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EVALUATION OF ADVANCED SILICA PACKINGS FOR THE SEPARATION OF BIOPOLYMERS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

II. PERFORMANCE OF NON-POROUS MONODISPERSE 1.5-μm SILICA BEADS IN THE SEPARATION OF PROTEINS BY REVERSED-PHASE GRADIENT ELUTION HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Columns of dimensions 36 × 8 mm I.D., packed with *n*-octyl-bonded non-porous monodisperse silica particles of diameter 1.5 μm ± 2%, were tested for their performance in the reversed-phase gradient elution of proteins, employing methanol, acetonitrile, 2-propanol and 1-propanol as organic solvents and 0.01–0.02 *M* trifluoroacetic acid or perchloric acid as eluents. The retention of proteins over a wide range of volume fractions of the organic solvent modifier follows the same dependences as are observed on porous reversed-phase packings, with one noticeable exception: the slope of the linear dependence of the logarithmic capacity factors on the composition of the binary eluent yields values between 4 and 7 for the proteins studied, whereas *S* is usually a function of the molecular weight and approaches values of up to 100. This peculiarity is assumed to be associated with the retention of proteins on a surface with easily accessible *n*-alkyl ligands and free from any pore diffusion effects. The 1.5-μm particles provide an excellent column performance, enhancing both the resolution and the detection sensitivity. Gradient times of 3 min and flow-rates of 1.5 ml/min allow the rapid separation of proteins. The sample capacity was found to be *ca.* 1 mg of protein per column volume, without a loss of resolution.

INTRODUCTION

The separation of peptides and proteins by reversed-phase gradient elution high-performance liquid chromatography (HPLC) has gained considerable interest,

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both in the theoretical aspects and in terms of application[1–20]. Employing acidic aqueous–organic eluents of low ionic strength and *n*-alkyl-bonded silica packings, the major target parameters are high resolution, high peak capacity, high mass recovery and maintenance of biological activity. The early stages of HPLC in this context were characterized by a more or less empirical selection of stationary and mobile phase compositions, column configurations and operation variables, and hence the conclusions derived therefrom were often contradictory[3–23]. Recently, more profound and comprehensive models, describing the retention and kinetics of peptides and proteins, have been elaborated and tested experimentally for their validity and limitations[3,23–27]. On the basis of these results, much more rigorous separation strategies and optimization procedures have been developed.

In addition to mobile phase composition, the column configuration is known to play a dominant role in resolution, peak capacity, sample capacity, detection sensitivity, mass recovery and biological activity[24]. The expression "column configuration" embraces the column dimensions, i.e., length (L) and bore (d_c), the average particle diameter of the packings (d_p), the pore diameter of the packing (p_d), the type of bonded *n*-alkyl ligands (L), the ligand density (α) and the accessible surface area of the ligands (A_L)[24,26,28]. Currently, column lengths vary from 80 to 250 mm, depending on the desired peak capacity, with column bores of 4–6 mm. Average particle diameters of reversed-phase packings, mainly of the *n*-octyl- and *n*-octadecyl-bonded type, range from 5 to 10 μm. The optimum particle size for peptides with a molecular weight (M) of 500–20 000 daltons has been predicted to lie around 2 μm and smaller[23]. Reversed-phase columns packed with 2-μm particles have been described by Dewaele and Verzele[29], but have not yet been applied to peptide separations. Based on an optimization model, Stadalius *et al.*[23] recommended packings with an average pore diameter of 15 nm as most suitable for peptides of M up to 20 000 daltons, and 30–50 nm for larger proteins. Hearn and Grego[26], evaluating stationary phase effects in peptide separations, tested a series of *n*-octyl- and *n*-octadecyl-bonded packings with pore diameters of 7.3–30 nm, and Engelhardt and Müller[28] extended the range of the nominal pore diameter to 100 nm.

Recently, Warren and Bidlingmeyer[30] compared various commercial reversed-phase packings, with nominal pore diameters of 5–30 nm, with respect to resolution, peak width and sample capacity for proteins. In the characterization of the pore size of a packing, the terms narrow and wide pore are often used without a clear definition. In order to avoid further confusion, it would make sense for chromatographers to adopt the IUPAC recommendation, which classifies the porous packings into three categories according to the average pore size[31]:

microporous	$p_d < 2$ nm;
mesoporous	$2 < p_d < 50$ nm;
macroporous	$p_d > 50$ nm.

