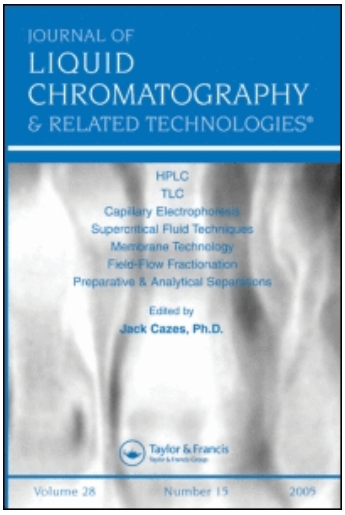

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

On the need for "Wide-Pores" in the Reversed-Phase Liquid Chromato-Graphic Separation of Proteins

F. Vincent Warren Jr.[a]; Brian A. Bidlingmeyer[a]
[a] Waters Associates, Milford, Massachusetts

To cite this Article Warren Jr., F. Vincent and Bidlingmeyer, Brian A.(1985) 'On the need for "Wide-Pores" in the Reversed-Phase Liquid Chromato- Graphic Separation of Proteins', Journal of Liquid Chromatography & Related Technologies, 8: 4, 619 — 634
To link to this Article: DOI: 10.1080/01483918508067108
URL: http://dx.doi.org/10.1080/01483918508067108

JOURNAL OF LIQUID CHROMATOGRAPHY, 8(4), 619-634 (1985)

ON THE NEED FOR "WIDE-PORES" IN THE REVERSED-PHASE LIQUID CHROMATOGRAPHIC SEPARATION OF PROTEINS

F. Vincent Warren, Jr. and Brian A. Bidlingmeyer
Waters Associates
34 Maple Street
Milford, Massachusetts 01757

ABSTRACT

Pore size information for 13 commercially available reversed-phase columns is determined by application of the GPC technique described by Halasz and Martin. In most cases, the experimental median pore diameter (MPD) is in good agreement with the nominal pore size for the packing material, although the discrepancies are large in a few cases. The measured MPDs are used as a basis for identifying whether pore size is a major contributor to resolution, peak width and sample capacity in the reversed-phase separation of proteins on commercially available columns.

INTRODUCTION

Successful separations of proteins with good recovery using conventional pore size columns continue to be reported (9-12), and it is not immediately clear that wide pore materials will always be preferred for protein separations. Certainly, the use of Reversed-Phase Liquid Chromatography (RPLC) for the separation of biological materials including peptides and proteins has steadily increased in recent years (1), but the nature and extent of pore size effects for the commercial RPLC columns available for separation of large proteins (MW 50,000) have not yet been fully clarified. Pore size is frequently suggested as an important factor in determining resolution and

 0148-3919/85/0804-0619$3.50/0

efficiency (2-7) as well as the maximum sample load (3) and the recovery of injected biological material (1, 4-5). Unfortunately, several existing reports consist of isolated comparisons in which several variables are simultaneously altered (2-4, 7), thus complicating the interpretation of results. Comprehensive studies of closely related packing materials under carefully controlled conditions are just beginning to appear (5-6, 8). The frequent claim regarding pore size is that "wide-pore" packings (eg. 300A, 500A) outperform conventional 60-100A materials in RPLC separations with regard to resolution, sample capacity and recovery. The findings which support this claim, although preliminary, have been sufficient to arouse a considerable upsurge of interest in wide-pore packings for RPLC and to stimulate the offerings of wide-pore columns by a number of manufacturers.

One of the significant problems with existing studies of pore size effects in RPLC is the absence of the "in situ" measurement of pore size of the actual column used in the study. Most reports have relied upon the silica manufacturer for the pore size information. The value of this source may not be adequate for a thorough comparison, particularly if a nominal pore size designation is all that can be obtained. There are several cautions associated with the use of such a value. The nominal pore size (NPS) is an "average" or "typical" value which gives no indication of batch to batch variations. In addition, a standard method for pore size measurements has not been agreed upon, and the method used to determine the NPS may not be stated by the manufacturer. If the Wheeler equation is used to determine the NPS (14) then the stated value gives only a rough indication (13) of the mean of the pore size distribution (PSD). In our experience, some NPS values have been as much as 100 percent greater than the experimentally determined mean pore size (15-16), indicating the inadvisability of relying on nominal pore size values

The two classical techniques for the direct measurement of pore size distributions are mercury intrusion (porisimetry) and gas condensation/ evaporation (13). Neither technique has been practical for most chromatographic laboratories, partly because of the expense of owning and maintaining the required equipment. In addition, both techniques are intended for dry, rigid solids and their ability to effectively characterize chemically modified materials is not clear. The manner in which these measurements are made is not representative of the type of interactions which occur in chromatographic systems.

