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LARGER-DIAMETER SMALL-PARTICLE COLUMNS FOR FAST HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATIONS WITH CONVENTIONAL EQUIPMENT

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SUMMARY

High-performance liquid chromatographic columns packed with 3-μm particles can achieve identical separations as for larger-particle columns (*e.g.*, 5–6-μm particles) in about half the total time. However these 3-μm columns also produce narrower solute bands that require very-low-dead-volume liquid chromatographic systems. This paper describes a new column design (DuPont Golden Zorbax Series®) based on wider-diameter columns (8 × 0.62 cm) plus 3-μm particles of various compositions (silica, C_8, C_{18}). These new columns provide all of the advantages of 3-μm-particle columns and can be used with present liquid chromatographic equipment, usually without any system modifications. A number of fundamental questions relating to the use of these new columns were examined experimentally and shown not to compromise their performance. A number of applications of Golden Zorbax Series columns are presented, including comparisons of separations with corresponding 6-μm columns. Relative retention was found to be generally identical on both 3- and 6-μm columns.

INTRODUCTION

The past 15 years have seen a continuing improvement in the performance of columns for liquid chromatography (LC). Emphasis is usually given to the achievement of some minimum plate number N (as required for a given separation problem) in the shortest time, without exceeding some maximum pressure for the system. The theory of such separations has been understood in general terms since the mid-1960s (*e.g.*, refs. 1–5), leading to straightforward optimization schemes of various kinds (*e.g.*, refs. 5–9). Generally the most effective approach to reduced separation time or increased N is to reduce particle diameter[10,11]. Columns with particles as small as 5 μm have been in routine use for several years, and research on smaller particle

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columns has been underway since the mid-1970s (*e.g.*, refs. 7 and 12–14). Theory predicts[5] that a particle diameter d_p of about 2 μm will be optimum for many separations. Recently columns with 3-μm particles have been described[15–20] which are now commercially available.

Columns with sub-5-μm particles should be generally useful for increasing the speed of analytical LC separations, especially for macromolecular samples such as proteins and for separations which do not require a very large N value (*e.g.*, ref. 21). However, these possibilities are compromised by certain practical considerations, by other problems that are peculiar to very-small-particle (VSP, *i.e.*, sub-5 μm) columns and by a lack of experience in the routine use of such columns. Some specific issues relating to the use of VSP columns are as follows:

(1) As d_p is decreased, the column length L must be proportionately reduced to allow reasonable flow-rates and to maintain an acceptable pressure drop across the column. These changes in d_p and L lead to a corresponding decrease in column volume and the volume of eluted sample bands. As a result, the LC system must be redesigned or substantially modified for minimum extra-column volume and reduced detector time constant[15].

(2) Frictional heating of the mobile phase during flow through a column is greater for smaller d_p and can lead to a decrease in column efficiency[16,22,23]. This effect can be minimized by separate adjustment of the temperatures of column and incoming mobile phase, but this is experimentally inconvenient or restrictive.

(3) LC columns are usually exposed to particulates that are present in the sample or arise from abrasion of seals in the pump or injection valve. VSP columns are more prone to pluggage by these particulates (usually due to smaller-pore frits).

(4) Column loadability can be expressed as the maximum weight of sample injected without a decrease of more than 20% of the original column N value. Previous studies[24,25] suggest that column loadability decreases with decrease in d_p, implying that VSP columns will exhibit limited analytical sensitivity and restricted utility for semi-preparative separations.

(5) The separation of "real" samples on VSP columns may eventually be limited by kinetic resistance to binding/release of solute from the stationary phase (slow interfacial mass transfer). A major contribution of this effect was shown for a 5-μm column[26], from which it might be concluded that 3-μm particles will be less useful than predicted by theory.

This paper describes a different approach to the exploitation of VSP columns in LC. We have elected to increase column internal diameter from the commonly used 0.46 to 0.62 cm. The resulting 1.8-fold increase in column volume and the volume of sample bands (other factors being equal) allows such columns to be used with little or no modification of conventional LC systems and negligible degradation of column performance. At the same time we have investigated the various problems of VSP columns cited above and have shown that these problems do not significantly limit the performance of these new (0.62 cm) columns. Our studies also provide additional insight into the characteristics of other VSP columns.

EXPERIMENTAL

Equipment

A standard Model 8800 Liquid Chromatography System (DuPont Instru-

ments, Wilmington, DE, U.S.A.) was used in the present studies, with data reduced by a PDP-10 based computer system[27]. For experiments involving flow-rates in excess of 10 ml/min, a Model DST-122 pneumatic-amplifier pump (Haskell Engineering, Burbank, CA, U.S.A.) was used. The columns and mobile phase were thermostated (forced-air oven) at the same temperature, with fixed photometric detection at 254 nm. The detector cell volume was 8 μl.

Materials

Columns were slurry-packed using a proprietary process; column blanks were 8.0 × 0.62 cm (polished SS 316, Bishop Tube, Frazer, PA, U.S.A.), with conventional low-dead-volume end-fittings (Parker-Hannifin, Huntsville, AL, U.S.A.) and column closures consisting of 2.8-μm nominal porosity mats (Brunswick, Deland, FL, U.S.A.), secured with Teflon® gaskets. The column packing material was based on Zorbax® porous silica[28,29], in some cases bonded with dimethyloctyl- or dimethyloctadecylchlorosilane (*e.g.*, ref. 21, Ch. 7). The particle diameter of the various column packings was obtained by averaging results from scanning electron microscopy (SEM), transmission electron microscopy (TEM) and column permeability[13]. The results of these various measurements agreed within an overall spread of ± 5% or less. The averaged d_p value for the nominal 3-μm particles was 3.04 μm and for the nominal 6-μm particles it was 5.67 μm. The distribution of particle sizes for a given packing was better than ± 10% of the mean diameter for 90% of the particles. Columns equivalent to these various 3-μm-particle columns of Table I are now available from DuPont under the name Golden Zorbax Series®.

Mobile phases were composed of acetonitrile–water mixtures; the water was distilled and deionized and the acetonitrile was of HPLC grade (Baker, Phillipsburg, NJ, U.S.A.). Di-*n*-pentyl phthalate, di-*n*-octyl phthalate or 4-bromoacetanilide was used as the solute (Chem Services, West Chester, PA, U.S.A.).

Methods and data reduction

Samples were injected manually and the analog detector signal was transmitted to a data buffer system which employed an analogue-to-digital converter with 10 Hz bandpass. Data were sampled at a rate of 10 points/sec for flow-rates less than 2 ml/min and at 30 points/sec at higher flow-rates. The time constant of the detector had a nominal value of 0.1 sec, and the data-reduction system was found to have a time constant of 0.17 sec. From this a time constant of 0.20 sec could be approximated for the overall HPLC–computer system. The determination of values of the theoretical plate number N and plate height H is described in the following section. All values are based on second-moment calculations[30]. Resulting values of reduced plate height (h) and reduced mobile phase velocity (v) were then fitted by least-squares regression to the Knox and Van Deemter equations.

RESULTS AND DISCUSSION

Column efficiency

Characteristics of the LC system. Column N values were determined for various separation conditions as described in the preceding section. An unmodified DuPont Model 8800 LC system was used to test the concept of the present wide-diameter LC

columns, *i.e.*, use of these columns with standard LC equipment, without major modification to reduce extra-column band broadening. The extra-column band broadening of the Model 8800 was determined as follows. A 8.0-cm length of 0.01-in. I.D. tubing was substituted for the column, samples were injected in the usual manner and the second-moment bandwidths were determined in time units σ_{ec} (1 standard deviation). The product of σ_{ec} and flow-rate F is then the extra-column contribution to band volume. Values of $\sigma_{ec}F$ were determined as 0.0158 ml at F = 0.24 ml/min and 0.0163 ml at F = 0.41 ml/min. Other studies have shown[15] that $\sigma_{ec}F$ is weakly dependent on flow-rate over the range $0.5 < F < 20$ ml/min; we assume here that $\sigma_{ec}F$ can be assumed to be constant (0.016 ml) to a first approximation.

The time constant τ of the DuPont Model 8800 system is 0.1 sec for interfacing to a high-speed data processing unit and 0.25 sec for fast analog (recorder) output with filtering of the signal. In the present studies the detector-integrator output was fed directly to the data system described in the preceding section, which increased the overall time constant to about 0.20 sec. Attempts to measure the chromatographic system time constant directly gave anomalous results: therefore we simply restricted the mobile phase flow-rate and values of k' to a range where the time constant had a negligible effect on values of H.

