ELSEVIER

Journal of Chromatography A, 793 (1998) 31–46

JOURNAL OF CHROMATOGRAPHY A

Influence of sample mass on the performance of reversed-phase columns in the analysis of strongly basic compounds by high-performance liquid chromatography

David Victor McCalley

Department of Chemical and Physical Sciences, University of the West of England, Frenchay, Bristol BS16 1QY, UK

Received 10 June 1997; received in revised form 25 August 1997; accepted 1 September 1997

Abstract

The overloading behaviour of two different silica-based ODS columns was investigated using six basic probe compounds with methanolic mobile phases buffered at pH 3.0 and pH 7.0. At pH 3.0, peak shape deteriorated seriously in most cases when the sample mass was increased above about 0.5 μg, as has been reported previously. The rate at which efficiency loss occurred varied with the solute and column, as reflected in values for the saturation capacity w_s calculated for the solute/column combination. The model proposed by Snyder and co-workers was shown to allow prediction of the column overload profile for five of the basic solutes, although pyridine gave anomalous behaviour. Whereas peak shapes at pH 7.0 were generally worse than at pH 3.0, no deterioration was shown with sample masses up to at least 10 μg. In some cases peak shape showed significant improvement as sample mass was increased. Thus, at high sample loading, analysis at pH 7.0 may be preferred for some solutes. It was shown that while overloading has not influenced our previous column evaluation studies, great care is needed to avoid confounding the results of such studies by these effects. © 1998 Elsevier Science B.V.

Keywords: Stationary phases, LC; Column overloading; Sample mass; Basic compounds; Pharmaceuticals

1. Introduction

The analysis of basic compounds using reversed-phase high-performance liquid chromatography (RP-HPLC) continues to receive much attention, due to problems of poor peak shape, which are generally attributed to detrimental interaction of these analytes with underivatised column silanol groups [1]. The considerable interest in this area is partially due to the large number of important pharmaceuticals and other clinically significant compounds which possess basic groups. Recently, we have performed evaluations of some RP columns specifically recommended by their manufacturers for the analysis of bases, using a set of probes containing various structural features and differing pK_a, with phosphate buffers at pH 3.0 and 7.0 modified with methanol, acetonitrile or tetrahydrofuran (THF) [2,3]. A difficulty with these evaluations is the possibility of overloading silanol groups. Due to their differing properties, different types of RP column yield different retention factors (k') for the same basic analytes when using identical mobile phases. Since k' is the ratio of the amount of analyte in the stationary phase divided by that in the mobile phase, injection of even the same mass of analyte can give rise to variable overloading effects if k' is different. Furthermore, differences in k' for the same analyte on different columns can give

PII S0021-9673(97)00898-4

other problems such as variable influence of extra-column volumes. Adjusting the organic modifier concentration to give the same k' for a given solute on each column is a possible solution to these problems. However, this strategy can introduce other variables such as differences in degree of ionisation of the analyte and buffer, and differences in solvation of the stationary phase [4,5]. Thus, in our evaluation studies, we have preferred to use identical mobile phases with each column, in conjunction with small analyte mass (0.2 μg) and volume (2 μl) to limit these effects. The injected sample mass was chosen after consideration of the work of Snyder and coworkers, who suggested that overloading may occur with injection of greater than about 0.5 μg of basic compound on a 25 cm column. Sample sizes 50–100 times larger could apparently be injected for non-basic compounds [6]. However, some recent publications report the use of much higher quantities of probe compound in column evaluation studies; Vervoort et al. [7] utilised 2 μg injections of five proprietary basic pharmaceuticals and Cruz et al. [8] 10 μg of benzylamine (together with other probes). Despite the wealth of literature on overloading in preparative chromatography, relatively little work on these effects has been published with particular reference to basic compounds in analytical RP chromatography. In another report, Snyder and coworkers discussed a theoretical model for prediction of overloading effects and included work on some basic solutes using an acidic phosphate buffer in conjunction with methanol [9]. A simplified description of this theory [10] and combination with the overload model proposed by Knox and Pyper [11] has also been presented. This model allows prediction of the complete overload profile of a column potentially from only two measurements of column efficiency made at low and high column loading. The aims of the present study can be summarised as follows:

(a) To investigate overloading effects on RP columns using a wider range of basic compounds than has been reported previously.

(b) To study the effect of overloading on k', the column efficiency (by measuring the plate number N), and also on peak asymmetry factor (A_s); the latter measurement of column performance has received very little attention. Also, to study briefly the effect of varying k' (measured for small sample mass) by means of adjusting modifier concentration, on overloading effects.

(c) To investigate loading effects with the same columns and the same basic solutes, using both ''high'' and ''low'' pH values (as determined by the generally accepted pH stability of such columns [2]).

(d) To investigate if significant differences existed in the sample capacity of columns assessed as giving ''excellent'' or only ''moderate'' performance with small amounts of the same basic analytes as used previously [2,3].

(e) To ascertain whether the results of this investigation fit the theoretical model proposed for prediction of the overload profile of these columns [10].

We hoped in addition that the results of the current investigation would provide practical recommendations for selection of the optimum sample mass for analysis of some important pharmaceuticals and other basic compounds. Finally, this study should provide guidelines for the proper conduct of column evaluation tests, so that variable overloading effects can be minimised.

2. Experimental

The HPLC system consisted of P200 pump, UV 100 detector (time constant 0.05 s, 5 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. N was determined from peak widths at half height ($w_{0.5}$) using the formula $N=5.54[t_r/w_{0.5}]^2$. For heavily overloaded peaks, we used the right angle triangle approximation and calculated column efficiency as shown in Ref. [10]. A_s was calculated at 10% of the peak height from the ratio of the widths of the rear and front sides of the peak; all measurements were made using a Model 2000 data station (Trivector, Bedford, UK). All results were the mean of at least duplicate injections. The columns used were Inertsil ODS-2, 25×0.46 cm I.D. (GL Sciences, Tokyo, Japan) and Kromasil C_{18}, 25×0.46 cm I.D. (Anachem, Luton, UK). Surface area, carbon loading and pore diameter

of these phases was recorded previously [2]. Columns were operated using a flow-rate of 1.0 cm^3 min^{-1}. All analyses were performed at 30°C with the column thermostatted in a block heater (Model 7980, Jones Chromatography, Hengoed, UK). Buffers were prepared by dissolving the appropriate quantity of KH_2PO_4 in pure water, and adjusting the pH with concentrated phosphoric acid (for pH 3.0 buffer) or KOH solution of the same molar concentration (for pH 7.0 buffer), in order to maintain $[K^+]$ constant. Buffer pH was measured before addition of the organic modifier. Injection of uracil using a mobile phase of methanol–water (55:45) was used to estimate column void volume. All analytes were obtained from Sigma–Aldrich (Poole, UK). Solutes were injected singly, rather than in mixtures and sufficient time was left between injections to allow complete elution of the solute from the column [2]. At least 150 column volumes were purged through before use with each new mobile phase.

3. Results and discussion

For this study, we selected two columns, Inertsil ODS-2 and Kromasil C_{18} from those well-characterised by our previous studies, in conjunction with some of the same basic analytes [2,3]. These studies indicated that the relative performance of a set of columns may vary with the pH used for the evaluation. From a set of eight columns selected by recommendation by their manufacturer as being especially suited for the analysis of basic compounds, Inertsil ODS-2 was found to give excellent performance for the set of probe compounds used with phosphate buffer at pH 3.0, while giving reasonable performance using pH 7.0 phosphate buffer. Kromasil C_{18} performed the least well of the eight columns both at pH 7.0 and 3.0; however, we believe that newer versions of this column material show improved performance with basic compounds. Furthermore, despite our use of a range of compounds with different structural features, it is conceivable that different results would be obtained using a different set of probe compounds. pH 3.0 and pH 7.0 were again chosen as the minimum and maximum values under which the columns showed reasonable long-term stability, at least when using phosphate buffers and relatively low temperatures [3].