Recently, a simple method has been introduced for the measurement of pore size information based on gel permeation chromatography (GPC). This method, introduced in 1975 by Halasz (17) and described more fully later (18-24), uses polystyrene standards as pore size probes and should be more indicative of the molecular diffusion which takes place in RPLC separations. The "in-situ" measurements can be completed in a few hours using an isocratic liquid chromatograph, which is commonly available to most chromatographers, and the necessary calculations are easily performed.

In this paper, we investigate the relationship between the pore size of commercially available columns and the performance of those columns for the separation of proteins. The Halasz method is applied to thirteen silica-based packings for RPLC, some of which are specifically marketed for the separation of proteins. The median pore diameter (MPD), thus determined, is used to characterize the PSD of each material. Each column is then used for the separation of five protein standards ranging in molecular weight from 3,300 to 67,000. Comparisons are made on the basis of retention, resolution, efficiency and sample capacity in an attempt to determine correlations between these factors and the MPD of the various column packings.

It should be noted that every effort was made to control as many variables as possible. Most materials were purchased in bulk and packed into identical containers by the same operator, although in some cases this was not possible. With one exception, C18 bonded phases were employed, and an identical gradient was used for the elution of the proteins from all columns. However, variables such as silica type, carbon load, degree of end-capping and pre-treatment of the silica cannot be controlled in any study which surveys a range of commercially produced columns. Since the purpose of this study is to see if wide pores are the "magic" ingredient for good protein separations and not to make a definitive statement regarding pore size effects, the complications introduced by these additional variables are acceptable. Also, this is the way a researcher must presently choose between commercially available packings.

MATERIALS AND METHODS

Preparation of Columns. Where available, bulk C18 packing materials of nominal 10μ designation were purchased from various manufacturers and used as received. Bulk materials were custom packed into 8mm ID x 10cm polyethylene

Radial-PAK™ cartridges (P/N 85820, Waters Associates, Milford, MA) by a single operator. In the remaining cases, (see Table I) packed steel columns were purchased. All columns were conditioned with several column volumes of tetrahydrofuran (THF) before determination of the median pore diameter. For convenience, columns will be referred to throughout this paper by the letter designations shown in Table 1. Columns K and L were not available with a C_{18} bonded phase. The bonded phase for column K is proprietary. Column A contains a pellicular packing, and column C was packed with a preparative material. Columns B,F, and J could not be obtained in a 10 micron particle size. It should be emphasized that the column packing materials studied consist of a single example of each type, and may not be adequately represent other samples of the same material due to variability from batch to batch.

Preparation of Eluents and Standards. Eluents were prepared from LC grade isopropanol (Waters) and water purified with a Milli-Q system (Millipore, Bedford, MA). Solvent A consisted of 0.1 percent trifluoroacetic acid (TFA) (Pierce, Rockford, Ill.) in water. Solvent B was neat isopropanol. Protein standards contained approximately 1.5mg per ml of solvent A of each of the following: insulin, chain β oxidized, MW 3.3K (Schwartz-Mann, Orangeburg, NY), ribonuclease a, MW 13.7K (Sigma, St. Louis, MO), ovalbumin, MW 45K (Sigma), bovine serum albumin, MW 67K (Sigma) and aldolase, MW 158K (Sigma). Uracil was included in all samples to serve as an unretained solute. All proteins were stored frozen when not in use and were used as received from the supplier. Standard solutions were prepared weekly and unused portions were stored frozen. Fresh aliquots were thawed every 1-2 days for chromatographic use.

For the MPD determination, UV-stabilized LC grade tetrahydrofuran (Waters) was used. Polystyrene standards (Waters) and n-hydrocarbons from various sources were prepared as dilute solutions in THF. Concentrations were approximately 0.03 percent (w/v) for the polystyrenes and 0.15 percent for the hydrocarbons.