The extra-column and time-constant contributions to total band broadening can be derived as follows. The column plate number N and plate height H are:

$$N = (t_R/\sigma)^2 \tag{1}$$

and

$$H = L/N = (L/t_R^2)\,\sigma^2 \tag{2}$$

The retention time t_R and bandwidth σ (1 standard deviation) are in seconds; the column length L is in cm. The total bandwidth σ can be expressed as contributions (sec) from the column (σ_c), extra-column band broadening (σ_{ec}) and the system time constant τ (*e.g.*, ref. 30):

$$\sigma^2 = \sigma_c^2 + \sigma_{ec}^2 + \tau^2 \tag{3}$$

If the retention time t_R is expressed as

$$t_R = V_m(1 + k')/F \tag{4}$$

where V_m is the volume of mobile phase within the column (ml), k' is the solute capacity factor and the flow-rate F is in ml/sec, then we can write

$$H = H_c + H_{ec} + H_\tau \tag{5}$$

where the apparent plate height H of the observed band is the sum of contributions from the column (H_c), extra-column broadening (H_{ec}) and the system time constant

(H_τ). We desire to minimize the values of H_{ec} and H_τ, which are given as

$$H_{ec} = L[(\sigma_{ec}F)/V_m(1 + k')]^2 \quad (5a)$$

and

$$H_\tau = L[F\tau/V_m(1 + k')]^2 \quad (5b)$$

The quantity $\sigma_{ec}F$ is assumed to be equal to 0.016 ml for the Model 8800 and the value of τ depends on how the LC system is interfaced to the data system.

Column performance. One of the problems in preparing highly efficient VSP columns is the initial production of particles that fall within a narrow range of d_p values[13]. The Zorbax particles used as the starting material for the present column packings[10,28,29] can be synthesized directly in narrow particle-size distributions, without the need for particle fractionation. This is illustrated in Fig. 1; which shows SEM photographs of samples of the 3- and 6-μm Zorbax particles used in the present study.

Column plate numbers (N) were determined for various combinations of sample and mobile phase composition, as a function of flow-rate, F. The results were generalized as plots of reduced plate height h *vs.* reduced velocity v[21], as illustrated in Fig. 2. It was found that plots as in Fig. 2 could be fitted with reasonable precision to the so-called Knox equation:

$$h = Av^{1/3} + B/v + Cv \quad (6)$$

The quantities A, B and C are often roughly constant for "good" columns, as discussed in ref. 21 on p. 836; typical values of these parameters are $A = 1$, $B = 2$ and $C = 0.05$[31,32]. Presentation of column N values in terms of h allows a general comparison of column performance with other columns described in the literature.

We will first consider values of H_c for the present columns, *i.e.*, plate height values corrected for contributions from H_{ec} and H_τ (eqn. 5). Values of H_c allow us to examine column performance *per se*, apart from the limitations of the LC system. The term $Av^{1/3}$ in eqn. 6 is of primary interest in this respect, as it is mainly a function of how well the column is packed. This latter term is also of major importance in determining H_c when separation conditions (mainly F) are adjusted for optimum column efficiency. Table I summarizes plate height data expressed in terms of eqn. 6 for the five column-types studied by us. These data are for a solute with $k' = 11$, in order to minimize the effect of H_{ec} and maximize the reliability of resulting parameters in eqn. 6.

Values of A in Table I are 0.50–0.59 for the bonded-phase columns and 0.73–0.79 for the silica columns. By comparison, A values for previous 5–10-μm packings have generally fallen within the range 0.5–1.5[31]. The smallest A value ever reported[32] was for a column of 480-μm glass beads, with $A = 0.37$. Thus the bonded-phase columns in Table I appear to be about as well packed as any previously reported column, and close to the ultimate limit suggested by the 480-μm glass bead data. It therefore appears that particles as small as 3-μm in diameter present no special problems in the packing of efficient, larger-diameter columns. This is further

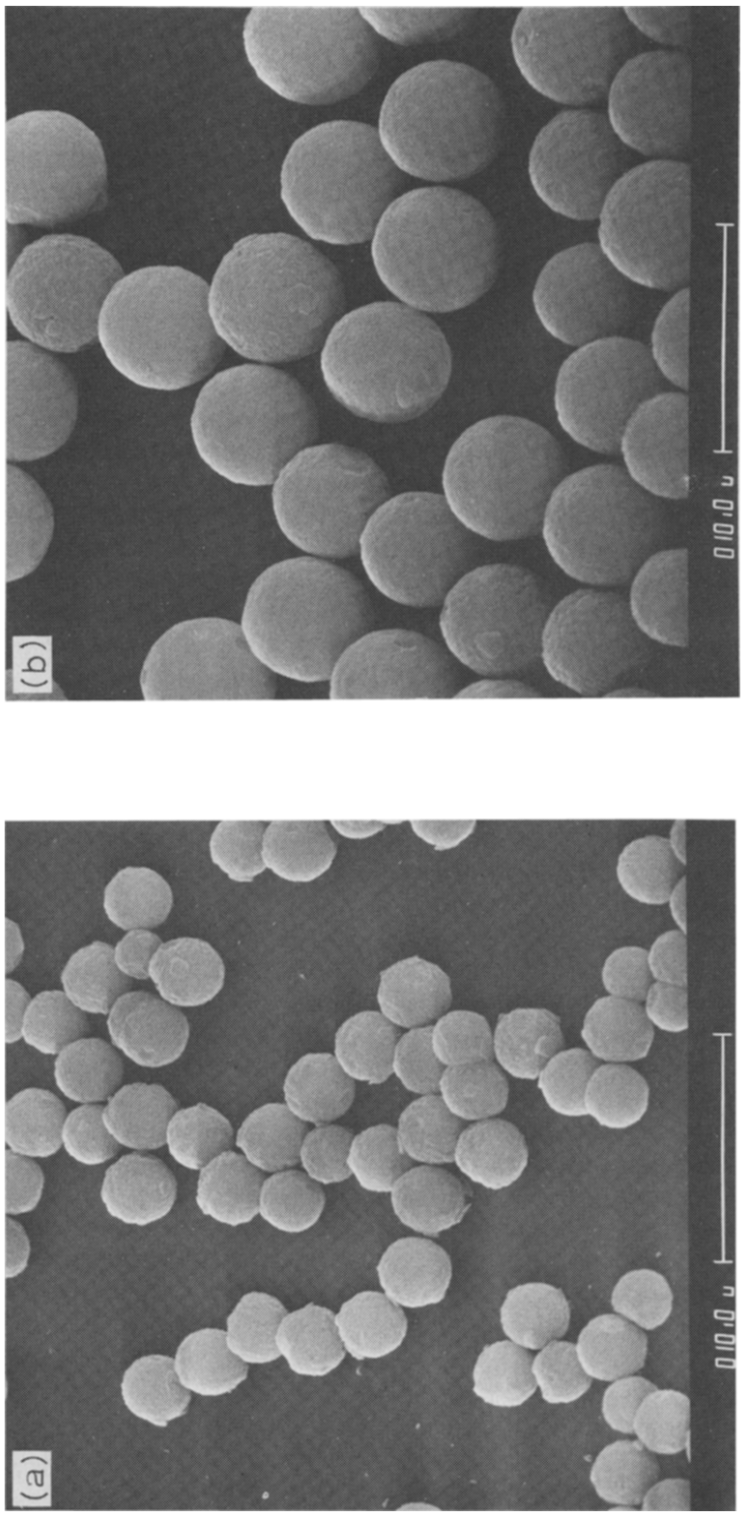


Fig. 1. Scanning electron microscopy (SEM) photographs of Zorbax particles: (a) 3-μm particles; (b) 6-μm particles.

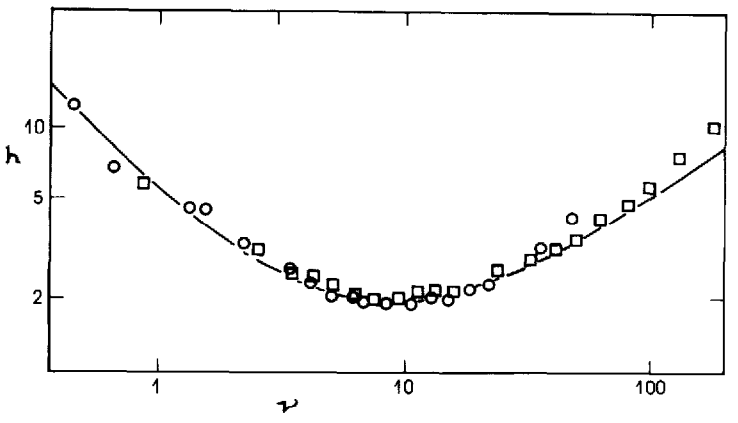


Fig. 2. Experimental plots of reduced plate height *vs.* reduced mobile phase velocity for 3-μm C_8 column (○) and 6-μm C_8 column (□). Di-*n*-octyl phthalate as solute (k' = 11.3–11.9), acetonitrile–water (85:15) as mobile phase, 25°C. Solid curve is from eqn. 6 with average coefficients from Table I (A = 0.545, B = 5.1, C = 0.0285).

illustrated in Table II by h values measured at the optimum value of v for various solutes and the columns in Table I. We see that minimum plate height values of 1.7–2.2 particle diameters are found after correcting for extra-column effects. Many columns similar to those in Tables I and II were packed, showing consistently low values of h at the optimum reduced velocity.

