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A comparison of overload behaviour for some sub 2 μm totally porous and sub 3 μm shell particle columns with ionised solutes

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ABSTRACT

The overloading performance of some 2.7 μm shell and sub 2 μm totally porous columns, including one pair manufactured from similar materials with similar bonding chemistries, was compared using strongly acidic and basic probe compounds. In general, the capacity of shell particles was not greatly reduced, despite containing a smaller porous volume. Nevertheless, at low pH, both types of column were overloaded by only small concentrations of ionised solute. Considerable improvement could be gained by increasing the buffer concentration, although sensitivity in mass spectrometric detection may be compromised. The capacity of columns of different internal diameter may not be directly compared merely by scaling the injection volumes, as it is possible that the sample is not homogeneously distributed across the column radius, especially in larger diameter columns, where the sample may travel preferentially through a central core of the packing. A totally porous charged surface hybrid phase gave much improved loading properties of the basic probe in low ionic strength mobile phases such as formic acid, often used in mass spectrometry. However, its relative advantage over conventional phases was reduced as the mobile phase ionic strength was increased. Furthermore, acidic compounds may give tailing on this phase. At pH 7, all columns tested showed evidence of interaction with ionised silanols; peak shapes improved as the buffer concentration was increased. Column efficiency first increased and then decreased as solute concentration was increased at constant buffer concentration, which can be attributed to the decreasing proportion of solute molecules retained by the ion exchange process.

1. Introduction

Superficially porous or "shell" columns of diameters <3 μm have attracted considerable attention since the publication of the pioneering study by Kirkland et al. [1]. While the concept of so-called pellicular particles, in which a porous layer of separation medium is coated on a non-porous core has been utilised in LC separations for over 40 years [2], the newly developed material ("Halo") had in addition to a much smaller particle diameter (d_p = 2.7 μm), a value of ρ, the ratio of the diameter of the solid core divided by the diameter of the whole particle of ~0.63. This value compares with a figure close to 1.0 for the original 50 μm pellicular particles. Thus, these new particles could be regarded as having a thick shell, as compared with the thin shells of the earlier particle design. The advantage of these new shell columns is that they can give efficiencies very similar to that of sub 2 μm totally porous particles while generating pressures of around half or less [3]. The smaller back pressures result from the pressure drop on the column being inversely proportional to the square of the particle diameter [4]. These high efficiency columns offer considerable potential for analysis of complex samples in biomedicine, or for rapid separations of pharmaceuticals.

Gritti and Guiochon have explored in detail the reasons for the high efficiencies of shell particle columns [5]. The *A* term describing eddy diffusion in van Deemter type equations used to model the effect of various parameters on column efficiency is apparently reduced by as much as 40% compared with totally porous particles of similar dimensions [6]. Much interest has centred over whether this reduction is due to the narrow particle size distribution of shell particles or whether it is due to better packing properties, perhaps caused by the roughness of the particles, causing them to move less against each other [6–8]. This restriction may cause the distribution of particles from the centre of the column to the walls to be more homogeneous than that obtained with classical porous particles. As 50% or more of the reduced plate height of these columns is contributed by the *A* term [6], clearly its smaller value in shell particle columns is a major factor in their good performance. The *B* term in shell particles is reduced by 20–30% or more compared with totally porous particles [9–11] but the decrease in overall reduced plate height may be only ~10%. The contribution of the *C* term to overall

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plate height for these very small particle columns is very low and therefore is not a major factor, at least not for small molecules, contrary to original suppositions concerning the performance of shell particles. Mass transfer may however be more important when higher molecular weight solutes with lower mobile phase diffusion coefficients are used. Furthermore, heat friction effects, which can cause a drastic increase in plate height at high mobile phase velocity are reduced in shell and totally porous particles of the same size, due to the greater heat conductivity of the solid core [3,12]. Clearly, when comparing sub 2 μm totally porous with shell particles of larger diameter, heat friction effects are more important for the smaller particles, due to the higher power generated by the higher back pressure [3,9,13–15]. Besides being influenced by pressure and flow, heat friction effects are also influenced when high concentrations of acetonitrile are used in the mobile phase, resulting in its lower thermal conductivity and thus reduced opportunity for heat dissipation.

Shell columns would appear to have few drawbacks. A possible disadvantage is that only a proportion of the particle volume is porous, potentially leading to poor sample capacity. Simple geometry shows that the porous volume of the Halo particle with core diameter of 1.7 μm and overall particle diameter 2.7 μm represents ~75% of its total volume if the particles are regarded as perfect spheres, or 61% for another popular variety of these particles (Kinetex, core diameter 1.9 μm, overall diameter 2.6 μm). Nevertheless, concerns still remain about the capacity of materials that are partially composed of an impervious core, and simple calculations may not reflect other differences in particle morphology between totally and partially porous packings. Overloading is of great concern for ionised compounds, which may exhibit reduction in column efficiency with solute loads up to 100 times smaller than for neutrals [16–20]. The problem is compounded by the extremely high efficiency of both sub 2 μm porous and sub 3 μm shell columns, which results in narrow bands and thus elevated sample concentration in a given volume of packing material. Therefore, it was found to be barely possible to achieve maximum efficiency for fully ionised acidic and basic compounds on these columns with 20 mM ammonium formate or phosphate buffers at pH 3.0 (conditions typical for their analysis) at sufficiently low levels which still permitted their determination with reasonable signal/noise on UV detectors [3]. With low ionic strength formic acid solutions (0.1%, v/v), favoured by mass spectroscopists due to superior detection properties, it proved impossible to use low enough concentrations of strong acids and bases such that no overload occurred but that detection was still unproblematic. This previous study compared overload between a shell (Phenomenex Kinetex C18) and a totally porous column (Waters BEH C18) from different manufacturers and of different dimensions (0.46 cm and 0.21 cm, respectively). At the time the study was performed, sub 2 μm totally porous and sub 3 μm shell particles were not available from the same manufacturer. This study was also confined to the use of low pH buffers, as these conditions tend to give the best peak shape with ionised compounds. A conclusion of the study was that the capacity of shell columns was somewhat reduced, broadly in line with the decrease in the porous volume of the packing.

The aims of the present study can be summarised as follows:

(a) To study overloading of strong acids and bases on totally porous columns and shell columns of the same dimensions (10 cm × 0.21 cm) obtained from the same manufacturer (Agilent), as well as columns of different dimensions from different manufacturers, both at low pH (3.0) and intermediate pH (7.0). According to Agilent, Zorbax Eclipse Plus C18 and Agilent Poroshell 120 EC-C18 columns have equivalent selectivity across a wide range of pH and mobile phase conditions, and both columns are manufactured using similar materials with similar proprietary bonding chemistries [21].
(b) To explore the effect of increasing buffer strength on overload, as a potential means of reducing the problem for these high efficiency columns.
(c) To investigate the performance of so-called charge-stabilised hybrid columns [22]. These columns have recently become commercially available, and contain a small number of positively charged groups on the phase surface. It is claimed that they give much better performance for charged basic compounds when used with low ionic strength mobile phases (such as formic acid solutions) favoured by mass spectroscopists as they give rise to better detection sensitivity.