Determination of Column Efficiency. The plate count of each column was determined prior to any other chromatography. A test mix containing benzene, uracil and acenapthene was eluted using an eluent consisting of 60/40 acetonitrile/water at a flow rate of 1.0 ml/min. A chart speed of 5.0 cm/min was used during plate count determination. Plate counts were then calculated on the basis of manual measurement, using both tangent and 5-sigma methods.

TABLE I: COLUMNS STUDIED

COLUMN	BONDED PHASE	PARTICLE SIZE (μ)	COLUMN* CONTAINER	PORE VOLUME (ml)	MEAN PORE** DIAMETER (A)	NAME	°SOURCE
A	C18	35-70***	x	0.35	59	CORASIL C18	1
B	C18	5	x	0.89	68	NOVAPAK C18	1
C	C18	55-105	x	1.89	69	PREP BONDAPAK C18	1
D	C18	10	x	2.22	100	μBONDAPAK C18	1
E	C18	10	x	1.62	81	UNIVERSIL C18	2
F	C18	5	x	1.93	245	SYNCHROM RP-P	3
G	C18	10	x	1.86	251	VYDAC 218 TP	4
H	C18	10	x	2.10	263	BAKERBOND WIDEPORE C18	5
I	C18	10	x	2.13	251	(EXPERIMENTAL)	1
J	C18	5	y	1.30	219	SUPELCO LC318	6
K		10	z	0.61	275	BROWNLEE RP300	7
L	C8	10	y	1.96	162	WHATMAN PROTESIL C8	8
M	C18	5	y	1.34	99	IBM OCTADECYL	9

* x = Radial-Pak cartridge 10 x 0.8 cm
y = Steel 25 x 0.46 cm
z = Steel 10 x 0.46 cm

** Experimental MPD value (see text)
*** Pellicular Packing

° 1 = Waters Associates, Milford, MA.
2 = Universal Scientific, Atlanta, GA
3 = SynChrom, Linden, IN
4 = The Sep/a/ra/tions Group, Hesperia, CA
5 = J.T. Baker Research Products, Phillipsburg, NJ
6 = Supelco, Bellefonte, PA
7 = Brownlee Labs, Santa Clara, CA
8 = Whatman Chemical Separation, Clifton, NJ
9 = IBM Instruments, Wallingford, CT

Measurement of Median Pore Diameters. The procedure for using GPC to measure pore size distribution is described in detail elsewhere (16-29). The value of pore size, ø, corresponding to the 50 percent value in the cumulative distribution is taken as the median pore diameter.

Chromatographic System. Gradient chromatography was performed using a Waters Associates Model 244 ALC, including two M6000A Solvent Delivery Systems, an M440 two channel UV-Visible Detector operating at 254nm and 280nm, and a M401 Differential Refractometer (for PSD measurements only). Runs were directed by a Model 730 System Controller (Waters) with injections made by a Model 710B WISP™ Auto Sampler. Detector outputs were monitored by a model 730 Data Module printer/plotter/integrator (Waters). The last three components were connected via the Intelink system.

In order to insure that each column was evaluated fairly, using solvent conditions which would give reasonable resolution of the protein test mixture, two gradients were used. Gradient G1 was used for all columns, with G2 applied only to those columns unable to resolve bovine serum albumin and aldolase with G1. Except for two columns which failed to elute some of the proteins (see below), all columns could successfully resolve the five proteins with one of the two gradients. Gradient G1 was linear from 0 percent B to 50 percent B in 35 minutes. Gradient G2 was linear from 0 percent B to 25 percent B in 5 minutes, followed by a 30 minute linear segment from 25 percent B to 50 percent B.

RESULTS AND DISCUSSION

The experimentally determined MPD for each column is indicated in Table I. For those cases in which a manufacturer-supplied NPS value was available, a comparison of NPS and MPD values is given in Table II. The relative deviations range from 9-100 percent, indicating the approximate nature of some NPS claims. A small deviation is expected, due in part to the fact that NPS values are generally based on unbonded silica, while MPD values are determined using the bonded phase. For a C_{18} phase, Halasz estimates a 20A reduction of the pore diameter (15). Batch-to-batch variability and experimental uncertainties may also contribute to a deviation between the NPS and MPD values, but several of the deviations shown in Table II are too large to be explained by these contributions.

