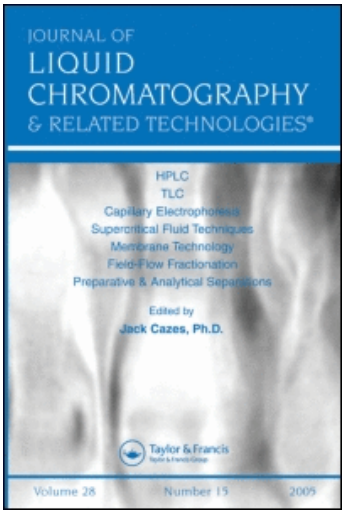

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SOME SAMPLING EFFECTS IN LIQUID CHROMATOGRAPHY

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ABSTRACT

The relationship between column packing particle size and column efficiency under mass overload conditions was examined with three silica-based packing materials of moderate surface area (S_{BET} range 200-500 $m^2 g^{-1}$). An equivalent relation between reduced plate height, H/dp, and relative sample load, sample mass/mass packing, was shown for all materials examined, both in the adsorption and reversed phase modes of chromatography over a narrow range of reduced velocity, udp/Dm. The data were used in a simple calculation to establish the minimum dimensions of columns packed with dp=5μm and 20μm particles for a typical semi-preparative separation of a mixture of 20 mg each of two substances.

A short study on the effect of variation of reduced velocity on reduced plate height at 'analytical' and semipreparative sample loadings showed that performance of LC columns in the preparative mode could be described reasonably well as a three dimensional surface in which the Y axis is reduced plate height, the X axis is reduced load, and the Z axis is reduced velocity.

Some aspects of the performance of a 'curtain-flow' preparative precolumn/column system are described along with an outline of the principles of design of this apparatus.

INTRODUCTION

Over the past few years several groups[1,2,3,4,5,6] have looked in detail at the relationship of the resolving power of a

chromatographic column to the mass (and volume of the sample solution) of the sample. Although this relationship, the basis of preparative applications of liquid chromatography (LC), is reasonably well understood, there are some contradictions in the data, particularly those of Done[1] and Kraak, et al.[2] It was these contradictions, which suggested that large particle column packings (dp>20μm) and reverse-phase (bonded) column packings were less affected by mass overload than small particle (dp 5-10 μm) adsorbent packings, that led to part of this study.

The LC parameter insufficiently appreciated (except by Done[1] and Kraak[2]) is the linear velocity of the eluent, since it is well understood that resolving power is a function of both flow rate and column packing particle size. Expression 1 has been derived by several workers[7,8] and shows that resolution, Rs, of two eluites is proportional to the square root of the number of theoretical plates, N.

$$(1) \qquad Rs = \tfrac{1}{2}\left[\frac{1-\alpha}{1+\alpha}\right]\left[\frac{\bar{K}'}{1+\bar{K}'}\right] N^{\frac{1}{2}}$$

where $\alpha = \dfrac{K'_2}{K'_1}$ and $\bar{K}' = \dfrac{K'_1 + K'_2}{2}$. N is defined as Z/H, where Z is the column length and H is the height of a theoretical plate, so Rs must be proportional to the reciprocal of the square root of H.

$$(2) \qquad Rs \sim (H)^{-\frac{1}{2}}$$

This fundamental parameter of liquid chromatography, H, can be more readily used in reduced form, $h = \dfrac{H}{dp}$, and Knox[9] has shown that, for a point injection of a negligible amount

of sample the semi-empirical equation:

$$(3) \qquad h = \frac{B}{\nu} + A\nu^{1/3} + C\nu$$

describes the relation between h and the reduced linear velocity, $\nu = \frac{udp}{Dm}$, where u is the linear velocity of the eluent, and Dm is the diffusion constant of the sample in the eluent phase. Since H is directly proportional to dp and is also a complex function of u, clearly both these variables must be considered in any study of sample overload effects. Well packed columns show minimum H values in the range between 1.5 and 3.0 particle diameters at reduced velocities, ν, in the range between 2 and 8. Accordingly, each of the columns in this study was operated at a flow rate appropriate to this range of reduced velocity values.

Furthermore, in order to reduce errors due to variable effects of sampling, connectors, and detector cells on effective resolving power, the bulk of these data were collected with eluites having a restricted retention range, i.e., K' between 2 and 3.

EXPERIMENTAL

General Methods and Materials

Most of the chromatographic columns and septum injectors used in these experiments were made in this department to a design similar to that now marketed by Shandon Southern Products Ltd (Runcorn, England). Samples were usually injected in either on-stream or stop-flow modes by syringes purchased from SGE, Ltd (London, England), although some data were also collected with sampling systems using Rheodyne Model 7120 (Rheodyne Inc., Berkeley, CA, USA) and Specac P/N30.100

(Analytical Accessories Ltd., Orpington, Kent, England) injection valves. Laboratory-made fixed wavelength detectors (Cell volume 8μl) and gas-driven pneumatic amplifier pumps completed the systems used in this study.

Column packings were derived from totally porous silica gel of two types: (1) SOTLC grade ground macroparticulate (dp=1-40μm, S_{BET} = 500 $m^2 g^{-1}$) silica gel (Whatman Lab Sales Ltd., Balston, England) and Hypersil spherical (dp 5-9 μm, S_{BET} = 200 $m^2 g^{-1}$) silica gel (Shandon Southern Products, Ltd). The ground silica was fractionated by hydraulic elutriation into size fractions in steps of doubled flow rate, and these isolated fractions were compared to samples of Partisil 10 and examined under a microscope to determine the equivalent (spherical) diameter. The Hypersil was obtained in three mean particle diameter ranges (5, 7.5 and 9 μm) as well as in its octadecylsilylated version, ODS-Hypersil (dp 5μm and 7.5μm).

