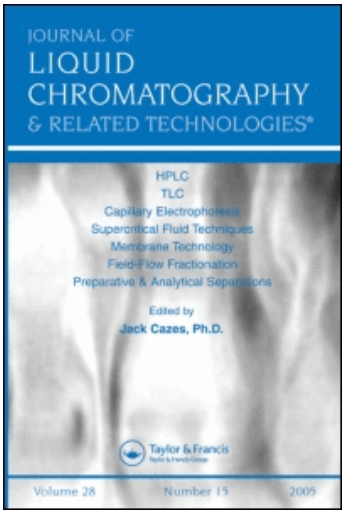

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Non-Porous Silica Supports for High Performance Liquid Chromatography

Larry F. Colwell[a]; Richard A. Hartwick[a]
[a] Department of Chemistry, Rutgers University Piscataway, New Jersey

To cite this Article Colwell, Larry F. and Hartwick, Richard A.(1987) 'Non-Porous Silica Supports for High Performance Liquid Chromatography', Journal of Liquid Chromatography & Related Technologies, 10: 12, 2721 — 2744
To link to this Article: DOI: 10.1080/01483918708066822
URL: http://dx.doi.org/10.1080/01483918708066822

JOURNAL OF LIQUID CHROMATOGRAPHY, 10(12), 2721-2744 (1987)

NON-POROUS SILICA SUPPORTS FOR HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Larry F. Colwell and Richard A. Hartwick*
Department of Chemistry
Rutgers University
Piscataway, New Jersey 08854

ABSTRACT

The physical and chromatographic properties of two types of non-porous HPLC support materials were examined. Loading capacities of non-porous supports were about 10 fold less than of wide-pore gels, while their physical strength was much greater, suggesting that non-porous supports could be useful for polymer separations. Such materials appeared to be less suitable for low-molecular-weight solutes, except for fundamental retention studies, where the absence of pore structure offers a less ambiguous support matrix.

INTRODUCTION

Small Diameter Solid Spheres as HPLC Supports

It has been suggested that the minimum practical particle diameter for HPLC supports is approximately 2 micrometers (1-5) due primarily to the adverse effects of thermal gradients resulting from frictional heat. Dewaele and Verzele (6) reported that at moderate flow-rates, reversed-phase columns packed with 2 um particles did not demonstrate a loss of efficiency due to thermal effects. However, they did note that the columns deteriorated unless a silica pre-

saturation column was used. Support particle size has recently been extended into the submicron range (7) through the use of non-porous silica spheres. Such spheres (typically ca. 0.2 um diameter) have been widely used in ceramics engineering and related fields (8-10) as models for the study of packing structure composite strength. Unger has recently reported the separation of proteins with a column packed with 1.5 um solid spheres (11), thus demonstrating that proteins can be successfully separated with non-porous supports.

Small solid spheres should, in theory, be very attractive for macromolecular separations. The value of such supports for macromolecular separations lies not so much in their small diameter, but rather their lack of pore structure. There have been many investigations into the effects of pore structure on protein separations. The adverse effect of a restrictive porous network on sample loading (12-17) and recovery (13,14) have been well documented. The development of supports with pore diameters >30 nm has greatly reduced this problem. However, these "wide-pore" supports are much more fragile (18) than narrower pore silica gels, making them difficult to pack and operate. Additionally, there is always the possibility that sub-populations of narrower pores will be present that might affect the chromatographic properties of the material. Non-porous supports overcome both these deficiencies, albeit at the price of diminished loading capacities. In addition, non-porous supports present an ideal surface upon which to study the fundamental retention mechanisms of both polymers and low molecular weight solutes, since both the kinetic and thermodynamic properties of the phase interaction itself can be studied apart from the effects of pore structure.

Required Pore Sizes for Protein Separations

In the analysis of proteins, the size of the analyte molecule is a major factor in determining what type of chromatographic support is to be employed. The subject of pore size has long been a focal point in the discussion of the reversed-phase analysis of proteins (12-17). The molecular diameter of a random coil protein can be estimated (in

angstroms) as approximately 80% of the square root of its molecular weight (19). Globular protein diameters can be calculated from their molecular weight by assuming a constant specific volume of 0.73 g/mL (20). Table 1 gives a comparison of the effective molecular diameters of both types of proteins as a function of molecular weight. It can be seen that while the estimated diameters of globular proteins would rarely exceed 150 A for proteins up to MW = 1 x 10^6 daltons, the effective diameter of the random coil proteins can eclipse the pore diameter of most packings at relatively low molecular weights. In contrast, for solid spheres if the narrowest channel is estimated as the diameter formed by the opening between surfaces of three tangent spheres, and assuming that the channel must be at least four times the diameter of the analyte, a column packed with 2 micron particles could be used to analyze random coil proteins up to 10^6 daltons.

A major concern in the reversed-phased analysis of proteins is that of poor sample recovery that cannot be attributed to solvent denaturing. Although this problem is often attributed to non-specific adsorption, the results of several studies on the effect of pore size suggest that both protein recovery (13,14) and the sample capacity (12-16) improve with increasing pore size. Given that the distribution of pore sizes for most packings is often rather large, it is plausible that small pores are at least partially responsible for some of the recovery problems typically associated with proteins. It has been suggested that the retention mechanism of certain proteins can involve a reversible structure change (25), where the protein molecules are adsorbed in their native form, while unfolding at the solvent strength that causes their elution. These facts would suggest the possibility of proteins becoming "trapped" in organic-rich pores of the "proper" diameter. The availability of completely non-porous supports with surface chemistry similar to existing gels will make possible the testing of such hypotheses.

Diminished Phase Ratios of Non-Porous Spheres

One of the most obvious concerns with non-porous, or from another point of view, "infinite pore diameter" supports, is that of reduced

Table 1. Estimated Molecular Diameters for Random Coil and Globular Proteins.