TABLE II: NOMINAL PORE SIZE VS. MEAN PORE DIAMETER BY GPC

	NPS*(Å)	MPD(Å)	Deviation
H	330	263	67
G	330	251	79
K	300	275	25
F	300	245	55
J	300	219	81
L	300	162	138
E	"WIDE"	81	-
D	125	100	25
B	90	68	22
A	NA**	59	-
C	NA	69	-
I	NA	251	-
M	NA	99	-

* value claimed by the manufacturer

** not available or proprietary

Retentivity. Figure 1 shows a typical chromatogram, obtained using a column having one of the smallest median pore diameters. The same elution pattern was observed for all columns, with two exceptions: columns E and M failed to elute three of the five test proteins under any eluent conditions. The reason for this behavior is not clear. Figure 2 provides a clearer comparison of the elution behavior observed for those columns which gave resolution of all five proteins using gradient G1 (see experimental section). The parameter G' used as the basis of comparison is identical to the commonly used k'. While k' is not strictly applicable to gradient elution, values of G' are still useful for judging similarity of elution behavior. All columns except for K and E are seen to behave quite similarly. Due to a lack of information regarding the proprietary phase used with column K, it is not possible to rationalize the unusual retentivity of that column. The non-elution exhibited by column E (and similarly by column M) is even more

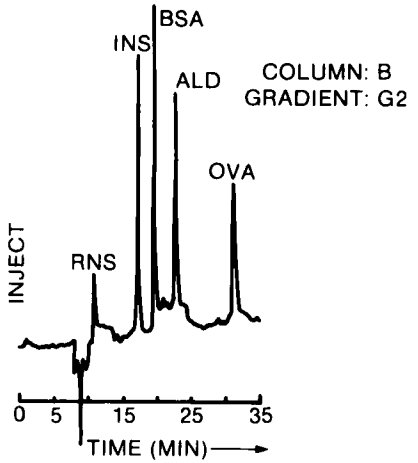

FIGURE 1 Typical Chromatogram.

disturbing. Factors such as carbon load or degree of end-capping may explain the observed behavior, but to our knowledge, studies which could shed light on this problem have yet to be performed.

Resolution. The chromatograms presented in Figure 3 demonstrate that the retention and resolution of proteins is not a simple function of the experimentally determined MPD. Column D is a general purpose reversed phase column having a similar MPD to that of Column E, a "wide-pore" column which is specifically marketed for the separation of proteins. The chromatography obtained with Column D, while clearly superior to that of Column E, is not obviously inferior to the results obtained using Column H for which the measured MPD is almost fourfold larger. The coelution of BSA and INS with Column D is readily eliminated by the use of gradient G2.

The correlation between MPD and resolution is displayed more clearly in Figure 4, which plots the resolution of two proteins, β-insulin and bovine serum albumin, for nine columns as a function of MPD. Gradient G1 was used in every case. While the overall trend is toward improved resolution as MPD increases, there are counter-examples present which cloud the correlation. Specifically, the column having the smallest MPD of the set provides a resolution nearly equal to that obtained with columns having a much larger