2. Experimental

Most experiments were performed on a model 1290 ultra-high pressure LC (UHPLC) from Agilent Technologies (Waldbronn, Germany) including Chemstation (data collection rate 80 Hz), autosampler, binary pump, and photodiode array (PDA) detector equipped with a flow cell that according to the manufacturer had a dispersion volume of 1.0 μL. Sample volume was 1.0 μL for 0.21 cm i.d. and 4.8 μL for 0.46 cm i.d. columns unless stated otherwise. The system incorporated a 340 mm × 0.075 mm inlet and 220 mm × 0.075 mm outlet capillary (obtained from Agilent), which is a lower volume modification to the standard system. Some comparative measurements of instrumental bandspreading were also carried out on a Waters Acquity UHPLC (Milford, MA, USA) that incorporated Empower data handling (data collection rate 80 Hz, fast filter 0.025 s), binary solvent manager, PDA detector with 0.5 μL flow cell, and sample injector using 1 μL injections. At least duplicate sample injections were made in all cases. The columns used (all 10 cm length and either 0.21 cm or 0.46 cm i.d.) were Zorbax Eclipse Plus C18 particle diameter (d_p) = 1.8 μm, surface area 160 m^2/g and Poroshell 120 EC-C18 d_p = 2.7 μm, core diameter 1.7 μm, shell thickness 0.5 μm, surface area of porous material 260 m^2/g (Agilent), 75% of particle volume porous, Acquity BEH C18, d_p = 1.7 μm surface area 181 m^2/g and Acquity CSH C18 d_p = 1.7 μm surface area 183 m^2/g (Waters), Kinetex C18 d_p = 2.6 μm, core diameter 1.9 μm, shell thickness 0.35 μm, surface area of porous material 211 m^2/g, 61% of particle volume porous (Phenomenex, Torrance, USA), Halo C18 d_p = 2.7 μm, core diameter 1.7 μm, shell thickness 0.5 μm, surface area of porous material 220 m^2/g, 75% of particle volume porous (AMT, Wilmington, USA). Surface area data were manufacturer's estimates apart from that of Kinetex [23]. Column temperature was maintained at 25 °C in all experiments. All solutes and mobile phase constituents were obtained from Sigma–Aldrich (Poole, U.K.). A flow rate of 0.3 mL/min was used with 0.21 cm i.d. columns, and 1.0 mL/min for 0.46 cm i.d. columns, which were close to the optimum flow for these solutes under the experimental conditions. Due to the broad optimum in the plate height vs. flow rate curves, it would have been possible to use higher flow rates, but such operation at higher pressures might have been detrimental to the long term performance of the columns, which had to be preserved over a number of different experiments. The pH 3.0 ammonium formate buffer was prepared by adjusting, e.g. a 20 mM solution of the salt with formic acid. The pH 7.0 phosphate buffer was prepared by adjusting 20 mM K_2HPO_4 with KOH.

3. Results and discussion

3.1. Instrumental bandspreading

Instrumental effects must be considered very carefully when using relatively short, highly efficient columns as peak variances

produced are comparable with those of the instrument itself. Column peak variances decrease in magnitude as efficiency increases, as k decreases, as column length and particularly as column internal diameter decrease. Thus, it is possible to operate highly efficient 10 cm × 0.46 cm shell columns of 25,000 theoretical plates at $k > 2$ even on conventional LC systems, if these are simply modified by use of small volume detector cells and narrow bore connecting tubing. In contrast, the operation of 5 cm × 0.21 cm columns of 12,500 plates even at $k = 2$ presents a serious challenge even to the most modern UHPLC systems which have been designed with reduction in instrument bandspreading in mind [24].

Gritti and Guiochon have investigated in detail the measurement of peak variance and instrumental contributions to band broadening [25]. They concluded that measurements at half peak height are inaccurate, as they do not reflect the tailing profiles of peaks obtained when the common method of replacing the column by a zero dead volume (ZDV) connector is utilised. They pointed out that the ZDV method does not simulate the (high pressure) conditions under which the actual column is operated although adding capillary restrictors to increase the pressure will itself increase the bandspreading. The same authors examined the bandspreading effects of the instrument (Agilent 1290) also used in the present study [26]. Bandspreading caused by the detector cell was identified as a major contribution. The authors explained the increase in instrumental dispersion as flow rate increased (as is normally observed in such measurements) as being due to radial velocity gradients in the capillaries, which do not have sufficient time to relax at high flow velocities.

Fig. 1 shows bandspreading of the Agilent instrument (with the 0.075 mm diameter capillaries modification) measured with a ZDV fitting. The 1.0 μL dispersion volume cell was used because the low dispersion cell (dispersion volume 600 nL) was not available at the time these measurements were made. Calculations were performed using σ measured at 4.4% of peak height for four different compounds as a function of the acetonitrile content of the mobile phase, using a constant flow rate of 0.3 mL/min (as used in subsequent experiments on overloading). For uracil and naphthalene-2-sulfonic acid (2-NSA), bandspreading remained below 10 μL^2 over most of the solvent composition range (0–90% ACN, no buffer). For naphtho-[2,3-a] pyrene and nortriptyline however, bandspreading increased substantially at 30% ACN, and peaks became severely distorted at lower concentrations of organic solvent. All solutes were completely dissolved in the exact mobile phase employed for each experiment. For naphthopyrene, the reduced solubility of the compound in low concentrations of ACN is likely to be a contributory a factor, but nortriptyline (used as the hydrochloride salt) was readily soluble even in 15% ACN. For the Acquity system, the bandspreading for uracil and 2-NSA was somewhat lower than on the Agilent instrument, but similar results were obtained on this instrument for the more hydrophobic compounds naphthopyrene and nortriptyline in mobile phases of low organic content. Fig. 2(a) compares bandspreading for nortriptyline when the mobile phase was buffered with 20 mM ammonium formate pH 3.0 or contained no buffer on both instruments. The bandspreading for nortriptyline was considerably reduced in the buffered mobile phase, although it remains higher than values for uracil and 2-NSA shown in Fig. 1. The possibility of adsorption of compounds on instrument components cannot be entirely discounted. Bandspreading for 2-NSA in aqueous-ACN solutions with and without buffer is shown in Fig. 2(b). Evidently, there are considerable variations in bandspreading, depending on the particular solute, and mobile phase chosen. The bandspreading of these instruments is clearly sufficient to affect measurements from these high efficiency columns [24]. Considering these variations and the cautions expressed by Gritti and Guiochon about these correction procedures, all experiments were performed using high k for the solutes

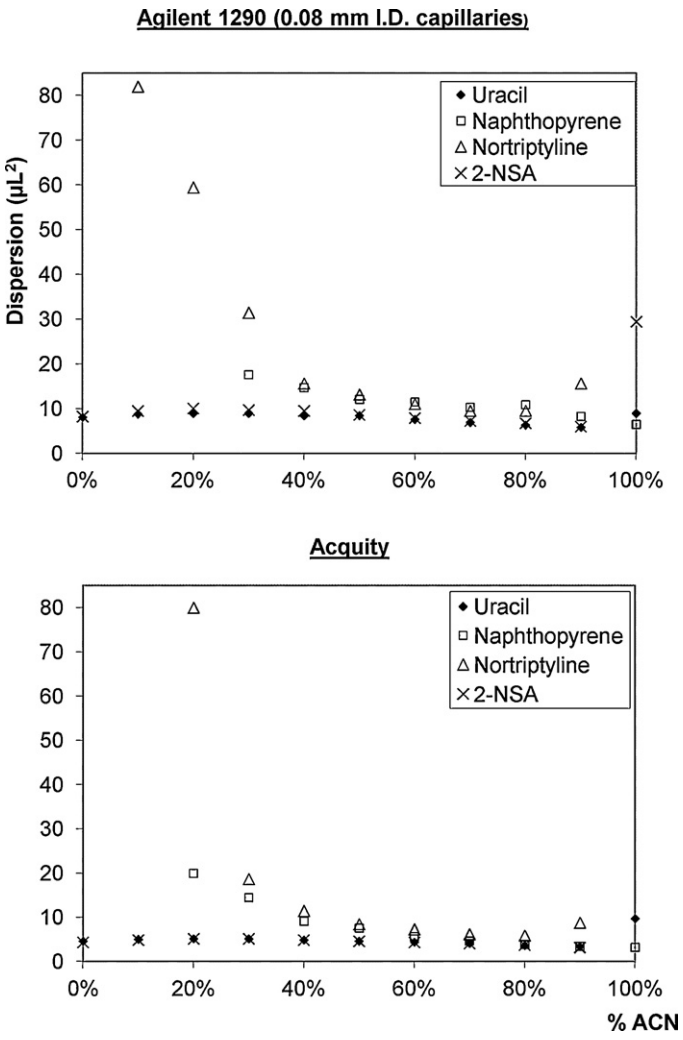

Fig. 1. Measurement of instrumental dispersion of Agilent 1290 and Waters Acquity UHPLC systems (replacing column with a ZDV connector) and using different solutes and variation of the ACN concentration in ACN-water mobile phase. Peak width (σ) monitored at 4.4% of peak height. Flow rate = 0.3 mL/min.

