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Review

The challenges of the analysis of basic compounds by high performance liquid chromatography: Some possible approaches for improved separations

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ABSTRACT

This review considers some of the difficulties encountered with the analysis of ionised bases using reversed-phase chromatography, such as detrimental interaction with column silanol groups, and overloading which both lead to poor peak shapes. Methods of overcoming these problems in reversed-phase (RP) separations, by judicious selection of the column and mobile phase conditions, are discussed. Hydrophilic interaction chromatography is considered as an alternative method for the separation of some basic compounds.

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1. Introduction

The analysis of basic compounds by high performance liquid chromatography (HPLC) continues to be of interest, as over 70% of pharmaceuticals are bases (with about 20% being acids) [1–3]. A large number of compounds of biomedical and biological significance are also bases. Reversed-phase (RP) separations are by far the most common in liquid chromatography (LC), due to advantages that include ease of use with gradient elution, compatibility with aqueous samples, versatility of the retention mechanism allowing changes in the separation to be brought about by changes in pH, organic modifier or additives, and long experience with the technique, allowing the rapid establishment of suitable experimental conditions for the analysis of a given sample [4]. Nevertheless, it has been recognised for a long time that the analysis of basic compounds poses particular difficulties in RP separations. Many of these problems are associated with the complex structure of the surface in silica-based RP packings, shown in Fig. 1. The surface concentration of silanols on bare silica is reported to be about 8.0 μmol m^{-2} [5]. C18 ligands are too bulky to react completely with all silanols; thus, a maximum coverage of 4–4.5 μmol m^{-2} can be achieved. A further number of reactive silanols can be "endcapped" by reaction with smaller silylating agents such as trimethylchlorosilane, but as many as 50% of the original silanol groups remain unreacted on a typical RP column. The average pK_a of these silanol groups is around 7.1, but their acidity can be enhanced by the presence of metal impurities in the silica. Some groups appear to be sufficiently acidic that their ionisation cannot be entirely suppressed using acidic mobile phases with a pH within the stability limit of typical RP columns (2.5–7.5). Over this range of operational pH values, basic compounds are likely to be ionised, leading to ionic interactions with ionised silanol groups.

$$BH^+ + SiO^-M^+ \rightarrow SiO^-BH^+ + M^+ \quad (1)$$

where BH^+ represents the protonated base, and M^+ the mobile phase buffer cation. The problem of poor column efficiency (N) and exponentially tailing peaks shown by small quantities of bases is often attributed to this mixed mechanism process of hydrophobic interaction and ion-exchange with the silanols. The slower sorption–desorption kinetics of silanol ion-exchange sites (kinetic tailing) with sample ions may be responsible [6], which will occur regardless of sample size. The simple existence of two retention processes cannot *per se* be the sole cause of tailing, as mixed-mode phases with carboxylic acid functions embedded within a hydrophobic chain can show excellent peak symmetry for bases [7]. However, the kinetics of interaction of such embedded groups, and the stereochemistry around the active site, could be completely different from that of ionised silanols, which may be buried beneath the hydrophobic chains on classical C18 phases. Instead of simple ion-exchange sites, Neue et al. [8] have proposed the existence of strong synergistic sites with combined RP and ion-exchange properties. The overall retention for bases was described by the equation:

$$k = k_{RP} + k_{IX} + k^*_{RP}k^*_{IX} \quad (2)$$

where k is the total retention, k_{RP} is the hydrophobic contribution, k_{IX} is the ion-exchange contribution from surface silanols, and $k^*_{RP}k^*_{IX}$ is a multiplicative contribution of both processes. These synergistic sites could correspond to the subset of very high-energy sites with slow kinetics which have been long suspected to be the cause of exponential tailing for bases, as they appear to be dominant in the retention process. It was shown that this type of tailing is not responsive to small changes in sample load in RP–LC at low pH [6]. This result might indicate that exponential tailing is not caused by overload of a small number of strong sites on the column. In contrast, overload often gives rise to right-angled triangle peak shapes when ionisation of silanols is suppressed in RP–LC when working at low pH. Overload tailing still occurs even for the most modern columns operated under conditions where there are no or a negligible number of ionised silanols on the column surface.

It was recognised more than 20 years ago that bonded phases synthesised from pure silica (Type B phases) made from the hydrolysis of metal-free tetraalkoxysilanes resulted in reduced silanol acidity, and their use has considerably improved the analysis of bases [9]. Only small contamination of such materials occurs during the processing of such packings, or from the water used in the hydrolysis. Nevertheless, some other features of the analysis of these solutes (such as overloading) remain problematic, and these issues have not been resolved by the use of high-purity silica.

Already in 1988, Snyder and co-workers [10] had reviewed the problems of analysis of basic solutes and had proposed some possible solutions. The following recommendations were made:

(a) Judicious selection of the column to reduce the number of available acidic sites.
(b) Reduction of the mobile phase pH to suppress ionisation of the silanols.
(c) Increasing the mobile phase pH above the analyte pK_a, such that the analyte is unprotonated.
(d) Addition of a silanol blocker such as triethylamine to the mobile phase to interact preferentially with ionised silanols.
(e) Reduction of the sample concentration to alleviate the saturation of the acidic sites.

Most of the arguments in this paper remain true more than 20 years later, and these conclusions can be used as a simple guide for the chromatographer aiming to achieve the best separations for basic solutes. Perhaps only the use of silanol blocking agents has fallen somewhat out of favour, as these are less necessary with modern high-purity silica phases, and can also have some undesirable effects. Such effects include the generation of additional background in HPLC–MS, the difficulty of removal from the stationary phase after use leading to permanent alteration of its properties, and even chemical reaction with some solute types. This topic, and some other well-known aspects of the chromatography of bases have been covered adequately in earlier reviews [11–13]. However, other features of the chromatography of these "difficult" compounds are still extensively debated in the literature, for example, the problem of their ready overloading in RP separations. This review will concentrate on the latest research in these topics, while attempting to summarise briefly previous findings. Thus, it will consider RP column choice by use of evaluation data obtained from the Tanaka and the Snyder "hydrophobic subtraction" tests; current theories and the effect of overload for ionised solutes; the use of high pH to improve peak shape; whether temperature is a useful parameter in improving peak shape; and finally whether other separation mechanisms such as HILIC can provide a viable alternative to RP–LC for the analysis of bases.

2. Choice of column

2.1. Column testing procedures

The selection of an appropriate RP column for the analysis of bases can be a daunting task, as now many hundreds are commercially available, with a considerable number recommended especially by their manufacturers for the analysis of basic solutes. Nevertheless, several databases are now available where a large number of different columns have been subjected to the same test procedure by the same group of workers on the same or similar

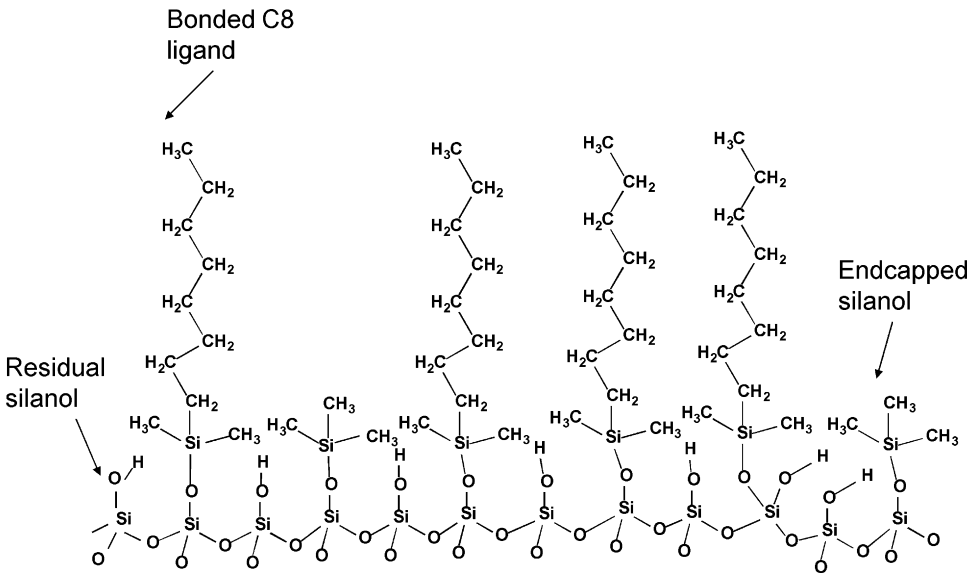


Fig. 1. Structures present on a typical RP monomeric-bonded silica (C8) endcapped with trimethylsilyl groups. After U.D. Neue, "Silica Gel and its derivatization for Liquid Chromatography", in "Encyclopedia of Analytical Chemistry", R.A. Meyers, Ed., John Wiley & Sons, Ltd., Chichester (2000) 11450–11472.

instruments, allowing a useful and objective comparison of performance to be made. A question arises as to the validity of databases constructed by evaluation of only a few or even a single column of a given type, as to whether the results obtained may be truly representative of the performance of this brand, due to column to column and batch to batch variations. However, a careful study [14,15] has suggested that columns from major manufacturers actually show a rather high degree of reproducibility, probably resulting from the use of stringent quality control procedures. Indeed, the industry is likely to be self-regulating to a degree, as dissatisfied customers would switch to the use of more reproducible brands. Tight retention specifications exist in the HPLC user environment, especially in the pharmaceutical industry, and changes in the column can jeopardise product release. However, it is possible that a manufacturer could be forced to change the sourcing of a production raw material, which might occur for example, if the column manufacturer does not make their own silica. Thus, under some circumstances, a recently purchased column may not behave in the same way as one tested several years beforehand. Nevertheless, we believe that such situations are rare, and in most cases, manufacturers strive to maintain the reproducibility of their products over a long period of time, as many customers have established methods on a given brand of phase. It appears more common to introduce a new name or name variant of an existing phase to mark definitively such changes or improvements to the production process. Taking this factor, and the reasonable reproducibility of commercial columns into account, it seems that the results of tests on a particular brand of column would generally reflect the performance of that brand throughout the product lifetime.

Both of the column evaluation methods described in detail below incorporate strongly basic compounds as test probes. In each test, their retention is monitored at low and intermediate pH values. Columns which give relatively low retention of basic probes are also likely to give higher efficiency for basic solutes, as shown by correlation studies for at least one of the procedures (see below).

2.2. The Tanaka test and the Snyder hydrophobic subtraction procedure. Comparison of results with direct peak shape measurements

While many different column testing methods have been developed, two have become prominent and have the distinct advantage that databases of results for many hundreds, rather than just a few columns, are available. The Tanaka method [16] and the hydrophobic subtraction procedure developed by Snyder et al. [17] both incorporate tests which allow a user to select phases that are likely to be suitable for the separation of basic compounds. We will consider here the Tanaka method as adapted and applied by Euerby and Petersson [18] to the evaluation of over 200 commercial columns that can be compared on a freely available program from Advanced Chemistry Development [19]. These databases appear to be updated periodically; for instance, the ACD database contains evaluations of recently introduced sub-2 μm phases. An alternative adaptation of the Tanaka procedure and its application to a large number of different stationary phases has also been made [20], and data are again freely available [21]. A fourth testing scheme is that published by the US Pharmacopeia. This protocol is an adaptation of the work of Sander and Wise [22]. For activity towards bases, this method uses the tailing factor of amitriptyline (the same probe as used in the Snyder–Dolan procedure). At the time of writing, the database contained fewer columns than the two major procedures (∼100) and will not be considered further here. However, data for both this procedure and the Snyder–Dolan (S–D) method are available on the USP website [23].

In the Tanaka–Euerby (T–E) procedure, columns are tested by measurement of k for pentylbenzene as a measure of surface area and surface coverage; hydrophobic selectivity from the ratio of k (pentylbenzene)/k (butylbenzene); shape selectivity from k (triphenylene)/k (o-terphenyl); hydrogen bonding capacity from k (caffeine)/k (phenol) in unbuffered methanol–water; total ion-exchange capacity from k (benzylamine)/k (phenol in methanol–phosphate buffer pH 7.6; and acidic ion-exchange capacity from k (benzylamine)/k (phenol) in methanol–phosphate buffer pH 2.7. The latter three tests are of particular interest for the analysis of basic solutes. The program [19] allows the comparison of the similarities and differences between various columns, and permits the separate weighting of the various factors—for example, columns can be ranked according solely to their total ion capacity at pH 7.6 if so desired. The S–D model recognises that hydrophobic retention is the dominant process in RP chromatography, and in the absence of other retention mechanisms, plots of log k for one column versus another should be a straight line. However, these other mechanisms give rise to scatter in the plots. Clearly, ion-exchange and hydrogen bonding are important contributors to the retention

Table 1
Evaluation of some selected RP columns by two different procedures. For details on the procedure, see text.

Column name	*k* pentylbz	*k*(pentbz)/*k* (butbz)	*k*(triphen)/*k*(terph)	*k*(caff)/*k*(phen)	*k*(bzm)/*k*(phen)2.7	*k*(bzm)/*k*(phen)7.6
Tanaka–Euerby procedure						
Chromolith	4.22	1.24	1.31	0.48	0.12	0.63
Discovery Amide	1.65	1.35	1.81	0.49	0.19	0.44
Discovery C18	3.32	1.48	1.51	0.39	0.10	0.28
Inertsil ODS-3	7.74	1.45	1.29	0.48	0.01	0.29
Resolve C18	2.40	1.46	1.59	1.29	1.23	4.06
Spherisorb ODS-2	3.00	1.51	1.56	0.59	0.23	0.76
Symmetry C18	6.51	1.46	1.49	0.41	0.01	0.68
Symmetry Shield RP18	4.66	1.41	2.22	0.27	0.04	0.20
Xterra MS C18	3.52	1.42	1.26	0.42	0.10	0.35
Xterra RP18	2.38	1.29	1.83	0.33	0.07	0.20

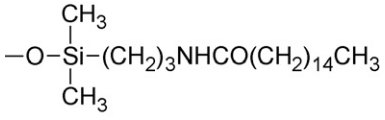
	H	*S*	*A*	*B*	*C*(2.8)	*C*(7.0)
Snyder procedure						
Chromolith	1.003	0.029	0.008	−0.014	0.103	0.187
Discovery Amide	0.720	0.013	−0.625	0.218	−0.092	−0.025
Discovery C18	0.984	0.027	−0.128	0.004	0.176	0.153
Inertsil ODS-3	0.990	0.022	−0.146	−0.023	−0.474	−0.334
Resolve C18	0.968	−0.127	0.335	−0.046	1.921	2.144
Spherisorb ODS-2	0.962	−0.076	0.07	0.034	0.908	1.263
Symmetry C18	1.052	0.063	0.018	−0.021	−0.302	0.123
Symmetry Shield RP18	0.850	0.027	−0.411	0.093	−0.728	0.136
Xterra MS C18	0.984	0.012	−0.143	−0.015	0.133	0.051
Xterra RP18	0.757	−0.043	−0.483	0.097	−0.170	−0.173

of basic solutes. The general equation for retention in the model is:

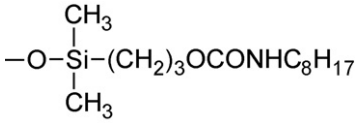
$$\log \alpha = \log k/\log k \text{ (ethylbenzene)} = \underset{\text{hydrophobic}}{\eta' H} - \underset{\text{steric resistance (to bulky interactions)}}{\sigma' S^*} + \underset{\text{column H-bond acidity (non-ionised silanols)}}{\beta' A} + \underset{\text{H-bond basicity (from sorbed water)}}{\alpha' B} + \underset{\text{ion interaction (ionised silanols)}}{\kappa' C} \quad (3)$$

Ethylbenzene is used as a non-polar reference solute. Greek letters represent empirical, eluent- and temperature- dependent properties of the *solute*, which are relative to the values for ethylbenzene, for which all solute parameters are identically zero. The selection of the optimum probes for evaluation of each retention mode has been made from detailed studies. Bold capitals represent eluent- and temperature-independent properties of the *column*; these values are relative to a hypothetical average Type B C18 column. Any column which behaves identically to this hypothetical reference column will have $H = 1$ and all other values $S^*, A, B, C = 0$. The dataset of columns evaluated by this procedure is even larger than that for the T–E procedure and presently extends to at least 400 columns. In some versions of the program, different weightings can be assigned to each evaluation parameter, as in the Euerby procedure.

