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Journal of Chromatography A, 828 (1998) 407–420

JOURNAL OF CHROMATOGRAPHY A

High-performance liquid chromatography of basic compounds Problems, possible solutions and tests of reversed-phase columns

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Abstract

Column testing in unbuffered mobile phases is adequate for identifying very active impure silicas used in reversed-phase chromatography, but inadequate for evaluating new generation phases, which still show considerable differences in activity towards strong bases. Overloading can seriously confound test results; buffered acetonitrile and tetrahydrofuran (THF) produce results similar to those previously shown for methanol. Principal component analysis (PCA) of a large data set indicates that relative performance for a given modifier at high and low pH is different. At a given pH, relative performance with methanol and acetonitrile is fairly similar, but distinct with THF. PCA also allows selection of a range of compounds assessing overall column performance; the use of a single probe for evaluation of activity towards bases is clearly inadequate. The newest columns give considerably improved peak shape for bases; the low back pressure generated by some allows coupled columns at least 75 cm long to be used, generating efficiencies similar to capillary electrochromatography.

Keywords: Stationary phases, LC; Column overloading; Principal component analysis; Chemometrics; Basic compounds; Pharmaceuticals

1. Introduction

Although high-performance liquid chromatography (HPLC) is now considered to be a mature separation method, there are still a number of areas in which problems are encountered, and where further research is desirable. One of the most important of these is the analysis of basic compounds, which can interact undesirably with the column surface of silica-based reversed-phase (RP) materials, giving rise to tailing peaks, poor quantitation, irreproducible retention times and even complete retention of some solutes. The problem is thought to be caused by interactions with column silanols. Additionally, interactions with metallic impurities may occur, or alternatively, silanophilic interactions may be enhanced by the presence of these impurities. Although a new generation of RP materials based on much purer silicas has recently been introduced, there are still considerable variations in the activity of even these new materials [1,2]. Thus, it is essential that reliable test procedures are available which can assess the activity of these columns towards basic substances.

A large number of different test procedures have been suggested for column evaluation. The best known of these is probably the Engelhardt test which seeks to assess the properties of the column with regard to bases as well as acidic and neutral solutes [3,4]. Although some problems exist with the analysis of acidic compounds, we have concentrated in our work on the analysis of bases which give rise to

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0021-9673/98/$ – see front matter
PII: S0021-9673(98)00590-1

much greater difficulties; these compounds are particularly important in biomedical or pharmaceutical work. The aims of the present paper can be summarised as follows:

(a) To clarify some important precautions necessary for column testing with basic compounds. It is vital that testing is carried out under closely controlled conditions, and that column evaluation parameters are clearly defined and understood.

(b) To consider evidence for and against testing columns in unbuffered mobile phases.

(c) To investigate further the effects of overloading on column performance. In particular we wished to investigate if the difference in peak shape obtained using different modifiers could be explained by different overloading effects in these modifiers.

(d) To evaluate the large column performance data set obtained previously [1,2], using chemometric procedures to identify significant probes and conditions in order to minimize the number of tests.

(e) To investigate the performance of the latest HPLC columns and to compare briefly these results with those achievable by capillary electrochromatography.

2. Experimental

The HPLC system consisted of P200 pump, UV 100 detector (1-μl flow cell) operated at 265, 254 or 215 nm (Thermo Separation Products, San Jose, CA, USA) and 7725 valve injector with 2-μl loop (Rheodyne, Cotati, CA, USA). Connections were made with minimum lengths of 0.0127 cm I.D. tubing.

The eight 25 cm columns used were 1=Inertsil ODS, 2=Inertsil ODS-2, 3=Inertsil ODS-3 (GL Sciences, Tokyo, Japan) 4=Kromasil C_{18}, 5=Kromasil C_8, (Anachem, Luton, UK), 6=Symmetry C_{18} (Waters, Milford, MA, USA), 7=Supelco ABZ+ (Supelco, Bellefonte, PA, USA) and 8=Purospher (Merck, Darmstadt, Germany) and have been described in detail previously [1]. In addition, we used Discovery amide, 25×0.46 cm I.D., surface area 200 m^2 g^{-1}, pore size 18 nm, coverage 3.3 μmol m^{-2} (Supelco). N was determined from peak widths at half height ($w_{0.5}$) using the formula $N=5.54(t_r/w_{0.5})^2$, or using the Dorsey–Foley equation, $N_{df}=41.7(t_r/w_{0.1})^2/(A_s+1.25)$. A_s was calculated at 10% of the peak height from the ratio of the widths of the rear and front sides of the peak; all measurements were made using a Model 2000 data station (Trivector, Bedford, UK). All results were the mean of at least duplicate injections. Preparation of buffers was as described previously [1,2]. All analyses were performed at 30°C with the column thermostatted in a block heater (Model 7980, Jones Chromatography, Hengoed, UK). Test solutes included codeine (pK_a=8.0), quinine (8.5), procainamide (9.2), diphenhydramine (9.0), nortriptyline (10.0), nicotine (7.9), amphetamine (9.9), pyridine (5.2) and benzylamine (9.3). Pattern recognition was performed using principal component analysis (PCA). Some authors recommend log scaling of raw data to account for variation, which under some circumstances can reveal hidden trends [5]. A difficulty of the data in this paper is that some values of k are 0; for the purpose of pattern recognition such numbers may be replaced by a small positive value. We chose to replace 0 values by half the minimum positive value for a particular parameter where appropriate. It is important to recognise that these values occur only occasionally. One advantage of log scaling is that very large values do not have an undue influence on data analysis. For instance, pyridine in tetrahydrofuran (THF) at pH 3.0 gave $k=0.31$ for one column but <0.05 for all others. Without log scaling, this particular parameter may be distorted. It was found that log scaling made only a small difference to the PCA results, which can be seen by comparing plots shown in the present paper with those published previously, where the data was not log scaled [6]. However, in some cases the loadings plots do improve; for instance, there is a clearer discrimination of k and A_s values. After log scaling and before PCA, the logarithms are standardised because the parameters are on different scales; for example N values are much larger than k values. The data are then centred and PCA scores and loadings plots computed as described previously [6]. All PCA calculations were carried out in Microsoft Excel.

3. Results and discussion

3.1. Basic precautions in column testing

In addition to normal precautions of thermostatting the column and limiting of extra column effects

(very important for peaks of low k), we recommend consideration of the following points:

(a) Computation of peak parameters. Various equations have been used to calculate column efficiency. In our work we have used the simple half height method which gives exaggerated values of the number of theoretical plates for tailing peaks, and the Dorsey–Foley efficiency parameter, which has been shown to give a reasonable estimation of true column efficiency [7]. In the present study, we hoped to determine whether use of the commonly used half height method could introduce serious errors in the ranking of columns i.e., in the relative column efficiency, or whether there was simply a difference in the magnitude of the values. There is also some confusion in the literature between the asymmetry factor (defined above) and the US Pharmacopoeia symmetry factor (S_f) or tailing factor which is defined as the ratio of the width of the peak (measured usually at 5% of peak height) to twice the width of the front side of the peak [8]. Simple algebra shows that $A_s=2S_f-1$, i.e., a symmetry factor of 1.5 implies an asymmetry factor of 2.0; however this relationship is only approximate since the measurements are not made at the same fractional peak height. While either method is acceptable, it is important that the same calculations are used when comparing results; in this work we quote asymmetry factor measurements.

