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Rationalisation of unusual changes in efficiency and retention with temperature shown for bases in reversed-phase high-performance liquid chromatography at intermediate pH

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Abstract

Despite the possibility of poorer peak shapes, analysis of pharmaceuticals and other bases using reversed-phase high-performance liquid chromatography (RP-HPLC) at intermediate pH gives useful increases in retention, selectivity and column loading capacity compared with low pH. Retention times of some bases showed anomalous increases with column temperature. Peak shapes for bases improved significantly at elevated temperature (up to 70 °C), with the weakest bases studied (pK_a ∼ 8) giving greater improvement than the strongest bases (pK_a ∼ 10). In contrast, quaternary ammonium compounds showed reduced retention with increasing column temperature (normal behaviour) and only modest improvements in peak shape. Considering these results, and pK_a measurements of the bases made using capillary electrophoresis, it appears that increases in retention and improvement in efficiency may be influenced significantly by reduction in the pK_a of bases with temperature, leading to reduction in protonation. It is less likely that efficiency improvements are due to the speeding up of the kinetics of silanol ion exchange, at least in the temperature range studied here.

Keywords: Retention behaviour; Temperature effects; pH; Column efficiency

1. Introduction

Separation scientists continually attempt to improve the reversed-phase high-performance liquid chromatography (RP-HPLC) analysis of bases, which is a difficult challenge due to the multiplicity of factors capable of influencing retention and peak shape [1]. The main problem probably lies with silanols that remain on the silica surface after the bonding process which introduces ligands such as octadecylsilyl (ODS) groups on to the phase [2,3]. These silanols may be partially or fully ionised over the pH range of operation of silica-based RP columns. Moderately strong bases can be partially or fully protonated over the same pH range. Thus, ionic interactions are possible in addition to the relatively weak hydrophobic interactions with ODS ligands, giving in some cases severe peak tailing [4–6].

Considerable advances have been made in improving the inertness of stationary phases by column manufacturers, based on knowledge of the factors that influence column activity gained from the fundamental studies of Kirkland and co-workers [7,8]. Nevertheless, the separation of bases is still mostly performed at low pH where silanol ionisation is suppressed. However, low pH may produce reduced retention of the relatively hydrophilic protonated bases; in addition, overloading occurs much more readily at low pH, probably due to mutual repulsion of protonated species [9–11]. In contrast, retention, selectivity and loading capacity of columns can be improved at pH 7, although peak shapes can be inferior.

Elevated temperature was shown to give substantial improvement in peak shapes for bases, with improvements relatively greater for analyses at pH 7 compared with pH 3 [12,13]. This finding indicated that the improvement could not be attributed entirely to increased solute diffusion at higher temperatures. Such an effect should influence results equally at both pH values. Guiochon and co-workers [14] suggested that peak shape improvements with temperature could be the result of speeding up the kinetics of the slow interactions in a dual retention process (e.g. the kinetics of

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silanol ionic interactions in RP-HPLC, although this system was not specifically studied). However, an additional factor is that the pK_a of bases generally decreases with increasing temperature [15–19]. At constant pH, as the strength of the base decreases (pK_a becomes smaller), bases will become less protonated (increasingly neutral) which will result in increased retention by hydrophobic processes. Alternatively, ionic retention caused by interactions between solute and silanols should be reduced. However, hydrophobic retention is usually the dominant retention mechanism using ODS phases, especially when pure ("Type B") silicas with low silanol activity are considered [12]. Supporting evidence for the latter hypothesis is shown by the anomalous increase in retention with temperature shown by some bases; normally, solute retention decreases at elevated temperature [12,20,21].

The aim of the present study was to rationalise the unusual increases in retention and column efficiency with temperature for bases at pH 7 using a range of moderately strong bases with a variety of pK_a values. Our previous studies of pK_a determination in aqueous–organic solutions using capillary electrophoresis have generated much useful data, which enables the estimation of base-protonation in the mobile phase at any temperature employed [18,22]. We have included in the present study quaternary ammonium compounds (R_4N^+), which can undergo cationic interactions with silanols but in contrast with bases, do not change their ionisation state with temperature.

2. Experimental

An 1100 HPLC system with Chemstation 8.0 was used (Agilent, Waldbronn, Germany). The UV detector (5 μl cell) was set to 210 nm. Connection tubing of minimum length with 0.01 cm i.d. was used in order to limit extra-column volume. The columns employed were Inertsil ODS 3V (5 μm) 25 cm × 0.46 cm i.d., surface area 430 m^2/g, %C = 15% (GL Sciences, Tokyo, Japan) and X-Terra RP_{18} (5 μm) 15 cm × 0.46 cm i.d., 170 m^2/g, %C = 15% (Waters, Milford, USA). Columns were equilibrated for at least 1 h prior to first injection when using different mobile phases and at least 0.5 h for thermal equilibration when using the same mobile phase. Temperature control was achieved by immersing the column and the valve injector with 5 μl loop (Rheodyne model 7725, Cotati USA) in a thermostatted water bath (model W14, Grant, Cambridge, UK). The mobile phase was preheated with a 3 m × 0.5 mm i.d. length stainless steel tubing to avoid temperature mismatch effects within the column [23]. The preheat tubing linked pump and injector, avoiding extra-column band broadening. Column dead time was determined at each temperature using uracil. Column efficiency was measured using the Dorsey–Foley equation $N_{df} = 41.7[t_r/w_{0.1}]^2/[A_s + 1.25]$, which was shown also by Kirkland and co-workers to give a better estimation of efficiency for asymmetric peaks [24,25]. $w_{0.1}$ is the peak width and A_s the asymmetry factor, the ratio of widths of the rear and the front sides of the peak, both measured at 10% peak height. All results were the average of at least duplicate injections.