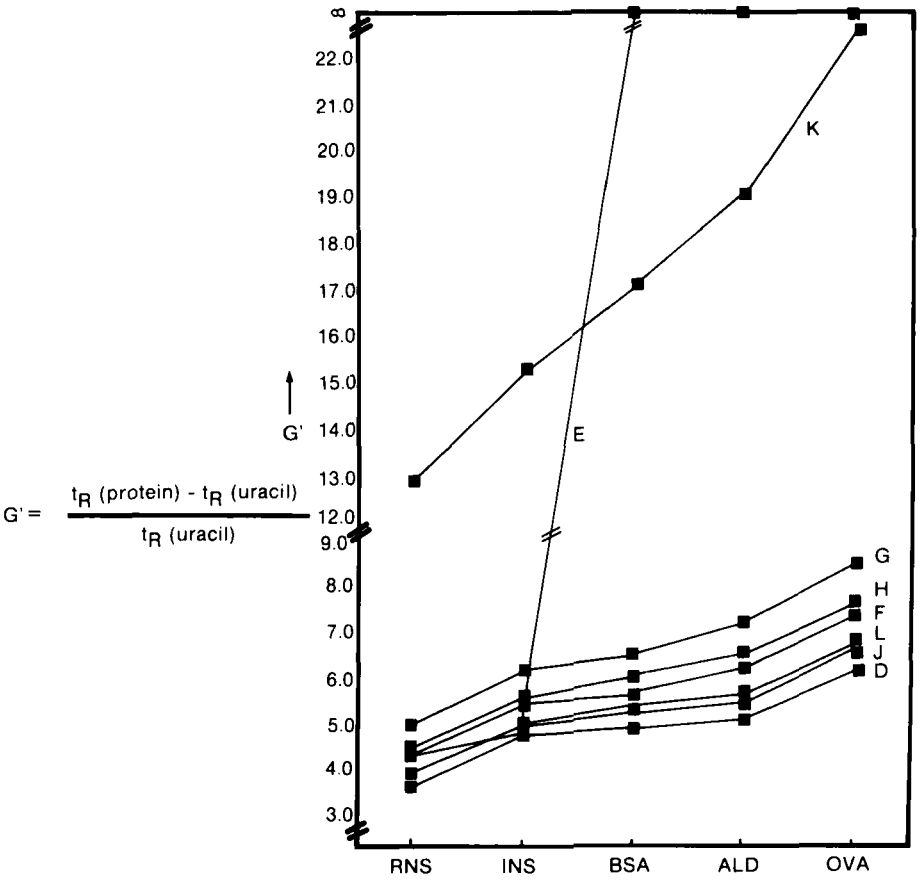

FIGURE 2 Retentivity of Columns for Protein Mixture.

MPD. Because the columns studied differ in more than their PSD characteristics, no definitive statement regarding resolution should be made on the basis of Figures 3 and 4. However, it is clear that the selection of a commercially available column for the separation of proteins cannot be based solely on pore size. Columns of small MPD (e.g. Column B) may offer excellent performance, while some wide pore materials (e.g. Column E) may fail to provide acceptable performance.

Peak Width. For all columns studied, the plate count was determined using a small molecule test mix. The influence of column efficiency (tangent plate

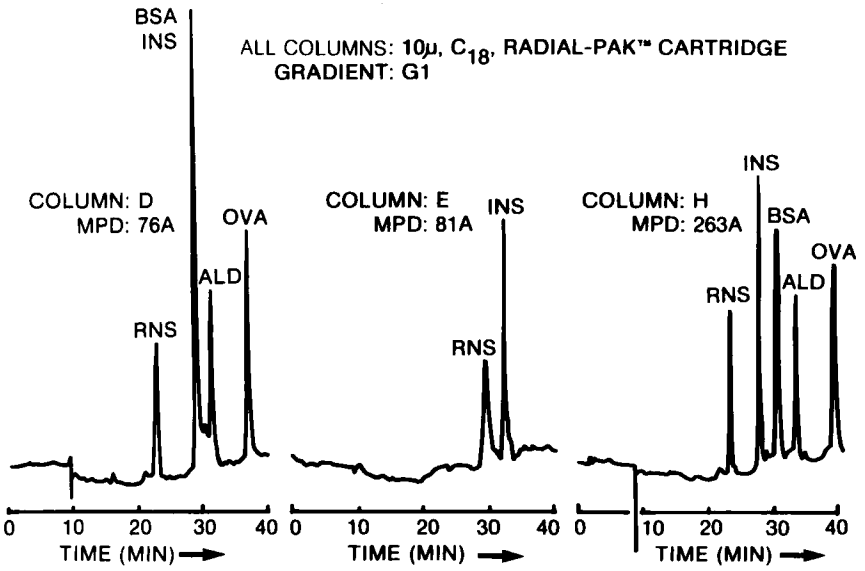

FIGURE 3 Lack of Influence of Mean Pore Diameter on Separation of Protein Mixture.

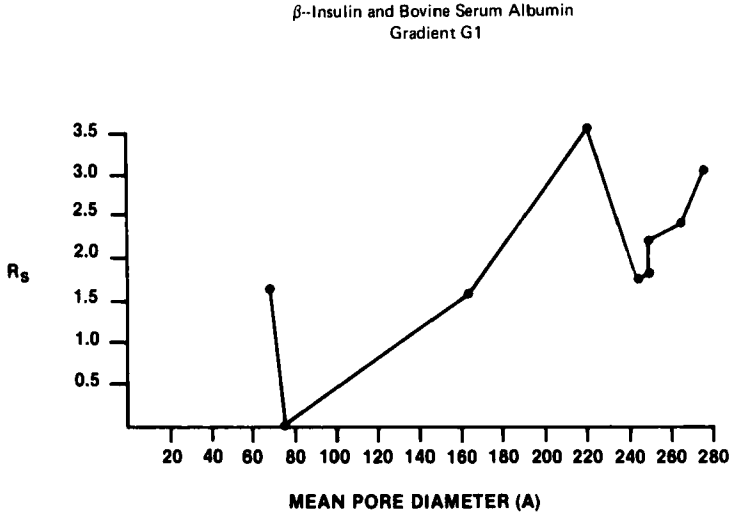

FIGURE 4 Influence of Mean Pore Diameter on Resolution.