3.1. Investigations of sample overloading at pH 3.0

The variation of column performance with sample mass w_x is shown in Fig. 1. For most compounds, sample mass injected ranged from 0.1 μg (log w_x = −1.0) to 20 μg or above (log w_x = 1.3 or greater). For pyridine, codeine, quinine, benzylamine and amphetamine, methanol–phosphate buffer pH 3.0 (30:70, v/v) was used as mobile phase; as previously, for nortriptyline methanol–phosphate buffer pH 3.0 (55:45, v/v) was used to compensate for the greater retention of this analyte [3]. The variation in efficiency is plotted as the ratio of the plate number divided by the maximum plate number. According to the work of Snyder et al. [9], maximum efficiency (N_0) should occur at lowest sample loading, thus a drop in N/N_0 is expected with increasing w_x. For five of the six compounds studied, this is indeed the case, and the shape of the graphs is very similar to previously reported results. Nevertheless, some analytes (e.g., codeine with Kromasil C_{18}) showed maximum efficiency around 0.5 μg of analyte (log w_x = −0.3) slightly above the lowest mass injected (0.1 μg). Also shown in our results is the variation of the ratio of the asymmetry factor to the minimum asymmetry factor, A_s/A_s(min). Although peak asymmetry has not been investigated in detail before in conjunction with overloading effects of basic compounds, it is clear, from Fig. 1, that in most cases increasing tailing in conjunction with decreasing efficiency occurs as sample mass is increased; the two curves thus form a crude trumpet shape. The results for pyridine, however, clearly depart from this pattern, especially when using Kromasil C_{18}. Here, maximum column efficiency was obtained for 1 μg of analyte (absolute value of N = 4700 plates, A_s = 3.56), with lower efficiency recorded at both lower and higher sample loading. The absolute values of A_s ranged from 7.0 to 0.84 with corresponding sample load w_x of 0.02 to 10 μg. Fig. 2 shows the peak shape obtained by injection of 5 μg of pyridine on to the Kromasil column. It is apparent that the peak shows simultaneous fronting and tailing; thus, great care is necessary in the consideration of asymmetry

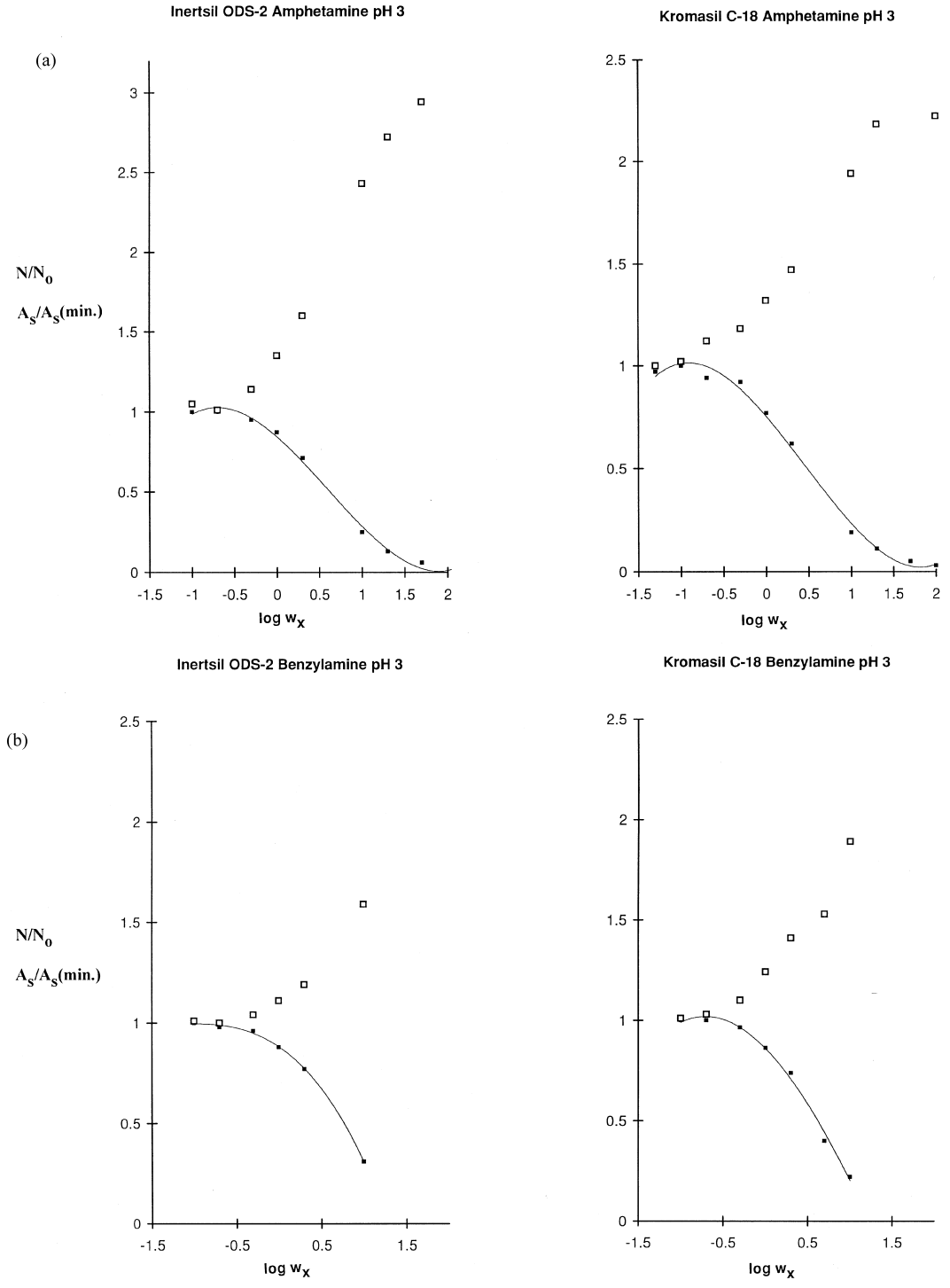

Fig. 1. Plots of N/N_0 (black squares) and A_s/A_s(min) (white squares) against log (sample mass, μg) for six basic solutes on two different ODS columns using methanol–0.0321 *M* phosphate buffer pH 3.0 (30:70, v/v) except for nortriptyline (methanol–0.05 *M* phosphate pH 3.0, 55:45, v/v). For other conditions, see Section 2.