Sample solutions were prepared in mobile phase at a concentration of about 0.02 g/l. The mobile phase was acetonitrile–phosphate buffer (40:60, v/v) and was premixed prior to analysis. The ${}^{s}_{w}$pH was measured in the aqueous–organic admixture. The ${}^{s}_{w}pK_a$ values of the bases at 25 °C and ionic strength 50 mM were obtained from [22] or for amitriptyline and protriptyline were determined at 30 °C and ionic strength 50 mM using a CE procedure similar to that described therein. ${}^{s}_{w}pK_a$ values at different temperatures were estimated using a temperature coefficient of −0.03 ${}^{s}_{w}pK_a$ units/K [18]. The pH meter used was an MP 220 from Mettler (Toledo, Spain) equipped with a Gelplas combination pH electrode from BDH (Poole, UK) with a single nylon junction containing saturated KCl. The meter was calibrated at 30 °C using aqueous buffer solutions of hydrogen phthalate and phosphate for pH 4.0 and 7.0, respectively. pH measurements were routinely performed at 30 °C. Mobile phases for use at higher temperature were produced taking into account the temperature coefficient of the phosphate buffer at its second pK_a 7.21 in aqueous solution (−0.0028 K^{-1} [17]). ${}^{s}_{w}$pH was checked rigorously at the temperature of operation of the mobile phase; good agreement was found with experimental values of ${}^{s}_{w}$pH within ±0.01 pH unit of the calculated value. These results suggest that temperature coefficients for phosphate buffer in the partially aqueous buffer are similar to those reported in the literature for aqueous buffer. However, we have not confirmed this result for other buffer systems. Despite the small temperature coefficient, buffers prepared for a certain temperature were only used in an interval of 10 K, e.g. the buffer prepared for use at 50 °C was used within 45–55 °C. Buffers and samples were filtered using membranes of 0.2 μm (Millipore, Molsheim, France) and 0.45 μm pore sizes (Chromacol, Herts, UK).

3. Results and discussion

Fig. 1 shows Van Deemter plots for two quaternary ammonium compounds obtained on Inertsil ODS 3V in the range 30–60 °C using a mobile phase with ${}^{s}_{w}$pH 7.8. The highest flow rates used in this experiment were chosen conservatively in such a way so as not to exceed the maximum allowed back-pressure of the column. For both solutes, the optimum flow rate increases with increasing temperature, as expected. This result can be attributed to the balance of increased solute diffusivity, which decreases the mass transfer term (*C* term) important at high flow rate, but increases longitudinal diffusion (*B* term) important at lower flow rate [13,26]. At the flow rate used for the remainder of the study (1 ml/min), the column performs better at higher temperature. The results are similar to those shown previously for bases [13]. If flow rates at or very near to the optimum flow

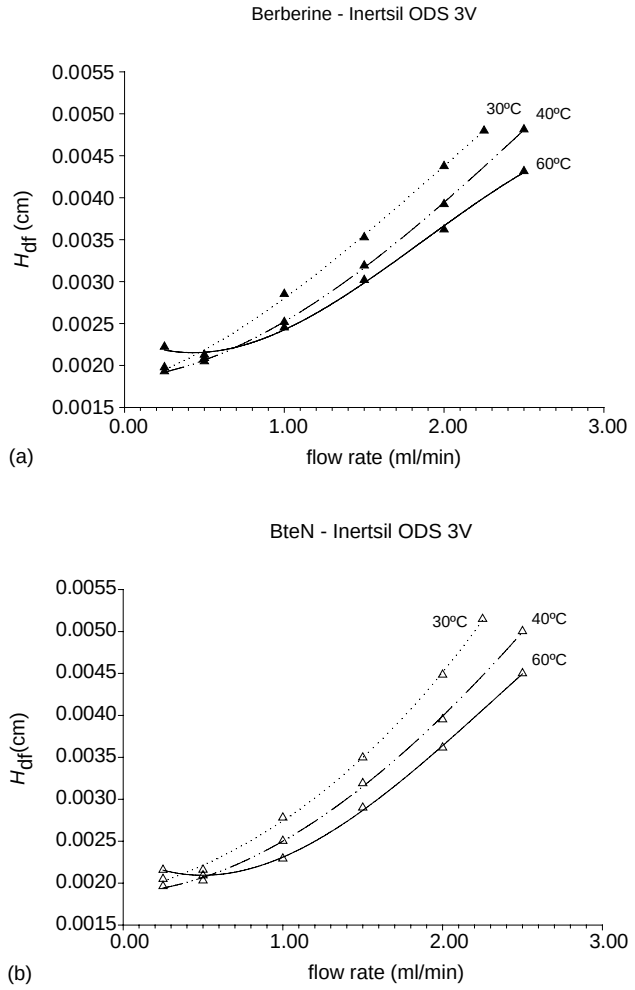

Fig. 1. Van Deemter plots, of plate height H_{df} against flow rate at temperatures between 30 and 60 °C of quaternary ammonium compounds berberine chloride (▲) and benzyl triethyl ammonium chloride (BteN, △). Mobile phase: $^{s}_{w}$pH 7.8, acetonitrile–phosphate buffer (40:60, v/v) with ionic strength (I) maintained at 0.05 M. Buffer concentration adjusted to maintain I constant at different temperatures. Sample volume 5 μl, sample mass 0.1 μg, UV detection at 210 nm. For other conditions see Section 2.

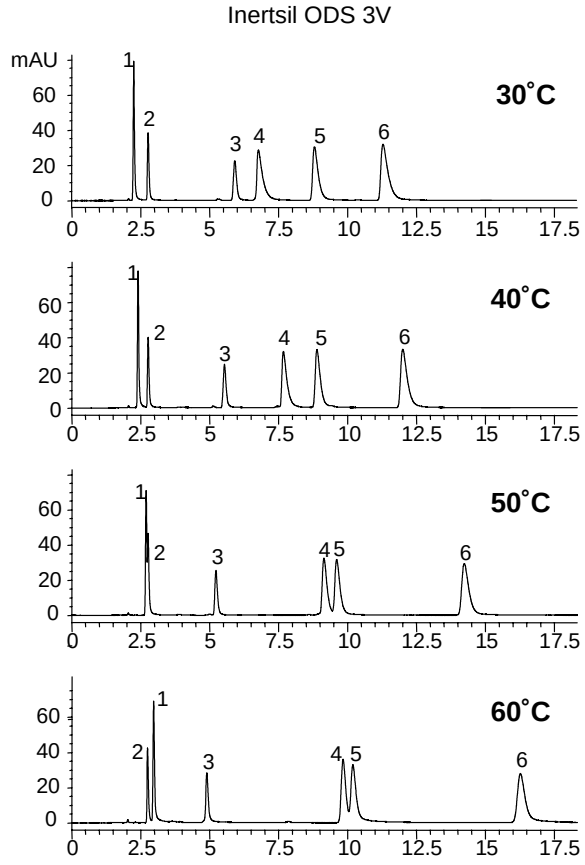

Fig. 2. Chromatograms obtained for sample mix at $^{s}_{w}$pH 7.8 using Inertsil ODS 3V in the temperature range 30–60 °C. Peaks: (1) benzylamine; (2) BteN; (3) berberine chloride; (4) quinine; (5) protriptyline; (6) nortriptyline. Flow rate: 1 ml/min. Other conditions as in Fig. 1.

for a particular solute are used, elevated temperature can have a more complex effect on peak shape. For instance, we found that the efficiency for benzene (which has a higher diffusion coefficient than these R_4N^+ compounds) remained virtually constant over the temperature range 20–60 °C due to the interplay of the B and C terms. This effect is not shown for these slower-diffusing solutes at 1 ml/min, which is clearly above their optimum flow rate.