($k \sim 10$) so that the peak variance was substantially increased relative to the instrumental contribution, and thus corrections could be avoided. Use of high k also facilitates simple comparisons of the effect of overload on efficiency, as the influence of k is greatly reduced (see below).

3.2. Overloading in low pH buffers – comparison of shell and totally porous columns

3.2.1. Theory

The capacity of a column is usually expressed as its saturation capacity, which can be loosely defined in frontal analysis as the maximum uptake of solute by the column before breakthrough occurs. Frontal analysis is a comprehensive, rigorous and accurate method for the determination of the total saturation capacity [27], but is time consuming. A simpler approximate method of assessing overload is by measurement of the reduction in efficiency that occurs with increasing amount of sample. An approximate expression for the thermodynamic contribution to column efficiency is [28]:

$$N_{th} = \frac{4}{L_f}\left(\frac{1+k_0}{k_0}\right)^2 \quad (1)$$

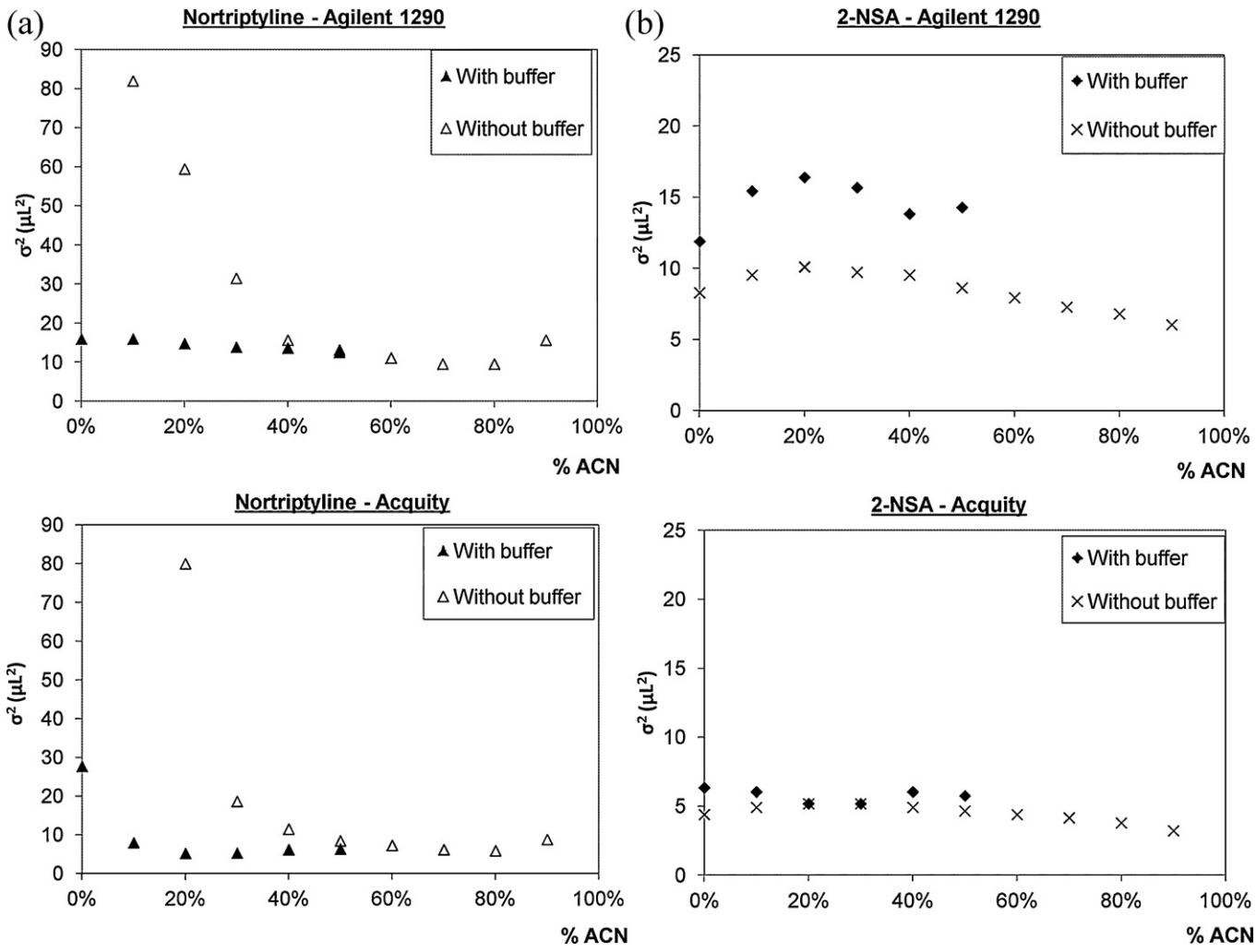

Fig. 2. (a) Comparison of instrumental dispersion on Agilent 1290 and Acquity for nortriptyline when using aqueous ACN with and without 20 mM ammonium formate buffer. Flow rate 0.3 mL/min; temperature 25 °C. (b) As (a) but with 2-NSA as solute.

k_0 is the retention factor for small sample mass, L_f is the loading factor (the sample mass divided by the saturation capacity w_s), and N is measured using the base peak width. The column HETP can be assumed to be the sum of two independent contributions which are due to the non-linear behaviour of the isotherm and to mass transfer kinetics [28,29]:

$$H = H_{th} + H_0 \tag{2}$$

Hence:

$$\frac{L}{N_{tot}} = \frac{L}{N_{th}} + \frac{L}{N_0} \tag{3}$$

$$N_{tot} = \frac{N_0}{1 + (N_0/N_{th})} \tag{4}$$

$$\frac{16t_r^2}{W^2} = \frac{N_0}{1 + (1/4)N_0L_f(k_0^2/(1+k_0)^2)} \tag{5}$$

$$W^2 = \frac{16t_0^2}{N_0(1+k_0)^2} + 4t_0^2k_0^2w_x/w_s \tag{6}$$

where t_0 is the void volume of the column and W the peak width at base of the overloaded peak.

This method, however only gives a very approximate estimate of w_s, which becomes increasingly incorrect as the loading factor increases, due partially to the approximation involved in Eq. (1). For ionised compounds, w_s measured in this way seems to correspond to that only of a very small subset of high energy sites which completely dominate the column performance, leading to rapid overload. The total saturation capacity of the column for ionised compounds, measured by the accurate method of frontal analysis, is at least 100 times larger, due to the presence of a very large number of low energy sites which remain barely occupied in analytical chromatography [18,27]. A much simpler empirical method to evaluate overloading through column efficiency measurements involves the measurement of two parameters, the limiting plate count N_0, that is observed when the sample amount is so small that a linear isotherm pertains, and the sample loading capacity $\omega_{0.5}$, that leads to a plate count half the value of N_0 [30]. The capacity can also be expressed as a concentration of sample $C_{0.5}$ that gives this reduction of efficiency for a specified injection volume. Differences in k affect simple plots of efficiency against sample load, as the fraction of the sample associated with the stationary phase at a given instant is given by $k/(1+k)$. However, the use of high and constant $k = 10$ for the experiments minimises the effects of small variations in k on the results. Overloading does not show a strong dependence on N_0, as the width of a band on the column is inversely proportional to the square root of efficiency, and the columns in this study generated rather similar N_0 (see Tables 1a and 1b). It should be stated clearly however that reference to "loadability" and "capacity" in the present work pertain mostly to the empirical measurement of $C_{0.5}$

Table 1a
Overloading of acid and base on various columns using ammonium formate buffer pH 3.0 in ACN. Columns all 10 cm × 0.21 cm ID.