M.W. (daltons)	Globular Proteins (Å)	Random coil Proteins (Å)
10,000	35	82
100,00	76	252
500,000	130	608
10^6	163	816
$5x10^6$	279	1,825
10^7	352	2,580

surface areas. With porous particles most of the surface is contained within the particle, and is related to the pore volume (V_p), BET surface area (S_{BET}) and pore diameter (d_{pore}) by;

$$\frac{40\ 000 * V_p\ (cm^3/g)}{S_{BET}\ (m^2/g)} = d_{pore}\ \text{(Angstroms)} \quad \text{Eq. 1}$$

Consequently, there is little variation in the surface area of particles with identical pore size and structure but different particle diameters. However, with non-porous particles the total surface area within a column is a function of particle size. The calculated surface area for 0.5 um particles with a specific gravity of 2.0 is 6.0 m^2/g. A consequence of this relationship is that columns of the same reduced length (L/d_p) will have the same total surface area, i.e., a 3.0 cm column packed with 1.0 micron spheres will have the same surface area as a 6 cm column packed with 2 um spheres.

A comparison of the surface area of non-porous spheres with some typical wide-pore supports (Table 2) shows an expected decrease in phase ratio of about 100 fold on a simple weight basis. This is

Table 2. Physical Properties of Solid Spheres and Wide-Pore Supports.

Silica	Particle diameter (μm)	Particle shape	Pore diameter (Å)	Surface area (m^2/g)	Density (g/cm^3)	Normalized area (m^2/cm^3)
Solid sphere	0.5	sphr.	N/A	6.0	2.0	12.0
Solid sphere	1.0	sphr.	N/A	3.0	2.0	6.0
Solid sphere	2.0	sphr.	N/A	1.5	2.0	3.0
LiChrosorb (100)	10	irrg.	100	297	0.36	106
Zorbax (100)	7	sphr.	100	139	0.66	92
Zorbax (150)	7	sphr.	150	99	0.68	67
Zorbax (300)	7	sphr.	300	39	0.72	28
Vydac TP	10	sphr.	330	82	0.5	41

offset somewhat by the fact that the particle density of the porous materials is less than 1/2 that of precipitated silica spheres. A 3-cm X 0.46-cm column packed with 1.0 um particles would have about 2 m^2 of total surface area, while the same column packed with Zorbax 300 A support would contain 7.2 m^2 of surface. A more realistic comparison to a 25-cm column yields a 30 fold difference. Data will be shown in the present work that support the observation that a loading capacity reduction of between 10-50 fold is to be expected when using small diameter non-porous spheres. This will limit the usefulness of non-porous to analytical analyses and small scale isolations.

Synthesis of Small Diameter Precipitated Silica Spheres

The procedures for producing silica spheres in the low um range was developed in the late 1960's. In 1968, Stober et al. (21) presented a detailed account of a set of reactions producing spherical particles with diameters that ranged from less than 0.05 um to 2.0 um. While the particle size distributions were very narrow at the very small diameters, the distributions increased substantially at the larger diameters, (i.e., >1 um). The particles were synthesized via the hydrolysis of alkyl silicates and the subsequent condensation of

silicic acid in alcoholic solutions. Ammonia concentration was used (empirically) to control the size and shape of the particles.

The purpose of our investigation was to prepare two types of non-porous supports, and to examine their chromatographic properties in relation to porous silica supports. The first type of non-porous support made was that of Stober (21) and Unger (11), which were synthesized de novo in the range of 0.2 to ca. 1.5 um in diameter. Difficulties in the synthesis of spheres larger than 2 um lead us to a second procedure, which was the production of solid spheres from conventional porous silica supports. This was accomplished by precipitating silica within the pores of the conventional porous silica particles until the surface of the particle was completely sealed. Unlike the Stober process which is restricted to the low micrometer range, the "pore plugging" technology can produce non-porous particles of any size in which porous silica is available. The resulting non-porous material will have essentially the same particle size distribution as the parent gel, with the mean diameter shifted upwards some small amount.

EXPERIMENTAL

Reagents and Instrumentation

Tetraethyl ortho-silane, n-amyl alcohol and reagent grade ammonium hydroxide were obtained from Fisher Scientific. Tetra n-butyl ortho-silane, tetrachlorosilane, trimethylchlorosilane, dimethyloctylchlorosilane and dimethyloctadecylchlorosilane were all obtained from Petrarch Chemicals. Baker HPLC-grade methanol and electro-grade isopropanol were purchased through Ace Scientific. Trifluoroacetic acid was obtained from Aldrich Chemical Company. The silica support used for pore plugging was 10 um Spherisorb (Phase Separations, Norwalk, CT 06854). Licrospher Si 300 was obtained from EM Laboratories Inc., Elmsford, NY. Whatman P/300 was donated by Whatman Inc., Clifton, NJ.

Ammoniacal alcohol solutions were prepared by bubbling ammonia gas from a tank through the alcohol at 0 °C. The ammonia concentration was determined by titration with 1 N hydrochloric acid.

Gradient HPLC analyses were performed with either a Hewlett Packard 1084B (Hewlitt Packard, Palo Alto, CA 94304) or a Varian 5560 (Varian Associates, Inc., Palo Alto, CA 94303) liquid chromatograph.

Synthesis of Solid Silica Spheres

Silica spheres were synthesized following the procedure outlined by Stober (21). After they were isolated from a reaction, particles were resuspended twice in methanol, twice in water, then neutralized to pH 2.0 with hydrochloric acid, washed twice more in water and methanol, suspended in dichloromethane and resettled. The dichloromethane was decanted and the particles were air dried and placed in a 110°C oven for 16 hours.

Synthesis of Plugged Pore Particles

Plugged-pore supports were prepared following the previously outlined procedure (22). Pore volume data supplied by the manufacturer was used to determine the quantity of tetraethyl orthosilane (TEOS) required to precipitate a volume of silica just sufficient to fill the pores. A volume of neat TEOS was mixed into the silica which was just equal to the pore volume, but which was usually less than half of the total TEOS required for total pore plugging. The particles were then suspended in a solution that was 1 part ammonium hydroxide and 4 parts 3:1 isopropanol:methanol. The suspension was stirred at room temperature with a paddle stirrer. After 24 hr, the remaining TEOS was added at a rate of 2 mL every 12 hr When the reactions were completed, the stirrer was stopped and the particles were allowed to settle. The particles were then processed in the same manner as the microspheres.