The types of *n*-alkyl ligands range from short chain, *e.g.*, trimethyl, to very long chains of C_{30}[26], but most studies were carried out on *n*-octyl- and *n*-octadecyl-bonded silicas. The ligand density varied from 2.0 to 4.0 μmol/m^2, depending on the modification procedure (for comparison, see refs. 26 and 28).

It appears that the existing packings and columns largely satisfy the predicted requirements. However, the application of mesoporous or even macroporous reversed-phase packings in peptide and protein separations still has drawbacks in terms of low performance, mass recovery and biological activity, and they often exhibit certain anomalies[26]. The phenomena are to some extent associated with the kinetics of biopolymer solutes migrating through the pore system of reversed-phase packings. The trend to enlarge the average pore diameter of the packings and, concurrently, to reduce the average particle size suggests the use of microparticulate non-porous packings, where the loss in internal surface area is counterbalanced by an appropriate reduction of the average particle diameter. The concept of employing non-porous packings was introduced by us in 1984[32] for the design of improved affinity packings and can also be adapted to other modes of HPLC. Assuming a true solid density of silica of 2200 kg/m^3 and spheres of uniform diameter (d_p = 1 μm or 0.1 μm), the external specific surface area, a_S (ext), is calculated to be 2.7 or 27.3 m^2/g[33]. Owing to the absence of pores, the external surface area of the particles, in either their native or bonded form, is then easily accessible and can be fully utilized in solute–ligand interactions of peptides and proteins.

This paper reports the use of non-porous, monodisperse 1.5-μm silica beads with bonded *n*-octyl ligands in the reversed-phase gradient elution of peptides and proteins, and compares the results with those obtained on meso- and macroporous packings.

EXPERIMENTAL

Chemicals, reagents and packings

Acetonitrile, methanol, 2-propanol and 1-propanol of HPLC grade and trifluoroacetic acid (TFA) and perchloric acid of reagent-grade were obtained from E. Merck (Darmstadt, F.R.G.). Water was quartz-distilled and deionized. Pure proteins from E. Merck were ribonuclease, cytochrome, lysozyme, albumin (bovine) and myoglobin; from Sigma (St. Louis, MO, U.S.A.) insulin and α-chymotrypsinogen; and from Boehringer (Mannheim, F.R.G.) aldolase, catalase and ovalbumin.

The packing was a non-porous silica of mean particle diameter $d_{p_{50}}$ = 1.5 μm ± 2%[34], which was converted into its *n*-octyl-bonded derivative with *n*-octyldimethylchlorosilane according to a procedure described elsewhere[35]. Particle size analysis was carried out by counting the particles from images obtained from both transmission and scanning electron microscopy and calculating the number average. For comparison we used a LiChrospher 1000 A RP-8 material (10 μm) from E. Merck.

The *n*-octyl-bonded silicas were slurry-packed into a column (36 mm × 8 mm I.D.) (Bischoff Analysentechnik und -geräte, Leonberg, F.R.G.) employing 2-propanol (2%, w/w) and applying a pressure of 100 MPa. The top- and end-fittings were made of paper filters, supplied by Schleicher & Schüll (Dassel, F.R.G.), and supported by No. 22800812 metal frits (Bischoff Analysentechnik und -geräte).

Apparatus

All chromatographic tests were performed on a Merck-Hitachi gradient HPLC system, consisting of two high-pressure pumps (No. 655-12), a dynamic high-pressure gradient mixer, a fluorescence detector (No. F 1000), an integrator (No. 655-61),

fitted with a processor A, and an injection system (No. 1000) from Rheodyne (Cotati, CA, U.S.A.) with a 20-μl sample loop.

