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# NEW PACKING AND COLUMN FOR FAST PROTEIN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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## SUMMARY

Macromolecules can be quickly and effectively separated using packings with very small (either non-porous or wide-pore) particles in short columns. However, exploitation of this capability has until now been hampered by a lack of commercial availability. The first commercially available 3-$\mu$m, 300 Å, bonded-phase, spherical silicas and the capabilities of a 1-cm column (5-cm columns are also commercially available) packed with these silicas at pressures used in high-performance liquid chromatography are described. The particle pore size and size distribution of the silicas are highly reproducible; the resulting bonded-phase silicas are efficient and yield high protein recoveries. The 1-cm column, which can handle normal sample loads (we used up to 50 $\mu$l), can be used for either extremely rapid or slower, more discriminating separations. With a fast gradient, a baseline separation of a standard protein mixture is achievable in less than 90 s with only standard commercial instrumentation. The slow-gradient separations produced by the 1-cm column are virtually indistinguishable from those of a similarly packed 5-cm column.

## INTRODUCTION

In an introduction to a study involving applications of 2-$\mu$m, 200-Å silica, Danielson and Kirkland[1] reviewed the concepts[2] and practices associated with the use of such small particles (diameter $< 5$ $\mu$m) for the separation of macromolecules. Because of improved mass transfer, such small particles should be able to separate macromolecules very quickly when packed into short columns[2]. Toward this end, Unger *et al.*[3] developed non-porous 1-$\mu$m silica microspheres and concluded that with them protein separations could indeed be fast and effective. However, the low load capacity of such non-porous particles limits their applicability, even aside from requirements for adequate peak retention and resolution[1]. Column length is another salient variable; Pearson[4] concluded that a column less than 1 cm long can provide not only increased recovery of but also sufficient resolution for molecules that are sufficiently different from each other. Stevens *et al.*[5] emphasized the importance of fast

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separations of proteins, peptides and other natural macromolecules in, for instance, certain clinical analyses.

Although small, wide-pore particles and short columns have great potential for the fast separation of macromolecules, their use has hitherto been severely hampered by a lack of commercial availability[1]. As a result of this study, a range of bonded phases, based on 3-μm, 300-Å silica, have become available and are marketed as Rexchrom (Regis, Morton Grove, IL, U.S.A.). We report here an evaluation of this first commercially available 3-μm, 300-Å bonded-phase, spherical silica, and the performance of a high-performance liquid chromatographic (HPLC) 1-cm column packed with this material (both 1- and 5-cm columns are commercially available).

## EXPERIMENTAL AND RESULTS

### *Materials*

Mobile phases were prepared with HPLC-grade water, acetonitrile, methanol (EM Science, Cherry Hill, NJ, U.S.A.) and trifluoroacetic acid (TFA) (Pierce, Rockford, IL, U.S.A.). Proteins (ribonuclease, insulin, lysozyme, myoglobin and ovalbumin) and dibutyl phthalate were purchased from Sigma (St. Louis, MO, U.S.A.).

Chromatographic experiments were carried out with standard commercial equipment. The Kratos (Ramsey, NJ, U.S.A.) gradient system used consisted of a Model 783G detector (flow-cell, 12 μl internal volume) that controls two Kratos Spectroflow 400 gradient pumps, and a dynamic high-pressure gradient mixer that combines the flow from the pumps. The internal diameter of the capillary tubing throughout the system was 0.01 in. Samples of 10 and 50 μl were injected with a Model 7010 injection valve (Rheodyne, Calabasas, CA, U.S.A.).

The 3-μm, 300-Å, wide-pore silica (Exsil A300) was specially manufactured and supplied by Exmere (Hawarden, Clwyd, U.K.). The particle-size distribution of this batch (1 kg) was determined by microscopy, and the mean diameter was found to be 3.2 μm, mode 3.1 μm, median 3.2 μm, with a standard deviation of 0.653 μm.

Size-exclusion chromatography (SEC) (Fig. 1) was carried out with polystyrene standards (Polymer Laboratories, Shropshire, U.K.) and tetrahydrofuran as mobile phase. The SEC data were transformed by the method developed by Jerebek[6] and showed a very narrow pore-size distribution with a mean pore diameter of 300 Å. Mercury porosimetry (Fig. 2) confirmed the extremely narrow pore-size distribution and indicated a mean pore diameter of 310 Å and a surface area of 97 $m^2/g$.

The pore volume, determined by titration with methanol by the method of Motlau[7], was 0.777 ml, compared with a differential volume of 0.79 ml found between 10 and 200 nm by mercury porosimetry.