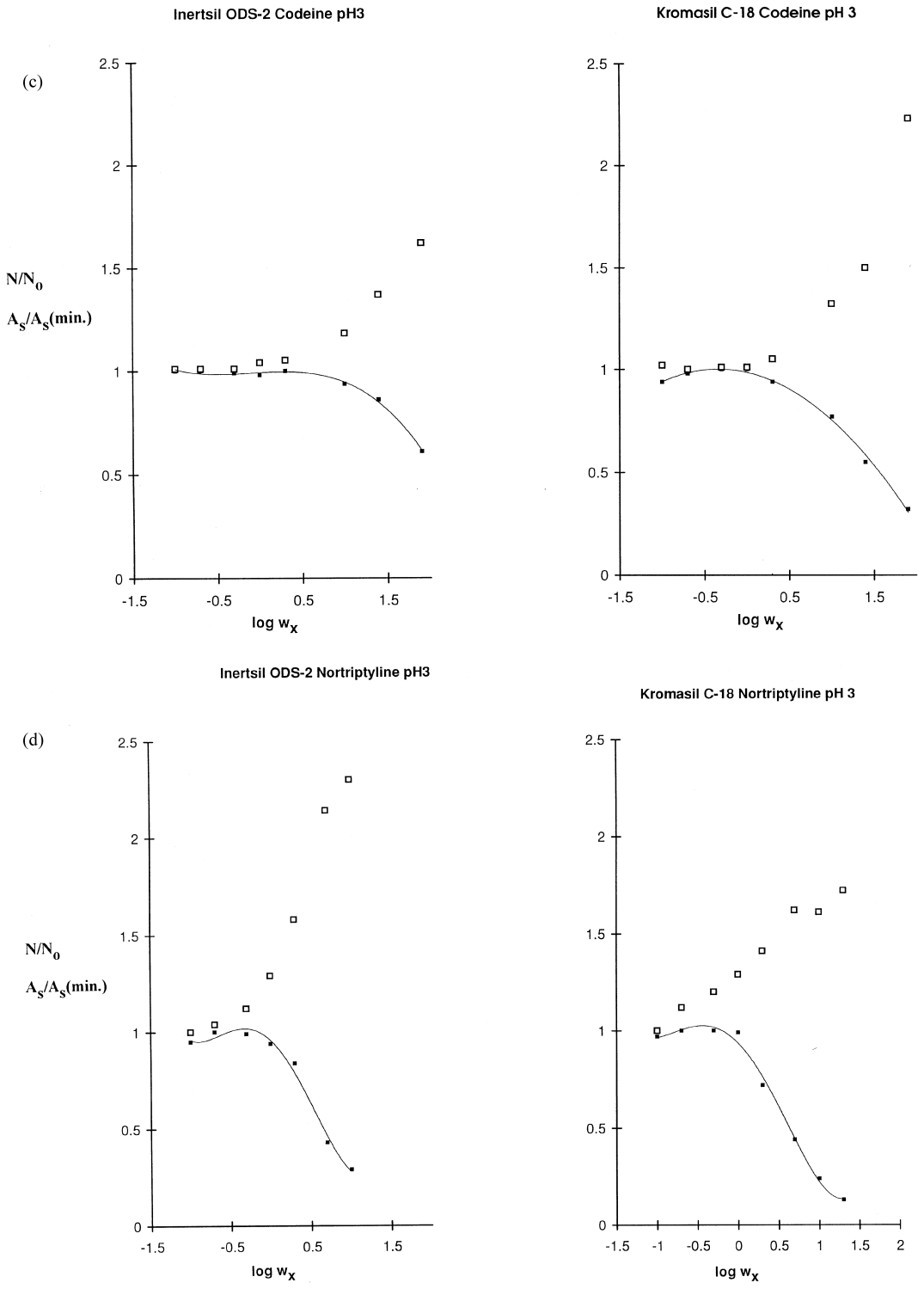

Fig. 1. (*continued*)

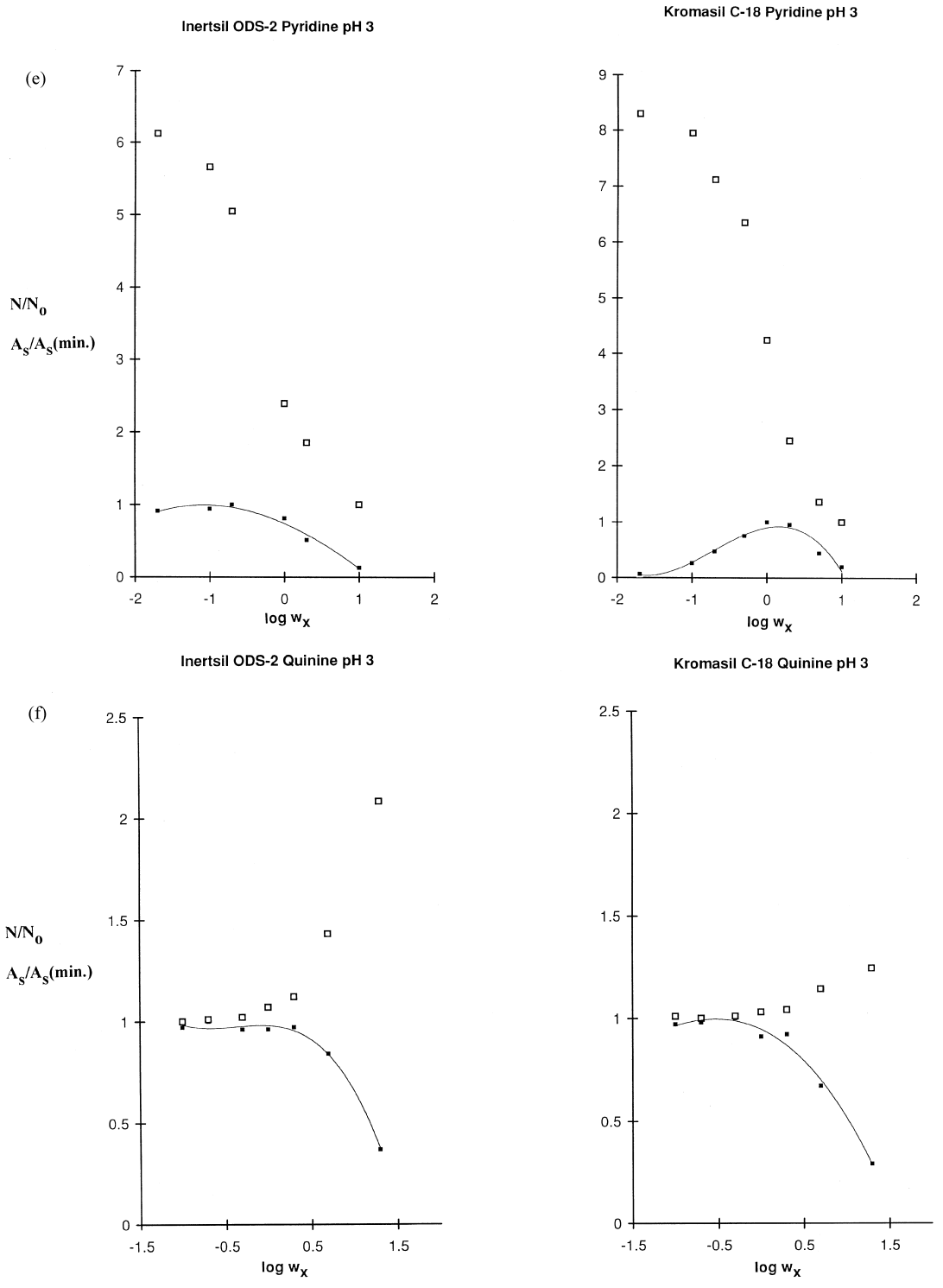

Fig. 1. (*continued*)