Results for some RP columns selected from each database are shown in Table 1. The T–E data show clearly that the older Type A bonded phases (Resolve C18 and Spherisorb ODS-2) give higher retention of benzylamine relative to phenol at pH 7.6 (alpha values 4.06 and 0.76, respectively) compared with newer Type B phases based on highly pure silica (Discovery C18 and Inertsil ODS-3, alpha values 0.28 and 0.29, respectively). Similarly with the S–D method, values of *C*(7.0) for Resolve C18 and Spherisorb ODS-2 are high (2.144 and 1.263, respectively) compared with Discovery C18 and Inertsil ODS-3 (0.153 and −0.334, respectively. Values of alpha (benzylamine/phenol) at pH 2.7 and values of *C*(2.8) are also higher for the Type A compared with the Type B phases using both procedures, indicating general agreement between them. Snyder and co-workers [24] have correlated a published dataset of "silanol activity" for a number of RP columns (measured by the average plate number for amitriptyline and pyridine with methanol-phosphate buffer pH 6.0) with values of C at pH 6.0, interpolated from *C*(2.8) and *C*(7.0). Columns with a high value of *C*(6.0) correlated with columns of high silanol activity, and those with low values of *C*(6.0) with low silanol activity. In a later study [6] 95% of Type B columns (designated either on the basis of manufacturer claims, or on the date a column was first sold) were shown to have *C*(2.8) ≤0.25, while only 11% of Type A columns satisfied this criterion. Tailing of basic solutes (as measured by the asymmetry factor A_s) was minimal for columns with *C*(2.8) < 0.25 (i.e. Type B columns) and tended to increase for larger values of *C*(2.8). From Table 1, the Type A phases Resolve C18 and Spherisob-ODS-2, now identified as such due to values of *C*(2.8) ≥ 0.25, also give the highest values of hydrogen bonding acidity (parameter *A*, 0.335 and 0.07, respectively, determined from the retention of amide probe compounds). Similarly, these phases also gave the highest relative retention of caffeine/phenol in the Tanaka procedure (1.29 and 0.59, respectively). The data can also be used to compare the effect of other features, e.g. the performance of embedded polar group phases (EPG) and the equivalent conventional C18 phase, manufactured on the same silica. EPG phases include columns with embedded amide groups within the hydrocarbon chain:



or carbamate groups:



EPG phases have been proposed to give better peak shapes for the analysis of bases [24,27]. The incorporation of an EPG in XTerra RP18 reduces somewhat the Tanaka alpha (benzylamine/phenol) 7.6 parameter to 0.20, compared with 0.35 for the XTerra MS C18 column. Similarly, the S–D *C*(7.0) parameter is reduced to −0.173 for the EPG compared with 0.051 for the conventional phase. It is

possible that the reduced retention of benzylamine and other bases may be caused by a layer of water that is adsorbed close to the surface of EPG phases, providing some deactivating effect for the silanol groups [25,26]. Other authors have compared conventional and EPG phases bonded on the same type of silica, on the basis of peak shape measurements. It was found that on average, peak shapes were indeed improved on the latter phases [27]. Nevertheless, it appears that the EPG technology gives more improvement in performance with phases bonded on older impure silicas, rather than the modern Type B silicas [27]. This result seems to be reflected in the somewhat inconclusive data from Table 1 concerning the relative retention of bases on conventional and EPG phases. Thus the Discovery EPG phase (amide) has a slightly larger value of the T–E alpha (benzylamine/phenol) 7.6 parameter (0.44) compared with the regular C18 phase (0.28). In contrast, the S–D $C(7.0)$ parameter is smaller on Discovery Amide (−0.025) compared with Discovery C18 (0.153). Similarly, while the T–E procedure indicates a considerable lower value of alpha (benzylamine/phenol) at pH 7.6 for Symmetry Shield (0.2) compared with Symmetry C18 (0.68), the S–D $C(7.0)$ parameter for the EPG phases is slightly greater (0.136) compared with the regular phase (0.123). Euerby and Petersson pointed out that the extra retentiveness of phenols on EPG phases might invalidate the results of tests for silanophilic activity which involve the use of such solutes. They therefore suggested substituting benzyl alcohol for phenol in the Tanaka test. Benzyl alcohol has retention properties similar to those of phenol but does not show excess retention on EPG phases [28]. These particular comparisons point to some possible differences in the compatibility of column evaluations from either method.

The Hoogmartens group looked more generally at the compatibility of results from the S–D method and their own adaptation of the Tanaka procedure [29], finding a rather poor overall correlation between the two approaches. In a previous paper, this group had demonstrated a good correlation between their own method and the Euerby results. This latter finding is perhaps not surprising, as both are based on the Tanaka method. The problem of compatibility of the S–D and Tanaka methods may well be in the different mobile phase conditions and different probe solutes used in these tests. The S–D procedure uses the retention of the strong bases amitriptyline and nortriptyline in acetonitrile–phosphate buffer to calculate the cation-exchange term $C(2.8)$ and derives the value of $C(7.0)$ from the $C(2.8)$ results by multiplying by the ratio of the retention factors of the quaternary amine berberine at pH 7.0 and 2.8; the T–E benzylamine tests use methanol as the organic modifier. Indeed the use of these different modifiers may explain the somewhat different evaluations of the EPG phases by either method. Even using the same mobile phase conditions, McCalley and Brereton [27,30–32] showed that peak shape data was not consistent between different basic probes. Thus, for example there was little correlation between A_s for codeine and nortriptyline when using methanol–phosphate buffers at pH 3.0, whereas either of these solutes has been used as a single test compound to evaluate the relative silanol activity of different phases. One phase (Waters Symmetry Shield) gave, of 9 highly inert RP columns, the highest N and lowest A_s for nicotine using acetonitrile–phosphate buffer at pH 7.0 but the lowest efficiency for analysis of pyridine. Fig. 2 shows a principal components analysis (PCA) loadings plot for analysis of nine basic solutes on eight different RP columns using a mixture of methanol with a pH 3.0 buffer. Lines can be drawn from the centre of the plot to each data point. Parameters that are opposed (i.e. appear at 180°) measure equivalent but opposite trends. Thus N and A_s values are opposed, with efficiency increasing as asymmetry decreases, as expected. Parameters that are at 90°, like the asymmetry factors of pyridine and quinine, measure unrelated trends, and thus may be evaluating relatively different aspects of the detrimental interaction of bases with the column surface. Conversely, the asymmetry parameters of nortriptyline and diphenhydramine have a smaller angle between them, and may be measuring more related properties. It might therefore not be necessary to include both substances in a test mix for these particular mobile phase conditions. For overall evaluation of column properties exploring different aspects of detrimental interactions, a test mix could include five compounds: codeine, quinine, amphetamine, nortriptyline and pyridine. The ranking of columns at pH 7 using methanol was different from that at pH 7 using acetonitrile; note that these correspond to the different modifiers of the T–E and S–D evaluation schemes, respectively. Snyder and co-workers [6] also observed that the tailing of basic (cationic) solutes on a given column appeared to be solute specific, finding that values of A_s for the bases amitriptyline, nortriptyline, the quaternary compound berberine, and 4-*n*-hexylaniline correlated extremely poorly ($r^2 = 0.01$–0.19). The use of multiple basic test solutes and different mobile phase modifiers at different pH values would be a considerable task for the construction of these column evaluation databases. However, inclusion of a range of test compounds would undoubtedly improve the performance of these databases. It seems certain that these differences in test solutes and conditions contribute to the lack of correlation between the S–D and T–E tests.

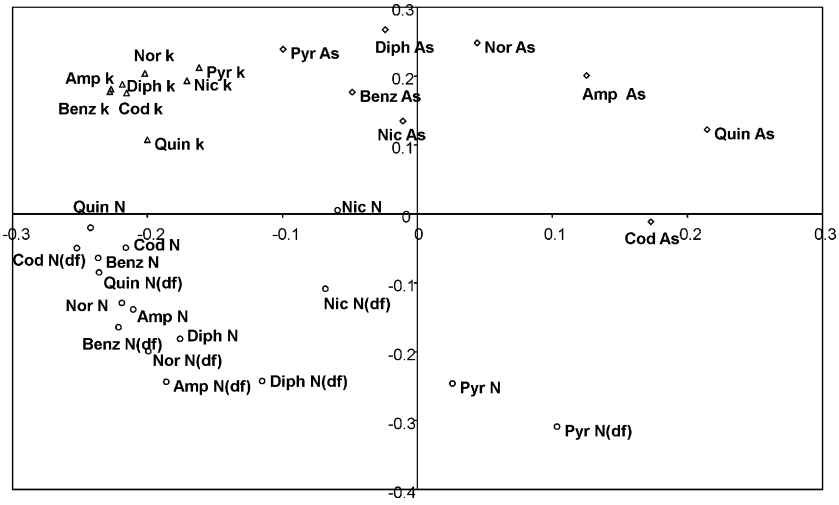


Fig. 2. PCA loadings plots based on retention factor (k), column efficiency (N), Dorsey–Foley column efficiency (N_{df}) and asymmetry factor (A_s). Data for eight different Type B reversed-phase columns and nine different probe compounds with methanol–phosphate buffer pH 3.0 as mobile phase. See [30].

There are other complexities involved with these tests. The presence of positively charged sites on some phases (see below) may also complicate the interpretation of results from these evaluation schemes. Finally, there may be other reasons for the selection of suitable columns for basic solutes. For example, EPG phases seem to generate more column bleed in combined HPLC–MS, and thus are less popular in these applications. A contributing factor appears to be the greater sensitivity of the mass spectrometer to (the same amount of) bleed from the EPG phase compared with that from conventional C18 phases. Nevertheless, it is also conceivable that the presence of a water layer close to the surface of the phase could promote its decomposition.

2.3. Monolithic silica columns

Monoliths are single pieces of chromatography material which can be externally clad with a plastic to yield columns of dimensions similar to those used for conventional particle-packed columns, or can be synthesised *in situ* in capillary format. Silica-based monoliths have found their place in the array of different RP columns available for the analysis of small molecules since their commercial introduction about 10 years ago. They have generated much interest, as they can give plate heights for neutral molecules similar to 3 μm particle-packed columns, while giving back pressures comparable with columns with particle size >10 μm [33].

Kele and Guiochon [34] found that the reproducibility of the commercial monoliths was high, matching values obtained for conventional packed columns. They used test mixtures containing weak bases tested in unbuffered methanol–water mobile phases, and stronger bases, tested in buffered mobile phases, at both low and intermediate pH. They found a small degree of tailing even for neutral solutes, quoting a USP tailing factor (T_f) for benzene of 1.3. McCalley [35] reported asymmetry factors (A_s) for weak bases and benzene as shown in Table 2, which are rather similar to values reported by the other group [34]. A_s is calculated at 10% of the peak height from the relationship:

$$A_s = \frac{b}{a} \tag{4}$$

where a is the leading edge, and b the trailing edge of the peak.

T_f is measured at 5% of the peak height from:

$$T_f = \frac{a+b}{2a} \tag{5}$$

Simple algebra shows that if measured at the same fractional peak height:

$$A_s = 2T_{f-1} \tag{6}$$

If the asymmetrical peak can be modelled with an exponentially modified Gaussian function, the following relationship applies [36]:

$$T_f = 0.6A_s + 0.4 \tag{7}$$

This relationship allows conversion of A_s = 1.39 reported in Table 2 for benzene at 1 mL/min to T_f = 1.23, in agreement with the figure of 1.3 quoted by Guiochon. McCalley [37] later compared column efficiencies (measured by both the half-height and Dorsey–Foley procedures) for nine different basic solutes on the commercial monolith and a number of different packed columns using acetonitrile combined with phosphate buffers at pH 2.7 and pH 7.0 (see Table 3). The Dorsey–Foley (DF) equation takes into account the peak asymmetry in the calculation of the column efficiency:

$$N_{DF} = 41.7\frac{(t_r/w_{0.1})^2}{(A_s + 1.25)} \tag{8}$$

In agreement with other studies [33], Table 3 shows that the monolith gave higher efficiency than the 5 μm packed columns tested, when efficiency was measured by the half-height method. However, the monolith is less competitive when the greater asymmetry of the peaks (average A_s = 2.01) is included in the DF method. Using Eq. (7), A_s = 2.01 converts to $T_f \sim 1.6$, a value similar to that given by Kele and Guiochon for the strong bases procainamide and benzylamine in acidic mobile phases (T_f = 1.5). The monolith gave the lowest number of plates using the DF equation when tested with strong bases at pH 7.0, due in part to the high average asymmetry of the peaks (4.33). Again these results are similar to the values of T_f = 3.7 for procainamide and 3.2 for amitriptyline reported by Kele and Guiochon. In agreement with these results, both the Tanaka alpha (benzylamine/phenol) parameter at pH 7.6, and the S–D $C(7.0)$ value are higher for the commercial monolith (Chromolith) than for the other Type B columns in Table 1, indicating quite a high silanophilic activity. No details of the preparation of the C18 monolith have been released by the manufacturer. Thus it is unknown whether the monolith is prepared, clad with PEEK and derivatised *in situ* or, alternatively, whether it is prepared and derivatised first, and clad afterwards. In the first case, some restrictions on the conditions of the derivatisation are likely to be imposed by the presence of the PEEK, which cannot be heated much above 60 °C, and is not compatible with some solvents [38]. However, some practical difficulties could also be envisaged for the second procedure. Tanaka and co-workers [39] obtained considerably improved peak shapes for bases using *in situ* derivatisation with the alternative reagent $C_{18}H_{37}Si(CH_3)_2N(C_2H_5)_2$ and endcapping with trimethylsilylimidazole. The asymmetry of the peaks of the strong bases procainamide and *N*-acetylprocainamide when chromatographed using methanol–phosphate buffer pH 7.6 were greatly reduced via this simple procedure. A particular quality of monoliths is their good mass transfer properties, yielding low values of the plate height even at high mobile phase flow velocity [40]. Fig. 3 shows van Deemter plots for weak bases and benzene in ACN–water, under conditions where the bases are probably not protonated. Small values of the C coefficient (~1.0 ms on average) are shown when the plate height is calculated from the half-height efficiency. Small frictional heating effects at their low operating pressures may contribute to the good performance shown [41]. The van Deemter plots have a more unusual profile when the DF effi-

Table 2
Asymmetry factors for weak bases and benzene as a function of flow rate. Mobile phase: ACN–water (40:60, v/v) column, Chromolith C18 (10 cm × 0.46 cm i.d.).

Flow rate (mL/min)	Pyridine	Aniline	4-Et aniline	Benzene
0.5	2.79	1.60	2.01	1.23
1	2.65	1.73	2.18	1.39
2	2.11	1.62	1.73	1.43
3	1.82	1.48	1.38	1.36
4	1.79	1.49	1.35	1.36
5	1.70	1.44	1.28	1.33

Table 3
(a) Comparison of average column efficiencies and asymmetry factors for nine different probe compounds for 3 particle packed columns and for a monolithic column (Chromolith) using ACN-phosphate buffe. Efficiency in plates/m.

Column	N (half-height)	N (Dorsey–Foley)	A_s
Buffer pH = 2.7			
Symmetry C18	89600	72600	1.34
Discovery C18	90900	65300	1.33
Symmetry 300	66700	47600	1.47
Chromolith	117600	53600	2.01
Buffer pH = 7.0			
Symmetry C18	23500	9300	4.26
Discovery C18	73300	36600	1.98
Eclipse XDB C8	50300	21700	4.19
Chromolith	25600	6900	4.33

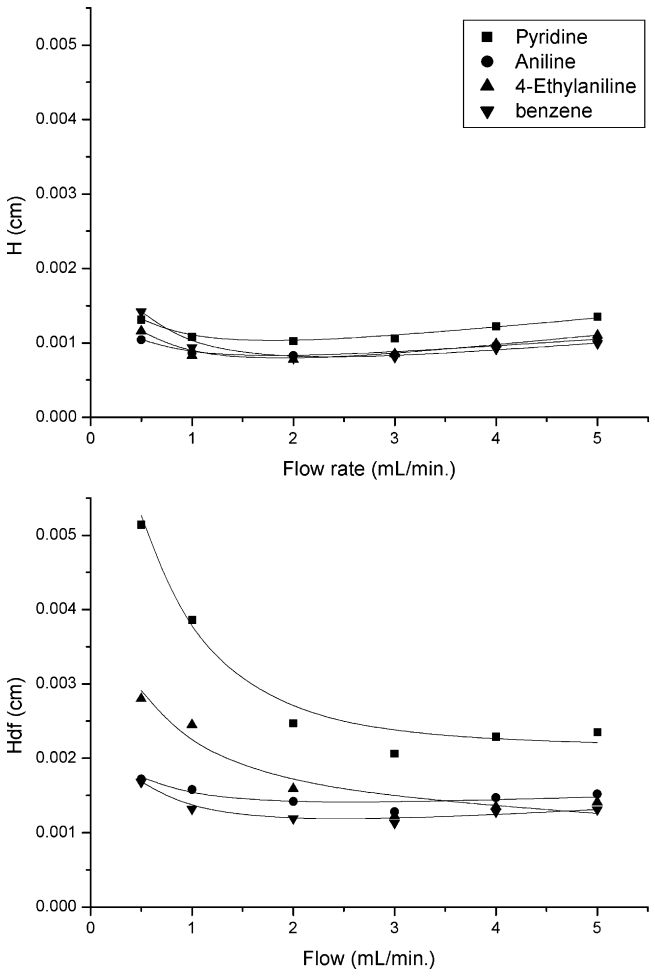


Fig. 3. van Deemter plots for weak bases and benzene on Chromolith RP-18 (10 cm × 0.46 cm i.d.) using plate height based on *N* at half-height, or the Dorsey–Foley method. Mobile phase: acetonitrile–water (40:60, v/v). (1 mL/min. corresponds to a linear velocity of 0.13 cm/s).

ciency is used to derive the plate height and there is more scatter, due to the poorer reproducibility of the DF efficiency. The shape of the curves can be partially explained by the decrease in asymmetry factor for the bases as the flow rate increases (see Table 2, Fig. 4). We presently have no explanation for this effect, which however, did not occur for the neutral compound benzene. The effect was noted both for weak bases in unbuffered mobile phases (Fig. 4a) and also for stronger bases in a mobile phase buffered at acid pH (Fig. 4b). The calculated values of the *C* coefficient are somewhat brought into question by this result, as it acts against the normal increase in plate height due to increased mass transfer resistance at higher flow rate. Generally similar results were found for the van Deemter curves of strong bases in a buffered mobile phase at low pH (results not shown). It is possible that the observed effects might be related to the radial inhomogeneity of the commercial monolith [42]. It can be concluded that the present generation of commercial monoliths shows reasonable but not exceptional performance for the analysis of basic solutes.