### *Methods*

By proprietary means to ensure a high protein recovery and a truly monomeric coating, an octadecyl stationary phase was bonded to the Exsil A300 silica to produce Rexchrom S3-300-ODS packing (Regis). Columns of 5 cm × 4.6 mm I.D. and 1 cm × 3.0 mm I.D. were packed with this material by proprietary means to achieve a high column efficiency with good peak symmetry. The efficiency of the columns was determined by the half-height method. With dibutyl phthalate (0.85 mg/ml) as the test

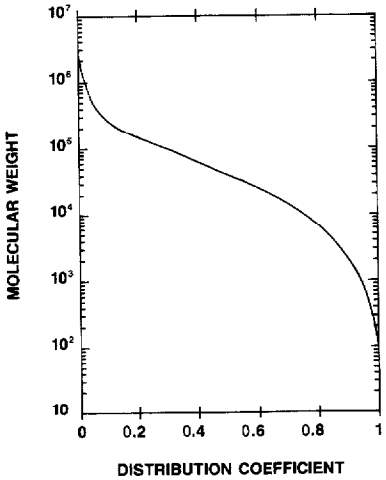


Fig. 1. Size-exclusion calibration graph for 3-$\mu$m, 300-Å silica (Exsil A300/3), obtained by using polystyrene standards in THF.

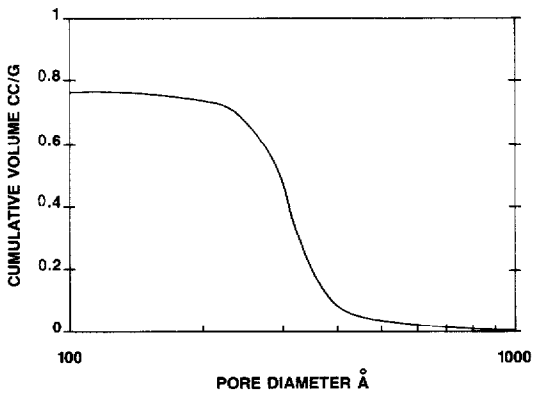


Fig. 2. Pore-size distribution of 3-$\mu$m, 300-Å silica (Exsil A300/3) obtained by mercury porosimetry.

substance, the column efficiency was measured with 70% methanol at a flow-rate of 0.5 ml/min with detection at 254 nm.

The protein test mixture was prepared by dissolving 15 mg of ribonuclease, 5 mg of insulin, 5 mg of lysozyme, 10 mg of myoglobin and 25 mg ovalbumin in 50 ml 0.1% TFA.

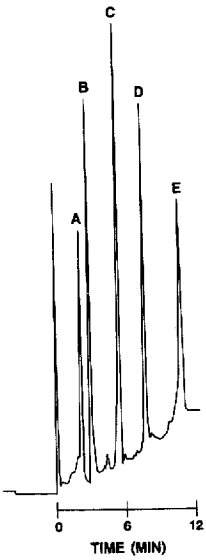


Fig. 3. Chromatogram of a five-component protein mixture: A = ribonuclease; B = insulin; C = lysozyme; D = myoglobin; E = ovalbumin. Column, 1 cm × 3.0 mm I.D. S3-300-ODS; gradient, from A–B (25:75) to 100% B in 25 min (A = 0.1% TFA, B = 0.1% TFA in 95% aq. acetonitrile; flow-rate, 1.0 ml/min; sample, 10 $\mu$l containing 12 $\mu$g of protein; pressure, 500 p.s.i.

For the chromatogram shown in Fig. 3, 10 $\mu$l of the test solution containing 12 $\mu$g of proteins were injected into a 1 cm × 3.0 mm I.D. column. For the chromatograms shown in Figs. 4 (1-cm column) and 5 (5-cm column), 50 $\mu$l of test solution containing 60 $\mu$g of proteins were injected. For the separation shown in Fig. 4, the sample was eluted with a combination of two linear gradients; this took only 2.25 min. Eluent A was 0.1% TFA and eluent B was 0.1% TFA in acetonitrile. The first gradient went from A–B (75:25) to A–B (25:75) in 1.00 min; the second proceeded to 100% in the next 1.25 min.

For Figs. 3 and 5, one 25-min linear gradient was used, from A–B (25:75) to 100% B, where eluent A was 0.1% TFA and eluent B was 0.1% TFA in 95% acetonitrile.