(b) Great care is necessary in the construction of mixtures of test compounds. Compounds eluting on the tail of a previous asymmetric peak may show unrealistically favourable results, presumably due to some deactivation effect by the preceding compound. Similarly, it is sometimes necessary to wait a considerable period of time between injections to prevent column ''loading'' effects [1], especially with compounds of low k. This makes the design of a single test mixture extremely difficult.

3.2. Testing in unbuffered mobile phases

The potential advantages of the use of unbuffered mobile phases are very considerable and include simplicity and reproducibility of mobile phase preparation – there is still much variation in the way buffered mobile phases are prepared and pH adjustment made, giving a source of irreproducibility. The Engelhardt test includes aniline, phenol, *N*,*N*-dimethylaniline and the isomeric toludines as test compounds which are chromatographed using methanol–water (49:51, w/w). Later versions of the test use also *p*-ethylaniline, which is suggested as a more stringent silanophilic probe. However, there are potentially serious problems with the use of unbuffered mobile phases.

(a) Test mixtures cannot include high pK_a compounds, as in that case peak shape effects may be caused merely by variable ionisation of the solutes in the mobile phase. We have noted these effects even with relatively weak bases (pK_a ca. 6.5). Alternatively, many studies have shown that the most significant problems in the analysis of bases (with buffered mobile phases) arise with compounds of considerably higher pK_a. Therefore, the permissible test compounds tend to be not very demanding; in our hands, the Engelhardt test provides an excellent way of distinguishing very active columns from the rest, as reasonable columns elute aniline before phenol, fail to resolve the isomeric toluidines, and give good peak shape for aniline, *N*,*N*′-dimethylaniline and *p*-ethylaniline. However, in our experience, this test fails to distinguish even rather moderate from excellent columns. Furthermore, a recent grading of columns [3] showed at least a dozen (and the present authors could name at least a dozen more) which produced asymmetry factors of 1.1–1.5 even with the supposedly more stringent test compound *p*-ethylaniline when used with unbuffered methanol–water (49:51, w/w). And yet, in the present authors' experience, these same columns still show very marked differences in their behaviour towards more stringent test compounds in buffered mobile phases. Pyridine seems to be a rare example of a low pK_a compound which can be used in unbuffered mobile phases which produces considerable differences in asymmetry even with ''new generation'' RP materials based on pure silica [1,9]. It is possible that favourable stereochemistry of the molecule allows penetration of this solute to the column surface. However, it is shown conclusively below that a single compound is insufficient for meaningful column evaluation.

(b) The behaviour of a column can depend on the history of its use, and long equilibration times may be experienced with unbuffered mobile phases. We always use new columns for evaluation but even

packing or storage solvents may influence the results of the test.

(c) Another serious problem with the use of unbuffered mobile phases is that they are unrepresentative and are remote from usual practice. Because of this, test results must be related to routinely used conditions (ie with buffered mobile phases). There is little published data which attempts to relate success (or failure!) of a given column with low pK_a bases in unbuffered mobile phases to analysis of high pK_a bases in buffered mobile phases. It is questionable how tests in a mobile phase of indeterminate pH could predict column performance in buffered mobile phases at both the low and high end of the useful pH range of typical columns (pH 3.0 and 7.0, respectively).

3.3. Testing in buffered mobile phases

3.3.1. Influence of organic solvents on mobile phase pH

Although it is generally accepted that pH should be measured prior to addition of organic modifier, the modifier may still influence dissociation of buffer compounds and the pK_a of both analytes and column silanols. Care is therefore necessary in the interpretation of data; for instance pyridine (aq. pK_a=5.2) is half protonated in 55% methanol only when the pH of the phosphate buffer is 3.2 [10].

3.3.2. Column overloading effects

Recently, we have shown that the overloading behaviour of RP columns when using basic compounds depends markedly on the pH of the mobile phase [11]. Significant deterioration (reduction of *N* by 10%) was found to occur at pH 3.0 in some cases with as little as 0.5 μg of injected compound. We did not find large differences in the sample capacity for a given basic compound from column-to-column and our results are in agreement with the values given by Snyder and co-workers [12,13]. The column saturation capacity for different solutes however, varied considerably. Overloading at pH 3.0 is also highly dependent on *k* of the analyte, in accord with the theoretical description proposed previously [13,14]. Because the same solute with the same mobile phase can produce variable overloading due to differences in *k* values on different columns, it is sensible to use sample masses well below those expected to produce overloading for any basic compound; in our evaluations, we have used 0.2 μg of analyte although it may be prudent to reduce this even further to 0.1 μg. These amounts are much smaller than those used by some other scientists for column testing [15,16]. While injection of small sample amounts is important for column testing, of course this may not be possible in daily practice; furthermore it would be useful to have more information as to whether some columns are more easily overloaded than others, although relatively little work has been published on this subject for basic compounds. At pH 7.0, overloading effects are completely different, with peak shapes often showing no deterioration until the injection of at least 20 μg. It appears that column efficiency even improves in some cases (particularly with more active columns) up to this 20 μg level, and variations in asymmetry factor can also be obtained. It again seems prudent to use small fixed amounts of analyte to minimise these variations. Whereas our previously published results used phosphate buffers modified only with methanol [11], Fig. 1 indicates that similar results are obtained with other modifiers both at pH 3.0 and 7.0. Fig. 1a and b show the overloading profile of benzylamine using phosphate buffer, pH 3.0 modified with THF and with acetonitrile, respectively. The organic modifier concentration was adjusted to give similar *k* in these alternative solvents as had been obtained with benzylamine using methanol [11]. With all three modifiers, similar trumpet shaped plots were obtained for benzylamine, indicating rapidly increasing peak asymmetry and decreasing column efficiency as sample load increases. Fig. 1c shows the overloading profile for amphetamine using phosphate buffer, pH 3.0 modified with acetonitrile, which gives again very similar results to those obtained previously with methanol [11]. Furthermore, Table 1 shows that the column saturation capacity both for benzylamine and amphetamine changes very little when only the modifier is changed. Table 1 also indicates that quite small amounts of these solutes can produce a 10% reduction in column efficiency. Finally, Fig. 1d shows the overloading profile for amphetamine in phosphate buffer, pH 7.0 modified with acetonitrile. It is apparent that once again there is little difference from results obtained with methanol at pH 7.0 [11].