	Buffer conc. (mM)	A_s (smallest conc.)	N_0	$C_{0.5}$ (mg/L)
Zorbax				
Nortriptyline	5	1.21	21,100	19
	20	1.07	22,500	97
	100	1.01	22,400	300
2-NSA	5	1.50	19,800	37
	20	1.14	22,000	163
	100	1.12	21,900	472
Poroshell				
Nortriptyline	5	1.21	17,600	34
	10	1.32	17,100	61
	20	1.08	17,700	100
	40	1.04	16,900	179
	60	1.13	16,800	254
	100	1.07	16,400	360
2-NSA	5	1.31	17,200	36
	10	1.29	16,700	73
	20	0.93	17,600	128
	40	1.28	17,500	213
	60	1.20	16,500	316
	100	1.79	17,200	451
Halo				
Nortriptyline	20	1.00	15,700	80
2-NSA	20	1.06	15,400	122
Kinetex				
Nortriptyline	20	0.90	17,700	94
2-NSA	20	0.94	17,400	132
BEH				
Nortriptyline	20	1.32	21,200	103
2NSA	20	1.19	20,700	132

rather than the rigorous saturation capacity as derived from frontal analysis.

3.2.2. Comparison of the loading properties of Zorbax and Poroshell columns at low pH

We previously found somewhat reduced loading capacity on a shell column (Kinetex C18, Phenomenex) compared with that of the totally porous column (BEH C18, Waters) roughly in line with the smaller porous volume of the shell column resulting from the presence of the solid core [3]. The surface area of the porous silica in both packings was similar. However, other differences in the silica materials used to prepare these columns could confound the results; also the previous work compared columns of different i.d. with scaled injection volumes (see below). In the present study, we first compared totally porous (Zorbax Eclipse Plus) and shell (Poroshell) columns of the same i.d. (0.21 cm) from the same manufacturer (Agilent). Fig. 3 shows plots of column efficiency against concentration of the injected solution for the strong base nortriptyline (pK_a = 10.3) and the strong acid naphthalene-2-sulfonic acid (pK_a = 0.61) using pH 3.0 ammonium formate buffers (investigated due to mass spectrometer compatibility) in acetonitrile. According to their pK_a, both solutes are fully ionised. It was shown previously that ammonium formate and potassium phosphate buffers of similar ionic strength gave very similar overloading profiles for ionised acidic and basic solutes [3]. Using 5 mM buffer, both columns showed a rapid decrease in efficiency with increasing concentration of nortriptyline, that is characteristic of ionised compounds on C18 columns [16,17]. This behaviour can be explained by rapid overload of scarce strong interaction sites. It is possible that these sites might be undissociated silanols [12], but it seems unlikely that the sites are ionised on these Type B silica columns at low pH. An interesting observation from Fig. 3 is the remarkable similarity in overload profiles on both columns between the ionised acid and base, which strongly suggests a rather non-specific mechanism that does not involve ionised silanols. This behaviour is difficult to explain on the basis of interaction with strong sites, which might be expected to interact with acids and bases differently. An alternative explanation could be repulsion of ions of the same charge held on the phase surface, which may give rise to exclusion effects from the column pores [17,31,32]. The peak shapes of the acidic and basic probes were very good at low sample load at pH 3.0, both columns giving asymmetry factor (A_s) between 0.93 and 1.14 for a 20 mM buffer concentration (see Tables 1a and 1b). This result again suggests that ionised silanols, whose presence is often indicated by strong peak tailing, are mostly absent from these Type B phases at pH 3.0. It is possible that these were more inert than some of the phases studied by Marchand et al. [33], for which evidence for cation exchange (and presumably silanol ionisation) was found at the same low pH. This previous conclusion was also based in part on measurements of asymmetry factors obtained with an older LC system (Hewlett Packard 1090) with higher instrumental bandspreading than the modern instrument used in the present study. Despite some correction of the results in the previous study for this bandspreading, discrepancies between measurements on the two very different instruments could still arise.

$C_{0.5}$ for nortriptyline at pH 3.0 was rather similar on the Zorbax and Poroshell columns (97 and 100 mg/L), and for 2-NSA (163 and 128 mg/L) using 20 mM buffer. The ~20% lower efficiency of the shell column (Table 1a) should act to increase its loadability, but only by ~10%. w_s values (not shown) indicated a similar assessment of overloading being of the order of only a few tenths of a mg, as is typically found for ionised compounds. The surface area (SA) of the porous silica in Poroshell was 260 m^2/g in comparison with 160 m^2/g for Zorbax [34]. The radius of the solid core of Poroshell (1.7 μm) and the thickness of the porous layer (0.5 μm) indicate that ~75% of the particle volume is porous, suggesting even a slightly higher total column surface area for the shell column, which may partially explain these results. Nevertheless, the BET method for measuring SA uses nitrogen, which can access very small pores, whereas solute molecules may not. Furthermore, SA measurements are made on the bare silica prior to the ligand bonding process. Thus, some caution is necessary in such interpretations. The lower limiting efficiency (N_0) of the Poroshell column for both nortriptyline and 2-NSA in the same buffer may be attributable to packing effects with this material in 0.21 cm i.d. formats, as 0.46 cm columns generally give higher efficiency [3].

Table 1b
Overloading of acid and base using ammonium formate buffer pH 3. Columns all 10 cm × 0.46 cm ID Injection volume 4.8 μL.

	Buffer conc. (mM)	A_s (smallest conc.)	N_0	$C_{0.5}$ (mg/L)
Kinetex				
Nortriptyline	20	1.09	22,200	53
2-NSA	20	1.20	21,600	69
Poroshell				
Nortriptyline	20	1.15	20,200	69
2-NSA	20	1.07	17,900	110

3.2.3. Effect of buffer concentration on overloading

The effect of changing the ammonium formate buffer concentration over the range 5 mM to 100 mM at pH 3.0 is shown in Fig. 3 and Tables 1a and 1b. Major increases in capacity for nortriptyline and 2-NSA of at least an order of magnitude using $C_{0.5}$ or w_s values were indicated for both Zorbax and Poroshell columns. For a 5 mM buffer concentration, Tables 1a and 1b indicates $C_{0.5}$ < 40 mg/L, i.e. a loss in efficiency of at least 50% occurs for both solutes on either column when only 1 μL of a 40 mg/L solution was injected. Low concentrations of buffer are often used to improve detection sensitivity with mass spectrometry (MS). Nevertheless, capacity of the columns with even 5 mM ammonium formate buffers (prepared by