2.4. Slow column equilibration. Anion-exchange behaviour of alkylsilica RP columns

While silica-based RP columns possess underivatised silanol groups that can become ionised at neutral or high pH giving negative sites capable of exchanging cations, some bonded phases have positively charged sites that can exchange anions. For example, some types of EPG phase, specified as amide phases by their manufacturer (or believed to be amide phases) give strong retention of acidic probes [28]. It was suggested that this property was likely to result from a high concentration of amino functionalities, presumed to be as a result of a two-step bonding technology involving bonding of such functionalities in the first step. Furthermore, some phases have used endcapping reagents with amino functionalities, which generate considerable anion-exchange character. However, positively charged sites may also exist on some conventional Type B C18 columns (i.e. without embedded polar groups or amino endcapping). It seems that these phases also exhibit a slow equilibration of retention for ionised solutes, when the column is first exposed to buffered mobile phase at low pH [43]. Whereas non-ionised compounds exhibit constant retention times within around 20 min of the beginning of mobile phase flow, the retention of ionised compounds can continue to change (by 20% or more) for several hours before solute retention times became constant (within ±1%). The retention times of cationic and anionic solutes drift in opposite directions—the retention of protonated bases and quaternary compounds drifts to lower retention, while the retention of acidic solutes drifts to higher retention. If the mobile phase pH was changed from low to high and back again, an even longer time may be required before the column reaches equilibrium again at low pH. The speed of column equilibration for ionised solutes was found to vary significantly among different RP columns, and was not affected by flow rate. Therefore, it was proposed that column equilibration could be effected by storing the column in mobile phase prior to use (static equilibration). Rosés and co-workers first demonstrated the presence of positive charges on the surface of the RP column Symmetry C18 (Waters) by the anion-exchange retention of nitrate ion as a function of mobile phase ionic strength

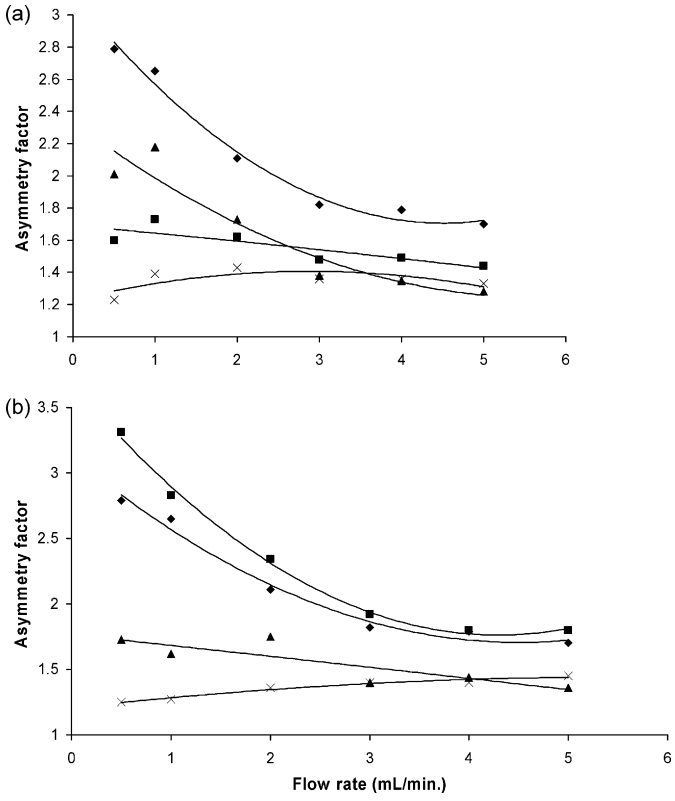


Fig. 4. Variation of asymmetry factor with flow rate. (a) Mobile phase: acetonitrile–water (40:60, v/v). Peak identities: pyridine (diamonds), aniline (squares), 4-ethylaniline (triangles), benzene (crosses). (b) Mobile phase: acetonitrile–phosphate buffer pH 3.0 (30:70, v/v). Peak identities: pyridine (diamonds), quinine (squares), nortriptyline (triangles), benzene (crosses).

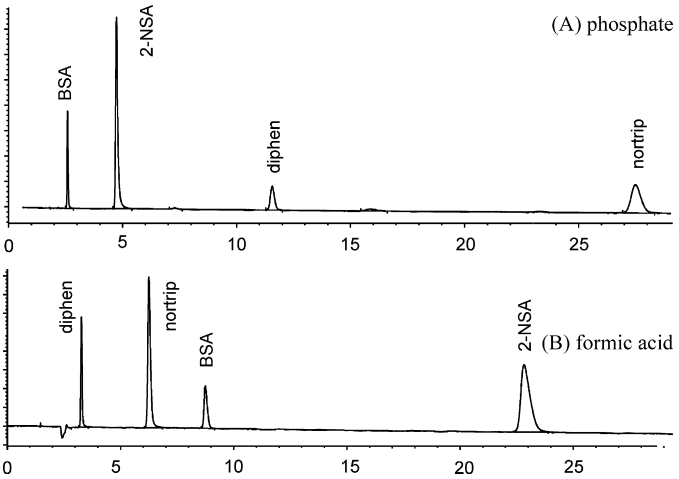


Fig. 5. Separation of acidic and basic compounds on a Symmetry 100 column. Mobile phase (a) ACN–0.02 M phosphate buffer pH 2.7 (28:72, v/v); (b) ACN–0.02 M formic acid, pH 2.7 (28:72, v/v). Flow rate 1.0 mL/min. Detection UV at 215 nm. Column temperature 30 °C. See [45].

[44]. McCalley [45] showed unusual differences in retention of acidic and basic solutes when using formic acid compared with phosphate buffers at the same pH. On one variety of ODS packing (Symmetry C18), the acids eluted before the bases in phosphate buffer but after the bases in formic acid buffer at the same pH, giving a dramatic selectivity change (see Fig. 5). This effect was also attributed to the existence of anionic sites on the surface of this phase. Positive charges may exist due to residues remaining of certain basic catalysts used in the synthesis of some phases. While pyridine is the classic basic catalyst used in the synthesis of bonded silica phases [46], the particular catalyst employed by a given manufacturer is rarely, if ever, revealed. At low ionic strength, repulsion of cationic solutes from these sites takes place, decreasing the retention of basic solutes; conversely retention of acid solutes occurs. As the ionic strength of the buffer is raised (e.g. by changing from formic acid to phosphate buffers) the charged column sites are increasingly screened from these interactions, leading to increased retention of protonated bases and reduced retention of acids. Loeser and Drumm [47,48] similarly showed that cationic solutes often give increased retention when mobile phase salt content is increased, explaining this result as due to a cation exclusion mechanism, rather than to chaotropic or ion-pairing, to which this result is more normally attributed. Their studies used a buffer anion (dihydrogen phosphate as used also in [45]) that exhibits minimal chaotropic/ion-pairing effects. Glycinamide (in protonated form, assumed to have negligible RP retention in 33% ACN) was used as a cationic void volume marker, with uracil as a neutral marker, assuming that the differences in retention volumes between these markers would reflect an excluded volume inaccessible to cationic analytes. At the lowest ionic strength tested (1.4 mM) it appeared that as much as 80% of the pore volume became inaccessible to the glycinamide cation, and three model cationic analytes showed retention loss approximately proportional to the excluded volume as ionic strength was decreased. Plots of k (for nitrate ion) vs the reciprocal of competing anion concentration in the mobile phase [$H_2PO_4^-$] were linear as expected from the relationship:

$$k = \frac{K}{[A^-]} \quad (9)$$

where [A^-] is the concentration of competing anion in the mobile phase, and K is a combined constant that reflects both the ion-exchange equilibrium constant and the ion-exchange capacity of the column. The RP columns Symmetry, Gemini and XTerra MS had significant positive charges at both pH 3 and pH 4.5. From the slope of the k vs [A^-] plot it appeared that Gemini and Symmetry had similar cation-exchange properties, which were about 10 times larger than for XTerra MS. In contrast, Zorbax SB C18 appeared to be essentially neutral at pH 3.0 and became negative at pH 4.5, as expected from ionisation of silanols. The authors also concluded that the loading capacity of the column could be expected to decrease if a large proportion of the stationary phase was inaccessible to the solute. A different study compared the loading capacity for bases on a custom-prepared classical pyridine-catalysed XTerra MS column (which had no positive charges on the surface) with that of a commercially available XTerra MS column. This study showed little difference in the (apparent) saturation capacity for bases, indicating these positive column charges were not the major source of rapid overloading, at least not on this phase (see Section 4 below). However, it is possible that different results would be obtained on the Symmetry C18 phase, which has much greater anion-exchange properties. It is possible that the contamination of the packing by metals, especially from the frits, might contribute to cation-exchange behaviour of some phases [49].

In later work, Marchand and Snyder [50] measured the extent of anion-exchange behaviour using nitrate ion as a probe. It was found that all but 3 of the 14 columns studied carried a significant positive charge. The relative positive charge correlated approximately with the relative cation-exchange behaviour as measured by the $C(2.8)$ values of the hydrophobic subtraction model and the slow column equilibration effect. In this study which used a lower concentration of buffer (33% ACN and 9.8 mM buffer vs 50% ACN and 60 mM previously), changes in retention time were greater, but the equilibration time was reduced to within an hour or two. The authors considered that the presence of these positive charges could explain why, if Type B RP columns have very few negative charges on their surface at low pH, there is nevertheless considerable variation in the values of $C(2.8)$ for columns tested by the hydrophobic subtraction procedure. For Type B columns, C −2.8 varies over the range $-0.6 < C$ −2.8 < 0.4, whereas it should be otherwise much more consistent. The presence of cation-exchange sites would reduce the retention of positively charged sample ions by ion exclusion, resulting in lower values of C −2.8. It is uncertain how many columns are affected by anion-exchange behaviour, although it was noted that most C18 columns with very low values of C −2.8 are made by just two column manufacturers. This observation strengthens the hypothesis that the effect is due to the use of a particular catalyst in the production process.

Clearly, the problem of slow column equilibration is yet another difficulty and complication that confronts the analyst in the determination of basic compounds. It can produce totally unexpected changes in the selectivity of a separation dependent on mobile phase ionic strength, as demonstrated in Fig. 5. However, if the analyst is aware of this effect, it can be overcome relatively easily, either by choice of phase, by static equilibration of the column prior to use, or by storing the column in the mobile phase, although this could lead to phase degradation if maintained for long periods. It should be noted that all columns tested by the S–D method were equilibrated prior to testing, so published results were not compromised by slow column equilibration.

2.5. Use of column materials other than silica

Silica has been the material of choice for LC columns for many years. However, the problems of silica (such as underivatised silanols on RP materials) have encouraged work on alternatives, whose use was covered briefly in an earlier review [13]. Basically, the conclusions of that review remain the same. A number of materials including purely polymeric columns, zirconia or titania based phases have been commercially available for many years as alternatives to silica. However, these materials still seem to

suffer from their own disadvantages. For example, while purely polymeric columns do not possess silanol groups, two types of such phases were shown to possess negatively charged groups on their surface that are apparently capable of producing tailing peaks at intermediate pH in the same way as found for silica columns ([51], see below). Furthermore, most polymeric columns have considerably lower efficiencies than silica-based columns. New types of polymeric column have recently been developed with improved efficiency, and it has been suggested that these will be much more competitive with silica-based columns in the future [52]. Zirconia columns, as developed originally by Carr and co-workers, have also shown some promise for the analysis of basic compounds [53,54]. They are often coated with organic polymers to confer RP properties, as silanisation is not useful due to the relative instability of the Zr–O–Si bond. These phases can produce useful selectivity differences from that provided by silica-ODS phases, for example when using phosphate buffers at pH 7. Buffer anions become adsorbed on the surface of the zirconia phase, providing cation-exchange sites which can provide the majority of the retention, compared with hydrophobic retention which dominates the properties of silica-ODS phases. More comparative studies of the properties of these new phases with silica-based phases are necessary. It is likely also that a considerable resistance exists within the chromatographic community to changing to a column material that may have unfamiliar and unexpected properties, compared with the wealth of knowledge and experience that has been accumulated with silica-based phases over the last 40 years or so. Thus, it seems that silica-based phases will remain the material of choice for the analysis of bases for the foreseeable future.

3. Choice of mobile phase

3.1. Choice of modifier

Some work has indicated that peak shape for basic compounds can be optimised by judicious choice of RP modifier (ACN, methanol or THF), although little recent work has been published on this subject. A consideration of earlier results [13] indicates that of the common RP solvents, THF may be advantageous in giving good peak shapes for basic compounds, in mobile phases buffered at both pH 3 and pH 7. Nevertheless, there is considerable resistance to the use of THF within the chromatographic community, possibly due to perceived hazards associated with the presence of organic peroxides in this solvent as well as other practical difficulties. Acetonitrile generates somewhat higher efficiencies than methanol at low pH, presumably due to its lower viscosity, thus higher mobile phase diffusion coefficients and improved efficiency that result when the column is operated at a fixed flow rate (typically one that is somewhat higher than the optimum flow velocity). At low pH, silanophilic interactions may be sufficiently suppressed such that the same effects are dominant for bases as with neutral solutes. At intermediate pH, isoeluotropic mixtures of methanol-buffer can give improved efficiency compared with acetonitrile-buffer. This may be because of some deactivating effect of methanol on ionised silanol groups [31], or could be merely due to the greater lowering of basic solute pK_a which takes place in the higher concentrations of methanol compared with acetonitrile necessary to achieve isoeluotropic mobile phases. Of course, there may be other reasons for choice of modifier, such as to achieve a particular selectivity in a separation.

3.2. Choice of mobile phase pH. Problem of reduced retention of bases at low pH

It is clear that superior peak shapes for bases are found at low compared with neutral pH, due to the suppression of the ionisation of column silanol groups. The evaluation data in Table 1 illustrates this finding; for example, much higher values of alpha (benzylamine/phenol) are obtained at pH 7.6 than at pH 2.7 in the T–E test, indicating higher silanophilic activity. In a study using nine basic probe solutes and nine 25 cm Type B columns, the average efficiency and asymmetry factors were 14,500 and 2.0, respectively, at pH 7.0 compared with 18,600 and 1.3, respectively, at pH 3.0 when using very small amounts of solute to avoid column overload [27]. Somewhat improved peak shapes at pH 7 were found in subsequent work when NH_4^+ was the buffer cation rather than K^+ [37].

A disadvantage of working at low pH is the low retention of hydrophilic bases such as catecholamines, drug metabolites and some drugs of abuse. Carr and co-workers developed a silica-based, hyper-crosslinked sulfonate-modified RP for separating highly hydrophilic compounds [55]. Use of highly aqueous eluents in conjunction with conventional RP columns may give rise to phase de-wetting, resulting in poor retention, low selectivity and irreproducibility, whereas the use of ion pair reagents to increase retention may render the separation incompatible with mass spectrometry, and also complicate preparative separations. Sulfonate-modified RP columns as synthesised by the Carr group increase retention by the hydrophobically assisted cation-exchange mechanism suggested in Eq. (2). The separation could be manipulated either by changing the type or concentration of ionic additive or organic modifier. The authors also showed useful selectivity differences between this phase and conventional RP columns. The column had, however, low ion-exchange capacity, thus avoiding the necessity for use of strong displacers at high concentrations. Indeed, the weakest cation-exchange displacer, the proton, was strong enough to elute all the highly hydrophilic amines studied, for example using TFA at a concentration of <0.1%, decreasing the background for both UV and MS detection. Neutral compounds were also quite well retained, due to interaction with the hydrophobic parts of the bonded chain. Despite the possible disadvantages of ion pair reagents mentioned above, Carr showed enhanced retention and efficiency of basic analytes on conventional RP columns by incorporating strong inorganic pairing ions (e.g. perchlorate) into the eluent [56]. Somewhat surprisingly, larger retention increases on addition of the anion were observed at higher concentrations of organic modifier in the mobile phase. It was believed that this phenomenon was caused by the decreased dielectric constant of the mobile phase as % ACN was raised, which should, for example, enhance the formation of classical ion pairs in the mobile phase.