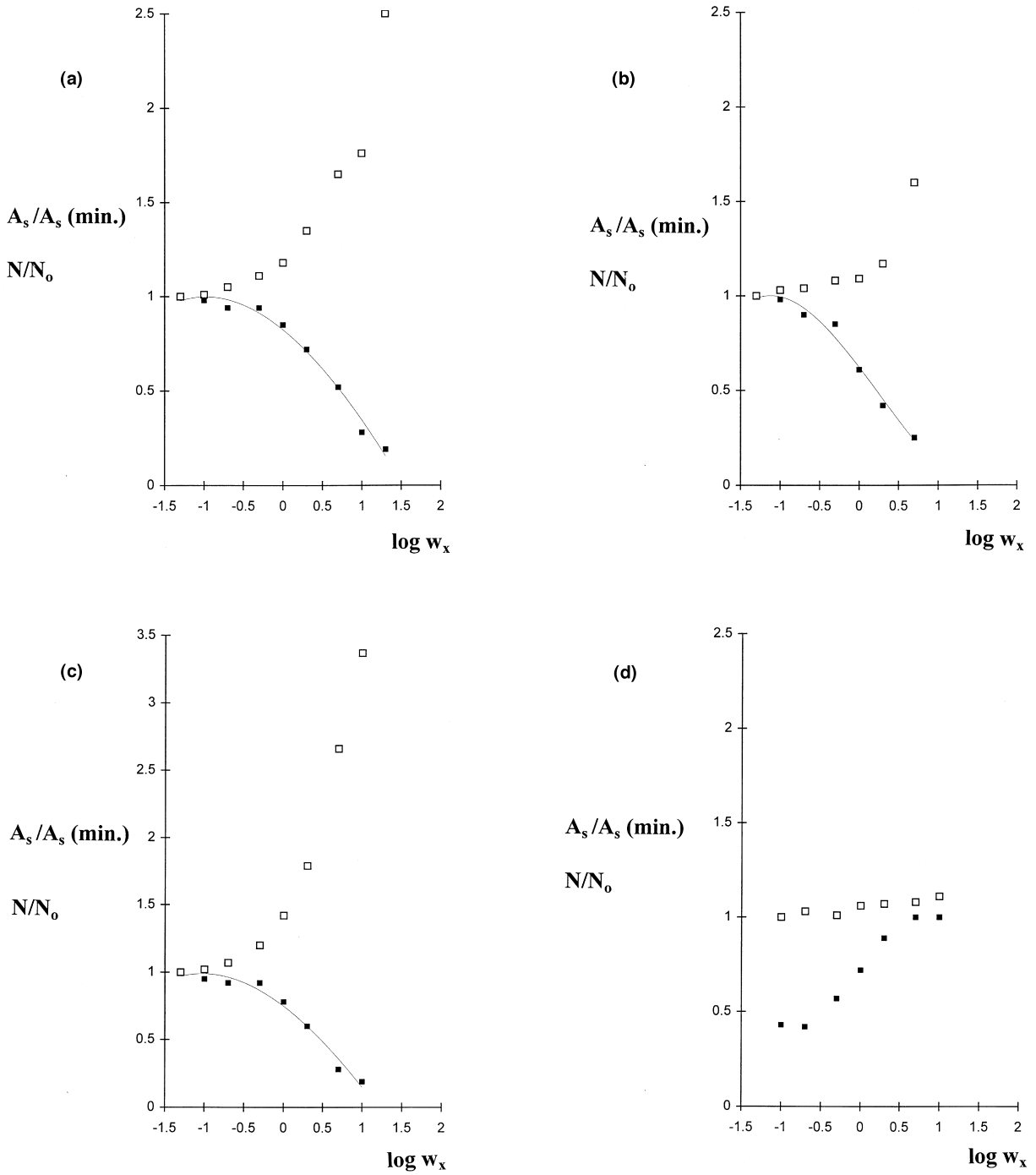

Fig. 1. Plots of N/N_0 (black squares) and A_s/A_s (min) (white squares) versus the logarithm of sample mass w_x (μg) for (a) benzylamine using THF–0.0243 *M* phosphate buffer, pH 3.0 (7.5:92.5, v/v); (b) benzylamine and (c) amphetamine using acetonitrile–0.0265 *M* phosphate buffer, pH 3.0 (15:85, v/v); (d) amphetamine using acetonitrile–0.0375 *M* phosphate buffer, pH 7.0 (40:60, v/v). Column: Inertsil ODS-2.

Table 1
Retention factor, column saturation capacity (w_s) and column efficiency using different organic modifiers in combination with phosphate buffer using Inertsil ODS-2; also sample masses which reduce efficiency to 50% and 90% of small mass value

Mobile phase	k	50% N (μg)	90% N (μg)	w_s (mg)
Benzylamine				
MeOH–phosphate buffer pH 3.0 (30:70, v/v)	0.27	5.7	0.9	1.2
ACN–phosphate buffer pH 3.0 (15:85, v/v)	0.42	1.7	0.3	0.8
THF–phosphate buffer pH 3.0 (7.5:92.5, v/v)	0.38	5.5	0.6	1.5
Amphetamine				
MeOH–phosphate buffer pH 3.0 (30:70, v/v)	2.1	4.2	0.7	5.4
ACN–phosphate buffer pH 3.0 (15:85, v/v)	1.5	3.2	0.4	5.3

Nevertheless, the overloading effects are completely different from those at pH 3.0, with an apparent increase in column efficiency with up to 10 μg of amphetamine using buffered acetonitrile. It would appear therefore that differences in peak shape shown by a solute when different modifiers are used [1,2] do not seem attributable to differences in overloading effects with these various modifiers.

3.3.3. Use of chemometrics to investigate influence of solute, modifier and pH

We have recently published a large data set containing evaluation parameters (including N, k and A_s) for eight columns, using up to 10 basic probe compounds, with three organic modifiers (methanol, acetonitrile and THF) at pH 3.0 and 7.0. Each column was also evaluated with an Engelhardt-type test both in unbuffered methanol and acetonitrile (i.e., eight different mobile phases per column) [1,2]. The performance parameters k, N, A_s were recorded at both pH 3.0 and pH 7.0; in addition, N_{df} values were recorded at pH 3.0. These detailed investigations were necessary in order to study the effect of a large number of variables. However, such comprehensive testing is laborious, generating as many as 200 separate data points per column. Thus, we used a chemometric procedure, PCA to help investigate data reduction by identifying those conditions and test solutes which have a significant influence. PCA finds the maximum variations in the data and forms new variables (''principal components''). Each of these new variables is orthogonal. The output of PCA is a scores plot, indicating in this case the correlation between columns, and a loadings plot which indicates the distribution of information in the data and, in this case, the relationship between the evaluation parameters.