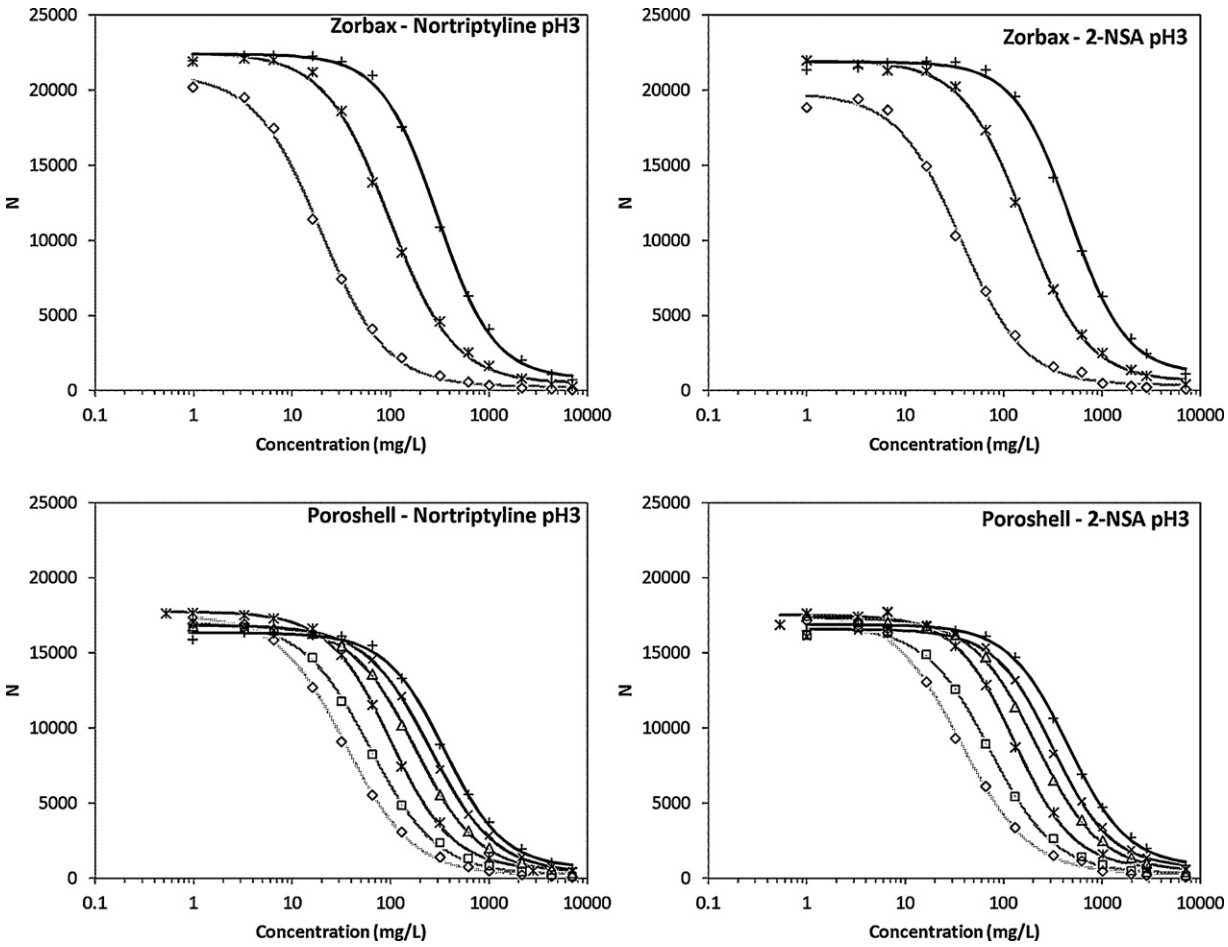

Fig. 3. Overloading plots of totally porous (Zorbax, d_p = 1.8 μm) and shell particle (Poroshell, d_p = 2.7 μm) columns. Sample volume 1 μL. Detection wavelength 254 nm. Mobile phase: (◊) 5 mM, (□) 10 mM, (*) 20 mM, (△) 40 mM, (×) 60 mM, (+) 100 mM ammonium formate in ~30% ACN for nortriptyline and ~11% ACN for 2-NSA (adjusted to maintain k = 10.2). Flow rate 0.3 mL/min.

adjusting a 5 mM salt concentration with formic acid) is superior to 0.1% formic acid solutions, particularly favoured for MS work, as found previously [3]; the ionic strength of these solutions is 6.1 and 2.2 mM/L, respectively. A possible rationale for improvements in $C_{0.5}$ and w_s with increasing ionic strength is that the buffer produces a "salting out" effect for ionised species on the weak column sites [35]. An alternative explanation is a decrease in Donnan exclusion with increased buffer concentration. Effectively, the increased buffer concentration screens the effect of repulsion of solute ions of the same charge. Clearly, high ammonium formate concentrations considerably alleviate the problem of overloading of both basic and acidic components in a strikingly similar fashion, but at the potential expense of mass spectrometer detection sensitivity, and a possible adverse effect on column lifetime.

3.2.4. Comparison of capacity of columns from different manufacturers

The capacity of Poroshell and Zorbax was compared with that of other shell materials Halo, Kinetex and with a totally porous BEH C18 column. Table 1a indicates very comparable loading properties of the two totally porous columns. Thus $C_{0.5}$ for nortriptyline at 20 mM buffer concentration was 97 mg/L for Zorbax and 103 mg/L for BEH C18. Similarly for 2-NSA values were 163 mg/L on Zorbax compared with 132 mg/L on BEH C18. These results appear to be in line with the similar surface areas of these materials (160 and 181 m^2/g for Zorbax and BEH, respectively). For the shell columns with nortriptyline, $C_{0.5}$ was 100 mg/L for Poroshell, 80 mg/L for Halo, 94 mg/L for Kinetex. For 2-NSA, results were in similar proportion. The values for Poroshell are a little higher than for the other shell columns, which may be in part due to the higher surface area of the porous silica in this packing, and the higher fractional porous volume compared with Kinetex; the capacity of Halo appeared slightly smaller than the other shell columns. However, the differences between the shell columns would hardly have a major impact on column selection on this basis. Indeed, the differences in loadability between the shell and totally porous columns are also rather small.

3.2.5. Comparison of capacity of 0.46 and 0.21 cm i.d. columns

Our previous study compared the loading properties of 0.46 cm i.d. shell and 0.21 cm totally porous columns. The larger diameter shell column was selected in the previous study for purely practical reasons, as higher efficiencies can presently be obtained compared with 0.21 columns of this type, presumably due to packing difficulties [3]. The injection volume was scaled, using 4.8 μL and 1.0 μL injections for the 0.46 and 0.21 cm i.d. columns respectively, and it was assumed that the distribution of the solute over the column cross section would be the same in both cases.

Table 1b shows values of $C_{0.5}$ for nortriptyline on the 0.46 cm i.d. Kinetex column (53 mg/L) which compares with 94 mg/L for the 0.21 cm column. This indicates a higher loading capacity per unit volume of the packing in the narrower column. Some of this difference is attributable to the 20% lower efficiency of the 0.21 cm column which may increase loadability by about 10%. The results

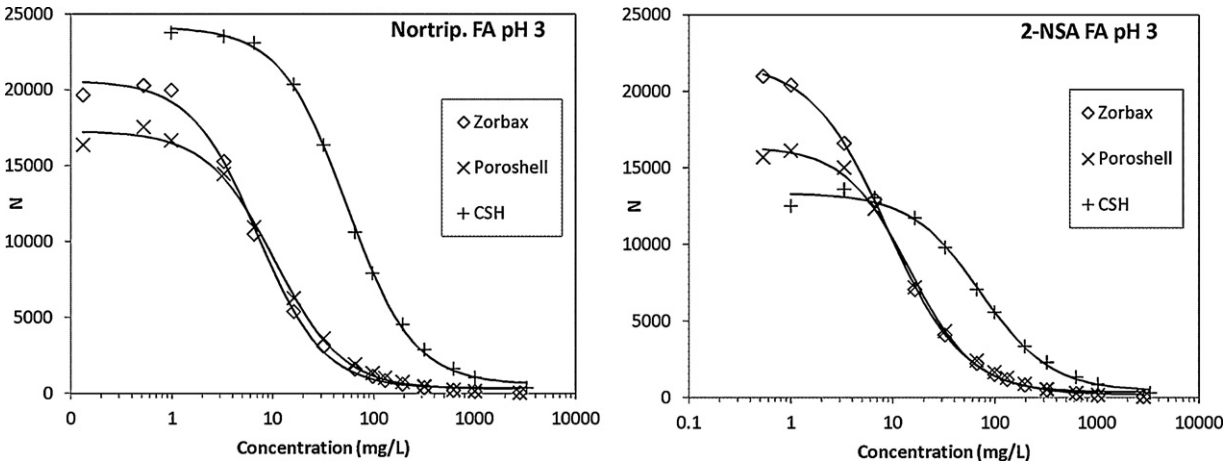

Fig. 4. Comparison of overloading for CSH, Poroshell and Zorbax columns using 0.1% formic acid in ACN. ACN concentrations for the CSH column ~22% for nortriptyline, ~21% for 2-NSA; ACN concentrations for Zorbax and Poroshell ~27% for nortriptyline, ~11% for 2-NSA. Other conditions as Fig. 3.

indicate that the capacity of the wider column per unit volume of packing was about 60% of that of compared with the narrow bore column for both nortriptyline and 2-NSA. Similar calculations for the Poroshell column indicated a smaller but still appreciable reduction in loadability.