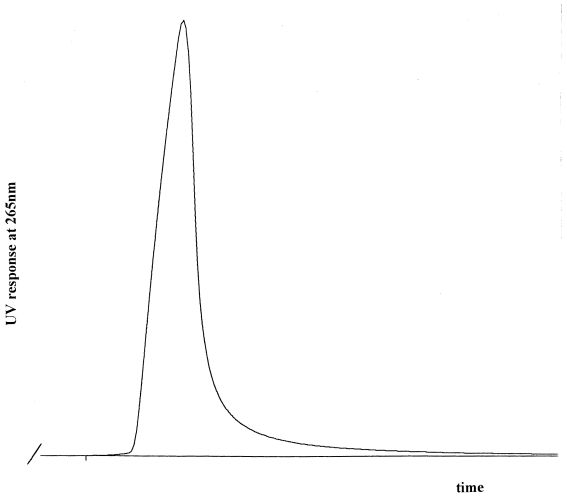

Fig. 2. Peak shape obtained by injection of 5 μg pyridine on Kromasil C_{18}. Mobile phase and other conditions as in Fig. 1.

factors of such peaks; we believe that N and A_s must both be quoted in assessment of peak shape since some peaks can give asymmetry factors close to 1.0 despite low efficiency. Inertsil ODS-2 gave rather similar behaviour with pyridine. Fig. 3 also shows plots for amphetamine on both columns of k'/k_0 (k_0 is the value of k' for a small solute mass) against the logarithm of $N_0^{0.5}[(k_0/(1+k_0)]w_x$ (note this can be regarded as a graph of k'/k_0 against log Cw_x, where C is a constant). Similar plots were obtained for codeine, quinine, benzylamine and nortriptyline, where retention time was found to decrease with sample loading as shown previously [9]. Fig. 3 shows a similar plot for pyridine on Kromasil C_{18}. In this case, unusually, retention increases with sample loading; similar results were obtained for pyridine on Inertsil ODS-2. It is possible that while five of the solutes exhibit behaviour that closely resembles the Langmuir isotherm [9], pyridine may show anti-Langmuir behaviour. This latter behaviour arises when interactions between solute molecules are relatively strong. As the amount of solute injected increases, these solute–solute interactions draw further molecules into the stationary phase, giving an increase in k' with concentration [12]. Anti-Langmuir behaviour is expected to give fronting peaks if the solute concentration is high enough. However, an alternative explanation is that slow kinetics in solute sorption–desorption may be involved [13], or that

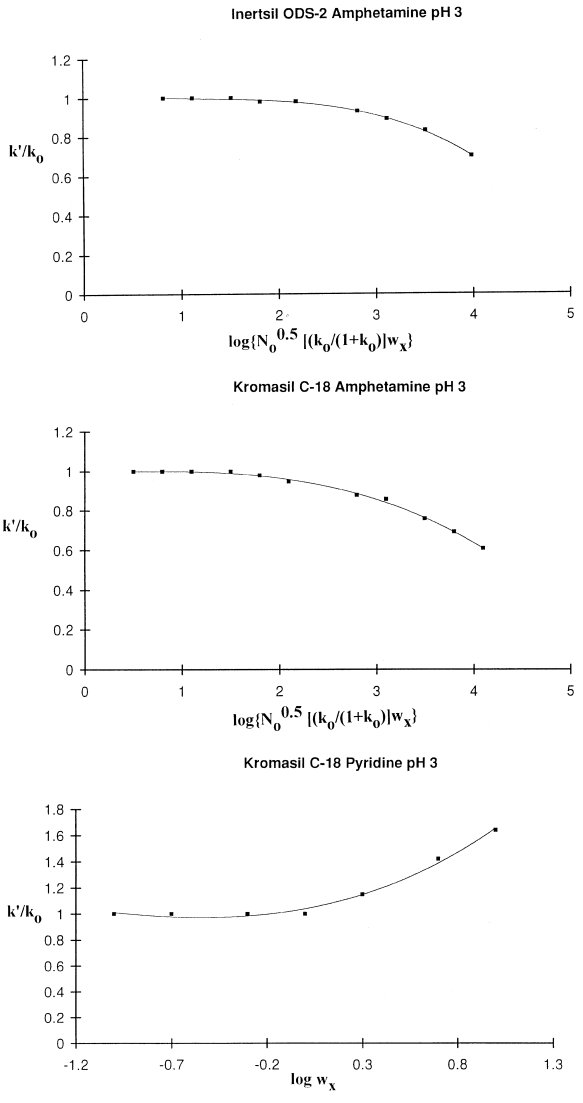

Fig. 3. (a) and (b) Plots of k'/k_0 against log $\{N_0^{0.5}[(k_0/(1+k_0)]w_x\}$ for amphetamine on Inertsil ODS-2 and Kromasil C_{18}; (c) plot of k'/k_0 against log w_x for pyridine. Mobile phase and other conditions as in Fig. 1.

the peak shape shown is a result of a combination of these factors.

The data shown in Figs. 1 and 3 can be used to estimate the column saturation capacity w_s, (in mg) which is the maximum column loading at large values of solute concentration in the mobile phase. The calculation of w_s is discussed in detail by Snyder and coworkers [9]. From Fig. 3, w_s can be

obtained from the decrease in k' with sample loading by comparison with published ''master curves''. The values of w_s for amphetamine calculated in this way were 4.6 mg for Inertsil ODS-2 and 2.3 mg for Kromasil C_{18}. However, as previously noted, the reduction in k' with sample loading occurs much less readily than the drop in N. The reduction in k' for injection of 0.1–25 μg of codeine on Kromasil C_{18} was less than 5%, whereas the simultaneous reduction in N was of the order of 50% over this range. Therefore, determination of w_s through reduction in k' values may demand high concentrations of injected solutes, which caused some problems of analyte solubility, especially since low injected volumes were used in the present study to avoid effects of extra-column band broadening. Thus we preferred to determine w_s through reduction in N values. This can be performed again by comparison with ''master curves'' [9] or by using the combination theory approach [10], where potentially, only two runs, a small sample run and a mass overloaded run where $N/N_0 < 0.5$ are necessary to predict column overload. In this latter approach, the equation

$$N/N_0 = 1/[1 + (3/8)w_{xn}] \quad (1)$$

was proposed to predict the variation in N with sample loading, where w_{xn}, the loading function for a given plate number as a function of sample size and conditions is given by

$$w_{xn} = N_0[(k_0/(1 + k_0)]^2(w_x/w_s) \quad (2)$$

We calculated the value of w_{xn} for sample masses where the measured values of N/N_0 had fallen typically to around 0.25 using Eq. (1). Using this value of w_{xn} and the appropriate sample mass, the value of w_s can be calculated using Eq. (2). Table 1 displays values of w_s calculated in this way. For amphetamine, the tabulated values of w_s are 5.4 mg and 3.5 mg on Inertsil ODS-2 and Kromasil C_{18}, respectively, which compare reasonably with those reported above for this compound using Fig. 3 and

Table 1
Retention factor, column saturation capacity, predicted and actual column efficiency using pH 3.0 buffer

Compound	k'	w_s (mg)	50% N (μg) (predicted)	50% N (μg) (actual)	90% N (μg) (predicted)	90% N (μg) (actual)
Inertsil ODS-2						
Pyridine	0.02	–	–	–	–	–
Amphetamine	1.25	5.4	3.3	4.2	0.4	0.7
Quinine	3.81	32	12	15	1.3	3.5
Benzylamine	0.27	1.2	5.6	5.7	0.6	0.9
Codeine	0.26	–	–	–	–	18
Nortriptyline[b]	5.10	15	4.1	4.2	0.5	1.3
Benzylamine[d]	0.92	2.6	2.1	3.7	0.2	0.6
Nortriptyline[a]	1.61	14	9	10	1.0	2.4
Nortriptyline[c]	14.7	16	2.7	3.3	0.3	0.8
Kromasil C_{18}						
Pyridine	0.12	–	–	–	–	–
Amphetamine	2.08	3.5	2.4	3.0	0.3	0.5
Quinine	6.41	11	8.2	10	0.9	1.5
Benzylamine	0.44	1.2	2.8	4.1	0.3	0.8
Codeine	0.44	8.8	31	38	3.5	3.2
Nortriptyline[b]	7.75	8.3	3.2	4.3	0.4	1.1