Observed column plate numbers. The data in Table II suggest that N should range between 12,000 and 15,000 plates for the present 3-μm columns (140,000–180,000 plates/m), in the absence of additional band broadening by the LC system and when operated at the optimum flow-rate for minimum h. Eqns. 5–5b allow us to predict *actual* N values as a function of column performance and the characteristics of the LC system. A model calculation summarized in Table III is of value in this connection. Here average column characteristics for the 3-μm particle columns in Table I are assumed, with a flow-rate for minimum h of V_m = 1.5 ml, F = 0.03 ml/sec (1.8 ml/min). A value of $\sigma_{ec}F$ equal to 0.016 ml is assumed, while τ is taken to be either 0.1 or 0.25 sec (values characteristic of the Model 8800 system). The value of h for the column in the absence of band broadening by the system is assumed to be equal to 2 particle diameters. Table III shows a negligible decrease in N for retained compounds as a result of the LC system time constant (0.1 sec). A minor decrease in N would be predicted for an analog signal (τ = 0.25 sec) calculated from a chromatogram on a strip-chart recorder, but this will not be of practical significance when automated data processing (τ = 0.1 sec) is used. The decrease in plate number is more significant as a result of the dead volume of the system (value of $\sigma_{ec}F$): a decrease in N of 17–37% for bands eluted with k' less than 1.5. The resulting decrease in resolution of early bands in the chromatogram is only marginally important, however. Thus, several considerations combine to minimize the loss in actual column plate number:

(1) For the columns in Table I it is found that the parameters A and C in eqn. 6 vary with k'[33]; as a result, an optimum value of F for compounds with k' = 2–5

TABLE I

CHARACTERISTICS OF 8.0 × 0.62 cm COLUMNS USED

Conditions selected in each case to yield a k' value of 12 ± 1; second moment data.

*Column**	*Knox parameters***			*Solute****	*Mobile phase*	*Error of fit*§	*Ø*§§	V_m (cm^3)
	A	*B*	*C*					
C_8, 3 μm§§§	0.50	5.2	0.030	DOP	Acetonitrile water (85:15)	0.26	700	1.25
C_8, 6 μm	0.59	5.0	0.027	DOP	Acetonitrile water (85:15)	0.07	470	1.31
C_{18}, 3 μm§§§	0.54	3.5	0.071	DOP	Acetonitrile water (81:9)	0.05	680	1.23
Silica, 3 μm§§§	0.79	1.3	0.32	PBA	CH_2Cl_2	0.02	680	1.73
Silica, 6 μm	0.73	1.3	0.22	PBA	CH_2Cl_2	0.05	760	1.96

* Nominal particle size; actual values 3.04 and 5.67 μm; pore diameters 6.0 nm for 3-μm columns, 7.5 nm for 6-μm columns.

** Corrected for H_{ec} (eqns. 5 and 5a).

*** DOP = di-*n*-octyl phthalate; PBA = *p*-bromoacetanilide.

§ RMS error of fit of data to eqn. 6 (least squares).

§§ Flow resistance parameter[17].

§§§ VSP (very-small-particle) columns.

TABLE II

EXPERIMENTAL VALUES OF MINIMUM PLATE HEIGHT h (SECOND MOMENTS) FOR COLUMNS IN TABLE I AND TWO DIFFERENT SOLUTES

Column	*Value of h at optimum flow-rate*			
	$k' = 2.4$*		$k' = 12$***	
	h	$h(corr)$**	h	$h(corr)$**
C_8, 3 μm	2.09	1.72	1.96	1.93
C_8, 6 μm	1.99	1.83	2.04	2.03
C_{18}, 3 μm	2.47	2.01	1.99	1.97
Silica, 3 μm	2.05	1.84	2.20	2.18
Silica, 6 μm	1.96	1.88	2.05	2.04

* Di-*n*-pentyl phthalate for C_8 and C_{18} columns; benzanilide for silica columns; mobile phases as in Table I.

** Corrected for H_{ec} (eqns. 5 and 5a).

*** Conditions as in Table I.

will result in $h > 2$ for compounds with smaller k' values. The percentage decrease in N predicted from Table III is then proportionately reduced.

(2) Separation conditions are usually adjusted to achieve k' values greater than 1 for all sample components, because of greater resolution at higher k' values (ref. 21, pp. 51–56).

(3) Second-moment calculations as in Table III tend to exaggerate the practical loss in resolution as a result of exponential (non-Gaussian) contributions to H_{ec}[34].

Decrease in N as a result of system band broadening becomes more serious for narrower columns. Thus eqns. 5a and 5b predict that H_{ec} and H_τ will be proportional to $1/V_m^2$, or the decrease in N as in Table III increases with $1/d_c^2$ (d_c is column internal diameter). Therefore, conventional 3-μm columns (*e.g.*, refs. 15–20) with dimensions

TABLE III

DEGRADATION OF COLUMN PERFORMANCE BY THE LC INSTRUMENT

3-μm columns in Table I operated at optimum flow-rate.

k'	*Decrease in N (%) due to system band broadening*		
	Extra-column (H_{ec})*	*Time-constant* (H_τ)**	
		$\tau = 0.1$	$\tau = 0.25$
0.5	−37	−2	−13
1.5	−17	−1	− 5
5.0	− 4	0	0

* Eqns. 5 and 5a; $\sigma_{ec}F = 0.016$ ml, $V_m = 1.5$ ml, $L = 8$ cm; $H_c = 2 \times 0.000304 = 0.000608$ cm.

** Eqns. 5 and 5b; conditions as in first footnote.

of 7.5–10 × 0.46 cm are much more susceptible to extra-column band broadening than the 8.0 × 0.62 cm columns in Table I.

In conclusion, it appears that the VSP columns in Table I can be used with LC systems such as the Du Pont Model 8800, using 8-μl detector flow cells and conventional sample-injection valves (see below), provided that the plumbing between the injector, column and detector is well designed. Under these conditions, little practical loss in column performance will result.

Sample size effects

As the amount of sample injected increases, there is eventually a corresponding decrease in column plate number. This effect can be dependent on the volume of sample solution, the mass of sample injected and the concentration of the sample solution. Each of these factors was studied for the 3- and 6-μm C_8 columns of Table I.

Sample volume. It is good practice to dissolve the sample in the mobile phase, and in this case the volume-variance of the sample band will be increased from $(\sigma F)^2$ to $[(\sigma F)^2 + \sigma^2]$ (ref. 9), where

$$\sigma_s^2 = V_s^2/\lambda^2 \tag{7}$$

V_s is the volume of sample injected and $\lambda^2 = 12$ for the case of plug flow on to the column[2]. For the usual case of a filled loop in a sample valve, $\lambda^2 = 4$–5[9,35] because of laminar flow of the sample from the loop, thus increasing the effect of sample volume on band width. We studied sample volume effects by injecting a fixed mass of sample (2 μg) dissolved in varying volumes of mobile phase, using a Rheodyne Model 7125 sample valve with a 50-μl loop. The results of this study for our 3-μm C_8 column are illustrated in Fig. 3 for two different solutes: di-*n*-pentyl phthalate (k' = 2.6) and di-*n*-octyl phthalate (k' = 12.1). The column plate number is plotted

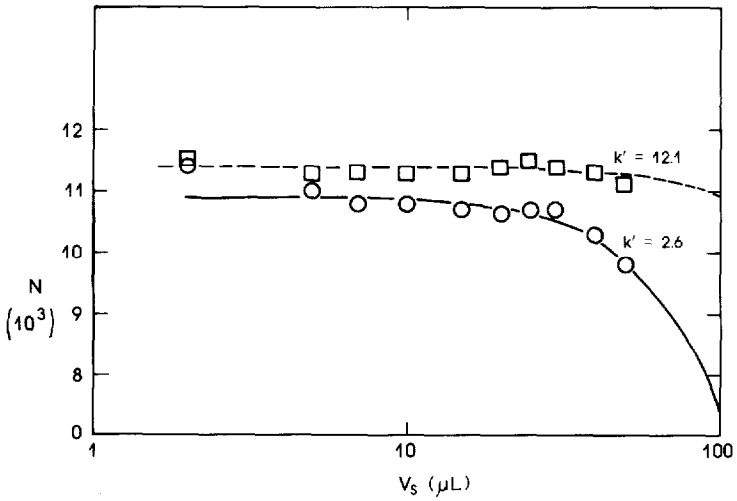


Fig. 3. Effect of sample volume on column plate number N. Data for 3-μm C_8 column, di-*n*-octyl phthalate (k' = 12.1) and di-*n*-pentyl phthalate (k' = 2.6) solutes; other conditions as in Fig. 2. The solid and broken lines are calculated curves from eqn. 7 with $\lambda^2 = 12$.

against sample volume V_s, and the curves are calculated from eqn. 7 for $\lambda^2 = 12$.