3.3.3.1. PCA at pH 3.0

Our previous results indicated that at pH 3.0, small differences in peak asymmetry occurred when comparing methanol or acetonitrile as modifier, although higher column efficiency for most basic compounds was found with acetonitrile as is normally found for neutral compounds. This result is probably due to the reduced viscosity of such mobile phases modified with acetonitrile. Significant reduction in peak asymmetry was found with THF. However, it is not easy to visualise differences in relative column performance in these different solvents. For instance, do all columns show more or less the same improvement in performance with THF? If so, testing and ranking of columns could be performed with any one chosen modifier and the results with another modifier could be predicted. Computing of PCA scores plots for individual isoeluotropic mobile phases modified with methanol, acetonitrile and THF readily indicates columns showing similar or widely different behaviour. Fig. 2a shows a scores plot for methanol at pH 3.0 obtained by considering four performance parameters (k, N, N_{df} and A_s) reported previously and eight different test compounds [2]. These PCA calculations were repeated separately for acetonitrile and THF. Clustering of columns in methanol, and acetonitrile (not shown) produced very similar results, but the scores plot for THF shown in Fig. 2b shows significant differences [6]. This is confirmed by the ranking of columns according to the mean asymmetry factor for eight different solutes in

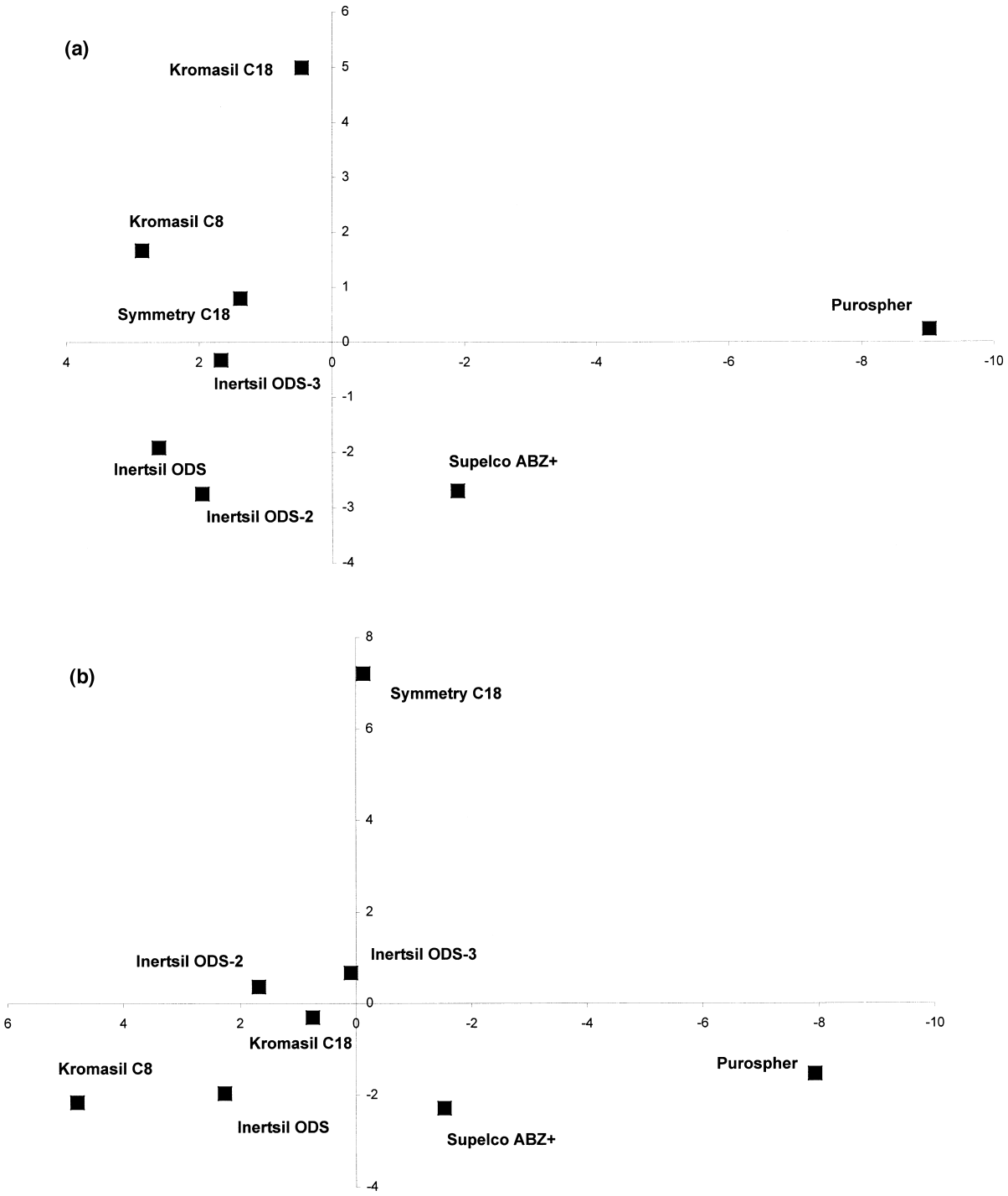

Fig. 2. (a) Scores plot methanol–phosphate buffer, pH 3.0 (30:70, v/v). (b) Scores plot THF–phosphate buffer, pH 3.0 (75:92.5, v/v).

methanol (obtained from the raw data, [2]) which was:

1 (lowest asymmetry) < 2 < 7 < 3 < 8 < 6 < 5 < 4

in acetonitrile it was:

1 (lowest asymmetry) < 2 < 3 < 7 < 8 < 5 < 6 < 4

which is very similar. However, in THF the ranking was

5 (lowest asymmetry) < 1 < 4 < 3 < 8 < 7 < 2 < 6

which is considerably different.

It seems that rankings in THF are different due to substantially different degrees of improvement in performance with this solvent among columns [2]. Thus, it appears that testing a column in methanol or acetonitrile could not be used to predict reliably its performance in THF. However, THF is rarely used due to practical difficulties and hazards. It can be concluded that in general, testing columns at pH 3.0 with only one chosen modifier, methanol or acetonitrile, is necessary (although mixing results obtained from each modifier is not recommended because of differences in the absolute values of eg column efficiency). More information can be obtained from PCA loadings plots which are shown for methanol–phosphate buffer, pH 3.0 and 7.0 in Fig. 3a and b, respectively. Parameters that are at 180° measure equivalent but opposite trends and have a correlation coefficient, r, close to -1.0; it may not be necessary to measure both parameters. Parameters that are at 90° measure unique properties ($r=0$), so must be retained in column evaluation. Parameters that measure related trends have a small angle between them and have r close to 1.0. These parameters are likely to duplicate information, allowing one parameter to be discarded. A way of minimising the amount of data might be to measure either column efficiency or asymmetry values but not both. The validity of this approach can be checked for methanol at pH 3.0 from Fig. 3a, and from a consideration of the correlation coefficients between parameters. Although in some cases A_s and N values are diametrically opposed for a given solute (i.e., increase in peak asymmetry is accompanied by a directly related decrease in column efficiency), this is not always the case. The mean value of r between N and A_s for all eight solutes was -0.44; thus it appears necessary to retain both of these evaluation parameters. On the other hand, there are only small angles between N and N_{df} measurements for a given solute, and the mean value of r between N and N_{df} for all eight solutes was about 0.9. It would appear that while there can be considerable differences in the absolute values of N and N_{df} the relative values of column efficiency are fairly similar. It thus seems unnecessary, at least at pH 3.0, to measure N_{df} for column comparison purposes. The loadings plots can also be used in reduction of the number of test compounds by selection of those measuring apparently unrelated properties. Fig. 3a shows that asymmetry values for pyridine, codeine, quinine, amphetamine and nortriptyline cover a range of column ''properties'' (their vectors give a roughly evenly spaced span of the upper right hand quadrant of the plot). For example, there is an angle of almost 90° between the vectors for asymmetry of nortriptyline and codeine, indicating that there is almost no correlation between these quantities; indeed, the correlation coefficient between the eight pairs of values obtained on the eight different HPLC columns is only 0.13. Alternatively, there is a closer correlation between asymmetry values for codeine and quinine ($r=0.67$). A similar relationship between N values is also apparent for these five probes in the lower left hand quadrant. Values of column efficiency and A_s for nicotine, and to a lesser extent benzylamine are close to the centre of the plot. Variables near the centre do not describe the pattern illustrated by two PCs, and are likely to show high loadings on the third or later principal component. Indeed Fig. 4 shows a high weighting of these parameters for nicotine on the 3rd PC (y axis) – note both column efficiency and asymmetry factor for nicotine are far from the centre along the 3rd PC, which does not, however, give much separation of the parameters for other probes. Thus nicotine may also measure distinct column properties. Similar results were obtained for PC loadings plots for nicotine with acetonitrile at pH 3.0, and in general the loadings plots showed broad similarity with those for methanol for all test compounds [6].