Mobile phase methanol–0.032 *M* phosphate buffer pH 3.0 (30:70, v/v) except:
[a] 65:35 methanol–0.064 *M* phosphate pH 3.0.
[b] 55:45 methanol–0.050 *M* phosphate pH 3.0.
[c] 45:55 methanol–0.041 *M* phosphate pH 3.0.
[d] 15:85 methanol–0.026 *M* phosphate pH 3.0.
Values of w_s were determined using the ''combination theory'' approach; see Section 3.1 and Ref. [10].

the alternative ''master curves'' approach [9]. Using Table 1 values of w_s (combination theory approach) it is possible to predict N for any value of the sample loading. Table 1 compares the sample mass predicted to give 10% and 50% loss in column efficiency with the actual values derived from Fig. 1. Results are not given for pyridine due to its anomalous behaviour. Furthermore, it was not possible to determine the mass required to give 50% loss of efficiency for codeine on Inertsil ODS-2 due to the high sample concentration required which gave solubility problems. It can be seen that the agreement between actual and predicted values is reasonable, although in most cases, loss of efficiency is somewhat less extreme than predicted. Clearly, there are large differences in the saturation capacity of a given column for different basic solutes. Thus, while k' for benzylamine and codeine are virtually the same on Inertsil ODS-2, it takes about 20 times as much sample to produce a 10% reduction in column efficiency for codeine than benzylamine on this column. Values of w_s ranged from 1–32 mg using Inertsil ODS-2; these can be compared with the value of 1 mg for angiotensin II, and over 60 mg for benzyl alcohol reported previously for Zorbax ODS [9]. It is possible that the range of w_s for basic compounds thus extends from small values right up to those normally found for neutral compounds. Comparison of w_s for Inertsil ODS-2 and Kromasil C_{18} shows that the latter column gives generally smaller values. The average column performance for 0.2 μg injections of eight basic solutes reported previously [3] using the same mobile phases (average $N=9100$, $A_s=2.40$) for Kromasil C_{18} was worse than for Inertsil ODS-2 (average $N=11\,400$, $A_s=1.58$). However, Table 1 and Fig. 1 indicate that use of 0.2 μg of solute produces no measurable loss in column efficiency for the solutes studied in our previous work and that sample overloading has not influenced the results of our column comparisons. Thus, it appears that at least in this case, the differences in column performance are not due to overloading effects. Table 1 indicates broad agreement with results of Snyder and coworkers [6] who proposed that overloading would occur on standard columns when the injected mass exceeded 0.5 μg. Nevertheless, it does indicate the necessity of restricting sample mass in column comparisons, and that the values used by some other workers are probably too high. The confirmation of this result would also imply that overloading of basic compounds in RP chromatography at low pH is a much more serious problem than generally thought, especially when considering that 0.5 μg corresponds to the injection of a solution of concentration only 25 mg l^{-1} if 20 μl of sample is injected, as is often the case.

As indicated by theory above, sample overloading should occur more readily at high k_0 than low k_0, since w_{xn} increases with k_0. This is demonstrated experimentally in Table 2 which shows the variation in N/N_0 with sample mass for nortriptyline, using

Table 2
Effect of solvent strength on overloading of Inertsil ODS-2 using nortriptyline as solute

Sample mass (μg)	MeOH–buffer (65:35)[a] ($k_0'=1.6$)		MeOH–buffer (55:45)[b] ($k_0'=5.1$)		MeOH–buffer (45:55)[c] ($k_0'=14.7$)	
	N/N_0	A_s	N/N_0	A_s	N/N_0	A_s
0.1	1.0	1.21	0.94	1.13	0.93	1.13
0.2	1.0	1.20	1.0	1.18	1.0	1.11
0.5	1.0	1.25	0.99	1.27	0.92	1.36
1.0	0.99	1.32	0.94	1.46	0.86	1.56
2.0	0.90	1.44	0.84	1.79	0.73	2.01
5.0	0.75	1.78	0.59	2.42	–[d]	–[d]
10	0.57	2.01	0.29	2.60	0.21	2.94

[a] Mobile phase: methanol–0.064 *M* phosphate buffer pH 3.0 (65:35, v/v).
[b] Mobile phase: methanol–0.050 *M* phosphate buffer pH 3.0 (55:45, v/v).
[c] Mobile phase: methanol–0.041 *M* phosphate buffer pH 3.0 (45:55, v/v).
[d] Data point not obtained.

mobile phases containing different concentrations of methanol yielding k_0 values from 1.6 to 14.7. Thus 10 μg of nortriptyline gives a drop in efficiency to about 60% of its small mass value with methanol–buffer (65:35). However, the same mass of solute causes a drop to about 30% with methanol–buffer (55:45) and only about 20% with methanol–buffer (45:55). Similar results were obtained with benzylamine as shown in Table 3. It would be useful to apply values of w_s calculated from experiments using a given mobile phase to predict overloading effects at for example, higher k' values generated by use of mobile phases with a reduced organic modifier content. For example, if a compound such as benzylamine with $w_s = 1$ mg had $k' = 10$, a 10% loss in efficiency would be predicted by theory if the sample mass was only 0.025 μg. It was shown previously [9] that values of w_s were approximately constant for benzyl alcohol when determined with acidic phosphate buffers modified with concentrations of between 20–40% methanol or acetonitrile. Nevertheless, w_s can change for basic compounds dependent on mobile phase additives or pH [10], and it should be noted that changing the modifier concentration can alter the effective pH of the buffer and the ionisation state of the solute, even if the pH of the aqueous component before modifier addition is the same [4,5]. Table 1 shows some calculated results for nortriptyline and benzylamine with different modifier concentrations (based on the experimental data in Fig. 1 and Tables 2 and 3), using Inertsil ODS-2. The change in column loadability with solvent strength and k_0 is again illustrated. While 10% loss in N occurs with 0.8 μg of nortriptyline using methanol–buffer (45:55, v/v), three times this amount can be injected before similar loss in efficiency occurs for methanol–buffer (65:35, v/v). Whereas w_s calculated for nortriptyline is very similar for modifier concentrations of between 45–65% methanol; Table 1), there is some variation in the result for benzylamine when using 15% instead of 30% methanol giving values of 2.6 mg and 1.2 mg, respectively. However, this result may reflect the greater proportional change in modifier concentration employed compared with nortriptyline. Thus, there may be some difficulty in using values of w_s to predict overload using mobile phases other than that used for its determination. Nevertheless, it is possible that for some analytes with low column saturation capacities when using mobile phases generating high k' values, some overloading may occur even using masses of 0.2 μg even if values predicted by theory appear to be somewhat pessimistic especially at the 10% efficiency loss level. Thus, it is a recommendation of the present study that careful checks should be performed in column evaluation studies at pH 3.0 to ensure that no significant gain in column efficiency is obtained by decreasing sample mass.