Several conclusions can be drawn from the data in Fig. 3. First, the calculated curves (solid lines) provide a good fit to the actual data. As λ^2 is taken as 12, this implies plug flow of sample on to the column. Apparently this is the result of our using the technique of partial filling of the sample loop by the sample, with back-flushing of the sample on to the column (which occurs with the usual sample injector designs). Thus, spreading of the sample plug during filling of the loop is cancelled by contraction of the plug during injection in the reverse direction (laminar flow in both directions). Injection of an air-bubble with the sample produces a similar result ($\lambda^2 = 12$) (ref. 36). Second, it is possible to inject large sample volumes, before significant further spreading of the sample band occurs as a result of volume overloading of the column. If a 10% decrease in N is allowable as a result of volume overload, then about 40 μl of a solute with $k' = 2.6$ can be injected. We can also estimate from Fig. 2 and eqn. 7 that the maximum sample volume for $k' = 0.5$ would be reduced to 20 μl. Finally, if complete filling of the sample loop is used, λ^2 will be closer to 5 than to 12, with a reduction in maximum sample volume (*vs.* partial filling of the loop) by a factor of 0.65, or a sample volume of 13 μl, for a sample with $k' = 0.5$. Fortunately, this volume is still compatible with conventional sample injectors which allow the use of sample loops as small as 10 μl. As a practical alternative, the partial fill technique (with use of an internal standard to correct for imprecise injection of sample into the loop) can be used with larger sample loops, provided the volume of sample injected is no greater than 20 μl.

Sample mass. The mass of sample injected can affect N values at sample sizes too small to result in changes in k'. Thus one study[24] showed that N can be reduced by as much as 30% when the mass of sample injected exceeds 2 μg per gram of column packing. The same study showed that the maximum allowable sample mass (with acceptable decrease in N) is smaller for columns packed with smaller particles. More recently a theoretical analysis of maximum sample mass has been given[25], with the conclusion that the mass $M_{max}^{10\%}$ for a 10% increase in bandwidth (and a 20% decrease in N) is predictable as

$$M_{max}^{10\%} = 2[(1 + k')/k']^2 \, W_s/N \tag{8}$$

where k' is the capacity factor for a small sample mass and W_s is the weight of sample taken up by the column at saturation (roughly 0.35 mg of sample per m^2 of stationary phase surface). Eqn. 8 predicts considerably larger values of $M_{max}^{10\%}$ than are reported in ref. 24, but this may be due to on-column injection of sample in ref. 24, with a resulting reduction in the amount of stationary phase seen initially by the sample. Both studies[24,25] show agreement of experiment and theory in predicting (a) smaller values of $M_{max}^{10\%}$ as d_p decreases (due to increase in N) and (b) a decrease in $M_{max}^{10\%}$ as k' increases. A further variable in defining mass overload in an LC system is the observation[37] that in one case mass overload occurred at higher sample sizes for more dilute samples, *i.e.*, use of larger sample volumes gave less reduction in N for the sample mass. We have accordingly investigated mass overloading of the present columns in terms of these previous findings[24,25,37].

Fig. 4 shows data for N *versus* sample mass for our 3- and 6-μm C_8 columns, for two different k' values (2.4 and 11); data points are shown only for the 3-μm

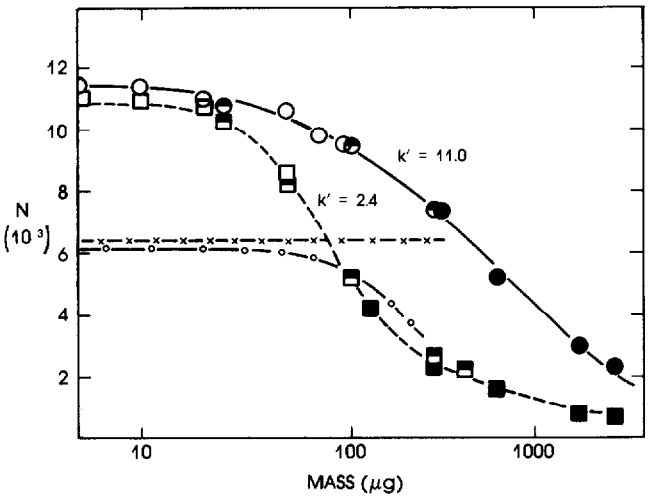


Fig. 4. Variation of column plate number N with sample mass. Conditions as in Fig. 2, except:

Solute	V_s (μl)			d_p (μm)	k'
	2	*10*	*50*		
DPP	□	◒	■	3	2.6
DOP	○	◓	●	3	12.1
DPP		◦		6	2.6
DOP		×		6	12.1

column, with experimental curves shown for the 6-μm-particle C_8 column for $k' = 2.4$ (○) and $k' = 11$ (×). The effect of solute concentration on mass loadability was studied at the same time, by injecting samples in different volumes of mobile phase (2, 10 or 50 μl). Concerning the effect of sample concentration, the data in Fig. 4 shows that injection of a given mass (*e.g.*, 250 μg) yielded the same value of N (7400, $k' = 11$; 2500, $k' = 2.4$) regardless of whether the sample volume was 10 or 50 μl. We therefore conclude that sample concentration *per se* has little effect on the mass loadability of these columns in the mass range used in this study.

The value of $M_{\text{max}}^{10\%}$ can be derived from these plots with the results given in Table IV.

TABLE IV

OBSERVED AND CALCULATED $M_{\text{max}}^{10\%}$ VALUES FOR COLUMNS WITH PARTICLES OF DIFFERENT DIAMETER

Column d_p (μm)	*k'*	*Observed $M_{max}^{10\%}$ (μg)*	*Calculated $M_{max}^{10\%}$ (eqn. 8) (μg)*
3	2.4	45	70
3	11	115	40
6	2.4	125	125
6	11	>250	70

The mass loadability of the 6-μm column is seen to be about double that of the 3-μm column, in accordance with eqn. 8 (N for the 3-μm column is double that for the 6-μm column). Eqn. 8 (with W_s estimated to be 0.19 g, from 550 m^2 of stationary phase surface per column) yields calculated values of $M_{max}^{10\%}$ which are in rough agreement with values found. However, eqn. 8 predicts a decrease in mass loadability as k' increases, whereas the opposite was found. We are not inclined to attribute much fundamental significance to these differences between theory and experiment. Emphasis should instead be given to the appreciable mass loadability of these columns, *i.e.*, about 50 μg of solute for the 3-μm column. Presumably mass loadability is determined largely by the amounts of *individual* solutes in the sample, so that total sample size could be significantly larger than 50 μg.

While it is encouraging that the mass loadability of the present 3-μm particle columns is reasonably large, it is of interest to consider the detection limits possible with these columns *vs.* their larger particle counterparts. Cooke *et al.*[16] have given perhaps the best analysis of this question, taking both bandwidth and detector characteristics into account. Their conclusion is that detection sensitivity is comparable for both 3- and 5-μm particle columns, even though small-volume flow cells were required for the 3-μm column in their study. Two additional factors must be considered, however, in the case of the present wider diameter columns. First, the same detector flow cell (1.0 × 0.1 cm) can be used for both 3- and 5-μm columns. Second, the mass loadability of the column can limit detection sensitivity for some samples. Eqn. 8 predicts that the mass loadability will be similar for columns with similar volume and N values (*i.e.*, 8.0 × 0.62 cm, 3-μm column *vs.* 15 × 0.46 cm, 5-μm column), and eqn. 1 states that the band volumes will be similar for these two columns when the column dead volume V_m is the same (which is the case for an 8 × 0.62 cm *vs.* a 15 × 0.46 cm column). If the same detector is used in each case, the detection sensitivity for the 3-μm *vs.* 5-μm columns should then be comparable. Thus our conclusion is that the present 8.0 × 0.62 cm, 3-μm particle columns used with conventional HPLC equipment will give about the same detection limits as would 15 × 0.46 cm, 5-μm particle columns in the same equipment.

The present data and eqn. 8 bear also on another question related to mass loadability and ultimate detection limits in LC. It had been claimed earlier[38,39] that the concentration rather than the solute mass in the injected sample is the fundamental restriction to increasing sample size for greater detectability. This view is inconsistent with chromatographic theory[40]; the present data as well as the analysis in ref. 25 support the conclusion that sample mass is of major importance in this respect.

Frictional heating and temperature effects

Various workers (*e.g.*, refs. 13, 22 and 23) have recognized that as d_p is decreased and mobile phase flow-rates are maintained roughly equal, there will be increased frictional heating of the mobile phase during its passage through the column. This will lead ultimately to a decrease in column N values as a result of the radial thermal gradients thereby produced. Cooke *et al.*[16] confirmed this effect experimentally for a 7.5 × 0.46 cm column of 3-μm particles. A rapid increase in H was observed as the mobile phase flow-rate was increased beyond 2 ml/min, and the effect could be largely eliminated by pre-cooling the mobile phase entering the column, thereby counteracting the frictional thermal gradients within the column.