While low pH analysis is clearly favoured for most columns, recent studies have indicated that there is little difference in the efficiency of some hybrid columns at low and intermediate pH [57]. Fig. 6a shows a plot of N vs sample mass for the quaternary ammonium compound benzyltriethylammonium chloride on an XTerra RP-18 column at $^{s}_{w}$pH 3.2 and $^{s}_{w}$pH 7.4 over a wide range of sample mass (0.04–20 μg). The subscript w refers to standardisation of the pH meter in aqueous buffers, and the superscript s to the measurement of the pH in the aqueous–organic solution (for a fuller discussion of pH and pK_a measurements in aqueous–organic solution, see [13]). The ionisation of quaternary compounds is independent of pH; thus, any changes in performance must be due to changes in the column. For the hybrid phase, there is virtually no difference in the results over the mass range studied at either pH, indicating that little change occurs in the state of ionisation of the column groups at intermediate pH. In contrast, Fig. 6(b) shows the performance of an average Type B column (Inertsil ODS-2), indicating that the performance across the entire range of sample mass is considerably reduced at intermediate compared with low pH, presumably due to silanol ionisation. The consistency of performance at low and intermediate pH on the hybrid column was observed for several basic solutes in addition to quaternary compounds. A different study [44] suggests that there are no ionised silanols on the

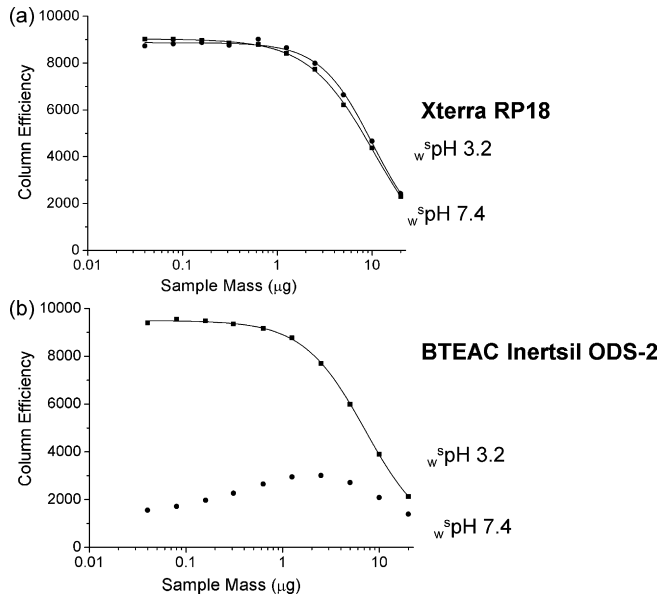


Fig. 6. Variation in efficiency with mass of benzyltriethylammonium chloride on XTerra RP18 (a) and Inertsil ODS-2 (b). Mobile phase: acetonitrile–phosphate buffer $_w^w$pH 2.7 (20:80,v/v), $_w^s$pH 3.2 (squares) $_w^s$pH 7.4 (circles). See [57].

surface of some inorganic–organic hybrid phases at low or even neutral pH. The retention of lithium ions on such a hybrid phase does not increase as the pH is increased from acidic to at least intermediate pH, in contrast to the behaviour of older RP columns, where Li^+ shows a continuous increase as pH is raised above 2, due to the gradual ionisation of silanol groups (see Fig. 7). It is possible that the pK_a of the silanols is raised in the more organic environment in which the silanol groups are situated in these hybrid phases.

McCalley and co-workers compared peak shapes for bases on a hybrid column (XTerra RP18) from low pH to $_w^s$pH 10 [57]. Hybrid phases are stable to higher pH than pure silica-based phases. At $_w^s$pH 10 slight improvements were noted in *N* for some solutes of relatively low pK_a e.g. amitriptyline ($_w^s$pK_a 8.9), but quite substantial deterioration for higher pK_a solutes such as amphetamine ($_w^s$pK_a 9.8). Similar results were obtained for the newer bridged ethyl hybrid version of phase [58]. It appears that at high pH, the silanols start to ionise and unless the mobile phase pH is considerably in excess of the solute pK_a, detrimental ionic interactions can still occur. Thus, increased peak tailing and reduced column efficiency was obtained for some strongly basic solutes even at $_w^s$pH 12.0 compared with the usual low pH operation. These results are discussed in more detail below in Section 4.

Despite the apparently favourable properties of these hybrids, they do have some drawbacks. The methyl hybrid has reduced efficiency compared with pure silica-based phases at low pH (when the latter do not suffer from strong silanophilic interactions) and it has less favourable mass transfer properties, leading to poorer efficiency at high mobile phase flow rates [12]. It is possible that these features may have been resolved in the newer bridged ethyl hybrid phase (Waters Corporation).

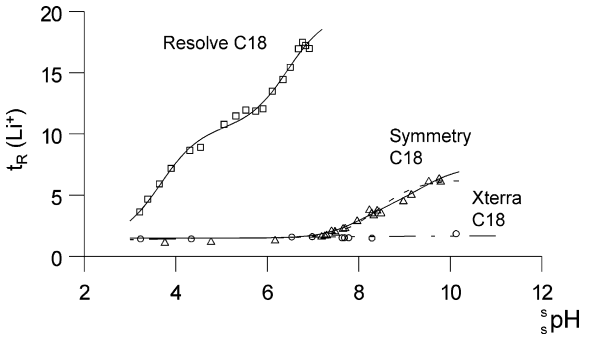


Fig. 7. Plot of retention of Li+ against $_s^s$pH for various Waters columns (after [44]).

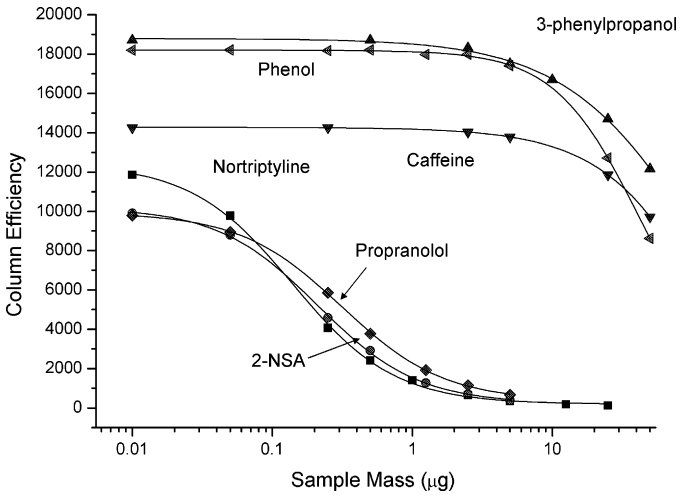


Fig. 8. Plot of column efficiency against sample mass for three neutral compounds (3-phenylpropanol, caffeine, phenol), and three charged compounds (propranolol, nortriptyline, 2-naphthalenesulfonic acid) on XTerra MS C18 (15 cm × 0.46 cm, 3.5 μm particles). Mobile phase: 0.02 M formic acid $_w^w$pH 2.7 in ACN–water (28:72, v/v) except for caffeine (12.5:87.5, v/v). Flow: 1 mL/min. After [60].

4. Overloading

4.1. Overview of the problem

Overloading is the deterioration in column performance that results from increasing sample mass or volume. The latter problem can easily be eliminated by reducing the injected volume. It is usually possible to increase concomitantly the concentration of the sample if necessary for detection. However, mass overload is more intractable as it can occur with extremely small amounts, at levels where detection sensitivity is compromised if the injected amount is reduced. After the use of pure silica had become common in the manufacture of RP columns, limiting the undesirable interactions of protonated bases with ionised silanols, overloading is considered by some researchers to be one of the main causes of the difference in performance of different types of phase in analytical chromatography [59]. Furthermore, considerably more information can be derived on the retention mechanism from consideration of the peak shape for a wide range of solute mass, rather than a single injection of small mass, emphasising the importance of these studies.

Fig. 8 shows a plot of column efficiency (measured from the peak width at half height) against sample mass for three polar (but neutral) compounds (phenol, caffeine and 3-phenylpropanol), two strong bases (nortriptyline and propranolol), and one strongly acidic compound (2-naphthalenesulfonic acid) [60]. The bases and acid are completely ionised under the mobile phase conditions (acetonitrile–water 28:72 (v/v) containing 0.02 M formic acid pH 2.7). While some deterioration in *N* occurs for the neutral compounds when the sample mass exceeds ~10 μg, a similar deterioration in the peak shape of both the acids and bases occurs when loads greater than ~0.1 μg are introduced, a figure some two orders of magnitude less. Clearly, there can be serious problems with the overloading of ionised bases.

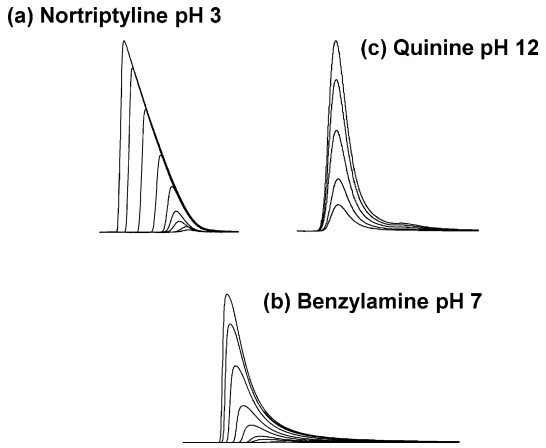


Fig. 9. Variation of peak shape with sample mass on purely polymeric columns. (a) 0.1–20 μg nortriptyline on Asahipak ODP-50 (12.5 cm × 0.46 cm i.d.), mobile phase: acetonitrile–phosphate buffer pH 3.0 (25:75, v/v), (b) 0.1–20 μg benzylamine on PRP-1, mobile phase: acetonitrile–phosphate buffer pH 7.0 (15:85, v/v) and (c) 2.6–20 μg quinine on PRP-1, mobile phase: acetonitrile–phosphate buffer pH 12.0 (43:57, v/v). Flow rate 1 mL/min. in each case. See [51].

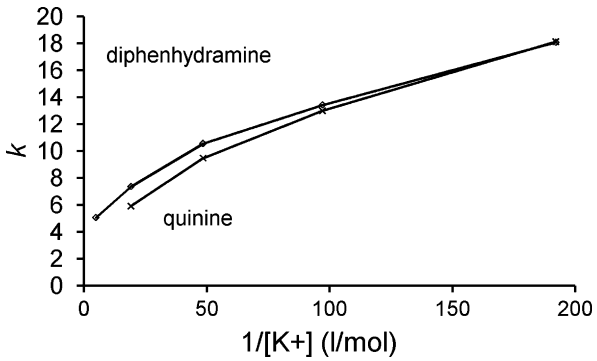


Fig. 10. Plot of k vs the reciprocal of buffer cation concentration for diphenhydramine (diamonds) and quinine (crosses) on PRP-1, mobile phase: acetonitrile–phosphate buffer pH 7.0 (40:60, v/v). See [51].

4.2. Possible causes of overloading

The reasons for the unusual overload behaviour of ionised acids and bases have been much debated in the literature. Snyder and co-workers, in studies performed more than 20 years ago with Type A phases (made of rather impure silicas, having a relatively high concentration of acidic silanols even at low pH) proposed that ionised silanols provided a relatively small number of strong retention sites for protonated bases. These are filled rapidly, prior to the filling of a large number of weak hydrophobic sites situated on the hydrophobic ligands of the RP column, which are the principal sites available for retention of neutral compounds. Using frontal analysis, it was shown that the total saturation capacity of the phase was nevertheless similar for both ionised and neutral compounds [61–63]. It is possible that the strong sites on this type of silica may relate to the "hydrophobically assisted" ionic retention sites [8] of Eq. (2).

Häglund and Ståhlberg studied the overloading of anionic solutes, in order to avoid the possibility of silanophilic interactions experienced by ionised basic compounds [64]. They explained the increased overloading of these ionised solutes as being due to mutual repulsion of the similarly charged ions occurring on the surface of the stationary phase, a possibility that had also been previously suggested by Snyder. Following the work of Ståhlberg, McCalley and co-workers [51] studied the overloading of ionised bases on purely polymeric columns such as Hamilton PRP-1 made of polystyrene–divinylbenzene, which clearly contain no silanols. Fig. 9(a) shows overloaded peaks profiles for the strong base nortriptyline on a polymeric Asahipak ODP-50 column using acetonitrile–phosphate buffer pH 3.0, which show the typical overloaded peak shapes as given also on silica-based columns. As usual, retention times decrease with sample load. Values of the column "saturation capacity" were calculated according to the method of Snyder et al. [62] from the equation:

$$W_{\text{base}}^2 = \frac{16t_0^2(1+k_0)^2}{N_0} + \frac{6t_0^2k_0^2w_x}{w_s} \quad (9')$$

where W_{base} is the peak width at base, w_x is the sample mass, t_0 is the column dead time, k_0 and N_0 are the retention factor and column efficiency of a very small sample mass (non-overloaded peak), and w_s is the column saturation capacity. Note that the saturation capacity calculated in this way may correspond to the saturation of an important population of scarce strong sites, which dominate the performance of the column, but ignore the loading of the plentiful weak sites, which are only filled afterwards—see below. They can be referred to as "apparent saturation capacities". As results were similar on both silica-ODS and purely polymeric columns, it appears that a common overload mechanism was in operation for both, and therefore could not involve silanols. It was concluded that the Ståhlberg interpretation of mutual repulsion of solutes of the same charge was most likely to be responsible for overload, as this mechanism could occur on any surface. However, some caution is necessary in the interpretation of results from these purely polymeric phases, as they possess anionic sites at pH 7, giving behaviour apparently analogous to the ionised silanols of silica-based columns (see above). Fig. 9(b) shows exponential peak tailing for the analysis of benzylamine at pH 7 on a polymeric PRP-1 column (Hamilton) using acetonitrile–phosphate buffer pH 7. Fig. 10 shows plots of k vs the reciprocal of buffer cation concentration on the same column, showing that retention is reduced as the buffer concentration increases. Retention sites for cations may be due to the incorporation of acid moieties on the stationary phase, resulting from catalysts used in their production. However, these groups are not ionised at pH 3.0 [51]. This work also showed that the overload trends of efficiency vs sample mass did not differ substantially when N is measured by the half-height or statistical moments procedure, which gives a more accurate value for non-Gaussian shaped peaks. In further studies, using a hybrid silica column at low pH (on which there are presumed to be no ionised silanols—see above) McCalley and co-workers confirmed also that the right-angled triangle peak shapes typical for overload were exactly similar for ionised bases (e.g. propranolol) and acids (e.g. 2-naphthalenesulfonic acid) as shown in Fig. 11 [60]. These two compounds have very similar retention; also there is little evidence of the exponential tailing typical of the interaction of protonated bases with ionised silanols for either solute. This study again indicates that ionised silanols were unlikely to be the source of the overloading, which clearly occurs in a similar fashion on hybrid columns, conventional silica-based ODS phases, and purely polymeric columns. Nevertheless, the overload of ionised silanols as described originally by Snyder remains a possible contribution to the overloading mechanism on some older (Type A) stationary phases.

Carr and co-workers [59] reported a new approach to the determination of column overload characteristics, again based on efficiency measurements. They developed a practical method for the precise measurement of the plate count at low sample load and a column overload parameter using a simple but mathematically rigorous model of Langmuirian non-linear chromatography. The concept of the *limiting plate count* (N_0) was introduced, a value of the column efficiency for a very small quantity of cationic analyte,

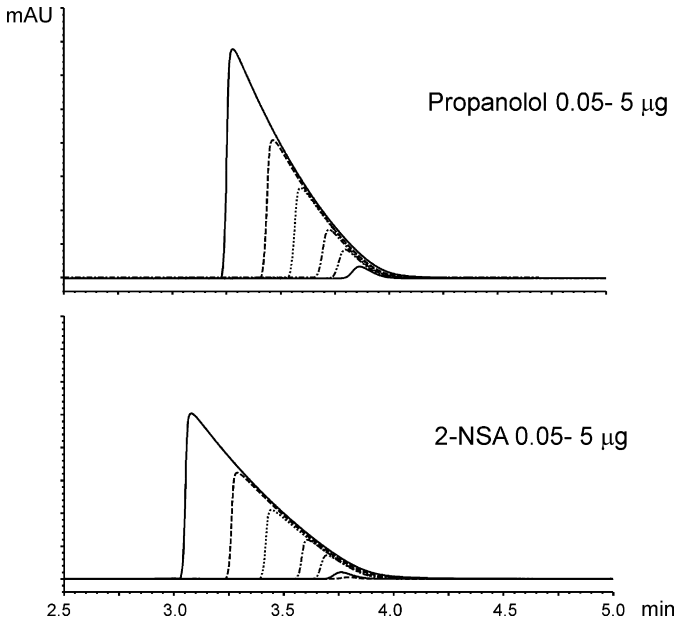


Fig. 11. Overlaid chromatograms on XTerra MS (15 cm × 0.46 cm i.d., 3.5 μm particles) for propranolol (0.05–5 μg) and 2-naphthalenesulfonic acid, mobile phase: 0.02 M formic acid in acetonitrile–water (28:72, v/v). Flow rate 1 mL/min. See [60].

derived from values of N at higher sample load using a non-linear fitting procedure to a universal overload curve obtained from the theoretical model. In this way, the precision and accuracy of values of N_0 are not affected by the problem of noise which can result from their direct empirical measurement, when the sample concentration is of necessity very small. Plate counts were calculated from the square of the first theoretical moment of the peak divided by the second theoretical moment. This measure of column efficiency ("statistical moments method") is available from some data stations (e.g. as marketed by Agilent Technologies, Waldbronn, Germany). Nevertheless, reasonable agreement with the model could still be obtained using column efficiency derived from the peak width at half height. The analytical sample loading capacity $\omega_{0.5}$ was defined as the sample load required for the plate count to decrease to half of its limiting (i.e. N_0) value. Values of N/N_0 plotted against $\omega/\omega_{0.5}$ were shown to fall very closely on a single master curve independent of k and N_0, exactly as predicted by a theoretical treatment based on the Wade–Lucy–Carr model of overload. The sample load capacity $\omega_{0.5}$ was shown to be dependent on the particular C18 stationary phase used, and higher capacity appeared to be related to larger surface area of the stationary phase. It was proposed that because N_0 did not show a large dependence on the particular stationary phase for matched column, particle size and flow rate, the differences in efficiency for basic solutes between various stationary phases could be largely due to differences in loading capacity.