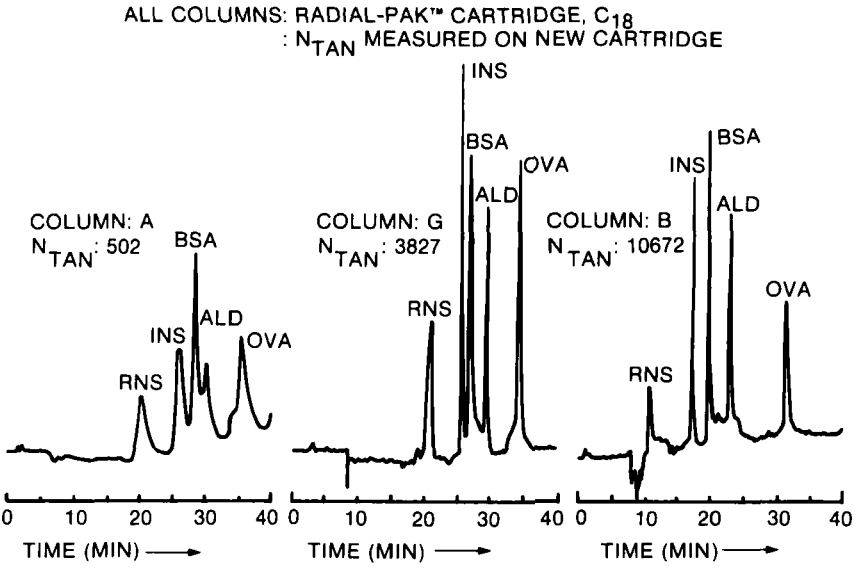

FIGURE 5 Influence of Column Efficiency on Separation of Protein Mixture.

count) on resolution and peak width is indicated by the three examples shown in Figure 5. Column A, containing a pellicular material, offered only 500 plates. Resolution is adequate, but peaks are notably broader than for Columns G and B. The difference in peak width seen in the chromatograms for Columns B and G, however, is less dramatic, despite the nearly threefold difference in plate count between these two columns. From this data it appears that high column efficiency is much less important for the resolution of proteins than for small molecules. This lack of correlation between plate count and resolution of proteins has previously been noted (6).

Increasing the column efficiency beyond a certain point yields diminishing improvements in chromatographic performance for proteins. Figure 6 reinforces this conclusion. Ovalbumin and β-insulin standards were eluted from seven columns, with the peak widths at half-height measured in each experiment. The resulting widths are plotted in Figure 6 as a function of both the tangent plate count and the MPD. The plate count plot shows that peak width decreases as column efficiency improves until a value of 1,000 plates is obtained. Beyond this point there is little change in peak width, even for a column having more than 10,000 theoretical plates.

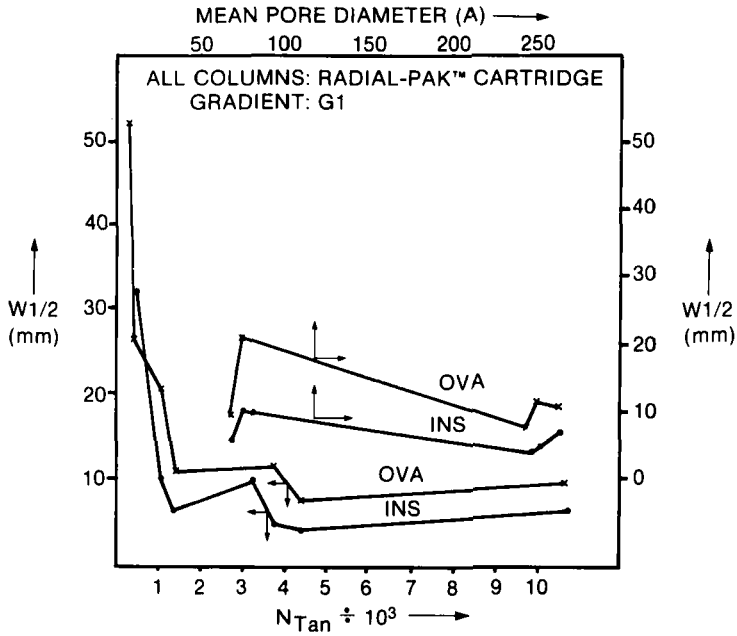

FIGURE 6 Influence of Small Molecule Plate Count and Mean Pore Diameter on Protein Peak Width.