3.3.3.2. PCA at pH 7.0

We noted previously [1] that while at pH 3.0, similar peak asymmetries for basic compounds were

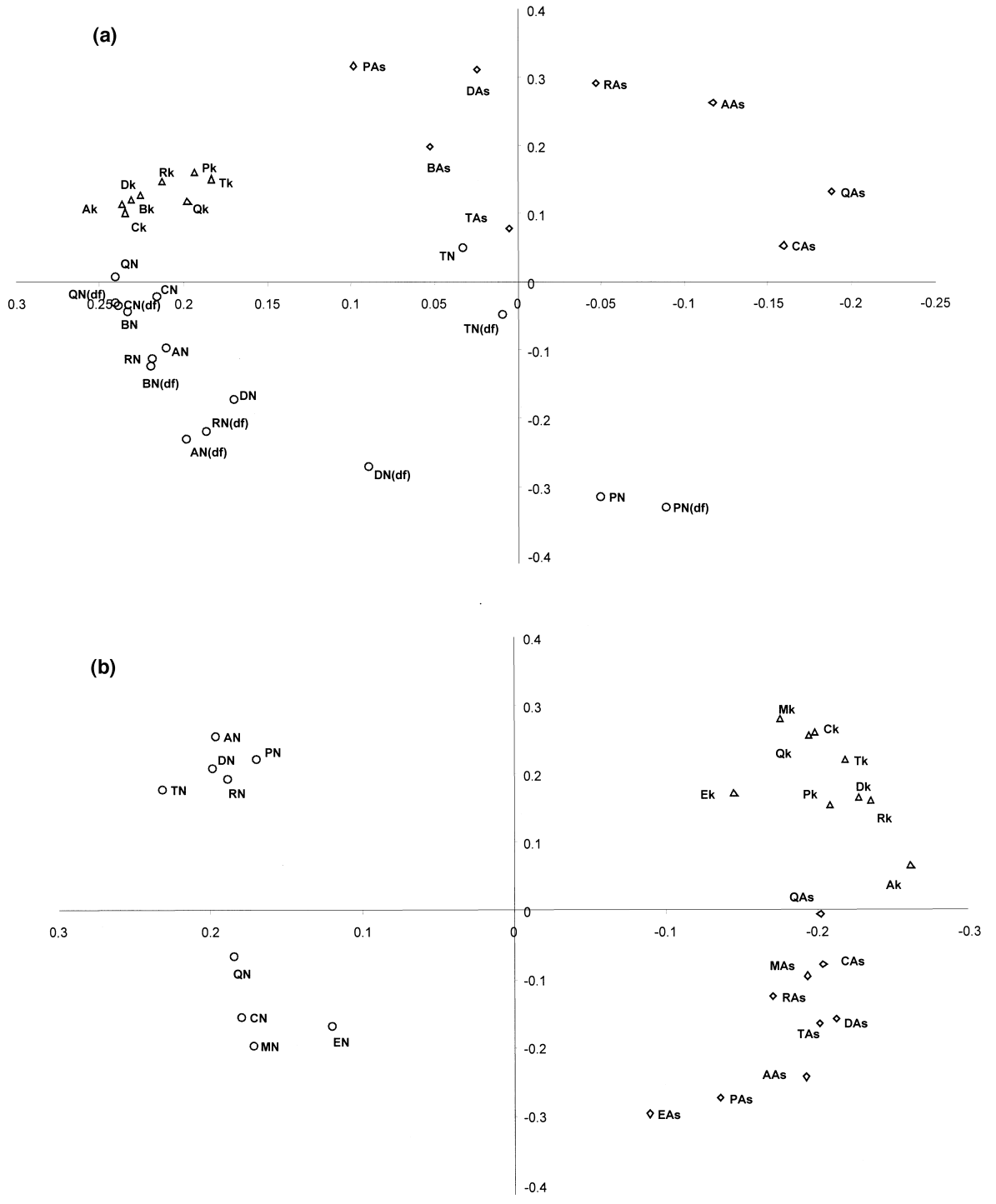

Fig. 3. (a) Loadings plot methanol–phosphate buffer, pH 3.0 (30:70, v/v). (b) Loadings plot methanol–phosphate buffer, pH 7.0 (65:35, v/v). P = Pyridine, T = nicotine, A = amphetamine, C = codeine, D = diphenhydramine, R = nortriptyline, M = procainamide, Q = quinine, B = benzylamine, E = 2-[*N*-methyl-*N*-(2 pyridyl)-amino]ethanol. *N* = Column efficiency, *N*(df) = Dorsey–Foley efficiency, *k* = retention factor, As = asymmetry factor.