Stationary Phase Bonding and Column Packing

All of the bonding was performed using the method developed by Berendsen (23). The silica was activated by drying in a 120°C oven for 24 hours. Just prior to bonding, the silica was placed in a microwave oven for 30 min at the high setting. The dried silica was reacted with a monochlorosilane in dry toluene using pyridine as an HCl-scavenging catalyst. The reaction was stirred at 80°C for a period of 16 hours. The bonded particles were then washed with toluene, methylene chloride, methanol, 50 % methanol/water, methanol, methylene chloride and dried at 110 °C. Gas chromatography (24) was used to measure the amount of bonded trimethylsilane.

All columns with 10 um porous particles were packed upward at 8 000 psig from a stirred reservoir using chloroform as the slurry solvent and methanol as the charging fluid. The solid sphere particles were packed downward at 13 000 psig using a 50-cm length of high-pressure tubing as the reservoir.

An attempt to pack the 10 um Si-300 at 5000 psig with a constant pressure pump resulted in crushed particles, as evidenced by a high back-pressure. A second column was then packed using a Waters M-6000. The pump was run at 10 mL/min until the pressure reached 4 000 psig, then the flow was gradually reduced to maintain a constant pressure. The Si-500 and Si-1000 supports were also packed using this procedure.

Chromatography

Gradient analyses of the test proteins were performed at ambient with a linear gradient run from 0-60% of solvent B with a slope of 2% B per mL of mobile phase. Solvent A consisted of 0.1% trifluoroacetic acid in distilled water. Solvent B was neat isopropanol. The run time was adjusted to maintain the same gradient slope at different flow rates. Standards were prepared by dissolving a weighed amount in solvent A.

RESULTS AND DISCUSSION

Silica Spheres Synthesis

The reactions described in the Experimental section produced spheres of uniform size, as illustrated in the photomicrograph shown in Figure 1. Depending upon batch conditions, particles between ca. 0.2 to 1.5 um could be grown. Particles up to 1.5 um were made by reacting tetrabutoxy ortho-silane (TBOS) in 3:1 isopropanol:methanol that was 4.5 M water and 4.6 M ammonia.

Particles in the range between 0.2-0.4 um were narrowly dispersed in diameter, with a relative standard deviation (RSD) averaging about

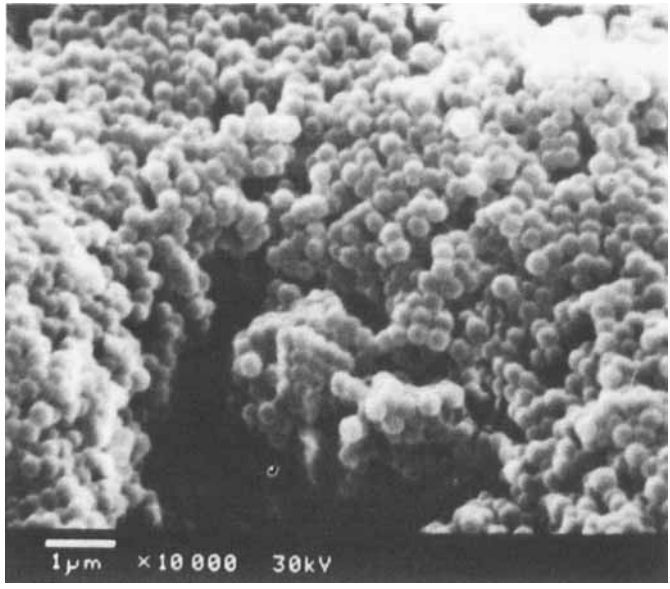

Figure 1: Scanning electron micrograph of 0.2 micrometer non-porous microspheres produced by the Stober process (21).

10%. The %RSD of particles in the range of 1 um increased to an average of about 25%. In general, it was found that the particle-size distribution increased significantly with increasing particle diameter, a situation also observed by Stober (21) in his original work. The actual particle size distributions of the materials produced by Unger (35) were not reported, however examination of the electron micrographs published indicate particle distributions similar in magnitude to those observed here.

Particle Size Determination

The low dispersion and spherical nature of these solid particles makes them ideal for size analysis by sedimentation. A comparison of particle diameters obtained by sedimentation with scanning electron microscopy (SEM) and Galai CIS-1 data (Table 3) shows good agreement among the three techniques. The diameters measured by sedimentation were slightly lower (ca. 10%), probably due to the bias introduced by measuring the fall velocity at the upper visible-edge of the band rather than at the center.

Surface Area Measurement

The use of TMS bonding for surface-area estimates was validated by calibration of several batches of precipitated silica spheres against the nitrogen BET method. The surface area was estimated by assuming a sterically-limited coverage of 4 micromoles/m^2 for the bonded TMS molecules (23). Gas chromatography (24) was used to determine the amount of TMS bonded. The average area of three different porous gel samples, each analyzed in duplicate by the TMS analysis was 208 +/- 5 m^2/g vs. 202 +/- 6 m^2/g for the nitrogen BET measurements. Similar comparisons for batches of the plugged-pore materials yielded 3 m^2/g for the nitrogen BET and 1 m^2/g for the TMS bonding.

Table 3. Comparison of Particle Diameters obtained by Sedimentation with SEM and Galai CIS-1 Results.