All the 'analytical' size columns (Ø 4.6, 5.0, and 7.0 mm) were packed by the upward flow slurry packing method of Bristow, et al.[10] using methanol for suspension of silica packings and acetone for suspension of ODS-Hypersil. The larger bore (up to 1000 x Ø 22 mm) columns were packed in sections by a downward flow slurry method; the silica packing was suspended in dilute aqueous ammonia solution. These larger columns all gave reduced plate heights between 2.5 and 4 with 'analytical' size samples, as opposed to the slightly better range of 1.8 to 3.5 for the smaller columns packed by the upward flow technique.

Nearly all the adsorption chromatography separations were carried out with one batch of petroleum ether (boiling range 80-100°C), to which small amounts of AR acetone, ethyl acetate, and propan-2-ol were added as required. The hydrocarbon

solvent was recovered by washing 3x with an equal volume of water, drying with anhydrous magnesium sulphate, and passage (in 1.5 litre lots) through a short column (100-150 x Ø 35 mm) of chromatographic alumina (100-200 m) when acetone and propanol modifiers were used. Methanol was HPLC grade from Rathburn Chemicals Ltd. (Walkerburn, Scotland).

'Curtain Flow' Large Column Systems

All the large bore (Ø 9.5-Ø 22) columns were made to a design which incorporated a main column top assembly into which a precolumn of equivalent or smaller bore could be inserted, as shown in Figure 1. This system was devised after observation of the all-glass preparative LC apparatus in use in this department. It appeared that sample zones that were well shaped in the 'small' precolumn (200 x Ø 15 mm) of this glass system were frequently distorted badly on entry (via precolumn exit frit, connecting tube, and column top frit) to the main column (1000 x Ø 25 mm). Accordingly, the coupling between the precolumn and the column proper was made as direct as possible, with only a thin woven stainless steel cloth disc separating the two packed beds, but still allowing space for an annular outlet/inlet port connected to waste or to a pump via a three way valve.

This system was tested in three forms: (1) a minimum length, Ø 9.5 mm precolumn was attached to a septum injector for column efficiency testing; (2) a 120 x Ø 9.5 mm precolumn with ca 5 cm^3 liquid capacity was used in place of (1), and (3) samples were loaded through a 120 x Ø 22 mm precolumn with ca 34 cm^3 liquid capacity. Analytical scale samples were injected into the flowing stream, but large volumes (up to the holding capacity of the precolumn) were injected with flow off

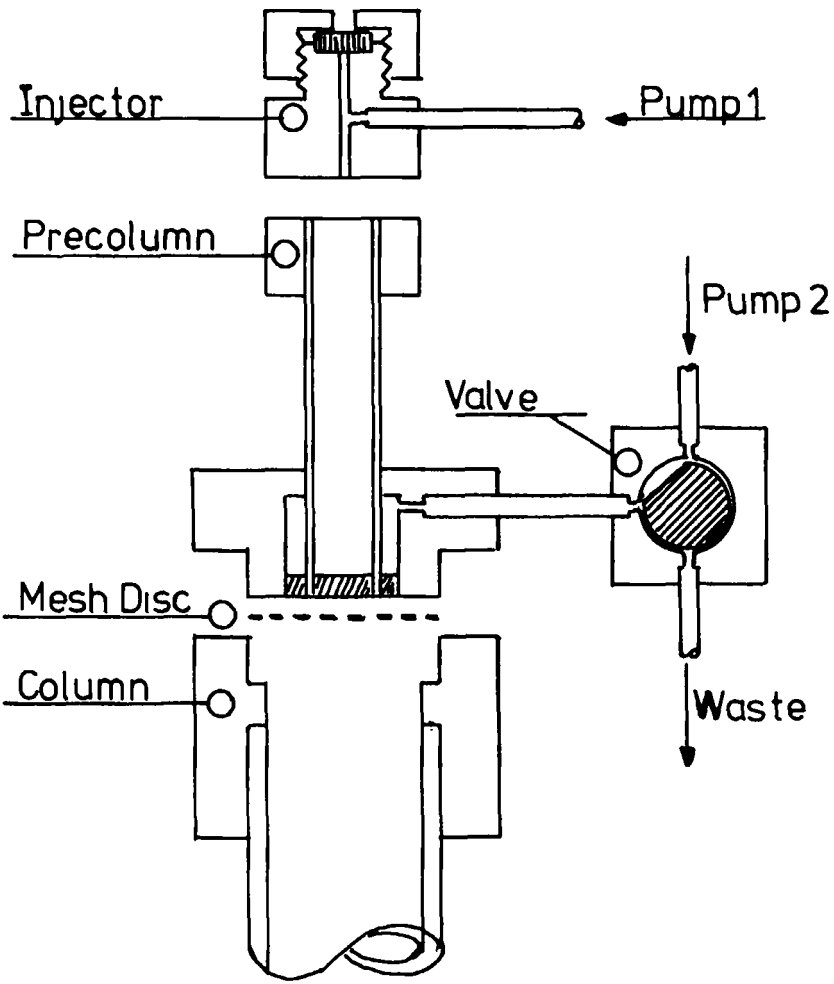

FIGURE 1. Curtain Flow Pre-column/Column Assembly

and the three way valve open to waste, so that the only viscous resistance to sample loading was that of the precolumn.

However, even with the minimised viscous resistance of the above system, loading large (>1 cm^3) volume of solutions of analytes dissolved in the relatively low viscosity solvents

used in adsorptive chromatography always led to leakage in conventional all-glass syringes. A gas-ballasted loading tube attachment (cf. Fig. 2) which coupled to the top of the Ø 9.5 mm