Fig. 2 shows chromatograms for a mixture of four bases and two quaternary ammonium compounds over the temperature range 30–60 °C at $^{s}_{w}$pH 7.8. It is immediately apparent that useful selectivity differences can be obtained by changing the temperature. For example, the order of elution of peaks 1 and 2 is reversed at 30 and 60 °C and the retention of peaks 3 and 4 moves in opposite directions as temperature is increased. Van't Hoff plots for these compounds, the additional base amitriptyline, and the neutral benzene on both Inertsil ODS 3V and X-Terra RP_{18} over the range 30–60 °C at $^{s}_{w}$pH 7.8 are shown in Fig. 3. The compounds were injected in this case singly, to prevent any interference, which could result from injection of mixtures as shown in Fig. 2. The Van't Hoff plot shows "normal behaviour" for benzene and the R_4N^+ compounds, i.e. retention decreases with increasing temperature (T) according to the equation:

$$\ln k = \frac{-\Delta H^\circ}{RT} + \frac{\Delta S^\circ}{R} + \ln \phi \quad (1)$$

where ΔH° and ΔS° are the changes in enthalpy and entropy, respectively, that result from the transfer of one molecule of solute from the mobile phase to stationary phase, R the gas constant and ϕ the volumetric phase ratio [21]. Benzene on both columns gave $-\Delta H^\circ = 11.2$ kJ/mol (2.7 kcal/mol), which is in agreement with earlier findings for benzene on Inertsil ODS 3V ($-\Delta H^\circ = 2.5$ kcal/mol) [12].

In contrast, the bases amitriptyline, benzylamine, nortriptyline and quinine experience continuous retention increase with increasing temperature. This behaviour could be due to temperature-dependent pK_a shifts (typically about −0.03 pK_a units/K [17,18]). For example, at ionic strength 50 mM and 40% acetonitrile content in the mobile phase, the pK_a of nortriptyline at 30 °C ($^{s}_{w}$pK_a 9.72) reduces by

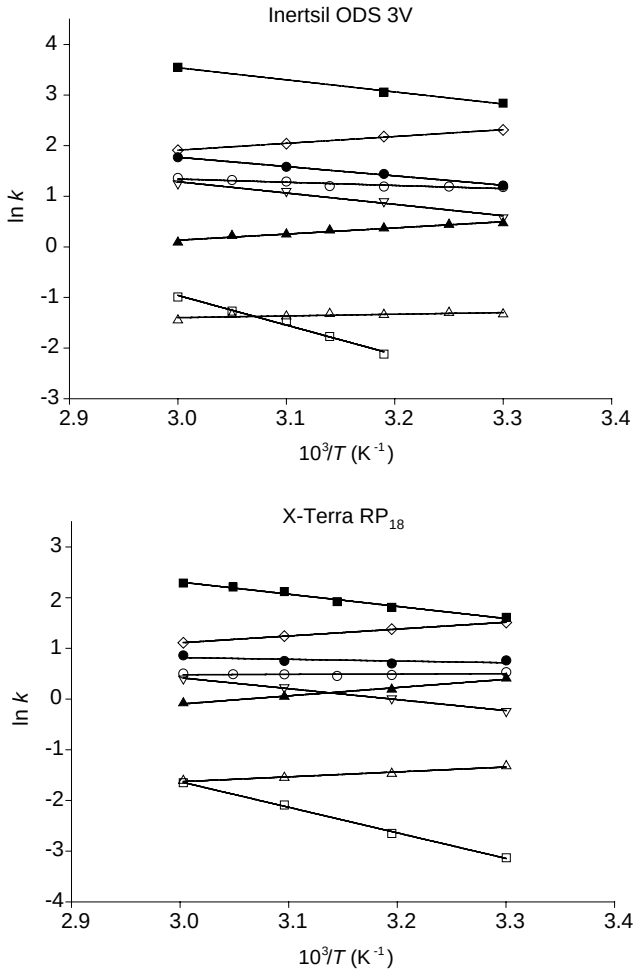

Fig. 3. Van't Hoff plots, illustrating the variation of retention k with increasing temperature T at ${}^{s}_{w}$pH 7.8 for benzene (◇), berberine chloride (▲), BteN (△), nortriptyline (●), quinine (▽), benzylamine (□), protriptyline (○) and amitriptyline (■). Flow rate: 1 ml/min. Other conditions as in Fig. 1.

about 0.9 pK_a units to ${}^{s}_{w}$pK_a 8.82 at 60 °C. Thus, at constant ${}^{s}_{w}$pH 7.8 nortriptyline is 99% protonated (BH^+) at 30 °C, which reduces to 91% BH^+ at 60 °C, as estimated by the Hendersson–Hasselbalch equation [27]. Table 1 contains more ionisation information also for the other bases for the range 30–60 °C at ${}^{s}_{w}$pH 7.8. It appears that a relatively small reduction in %BH^+ can effectively increase the hydrophobic retention of the solute, which counteracts the usual decrease in retention with temperature. This result is not altogether surprising considering that the retention of the protonated base should be considerably smaller than that of the corresponding neutral molecule. The sensitivity of retention to this effect is well illustrated in Fig. 4, which shows magnified Van't Hoff curves of protriptyline. An additional data point obtained at 71 °C is included in these plots, which are clearly not linear as would be expected from Eq. (1). Protriptyline is a somewhat stronger base and thus changes in its protonation state are smaller than for nortriptyline (Table 1). On Inertsil ODS 3V changes in k are moderate in the range

Table 1
${}^{s}_{w}$pK_a for the bases at 30 °C in acetonitrile–buffer (40:60, v/v) with ionic strength nominally 0.05 M

${}^{s}_{w}$pH 7.80	${}^{s}_{w}$pK_a at 30 °C	%BH^+			
		30 °C	40 °C	50 °C	60 °C
Amitriptyline	8.78	91	83	71	55
Benzylamine	8.96	94	88	78	65
Nortriptyline	9.72	99	98	95	91
Quinine	8.32	77	62	45	29
Protriptyline	10.02	99	99	98	95

These were obtained from [22] or measured using the CE procedure described therein. A temperature coefficient, −0.03 pK_a units/K [18], was used to correct for temperature-induced pK_a shifts of the bases in order to obtain %BH^+ at each temperature. %BH^+ reflects the proportion of protonated base relative to total base (i.e. unprotonated + protonated base) at ${}^{s}_{w}$pH.