Chromatographic procedures

Solvents and eluents were degassed by flushing with helium. The pH of the TFA (10 m*M*) and perchloric acid (20 m*M*) solution was adjusted to 2.0, measured with a digital pH meter (No. 646) from Knick (Frankfurt/M, F.R.G.). the *n*-octyl-bonded silica columns were equilibrated with the new eluents within 20 min at a flow-rate of 1.5 ml/min. The column temperatures were 298 K. The sample sizes varied from nanograms to milligrams, but generally they were 0.1–5.0 μg. The column-dead volume was determined with acetone. The gradient dwell time was 1.03 min at a flow-rate of 1.5 ml/min.

The following eluents were employed for gradient elution: (A) 20 m*M* perchloric acid (pH 2)–(B) acetonitrile–20 m*M* perchloric acid (pH 2) (A:B = 75:25, v/v); (A) 10 m*M* TFA (pH 2)–(B) acetonitrile–10 m*M* TFA (pH 2) (A:B = 75:25, v/v); (A) 10 m*M* TFA (pH 2)–(B) methanol–10 m*M* TFA (pH 2) (A:B = 75:25, v/v); (A) 10 m*M* TFA (pH 2)–(B) 2-propanol–10 m*M* TFA (pH 2) (A:B = 75:25, v/v); (A) 10 m*M* TFA (pH 2)–(B) 1-propanol–10 m*M* TFA (pH 2) (A:B = 75:25, v/v); (A) 50 m*M* TFA (pH 1.4)–(B) 1-propanol–50 m*M* TFA (pH 1.4) (A:B = 75:25 v/v).

RESULTS AND DISCUSSION

Theoretical considerations

The retention of polypeptides in reversed-phase HPLC is based on several interaction phenomena, which are associated with the accessible nonpolar *n*-alkyl and polar hydroxyl surface sites of reversed-phase packings. Hence, the capacity factor of a given solute is expressed by the sum of the capacity factors from solvophobic, silanophilic and size-exclusion retention[26]:

$$k' = \rho_{so} k'_{so} + \rho_{si} + \rho_{ex} k'_{ex} \quad (1)$$

where ρ_{so}, ρ_{si}, and ρ_{ex} are the weighed molar fractions of the solute in each mode. For non-porous, reversed-phase packings, the size exclusion term $\rho_{ex}k'_{ex}$ is inapplicable. However, when using columns packed with 1.5-μm non-porous particles, the exclusion arising from interstitial channels must be taken into account. According to Giddings[36] and to Guiochon and Martin[37], the average particle diameter (d_p) should be 50 times larger than the diameter of the excluded molecule in order to avoid interstitial size-exclusion effects. The minimum average particle diameters, $d_{p(min)}$, for globular proteins and random-coil proteins with a molecular mass of 10^5 dalton (10^6 dalton) are calculated to be 0.38 (0.82) μm and 1.29 (4.08) μm, respectively[37]. Thus, size exclusion in the interstitial voids of a column packed with monodisperse particles of d_p 1.5 μm can be neglected for globular proteins. In the context of size exclusion phenomena it is worth evaluating the magnitude of the average pore width of the interstitial voids in this packed column. For an assembly of packed silica spheres the average pore diameter, *i.e.*, the equivalent of the diameter of the circle describing the pore opening, is calculated from the equation[38]

$$p_d = 1027.4 v_p d_p \quad (2)$$

where v_p is the specific pore volume in ml/g and d_p is the average particle diameter in μm. An interstitial porosity of $\varepsilon_0 = 0.26$ (0.40) will result in a specific interstitial pore volume of 0.16 (0.30) ml/g. Inserting these values into eqn. 2 with $d_p = 1.5$ μm, $p_d = 0.25$ (0.45) μm. As the packed columns showed an interstitial porosity of $\varepsilon_0 \approx 0.35$, the p_d value reaches about 0.40 μm.

For binary eluents with increasing organic solvent ratio, φ, from 0 to 0.8, the dependence of k' on φ is described by

$$\log k' = \log k'_w - S\varphi \tag{3}$$

where k'_w is the extrapolated capacity factor for pure water and S is the slope of the function[26]. A first approximation of S is given by[24]

$$S = 0.48M^{0.44} \tag{4}$$

where M is the molecular weight of the solute. For low-molecular-weight peptides, S is of the order of 10, but reaches values of up to 100 for large proteins[23,26]. Accordingly, the steepness of the plot of $\log k'$ *vs.* φ increases with increasing molecular weight, *i.e.*, slight variations in the eluent composition will result in a marked effect on retention, and a small, so-called elution window appears at the minimum of the bimodal curve[25].