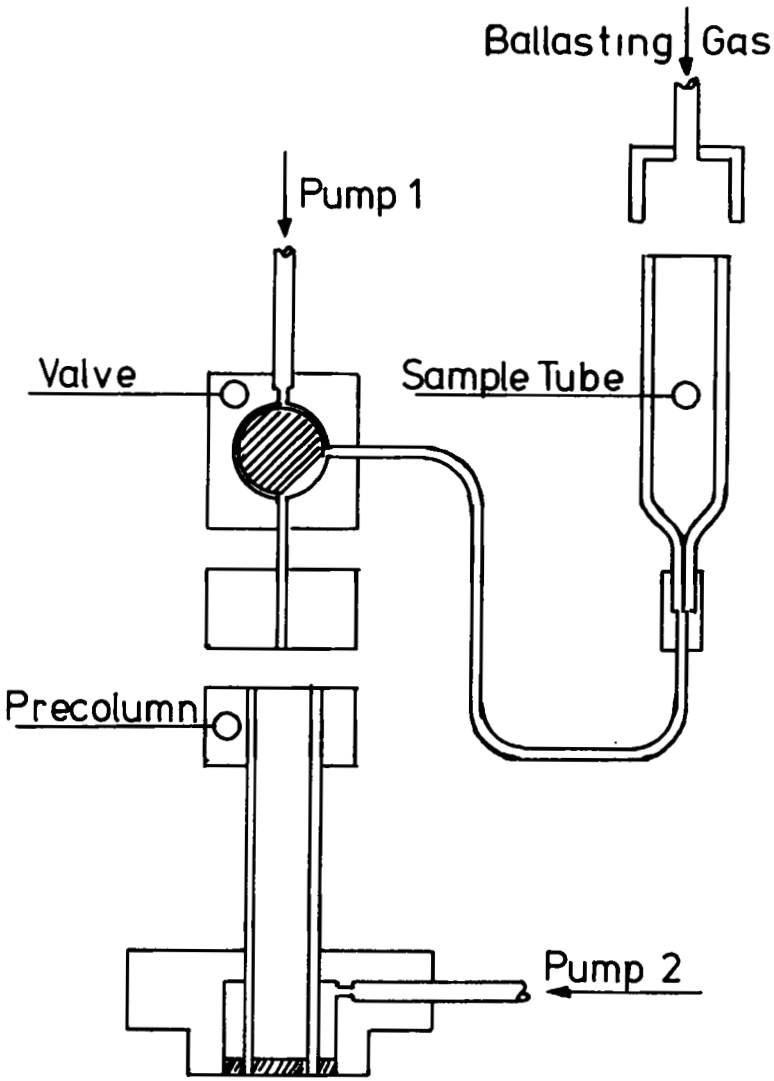

FIGURE 2. Gas-pressurised Loading Tube Attachment

precolumn via a short Ø 0.5 mm capillary tube (similar to that described by Godbille and Devaux[4]) was used in several experiments to avoid leakage from syringes.

RESULTS

Large Bore Column/Precolumn Systems

A first set of experiments in this study were done on a commercial fluorenone sample, which proved to contain several impurities which were suffiently coloured to allow detection at 436 nm and thereby avoid difficulties with the aromatic and unsaturated impurities in the petroleum ether solvent (which did not transmit light at wavelengths below 270 nm). Small scale injections via the shortest precolumn attached to a 1000X Ø 22 mm column packed with dp 30μm silica gel showed that 8,000 plates were generated at a reduced velocity of 8, giving a reduced plate height of 4. Slightly better values of h in the 2.8-4 range were subsequently shown with several columns of smaller dimensions (1000 and 250 x Ø 16 mm and 400 and 250x Ø 9.5 mm) at similar reduced velocities (flow rates were: Ø 22 mm, 7 $cm^3 mm^{-1}$; Ø 16 mm, with 22μm particles, 5 $cm^3 min^{-1}$; Ø 9.5 mm with 16μm particles, 2.5 $cm^3 min^{-1}$).

These results could not be obtained without recourse to the 'curtain flow' facility provided by the second inlet at the precolumn/column coupling. Flow down the main column was as stated above, but was split via the injector at the precolumn top and the second, lower inlet according to the ratio of the [precolumn]:[column-precolumn] cross sectional areas. Samples could be injected via the short precolumn carrying the full flow, but all peaks were then very badly skewed toward the trailing edge and resolving power was sharply reduced with

Ø 22 and Ø 16 mm columns. Direct injection into an equal diameter precolumn/column system gave the expected good results, even when the annular second inlet was incorporated (but not used). Figure 3 shows the effects of curtain flow on the elution pattern from a Ø 22 mm column.

A Ø 9.5 mm glass precolumn/column assembly based on the above design was made (glass tubes cemented into stainless steel end fittings) so that sample zone shapes could be

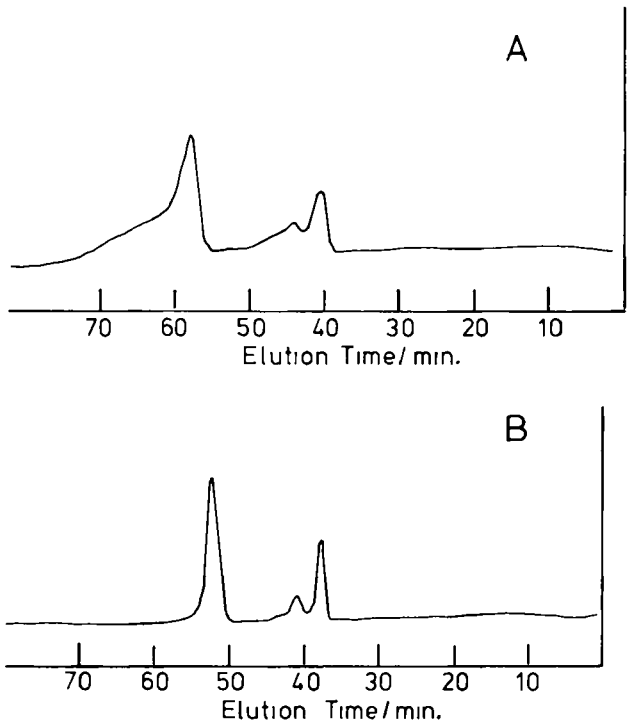

FIGURE 3. Curtain Flow Sample Loading Effects (column 1000 mm x Ø 22 mm). A; Crude fluorenone (45 mg.), single pump, 9 $cm^3 min^{-1}$. B; Crude fluorenone (25 mg), two pumps, 7 $cm^3 min^{-1}$ through side inlet and 3 $cm^3 min^{-1}$ through Ø 9.5 precolumn.

examined visually. The fundamental importance of the sampling procedure to the effective resolving power of large column/preparative systems was soon evident, since axial zone asymmetry could be readily seen (with fluorenone, azobenzene, or 4-aminoazobenzene samples) at virtually all loading levels used. Equally, the initiation of mass-overload conditions could be seen as a widening of the sample zone immediately after its introduction. One fact quickly established was that if precolumn top condition was not optimal for analytical scale injection, then the situation did not improve at any load level, so careful column preparation proved to be just as important in large scale chromatography as it is in analytical scale separations.