	Particle Size (microns)		
Sample	SEM	CIS-1	Sedimentation
44-15-0	0.9	1.1	0.8
44-15-1	0.9	1.1	0.8
44-20-0	0.9	1.0	0.8
59-18-1	*	1.2	1.0
59-23-3	*	1.4	1.3
59-24-0	*	1.6	1.4
59-29-2	*	1.1	1.0
59-33-1	*	1.5	1.3

Protein Separations

A primary application of non-porous supports is in the separation of macromolecules. The gradient separation of three protein standards (Figure 2) on a C_{18} phase bonded to 1.5 um solid spheres yielded retention values comparable to a column packed with a porous support of 30 nm mean pore diameter. Even though the 1.5 um particle column was eight times shorter with a total of fifty times less surface area, the retention values were not significantly different. The fact that the surface area of the support appears to have little effect on the separation is consistent with the fact that proteins, and other strongly adsorbed macromolecules, tend to move quite slowly under isocratic conditions, but then elute with a substantial velocity ($k' <= 2$) over a small change in mobile phase composition. This suggests that the elution of proteins may be more of a solvent-driven phenomenon, as suggested by Sadler et al. (25). Armstrong has reported similar behavior for the non-aqueous separation of polystyrene (26-28). Others have argued that critical adsorption theory is not necessary to describe the retention of macromolecules (29,30). A multi-site adsorption mechanism (31) has also been proposed to account for the nearly "on-off" retention behavior of proteins typically observed under reversed-phase conditions.

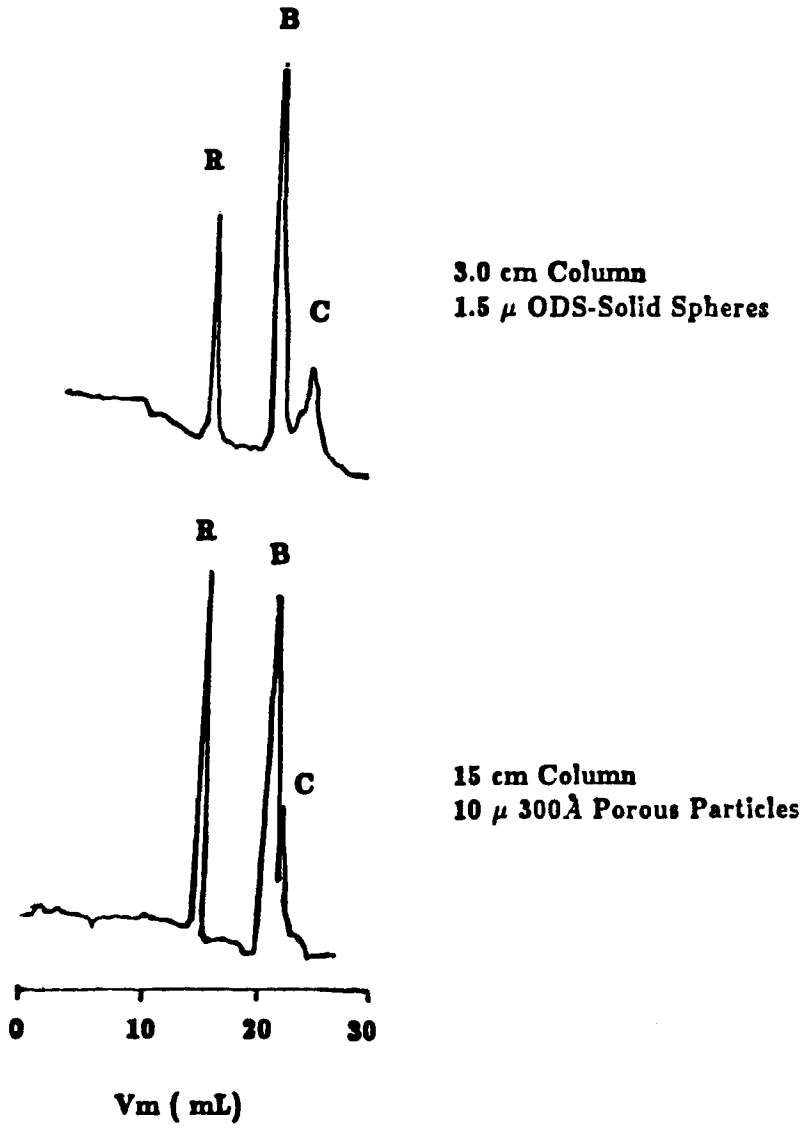

Figure 2: Comparison of a equivalent gradient separations of protein standards with a 3.0 x 0.46 cm column packed with a 1.5 micron non-porous support (C_{18} stationary phase), upper figure, and a 15 x 0.46 cm column packed with 10 um Partisil, pore diameter 30 nm, also bonded with C_{18} under identical conditions. R = Ribonuclease A; B = Bovine Serum Albumin; C = Chymotrysogenin.

Table 4. Effect of Column Length and Particle Size on Protein Retention.

Column Length (cm)	Support	Particle Size (μm)	Retention Volume (mL)		
			RNASE	BSA	CHYM
25	PP-ODS	10	13.0	18.8	27.7
15	300 Å-ODS	10	14.6	21.3	22.2
15	PP-ODS	10	11.7	18.0	20.0
10	80 Å-ODS	3	16.5	21.0	26.0
4	SS-ODS	3	10.1	15.9	19.6
3	300 Å-ODS	10	10.4	13.8	14.7
3	SS-ODS	1.5	14.9	17.8	20.0
1	SS-ODS	0.6	10.3	14.1	16.0

Gradient Conditions: 0-60% B; 2% B ml^{-1}; A= 0.1% TFA/H_2O; B= 2-PrOH; RNASE= Ribonuclease A; BSA= Bovine Serum Albumin; CHYM= Chymotrypsogenin A; PP= Plugged Pore Support; SS= Solid Spheres.

A comparison of the results obtained with columns of various lengths packed with particles ranging from 0.6 to 10 um was performed under constant chromatographic conditions. The results (Table 4) suggest that neither column length or total intra-particle surface area are highly significant factors in the retention of proteins separated by reversed-phase gradient elution chromatography. This behavior is also consistent with the previous observation that the retention of proteins seems to be largely a function of the composition of the mobile phase.