In an extensive series of publications, Gritti and Guiochon [65–70] have investigated column overload, through rigorous evaluation of the adsorption isotherms (mostly obtained using frontal analysis) and calculating the adsorption energy distribution. They claimed that while this is the slowest and most costly method, it is also the most accurate. It involves the stepwise replacement of the mobile phase with analyte streams of different concentrations, and determining the resulting breakthrough curves. The adsorbed amount of solute is calculated from the solution concentration and from the time when the analyte solution is introduced and when the plateau recorded on the detector is reached. For overloading of propranolol on the hybrid phase XTerra MS [65], they concluded that the surface consisted of two different types of site with different adsorption energies and different saturation capacities—plentiful low-energy sites and much less common high-energy sites. The high-energy sites are filled first. They have a very low capacity and thus readily lead to deterioration in peak shape with increasing sample load. Only when these high-energy sites are mostly or completely filled do the low-energy sites begin to become occupied. High-energy sites were considered as unlikely to be free silanol groups, because the difference in energy between these and the low-energy sites was too small (<10 kJ/mol). They also considered that while the saturation capacity of the high-energy sites was low (they are easily overloaded) their capacity was still too high for them to be ionised silanols, considering the very low density of these sites on the hybrid column (see above). They suggested instead that the two sites corresponded to two different environments in and around the alkyl ligands bonded to the adsorbent surface, with the low-energy sites corresponding to simple interactions with these ligands. According to the authors, the increase in saturation capacity obtained when increasing the concentration of KCl in the mobile phase could be due to a decrease in repulsive interactions between the charges of the adsorbed cations and the decrease in the surface potential. These authors also studied the adsorption of the strong base nortriptyline [66], replicating the conditions used in previous work by McCalley [45] on Discovery C18 (Supelco). The authors deduced that in buffered mobile phases at acid pH, three types of site existed of high, medium and low energy on this column. Due to the high-energy difference between the highest and lowest energy sites, the former might be ionised silanols giving strong electrostatic interactions. The total saturation capacity of the column was found to be of the same magnitude for nortriptyline as for neutral compounds, similar to the results previously found by Snyder (see above). However, this large saturation capacity was deemed not practically useful due to the low saturation capacity of the high-energy sites, which dominate the column performance. In a later publication [67], the authors appeared to contradict the hypothesis that ionised silanols were the source of the high-energy sites, apparently in the light of the experiments by Rosés and co-workers [44] indicating that there were few if any ionised silanols on Type B phases at low pH. In this paper they reported saturation capacities were in the ratio 1:20:300 for three sites of strong, intermediate and weak energy. Subsequently, these authors [68] studied the overloading of caffeine, phenol, propranolol and 2-naphthalenesulfonic acid, concluding that between two and four adsorption sites were involved in the retention of these solutes. The number of sites found depended on the particular solute. Only the highest energy sites q_s4 apparently involve electrostatic interactions. They stressed the heterogeneous nature of the adsorption mechanism in RP–LC [69], explaining that active high-energy adsorption sites are filled first as the sample load increases, so that retention rapidly decreases, and peak tailing results. On the XTerra MS C18 hybrid phase, caffeine and phenol (neutral polar compounds) adsorb onto a large number of weak adsorbent sites at the interface of the C18 layer and more strongly onto a small number of sites located deeper inside the C18 layer. The difference in adsorption energy between these sites was small (maximum ~7.5 kJ/mol). There was no apparent difference in the adsorption mechanism of ionised acids and bases (naphthalene sulfonate and nortriptylinium) with both showing evidence of three adsorption sites. This result was in agreement with the overload profiles found by McCalley as shown in Fig. 11. There was a much higher difference in energy between the highest and lowest adsorption sites (15–20 kJ/mol) compared with the result for the neutral polar compounds. The highest energy sites were very few in number, having a saturation capacity two to three orders of magnitude lower than the total saturation capacity of the phase. In these recent studies, the authors expressed considerable doubt over the theory of charge repulsion of adsorbed compounds, claiming that it could not satisfactorily explain the

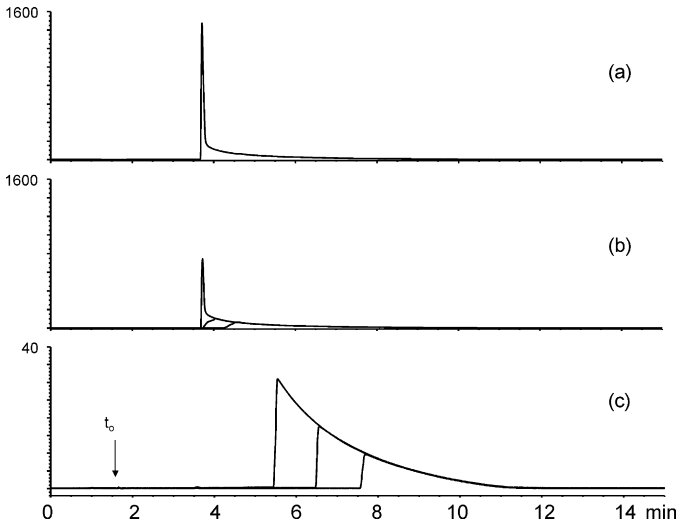


Fig. 12. Chromatograms for nortriptyline on XTerra MS C18 for (a) 250 μg, (b) 100, 150, 200 μg and (c) 12.5, 25, 50 μg. t_0 indicates the column dead time. Mobile phase: 0.02 M formic acid in acetonitrile–water (28:72, v/v). See [60].

band shapes for these charged bases when in the presence of their conjugated neutral species [70]. They concluded instead that the adsorption of ionisable samples in RP–LC must involve a very small number of ion-exchange or ion-dipole sites which constitute the easily overloaded strong sites that they have postulated previously. However, they stated that the nature of these sites is still obscure, and they admit that conclusions regarding their nature would be hasty, especially as the hybrid material they used (Waters XBridge C18) appears to contain very few silanol groups and their pK_a is rather high. Ionised silanols thus seemed not responsible for the adsorption behaviour they found for aniline. They speculated that solute ions may strongly interact with the accessible bare silica surface, competing with water, the organic modifier and the other co-ions and counter ions present in the eluent.

Some supporting evidence for the idea that multiple sites are involved in overloading has been shown by others (see Fig. 12 [60]). A sharp, L-shaped peak with a long tail was given by injection of very large amounts of the basic drug nortriptyline on the inert hybrid phase XTerra MS C18 at low pH. The peak appears rather suddenly as a mass of ~150 μg was introduced. It is possible that this peak corresponds to the filling of the high capacity weak sites of this column, after the strong sites have been fully saturated. Further increases in the sample mass do not produce the decreases in retention time observed when sample mass is increased in the lower range, possibly as these increases do not saturate these weak sites. Nevertheless, other interpretations of Fig. 12 are possible. A complication is that the mobile phase buffer is substantially overloaded in these experiments by the sample. For example, the sharp peak at lowest retention may correspond to nortriptyline hydrochloride, where the chloride counter-ion has never been replaced by the mobile phase buffer anion (formic acid), whereas the long tailing peak is the fraction of the injected nortriptyline that migrated with the buffer counter-ion. Clearly, the separation ability of the column at these high loads is hardly of practical use, due to the long tail of the peaks (even though this is hardly evident at the low detector sensitivity necessary to record these results). Similar results were produced by heavy overload of the column with the acid p-xylenesulfonic acid [60].

4.3. Effect of buffer anion on overload

The nature of the buffer anion on the column overloading properties has been reported in a few studies. One study compared the overload of both ionised acids and bases on a number of different RP columns, using phosphate and low ionic strength buffers such as formic acid, recommended for mass spectrometric detection due to their volatility [45]. The author had noted previously that peak shapes for bases in formic acid buffers were considerably worse than in phosphate buffers of the same concentration [37]. The apparent saturation capacities in 0.02 M formic acid were on average less than 15% of the values in 0.02 M phosphate buffers and were also considerably reduced for acidic solutes in formic acid. This was the first time that a change in the useable sample capacity for ionised solutes using different buffers at the same pH had been reported in RP–LC. The ionic strength of the formic acid buffers was less than 10% of that of the phosphate buffers according to the method described for their preparation [45]. Therefore, the poorer efficiencies in formic acid previously reported could at least in part be due to greater overloading in this buffer. Further studies showed that increasing the ionic strength of phosphate buffers by the addition of KCl produced further increases in the apparent saturation capacity for basic and acidic solutes. The author concluded that increased mutual repulsion of ions could take place in low ionic strength buffers, as solute ions are less "screened" from one another.

Carr and co-workers [59] also showed that sample loading capacity was dependent on the nature of the mobile phase, being much lower with additives such as formic acid, in agreement with the previous results of McCalley [45]. For example, values of $\omega_{0.5}$ were around 15 times smaller in acetonitrile–water containing 0.05% formic acid compared with a similar buffer but with 0.1% formic acid containing 20 mM sodium acetate (i.e. considerably higher ionic strength). The Carr group have looked extensively at the role of ion-pairing in the retention and overloading of basic solutes. They measured ion pair formation constants by capillary electrophoresis [71], noting that the extent of ion-pairing in the mobile phase was not large with only 15%, 6% and 3% of the analyte present as ion pairs in 20 mM hexafluorophosphate, perchlorate and TFA, respectively. The relative contribution of the classical ion pair mechanism and the dynamic ion-exchange mechanism to the retention of cationic drugs was also considered [72]. The same group later looked at the effect of different anionic additives on the overloading of solute cations, which included the strong bases nortriptyline, desipramine, metoprolol and propranolol which have pK_a > 9 [73]. These solutes can therefore be assumed to be completely charged under the conditions of the experiments (pH 2.8 and 4.8). Using very small amounts of cationic analytes where the analyte exhibits the maximum plate count, they found that the type and concentration of the anionic additive had little effect on the column efficiency. Furthermore, the type and concentration of the anionic additive had only a small effect on the separation selectivity for the cations studied. However, the nature of the anion had a profound effect on the overloading profile of cationic solutes, with the effect of load on efficiency being strongly diminished by use of a stronger ion-pairing additive (at the same anion concentration), or by increasing the concentration of a particular anion as follows (see Figs. 13 and 14):

Cl^- (least effective) < CF_3COO^- < ClO_4^- < PF_6^- (most effective).
0 mM (least effective) < 40 mM < 60 mM (most effective).

The order of effectiveness of the anions corresponded to the order of ion pair constants determined by capillary electrophoresis previously [71]. The effect of concentration of the anion was rationalised as being due to an increase in the number of ion-exchange sites available, as more anions become adsorbed to the stationary phase. At the same time, the formation of a neutral ion paired analyte increases the amount of cation which can be loaded on to the stationary phase by allowing a greater proportion to be present

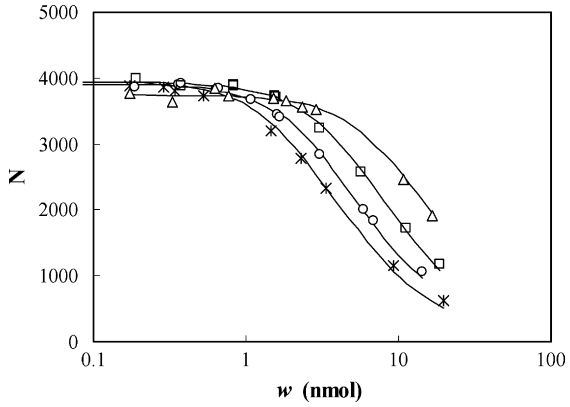


Fig. 13. Effect of anion type and amount injected on plate count of amitriptyline. Conditions: ACN–water (35:65, v/v), containing 20 mM sodium acetate at pH 4.8 and 20 mM NaX (X = Cl^-, $CF3COO^-$, $ClO4^-$, $PF6^-$). Plot legends, $PF6^-$ (triangles), ClO_4^- (squares), $CF3COO^-$ (circles) Cl^- (stars). Symbols, experimental data; lines, predicted data. See [73].

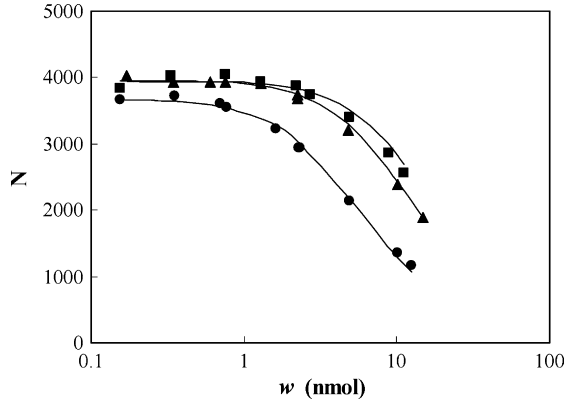


Fig. 14. Influence of concentration of sodium perchlorate on plots of efficiency against amount of amitriptyline. Mobile phase: acetonitrile–water containing 20 mM sodium acetate buffer pH 4.8. 60 mM (squares), 40 mM (triangles), 0 mM (circles). Constant ionic strength maintained in each case by addition of NaCl. Lines represent predicted data. See [73].

as an electrically neutral (ion paired) species. According to the authors, the anionic additives diminish the effect of mutual repulsion between the solute cations held on the stationary phase by both processes. The authors also noted that while chloride is a weak ion-pairing reagent, and does not sorb at all well to the stationary phase, increased chloride concentration significantly improved the sample loading capacity, possibly by decreasing the strength of electrostatic interactions between analyte cations by "screening" their mutual repulsion. This is in agreement with the Ståhlberg model.

Similar to these findings of the influence of buffer anion on overload, similar apparent saturation capacities were obtained for the basic drug nortriptyline using either 0.02 M phosphate or a considerably lower concentration of TFA (0.008 M). This result was attributed to ion-pairing with TFA [13,60]. Similarly, the considerably improved peak shapes of peptides obtained with acetonitrile-TFA mobile phases compared with those buffered with formic acid at the same concentration was attributed to the higher ionic strength and ion-pairing properties of TFA [74].

4.4. Overloading on mixed-mode reversed-phase/cation-exchange columns

Although mixed-mode ion-exchange/RP columns have been commercially available for many years, silica-based phases possessing ionic cation-exchange functionality embedded within a hydrophobic ligand have become available more recently [7]. These phases give very good peak shapes for basic compounds under the usual low pH mobile phase conditions. Another advantage of these phases is the superior loading capacity for ionised bases compared with conventional RP columns. A similar improvement was noted for columns containing a mixture of discrete RP and cation-exchange particles. In some cases, increases in the apparent saturation capacity of the phases by a factor of about 10 times were noted. The result could be attributed to the provision of extra ionic retention sites on the phase surface. Alternatively, if overloading is interpreted on the basis of mutual repulsion of solute ions, then the column ionic sites could neutralise or partially neutralise the charge of the solute ions, lessening the effects of this repulsion.

4.5. Effect of buffer pH on overloading

The peak shapes for small amounts of basic solute under a variety of conditions in RP–LC have been described above. Some early work compared overload of columns at low pH and pH 7 using sample masses up to 20 μg [75]. Deterioration in column efficiency with sample load was considerably less at pH 7. A possible explanation of this effect is that ionised silanols act as extra retention sites on the phase, increasing the column capacity. Nevertheless, peak shapes were extremely poor for most columns tested at pH 7, even for very small sample loads, presumably due to these ionic interactions which lead to kinetic tailing. Thus, the significance of these results is reduced. Newer phases, especially the hybrid discussed above (see Fig. 6) give better results at intermediate pH. More recent work by the same group [57] showed that the effects of overloading on some moderately basic compounds (amitriptyline) was gradually reduced as the mobile phase pH was raised from ${}^{s}_{w}$pH 3.4 to ${}^{s}_{w}$pH 10.1, using the hybrid phase XTerra RP18 (see Fig. 15). The ${}^{s}_{w}pK_a$ of the solute is 8.9, estimated in 35% acetonitrile from measurements in aqueous organic phases by capillary electrophoresis [76]. The degree of protonation of the solute is gradually reduced from 100% to only 7% as the pH was increased over this range. The result is easily explained on the idea that solute ionic repulsion is reduced as the pH increases. Alternatively, as the proportion of the solute which is charged is reduced, there could be less interaction with the strong column sites proposed in the Guiochon hypothesis. For amitriptyline, it seems feasible that ionic

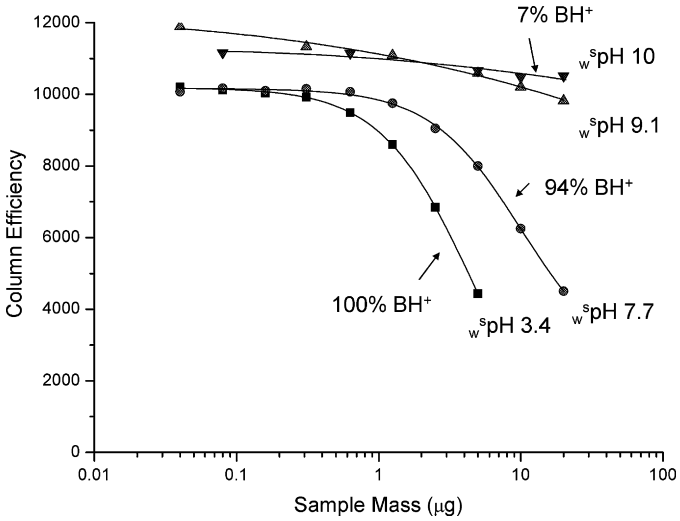


Fig. 15. Plot of efficiency vs sample mass for amitriptyline (${}^{s}_{w}pK_a$ 8.9) at various pH values using acetonitrile–phosphate buffers (35:65, v/v). Flow 1 mL/min. Column XTerra RP-18, 15 cm × 0.46 cm i.d., 5 μm particles. See [57].