Hearn and Grego[26] found nearly constant S values of *ca.* 10 for phenylalanine oligomers on *n*-octyl- and *n*-octadecyl-bonded silica of average pore size (7.3–30 nm), whereas anomalies arose for proteins, *e.g.*, hen lysozyme, sperm whale apomyoglobin and bovine serum albumin. Assuming normally behaved bonded phases with accessible ligand sites, the capacity factor of peptide and protein should increase linearly with the ligand density per unit column volume, or with the total hydrophobic surface area of ligands per unit column volume. In gradient elution, the median capacity factor, $\bar{k}$, is given by[24]

$$\bar{k} = t_G(F/1.15)V_m\Delta\varphi S \tag{5}$$

where t_G is the gradient time, F the volume flow-rate, V_m the column dead-volume, $\Delta\varphi$ the change in volume fraction of strong solvent during gradient elution and S the slope of the relationship $\log k$ *vs.* φ.

The optimum value of $\bar{k}$ ranges from 2 to 10. Adjustment of $\bar{k}$ is achieved by changing the flow-rate (F), and V_m, which is proportional to the column length (L) at a given column diameter. When a column is packed with non-porous particles to an interstitial column porosity ε_0 of, say, 0.40, V_m is half for a column packed with porous particles of the same particle diameter, *i.e.*, in the latter instance the total column porosity amounts to $\varepsilon_t = \varepsilon_0 + \varepsilon_i = 0.4 + 0.4 = 0.8$. This allows a decrease in the flow-rate by a factor of two for a column packed with non-porous beads, under otherwise constant conditions.

Optimum flow-rates with respect to maximum plate number correspond to a reduced linear velocity of $v = 3$–10 for silica packings, where v is given by[37]

$$v = \frac{u_0 d_p}{D_m} \tag{6}$$

TABLE I

VALUES OF D_m CALCULATED ACCORDING TO THE STOKES–EINSTEIN EQUATION[37]

M	D_m ($m^2s \times 10^{-10}$)
10^2	5.27
10^3	2.44
10^4	1.13
10^5	0.53
10^6	0.24

where u_0 is the linear velocity of the eluent, d_p the average particle diameter and D_m the diffusion coefficient of the solute in the eluent. For a column packed with non-porous particles, u_0 corresponds to the interstitial linear velocity. At a given D_m, a reduction of d_p from 5 μm to 1.5 μm results in a three-fold increase in u_0, achieving maximum performance.

At a given particle diameter (d_p), the optimum interstitial velocity (u_0) is proportional to the diffusion coefficient (see eqn. 6). For globular proteins at a solvent viscosity of 1 cP, a solvent molecular weight of 41 and a temperature of 293 K, the D_m values given in Table I are obtained according to the Stokes–Einstein equation[37]. In other words, D_m changes by a factor of 10 for solutes with M between 10^3 and 10^6 or between 10^2 and 10^5.

On adopting an optimum flow-rate for the separation of peptides and proteins on non-porous, small-particle columns, the advantage gained by an increase in F on reducing d_p is more than compensated for by the low diffusion coefficient when high-molecular weight solutes are chromatographed.

In order to maintain the gradient volume, $V_G = t_G F$, and hence k constant (see eqn. 1), a low flow-rate for optimum performance requires a long gradient time, t_G. As the detection sensitivity is related to the peak height (PH) and proportional to the term

$$PH \propto N^{1/2}/V_m\,(\bar{k}/2 + 1) \tag{7}$$

and increases with decreasing F, $\bar{k}$ and t_G, a compromise must be made between t_G and F in order to maintain a constant v_G.

The peak capacity, PC, is another relevant quantity, which is given by[24]

$$PC = (2.3/4)(S\Delta\varphi)N^{1/2}(\bar{k}/1 + \bar{k}) \tag{8}$$

Therefore PC is proportional to the slope, S, and for a fixed $\bar{k}$ and $\Delta\varphi$ varies only with the square root of N.