Differences in $C_{0.5}$ between the columns of different diameter might be due to errors in the volumetric metering of the sample solution by the instrument. To investigate this possibility, the determination was repeated on the 0.46 cm Kinetex column with a 1.0 μL instead of a 4.8 μL sample volume. However, it was found that $C_{0.5}$ increased by almost exactly a factor of 4.8 as expected (results not shown), showing that volumetric errors were not responsible. It is conceivable instead that different distribution of the injected sample may take place in the 0.46 and 0.21 cm i.d. columns even though the injected volume was scaled (4.8 μL and 1.0 μL, respectively). The inlet design may not ensure even distribution of the sample plug over the whole of the column cross-section. It is conceivable that differences between brands could be the result of differences in column hardware design. Sample distribution across the column radius might be more homogeneous in narrower columns. If this was the case, then the sample might travel preferentially through a central core of the 0.46 cm column, producing an enhanced overloading effect compared with the narrow bore column, as well as a higher N_0, as the influence of wall effects would be reduced. It is also possible that this effect could be solute dependent. Gritti and Guiochon [25] have suggested that 0.46 cm columns are more radially homogeneous than narrower bore columns, and that wall effects barely affect the packing structure in the central region of such columns. Our finding may compound this effect.

3.2.6. Overloading at low pH for charged surface hybrid columns

A catastrophic deterioration in efficiency with sample load occurred for both types of small particle column with ionised acids and bases when using low ionic strength additives such as 0.1% formic acid in the mobile phase [3]. This observation has been confirmed by other workers [36]. Nevertheless, there is still much interest in the use of these additives for LC–MS [37], due to the increased sensitivity of detection and the ease of mobile phase preparation compared with buffers that contain volatile salts. In recognition of this problem, a "charged surface hybrid" (CSH) C18 column was recently introduced. A low level of basic groups is deliberately introduced into the phase, giving it a positive charge that is maintained when operated under acidic conditions [22]. Although it is claimed that these groups stabilise the charge on the phase surface (presumably making it less susceptible to fluctuations), no further explanation of its mechanism has been offered to date. At present, this type of column is not available in a shell format.

Fig. 4 shows plots of column efficiency vs. sample mass for Zorbax (fully porous), Poroshell and the CSH column (fully porous) for nortriptyline using 0.1% (v/v) formic acid in ACN. For the first two columns, an extremely rapid deterioration in efficiency as sample load increased was shown. Values of $C_{0.5}$ were only 7 and 10 mg/L for nortriptyline, and 9 and 14 mg/L for 2-NSA for Zorbax and Poroshell respectively (see Table 2), considerably smaller even than with 5 mM ammonium formate buffer. Similar low w_s values of the order of tens of micrograms were recorded. $C_{0.5}$ values were comparable to those for BEH C18 (totally porous), and Kinetex C18 shell particles reported previously in similar mobile phases [3]. However, Fig. 4 shows that considerably better performance was obtained for the CSH column giving $C_{0.5}$ 54 mg/L. This capacity was greater than that of the Zorbax and Poroshell columns even when used with 5 mM ammonium formate buffer, which has considerably higher ionic strength (see above, Table 1a). The performance in formic acid is thus a major advantage of this type of phase. The ACN concentration necessary to elute nortriptyline at the same k with the CSH column using formic acid (~22%, v/v) was considerably less than required for the Zorbax and Poroshell columns (~27%, v/v) indicative of the presence of the positive charges on the surface of the phase, which might be expected to produce some repulsion effects on cationic species which have charge of the same sign. It is difficult to explain therefore the loading behaviour of this phase in terms of the "solute charge repulsion" theory, which would suggest overloading of positively charged solutes should be increased on such a phase. Table 2 shows that the advantages of CSH for nortriptyline continue when $C_{0.5}$ values are compared with the other columns for 5 mM ammonium formate buffer. Nevertheless, as the buffer concentration increased, the relative advantage of the CSH column decreased progressively, until at a buffer concentration of 100 mM, $C_{0.5}$ values were 333 mg/L on CSH, 300 mg/L on Zorbax, 360 mg/L on Poroshell.

A limitation of the CSH column was its performance for 2-NSA. Using formic acid, it gave the lowest efficiency for small sample mass of all 3 columns (13,400 plates), together with tailing peaks (A_s = 1.81). Nevertheless, at higher sample concentrations of 2-NSA (e.g. 100 mg/L), efficiency was somewhat better than for the other columns (see Fig. 4). The presence of positive charges was again indicated by the use of a higher concentration of ACN (~21%, v/v) necessary to elute 2-NSA on the CSH column with k = 10 compared with ~11% for the Zorbax and Poroshell columns when using 0.1% formic acid in the mobile phase. This result indicates ionic

Table 2
Overloading of acid and base on CSH, Zorbax and Poroshell columns using acid buffers.

	Buffer	A_s (smallest conc.)	N_0	$C_{0.5}$ (mg/L)
CSH column				
Nortriptyline	0.1% formic acid	1.31	24,200	54
	5 mM AF pH 3	1.35	21,900	63
	20 mM AF pH 3	1.17	24,200	131
	100 mM AF pH 3	1.23	21,900	333
2-NSA	0.1% formic acid	1.81	13,400	72
	5 mM AF pH 3	1.82	9500	139
	20 mM AF pH 3	1.47	18,100	178
	100 mM AF pH 3	1.49	18,400	414
Zorbax				
Nortriptyline	0.1% formic acid	1.54	20,600	7
2-NSA	0.1% formic acid	1.47	21,900	9
Poroshell				
Nortriptyline	0.1% formic acid	1.37	17,300	10
2-NSA	0.1% formic acid	1.38	16,400	14

retention of the anionic solute on the positively charged column groups. Poorer performance for small sample concentration of 2-NSA was also shown using 5 mM ammonium formate pH 3.0 rather than formic acid, giving 9500 plates and A_s = 1.82 (see Table 2). However, as shown in Fig. 5, increasing the ammonium formate concentration to 20 or 100 mM gave considerable improvements in N_0, indicating that this column can also give loading performance equivalent to the conventional columns for acidic solutes under these conditions. The results for the acid are consistent with a mixed mechanism process involving ion-exchange of the acid on the positively charged column sites, in a similar but converse way to the interactions of protonated basic compounds on negatively charged (silanol) sites. As the buffer concentration is increased, the influence of ion exchange is diminished.

3.3. Overloading in neutral pH buffers

The overloading performance of C18 columns at pH 7.0 is important, as use of intermediate pH can introduce different selectivity in a separation due to variation of the degree of ionisation of the ionisable components in a mixture. For weak bases, pH 7.0 can enable some solutes to be analysed in the neutral state, with advantages in peak shape and overloading performance [38]. Fig. 6 and Table 3 show overloading data for nortriptyline and 2-NSA on the Zorbax column using potassium phosphate buffer pH 7.0 of concentrations 5, 20 and 100 mM. For nortriptyline with a given buffer concentration (e.g. 20 mM phosphate), efficiency actually increased at first with increasing solute concentration, reaching a maximum value, which then declined with higher solute concentration. Table 3 records the maximum efficiency and asymmetry factor at this point for nortriptyline and 2-NSA. For nortriptyline, maximum efficiency on Zorbax was around 11,000 plates with A_s = 3.8 using 5 mM buffer, which represents about half the plate count obtained with pH 3.0 buffer (N_0 = 21,100 plates, Tables 1a and 1b). Increasing the buffer concentration from 5 to 100 mM increased the maximum efficiency from 11,300 plates (A_s = 3.82) to 16,500 plates (A_s = 1.75). The behaviour is indicative of ionisation of some silanol groups at the higher pH which produce detrimental secondary interactions with solute ions of opposite charge. In contrast, Tables 1a and 1b shows that increasing the buffer concentration over the same range at pH 3.0 produced only minor changes in N_0 and A_s for nortriptyline, and A_s values at pH 3.0 for this compound were close to 1.0 on both Zorbax and Poroshell. Previous studies have shown that increased silanol ionisation is linked with decreased efficiency for bases, although the complex stereochemistry of the sample surface is also likely to be important [39,40]. Peak shape for bases can be considered to result from a combination of cation exchange and non cation exchange processes. As the latter process prevails when solute concentration increases, it will overwhelm the tailing produced by cation exchange. Similarly, at high buffer concentrations, cation-exchange becomes less important, and its contribution to peak tailing becomes less significant.