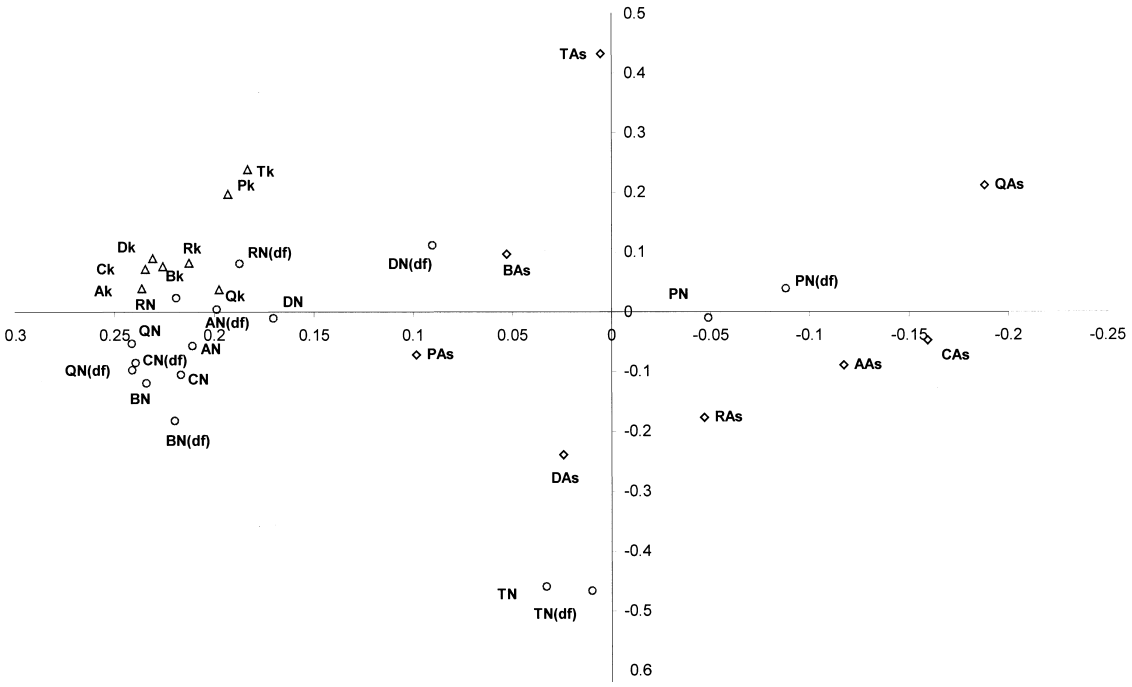

Fig. 4. Loadings plot methanol–phosphate buffer, pH 3.0. PC1 (*x* axis) vs. PC3 (*y* axis).

obtained using methanol or acetonitrile, most columns gave considerably worse peak shape in acetonitrile rather than methanol using pH 7.0 buffer. At pH 7.0, THF gave significant improvement in the performance of all columns. For practical analysis of basic compounds these results are important and indicate that methanol may often be a better choice of modifier than acetonitrile and more thought should be given to the possibilities of using THF, despite its practical difficulties and hazards. Again, it should be stressed that for column testing however, the performance of one column relative to another is more important than across the board improvement or decline. Additionally, whereas column performance is generally worse at pH 7.0 than at pH 3.0 it is difficult to conclude from mere inspection of the raw data whether there are significant or only minor changes in the relative clustering of columns at the different pH values. Is the best column at one pH likely to be also the best column at a different pH? Fig. 5a shows the scores plot at pH 7.0 with methanolic mobile phase based on the performance parameters k, N and A_s and nine different test compounds. A comparison of Fig. 5a with Fig. 2a shows that there are significant differences in the scores plots for methanol when used with pH 7.0 buffer compared with pH 3.0. For instance, while the three Inertsil columns are closely grouped along the first principal component at both pH values, the positions of both Symmetry C_{18} and Kromasil C_{18} are considerably different in the two plots. It is important to realise that the first principal component (*x* axis) is much more significant than the second (*y* axis), so differences between the columns along the second principal component are not as consequential. The rankings of the columns at pH 7.0 using methanol as modifier according to the mean asymmetry (obtained from the raw data, [1]) is

7 (lowest asymmetry) <

$3 < 1 < 6 < 2 < 8 < 5 <$ 4(highest asymmetry)

which is clearly different from that for pH 3.0 with methanol, shown above; this confirms the results of the PCA score plots. These results suggest strongly that column testing must be carried out at different

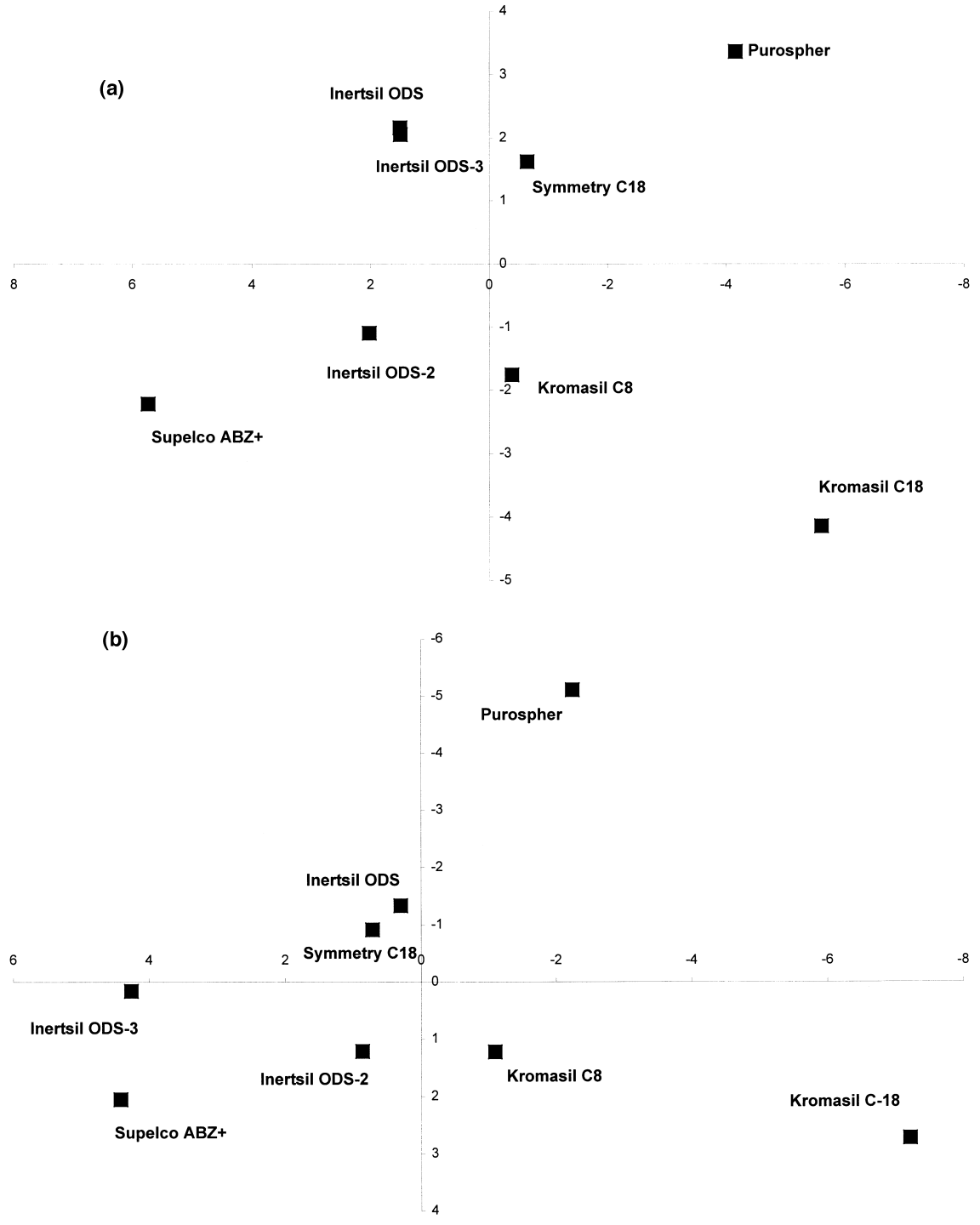

Fig. 5. (a) Scores plot methanol–phosphate buffer, pH 7.0 (65:35, v/v). (b) Scores plot acetonitrile–phosphate buffer, pH 7.0 (40:60, v/v).