Determination of column efficiencies, especially for protein separations, proved to be futile. Reliable data for low molecular weight test solutes were difficult to obtain, and were not always a reliable indicator of column efficiency for protein separations. In addition, since we were dealing with several new materials, it could not be determined if columns which performed poorly did so because of poor packing techniques, or because of intrinsic performance properties. Further detailed investigations remain underway to study the intrinsic efficiency properties of the non-porous materials developed here.

Since protein separations must be performed with gradient elution, only qualitative evaluations of the overall performance of non-porous spheres could be obtained. Once the proper packing procedures for small diameter solids were determined, reasonably good gradient separations of proteins could be obtained. For example, a 1-cm column packed with 0.6 um solid spheres produced separations of better resolution than a 3-cm column containing 10 um diameter particles of 30 nm pore diameter (Figure 3, Table 5). These data suggest that short columns packed with small particles are capable of achieving gradient elution protein separations as well as conventional columns. Since the peak volumes of the short columns are smaller, they may be preferred for isolations where small amounts of protein are involved.

Effective Surface Area of Porous and Non-porous Supports

Systematic studies were conducted to compare the retention of bovine serum albumin (BSA) on supports of various pore sizes. A series of 15-cm x 4.6-mm columns was packed with 10 um supports of various pore sizes, all bonded in the same manner with monofunctional C_8 silane. The phases were carefully analyzed for bonded phase coverage. The supports covered a range of pore sizes diameters from 6 nm to 100 nm (Table 6).

The retention of BSA was found to be largely independent of the surface area of the support. At 38% acetonitrile/water, BSA eluted unretained from all of the columns. At 37% acetonitrile/water, no elution was observed for times of over 1 hour. Similar observations were made by Norde et al. (32), who studied the adsorption and desorption isotherm of human serum albumin on surfaces that differed with respect to electrical charge and hydrophobicity.

There was one difference observed in the chromatography obtained with the various supports. At 38% acetonitrile, supports with pores below 50 nm produced split peaks. The leading peak eluted in the extraparticle void volume while the second peak eluted closer to t_M.

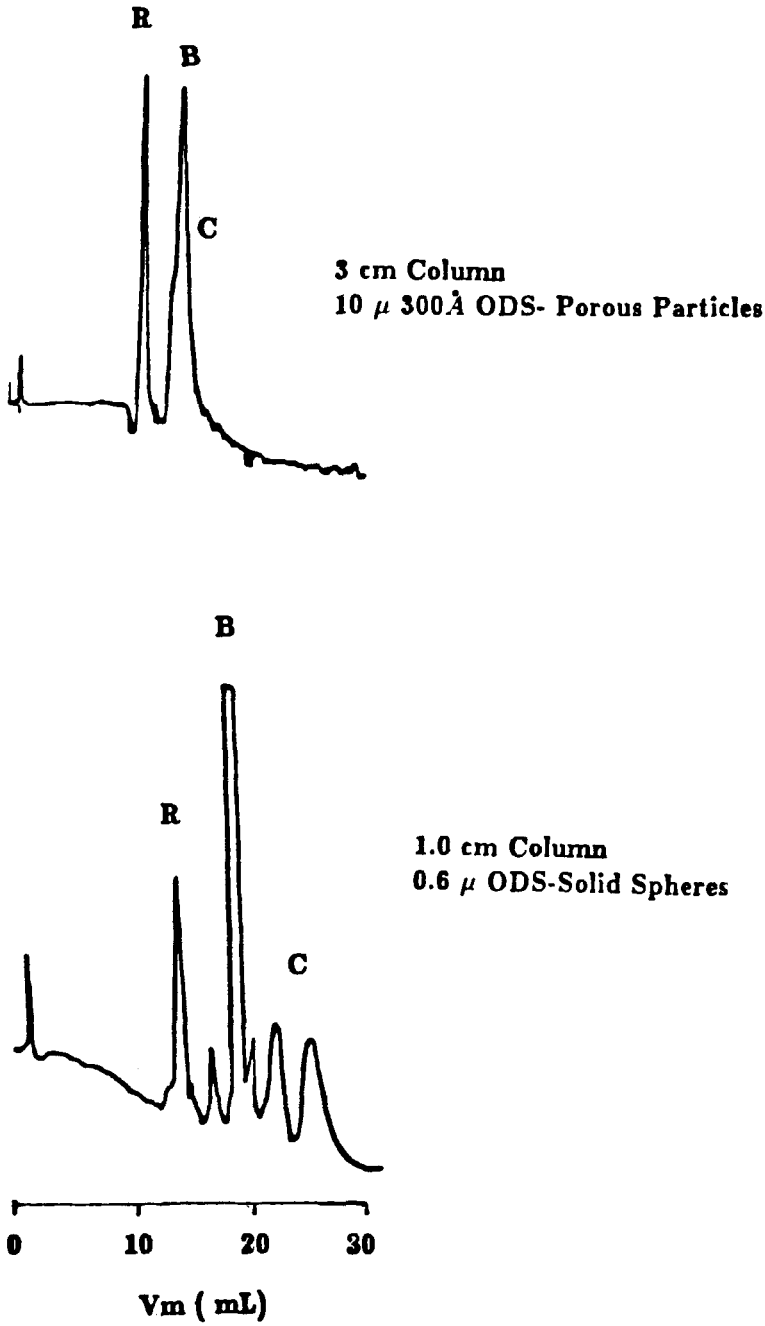

Figure 3: Gradient separations of protein standards with a 3.0 x 0.46 cm column packed with 10 um, 30 nm pore diameter C_{18} Partisil (upper chromatogram) compared to a 1.0 x 0.46 cm column packed with 1.5 micron non-porous C_{18} bonded support. R = Ribonuclease A; B = Bovine Serum Albumin; C = Chymotrysogenin.

Table 5. Effect of Column Length and Particle Size on Protein Resolution.