## DISCUSSION

### *Silica*

The previously outlined advantages that accrue from the development of

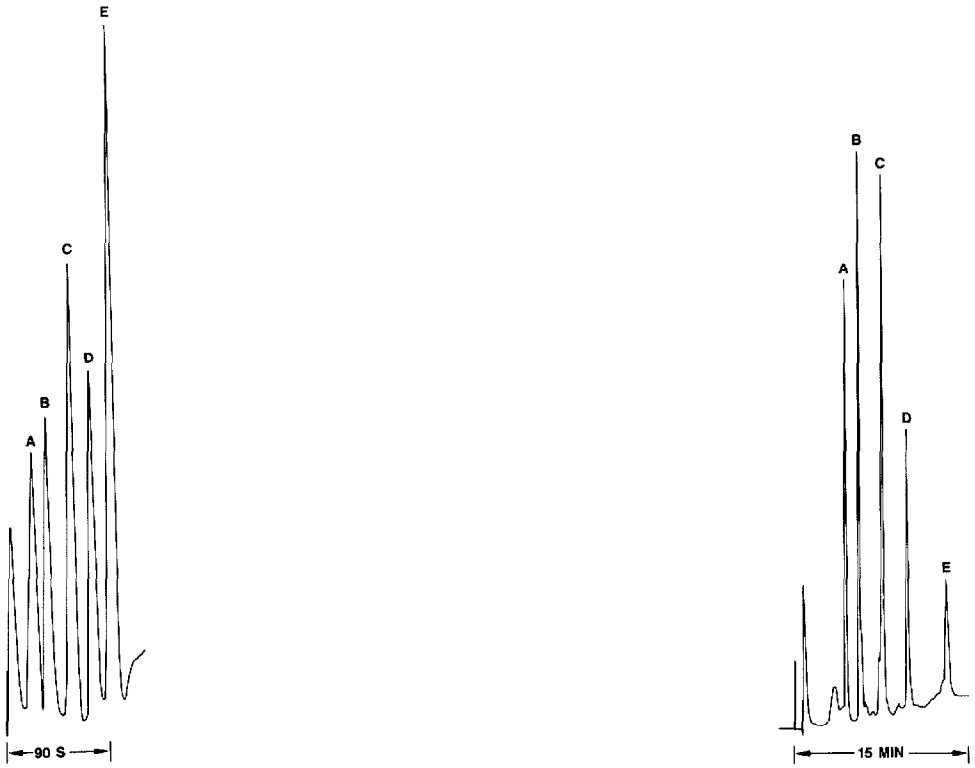


Fig. 4. Chromatogram of the same five-component protein mixture as in Fig. 3 with the same column but with a gradient from A–B (75:25) to A–B (25:75) in 1.00 min, then to 100% B in the next 1.25 min (A and B as in Fig. 3). Flow-rate, 3.5 ml/min; sample, 50 $\mu$l containing 60 $\mu$g of protein; pressure, 1900 p.s.i.

Fig. 5. Chromatogram of the same five-component protein mixture as in Fig. 3 with a 5 cm × 4.6 mm I.D. S3-300-ODS column and the same gradient as in Fig. 3. Flow-rate, 1.0 ml/min; sample, 50 $\mu$l containing 60 $\mu$g of protein; pressure, 1400 p.s.i.

a wide-pore silica based on 3-$\mu$m particles prompted Exmere to carry out a feasibility study on its manufacture as part of a joint study between Exmere and Regis. The proprietary processes developed by Exmere for the manufacture of 5- and 10-$\mu$m wide-pore silicas (Exsil A300) were extended to the preparation of 3-$\mu$m, 300-Å silica. The resulting silica was used in the work reported here.

The intrinsic properties of this new 3-$\mu$m silica were found to be identical with those of the larger diameter products. The extremely narrow pore-size distribution established for the Exsil A300 5-$\mu$m product (which compared favorably in this regard with competitive 5-$\mu$m, 300-Å silicas) was found to be maintained in the new 3-$\mu$m silica.

*Bonding.* Having found that the bonding procedures that heretofore had led to satisfactorily high chromatographic efficiency did not yield satisfactory protein recovery, we re-examined and recast the bonding methods in every detail. The new methods finally adopted, including end-capping, yield a strictly monomeric stationary phase, with a carbon load of 5.75% for ODS. Perhaps because each part of this strictly monomeric coating participates with equal effect in chromatography, proteins are retained more strongly by this packing than they are by comparable products. We found that the reproducibility of each stage of the bonding process could not be taken for granted but had to be, and was, separately established.

The 80 000 plates/m chromatographic efficiency of the new 3-$\mu$m, 300-Å ODS packing is much higher than that of other commercially available products. Nevertheless, it is much lower than the comparably tested, *ca.* 125 000 plates/m efficiency of the 3-$\mu$m, 100 Å ODS packing that we have bonded in exactly the same way. We cannot at present explain the discrepancy.

*Performance of the 1-cm column*

Fig. 4 illustrates both the rapid resolving ability of the 1-cm column and its high capacity. A baseline separation of the protein test mixture was achieved in less than 90 s with a sample load of 50 $\mu$l, containing 60 $\mu$g of protein.

Fig. 3 shows the relatively high resolution achievable with the 1-cm column when a slower gradient is employed. Indeed, it is not easy to distinguish between the chromatogram in Fig. 3, obtained with the 1-cm column, and that in Fig. 5, obtained with the 5-cm column. These results support Pearson's[4] conclusion that very short columns can provide sufficient resolution for pairs of molecules that show adequate differences in retention indices.

Shorter columns have another significant advantage over longer columns, *viz.* the back-pressure was 500 p.s.i. for the 1-cm column (Fig. 3) but 1400 p.s.i. for the 5-cm column (Fig. 5). Because these columns can easily withstand imposed pressures of 5000 p.s.i., flow-rates much higher than the 0.5 ml/min actually used could be tolerated. Under constant use and testing for 4 days, the columns performed consistently. No increase in back-pressure or loss of resolution was observed.

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