We have not observed such large increases in H for our 3-μm columns at higher flow-rates, as can be seen from Fig. 2 and the C values in Table I for the C_8 columns; these values are both low (equal 0.03) and constant for particles of different size. The much larger C values for the silica packings in Table I cannot be attributed to frictional effects, as both C_8 and silica packings are predicted[41] to give similar thermal gradients (but see ref. 33). One possibility[41] is that our use of air-bath thermostating results in more nearly adiabatic operation of the column, with a resulting decrease in the thermal gradients within the column that cause a decrease in N. Examination of Fig. 2 shows some tendency for the values of H to increase faster than theory predicts, in the case of data collected at very high flow-rates (arrows labeled i and ii). However, the effect is small and occurs only for flow-rates in excess of 10 ml/min.

Slow interfacial mass transfer

Slow interfacial mass transfer has been examined both theoretically and experimentally[26], and certain predictions about its practical consequences can be made: (1) it should increase in importance for small values of d_p; (2) it should result in an increase of C *vs.* the case of fast interfacial mass transfer; (3) this effect should generally be greatest for the most strongly retained solutes, having larger k' values; (4) solutes with numerous interactive functional groups in the molecule (*i.e.*, polar and/or acidic or basic groups) will generally exhibit the slowest mass transfer across the interface. Thus, lower plate numbers might be expected for the combination of small-particle columns and "real" multifunctional samples.

The data in Table I suggest that interfacial mass transfer is not slow for the C_8 columns, where comparable values of C are found for both the 3-μm (0.030) and 6-μm packings (0.027). The larger C value for the 3-μm silica (0.32) *vs.* the 6-μm silica (0.22) might be attributed to slow interfacial mass transfer, but other work[33] suggests that small differences in particle pore diameter are involved here (6.0-nm pores for 3-μm particles *vs.* 7.5-nm pores for 6-μm particles).

Previous work suggests that slow interfacial mass transfer leads to band tailing in addition to a decrease in plate number, and such effects are commonly observed for various polar compounds and silica-based packings. However, it is also found that such tailing and reduction in N can generally be eliminated by chemical means, *e.g.*, addition to the mobile phase of amines or various buffers, variation of pH, etc. A few examples further suggest that this will prove true for most practical separations. Thus, one study[42] achieved minimum h values of about 2 with 6.6-μm packings for the very polar aminosulfonic acids used as dye intermediates. In that study ion pairing was used, which presumably provides chemical suppression of the original strong solute–silica interactions. Another study[43] reports plate height data for the reversed-phase separation of peptides as large as insulin (6000 daltons), on both 5- and 10-μm columns. There is fairly close agreement of the resulting h *vs.* v plots with each other and with data for the columns in Table I. As these peptide separations are carried out at large values of v, where the C term in eqn. 6 is dominant, this implies little contribution of slow interfacial mass transfer.

Operational stability of 3-μm columns

VSP columns tend to have shorter lifetimes *vs.* larger-particle columns, unless

special precautions are taken. They are more easily plugged by particulates, and they have been reported to be more sensitive to hydraulic and temperature shock. In our experience the major problem in the case of well made 3-μm particle columns is pluggage of the frit and/or adjacent column packing at the column inlet. Thus all the measures routinely used with 5-μm and larger particle columns to avoid plugging become even more important for 3-μm columns. These include the use of pre-columns between pump and injector, guard columns between injector and column, in-line filters between pump and column and adequate filtering and/or clean-up of the sample. The extent to which some or all of these measures become necessary for reasonable column lifetime depends on various factors peculiar to the individual laboratory.

Where only particulate material is of concern, filtration of the sample with 0.5-μm filters can be combined with the use of in-line 0.5-μm filters between the pump and injector, and between the injector and column. The major concern with filters between the injector and column is that they should not appreciably increase the extra-column volume of the system. We have found several commercially available in-line filters (having σ_{ec} values of 0.005–0.010 ml) to be acceptable in this regard. One example is the Upchurch Precolumn filter (Upchurch Scientific, Oak Harbor, WA, U.S.A.) with a σ_{ec} of about 0.008 ml, measured at $k' = 2$.

In some cases we have observed column pluggage that appears to involve retention of strongly adsorbed compounds from the mobile phase and/or samples used with these columns. When the packing at the head of the column is strongly discolored and/or assumes a cement-like consistency, the use of guard columns between injector and column is advisable. Again the primary concern is the avoidance of large σ_{ec} values associated with the guard column, as reviewed in ref. 21, pp. 228–230.

Hydraulic shock to the column can arise as a result of the operation of the sample valve[44]. Slow rotation of the valve, especially in the automated injection mode, in combination with higher flow-rates and/or inherently unstable columns, can result in a severe decrease in plate number within 50–100 runs. We have subjected the columns described in Table I to long-term repetitive, automated sample injection with the Model 8800 system, without encountering this particular problem. When the problem of premature loss of plate number arises with small-particle columns, several corrective measures exist. The use of a bypass around the injector[44] has been suggested as a remedy that eliminates the problem. Increasing the speed of rotation of the sample valve, *e.g.*, by using helium rather than air or nitrogen in pneumatic actuators, is also effective.

We have investigated the stability of the present VSP columns to changes in temperature, and found the columns to be stable to temperature cycling between the limits of ambient and 60°C.

Some practical applications of the present 3-μm columns

The primary practical characteristic of small-particle columns is that they can provide faster separations (all other factors being equal) than their larger particle counterparts. This leads to the possibility of very fast separations of simple mixtures (*e.g.*, separation times of 1 min or less[15]), more rapid method development[16] or simply a general decrease in analysis time for most samples. The optimal usage of these columns may involve both replacement of older, slower separations and the use

of VSP columns for scouting purposes in method development. In either of these applications it is essential that the retention characteristics of columns of different particle size be essentially invariant. Finally, it is important that the same HPLC equipment can be used for both VSP and larger particle columns.

We have carried out several model separations which illustrate the ability of the present VSP columns to achieve the above objectives, using standard LC equipment such as the DuPont Models 850 and 8800. Because of the unique way in which Zorbax particles are synthesized[28,29], yielding a precisely defined and reproducible particle matrix, we believe that retention reproducibility (among columns of different d_p) should be particularly favorable with these columns. The approach followed to demonstrate that these requirements have been met by the present columns is as follows. An earlier separation that had been achieved on a 6-μm Zorbax C_8 column was repeated under similar conditions with the new 3-μm Golden Series Zorbax C_8 column in Table I. Small adjustments in mobile phase composition were

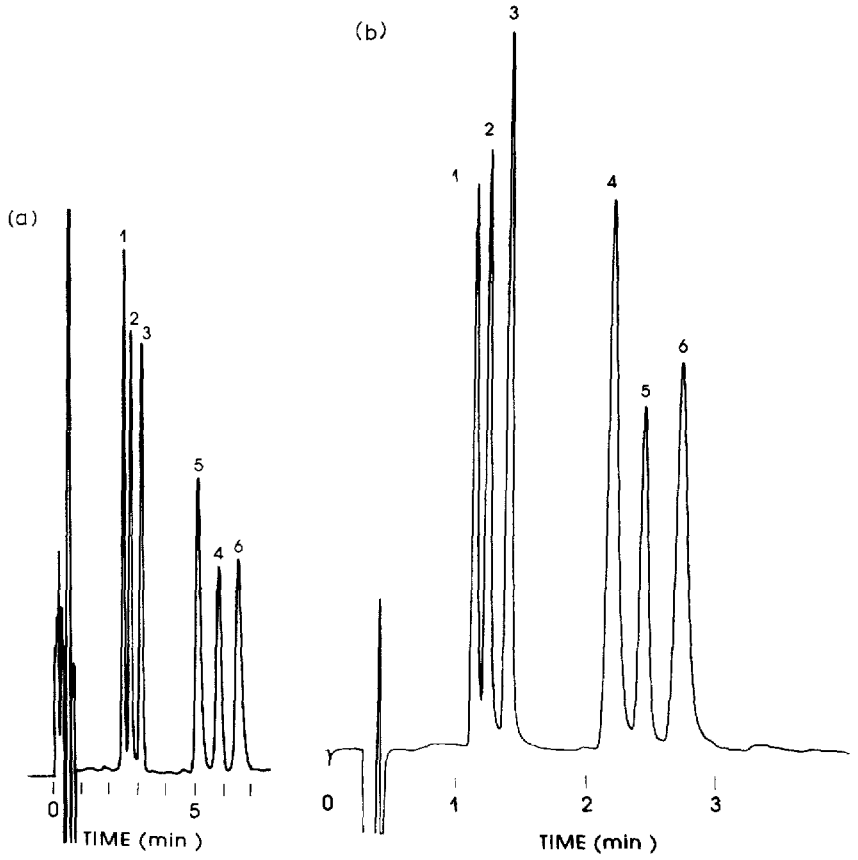


Fig. 5. Separation of steroid mixture: 3-μm wide-bore *vs.* 6-μm standard columns. (a) 6 μm Zorbax C_8 column, 15 × 0.46 cm, 50°C, UV detection at 254 nm, mobile phase acetonitrile–tetrahydrofuran–water (11:12:77); (b) 3-μm Golden Series Zorbax C_8 column, 8 × 0.62 cm, mobile phase acetonitrile–tetrahydrofuran–water (10:13:77); other conditions as in (a). Compounds: 1 = prednisone; 2 = cortisone; 3 = hydrocortisone; 4 = dexamethasone; 5 = corticosterone; 6 = cortexolone.