Column Length (cm)	Support	Particle Size (μm)	Retention (mL) RNASE	Volume (mL) BSA	RESOLUTION
25	PP-ODS	10	13.0	18.8	2.84
10	80 Å-ODS	3	16.5	21.0	1.20
15	300 Å-ODS	10	14.6	21.3	3.04
3	300 Å-ODS	10	10.4	13.8	1.42
3	SS-ODS	1.5	14.9	17.8	3.55
1	SS-ODS	0.6	10.3	14.1	4.8

Gradient Conditions: 0-60% B; 2% B ml^{-1}; A= 0.1% TFA/H_2O; B= 2-PrOH; RNASE= Ribonuclease A; BSA= Bovine Serum Albumin; PP= Plugged Pore Support; SS= Solid Spheres.

Table 6. Physical Data of Supports Used In Isocratic BSA Study.

SUPPORT	PORE SIZE (Å)	SURFACE AREA ($m^2\ g^{-1}$)	SURFACE AREA PER COLUMN (m^2)
Lichrosorb SI-60	60	398	623
Lichrospher SI-100	100	266	313
Lichrospher SI-500	500	59	68
Lichrospher SI-1000	1000	32	49
Vydac TP	330	82	192
1.5μ Solid Spheres	NA	3	1.5

Although it would seem that some type of kinetic process must be involved for this to occur, we are presently at a lost to explain this phenomenon. It is however produced by the pore network, since neither the very large pore supports nor the non-porous supports exhibited this type of behavior.

Denaturation of BSA Protein on Reversed-Phase Surfaces

Upon observing the "critical" type retention behavior of BSA on all of the reversed-phase columns, we were curious if any long-term structural changes might have occurred to the protein as a result of having been adsorbed to the surface. We also wished to determine if any such structural changes could be associated with differences in support pore structure.

The UV-Vis spectrum of BSA was used as an indicator of structural changes. Spectra of BSA were taken with a diode-array detector under two conditions. In the first, BSA was injected into a mobile phase of 40% acetonitrile, where no adsorption to the stationary phase was presumed to have occurred. These spectra were compared to those of BSA injected into 37% acetonitrile, followed by elution with 40% acetonitrile. Significant differences in the spectrum of BSA was consistently observed with this experiment. Typically, the UV spectrum of the peak that was retained and then eluted exhibited a much stronger absorption band at 280 nm (Figure 4), with a hypsochromic shift of about 4 nm in its absorption maximum. No attempt was made to determine if the BSA could be melted back to its original configuration, or how long such melting would take if it did occur.

This experiment indicated that BSA eluting at 40% acetonitrile had not interacted with the stationary phase in such a manner as to produce the long-lived spectral changes being monitored. This implies that on the average, few BSA molecules had adsorbed/desorbed to the surface under these conditions, and that the statistical ratio of molecules in the adsorbed/desorbed state must have approached 0, or at least was below the detection limit of our observations. Furthermore, this ratio changed from some large fraction, which we could not measure, to a very small fraction approaching 0, within less than a 1% change in mobile phase, which we felt represented about the lowest reliable change in mobile phase that we could readily monitor. Such observations, while not definitive, would argue against a normal retention mechanism based on probability distributions, but would be consistent with a critical solubility phenomenon.

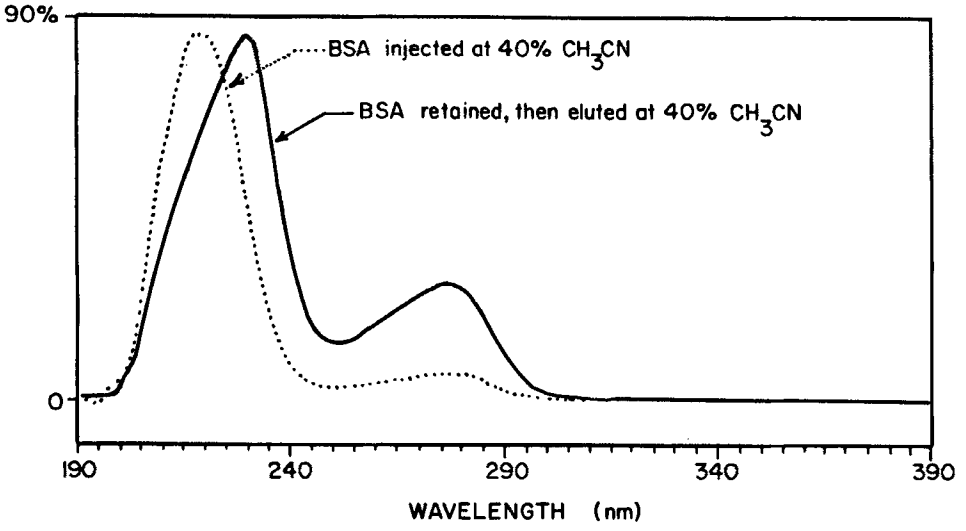

Figure 4: Diode-array UV-spectrum of retained and non-retained BSA peak using a 30 nm porous support, showing protein structural after adsorption on the C_{18} bonded surface. Similar spectral shifts were observed on both the porous and non-porous supports, indicating that surface hydrophobicity, rather than pore structure, was responsible for the denaturation.

It was found that little or no difference in spectral changes could be attributed to pore structure, or lack thereof, with porous and non-porous supports with C_8 bonded phases producing similar spectral shifts. Several authors have reported similar denaturation of proteins during the adsorption process (32-34). Through the use of circular dichroism, Norde et al. (32) found a 20% loss of alpha helix content of desorbed serum albumin. The degree of helix loss was independent of the method of desorption, suggesting that the loss was the result of structural rearrangement during adsorption. Further evidence of structural rearrangement was presented by Soria et al. (33), who used an immunoenzymological assay with a monoclonal antibody to bind an epitope present in the D domain of adsorbed fibrinogen that is inaccessible in the native conformation.