pH values. It should be noted that the data set at pH 7.0 did not include N_{df} measurements and only seven of the eight compounds used at pH 3.0. However, we have shown in detail [6] that these slight variations make little difference to the scores plots. A comparison between the scores plot for methanol and acetonitrile at pH 7.0 (Fig. 5a and b) reveal more differences between these organic modifiers than found at pH 3.0. The rankings of the columns with acetonitrile at pH 7.0 according to the mean column asymmetry factor based on all the test solutes was

3 (lowest asymmetry) < 7 < 6 < 8 < 2 < 1 < 5 < 4(highest asymmetry)

There is considerable argument to advocate separate testing using methanol and acetonitrile at pH 7.0, due to movements in the ranking of both columns 1 and 3. The scores plots Fig. 5a and b also show the movements of these columns both relative to the other 6 columns and to each other. Although it would appear that results with these two modifiers at pH 7.0 are reasonably similar, a firm decision on whether separate testing in each modifier is necessary requires further experimental data using different columns. Once again, there are greater differences in the plot for THF (not shown), indicating that a test performed in acetonitrile or methanol could not reliably predict the behaviour in THF.

Fig. 3b shows the corresponding loadings plot for methanol–phosphate buffer, pH 7.0. The mean correlation coefficient between N and A_s for the nine test compounds was -0.84. This would suggest it is more valid to measure only one of these parameters at pH 7.0. However, considering the results at pH 3.0 and the ease with which these parameters can be measured simultaneously using modern data stations, we advocate that both measurements should be made. Considering the asymmetry values shown in Fig. 3b, pyridine, amphetamine nicotine (which is not close to the centre at pH 7.0) nortriptyline, codeine and quinine are again a reasonable choice of probes in that they span the lower right hand quadrant of the loadings plot. An example of eliminating duplicate information is shown by the small angle between the vectors for A_s of diphenydramine and nortriptyline in Fig. 3b; there is little point in including both compounds in a test mix since the values are highly correlated ($r=0.96$). The same is true for procainamide, whose A_s is correlated highly with codeine ($r=0.97$). As at pH 3.0, the loadings plots broadly show similar relative results for acetonitrile (not shown) at pH 7.0 [6]. The choice of probes is a matter for debate because different probes may be more suitable under different mobile phase conditions: furthermore, amphetamine is a controlled drug and may be rejected by some scientists. However, PCA loadings plots can be used to select other combinations of probes to suit particular circumstances.

3.4. Performance of the latest columns

The inertness of the latest commercially available HPLC columns towards basic substances has been improved substantially, as well as considerable increases in column efficiency for neutral substances. Fig. 6 shows an evaluation of one of the latest columns in an unbuffered mobile phase indicating high efficiency and good asymmetry for acidic, basic and neutral compounds. The average value of N for pyridine, nicotine, amphetamine, codeine, notriptyline and quinine was 24 500 with average $A_s=$ 1.30 using acetonitrile–phosphate buffer, pH 3.0 (15:85, v/v) [for nortriptyline, acetonitrile–phosphate buffer (28:72, v/v)], representing excellent performance. For comparative purposes the average N was 17 400 with average $A_s=1.47$ using methanol–phosphate, buffer pH 7.0 (65:35, v/v), indicating still good results. Another advance is the low back pressures generated by some columns, allowing coupling of several 25 cm lengths without generating excessive back pressures. Fig. 7 shows a chromatogram obtained by coupling 3×25 cm Inertsil ODS-3 columns using short lengths of 0.0127 cm I.D. tubing to generate a 75 cm column which when run with acetonitrile–water (70:30, v/v) generates a back pressure of only 145 bar. The overall column efficiency showed no degradation and the plate count of the 75 cm column was merely the sum of the plate counts of the individual columns. Thus N, for benzene was over 70 000 and that for pyridine and aniline about 55 000 and 65 000 plates, respectively. This long column could be run with any combination of acetonitrile–buffer without generating excessive back pressure and giving virtually the same peak asymmetry as the individual columns; it should be noted that Inertsil ODS-3 gives very good peak

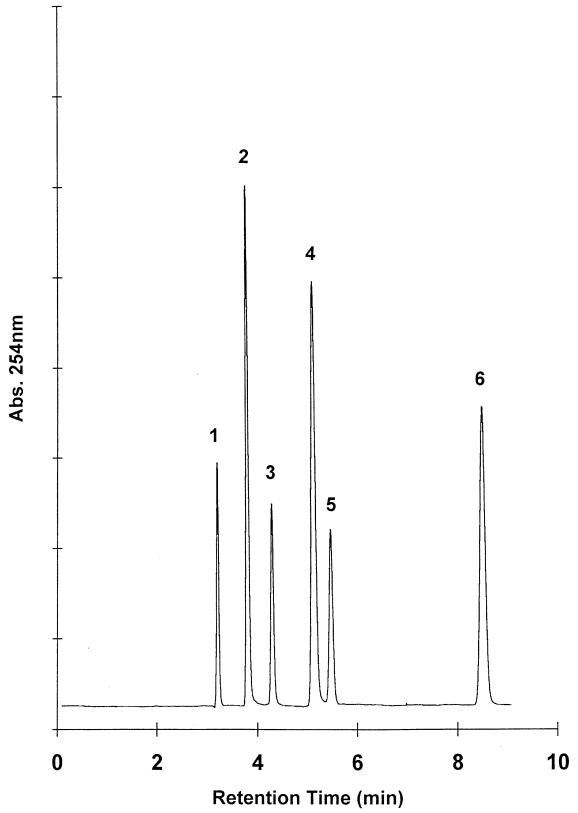

Fig. 6. Analysis of test compounds with Supelco Discovery Amide column. Mobile phase methanol–water (55:45 v/v). Peaks: 1=uracil (N=25 800, A_s=1.22), 2=pyridine (N=26 100, A_s=1.28), 3=aniline (N=27 800, A_s=1.22), 4=*o,m,p*-toluidines (co-eluted), 5=phenol (N=26 500, A_s=1.09) 6=benzene (N=30 200, A_s=1.04).