30–45 °C, where %BH^+ of protriptyline is about 99 and 98%, respectively, as calculated from the ${}^{s}_{w}$pK_a determined at 30 °C (Table 1). Further increasing temperature reduces %BH^+ to 95% at 60 °C, which however leads to a clearly

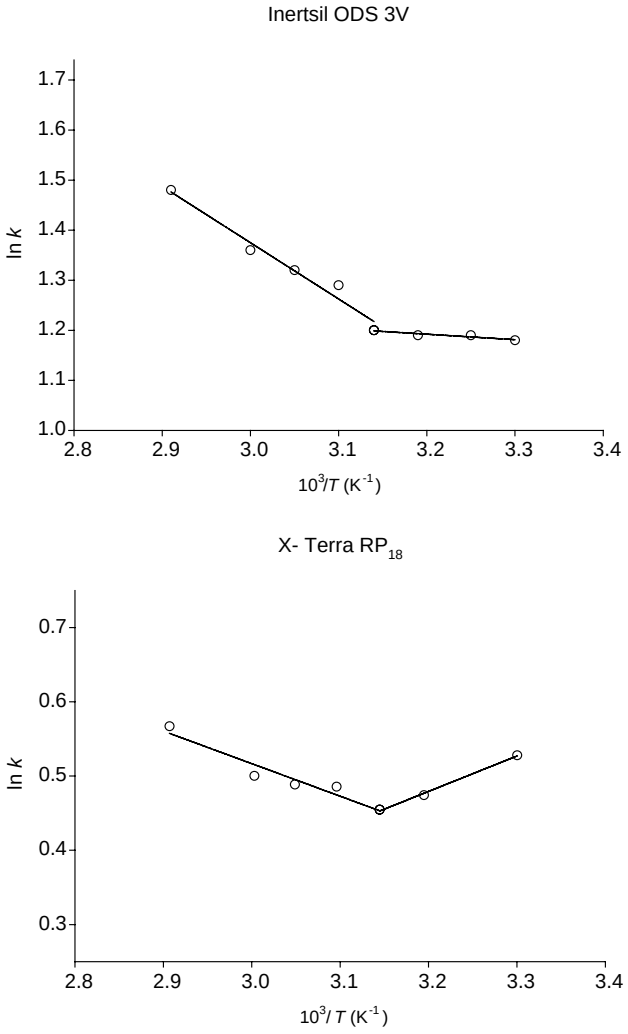

Fig. 4. Magnified Van't Hoff plots of protriptyline obtained at ${}^{s}_{w}$pH 7.8. Flow rate: 1 ml/min.

more pronounced negative slope of the Van't Hoff plot above 45 °C. Also on X-Terra RP_{18}, the curve has a negative slope at temperatures between 45 and 71 °C, but normal behaviour is exhibited in the range 30–45 °C. It seems thus likely that these pK_a changes and the resulting differences in percentage ionisation with temperature, give rise to the interesting selectivity variations as temperature is raised (Fig. 2). Generally, the lower the pK_a of the base in the group we studied, the closer is its value to the mobile phase pH. Thus, temperature-induced pK_a changes give more pronounced reductions in $\%BH^+$ and thus a greater effect on retention, as demonstrated by the slopes of the Van't Hoff plots.

It is possible to measure experimentally the retention of a compound as the free base and the totally protonated species. Using these data and knowing $^{s}_{w}pK_a$ of the solutes and $^{s}_{w}$pH of the mobile phase, it would be possible to calculate the expected retention time of the base and how it changes with temperature, and compare this with the results found experimentally [28]. However, a problem is the question of silanol retention, and how it might vary with temperature, which is clearly a confounding issue. Unfortunately, the effects of temperature and organic solvent on the pK_a of silanols have received very little mention in the literature. We intend to investigate these important effects in a future study. In the present work however, had changes in silanol ionisation caused major increases in retention with temperature for bases, then these increases in retention would also have occurred for quaternary ammonium compounds; this was not observed. It is possible that the high silanol-pK_a on X-Terra material would render changes in silanol ionisation with temperature unmarked at this pH studied [29]. Thus, we believe major changes in silanol ionisation have not greatly affected our results, but these effects definitely require further study.

Fig. 5 shows plots of column efficiency N_{df} versus temperature obtained at $^{s}_{w}$pH 7.8 on Inertsil ODS 3V and X-Terra RP_{18}. Table 2 contains corresponding retention factors obtained at 30 °C ($k_{30\,°C}$), efficiencies measured at 30 and 60 °C and their ratio $N_{df(60\,°C)}/N_{df(30\,°C)}$ and asymmetry factors measured at 30 °C ($A_{s(30\,°C)}$). As expected, benzene changes very little in efficiency with increasing temperature, due to its higher diffusion coefficient than the (higher molecular mass) bases and quaternary ammonium compounds which leads to significantly higher optimum flow rates for benzene [13]. Fig. 5 and Table 2 show all cationic solutes to increase in efficiency with increasing temperature. Clearly, some of this increase can be attributed to increase in solute diffusivity with increasing temperature as shown previously [13]. The ratios $N_{df(60\,°C)}/N_{df(30\,°C)}$ show the rate of efficiency increase to be smallest for R_4N^+ which are always completely charged (e.g. $N_{df(60\,°C)}/N_{df(30\,°C)}$ 1.1–1.2 for R_4N^+ on either column). These values are more similar to those shown for strong bases at low pH, where any change in pK_a does not affect the (already complete) protonation of the solute [13]. For bases, ratios $N_{df(60\,°C)}/N_{df(30\,°C)}$ were considerably larger, and greatest for compounds of lower