Sample Loading and Pore Accessibility

A sample loading study was also performed with bovine serum albumin using the same chromatographic conditions on a series of columns with various pore sizes, and on non-porous supports of various particle sizes. Column loading capacity was defined as the onset of peak splitting, indicated by the first appearance of a second peak at the approximate column dead-volume (16) with increasing injected mass. The results of this experiment are shown in Table 7.

Compared to columns packed with porous support, the effective sample capacity of the non-porous materials is less by roughly an order of magnitude. For example, a sample capacity of 21 mg has been reported for BSA on a 10 x 0.8 cm packed with one "wide-pore" support (16), versus our value of 1 mg for a 30 x 0.46 cm column packed with 1.5 um solid spheres. This is equivalent to a 7-fold reduction in total injection capacity for the solid-sphere material when normalized to column diameter, or a 21-fold reduction when normalized to column geometric volume.

When the sample loading is normalized to the the total nitrogen BET surface area, the role of pore structure on the available surface area becomes apparent. Using BSA as the test protein, load capacity per m^2 of (BET) surface area increases with pore size, up to a pore diameter of 50 nm, whereas the normalized loading of both wide-pore and non-porous particles become identical at 0.1 m^2/g. Unger also obtained a mass loadability of 0.1 m^2/g for a 1.5 um non-porous support (35). It is evident that BSA is not accessing all of the stationary phase surface for nominal pore diameters of up to 30 nm. Nominal pore diameters of 50 nm or greater were required before full access to the support surface was obtained for the BSA molecule.

Some care must be taken in interpreting these results however, since in real materials one is dealing with a pore-size distribution, and not a true pore-size. Based on the dimensions of a BSA molecule, it is likely that full access to 300 A channels was taking place, with the proteins being excluded from sub-populations of pores of

Table 7. Normalized Loading Capacity of Bovine Serum Albumin.

Column Support	Pore Size Å	Column Length (cm)	Column Surface Area (m^2)	Sample Loading (mg)	Normalized Loading (mg m^{-2})
Spherisorb (ODS)	60	15	786	0.44	5.6×10^{-4}
Lichrospher SI-300 (ODS)	300	25	518	11.1	2.1×10^{-2}
Lichrospher SI-500 (C-8)	500	15	68	8.6	0.1
Lichrospher SI-1000 (C-8)	1000	15	49	5.8	0.1
Plugged-Pore (ODS)	NA	25	4.0	0.54	0.1

significantly smaller diameter. Thus, the effects observed are likely to vary from one particular support to another, depending upon the pore size distributions present, and carte blanche implications as to the general utility of nominal 300 A silica gels for certain applications should be avoided. The same must be said of the many published reports regarding the role of pore diameter on macromolecular retention, most of which have made no attempt to quantify true pore size distributions.

Sample Recovery

Given the complexity and uncertainty in the interpretation of protein recovery, no attempt was made to devise exhaustive experiments to measure recovery. Instead, a qualitative indication of sample recovery was obtained by performing a blank gradient run immediately following the separation of a sample. When this experiment was performed with a non-porous support using sample loadings less than full capacity, no carry-over was observed in subsequent blank gradients. Even at a 250% overload injection of BSA, only a small "ghost" peak was observed (Figure 5) in the subsequent blank run. In contrast, a 50% capacity injection on a 30 nm support re-packed into the identical column hardware produced a proportionally larger ghost peak. This "ghosting" of the 30 nm pore support operated under normal

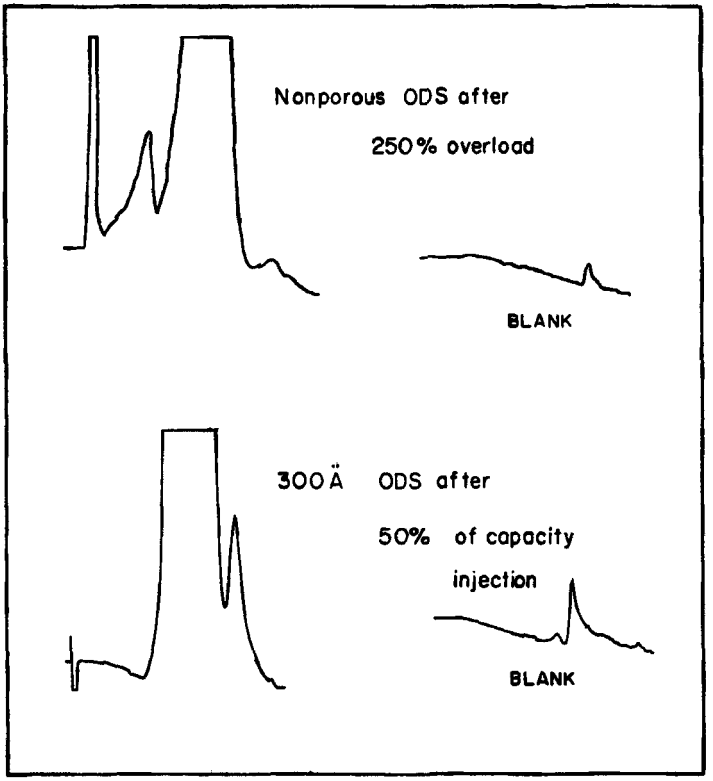

Figure 5: Sample carry-over of BSA after a 250% overload on a non-porous support and a 50% of capacity injection on a 30 nm pore size support.

loading conditions was taken as evidence that pore structure might play a role in sample recovery, along with active-site adsorption and other effects. Further experiments will be required to further isolate where and how protein was being lost in the system.

CONCLUSIONS

Non-porous supports present a viable alternative to the current generation of wide-pore silica gels. With the template synthetic

procedures developed, particle sizes for non-porous supports are not limited to 1-2 um. However, the weakness of the approach is that the particle size distribution will mirror that of the parent material. While porous silica gel was used as the seed material, it should be possible to use other less expensive and more uniform supports for such seeding, especially if the silica shell is made thick enough for good mechanical strength. Monodisperse polystyrene and latex particles are two materials that might be considered. It should also be possible to create a small diameter "pellicular" support by adding a thin porous layer of silica gel around the outside of the spheres, thus increasing the surface area by probably a factor of 10, with negligible reductions in mass transfer rates. The utility of such materials to low molecular weight solutes is doubtful however, since small diameter supports will usually be operated near their optimum reduced velocity within reasonable pressure drops, thus mitigating any stagnant mobile phase mass transfer advantages.