Elution of proteins from non-porous monodisperse 1.5-μm n-octyl-bonded silica columns

The experiments were intended as a first step towards obtaining chromatographic information on the novel packing, and to compare the results with those collected in previous investigations on porous reversed-phase silicas. Research focused on determining the effect of the eluent composition and gradient conditions on the retention of proteins, employing the same type of *n*-octyl-bonded silica and the

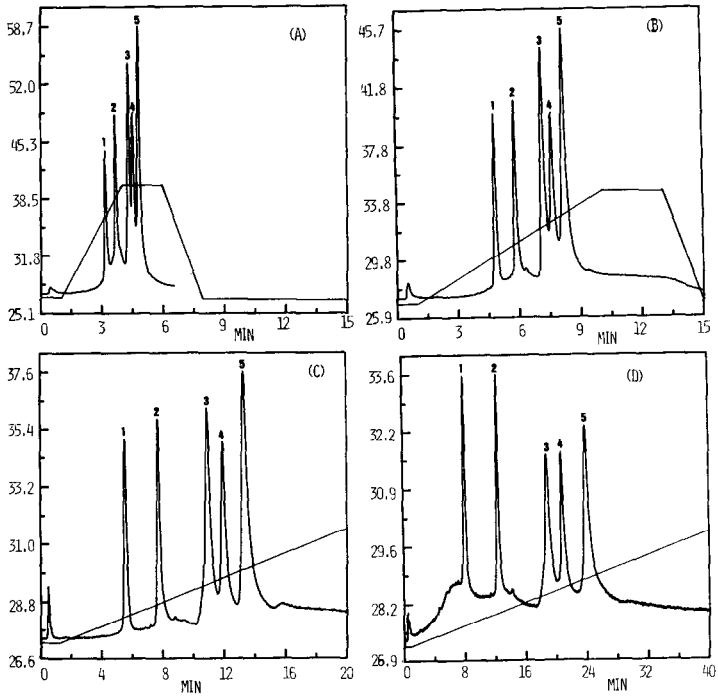

Fig. 1. Effect of gradient time, t_G, on the resolution of proteins on a 36 × 8 mm I.D. *n*-octyl-bonded reversed-phase silica column (d_p = 1.5 μm). The linear gradients were from 19 to 75% acetonitrile in 0.02 *M* perchloric acid. Peaks in order of elution: ribonuclease, cytochrome *c*, aldolase, catalase, ovalbumin (each *ca.* 1–5 μg). Fluorescence detection: $\lambda_{em.}$ = 280 nm, $\lambda_{ex.}$ = 340 nm. (A) t_G = 3 min; (B) t_G = 10 min; (C) t_G = 20 min; (D) t_G = 40 min; flow-rate, 1.5 ml/min. The ordinate values correspond to normalized peak height ratios.

same column dimensions in all instances. The elution of standard proteins from a 36 × 8 mm I.D. column was examined with organic solvents (acetonitrile, methanol, 1-propanol and 2-propanol) and low-ionic-strength acidic eluents, using TFA and perchloric acid as acids. The flow-rates varied from 0.2 to 1.5 ml/min and gradient elution times, t_G, from 3 to 40 min. Fig. 1 shows the chromatograms for the elution system acetonitrile–perchloric acid (pH 2) at four different gradient times and a flow-rate of 1.5 ml/min. The elution order of proteins follows the sequence ribonuclease < cytochrome *c* < aldolase < catalase < ovalbumin. The corresponding plot of log $k'_{i,app}$ *vs.* φ is based on these data; $k'_{i,app}$ is the apparent capacity factor of the protein given by[39]

$$k'_{i,app} = \frac{t_{g,i} - t_0}{t_0} \tag{9}$$

where $t_{g,i}$ is the retention time of a retained solute and t_0 the retention time of an unretained solute; φ is the content of the organic solvent. The term $k'_{i,app}$ can be related to the median capacity factor, $\bar{k}$, through the dependence

$$\bar{k} = \frac{2}{(\ln 10)b} \approx 0.5\, k'_{i,app} \tag{10}$$

for solutes that are eluted with constant b values, *i.e.*, for solutes that are eluted in the gradient mode. These S values, derived from $k'_{i,app}$ values, provide a reasonable approximation to the S values obtained from $\bar{k}$ *vs.* φ plots, although such a derivation does not take into account the curvature of the retention relationship. In a paper which is still in preparation we will describe a detailed analysis of the $\bar{k}$ *vs.* φ relationships for these systems.