When the optimal flow and loading conditions were worked out for the Ø 9.5 system, a short series of experiments aimed at establishing the effects of sample solvent polarity on separation was initiated. The expected loss of adsorbent activity was found, and not surprisingly recovery of activity took longer with more polar sampling solvents. Separating power was also diminished more or less as expected - an example of this sample solvent effect is shown in Figure 4, but in extreme cases this may lead to splitting of peaks such that zone identification becomes difficult.

The large bore column experiments were begun in the expectation that adsorbent capacity would simply be a function of the surface properties of a given column packing, but Kraak, et al,[2] suggested that particle diameter might affect capacity in ways unrelated to the previously described correlations of flowrate, particle diameter, and resolution. Since the basic concept of an immediately joined loading/pre-column stop-flow large volume injection system appeared to be practical,

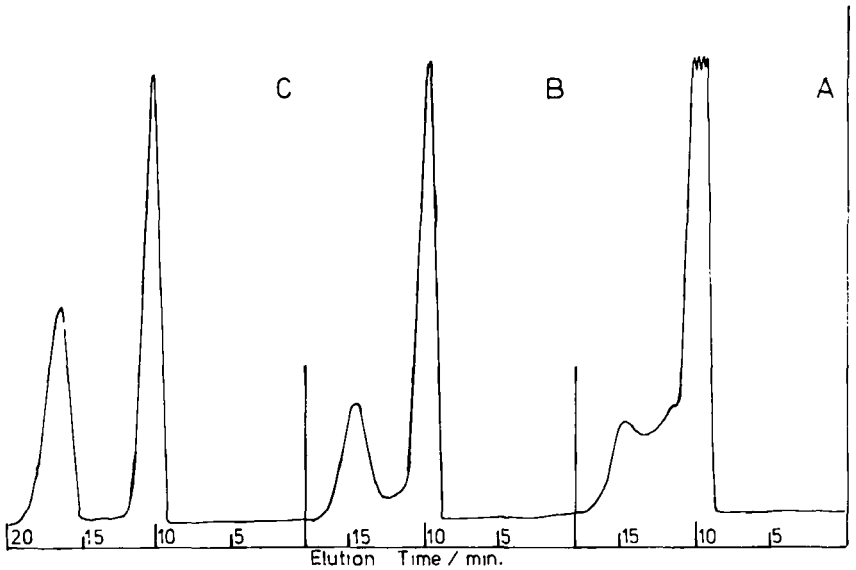

FIGURE 4. Sample Solvent Effects on Adsorbent Silica (column 350 mm x Ø 9.5 mm). Three sequential samples (1 mg, 0.5 mg, 0.5 mg) of azobenzene (cis most retained), sample solvents; A; 6:94 propan-2-ol: petroleum ether (1 cm^3) B; 6:94 propan-2-ol; petroleum ether (0.5 cm^3) C; column eluent (2:98 acetone:petroleum ether). F = 2 cm^3 $min.^{-1}$

it seemed sensible to clarify the relationship of particle diameter to column capacity with smaller columns (and in less time) before beginning a long series of large-scale experiments.

Particle Diameter Effects

SOTLC Fractions with spherical equivalent mean diameters of 5, 10 and 17μm were packed into columns (respectively 125 x Ø 5, 250 x Ø 5, and 250 x Ø 7 mm) and equili-

brated with a solution of propan-2-ol in 80-100° petroleum ether (5%, V:V), before a series of samples of 4-aminoazobenzene (k'=2.6) dissolved in the solvent were passed through each column. Although sample volume effects (cf Scott and Kucera[5]) were not systematically separated from sample mass effects, a reasonably smooth curve could be drawn through the data points on plots of either N or H versus relative sample load (expressed as μg sample/g packing in column).

The number of theoretical plates represented by each sample peak was calculated in at least two ways at load levels below 50μg/g. These were based on the assumptions of Gaussian peak shape, when the following relations apply:

$$(4) \qquad N = 16 \left(\frac{t_r}{4\sigma_t}\right)^2 = 5.55 \left(\frac{t_r}{w_{\frac{1}{2}}}\right)^2 = 18.6 \left(\frac{t_r}{w_{\frac{1}{10}}}\right)^2$$

where t_r = retention time of eluite, $4\sigma_t$ is the base width between lines drawn tangent to the inflection points of the leading and trailing edges of the peak, $w_{\frac{1}{2}}$ is the time width of the peak at half height, and $w_{\frac{1}{10}}$ is the time width of the peak at one tenth of peak height. H was calculated from the measured average value of N and the sample loading in μg/g was calculated from the known volume samples of known concentration after weighing the dried, extruded column packing material.

At relative loadings of 30-50μg/g adsorbent there is clear evidence of mass overload, and the three methods of measuring N begin to give quite different answers as the peak shape changes from pseudo-Gaussian to the rounded square wave characteristic (cf Scott and Kucera[5] and Huber, et al.[6]) of mass overload. Since resolution of square wave peaks

must be some function of the base width, the method of tangents was used to calculate N and hence H of the mass overload peaks.

The data generated in this type of experiment has been presented in several different ways[1,2,3,4,5,6] each of which has its own particular value. Thus, plotting N against load enables direct assessment of the resolving power of a column as a function of load, but only at a single volumetric flow rate. Figure 5 shows such a plot (at $\nu = 3$) for two 250 mm long columns of 10μm and 17μm SOTLC packing which may be compared with Ref.2, Fig. 8.

However, if we wish to compare column performance with a wide range of packing material sizes and independently of column length, another method of presenting the data, i. e. reduced plate height h as a function of reduced load is more useful. Figure 6 shows such a plot representing measurements on three columns, and demonstrates that this 'reduced' plot may well describe a relationship as generally applicable in LC as that between h and ν .

