modified Engelhardt mixture [1], showed that at the very least, all columns were well-packed and undamaged, yielding similar very high efficiencies and symmetrical peaks not only for neutral compounds, but also for phenol and aniline. Thus, despite these cautions, it still seems valid and of interest at least to *compare* the ranking of the (same) columns at the two pH values. At pH 7.0, wide differences between column performance were noted; for example, the best column (Inertsil ODS-3) gave a mean $A_s$ of 2.14 for all three modifiers and all nine solutes studied, which was less than half the value given by the worst column, even though all columns were pre-selected by their recommendation for use with bases. Table 1 indicates that inter-column variations in performance are considerably less at pH 3.0. This result is in agreement with an observation in a very recent study [16]. In general, the ranking of a column at pH 3.0 seems similar to its ranking at pH 7.0; for example, Inertsil ODS-3 shows very good performance at both values. However the ranking of Inertsil ODS, which was quite high at pH 7.0, is significantly enhanced at pH 3.0, due largely to improved performance with acetonitrile at pH 3.0. Alternatively, the ranking of Symmetry $C_{18}$ at pH 3.0 is not as favourable as would have been expected from the good results obtained at pH 7.0. Finally, a comparison of Fig. 1 and Fig. 2 (from [1]) shows (unsurprisingly when considering the results above) that the bar graphs generated, and thus column rankings for analysis of a given solute also give different results at pH 3.0 and pH 7.0. For example, while Symmetry $C_{18}$ gave excellent peak shape for quinine relative to other columns at pH 7.0, it would not appear to be the best choice for analysis of this solute at pH 3.0. Conversely, Inertsil ODS gave relatively poor performance for nortriptyline at pH 7.0, but probably the best performance for this solute at pH 3.0. Thus, there is not necessarily a direct relationship between the performance of a given column at different pH, either when considering its ranking for individual solutes, or when based on the average asymmetry of a range of compounds.

Thirdly, the question of solute-dependent performance for a given column can be considered. As mentioned above, some caution is necessary in interpretation of the data due to the stronger mobile phases used for nortriptyline and diphenhydramine. Individual solute asymmetry averaged over the eight columns at pH 3.0 shows a much reduced spread of values than obtained at pH 7.0. Mean solute $A_s$ for amphetamine ($pK_a$=9.9), codeine ($pK_a$=8.0), quinine ($pK_a$=8.5), benzylamine ($pK_a$=9.3) diphenhydramine ($pK_a$=9.0) and nortriptyline ($pK_a$=10.0) is rather similar, suggesting a smaller influence of solute on column performance. This may be because these solutes have a $pK_a$ of 8 or higher and basic groups responsible for tailing would be expected to be fully protonated at pH 3.0. At pH 7.0, considering also the strong influence of larger concentrations of organic modifier, the compounds might be expected to have different degrees of protonation. Pyridine ($pK_a$=5.2) gave significantly higher mean solute asymmetry than the other solutes at pH 3.0. It is more difficult at pH 3.0 than at pH 7.0 to find instances where results for two different solutes give contradictory indications of column quality. Most columns follow the general pattern of poor results for pyridine, but reasonable results for the other solutes (Fig. 1). Nevertheless, evaluation of Symmetry $C_{18}$ on the basis of results for nicotine would give an unfairly pessimistic indication of its quality; Kromasil $C_8$ gave relatively very good results for nicotine but poor results for pyridine (except using THF), again indicating solute-dependent performance [1,5]. Thus, as at pH 7.0, choice of test compounds can influence column ranking and the use of a wide range of different bases is again recommended.

Finally, the peak shape of a given solute at pH 3.0 can be compared with previous results at pH 7.0. Apart from pyridine, all solutes show considerably improved mean $A_s$ (average for eight columns) at pH 3.0, supporting the general recommendation for the analysis of bases at low pH [6]. In some cases, the reductions in $A_s$ are very large, for instance, nortriptyline with pH 3.0 buffer shows mean $A_s$ 1.52, 1.34 and 1.69 with methanol, THF and acetonitrile respectively, whereas the corresponding $A_s$ recorded at pH 7.0 were 4.49, 3.44 and 5.17. Indeed, nortriptyline does not seem a particularly discriminating test compound at pH 3.0 for these relatively inert columns, whereas wide inter-column differences were recorded for this solute at pH 7.0. The per-