From the plot of log $k'_{i,app}$ *vs.* φ the following slopes, S, were calculated: ribonuclease = 3.5, cytochrome c = 5.1, aldolase = 5.9, catalase = 6.8 and ovalbumin = 5.4. The expected values according to eqn. 4 were 30.5, 29.4, 53.5, 93.0 and 111.8, respectively. In order words, the S values on the non-porous packings were smaller by a factor of 10 than the values on porous packings reported in the literature[23,24,26]. Further, S appeared to be fairly independent of the molecular weight. One might speculate that the small slope, S, is associated with the low interactive surface area of the non-porous beads. Under isocratic reversed-phase elution conditions, the solute capacity factor, k'_i, is equal to

$$k'_i = V_s/V_m \, {}^cK_{i0} \tag{11}$$

where V_s and V_m are the volumes of the stationary and mobile phase, respectively, and ${}^cK_{i0}$ is the distribution coefficient of the solute at infinite dilution in concentration units. The volume of the stationary phase is proportional to the surface area of the packing per column volume at a constant n-alkyl ligand and ligand density. The column under investigation contained 3.3 g of non-porous n-octyl-bonded packing with a specific surface area of 2.2 m^2/g; this generated 7.3 m^2 per column volume (V_c = 1.8 ml). It should be noted that the derived packing density ρ_p amounted to 3.3 g per 1.8 ml = 1.83 g/ml; ρ_p for the non-porous material is higher by a factor of 3–4 than that for standard reversed-phase silicas[36]. A 30-nm pore-sized reversed-phase silica typically provides a packing density of 0.5 g/ml and a specific surface area of *ca.* 50 m^2/g. The specific surface area per column volume of 1.8 ml is equal to 45 m^2.

Assuming that the ligand densities of n-octyl groups on both the porous and non-porous silica are the same, the total hydrophobic surface area (A_L) per column volume differs by a factor of 6–7, which does not satisfactorily explain the ten-fold smaller slope, S, of the plot of log k' *vs.* φ for the non-porous silica. A more plausible explanation, as yet unverified, relates to the order of the distribution coefficient reflecting the chromatographic equilibrium. It is generally known that the equilibrium constant, K_{eq}, is equal to the ratio of the rate constants k_1 and k_2, where k_1 represents the adsorption and k_2 for the desorption step. As the n-alkyl ligands are located at the external surface area of the packing, and are thus easily accessible, a diffusion into and out of the pore system, as in the case of porous reversed-phase packings, does not occur. It is conceivable that the rapid diffusion affects the rate constants in such a way that lower equilibrium constants and distribution coefficients will result. Another explanation of the low S value might be the different strengths of solute–surface interactions of porous *vs.* non-porous reversed-phase silica associated with the curvature of surface or with the structure of the bonded ligand. It is conceivable that on porous reversed-phase silicas proteins interact via multi-side attachment whereas on a non-porous surface the probability of multi-side interaction is much

more reduced. All the mechanistic suggestions remain speculative at the moment as detailed examinations have not yet been carried out.

As the elution "window" of the log k_i' *vs.* φ function is usually narrow for proteins on porous reversed-phase packings, a small slope, S, as measured for the non-porous packings, extends the range of the separation capability. The resolution can be adjusted by manipulating the gradient time, t_G, at a constant flow-rate, F, given the slope, S, and constant V_m, according to eqn. 5 and exemplified in Fig. 1.

As expected, a change in organic solvent from acetonitrile to methanol increases the retention of proteins according to the strength of the solvents under otherwise constant conditions. Solvents stronger than acetonitrile reduce retention. Fig. 2a and b shows an example with 2-propanol and 1-propanol as organic solvents at constant t_G, F and pH when 0.01 M TFA is used. The retention of proteins is seen to decrease from 2-propanol to 1-propanol, which is in accord with published data[25]. On decreasing the pH from 2.0 (0.01 M TFA) to 1.4 (0.05 M TFA) under otherwise constant conditions (Fig. 2b and c), a further reduction in the retention is observed.