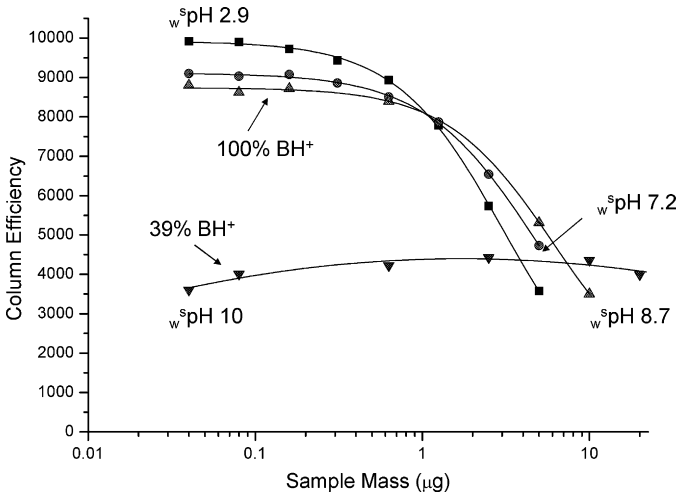


Fig. 16. Plot of efficiency against sample mass for amphetamine ($^{s}_{w}pK_a$ 9.8). Mobile phase: acetonitrile–phosphate buffer (10:90, v/v). Other conditions as Fig. 15. See [57].

interactions between solute and stationary phase may therefore be small, especially considering that this hybrid phase may not be extensively ionised, even at high pH. However, for amphetamine, a more strongly basic solute ($^{s}_{w}pK_a = 9.8$), poor results were obtained at $^{s}_{w}$pH 10, even using small sample mass (see Fig. 16). As noted above, stronger ionic interactions between the stationary phase and the solute, which is still almost 40% ionised at $^{s}_{w}$pH 10, may be responsible for this behaviour. However, it is noticeable that the efficiency hardly deteriorates further even when the sample mass is increased to 20 μg at this high pH, as was noted in previous studies [75]. These studies were extended to a high $^{s}_{w}$pH limit of 12, using the more recent XBridge phase (Waters), to see if the loading properties and peak shapes of small masses of stronger bases such as amphetamine could be improved at this still higher pH [58]. At $^{s}_{w}$pH 2.7 and $^{s}_{w}$pH 7.0, peak shape deteriorated with increasing sample mass according to the expected pattern, although some deterioration in efficiency and peak asymmetry for small sample mass is shown at pH 7 compared with $^{s}_{w}$pH 2.7 (see Fig. 17). At $^{s}_{w}$pH 10, peak shapes were poor even for small sample mass, and results were similar to those obtained previously on the methyl hybrid phase. At $^{s}_{w}$pH 11.0, unusual profiles of increasing followed by decreasing efficiency were obtained as sample mass was increased. The initial increase in N with sample mass could be explained by gradual deactivation of silanols that become ionised at high pH by the solute itself, allowing the rest of the sample to elute with increased efficiency. This deactivation might occur up to a point where overloading of the hydrophobic surface of the column occurs, at which point efficiency decreases in the normal fashion. At $^{s}_{w}$pH 12.0, the column loadability increased substantially, but more asymmetric peaks were still obtained for small sample mass of amphetamine ($A_s = 2.5$) compared with $^{s}_{w}$pH 2.7 ($A_s = 1.2$) even though calculations show that amphetamine is only 0.4% protonated at the higher pH. This result is interesting and warrants further investigation. However, it is likely that the proportion of ionised silanols on the column is quite high at pH 12.0.

Neue et al. [77] discussed the differences in preparative loadability between charged and uncharged forms of ionisable compounds; however, they stressed that their work was also important for analytical separations. If the identity of impurities present in a parent sample is desired, then a sufficient quantity of these impurities needs to be prepared in order that they can be collected or further examined. Clearly, an important example of such an application might be the identification of impurities in a pharmaceutical preparation, where a large amount of the active pharmaceuti-

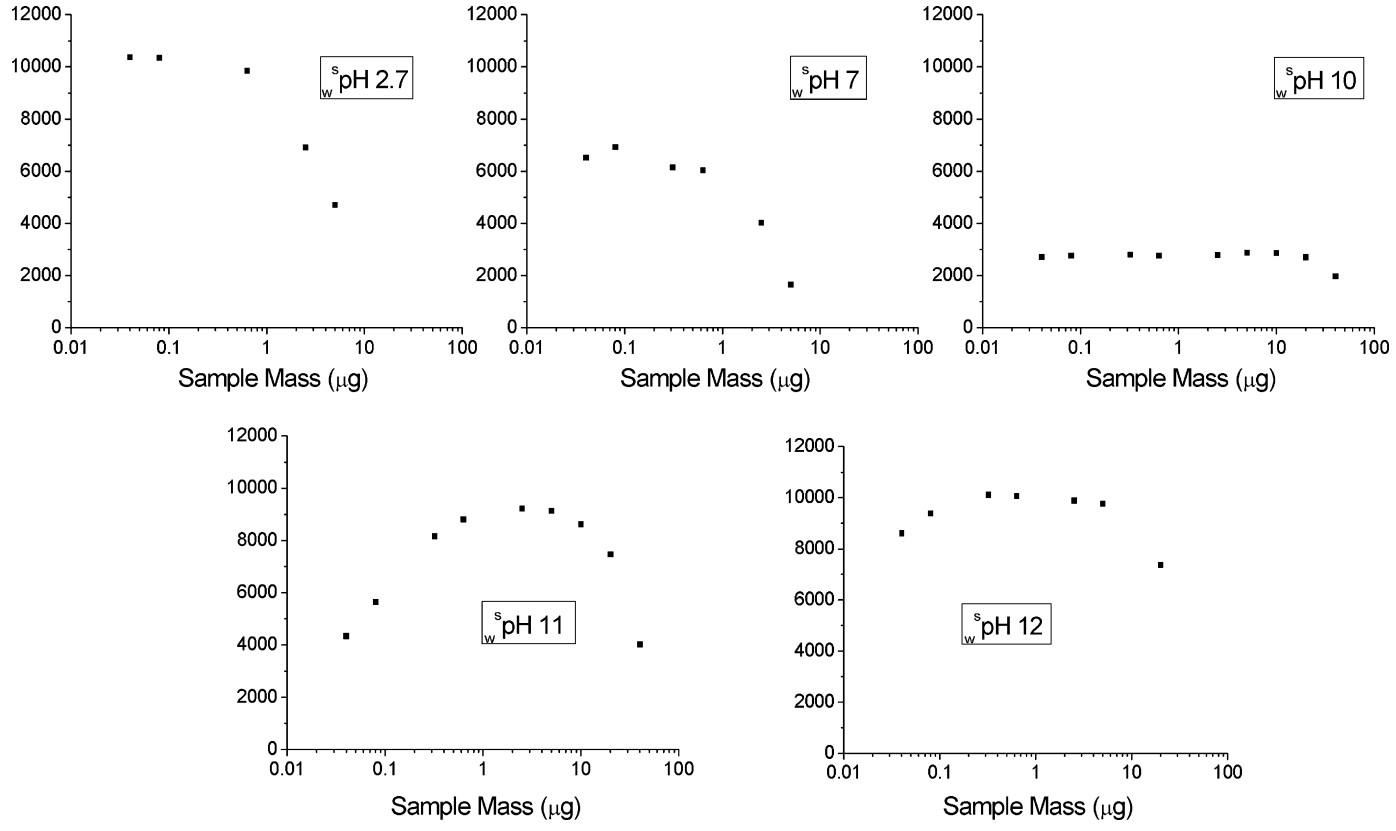


Fig. 17. Plots of efficiency vs sample mass for amphetamine on XBridge BEH (15 cm × 0.46 cm, 5 μm particles) at various pH values. Mobile phase: ACN–phosphate buffer (35:65, v/v) except at $^{s}_{w}$pH 2.7 (10:90, v/v), 1 mL/min. See [58].

cal ingredient (API) has to be injected in order to identify small amounts of closely related impurities. In this case, a broad overloaded peak from the API might effectively obscure these impurity peaks. Thus, it is important to maximise the loadability of the separation column. Theoretical treatment was based on a modification of the Langmuir isotherm that takes into account the mutual repulsion of ions adsorbed on the packing surface (according to the theory of Häglund and Ståhlberg [64]. This treatment predicted that the loadability of a compound in the ionic form is inferior to that of the same compound in the unionised form by a factor of 20 or more. The veracity of this prediction was demonstrated by experimental findings using XTerra MS C18 columns (Waters) with both acidic (e.g. oxacillin) and basic compounds (e.g. diphenhydramine). A mobile phase containing 100 mM formic acid or 100 mM ammonia was used for the acid and basic pH experiments respectively. Calculations indicated that the limiting sorption capacity was approximately one molecule associated with each column ligand. Thus they concluded that the preparative chromatography of acids should be carried out at acidic pH, and that of basic compounds carried out an alkaline pH, in order to maximise the loadability.

Fornstedt and co-workers [78] studied the adsorption isotherms of a neutral, an acidic, and a basic probe at pH 3, 7 and 11 on alkaline-stable C18 columns, under broad concentration ranges using frontal analysis and methodology similar to that of Guiochon. For the basic probe metoprolol, the adsorption isotherm was best described with a bi-Langmuir model (low-energy and high-energy sites) at ${}^{w}_{w}$pH 3 and 11. The total monolayer saturation capacity at pH 3 was about half that at pH 11. The capacity for the high-energy site was ~2–5% of the total capacity at pH 3, and ~20% at pH 11.

Gritti and Guiochon [79] studied a series of neutral polar compounds together with ionisable acids and bases over the pH range ${}^{s}_{w}$pK_a 2.6 to ${}^{s}_{w}$pK_a 11.3 using four different RP materials. They concluded that the neutral species of ionisable compounds adsorb weakly on type 1 sites that have a high density and low energy, while the ionic species (both acidic and basic) adsorb preferentially on a second type of site with high energy and low density. This explains for instance why bases at low pH tail, as the high-energy sites are easily overloaded by the protonated solute. There appeared to be little competition between adsorption of the ionic and neutral species when both were present, and the model they used did not take any such competition into account. They remarked that they had not yet tested whether the saturation capacity of the strong sites varied with the buffer concentration. Their work however, allowed prediction of the band profiles if the sample concentration exceeded the buffer concentration.

5. Temperature effects

The possible merits and limitations of temperature have been discussed extensively in the literature, which has recently been thoroughly reviewed [80]. It was noted that high temperatures are not widely used at present. Many workers merely use a temperature somewhat above ambient in order to thermostat the column efficiently. Medium temperatures (up to 100 °C) give noticeable benefits, and stationary phases and suitable equipment to handle such temperatures are now readily available. The use of still higher temperatures is at present restricted by the availability of suitable stationary phases (based on silica or alternative materials). A problem of high temperatures is the reduction in retention that usually occurs, which can result also in a loss of resolution, unless the composition of the mobile phase can be adjusted to compensate for this effect. The main advantages of high temperature (for neutral molecules) are the possibilities of increased analysis speed, and different selectivity compared with separations at room temperature. The landmark paper of Antia and Horváth in 1988 [81] gave a sound theoretical discussion of the effects of temperature on speed and efficiency. The optimum mobile phase velocity (u_{opt}) increases and the slope of the curve in the C term region of van Deemter plots at high velocity decreases with increasing temperature. This effect is due to the reduced mobile phase viscosity and increased solute diffusion coefficients at elevated temperature. As a result, fast analysis above u_{opt} is possible with little loss in efficiency [82,83]. Nevertheless, as pointed out by Heinisch, the retention factors of the solutes must be constant to enable a fair comparison with operation at low temperature. This can be achieved in RP separations in general by decreasing the concentration of organic modifier (%B) in the mobile phase as the temperature is raised. A decrease in %B increases the mobile phase viscosity. Another factor is that the pressure required to reach u_{opt} may actually increase somewhat at higher temperatures, as higher linear velocities are required to reach this optimum value. These factors lead to some moderation of the benefits of temperature. One clear fact, as shown by Antia and Horváth [81] (at least for simple neutral molecules), is that the minimum plate height remains virtually constant at u_{opt} as the temperature increases i.e. higher temperatures do not normally produce higher efficiencies on a given column (i.e. of fixed length). This remains true if very fast kinetics are assumed, but in a system with slow kinetics, such as in the chromatography of large molecules [56], sorption kinetics are often slow and adversely affect the column efficiency. The reduced form of the van Deemter equation can be written:

$$h = A + \frac{B}{\nu} + C\nu + D\nu^{2/3} + \frac{E}{k_d}\nu \quad (10)$$

where $E = 3D_m/8{d_p}^2$; where h is the reduced plate height, A–D are the van Deemter coefficients, ν is the reduced velocity ($=ud_p/D_m$), D_m is the diffusion coefficient of the solute in the mobile phase, k_d is the desorption rate constant and d_p is the particle size. Increases in temperature accelerate sorption coefficients, decreasing the value of the 5th term in this equation. Clearly, these effects could also influence the chromatography of basic compounds due to slow kinetics of interaction of protonated bases with ionised silanols. In linear chromatography (i.e. where sample overload is not a factor), Guiochon stated that poor peak shape may be due to the combination of slow kinetics of "strong" sites together with fast kinetics of "weak" sites. Increasing the temperature may increase the kinetics of the slow sites to approach that of the fast sites, giving an improvement in peak shape [84,85].

McCalley investigated the effect of temperature on the column efficiency of some basic compounds as a function of flow rate using buffered mobile phases at pH 3 and pH 7 and temperatures from 20 to 60 °C [35]. At pH 3.0, plots of H (using the Dorsey–Foley equation to take peak asymmetry into account) against flow velocity were flatter in the C term region at higher temperature, as expected from the arguments above. There appeared to be only small differences in the plate heights at flows close to the optimum as a function of column temperature. In contrast, at pH 7, major improvements in efficiency were noted for the strong bases quinine and nortriptyline as the temperature was raised (see Fig. 18). For example, the Dorsey–Foley plate height for quinine averages above 0.025 cm over the flow rate range studied at 20 °C, but is about an order of magnitude lower at 60 °C. Substantial improvements in efficiency at elevated temperature can also be seen for nortriptyline, another strong base. The weak base pyridine and the neutral compound benzene do not show these major improvements in efficiency. Pyridine is only weakly basic (${}^{w}_{w}$pK_a = 5.2 at 25 °C) and is likely to be present as a neutral molecule throughout the experiments. The improvement in efficiency for the strong bases could be attributed to an increase in sorption–desorption kinetics as described above.

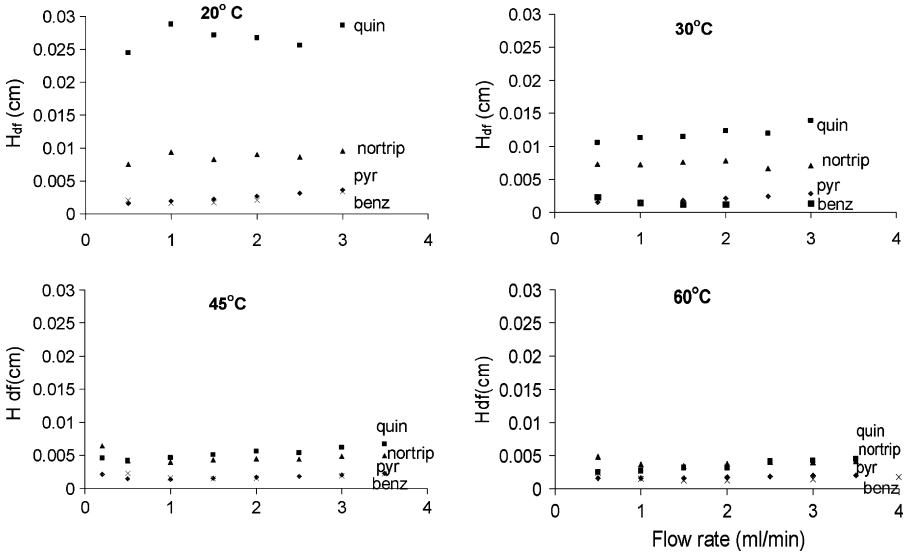


Fig. 18. Plots of plate height (calculated from the Dorsey–Foley efficiency) against flow rate for quinine, nortriptyline, pyridine and benzene at various temperatures. Mobile phase: acetonitrile–phosphate buffer pH 7.0 (35:65, v/v). Column: Inertsil ODS-3 (5 μm particle size). See [35].