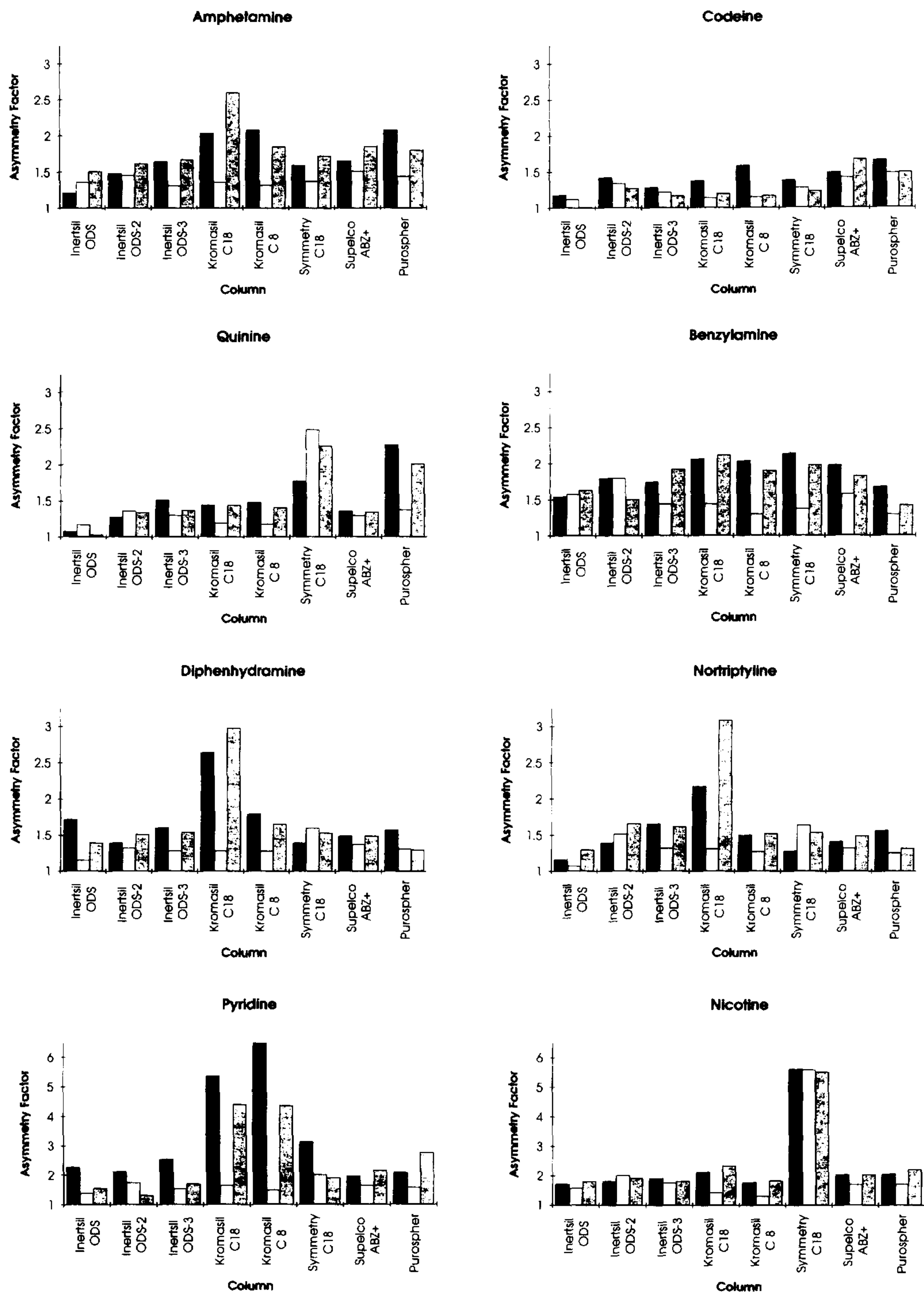


Fig. 1. Bar graphs of asymmetry factor for eight basic solutes with eight different RP columns using (black bars) methanol–phosphate buffer pH 3.0; (white bars) THF–phosphate pH 3.0; and (shaded bars) acetonitrile–phosphate pH 3.0. Note the $y$ axis scales for pyridine and nicotine differ by a factor of 2 from those for the other compounds. For other details, see Table 1 and Section 2.