The shaded zone on the diagram represents the total range of all data in this study and also comprehends the earlier results of Done[1] and Kraak[2] (up to the 100μg/g level). Interestingly, there is no evidence that the load vs reduced velocity relation is dependent either on particle diameter or on adsorbent surface area (within the 200-500 $m^2 g^{-1}$ range, but notice that retention changes with surface area are relatively minor up to 500 $m^2 g^{-1}$, cf. Guillemin[12]). The slope of the line defining the loading performance of a given column appears to be a consequence of fundamental chromatographic processes, in which the regularity of column packing defines not only the maximum possible resolving power with minimal

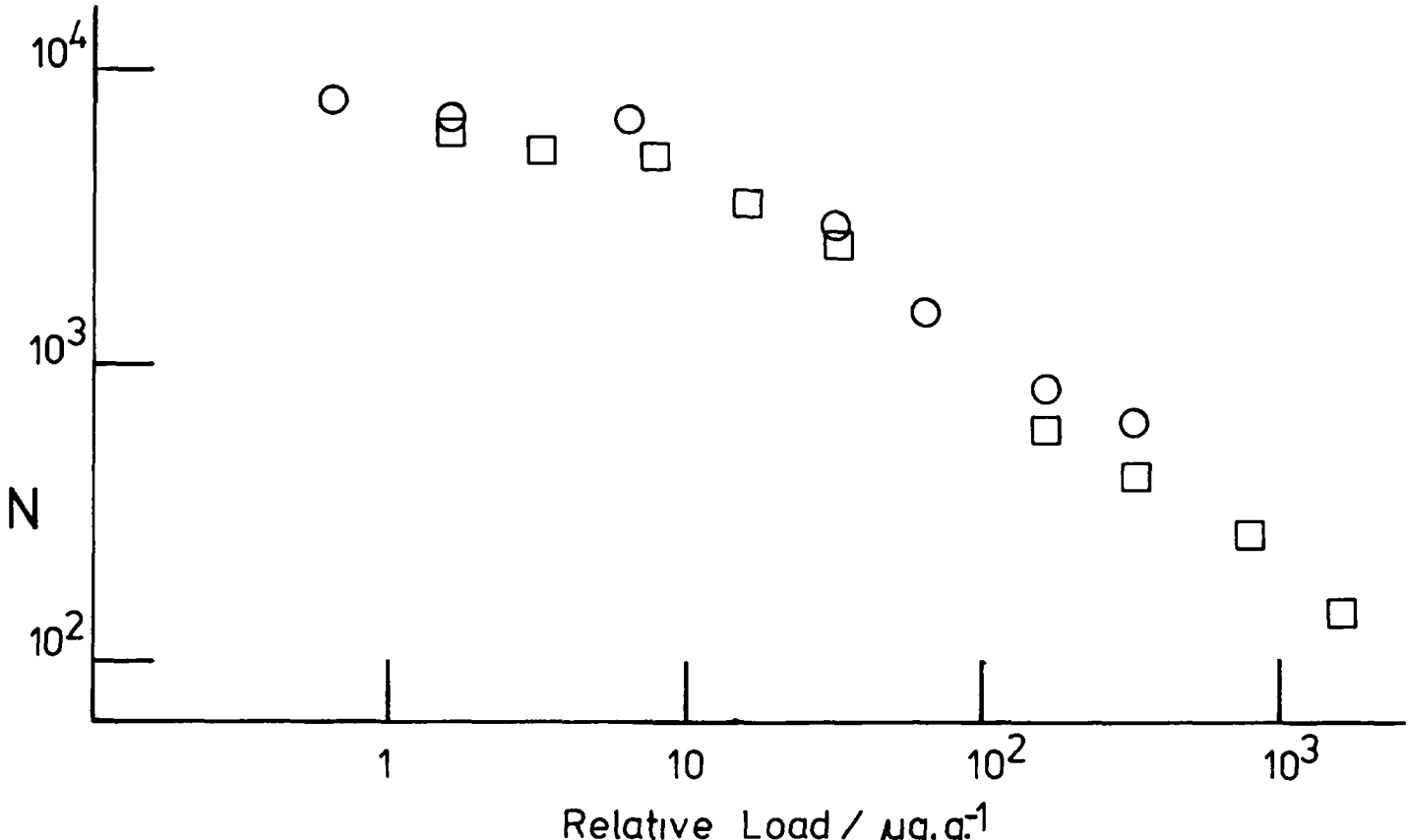

Figure 5. Relationship of Number of Theoretical Plates (Z=25 cm) to Sample Loading on SOTLC Solica Gel. Crude 4-aminoazobenzene eluted with 5:95 propan-2-ol: petroleum ether. □ dp=17μm; ○ dp=10μm.