The performance of Poroshell was comparable to that of Zorbax at pH 7.0. Thus, using 20 mM phosphate buffer, reduced maximum efficiency (13,700 plates) and increased asymmetry (A_s = 2.95) was obtained for nortriptyline. Indeed, broadly similar results at pH 7.0 were also obtained for all the other columns Kinetex, BEH and CSH although clearly there are some differences in silanol

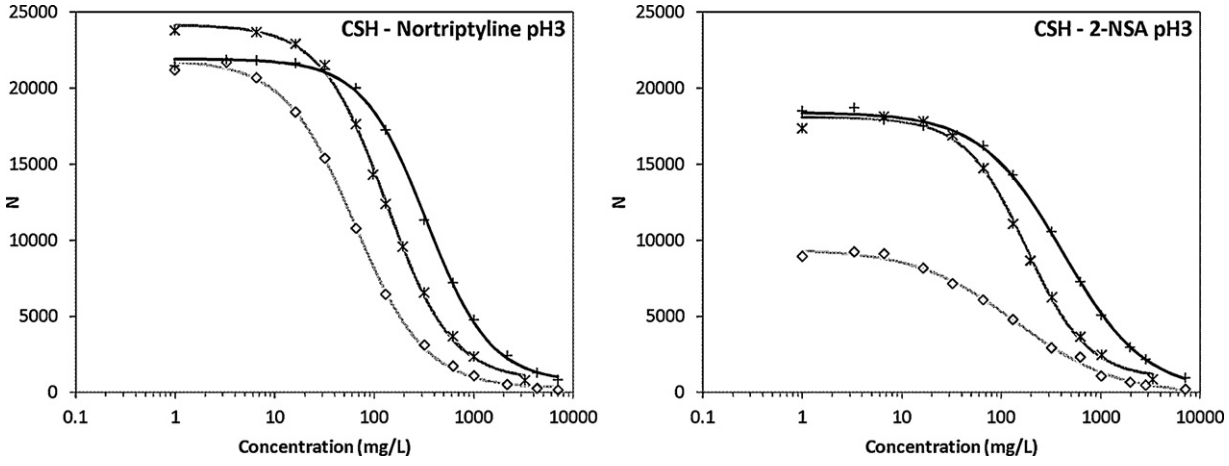

Fig. 5. Overloading plots of CSH column using 5–100 mM ammonium formate buffers in ACN (~30% for nortriptyline and ~12–16% for 2-NSA). Other conditions as Fig. 3.

Table 3
Overloading of acid and base on various columns using potassium phosphate buffer pH 7.0 in ACN.

	Buffer conc. (mM)	A_s at N_0 or N_{max}	N_0 or N (max.)[a]	$C_{0.5}$ (mg/L)
Zorbax				
Nortriptyline	5	3.82	11,300	[a]
	20	3.38	14,100	[a]
	100	1.75	16,500	[a]
2-NSA	5	1.15	22,700	57
	20	0.96	21,200	202
	100	1.01	20,400	606
Poroshell				
Nortriptyline	20	2.95	13,700	[a]
2-NSA	20	1.10	18,200	186
Kinetex				
Nortriptyline	20	2.22	11,900	[a]
2-NSA	20	0.96	17,000	201
BEH C18				
Nortriptyline	20	1.89	17,300	[a]
2-NSA	20	1.19	20,600	215
CSH				
Nortriptyline	20	6.11	8200	[a]
2-NSA	20	1.15	22,700	213

[a] For 2-NSA, the maximum efficiency was recorded at the lowest sample load. For nortriptyline, the maximum efficiency and the corresponding asymmetry are recorded irrespective of the sample load. Values of $W_{0.5}$ were not formally calculated for nortriptyline.

activity between the different columns [41]. All showed poor efficiency, asymmetric peaks and the same pattern of increasing and then decreasing efficiency as the concentration of nortriptyline was increased (Fig. 7). The apparent activity of the CSH column may indicate that the surface groups are no longer positively charged at pH 7, or that ionisation of silanols at this pH overwhelms the influence of these groups. The advantage of the hybrid phases is the potential of their use at more alkaline pH, where it may be possible to approach or exceed the pK_a of some basic solutes, reducing their ionisation and thus limiting problems of peak shape and

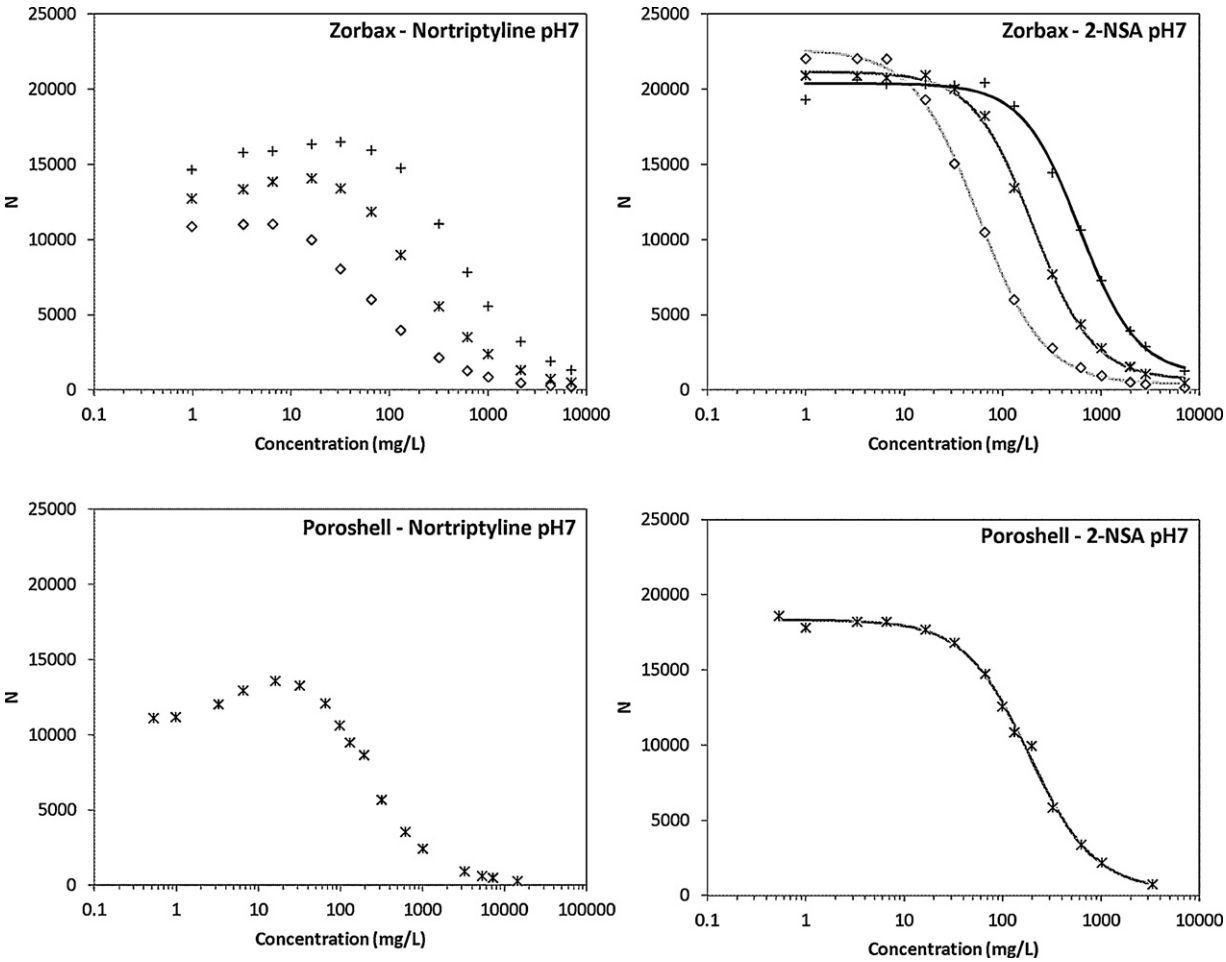

Fig. 6. Overloading plots for Zorbax and Poroshell columns using 20 mM potassium phosphate buffer pH 7.0 in ACN (~30% for nortriptyline, ~10% for 2-NSA). Other conditions as Fig. 3.