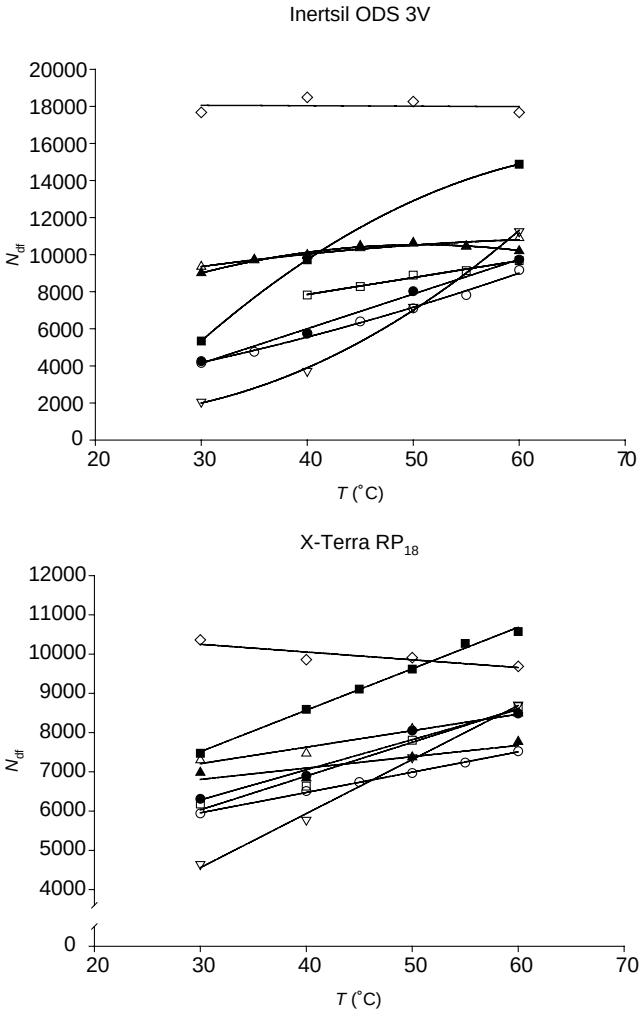

Fig. 5. Effect of temperature T on column efficiency N_{df} at $^{s}_{w}$pH 7.8. Symbols and conditions as in Fig. 3.

pK_a. For example, the strongest base we studied, protriptyline ($^{s}_{w}pK_{a(30\,°C)}$ 10.02) gave $N_{df(60\,°C)}/N_{df(30\,°C)} \sim 2.2$ on Inertsil ODS 3V whereas the structurally related amitriptyline $^{s}_{w}pK_{a(30\,°C)}$ 8.78 gave $N_{df(60\,°C)}/N_{df(30\,°C)} \sim 2.8$. Note the plate counts of both amitriptyline and protriptyline were rather similar at low temperature. The weakest base, quinine, gave a greater than five-fold increase in efficiency over this temperature range. At 60 °C, the efficiency for amitriptyline (about 15,000 plates) even approached efficiencies exhibited by the neutral compound benzene ($N_{df} \sim 18{,}000$). Similar results were obtained on X-Terra, if less pronounced. This result may be attributed to the already less severe column interactions of protonated bases with this phase, even at room temperature (although note that the efficiency of X-Terra, even for neutral compounds, can be significantly less than equivalent pure silica-based phases [30]). Nevertheless, the same pattern of results was obtained, with the greatest improvement in efficiency obtained for quinine, improvements for the tricyclic antidepressants in order of pK_a, and the smallest improvement for quaternary compounds. The disproportionate increase in efficiency with temperature shown

Table 2
Retention factor (k), efficiency (N_{df}), and asymmetry factor (A_s) obtained on Inertsil ODS 3V and X-Terra RP_{18} at 30 °C using mobile phases as given in Table 1

$^{s}_{w}$pH 7.80	Inertsil ODS 3V					X-Terra RP_{18}				
	$k_{30\,°C}$	$N_{df(30\,°C)}$	$N_{df(60\,°C)}$	$N_{df(60\,°C)}/N_{df(30\,°C)}$	$A_{s(30\,°C)}$	$k_{30\,°C}$	$N_{df(30\,°C)}$	$N_{df(60\,°C)}$	$N_{df(60\,°C)}/N_{df(30\,°C)}$	$A_{s(30\,°C)}$
Benzene	10	17800	17700	0.99	1.1	4.5	10400	9690	0.93	1.2
Berberine	1.6	9010	10200	1.1	1.5	1.5	6980	7770	1.1	1.3
BteN	0.27	9350	10900	1.2	1.5	0.27	7300	8490	1.2	1.3
Protriptyline	3.3	4150	9160	2.2	2.8	1.7	5940	7520	1.3	1.4
Nortriptyline	3.3	4250	9730	2.3	2.8	2.1	6310	8490	1.4	1.5
Amitriptyline	17	5340	14900	2.8	2.9	8.0	7470	10600	1.4	1.4
Quinine	1.8	2050	11200	5.5	3.6	1.8	4650	8710	1.9	1.5
Benzylamine	–	–	9680	–	–	0.04	6180	8660	1.4	1.4

Further is given the respective N_{df} at 60 °C and the ratio between $N_{df(60\,°C)}$ and $N_{df(30\,°C)}$.

by bases compared with quaternary ammonium compounds is evidence for the hypothesis that it is indeed pK_a changes of the solutes that give rise to these efficiency increases.

Fig. 6 shows the influence of temperature on peak symmetry. Both columns gave smaller A_s for benzene than for all cationic compounds, indicating detrimental silanol interactions to occur. As expected, benzene gave little variation in A_s with temperature. The A_s plots of R_4N^+ compounds almost parallel those of benzene. This result in itself suggests that the influence of faster kinetics at higher temperature may be relatively less important under the conditions of our study. However, the peak shapes of quaternary amines are reasonably good, even at low temperatures. It is possible that silanol interaction for quaternary amines is reduced due to the stereochemical effects in proximity to the charge bearing nitrogen atom, although we have studied such effects only for unprotonated bases previously [31,32]. In contrast, A_s for bases considerably reduces as temperature is raised. This reduction accounts in part for the increase in efficiency shown, since the asymmetry factor is incorporated in the Dorsey–Foley efficiency measurement (see Section 2). It appears that, the weaker the base (in the set of compounds we used), the more significant the A_s decrease with temperature, which is supportive of the pK_a change hypothesis. There is very little change observed in A_s for benzylamine, which might be due to lack of retention (e.g. $k_{60\,°C} = 0.37$ on Inertsil ODS 3V). Protriptyline also varies little in A_s on X-Terra RP_{18} despite reasonable retention ($k_{30\,°C} = 1.70$), which somewhat breaks the otherwise regular pattern shown by bases. However, in addition to low silanol activity of X-Terra at this pH compared to conventional silica RP columns [29], the high basicity of protriptyline indicates that little change in its ionisation state is expected over the temperature range investigated.