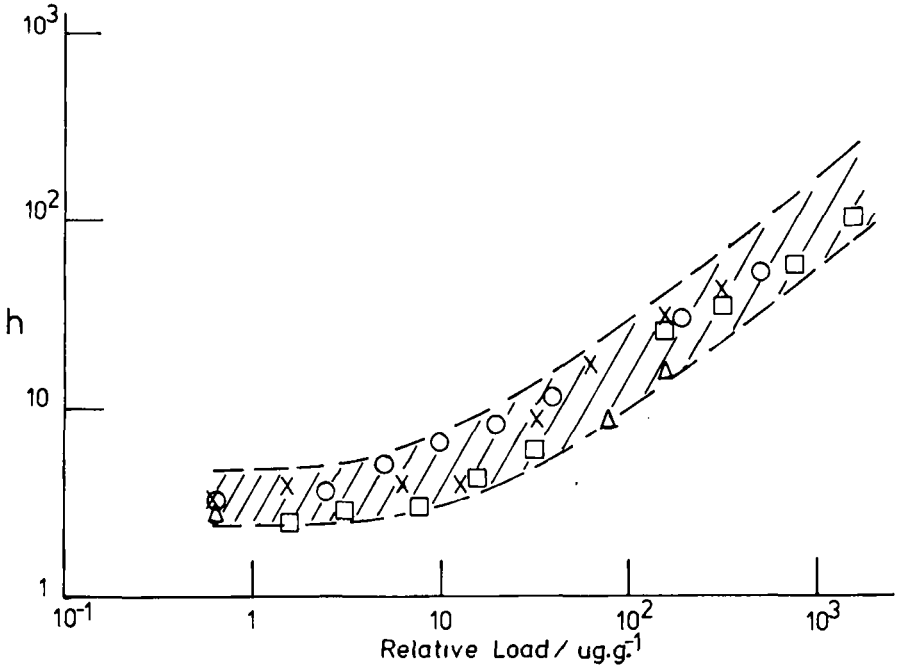

FIGURE 6. Relationship of Reduced Plate Height, h, to Relative Load on Columns of Silica Gel. Reduced velocity, ν, between 2 and 5. O, Hypersil, dp=7μm; Δ, SOTLC, dp=5μm; X, SOTLC, dp=10μm; $\square$, SOTLC, dp=17μm.

samples but also separation power at higher loading. Data from both the SOTLC and Hypersil columns produced what are effectively a series of parallel lines, all of which fall in the shaded zone of Fig. 6.

These findings contradict the anomalous results of Kraak[2] with 22.5μm particles; however, this earlier study used a reduced velocity in the large particle column that was $3\frac{1}{2}$ times that used with the small particle column.

The relation between h and ν might differ under mass overload conditions in such a way as to favour operation

at higher values of ν, which might explain Kraak's results. Accordingly, the dependence of h upon ν (over a restricted range of ν, 2-13) at load levels of 0.64μg/g and 128μg/g was measured on a 112 x ϕ 5 mm column of 5μm SOTLC silica. The admittedly incomplete data shown in Fig. 7 suggest that maximum column efficiency is attained at the same reduced velocity at both load levels and further that deviations from optimal linear velocities lead to more rapid loss of separating power at preparative load levels than at analytical levels.

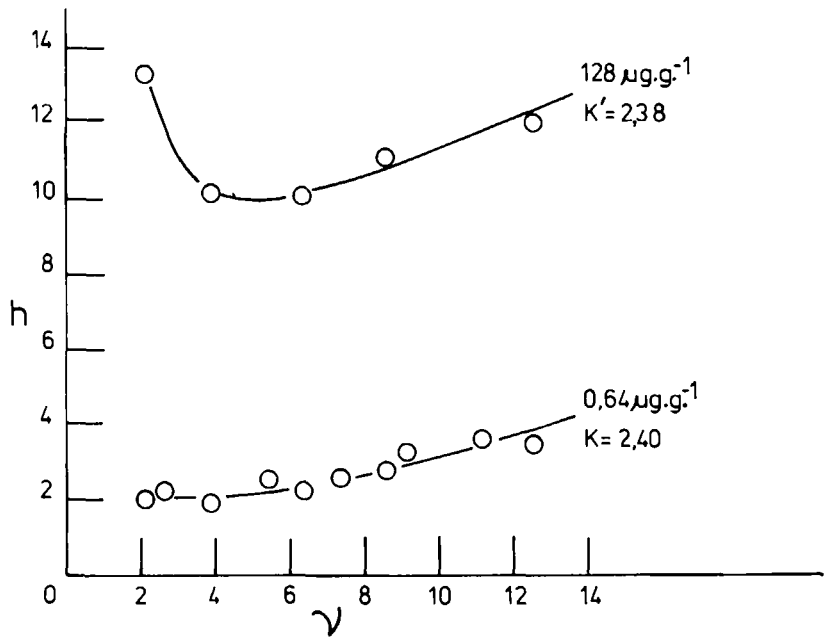

FIGURE 7. Relationship of Reduced Velocity, ν, and Reduced Plate Height, h, at two Different Load Levels. 4-Aminoazobenzene on SOTLC silica (dp=5μm, 112 mm x ϕ 5 mm).

Overload Effects in Reverse Phase LC

The other anomaly in the literature of loading effects was Done's[1] finding that alkyl bonded phase packings had a better response to sample overloads than do adsorbents (however, cf. Huber et al.[6]). The data presented in Figure 8 do not indicate any significant difference between conventional adsorbents and what may well be termed 'solvophobic' Horvath, et al.[11]) adsorbents in the h vs load relationship. These data were obtained at $\nu = 3$ with two different eluents (60:40 and 40:60 V:V methanol-water mixtures) using the phenol and cresol solutes of the earlier study, and do not show the large dependence on retention (K' range 0.6-2.2) found by Done.[1]

There was one major difference in the performance of 'reverse-phase' ODS packings compared to adsorbents at load levels in excess of 100μg/g, and this was the shapes of what were clearly mass-overloaded zones. Scott and Kucera[5] and many others have commented on the 'square wave' elution patterns characteristic of mass overload on adsorbents, but such patterns were never observed in this present study of alkylbonded silica gels. In all cases (at loads up to $10^4 \mu$g/g) the elution peaks retained the approximately Gaussian shapes expected from 'analytical' LC theory, as is clearly shown in Fig. 9. The two traces of Fig. 10 demonstrate another apparent difference in the performance of bonded phase columns, i.e., an enhanced sensitivity to the sampling solvent. The leading edge distortions of Fig. 10B show up as false peaks at higher load levels and/or with eluents of lesser water content (cf. Fig. 10A).