3.2. Investigations of sample overloading at pH 7.0

Low pH is generally preferred for the analysis of basic compounds due to better peak shapes which are usually obtained [3,14,15]. However, in some cases, useful selectivity differences in separation may result at pH 7.0; in addition, the retention of bases at pH 3.0 may be undesirably low. Furthermore, for some

Table 3
Effect of mobile phase strength on overloading of Inertsil ODS-2 using benzylamine as solute

Sample mass (μg)	MeOH–buffer (30:70)[a] ($k'_0 = 0.27$)		MeOH–buffer (15:85)[b] ($k'_0 = 0.92$)	
	N/N_0	A_s	N/N_0	A_s
0.1	1.0	1.45	1.0	1.30
0.2	0.98	1.45	0.97	1.34
0.5	0.95	1.51	0.93	1.47
1.0	0.88	1.61	0.82	1.60
2.0	0.76	1.73	0.67	1.87
10	0.31	2.31	0.17	2.86

[a] Mobile phase: methanol–0.064 *M* phosphate buffer pH 3.0 (30:70, v/v).
[b] Mobile phase: methanol–0.050 *M* phosphate buffer pH 3.0 (15:85, v/v).

bases which are unprotonated at pH 7.0, peak shapes may be better than at pH 3.0 [3]. Thus, further study of overloading effects at pH 7.0 is of interest. Injection of the same probe compounds over a similar loading range was repeated at pH 7.0, using adjustment in the mobile phase strength to compensate for the increased retention at higher pH. In a previous study using an Inertsil ODS column (which has different specification to the column studied here [4]), we showed that for injection of pyridine over the range 0.05–3.5 μg, efficiency remained approximately constant but asymmetry factor increased gradually with sample mass, either when using unbuffered methanol–water or methanol–phosphate buffer pH 7.0 as the mobile phase. The results of the present study are displayed in Fig. 4. Maximum values of N at pH 7.0 (obtained in some cases at high loading) were significantly lower for most solutes than maximum values at pH 3.0 (obtained with small sample masses), although pyridine and codeine showed comparable efficiency at pH 7.0 and 3.0 on each individual column. In general, the improved performance at pH 3.0 can be attributed to the non-dissociated state of (most) silanol groups, which reduces ion-exchange interactions with protonated bases. Of the compounds examined, pyridine has the lowest pK_a (5.2) and is therefore largely unprotonated at pH 7.0 unlike the stronger bases studied, again reducing ion-exchange and giving with some columns, superior peak shape to that at pH 3.0 [2,3]. The presence of relatively large concentrations of organic solvent can seriously affect the ionisation of both buffer and basic compounds [4,5]; it is necessary to use pH 3.2 phosphate buffer in a mixture with 55% methanol in order to half protonate pyridine, and in the present study a higher concentration of methanol (65%) was employed. Thus, results for overloading studies for pyridine should be considered with this factor in mind. All other compounds have a pK_a of 8.0 and above with values of 9.9 and 10.0 for amphetamine and nortriptyline, respectively [2]. Despite the presence of the organic solvent, these compounds are expected to be at least partially protonated at pH 7.0. Efficiency for some solutes on Kromasil C_{18} at pH 7.0 was particularly low, giving maximum N of about 200 plates for benzylamine and 300 plates for nortriptyline, in agreement with results obtained previously and consistent with strong interaction between ionised silanols and the protonated base. In comparison, on the same column at pH 3.0, maximum efficiency for the same solutes was 11 600 and 8900 plates, respectively. The efficiency on Kromasil C_{18} at pH 7.0 was too low to allow reliable determination of plate count and asymmetry for very small sample masses of benzylamine, nortriptyline and amphetamine, due to poor signal-to-noise ratio, thus some data points are missing in Fig. 4.

A comparison of Figs. 1 and 4 shows clearly that loading behaviour at pH 7.0 is very different from pH 3.0 using a comparable range of sample masses. Considering Inertsil ODS-2, efficiency remained approximately constant in some cases (e.g., for codeine and quinine) up to levels of at least 10 μg of solute. In other cases, efficiency increased steadily with sample amount up to or even beyond 10 μg of analyte (e.g., for benzylamine, nortriptyline and amphetamine). For pyridine, there is some evidence of deterioration of peak shape above 10 μg of analyte. The generally superior performance at acid pH is again demonstrated by results for amphetamine: 0.2 μg on the particular Inertsil ODS-2 column utilised in this study gave about 3500 theoretical plates with methanol–phosphate buffer pH 7.0, (comparable with the result obtained previously [2]) but 14 000 plates using pH 3.0 buffer. Table 4 shows the effect of increasing sample mass on these initial values. However, on increasing the mass to 10 μg, efficiency at pH 3.0 dropped to about 3500 plates, while at pH 7.0, efficiency increased to 5400 plates thus giving superior performance to that at pH 3.0. There was evidence of peak distortion (simultaneous fronting and tailing giving an apparent improvement in A_s) with 20 μg of amphetamine at pH 7.0. This unexpected superior efficiency at pH 7.0 at high sample loading was shown for some other probes. Thus, while pH 3.0 is generally recommended for the analysis of small quantities of basic analytes, the more rapid overloading of the column at pH 3.0 may destroy this advantage. For pyridine, where the performance is exceptionally, equivalent or improved at pH 7.0 compared with pH 3.0 even for small injected mass, increasing sample mass leads to considerably better results at pH 7.0 than pH 3.0. For Inertsil ODS-2, 10 μg of pyridine gave only about 900 plates using pH 3.0 buffer, whereas pH