formance for quinine shown in Table 1 compares very favourably with results we obtained about 10 years ago for this compound under very similar conditions [17]. In that study, the average $A_s$ for quinine using six different columns with acetonitrile–0.1 $M$ phosphate buffer pH 3.0 (15:85, v/v) was 3.44, compared with 1.53 in the present study (Table 1), showing the improvements which have been achieved by column manufacturers over this period. However, it is interesting to note that the stationary phases tested [17] are still in widespread use today. Comparing again the results in Table 1 with the previous study [1], it can be seen that exceptionally, pyridine gave similar or even worse performance at pH 3.0 (mean $A_s$ 3.24, 1.63, 2.52 with methanol, THF and acetonitrile, respectively), than at pH 7.0 (mean $A_s$ 2.18, 1.66, 2.74, respectively). Pyridine is unprotonated at pH 7.0, whereas all other analytes are at least partially protonated at this pH. Thus, it may be preferable, for those compounds with sufficiently low $pK_a$, to analyze them in their unprotonated form [18]; with the establishment of silica columns with higher pH stability, this may be a possibility even for higher $pK_a$ compounds, although more work is required in this area [19]. It is important to consider the influence of organic solvent on $pK_a$ values. It was shown by UV spectroscopic measurements [2,20] that it was necessary to use pH 3.2 phosphate buffer (pH measured prior to organic solvent addition) to half protonate pyridine when mixed with 55% methanol. With Inertsil ODS, use of 40% methanol instead of 30% methanol in combination with pH 3.0 buffer reduced $A_s$ from 2.27 to 1.56, whereas methanol–pH 7.0 buffer gave a value of 1.25 [1]. This may be due to a significant reduction of the protonation of pyridine in 40% methanol, which would be unexpected by consideration of its aqueous $pK_a$. Finally, a comparison of results at different pH shows that while pyridine is the most challenging probe at pH 3.0 whereas nortriptyline appears relatively undemanding, a virtually opposite result is obtained at pH 7.0. Thus, care is necessary in the choice of test compounds for use at a particular pH.

## 4. Conclusions

The difference in column performance caused by interchange of the common RP organic modifiers is considerably less at acid pH than at neutral pH. For a range of solutes, acetonitrile and methanol generated similar peak asymmetries, with the former generally preferred due to reduced viscosity and higher column efficiency. However, in accord with results at pH 7.0, THF gave significantly smaller $A_s$ than acetonitrile or methanol, and also the highest column efficiency when estimated by the Dorsey–Foley procedure.

At pH 3.0, some columns clearly performed better than others when an average value of $A_s$ is considered for a range of basic solutes. However, the inter-column differences were not as marked as at pH 7.0. Whereas, in general columns which performed well at neutral pH also performed well at acid pH, (and a similar conclusion can be reached for poorer columns), there is an indication that column rankings will change depending on the pH at which the test is carried out. This result obviously applies to individual solutes and a column which will produce the best result for a given compound at one pH will not automatically be the best at a different pH.

The spread of asymmetry values for different solutes on a given column is considerably reduced at pH 3.0 and also, the performance of columns is not as solute-dependent at pH 3.0 as it is at pH 7.0. However, it is still possible to choose solutes which would give particularly good or particularly bad performance on a given column. As before, testing columns with a wide range of unrelated basic compounds is recommended.

Most of the solutes studied (relatively high $pK_a$) gave improved results at low pH; for instance the analysis of nortriptyline, which provides a severe challenge for many columns at pH 7.0 appeared trivial at pH 3.0. However, it is possible that low $pK_a$ solutes (for example pyridine) are better analyzed in the unprotonated state, where this is possible within the pH limits of the column. Basic compounds which present the greatest challenge to columns at one pH are not necessarily the most challenging at any pH. Finally, comparison with results obtained about 10 years ago shows a marked improvement in the inertness of RP materials. Nevertheless, use of the Dorsey–Foley equation to approximate the true column efficiency indicates that the effect of peak tailing on resolution is still considerable, even under the generally favourable conditions of low pH.

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