A rather surprising point about the sensitivity of 'solvophobic' systems to sampling solvents is that this (measurable) deterioration of resolving power extends down

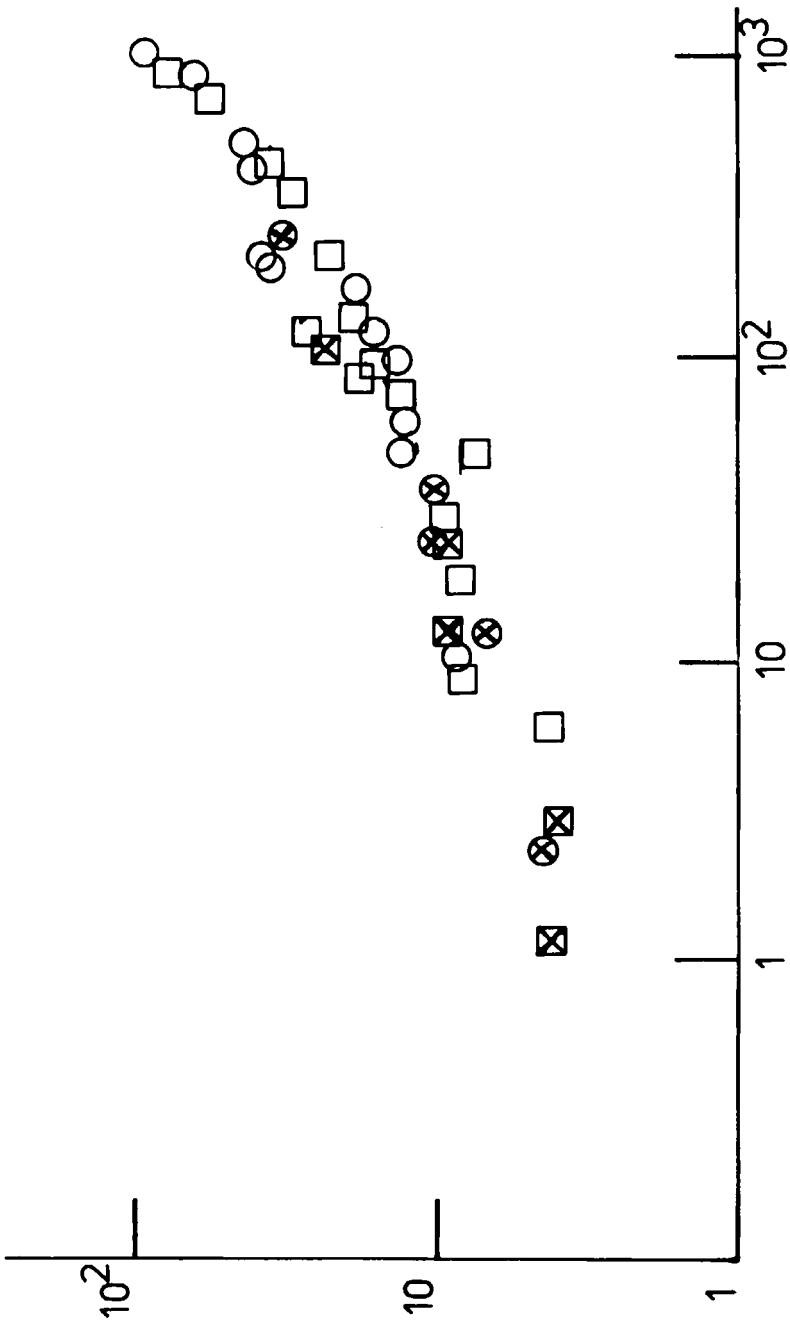

FIGURE 8. Relationship of Reduced Plate Height, h, and Relative Load for C_{18} Alkyl Bonded Silica. ODS Hypersil (dp=7.5µm, 112 mm x Ø 5 mm). ○, phenol, K'=1.26 (40:60 methanol:water); □, p-cresol, K'=3.10 (40:60 methanol:water); ⊗, phenol, K'=0.43 (60:40 methanol:water); ⊠, p-cresol, K'= 0.76 (60:40 methanol:water).

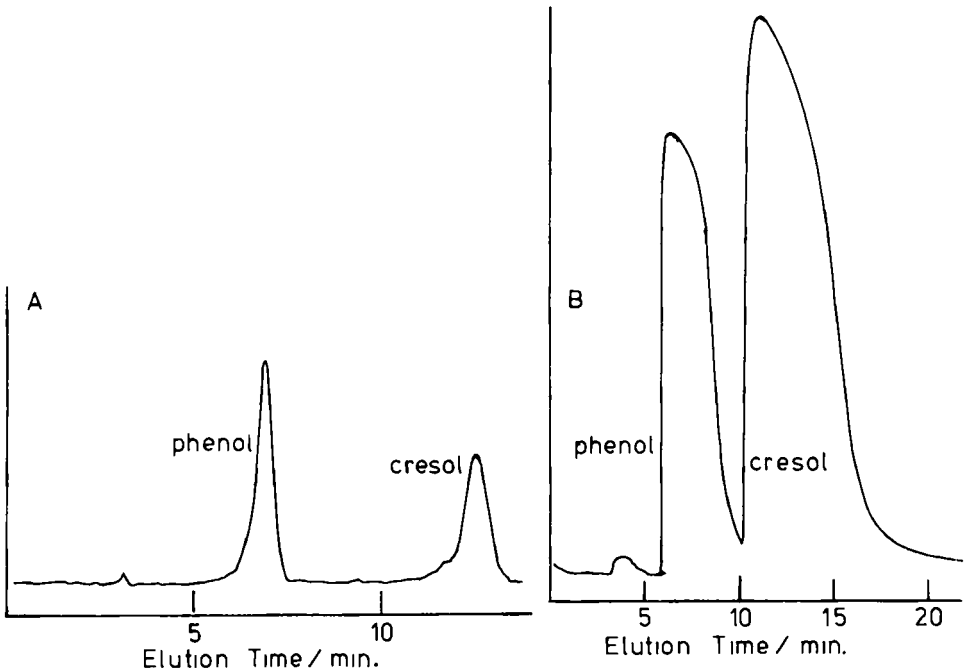

FIGURE 9. Effect of Mass Overload on Alkyl Bonded Silica. ODS Hypersil (dp=7.5μm, 112 mm x $\emptyset$ 5 mm) Eluent 40:60 methanol:water at 5 $cm^3 min^{-1}$. A; 50 μg phenol and 24 μg p-cresol dissolved in 4 μl ethanol. B; 6.36 mg phenol and 5.28 mg p-cresol dissolved in 0.6 cm^3 eluent.

to very small volumes indeed. The lowest value of ν attained with the column used for the measurements of Fig. 10 was just under 3 with a 1 μl sample (dissolved in eluent), but it was not possible to record values of ν below 3.2 (at K' = 3) for 1 μl samples of similar load dissolved in ethanol. The discrepancy in resolution between analyte samples dissolved in eluent and ethanol increased with increasing sample volume, and this effect should clearly be considered in any proposed mass (and volume) overload 'reverse phase' LC separations.