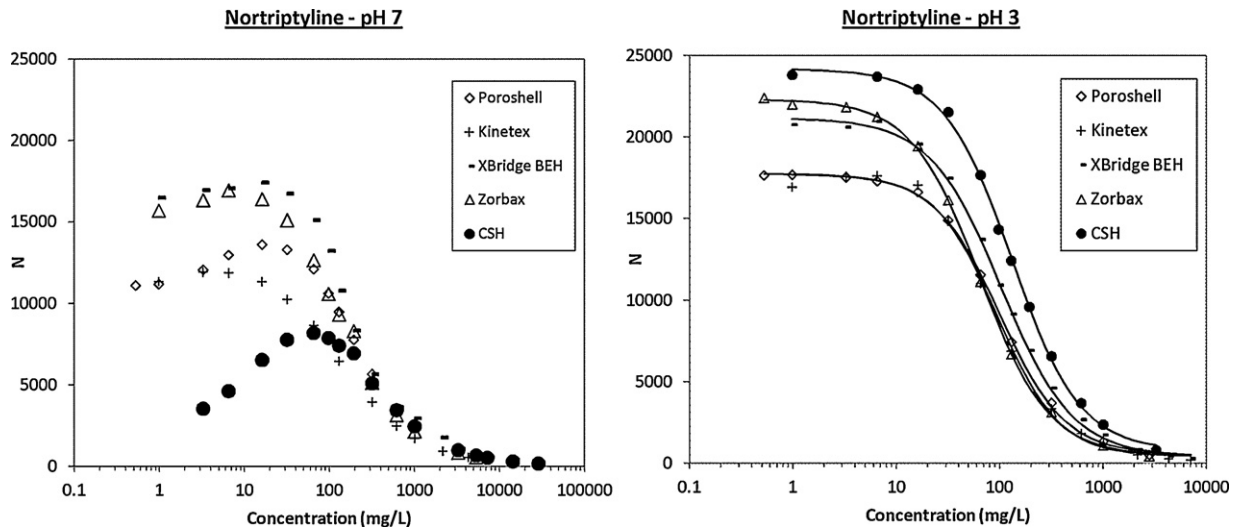

Fig. 7. Comparison of overloading plots for different columns using 20 mM potassium phosphate buffer pH 7.0 and pH 3.0 in ACN.

overload [38]. Analysis at pH 7.0 seems to represent a particularly difficult condition for peak shape, where ionisation of silanol groups is apparent on all the columns tested, while the mobile phase pH is not yet high enough for the ionisation of strongly basic solutes such as nortriptyline to be suppressed. Comparison of the performance of the columns at pH 3.0 and pH 7.0 in Fig. 7, clearly demonstrates the reduced performance at pH 7.0. It is possible however, that the performance of all the columns was somewhat compromised by their previous use at pH 3.0. Such use may preferentially remove short chain bonded ligands used to end-cap these columns and thus render the surface more active at the higher pH. Values of $C_{0.5}$ or w_s were not formally calculated for nortriptyline at pH 7.0. However, it is evident from Fig. 7 that the columns overload less readily at pH 7.0 than at pH 3.0. This result can be attributed partially to the poorer efficiencies recorded at pH 7.0, which distributes the solute over a greater portion of the column material, and to the presence of additional (ionic) retention sites at the higher pH. A further important factor is the higher ionic strength of the 20 mM pH 7.0 buffer (54 mmol/L) compared with the 20 mM pH 3.0 ammonium formate buffer (21 mmol/L), prepared as shown in Section 2.

In contrast to the results for nortriptyline, all columns in Table 3 showed excellent performance for 2-NSA at pH 7.0, with efficiencies and asymmetry factors similar to those recorded at pH 3.0. Clearly, the acidic solute is not adversely affected by the ionisation of column silanol groups at pH 7.0. Excellent performance was shown by the CSH column, again suggesting that the bonded groups are not positively charged at pH 7.0. Values of $C_{0.5}$ for 2-NSA increased with buffer concentration in a similar fashion at pH 7.0 as previously at pH 3.0, as shown for the Zorbax column in Fig. 6 and Table 3. $C_{0.5}$ for Zorbax with 2-NSA was similar but somewhat higher at pH 7.0 compared with pH 3.0 (202 and 163 mg/L respectively for a buffer concentration of 20 mM). Similar results were obtained for the same solute with Poroshell (186 and 128 mg/L respectively at 20 mM buffer concentration) and also for Kinetex, BEH and CSH. This increase can be attributed to the higher ionic strength of the pH 7.0 buffer (prepared as shown above).

4. Conclusions

Overloading can be problematic for ionised acidic and basic compounds on high efficiency sub 2 μm and sub 3 μm shell particle columns, partially as a result of their high efficiency and therefore concentration of the solute as a narrow band as it travels down the column. A comparison of shell and totally porous materials from the same manufacturer however, which used similar silica and similar proprietary bonding phase chemistry, showed relatively little difference in loading properties, despite the shell material used in the study having only 75% of the porous volume of the totally porous column. This result may partially be explained by the relatively high surface area of the silica in the porous coating. It is difficult to give a firm estimate of the effect of the non-porous core on loading, because of this difference in the stated surface areas of the porous silica in these particles, even in these chemically similar materials. Other factors could exist, other than the proportion of the particle volume that is porous. For example, it is conceivable that sample molecules do not penetrate appreciably into the very centre of totally porous particles. Nevertheless, the present work has shown practically that differences in the loading behaviour of shell columns from different manufacturers, and between shell and totally porous particle columns from different manufacturers, are generally small.

At low pH, the sample capacity can be increased considerably for both ionised acidic and basic solutes by increasing the buffer concentration. The concentration of solute needed to reduce the small mass efficiency by half, increased by at least an order of magnitude when ammonium formate buffer concentration was increased from 5 to 100 mM. In contrast, very poor performance was obtained using low ionic strength buffers like formic acid, which are favoured for high sensitivity mass spectrometer detection. Charged stabilised hybrid phases, which contain a controlled concentration of positively charged groups, gave much better overloading behaviour, which is of considerable advantage for the MS detection of cationic solutes. However, their advantage was reduced in buffers of higher ionic strength, giving overload behaviour rather similar to that of conventional columns in 100 mM ammonium formate pH 3.0. A disadvantage of the charge stabilised columns was the poorer efficiencies obtained for acidic solutes, especially when buffers of low ionic strength were used.

The capacity of 0.46 cm columns per unit volume of packing was lower than suggested by simple scaling of results from 0.21 cm columns. It may well be that the sample is less evenly distributed across the radius of a 0.46 cm column, causing it to travel in a relatively narrower core of the packing material compared with the 0.21 cm column, and mostly avoiding the wall region. This result may partially explain why 0.46 cm columns are more efficient than narrower columns, and why wall effects hardly affect their performance [25].

At pH 7.0, all columns examined showed evidence of detrimental interaction with ionised silanols when using the basic test probe. The same behaviour of the CSH column indicated that sites positively charged at low pH may be neutral at pH 7.0. Column efficiencies generally increased as the buffer concentration increased, which suppresses the ionic interactions with ionised silanols. For a given buffer concentration, column efficiency showed an increase followed by a decrease in efficiency as solute concentration was increased. This behaviour can be attributed to a greater proportion of the sample interacting with the phase through non-ionic processes, as the concentration of solute was increased. Sample capacities were slightly higher at pH 7.0 than at pH 3.0. This result may be attributed to the higher ionic strength of the pH 7.0 buffer used, but also to the lower column efficiencies, and the provision of extra ionic retention sites. In contrast to the performance for the basic solute, column efficiency at pH 7.0 was similar for the acid to that obtained using the low pH mobile phase.

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