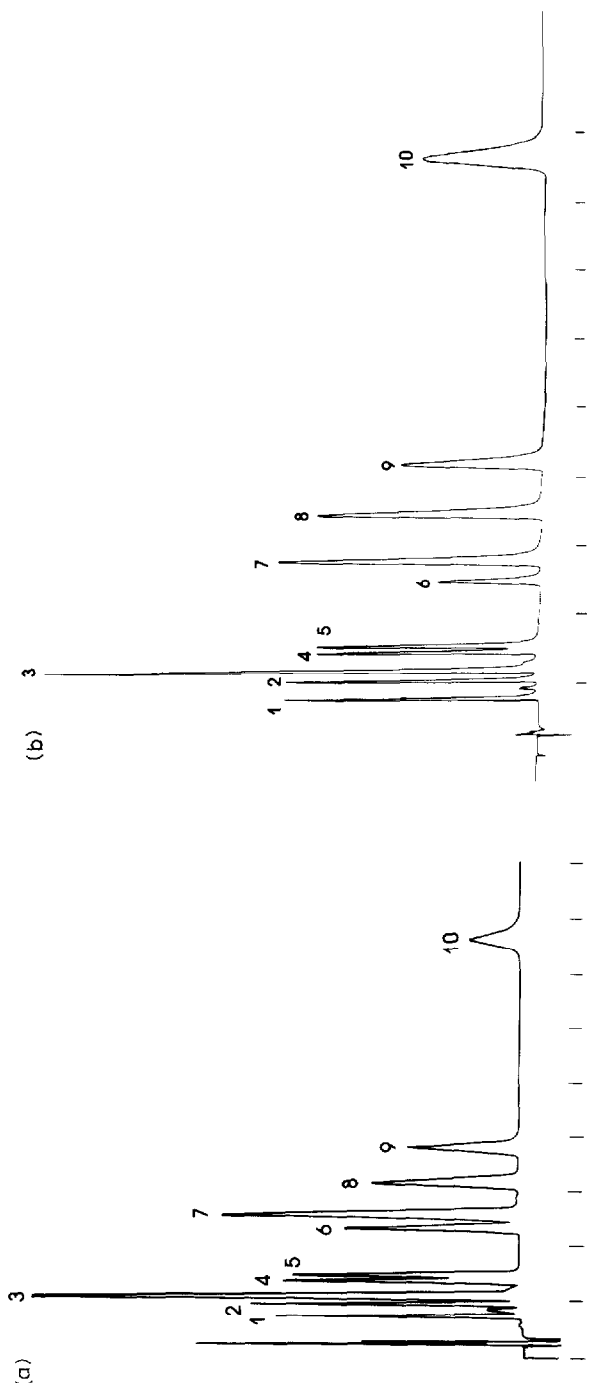


Fig. 6. Separation of phenol mixture: 3-μm wide-bore *vs.* 6-μm standard columns. (a) 6 μm Zorbax C_8 column, 15 × 0.46 cm, mobile phase 1% acetic acid/water–methanol–acetonitrile (63:34:3), other conditions as in Fig. 5a; (b) 3-μm Golden Series Zorbax C_8 column, 8 × 0.62 cm, other conditions as in Fig. 6a. Compounds: 1 = phenol; 2 = *p*-nitrophenol; 3 = 2,4-dinitrophenol; 4 = *o*-chlorophenol; 5 = *o*-nitrophenol; 6 = 2,4-dimethylphenol; 7 = 4,6-dinitro-*o*-cresol; 8 = 4-chloro-*m*-cresol; 9 = 2,4-dichlorophenol; 10 = 2,4,6-trichlorophenol.

allowed to duplicate the original separation as closely as possible, but other changes were avoided. The objective was to retain the resolution of the original separation on a 6-μm column while shortening separation time on the 3-μm column as much as possible.

Fig. 5a shows a separation(6-μm column) of a six-component steroid mixture, using a ternary solvent mobile phase (11:12:77 acetonitrile–tetrahydrofuran–water); the separation time is 7 min. Fig. 5b shows the separation on our 3-μm C_8 column of a similar sample, with essentially the same mobile phase (10:13:77 acetonitrile–tetrahydrofuran–water) to achieve similar relative retention and solute α values. The separation time is now only 3 min, or less than half that originally required with the 6-μm column.

Fig. 6a shows separation of a ten-component mixture of phenols on a 6-μm column, using a four-solvent mobile phase (62:34:3:1 water–methanol–acetonitrile–acetic acid). The separation time was 16 min. Fig. 6b shows the separation of a similar sample on the 3-μm column, with no adjustment to mobile phase compo-

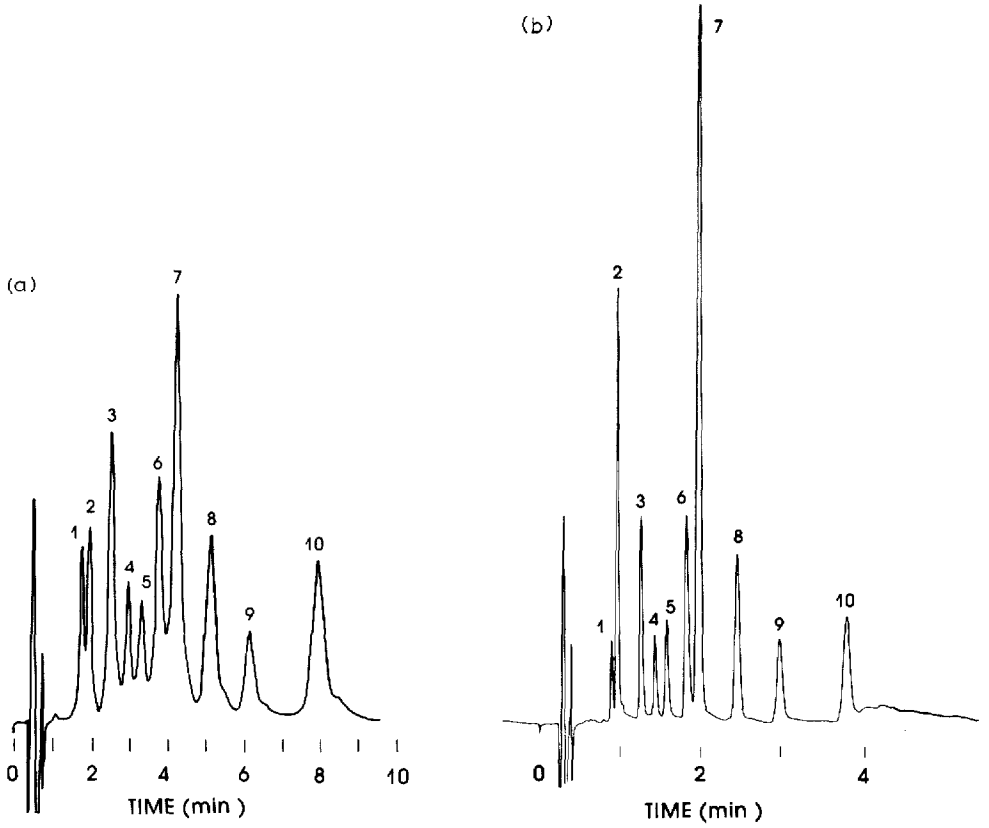


Fig. 7. Separation on herbicide mixture: 3-μm wide-bore *vs.* 6-μm standard columns. (a) 6-μm Zorbax C_8 column, 15 × 0.46 cm, mobile phase 1% acetic acid/water–methanol–acetonitrile–tetrahydrofuran (53:9:30:8), other conditions as in Fig. 5a; (b) 3-μm Golden Series Zorbax C_8 column, 8 × 0.62 cm, mobile phase 1% acetic acid/water–methanol–acetonitrile–tetrahydrofuran (53:9:31:7), other conditions as in Fig. 7a. Compounds: 1 = 2,4-D; 2 = Ramrod; 3 = 2,4,5-T; 4 = 2,4-DB; 5 = 2,4-D methyl ester; 6 = Silvex [2-(2,4,5-T) P-acid]; 7 = CIPC; 8 = 2,4,5-T methyl ester; 9 = 2,4-DB methyl ester; 10 = Silvex methyl ester.

sition. The separation time for the latter analysis is only 9 min, or about half that required earlier. In this case the resolution in Fig. 6b is somewhat better than that in Fig. 6a.