However, experiments with the quaternary compounds berberine and benzyltriethylammonium chloride showed only minor variations in the minimum plate height achievable at intermediate pH as a function of temperature. The quaternary compounds remain completely ionised under the conditions of the experiment. If faster sorption–desorption kinetics was the governing factor determining improved efficiency, the quaternaries should also show improved efficiency at elevated temperature. However, a complexity with this argument is that quaternary compounds showed generally better peak shape than the strong bases, indicating reduced interactions with ionised silanols. This result could be due to greater steric effects in proximity to the charge- bearing nitrogen atom, lessening detrimental interactions with ionised silanols. Thus, these quaternary compounds may not exactly model the behaviour of ionised bases. Alternatively, the results could be explained on the basis of reductions in the pK_a of the bases that occur as temperature is raised [86]. Reduction in pK_a by as much as 0.03 pK_a units per °C were reported [87]; thus the $^{s}_{w}$pK_a of quinine should drop from around 8.4 in the mobile phase used for the experiments in Fig. 18 at 20 °C, to 7.2 at 60 °C. In parallel, the % ionisation of the compound would drop from around 90% to less than 30% at 20° and 60°, respectively, thus providing a possible explanation for the improvement in peak shape. In agreement with this hypothesis, the retention of the bases benzylamine, quinine, nortriptyline and protriptyline was shown to *increase* with increasing temperature under conditions similar to those of Fig. 18 (intermediate pH), in contrast to the usual effect of decreasing retention shown for neutral compounds. As the base becomes deprotonated, hydrophobic retention (which is the dominant retention mechanism) increases as the retention of the neutral base is much higher than that of its conjugate acid. The quaternary ammonium compounds however, showed the normal decreases in retention with temperature, as their ionisation is unaffected by pH.

6. Hydrophilic interaction chromatography (HILIC)

In recent years, HILIC has emerged as a viable alternative technique to RP for the analysis of hydrophilic solutes. Its origins may be considerably earlier than some suppose. In 1941 Martin and Synge in their seminal paper on classical LC, used water-saturated chloroform as the mobile phase in combination with a silica column to separate amino acids, suggesting that the mechanism involved partition between a water layer on the stationary phase and the chloroform in the mobile phase [88]. It was later discovered that an immiscible organic solvent was not a prerequisite to achieve these separations. Alpert coined the name HILIC, and noted that charged basic groups in a solute led to pronounced hydrophilicity and retention. He considered that the retention mechanism consisted mostly of partitioning between the bulk mobile phase and a layer of mobile phase enriched with water, partially immobilised on the stationary phase [89] i.e. similar to the mechanism proposed by Martin. A recent review [90] concluded that both adsorption and the partition mechanism could contribute to retention in HILIC.

The acknowledged advantages of HILIC have been summarised [91] as:

(1) Good peak shapes can be obtained for bases.
(2) Mass spectrometer sensitivity is enhanced due to the high organic content in the mobile phase and the high efficiency of spraying and desolvation techniques.
(3) Direct injection can often be made of extracts eluted from C18 solid-phase extraction columns with solvents of high organic content, as these are weak solvents in HILIC.
(4) The order of elution of solutes is generally the opposite of that found in RP separations, giving useful alternative selectivity.
(5) Good retention of polar compounds is obtained in HILIC, whereas very poor retention is often obtained in RPHPLC.
(6) Higher flow rates are possible due to the high organic content of typical mobile phases.

Nevertheless, HILIC has some drawbacks compared with RP separations which include:

(1) The separation mechanism is at present somewhat less well understood than that of RP–LC. Thus, it may be difficult to predict the effect of change of conditions on the separation.
(2) The technique does not have the broad applicability of RP–LC. Analytes that are neutral and non-polar generally show very little retention. In addition, ionised acidic analytes (negatively charged ions) can also show little retention due to repulsion of the ion from negatively charged column silanol groups on some silica-based columns.
(3) HILIC is potentially an environmentally less friendly technique than RP–LC as it consumes much larger volumes of organic solvents.

(4) Columns can take a long time to equilibrate with the mobile phase, sometimes in excess of 1 h compared with ~15 min for RP–HPLC. However, the slow equilibration of some types of C18 column, which may require several hours prior to establishment of consistent retention times (see above), is a factor that also needs to be taken into account in some cases.

New advantages of HILIC that have emerged recently have included the use of long columns to achieve highly efficient separations, superior loading capacity to RP for charged basic solutes, and the potential of very fast analysis due to good mass transfer characteristics of the columns operated in mobile phases of much lower viscosity than typically used in RP–LC [91,92]. Many different types of HILIC column are now available, including the traditional bare silica column (of which some manufacturers produce a version especially for HILIC separations) and bonded phases with groups such as diol, amide, and zwitterions. Few detailed studies of the performance of these columns for bases have been published, apart from some recent studies with bare silica columns [91,92]; therefore, this review will largely concentrate on this type of HILIC column.

Table 4 shows retention factor, N and A_s for a mixture of acidic, basic and neutral solutes on a silica HILIC column using acetonitrile–ammonium formate buffer at pH 3.0. The acidic solutes (2-naphthalenesulfonic acid) and p-xylensulfonic acid have very low retention under these conditions, due to repulsion from ionised silanol groups on the column (note that this can sometimes be rectified by the use of lower pH mobile phases buffered with trifluoroacetic acid [91]). The ionised strong bases nortriptyline, diphenhydramine, benzylamine and procainamide gave excellent peak shapes (A_s 1.0–1.1) and column efficiencies of 22,000–25,000 theoretical plates (reduced plate height as low as 2.0). In our experience, this performance is significantly better than that of the most inert RP columns for these solutes. Retention of the bases was satisfactory, and can be increased by reducing the concentration of water in the mobile phase, as is normally the case for HILIC.

Fig. 19 compares chromatograms of the mixture of compounds in Table 4 on a 15 cm 3 μm porous silica column, a 15 cm 2.7 μm superficially porous (SP) silica column, and three SP silica columns coupled in series [92]. Superficially porous "pellicular" particles have been in use since the early days of HPLC, having typically a 1–2 μm porous core surrounding a solid non-porous core with overall particle diameter ~30 μm. More recent columns of this type have much smaller particle diameter (2.7 μm) with a 0.5 μm porous shell (Halo, AMT). Simple geometry shows that the volume of the porous layer is 75% of that of the total particle, so that the particles should not suffer from the serious overloading effects shown by the original pellicular particles. According to the manufacturer, these columns should show fast mass transfer through the thin porous layer, due to the short diffusion distance [93]. The pressures shown in Fig. 19 represent the total system pressure

Table 4
Values of retention factor (k), column efficiency (N) and asymmetry factor (A_s) for a mixture of acidic, basic and neutral solutes. Column: Atlantis silica (Waters) 25 cm × 0.46 cm i.d. Mobile phase: ACN–0.1M ammonium formate pH 3.0 (85:15, v/v).

Solute	k	N	A_s
Phenol	0.03	32100	1.1
2-Naphthalenesulfonic acid	0.07	28300	1.1
p-Xylenesulfonic acid	0.1	28100	1.0
Caffeine	0.4	34600	1.2
Nortriptyline	1.0	22100	1.0
Diphenhydramine	1.1	24600	1.0
Benzylamine	1.8	24300	1.0
Procainamide	3.0	22300	1.1

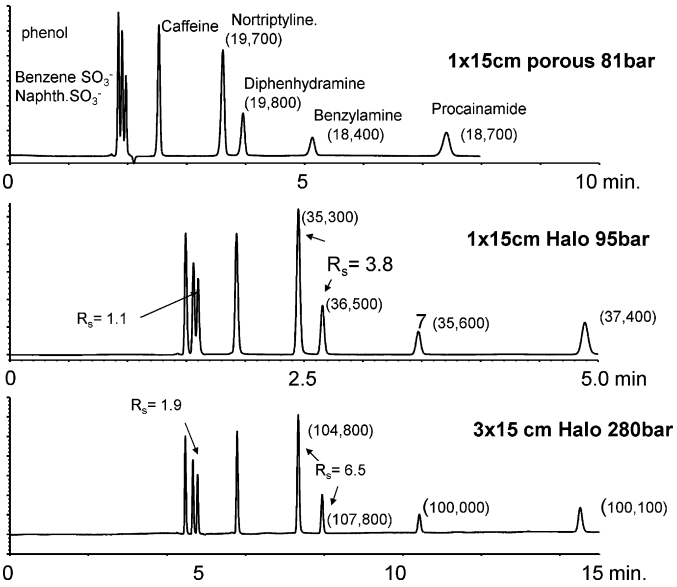


Fig. 19. Analysis of acidic, basic and neutral solutes on a 15 cm porous column (3 μm particles), a 15 cm superficially porous column (Halo, 2.7 μm particles) and three coupled Halo columns. Mobile phase: acetonitrile–ammonium formate buffer pH 3.0, 85:15, v/v). Column efficiencies in brackets. See [92].

including injector, connection tubing (of 0.01 cm i.d. to minimise dead volume) and detector (1 μL flow cell). The separation is satisfactory with the 3 μm porous column, giving almost 20,000 plates. However, the efficiency of the SP column is considerably higher, giving almost double the number of plates (up to 37,400) with only a small increase in back pressure (to 95 bar) compared with the totally porous column. Coupling of 3 cm × 15 cm columns generates in excess of 100,000 plates for the ionised bases nortriptyline, diphenhydramine, benzylamine and procainamide while the total system backpressure was still only 280 bar. The resolution of the 15 cm SP column was 3.8 for the peaks of nortriptyline and diphenhydramine, and 6.5 on the 45 cm column, comparing favourably with the theoretical value of 6.6, calculated from the resolution of a single column. These values confirm the similar efficiency and selectivity of the individual columns, and that little loss in efficiency results from the coupling process. Clearly it would be possible to extend the length of this column beyond 45 cm while operating at the same flow rate to gain even higher efficiency, or to optimise the analysis speed for the same column length by increasing the flow rate, while still remaining within the pressure limit of conventional HPLC systems (400 bar). Operating the column at maximum pressure is the basis of the kinetic plot method. For a desired analysis time and given maximum operating pressure, there exists a unique pair of values of the linear velocity, and the column length for a given column material-further information on these methods is given in [94] and references therein. Application of the kinetic plot method to the experimental results from a single 15 cm SP column indicates that using a flow rate of 1 mL/min, 165,000 theoretical plates could be obtained on a 70 cm column with a t_o time of ~430 s at an operating pressure of 400 bar, corresponding to an analysis time of ~22 min for a peak of $k = 2$. Fig. 20 shows plots of reduced plate height (H/d_p) against reduced interstitial velocity ($u_i d_p/D_m$ where u_i is the interstitial velocity) for the SP column at 30 °C and 50 °C compared with that of a totally porous 5 μm silica column at 30 °C for the four strong bases. The reduced interstitial velocity is a more fundamental measure of flow, and is more appropriate for comparison of performance of materials of different intraparticle porosity such as SP and totally porous particles. In contrast, the more generally used average linear velocity depends

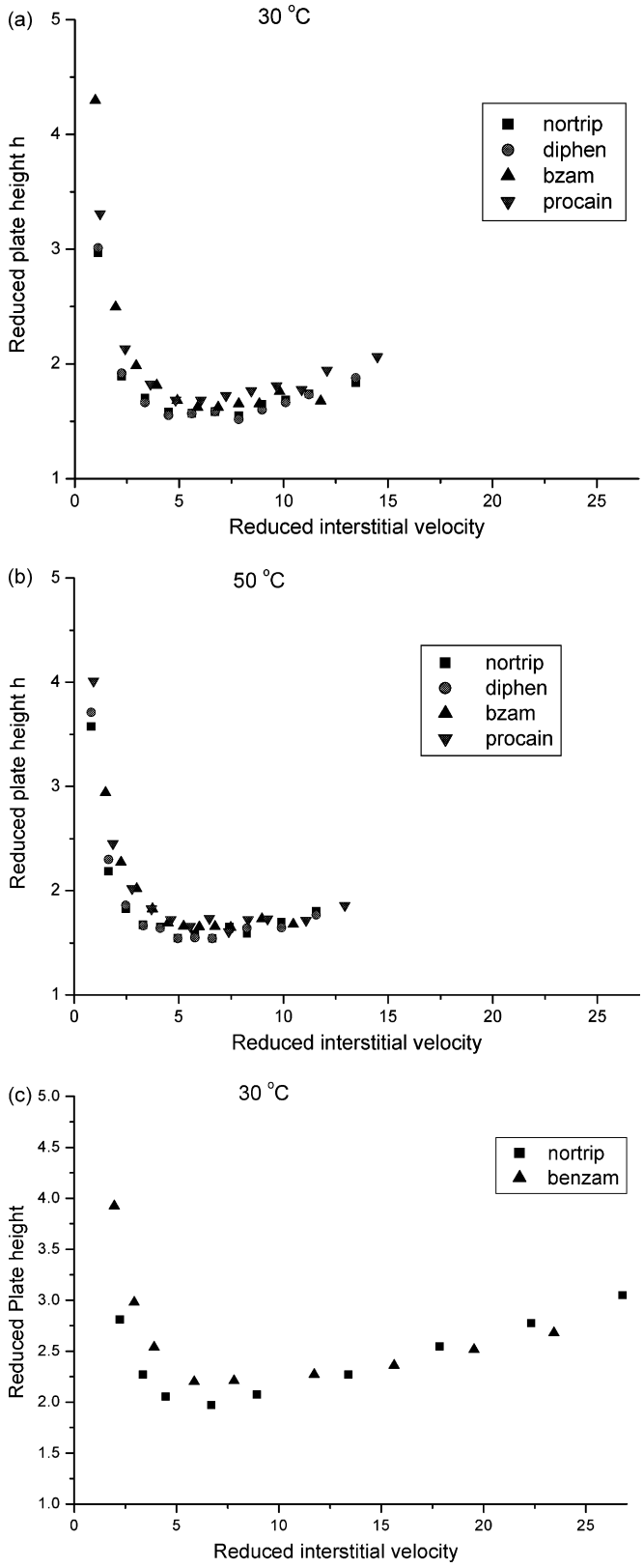


Fig. 20. Plots of reduced plate height (h) vs reduced interstitial velocity (ν) for bases on (a) 15 cm Halo silica at 30 °C (values of ν correspond to F = 0.25-3 mL/min). (b) Halo silica at 50 °C (values of ν correspond to F = 0.25–3.0 mL/min). (c) Atlantis silica (5 μm) at 30 °C (values of ν correspond to F = 0.25–3.5 mL/min). Mobile phase: ACN–0.1 M ammonium formate $^{w}_{w}$pH 3.0 (90:10, v/v). See [92].

Table 5
Fits to Knox plots $h = A\nu_i^{0.333} + B/\nu_i + C\nu_i$ (ν_i is the reduced interstitial velocity).

	A	B	C
30 °C fused core (2.7 μm)			
Nortriptyline	0.50	2.7	0.033
Diphenhydram	0.44	2.8	0.044
Benzylamine	0.44	3.7	0.036
Procainamide	0.48	3.3	0.044
30 °C porous (5 μm)			
Nortriptyline	0.61	4.2	0.038
Benzylamine	0.56	6.1	0.035

on the porous volume that can be explored by the probe. Values of D_m were measured using the Taylor–Aris procedure [92]. The plot for the SP column shows a minimum reduced plate height (h) of 1.5, indicating excellent performance for this material, and is similar to the value of h reported for the C18 version of the SP phase [95]. The excellent performance of this type of phase was attributed to exceptionally low eddy diffusion terms, possibly as a result of the good packing qualities of this material, and to lower values of the van Deemter B term. There is no evidence in Fig. 20 of an upturn in the curves at high reduced velocity as reported for the C18 phase [95]. In this paper, it was found that mass transfer terms of the C18 phase were surprisingly large, suggesting that the cause was a stationary film of mobile phase surrounding the rough external surface of the shell particles. The effect was apparently worse at 50 °C. Clearly, there is no evidence of this phenomenon on the bare silica version of the phase, and the curves at both temperatures virtually overlap, as would be expected for reduced plots. It could be that the nature of the HILIC mechanism somehow overcomes this apparent drawback of the C18 phase. The plots for the SP HILIC phase (Fig. 20) are also similar to those for the totally porous silica phase, although this 5 μm column shows minimum values of h around 2, a low value but still 33% larger than for the SP phase. Table 5 shows the coefficients A, B, and C obtained by curve fitting the data for the SP and fully porous columns at 30 °C to the Knox equation: $h = A\nu_i^{0.333} + \frac{B}{\nu_i} + C\nu_i$ where ν_i is the reduced interstitial velocity. Note that these reduced plots remove the effect of both particle size and solute diffusion coefficient on the curves. The values of C are little different between the two columns, casting doubt on the hypothesis that the improved performance of the SP column is due to superior mass transfer, at least for the small MW solutes used. Instead, Table 5 shows that both the A term and B term of the SP column are lower than that of the totally porous silica. This result is in full agreement with the publication of Guiochon and co-workers for C18 bonded superficially porous phases, who attributed their good performance to the narrow particle size distribution and good packing qualities of the particles (thus small A term) and their lower internal porosity, leading to lower axial diffusion (smaller B term) [96].