The 1.5-μm particles should provide excellent column performance when they are properly packed and the column is operated at the optimum flow-rate. Also, in order to utilize the column performance to the fullest, contributions to peak disper-

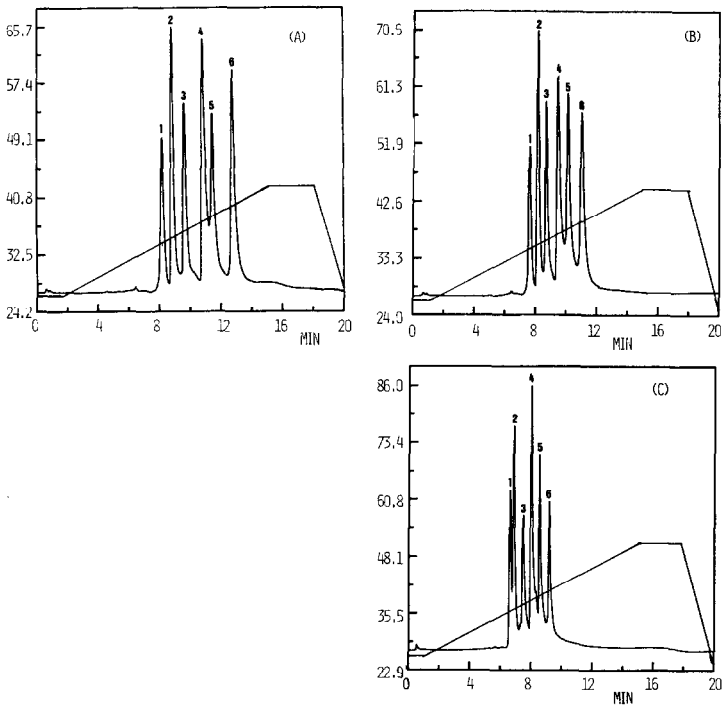

Fig. 2. Elution of proteins on a 36 × 8-mm I.D. *n*-octyl-bonded silica column (d_p = 1.5 μm). Linear gradients: (A) from 0 to 68% 2-propanol in 0.01 M TFA; (B) from 0 to 68% 1-propanol in 0.01 M TFA; (C) from 0 to 68% 1-propanol in 0.05 M TFA. The gradient time, t_G, was constant at 15 min. Peaks in order of elution: ribonuclease, insulin, cytochrome *c*, albumin, catalase, ovalbumin (each *ca.* 1–5 μg). Fluorescence detection: $\lambda_{em.}$ = 280 nm; $\lambda_{ex.}$ = 340 nm; flow-rate, 1.5 ml/min. Ordinate values correspond to normalized peak height ratios.

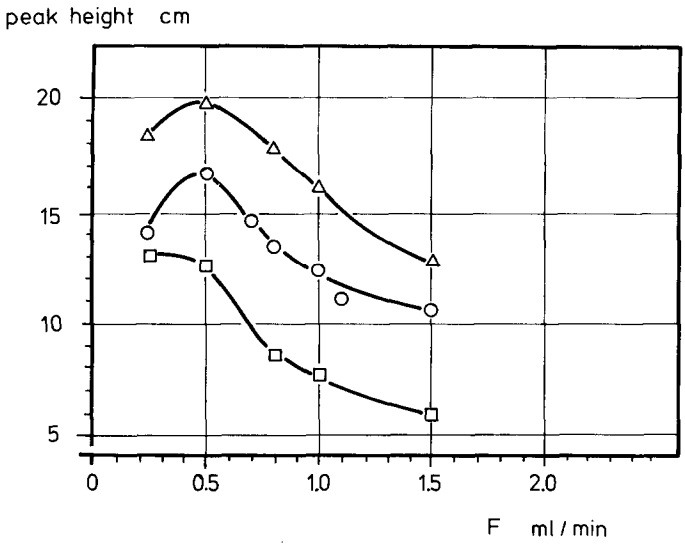

Fig. 3. Peak height of cytochrome *c* on a 36 × 8-mm I.D. *n*-octyl-bonded silica column (d_p = 1.5 μm), as a function of the eluent flow-rate at different gradient times, t_G, of 5 (△), 10 (○) and 20 (□) min. Conditions as in Fig. 1, except that acetonitrile–0.01 *M* TFA was employed.

sion caused by extra-column effects should be negligible. Studies on improved methods for the packing of these particles are still underway. At present, the column employed exhibits an interstitial porosity of $\varepsilon_0 = 0.35$, which is above the value of 0.27 corresponding to the densest packing of spheres of equal size. The tests were carried out with ordinary HPLC equipment, not adapted for any special purpose.