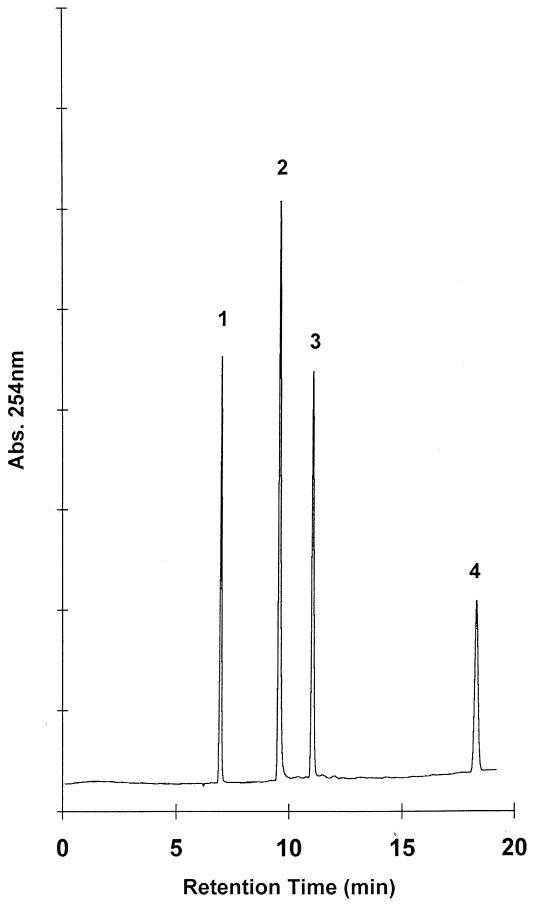

Fig. 7. Analysis of test compounds using Inertsil ODS-3 (75 cm). Mobile phase acetonitrile–water (70:30 v/v). Peaks: 1=uracil (N=48 500, A_s=1.31), 2=pyridine (N=55 000, A_s=1.40), 3=aniline (N=65 900, A_s=1.18) 4=benzene (N=70 300, A_s=1.08).

shapes for basic compounds with buffered mobile phases [1,2]. A problem with this approach is that increased analysis time is needed to achieve optimum resolution. Nevertheless, we believe that 50 cm columns generating up to 50 000 plates are viable. There has been much interest recently in the technique of capillary electrochromatography (CEC) for pharmaceutical analysis in that the technique gives high efficiency approaching that of capillary electrophoresis (CE), but retains both the selectivity advantage which HPLC has over CE and its simple applicability to the analysis of neutral compounds. A difficulty with CEC is low electroosmotic flow (EOF) with ''new generation'' phases, and bad peak shapes are still obtained with basic substances on standard CEC phases [17]. Thus CEC does not as yet provide a reliable alternative to HPLC in such applications. Our results above show that it is possible to generate similar numbers of theoretical plates for all compounds (including bases) in conventional HPLC to those shown in CEC, albeit at the expense of increased analysis time.

4. Conclusions

(1) Although testing of columns in unbuffered mobile phases seems attractive, there are severe difficulties inherent in such procedures. As a result,

we believe that such methods are useful only to reveal major differences between columns.

(2) pH measurement of the aqueous buffer prior to organic modifier addition still poses interpretation problems, since the modifier can have a considerable effect on buffer, solute and silanol ionisation.

(3) Columns must not be overloaded in comparative testing, especially at acidic pH when amounts as little as 0.5 μg may cause appreciable deterioration in peak shape. At pH 7.0, overloading does not occur until much larger amounts are injected. However, peak shape variations may still be obtained, and we recommend that the same small mass of sample is used. The nature of the organic modifier does not seem to affect overloading to any great extent.

(4) Use of PCA shows that: (a) relative performance is considerably different at pH 3.0 and pH 7.0. Thus, testing must be carried out at both pH values, or at the pH at which it is desired to operate the column. (b) At pH 3.0 isoeluotropic mobile phases modified with methanol or acetonitrile give different absolute values of evaluation parameters but similar results in terms of relative performance. Thus, either methanol or acetonitrile can be selected as a test modifier, but these cannot, be used to predict reliably the performance in THF at pH 3.0. At pH 7.0, there are somewhat greater differences between methanol and acetonitrile, with larger variations when THF is used. (c) Both efficiency and asymmetry measurements should be recorded, however, it is not essential, at least at pH 3.0, to use more accurate equations for N if relative performance is considered. Further study of use of equations such as the Dorsey–Foley equation, at pH 7.0 is necessary. (d) It is clearly erroneous to test columns with a single basic compound, or with closely related substances, due to extreme solute dependence. Pyridine, codeine, quinine, amphetamine, nortriptyline and nicotine allow a reasonable overall assessment at either pH 3.0 or 7.0, with methanol or acetonitrile.

(5) The latest columns can give average efficiencies as high as 25 000 plates in a 25 cm column at acid pH even for the challenging probes advocated. Some new materials allow the use of columns 75 cm long or greater without generating excess back pressure, giving as many as 70 000 theoretical plates on conventional HPLC instruments. This rivals the efficiencies produced in CEC which still has difficulties due to the problem of low EOF in phases suitable for the analysis of strong bases.

References

[1] D.V. McCalley, J. Chromatogr. A 738 (1996) 169.
[2] D.V. McCalley, J. Chromatogr. A 769 (1997) 169.
[3] H. Engelhardt, M. Arangio, T. Lobert, LC·GC Int. 10 (1997) 803.
[4] H. Engelhardt, M. Jungheim, Chromatographia 29 (1990) 59.
[5] R.G. Brereton (Ed.), Mutivariate Pattern Recognition in Chemometrics, Illustrated by Case Studies, Elsevier, Amsterdam, 1992.
[6] R.G. Brereton, D.V. McCalley, Analyst 123 (1998) 1175.
[7] W.W. Yau, S.W. Rementer, J.M. Boyajian, J.J. DeStefano, J.F. Graff, K.B. Lim, J.J. Kirkland, J. Chromatogr. 630 (1993) 69.
[8] United States Pharmacopeia, United States Pharmacopeial Convention, 1995.
[9] D.V. McCalley, J. Chromatogr. A 636 (1993) 213.
[10] D.V. McCalley, J. Chromatogr. A 708 (1995) 185.
[11] D.V. McCalley, J. Chromatogr. A 793 (1998) 31.
[12] D. Chan Leach, M.A. Stadalius, J.S. Berus, L.R. Snyder, LC·GC Int. 1 (1988) 22.
[13] J.E. Eble, R.L. Grob, P.E. Antle, L.R. Snyder, J. Chromatogr. 384 (1987) 45.
[14] L.R. Snyder, G.B. Cox, P.E. Antle, Chromatographia 24 (1987) 82.
[15] R. Vervoort, M.W. Derksen, A.J. Debets, J. Chromatogr. A 765 (1997) 157.
[16] E. Cruz, M.R. Euerby, C.M. Johnson, C.A. Hackett, Chromatographia 44 (1997) 151.
[17] T.M. Zimina, R.M. Smith, P. Myers, J. Chromatogr. A 758 (1997) 191.