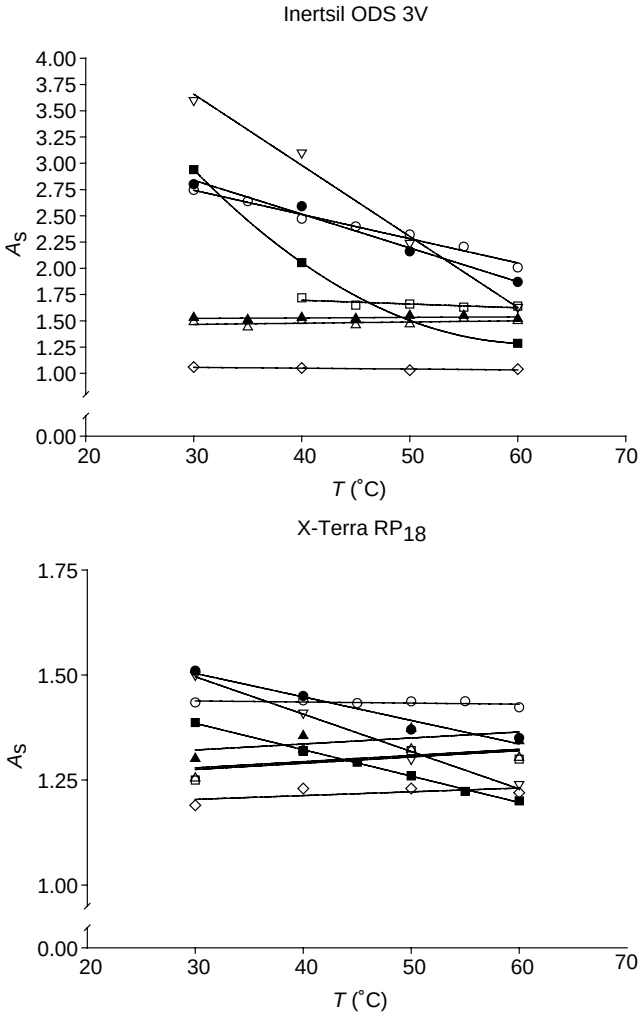

Fig. 6. Effect of temperature T on peak asymmetry A_s obtained at $^{s}_{w}$pH 7.8. Symbols and conditions as in Fig. 3.

Table 3
Ionisation information for bases, obtained as described in Table 1, in $^{s}_{w}$pH 7.0 acetonitrile–phosphate buffer (40:60, v/v)

$^{s}_{w}$pH 7.00	%BH^+				
	30 °C	40 °C	50 °C	60 °C	76 °C
Amitriptyline	98	97	94	88	72
Nortriptyline	100	100	99	99	96
Protriptyline	100	100	100	99	98

Phosphate buffer concentration adjusted to give $I = 0.05$ M with varying temperature.

Finally, the effect of temperature on retention and column performance at ${}^{s}_{w}$pH 7.0 was investigated. A somewhat larger temperature range (30–76 °C) was studied. A reduction in pH should give some changes in temperature-governed retention and peak shape patterns, if temperature effects on pK_a influence results. For example, protriptyline has a high ${}^{s}_{w}$pK_a (10.02) at 30 °C, and lowering the pH should ensure that the compound is almost fully protonated even at 60 °C, despite pK_a reduction with temperature. Thus, its behaviour should now more closely resemble that of R_4N^+ compounds. A complication is that lowering the pH will result in a lowering of the number of ionised silanol groups, possibly leading to somewhat better overall performance. Indeed, the quaternary compound berberine (ionisation does not vary with pH) gave on Inertsil at 30 °C, $N_{df} \sim 9000$ and $A_s = 1.5$ at ${}^{s}_{w}$pH 7.8, but $N_{df} \sim 10{,}200$ and $A_s = 1.4$ at ${}^{s}_{w}$pH 7.0. As before, X-Terra showed much smaller effects, again exhibiting its inertness over the pH range studied [29].

The ionisation information for bases at different temperatures and ${}^{s}_{w}$pH 7.0 can be found in Table 3. It can be seen that protriptyline should be in excess of 99% protonated even at 60 °C. Table 4 contains corresponding $k_{30\,°C}$, $N_{df(30\,°C)}$ and $N_{df(60\,°C)}$, their ratio $N_{df(60\,°C)}/N_{df(30\,°C)}$ and $A_{s(30\,°C)}$. Fig. 7 shows the corresponding Van't Hoff plots. Indeed, protriptyline now shows a more conventional plot with a positive slope similar to that of R_4N^+, rather than the complex pattern exhibited at ${}^{s}_{w}$pH 7.8 (compare Figs. 3 and 4). In contrast, calculations show the weaker base amitriptyline still experiences a considerable drop in protonation (from 98% at 30 °C to 88% at 60 °C). As expected, it gives the anomalous increase in retention with temperature, as found previously at ${}^{s}_{w}$pH 7.8. Nortriptyline is slightly less basic than protriptyline (Table 1); %BH^+ changes more significantly with temperature for nortriptyline from 100% at 30 °C to 96% at 76 °C (Table 3). Fig. 8 shows its expanded Van't Hoff curves. On Inertsil ODS 3V an unusual retention pattern is shown with a retention loss from 30 °C to about 55 °C followed by a retention increase at higher temperature,

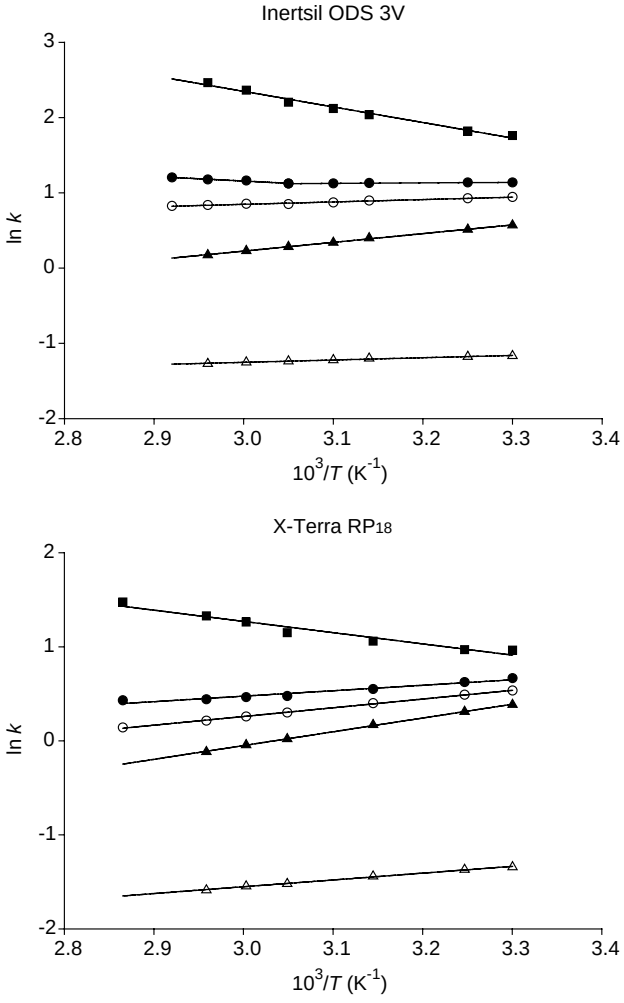

Fig. 7. Van't Hoff plots using mobile phase acetonitrile–phosphate buffer (40:60, v/v), ${}^{s}_{w}$pH 7.0. Phosphate buffer concentration adjusted to give I = 0.05 M with varying temperature. Symbols and other conditions as in Fig. 3.