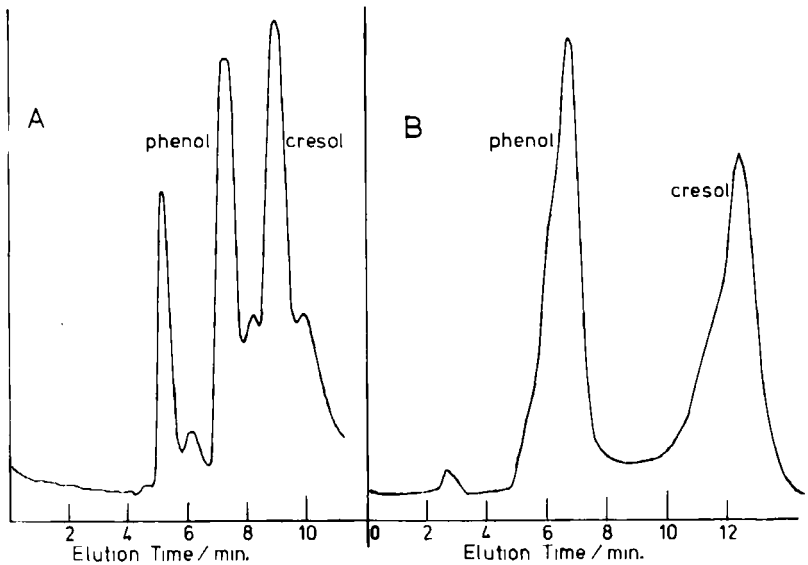

FIGURE 10. Peak Distortion Effects caused by Sample Solvent on Alkyl Bonded Silica. A; 242 μg phenol and 122 μg p-cresol dissolved in 0.1 c m^3 ethanol. Eluent: 60:40 methanol:water. B; 375 μg phenol and 183 μg p-cresol dissolved in 0.03 cm^3 ethanol. Eluent: 40:60 methanol:water.

DISCUSSION

The results reported here confirm the simplest hypothesis about column performance in LC under overload conditions: that a fundamental relationship between h, ν, and relative load is common to a range of adsorbent silica gel packings (varying both in particle diameter and surface area). Furthermore, some 'bonded phase' packings made from one of the above silica gel materials demonstrated essentially the same relationship between h and relative load.

Much more data needs to be collected before an accurate empirical description of the interrelation between h, ν, reduced load (and reduced sample volume?) similar to Eq. 3 can be written, but even the limited study described in this paper shows that column performance under overload conditions should be calculable from a knowledge of analytical scale resolving power.

However, since overload chromatography must introduce a third parameter, load, to the concepts of plate height and eluent velocity used by Knox[9] and others to describe analytical LC, the mathematical description of column performance must define a curved surface whose three-dimensional coordinates could be h, ν, and reduced load. When the characteristics of this surface are known, it will be possible to extrapolate analytical separations to overload conditions over the range of accessible eluent flow rates in such a way that accurate prediction of resolution will be possible. At the moment, all discussion on the question of whether large particles or small particles are 'better' for preparative purposes has an unsound, empirical basis, which is not a satisfactory state of affairs.

The data presented in this paper allow some sample calculations which may be useful. We will compare column dimensions required to achieve resolution, Rs, = 1.5 for 20 mg each of two analytes with average capacity factor, K' = 3, and selectivity $\alpha = \frac{K_2}{K'_1} = 1.10$ (N required = 3800 plates) for column packings of dp 5μm and 20μm. The further assumption is made that the reduced velocity is near the minimum on the plate height - velocity curve at a value of

$\gamma = 5$ with eluites whose diffusion constant in the eluent is 1.5×10^{-9} $m^2 s^{-1}$. Table 1 shows the results of such calculations for standard column lengths of 250, 500, and 1000 mm, assuming all the columns to be well enough packed that the load-reduced plate height relationship follows the lower limit shown in Fig. 6.

Table 1 suggests that separations on this scale are most efficiently (in time and materials) carried out on relatively long columns of moderate diameter with 5μm par-

TABLE I Column Parameters for Semipreparative LC

Function	dp = 5 m	dp = 20 m	Z /mm
Dcol/mm	36	108	250
F /$cm^3 min^{-1}$	58	131	"
load/$\mu g. g^{-1}$	141	15.8	"
V_R at k'=3, /cm^3	791	7143	"
Dcol/mm	16	41	500
F /$cm^3 min^{-1}$	11.4	25	"
load/$\mu g. g^{-1}$	363	55	"
V_R /cm^3	311	2053	"
Dcol /mm	7	18	1000
F /$cm^3 min^{-1}$	2.1	3.6	"
load/$\mu g. g.^{-1}$	1000	145	"
V /cm^3	116	780	"

ticles, confirming the study of Bristow.[13] The 20 mg sample was not a purely arbitrary choice, since current NMR spectrometers require this amount of material for analysis of C_{20}-C_{30} molecules. This spectral technique is at the same time the most useful and the most demanding (for amount of sample) method in current general use for structure determination of organic compounds, and hence sets the standard for what is usually termed semi-preparative HPLC.

This mode of LC is the largest scale use of the technique wherein manual sample injection and fraction collection are still practical, particularly if small particle packings are used. The truly preparative operations up to multigram scale samples really demand automation of fraction collection at the least, and methods optimisation calculations must include consideration of the consequences of automation as well as fundamentals of column performance. However, more complete understanding of column behaviour under mass (and volume) overload is clearly required for complete definition of preparative LC system performance and it is hoped that the concepts outlined in this study could lead to that understanding.

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