Fig. 7a shows the 8-min separation on a 6-μm column of a ten-component mixture of herbicides. A similar sample was separated with a 3-μm column, as shown in Fig. 7b, with a very minor change in mobile phase (1% increase in acetonitrile to 31% and a 1% decrease in tetrahydrofuran to 7%). The resulting separation shows generally better resolution in half the time (4 min).

Fig. 8a shows the separation of an eight-component mixture of photo-reactive chemicals, using a four-solvent mobile phase with a 6-μm C_8 column. The separation time is almost 8 min. Fig. 8b shows the separation of a similar sample in half the time (3.5 min) with equivalent resolution. Each of the separations so far discussed (Figs. 5, 6, 7 and 8) had been initially optimized (6-μm column) for resolution by the four-solvent optimization strategy of Glajch *et al.*[45], so that further improvements in separation involve column efficiency or theoretical plates per unit time.

VSP columns can also greatly speed up separations by gradient elution, al-

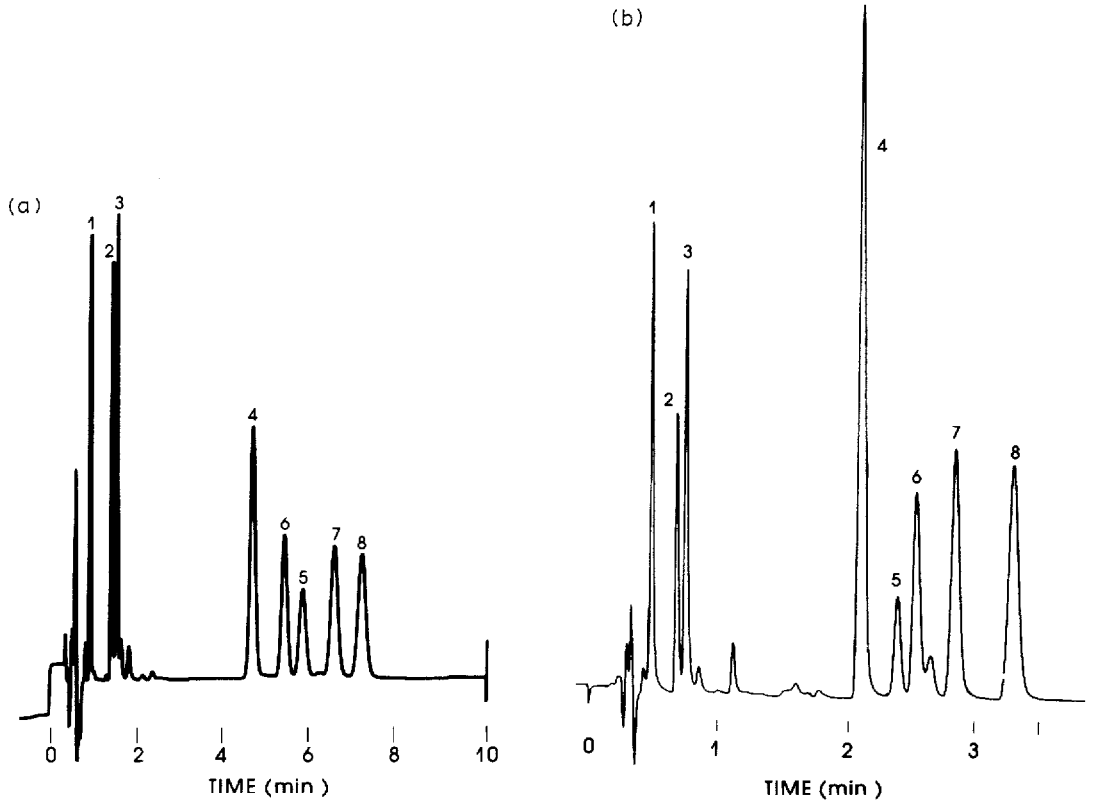


Fig. 8. Separation of photo-reactive chemicals: 3-μm wide-bore *vs.* 6-μm standard columns. (a) 6-μm Zorbax C_8 column, 15 × 0.46 cm, mobile phase methanol–acetonitrile–tetrahydrofuran–water (22:26:1:51), other conditions as in Fig. 5a; (b) 3-μm Golden Series Zorbax C_8 column, 8 × 0.62 cm, other conditions as in Fig. 8a. Compounds: 1 = *p*-methoxyphenol; 2 = diethylene glycol diacrylate; 3 = acetophenone; 4 = benzophenone; 5 = cyclohexylacrylate; 6 = 1,6-hexanedioldiacrylate; 7 = dimethoxyacetophenone; 8 = Michler's ketone.

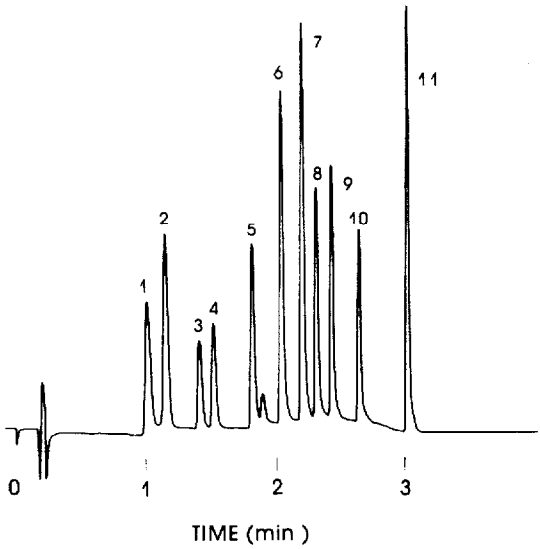
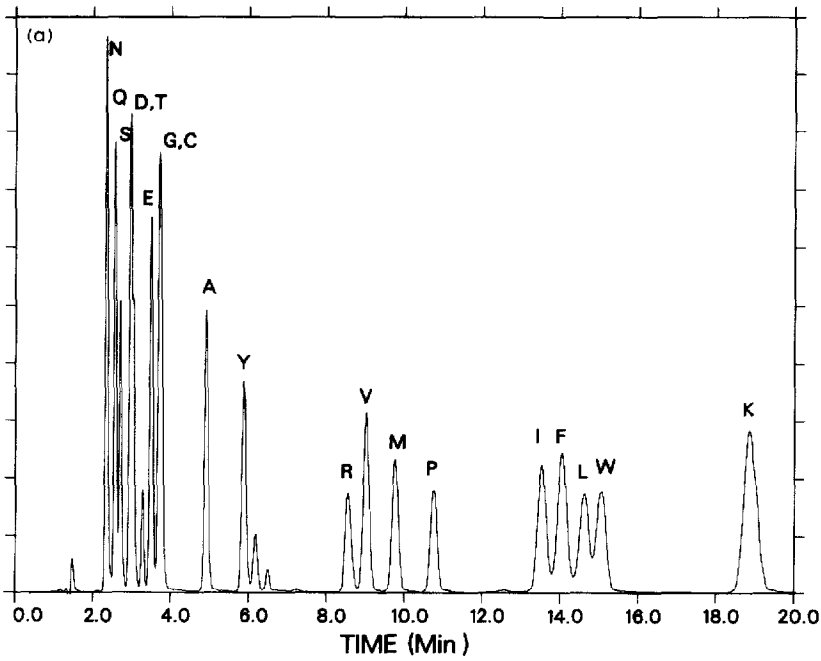


Fig. 9. Fast-gradient elution separation with 3-μm wide-bore column. Column, 3-μm Golden Series Zorbax ODS, 8 × 0.62 cm, gradient from 25 to 90% acetonitrile–water in 2 min, flow-rate 9 ml/min, pressure 350 bar. Compounds: 1 = benzaldehyde; 2 = acetophenone; 3 = nitrobenzene; 4 = benzene; 5 = toluene; 6 = ethylbenzene; 7 = cumene; 8 = *tert.*-butyl benzene; 9 = di-*n*-butyl phthalate; 10 = di-*n*-pentyl phthalate; 11 = di-*n*-octyl phthalate.

though the use of smaller volume columns means that the volume of the gradient mixer must be correspondingly reduced if the original gradient shape is to be preserved. The present 8.0 × 0.62 cm 3-μm columns have a volume equivalent to that of previous 15 × 0.46 cm 5-μm columns, and so can be used with the same gradient elution equipment, as illustrated in Fig. 9 An eleven-component synthetic sample is separated in 3 min on a standard DuPont Model 8800 system, with another 2 min required to re-equilibrate the column prior to the next sample injection (see discussion in ref. 21, Ch. 16). Thus gradient elution with the present 3-μm columns is potentially suitable for routine applications that require fast separation.