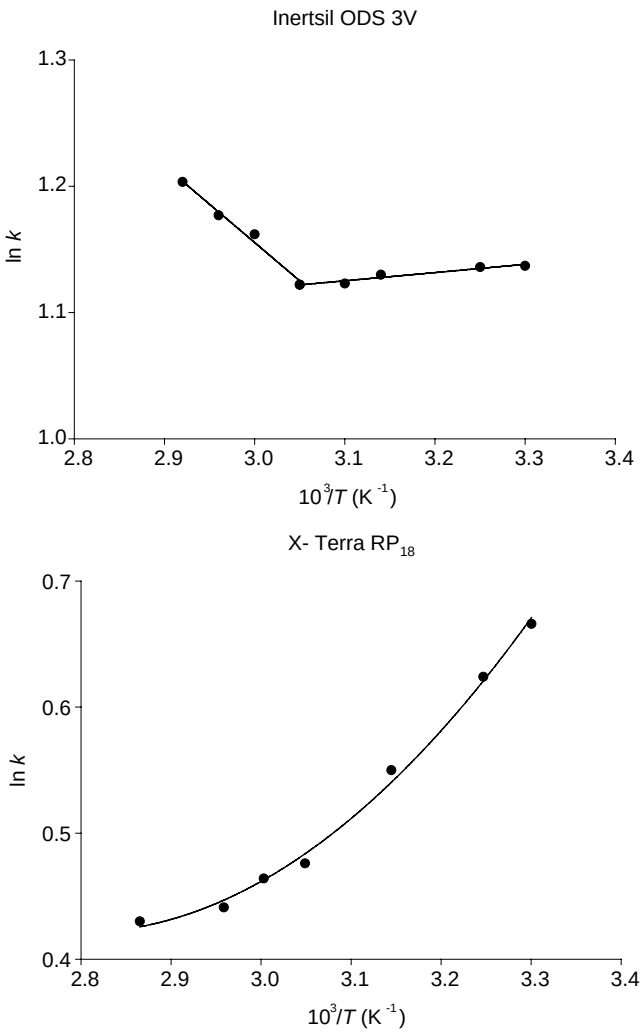

Fig. 8. Expansion of Van't Hoff plots of nortriptyline obtained at ${}^{s}_{w}$pH 7.0.

Table 4
As in Table 2, but mobile phase is $^{s}_{w}$pH 7.0, acetonitrile–phosphate buffer (40:60, v/v)

$^{s}_{w}$pH 7.00	Inertsil ODS 3V					X-Terra RP_{18}				
	$k_{30\,^\circ C}$	$N_{df(30\,^\circ C)}$	$N_{df(60\,^\circ C)}$	$N_{df(60\,^\circ C)}/N_{df(30\,^\circ C)}$	$A_{s(30\,^\circ C)}$	$k_{30\,^\circ C}$	$N_{df(30\,^\circ C)}$	$N_{df(60\,^\circ C)}$	$N_{df(60\,^\circ C)}/N_{df(30\,^\circ C)}$	$A_{s(30\,^\circ C)}$
Berberine	1.8	10200	11700	1.2	1.4	1.5	6890	7750	1.1	1.3
BteN	0.31	8910	10500	1.2	1.5	0.26	7030	8100	1.2	1.3
Protriptyline	2.6	8130	11800	1.5	1.7	1.7	6870	8210	1.2	1.3
Nortriptyline	3.1	7670	12100	1.6	1.9	2.0	6890	8460	1.2	1.4
Amitriptyline	5.8	7110	16200	2.3	2.2	2.6	7150	9840	1.4	1.4

I in the mobile phase was nominally 0.05 M.

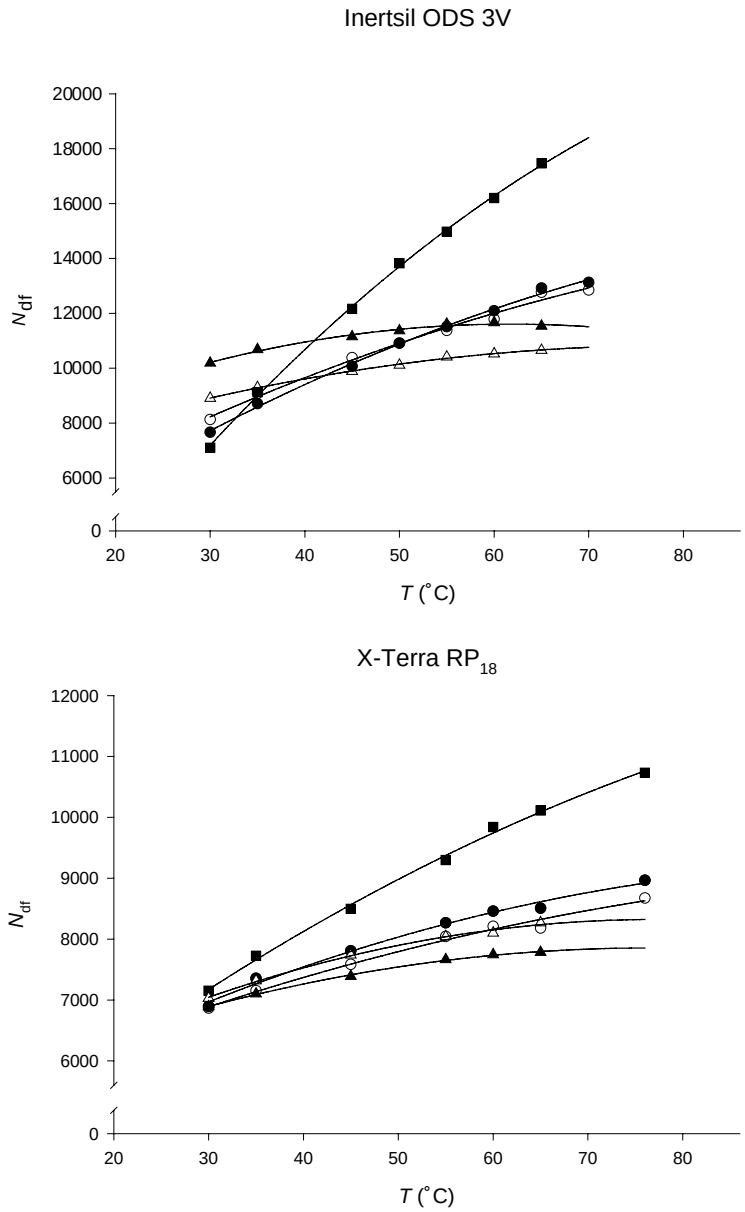

Fig. 9. Effect of temperature T on column efficiency N_{df} obtained at $^{s}_{w}$pH 7.0. Symbols as in Fig. 3 and conditions as in Fig. 7.