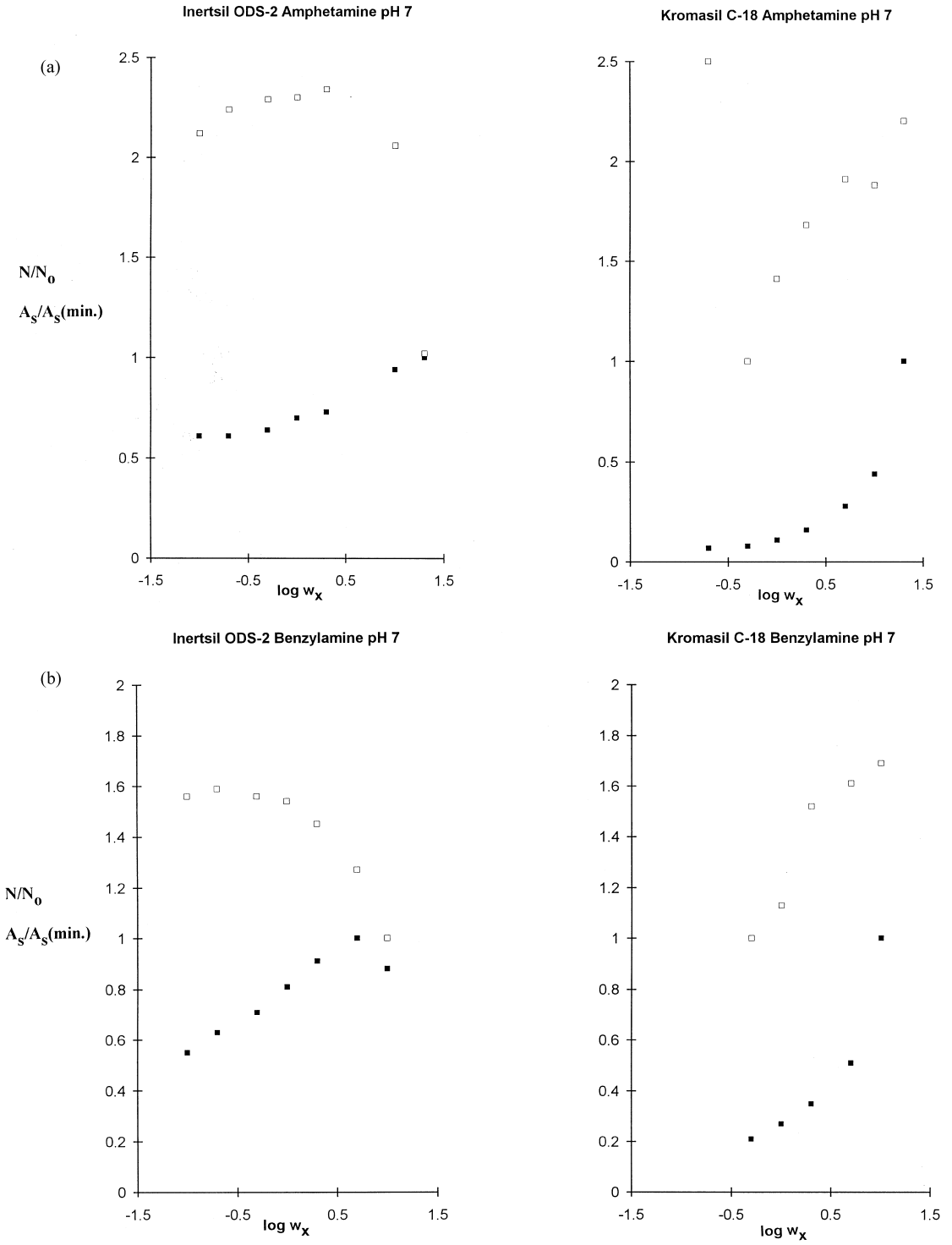

Fig. 4. Plots of N/N_0 (black squares) and A_s/A_s(min) (white squares) against log w_x for six basic solutes on two different ODS columns using methanol–0.064 *M* phosphate buffer pH 7.0 (65:35, v/v).

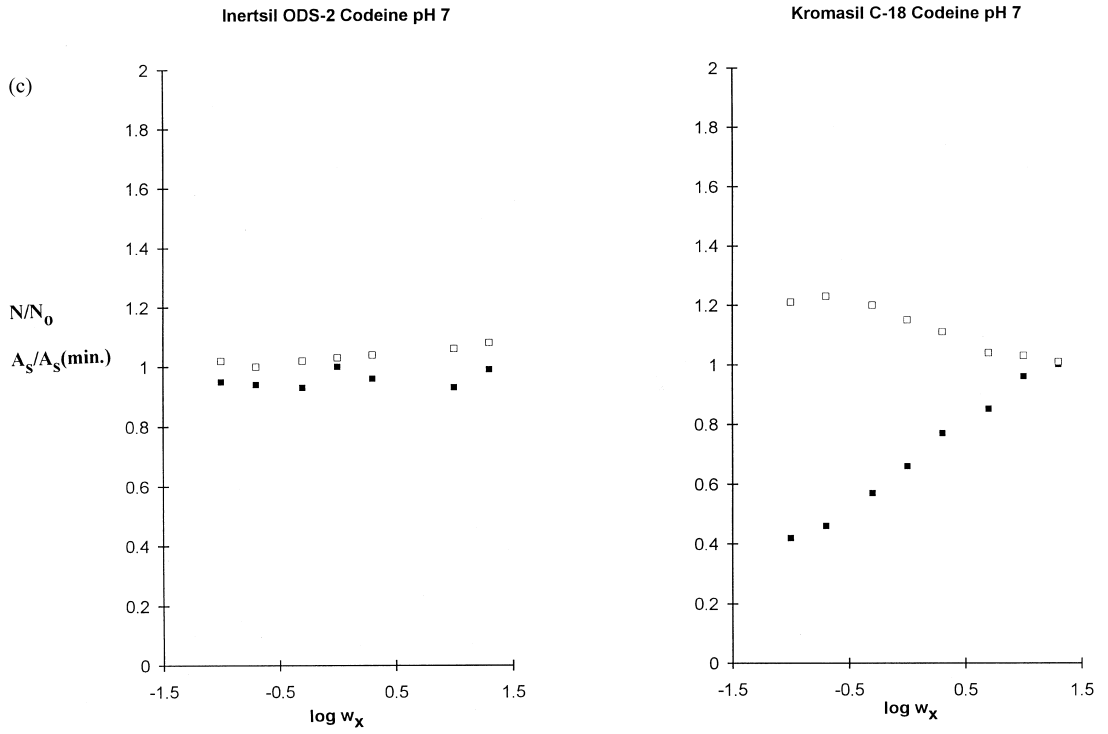

Fig. 4. (*continued*)