A final application of 3-μm packings in 8.0 × 0.62 cm columns is shown in Fig. 10 for the separation of the standard twenty-component mixture of phenylthiohydantoin (PTH) derivatives of amino acids as obtained from the sequencing of proteins. The isocratic separation of this mixture has been reported in a time of about 25 min, using optimized stationary and mobile phases[4-6]. The results of a non-optimized separation using a 6-μm phenethyl bonded-phase column are illustrated in Fig. 10a and b. All twenty compounds are reasonably well separated in 20 min. In Fig. 10c and d the same separation by means of an experimental 3-μm phenethyl column is shown, in a time of only 11 min. In addition, the excellent peak symmetry of most of these bands should be noted; the largest asymmetry factor (1.25) is observed for arginine, while other bands have asymmetry factors within an experimental error of 1.0.



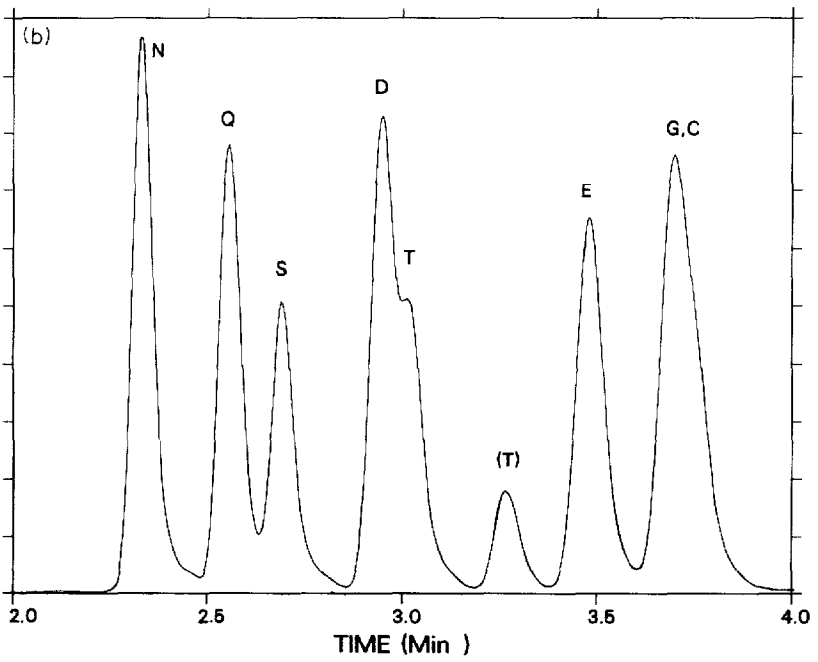


Fig. 10.

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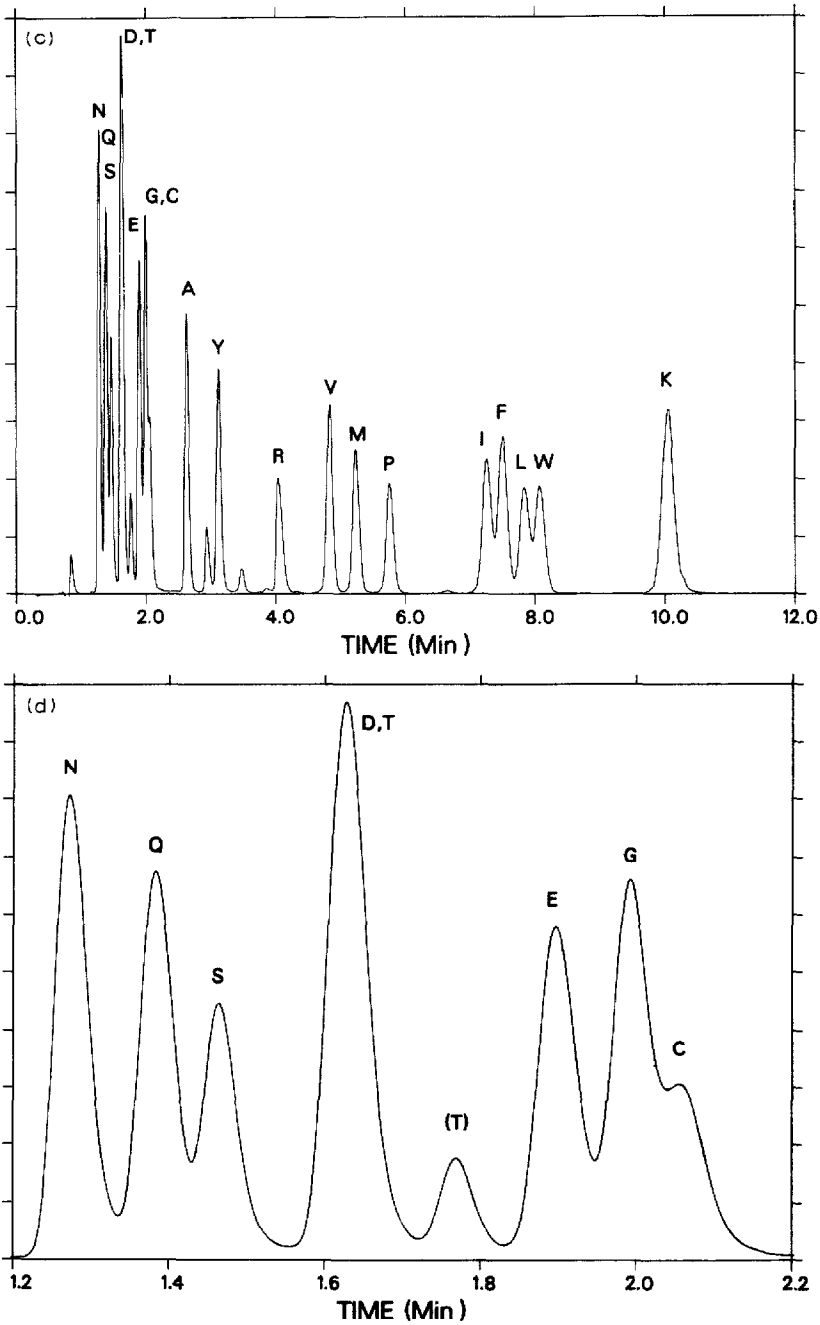

Fig. 10. Separation of 20 PTH amino acids: 3-μm wide-bore *vs.* 6-μm standard columns. (a) Experimental 6-μm Zorbax phenethyl column, 15 × 0.46 cm, flow-rate 2.0 ml/min, temperature 35°C, mobile phase acetonitrile–12 m*M* phosphate buffer (pH 3.2) (36:64); (b) expanded chromatogram from (a); (c) experimental 3-μm Zorbax phenethyl column, 8 × 0.62 cm, other conditions as in (a); (d) expanded chromatogram from (c). Compounds: A = alanine; C = carboxymethylcysteine; D = aspartic acid; E = glutamic acid; F = phenylalanine; G = glycine; I = isoleucine; K = lysine; L = leucine; M = methionine; N = asparagine; P = proline; Q = glutamine; R = arginine; S = serine; T = threonine; (T) = dehydrothreonine; V = valine; W = tryptophan; Y = tyrosine.

CONCLUSIONS

Columns with 3-μm particles based on Zorbax packings have been found to be stable and efficient when evaluated in various ways. Column performance as measured by the Knox equation (eqn. 6) shows values of the coefficient A in the range 0.5–0.7, and minimum reduced plate heights of about 2 particle diameters. The present wide-diameter column configuration allows the use of these columns with standard LC equipment, generally without modification (*e.g.*, DuPont Model 850 or 8800), and probably with no more than minor replumbing in less optimum cases. Thus the proven ability of 3-μm particle columns to greatly accelerate many LC separations (*e.g.*, refs. 7 and 12–20) is now routinely available to most laboratories. However, the greater tendency of these very small particle columns to become plugged by particulates requires additional precautionary measures: in-line filters, guard columns, sample clean-up, etc.

A number of fundamental questions have been raised previously concerning the ability of very small particle columns to perform as well as theory predicts. Some of these questions have been examined here, and it has been shown that there is no reason to believe that 3-μm particles cannot closely approach the performance suggested by theory. Frictional heating of the mobile phase has been shown experimentally and theoretically to be a possible problem than can limit column efficiency at higher flow-rates. However, using air-bath thermostating of the column and mobile phase, we have not seen any significant evidence for a decrease in column plate number as a result of such effects, when making use of 3-μm particles in combination with wider diameter columns.

Other studies have suggested that VSP columns would become overloaded by samples larger than a few micrograms. Although we find, in agreement with theory, that the maximum sample size decreases with d_p, it is still possible to inject 50–100 μg of sample on to 8.0 $\times$ 0.62 cm 3-μm columns without a significant decrease in plate number. Using the partial-loop-fill technique, sample volumes as large as 20 μl can be injected with little effect on N. Thus, conventional sample-injection valves can be used with these columns.

Finally, slow interfacial mass transfer was not observed with 3-μm Zorbax columns, and we believe that this effect can generally be eliminated by attention to the chemistry of the LC system. Thus, no fundamental impediments to the full exploitation of the potential of 3-μm particle columns were found. It appears that the combination of small-diameter particles with wider diameter columns opens up new capabilities for the routine HPLC laboratory.

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