For fast separations, it is of interest to compare the effect of flow rate on efficiency in HILIC and RP separations for columns of similar physical dimensions. Fig. 21 shows van Deemter plots for a neutral compound (caffeine), an acid (p-xylenesulfonic acid) and two basic compounds (benzylamine and nortriptyline) using these two different separation mechanisms. The HILIC separation used ACN–formate buffer pH 3.0 (90:10, v/v), whereas the RP separation used the same mobile phase but with a lower ACN concentration (10:90, v/v) for all compounds except the more hydrophobic nortriptyline (35:65 v/v). The RP column selected was Discovery C18, which gives good performance for basic compounds as evidenced by favourable data from the Tanaka and the Snyder test procedures (see Table 1) and also from comparative studies of peak shape [27]. Note that the curve for nortriptyline lies close to that for the other solutes in the RP separation (Fig. 21), indicating that the column

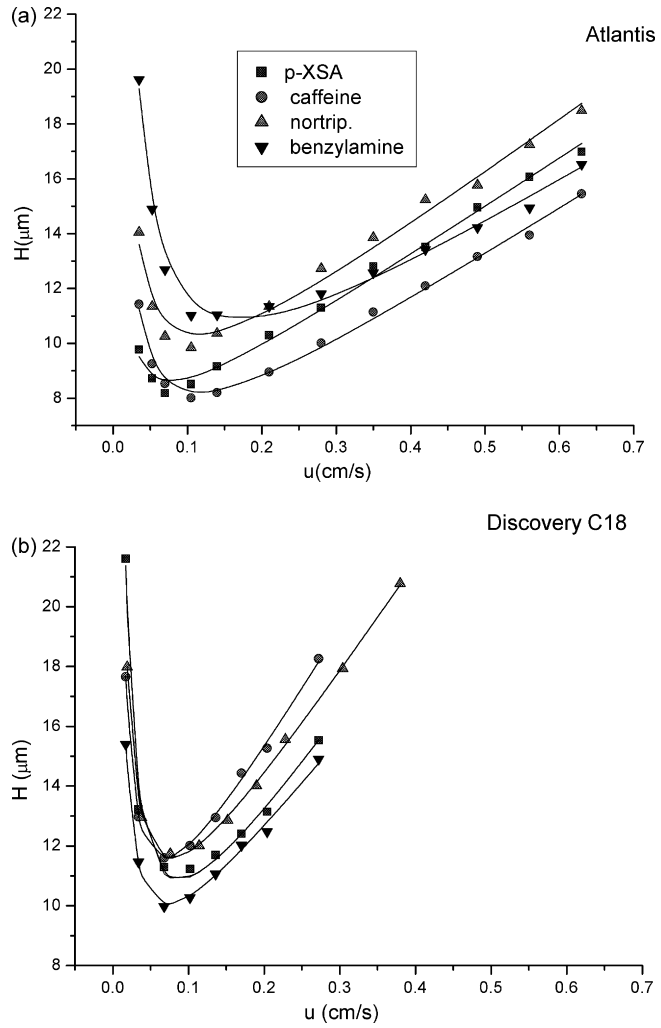


Fig. 21. (a) van Deemter plots on Atlantis silica for p-XSA, caffeine, nortriptyline, benzylamine mobile phase ACN–0.1 M $HCOONH_4$ $^{w}_{w}$pH 3.0 (90:10, v/v). Corresponding flow rate range 0.25–4.5 mL/min. (b) van Deemter plots on Discovery C18. Mobile phase 10% ACN in 0.025 M ammonium formate $^{w}_{w}$pH 3 (benzylamine, caffeine, p-XSA); 35% ACN in 0.025 M ammonium formate $^{w}_{w}$pH 3.0 (nortriptyline). See [91].

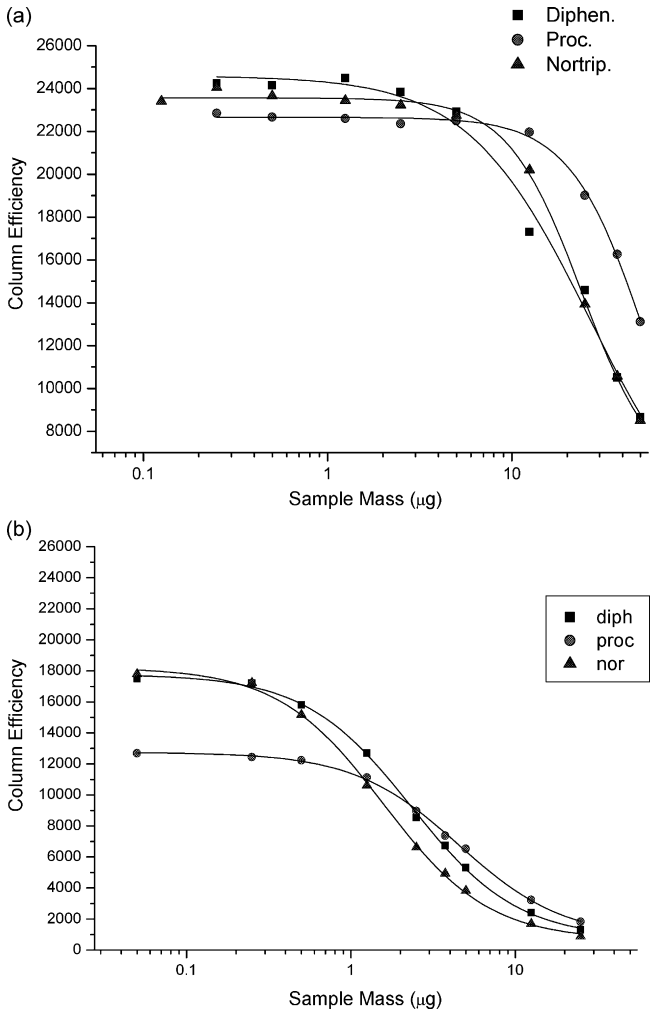


Fig. 22. (a) Plots of column efficiency (half-height method) vs mass for diphenhydramine (squares) and procainamide (circles) using ACN–0.1 M $HCOONH_4$ $^{w}_{w}$pH 3.0 (90:10, v/v) and for nortriptyline (triangles) with ACN–0.1 M $HCOONH_4$ $^{w}_{w}$pH 3.0 (92.5:7.5,v/v). (b) Plots of column efficiency (half-height method) vs mass on XTerra RP18 for diphenhydramine and nortriptyline, mobile phase 30% ACN in 0.025 M $HCOONH_4$ $^{w}_{w}$pH 3.0 and for procainamide using 0.025 M $HCOONH_4$ $^{w}_{w}$pH 3.0. See [91].

performance is not affected by any wetting effects of the stationary phase in the rather low concentrations of ACN used for the other solutes which are quite hydrophilic, and otherwise have low retention times. Neither are there any significant extracolumn effects, as a low dead volume system was used in conjunction with a 0.46 cm i.d. column. The poor retention of hydrophilic compounds is in itself a disadvantage for RP separations, whereas these solutes generally have very good retention in HILIC, at least on silica columns, where the retention is assisted by ion-exchange. The RP separation shows higher values of H_{min} (10–12 μm) compared with the HILIC plots (8–11 μm), lower values of the optimum velocity (0.075–0.1 cm/s compared with 0.1–0.15 cm/s) and steeper increase in the value of H at high flow rate, i.e. a larger C term. Values of C (obtained by curve fitting to the van Deemter equation) ranged from 3.2 to 4.3 ms for the RP separation compared with 1.7–2.0 ms for the HILIC separation. Clearly, the HILIC column could also be operated at about twice the maximum flow velocity for the RP separation within the imposed total system pressure limitation of ~300 bar. The low viscosity of the mobile phase gives rise to increased solute diffusivity according to the equation [97]:

$$D_{m,T} = D_{m,303}\frac{\eta_{303}}{\eta_T}\frac{T}{303} \quad (11)$$

where T is the absolute temperature, η_{303} and η_T are the viscosities of the mobile phase, $D_{m,T}$ and $D_{m,303}$ are the diffusion coefficients at 303 K and T, respectively. The viscosities of 10%, 35% and 90% ACN are approximately 0.90, 0.83 and 0.43 centipoise, respectively [98]. This result indicates that the diffusion coefficient of ionised basic solutes under typical HILIC conditions should be about twice those under typical RP conditions, explaining the lower C terms.

Overloading of ionised acids and bases is clearly a problem in RP separations; it is therefore of interest to compare overload with that in HILIC separations. Fig. 22(a) shows plots of efficiency vs sample mass for three basic compounds (diphenhydramine, procainamide and nortriptyline) on a bare silica column using acetonitrile–ammonium formate buffer pH 3. Fig. 22(b) shows similar plots but for a RP column using a similar mobile phase, with a smaller concentration of ACN, as is demanded in RP separations. The EPG column was selected due to the high hydrophilicity of procainamide. The column could be operated successfully in buffer alone (no ACN) without phase de-wetting that occurs with conventional RP columns. The ACN concentrations chosen for both RP and HILIC separations gave $k = 2.5$ or greater for each solute, where the effect of k on overload is reduced, as the proportion of solute held on the stationary phase is $k/(1+k)$. The overloading properties

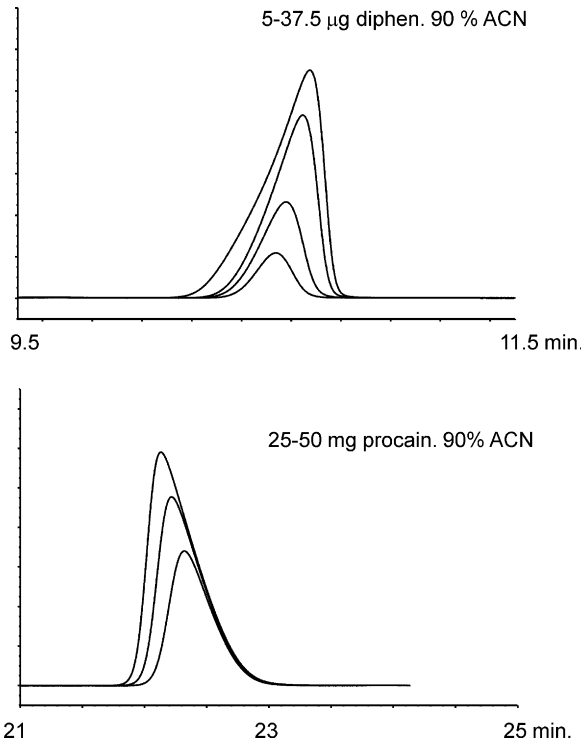


Fig. 23. Peak shapes for high loads of diphenhydramine and procainamide on Atlantis silica (25 cm × 0.46 cm i.d., 5 μm particle size). Mobile phase: ACN–0.1 M ammonium formate $^{w}_{w}$pH 3.0 (90:10, v/v). See [91].

of the RP column are somewhat improved compared with Fig. 8, which can be partially attributed to the higher ionic strength of the ammonium formate buffer used compared with formic acid. Nevertheless, a 10% loss in efficiency on the RP column still occurs when approximately 1 μg of these solutes is introduced. In comparison, a similar loss in efficiency was experienced in the HILIC separation for around 10 μg of solute, i.e. an order of magnitude higher than the RP separation. In the HILIC separation, nortriptyline and diphenhydramine showed increased fronting as sample load increased, whereas procainamide showed increased tailing with increasing load (see Fig. 23). Fronting could be due to anti-Langmuir adsorption behaviour, where solute–solute interactions cause increased retention of the peak maximum as the sample load increases. It is possible that solute–solute interactions for hydrophobic bases such as diphenhydramine and nortriptyline could be promoted in the environment of a bound layer of water on the stationary phase. In contrast, procainamide is a considerably more hydrophilic solute (shown by its high HILIC but low RP retention) so solute–solute interactions may not be promoted. In RP separations, under conditions where the mobile phase buffer concentration is sufficient for the quantity of sample injected, we have only observed tailing peaks for nortriptyline, characteristic of a Langmuir adsorption isotherm.

There has also been interest in the use of bare silica columns using aqueous–organic mobile phases but with organic solvent concentrations lower than typically used in HILIC. Bidlingmeyer et al. [99] separated organic amines with reasonable peak shape using ACN containing 4 mM ammonium phosphate pH 7.8. Over the ACN concentration range 30–70%, decreasing retention of lipophilic amines was observed as the ACN concentration was increased, the opposite of typical HILIC behaviour. Retention was attributed to a mixture of RP retention on the column siloxane groups together with ionic retention on ionised silanols. Euerby and co-workers achieved separations of reasonable efficiency for basic drugs using buffered aqueous mobile phases mostly containing about ~30% methanol, similarly attributing the mechanism as mostly ion-exchange but with a significant contribution of RP retention [100]. Very recently, Sandra and co-workers [101] showed high efficiency separations of some catecholamines and amino acids on bare silica columns using in some cases pure aqueous buffers (no organic solvent). Peak shapes were shown to be much improved for catecholamines compared with classical HILIC conditions. Clearly, this technique (although not classified as HILIC) could be useful in the separation of some solutes, and has the particularly advantages of being a "green" technique that avoids the environmental consequences of organic solvent use and disposal. Nevertheless, the disadvantage of the technique compared with HILIC appears to be the low optimum mobile phase velocities, as a consequence of reduced solute diffusion in purely or highly aqueous mobile phases.

7. Concluding remarks

Considerable difficulties remain in the analysis of basic compounds and opportunities exist for new solutions. These include the need for greater understanding and remediation of overloading, the instability of silica at extremes of pH which prevents exploitation of selectivity effects, and the existence of underivatised silanols on silica materials, which can give rise to undesirable interactions with basic solutes, especially under mobile phase conditions where their ionisation is not suppressed. The development of new, alternative, pH stable, non-silica-based materials (e.g. organic polymers) could provide a solution to many of these problems. Nevertheless, significant improvements have been made for analyses using silica-based columns in recent years.

7.1. Overloading

Considerable deterioration of column efficiency occurs for the injection of greater than about 0.5 μg of ionised basic solutes on standard-size columns, and is particularly difficult when low ionic strength mobile phases, such as formic acid, as preferred in mass spectrometry, are utilised. The causes of overloading remain a matter of debate, with some workers explaining the results on the basis of mutual repulsion of similarly charged ions on the stationary phase, while others consider that the overloading of scarce (and as yet unidentified) strong sites on the surface is involved. A better understanding of the overload mechanism would assist the design of improved stationary phases for analysis of these solutes. The overloading problem for bases of moderate pK_a can be considerably improved by working at high pH, where the base is relatively unionised. However, this approach is limited by silanol ionisation and column stability, as described above. Alternatively, improvements in loading capacity can be gained by the use of ion pair reagents, or with mixed-mode RP-ion-exchange columns.

7.2. Selection of mobile phase pH

The use of low pH has clear advantages in the separation of basic compounds, as it suppresses the ionisation of residual silanol groups, preventing their detrimental interaction with ionised bases. Modern Type B columns can still give quite poor peak shapes at intermediate pH. However, low pH can result in poor retention for ionised, particularly hydrophilic bases. Some inorganic–organic hybrid phases appear to show little silanol ionisation at intermediate pH, possibly due to raising of the silanol pK_a in the more organic environment of such phases. Thus, these phases maintain good peak shape for bases at intermediate pH. Analysis of bases at high pH (pH 10 or above) appears to give good results for solutes of moderate pK_a where the solute is mostly unionised at such pH values. However, ionisation of silanol groups occurs for

these phases, and for stronger bases, the residual ionisation of the solute can still give rise to detrimental interactions at quite high pH. Even at very high pH (e.g. pH 12), peak shape for small quantities of solute may still be inferior to those obtained at low pH. In addition, the stability of the column may be limited under these conditions.

7.3. Quality and choice of column

Major advances have been made in improving the purity of the base silica using for manufacturing RP columns. The acidity of residual silanols that remain after derivatisation has been substantially reduced, and in turn, the peak symmetry of small quantities of analysed bases has improved. Comprehensive databases of several hundred different columns evaluated by the Tanaka test, or the Snyder hydrophobic subtraction procedure are now available, allowing the user to choose a column of appropriate selectivity. In addition, data for the relative retention of benzylamine/phenol (from the Tanaka test) or the retention of amitriptyline/nortriptyline (from the Snyder test) can allow the selection of columns that are likely to give good peak shape for bases. Nevertheless, there are some differences in the evaluation of the same column by these different tests.

7.4. Temperature

The role of temperature in LC is now much better understood. Increased temperature does not generally give increased efficiency at the optimum flow rate for neutral compounds, but enables faster analysis, due to movement of the optimum flow velocity to higher values. Nevertheless, some real improvements in efficiency can occur for basic compounds at higher temperature and mobile phase pH close to the solute pK_a. It appears that this effect may be due to the lowering of the pK_a of basic solutes as temperature is raised, thus reducing the degree of protonation of bases, and their detrimental interactions with the surface. Increasing the temperature may also increase the kinetics of the slow sites to approach that of the fast sites, giving an improvement in peak shape.

7.5. Alternative separation mechanisms—e.g. HILIC

While HILIC is a much less flexible and less widely applicable technique than RP–LC, it can have considerable advantages for the separation of basic solutes. Due to the higher organic content and lower viscosities of the mobile phases used, HILIC can give better sensitivities in HPLC–MS. Longer columns can be used to gain high efficiency in reasonable analysis times, and loading properties for basic solutes may be superior to those for RP columns.

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