At a given particle size and a diffusion coefficient matching the molecular weight of the proteins, the intestitial velocity, u_0, can be estimated according to eqn. 6 to achieve maximum column performance. Setting d_p = 1.5 μm, $D_m = 10^{-10}$ m²/s and v = 10, u_0 is 1 mm/s. For the given column, u_0 corresponded to a volume flow-rate of 1.1 ml/min; hence the applied flow-rate is slightly above the optimum. Reduced apparent plate heights of proteins calculated from bandwidth data of the chromatogram indicated values between 2 and 9. Reduced plate heights of monomeric solutes assessed under isocratic conditions and acetonitrile–water as eluent gave h values of about 4–5. This means that optimum values around h = 2 were not yet achieved. This is due to the problems arising from agglomeration phenomena in packing these columns.

The expressions of eqns. 7 and 8 predict that the detection sensitivity, measured as the peak height, will increase with decreasing F, t_G and $\bar{k}$ (see also eqn. 5) under otherwise constant conditions. This is shown in Fig. 3, where the peak height of cytochrome *c* is plotted against the flow-rate at constant gradient time, t_G. At constant F the peak height increases when the gradient time, t_G, is reduced.

In accord with the rapid kinetics of proteins during migration in the column it is not surprising that the time required to equilibrate the column between individual runs was much shorter than for porous reversed-phase packings. Column stability, as far as it was tested, created no problems. In all the studies described here the same

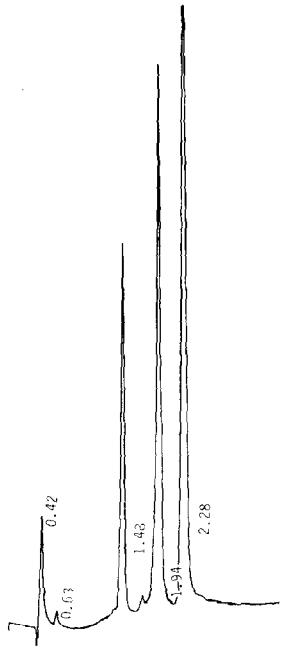

Fig. 4. Separation of ribonuclease (300 μg), lysozyme (190 μg) and myoglobin (500 μg) on a 36 × 8-mm I.D. *n*-octyl-bonded silica column (d_p = 1.5 μm). The linear gradients were from 20 to 75% acetonitrile in 0.01 *M* TFA for 3 min at a flow-rate of 1.5 ml/min; detection at 220 nm; 1.28 a.u.f.s.

column was used. Further, dissolution effects, expected because of the small particles, were not observed. It could be argued that the limited sample capacity of non-porous silica packings might restrict their application. Fig. 4 shows the separation of three proteins, ribonuclease, lysozyme and myoglobin (in amounts of 300, 190 and 500 μg, respectively), on a 36 × 8 mm I.D. column. At these concentrations, a slight decrease in retention times was observed, but the resolution of the proteins was nevertheless excellent. Tests in the low concentration range indicate that even 10-ng amounts of bovine albumin can be detected. This implies that non-porous reversed-phase silicas are suitable for the micropreparative isolation of proteins.

CONCLUSIONS

Although the non-porous *n*-octyl-bonded silica follows the well established pattern of a bimodal plot of logarithmic capacity factor *versus* the volume fraction of organic modifier, the low slopes of the dependences for high-molecular-weight proteins clearly indicate the use of these reversed-phase packings for the separation of larger proteins, both in analytical and isolation work. Resolution is additionally favoured by the high column performance due to the small particle size. The observed sample capacity was exceptionally high. Further investigations will show whether these particles, with an essential external surface, also provide high mass recoveries and maintenance of biological activity.

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