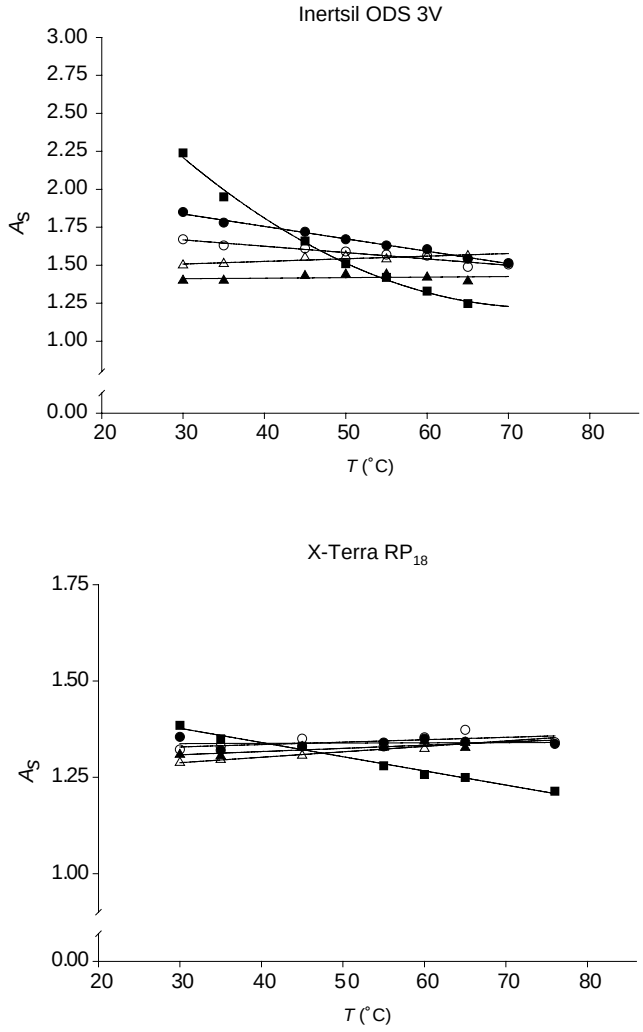

Fig. 10. Effect of temperature T on peak asymmetry A_s obtained at ${}^{s}_{w}$pH 7.0. Symbols as in Fig. 3 and conditions as in Fig. 7.

somewhat similar to the results for protriptyline at higher pH (Fig. 4). On X-Terra, an apparently more normal pattern of reducing retention with increasing temperature is indicated (Fig. 7) although the expanded plot shows some initial curvature.

Fig. 9 shows efficiency versus temperature plots obtained at ${}^{s}_{w}$pH 7.0. All solutes show increased efficiency with temperature. Results generally show the same trends as at ${}^{s}_{w}$pH 7.8. For example, Table 4 shows that the ratio $N_{df(60\,^{\circ}C)}/N_{df(30\,^{\circ}C)}$ is larger for all bases compared with R_4N^+ compounds. Furthermore, for protriptyline, nortriptyline and amitriptyline, the greatest improvement in efficiency with temperature occurs for the weakest base (amitriptyline); the smallest changes occur for protriptyline. The changes in efficiency with temperature are reflected also in changes in the asymmetry factor (Fig. 10); the largest improvement in asymmetry factor is for the weakest base amitriptyline.

The improvement in efficiency as shown by the ratio $N_{df(60\,^{\circ}C)}/N_{df(30\,^{\circ}C)}$ is generally greater at ${}^{s}_{w}$pH 7.8 compared with ${}^{s}_{w}$pH 7.0. For instance, the ratio for amitriptyline is 2.8 at ${}^{s}_{w}$pH 7.8 but 2.3 at ${}^{s}_{w}$pH 7.0 (Tables 2 and 4); these results parallel the proportionally greater change in percentage protonation of amitriptyline with temperature at ${}^{s}_{w}$pH 7.8 (Tables 1 and 3). In contrast, these ratios are almost identical for the quaternary compounds when compared at the different pH values. All of these results point to pK_a changes with temperature as being a significant contributor to increase in the efficiency of bases with column temperature at intermediate pH.

4. Conclusion

The retention and peak shapes of a number of different bases and quaternary ammonium compounds were studied as a function of temperature, flow rate and mobile phase pH, in order to rationalise the unusually large increases in column efficiency, and the anomalous increase in retention with temperature, observed previously. Improvements in efficiency with temperature were most pronounced for weaker bases, because their pK_a changes with temperature at constant pH result in the largest reduction in protonation with increased temperature. That is, neutral molecules have generally larger plate numbers. Quaternary compounds, which experience no change in ionisation with temperature, showed the smallest column efficiency improvement at higher temperatures. These solutes also exhibit generally higher efficiency-values than bases, possibly because of weaker interaction with ionised silanols due to steric considerations.

The anomalous increase in retention at higher temperatures of protonated bases has been shown to occur due to changes in pK_a with temperature that result in decreased solute ionisation with temperature. These changes in retention were very sensitive to even moderate changes in base-protonation and weaker bases in our study showed the greatest anomalous increase in retention with temperature.

Our results provide less evidence that acceleration of the kinetics of silanol interactions at elevated temperature is responsible for profound peak shape improvements shown for bases at intermediate pH. The overall detrimental effects of silanol interaction occurring at ${}^{s}_{w}$pH 7.8 were shown to be reduced at ${}^{s}_{w}$pH 7.0.

This work highlights the relevance of utilising temperature variance in practical analyses of bases. In separations where high selectivity is required (for instance, with very complex samples), intermediate pH in combination with elevated temperature exhibits a useful alternative to more commonly performed low pH separations; sufficiently raised temperature can yield column efficiencies for bases that can approach those of neutral compounds. If larger changes in retention are necessary, changing %BH^+ by varying temperature is less appropriate, since a change of one pK_a unit corresponds to a temperature difference of ~33 K assuming a temperature coefficient of about –0.03 K^{-1} (note, a maximum of

about 60 °C is usually recommended for most silica-based columns). However, temperature variations can yield subtle adjustments in retention and selectivity to further improve separation. It is also relatively easy to implement a change in temperature by a small instrumental adjustment compared with change in pH, which involves making up a different mobile phase.

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