The role of non-porous spheres in low molecular-weight separations would seem to be limited to theoretical studies, such as those involved with bonding densities, direct spectroscopic analyses of adsorbed solutes and in the isolation of stagnant mobile phase effects. In our opinion, the most significant role of non-porous supports, regardless of their particle diameter, will be in macromolecular separations, and in basic research into the retention mechanisms of both macromolecules and low molecular weight solutes.

Acknowledgments

This work was supported by the Center for Advanced Food Technology (CAFT), by a grant from the Petroleum Research Fund, 16161-AC3, by the Busch Memorial Fund and by a grant from the PQ Corporation. The support of Lawrence F. Colwell Jr. by a Merck & Co., Inc. educational fellowship is also gratefully acknowledged.

REFERENCES

1. H. Poppe and J. C Kraak, J. Chromatogr., 282, 399 (1983).

2. H. Poppe, J. C. Kraak, J. F. K. Huber and J. H. M. van den Berg, Chromatographia, 14, 515 (1981).

3. I. Halasz, R. Endele and J. Asshauer, J. Chromatogr., 112, 37 (1975).

4. J. Knox and M. Saleem, J. Chromatogr. Sci., 17, 614 (1969).

5. K. K. Unger, W. Messer and K. F. Krebs, J. Chromatogr., 149, 1 (1978).

6. C. Dewaele and M. Verzele, J. Chromatogr., 282, 341 (1983).

7. L. F. Colwell and R. A. Hartwick, "Non-Porous Silica Supports", presented at the 1985 Eastern Analytical Symposium, New York, November 1985.

8. S. C. Danforth, "Synthesis and Processing of Fine Ceramic Powders", Tokyo Inst. of Techn. Lecture Meeting on Fine Ceramics, Tokyo, Japan, Oct. 1985.

9. S. C. Danforth and J. S. Haggerty, J. Am. Ceram. Soc., 66, C-199 (1982).

10. R. L. Colbe and R. M. Cannon, "Current Paradigms in Powder Processing", Processing of Crystaline Ceramics, Ed. H. Palmour III, R. F. Davis and T. M. Hare, Plenum Press, 1978, p151-169.

11. K. K. Unger, G. Jilge, J. N. Kinkel and M. T. W. Hearn, J. Chromatogr., 359, 61 (1986).

12. B. N. Jones, R. V. Lewis, S. Paabo, K. Kopina, S. Kimura and S. Stein, J. Liq. Chromatogr., 3, 1373 (1980).

13. M. van der Rest, H. P. J. Bennet, S. Solomon and F. H. Glorieux, Biochem. J., 191, 253 (1980).

14. J. D. Pearson, W. C. Mahoney, M. A. Hermodson and F. G. Regnier, J. Chromatogr., 207, 325 (1981).

15. K. J. Wilson, E. van Wieringen, S. Klauser, M. W. Berchtold and G. J. Hughes, J. Chromatogr., 237, 407 (1982).

16. F. V. Warren, Jr. and B. A. Bidlingmeyer, J. Liq. Chromatogr., 8, 619 (1985).

17. R. V. Lewis, A. Fallon, S. Stein, K. D. Gibson and S. Underfriend, Anal. Biochem., 104, 153 (1980).

18. K. K. Unger, J. N. Kinkel, B. Anspach and H. Giesche, J. Chromatogr., 296, 3 (1984).

19. G. Guiochon and M. Martin, J. Chromatogr., 326, 3 (1985).

20. M. E. Young, P. A. Carroad and R. L. Bell, Biotechnol. Bioeng., 22, 947 (1980).

21. W. Stober, A. Fink and E. Bohn, J. Colloid Inter. Sci., 26, 62 (1968).

22. L. F. Colwell Jr. and R. A. Hartwick, HRC & CC, 9, 304 (1986).

23. G. E. Berendsen and L. de Galan, J. Liquid Chromatogr., 1, 403 (1978).

24. S. D. Fazio, S. A. Tomellini, S. Hsu, J. B. Crowther, T. V. Ragilone, T. R. Floyd and R. A. Hartwick, Anal. Chem., 57, 1559 (1985).

25. A. J. Sadler, R. Micanovic, G. E. Katzenstein, R. V. Lewis and C. R. Middaugh, J. Chromatogr., 317, 93 (1984).

26. D. W. Armstrong and K. H. Bui, Anal. Chem., 54, 706 (1982).

27. D. W. Armstrong, K. H. Bui and R. E. Boehm, J. Liq. Chromatogr., 6, 1 (1983).

28. D. W. Armstrong and R. E. Boehm, J. Chromatogr., 22, 378 (1984).

29. M. A. Quarry, M. A. Stadlius, T. H. Mourey and L. R. Snyder, J. Chromatogr., 358, 1 (1986).

30. M. A. Stadlius, M. A. Quarry, T. H. Mourey and L. R. Snyder, J. Chromatogr., 358, 17 (1986).

31. D. Pearson, N. T. Lin and F. E. Regnier, Anal. Biochem., 124, 217 (1982).

32. N. Norde, F. MacRitchie, G. Nowicka and J. Lyklema, J. Colloid Interface Sci., 112, 447 (1986).

33. J. Soria, C. Soria, M. Mirshanhi, C. Boucheix, A. Aurengo, J. Perrot, A. Bernadou, M. Samama and C. Rosenfeld, J. Colliod Interface Sci., 107, 204 (1985).

34. V. Sevastianov and O. Laksina, J. Colloid Interface Sci., 112, 279 (1986).

35. K.K. Unger, G. Jilge, R. Janzen, H. Giesche and J. N. Kinkel, Chromatographia, 22, 379 (1986).