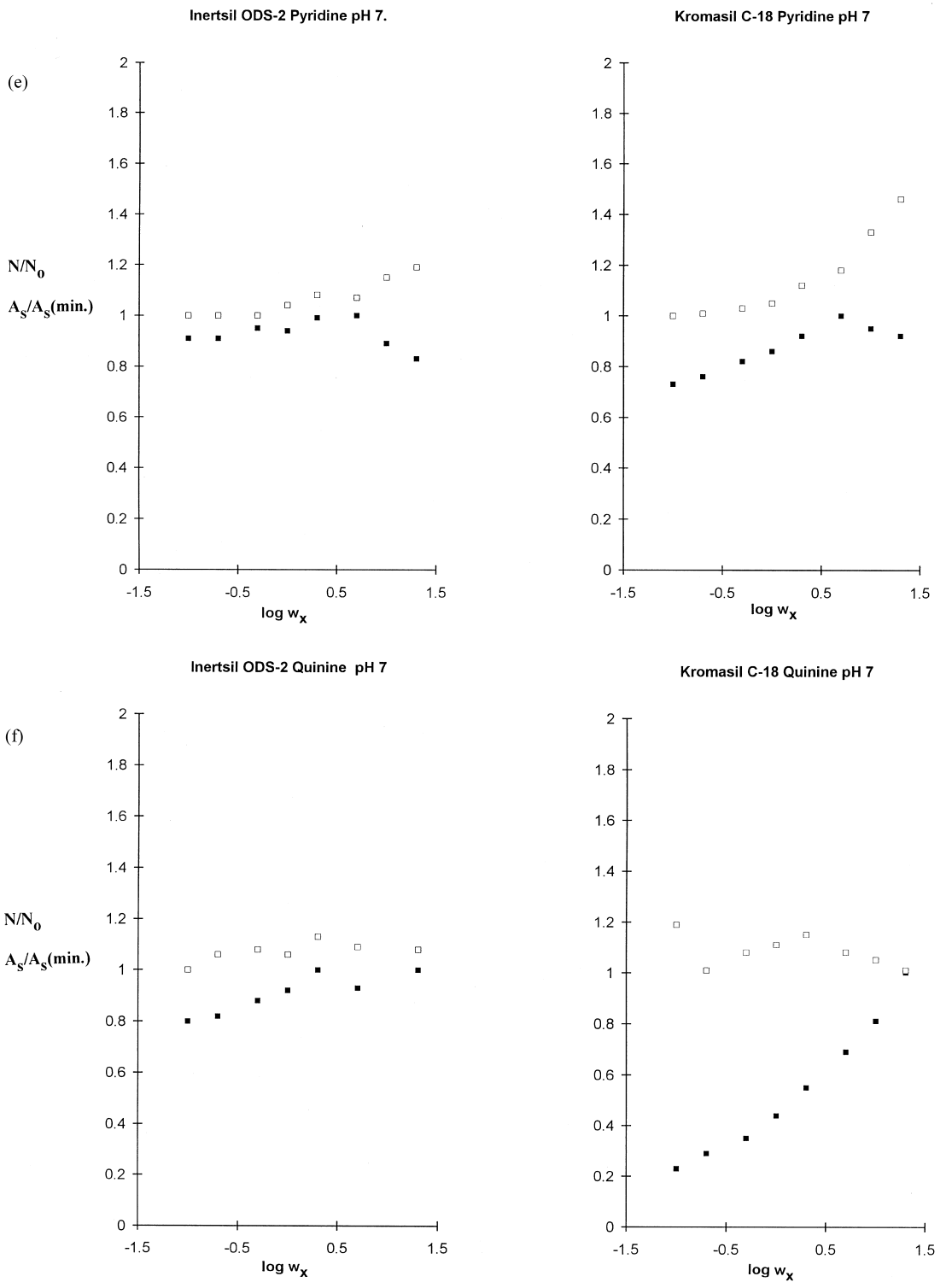

Fig. 4. (*continued*)

Table 4
Effect of sample mass on column performance for amphetamine using Inertsil ODS-2 at pH 3.0 and pH 7.0

Sample mass (μg)	pH 3.0[a]		pH 7.0[b]	
	N	A_s	N	A_s
0.1	14 000	1.36	3530	2.65
0.2	14 000	1.30	3570	2.80
0.5	13 400	1.48	3720	2.86
1.0	12 100	1.76	4050	2.87
2.0	9930	2.08	4260	2.92
10	3470	3.16	5430	2.57
20	1790	3.54	5810	1.25
50	841	3.82	–[c]	–[c]

[a] Mobile phase: methanol–0.0321 *M* phosphate buffer pH 3.0 (30:70, v/v).
[b] Mobile phase: methanol–0.0643 *M* phosphate buffer pH 7.0 (65:35, v/v).
[c] Data point not obtained.

7.0 on the same column gave nearly 6000 plates. This finding can be compared with 7200 plates and 5700 plates, respectively for 0.2 μg of this compound. This result could have some significance when considering the preparative separations of basic compounds under overload conditions. In general, for Kromasil C_{18}, the apparent improvement in column efficiency with sample loading is considerably more marked and this occurs also for codeine and quinine. For example, 20 μg of quinine yielded 1680 plates at pH 3.0 but 6830 plates at pH 7.0, whereas with small sample mass, performance at pH 3.0 was superior, as expected. This greater variation in peak shape at pH 7.0 for Kromasil C_{18} may be a consequence of the greater inertness towards bases shown by Inertsil ODS-2.

Using pH 3.0 buffer, it is likely that only a small proportion of silanols of unusually low pK_a remain dissociated; these are easily overloaded by small masses of basic analytes. However, at pH 7.0, a much larger number of the silanols on the column will be dissociated, thus it might be expected that overloading would occur much less readily than at pH 3.0 on the same column. It is difficult to explain the apparent improvement in N with sample mass that occurs in some cases. Nevertheless, if overloading effects are less important at pH 7.0 due to more ionised silanols, kinetic effects [13] are likely to be relatively more important. If kinetic tailing occurs for a small number of retention sites (and a small fraction of the solute molecules) then very bad tailing is predicted for a small sample mass. As sample size is increased, a larger fraction of the solute will be retained by non-silanol interactions and this part of the sample would elute with good efficiency. As the latter molecules become relatively more important (with increasing sample mass), the composite curve might give an improvement in N, especially when measured by the half-height method. However, pronounced tailing should still be evident in the composite curve.

We did not investigate the loading behaviour at pH 7.0 using masses greater than 20 μg. As mentioned previously, solubility problems were encountered in some cases due to the small injection volumes utilised. Furthermore, the principal aims of the present study were to address potential overloading problems in analytical scale, rather than preparative chromatography.

4. Conclusions

Overloading of ODS columns by basic solutes using methanolic mobile phases buffered at acid pH has been shown to follow the general pattern predicted by Snyder and coworkers [6,9,10]. Column efficiency is highest with small injected sample mass, but deteriorates above about 0.5 μg of injected solute. The rate of deterioration however, varies with the solute, and a range of values of column saturation capacity was obtained from 1 mg up to values which may approach those for neutral solutes. Overloading profiles can be predicted, using two injections, of small and large sample mass, from the model proposed. However, pyridine shows anomalous behaviour, which could be due to non-Langmuir adsorption of this compound, or to complex kinetic effects.

Values of w_s may change for basic compounds with mobile phase composition, due for example to the variation in ionisation of the buffer and solute with organic modifier concentration. Nevertheless, reduction in sample capacity as k' is increased by reducing the concentration of organic modifier was demonstrated.

Overloading takes place much less readily using equivalent mobile phases buffered at pH 7.0, rather

than at pH 3.0. This is probably due to the larger number of dissociated silanols which can undertake ion-exchange with the protonated bases. Peak shape can gradually improve (albeit from initially poor results) in some cases at pH 7.0 by injection of sample masses increasing up to at least 20 μg. Thus, while for small amounts of solute, pH 3.0 usually gives considerably better peak shapes for basic compounds, for larger sample masses, pH 7.0 may give superior results for some solutes.

It has been shown that overloading has not influenced the results of either of our previous comparative evaluations of column performance; therefore the difference in performance of individual columns for a given basic solute is not attributable to overloading effects. However, it appears that great care is necessary in comparative evaluation studies at pH 3.0; for exceptional solutes of low w_s under conditions giving high k' it is conceivable that sample masses even below the 0.2 μg used in our studies may produce variable overloading effects dependent on sample k'. Finally, particular care is necessary in comparison of results obtained for pyridine at pH 3.0, due to the considerable and unexpected variation in peak shape which can occur with sample mass.

References

[1] J. Nawrocki, J. Chromatogr. A 779 (1997) 29.
[2] D.V. McCalley, J. Chromatogr. A 738 (1996) 169.
[3] D.V. McCalley, J. Chromatogr. A 769 (1997) 169.
[4] D.V. McCalley, J. Chromatogr. A 664 (1994) 139.
[5] D.V. McCalley, J. Chromatogr. A 708 (1995) 185.
[6] D. Chan Leach, M.A. Stadalius, J.S. Berus, L.R. Snyder, LC·GC Int. 1 (1988) 22.
[7] R.J. Vervoort, M.W. Derksen, A.J. Debets, J. Chromatogr. A 765 (1997) 157.
[8] E. Cruz, M.R. Euerby, C.M. Johnson, C.A. Hackett, Chromatographia 44 (1997) 151.
[9] J.E. Eble, R.L. Grob, P.E. Antle, L.R. Snyder, J. Chromatogr. 384 (1987) 45.
[10] L.R. Snyder, G.B. Cox, P.E. Antle, Chromatographia 24 (1987) 82.
[11] J.H. Knox, H.M. Pyper, J. Chromatogr. 363 (1986) 1.
[12] P.A. Sewell, B. Clarke, Chromatographic Separations, Wiley, Chichester, 1987.
[13] T. Fornstedt, G. Zhong, G. Guiochon, J. Chromatogr. A 741 (1996) 1.
[14] L.R. Snyder, J.L. Glajch, J.J. Kirkland, Practical HPLC Method Development, Wiley, New York, 1988.
[15] D.V. McCalley, J. Chromatogr. 636 (1993) 213.