Also indicated in Figure 6 is a nearly complete lack of correlation between peak width and MPD. This contradicts some previous reports (4,5) which suggest that resolution of proteins is related to pore size characteristics. Neither this nor the previous reports represent studies which are sufficiently controlled in all variables to allow a clear judgement to be made concerning the role of pore size in reversed phase protein separations. The findings summarized in Figure 6 indicate that this is an open question, despite frequently voiced claims to the contrary.

Sample Capacity. In the chromatography of proteins, it is frequently necessary to load relatively large sample amounts onto a column so that a minor component of interest can be detected and/or isolated. The capacity of column packings to tolerate large sample loadings without degradation of chromatographic performance is therefore of great interest. To investigate the relationship between MPD and maximum sample load, three columns of varying MPD were selected. All columns were packed into identical containers, and

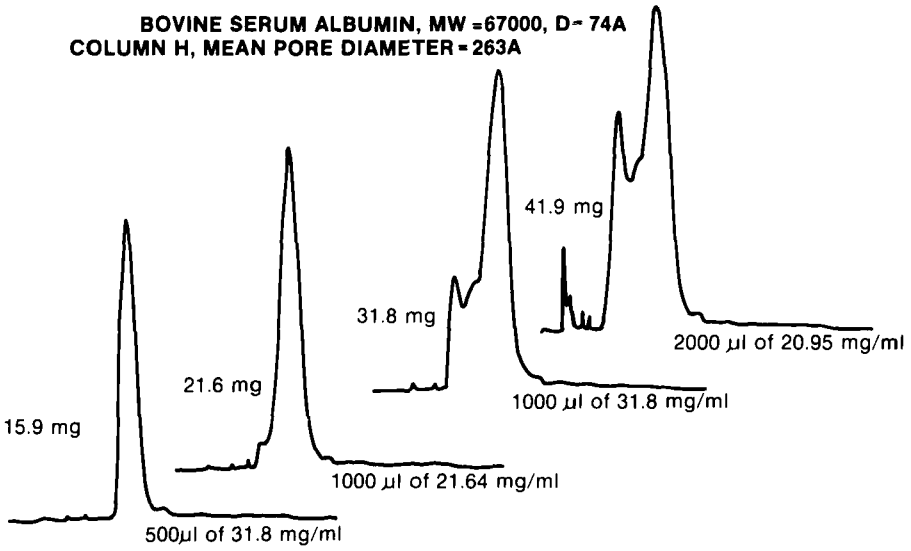

FIGURE 7 Sample Capacity Study.

gradient G2 was used in every case. Each was subjected to a series of injections containing increasing masses of BSA. The point of overload was taken as the point at which a second peak first appeared. This is demonstrated in the series of chromatograms presented in Figure 7. Injection of 15.9 mg of BSA gives a single peak, but a leading peak is seen upon injection of 21.5 mg. Injection of larger masses of BSA lead to further distortion of the major peak, until some BSA actually elutes at V_0 in the rightmost chromatogram. A maximum load of 21 mg is assigned to this column.

For the three columns studied, the maximum sample loads determined in this manner were 21 mg for column H, 10 mg for column B and 0.1 mg for column A. The overall trend observed was for sample capacity to increase with increasing MPD. However, other factors such as the pore volume, silica type and surface area differ for the columns studied so that the observed trend must be interpreted with caution. Still, the increase in sample capacity with MPD is sufficiently dramatic to suggest the need for additional investigation.

CONCLUSION

A simple technique for the determination of PSD information has been applied to a variety of packed columns. The use of experimentally determined MPD values allows a more confident assessment of pore size effects than is possible on the basis of the nominal pore sizes supplied by column manufacturers. It is anticipated that this technique for MPD determination will be useful to other chromatographers studying pore size effects. For the columns considered in this work, columns of nominal 300A pore size showed measured MPD values of 162A-275A.

It should be emphasized that columns of both conventional and "wide" pore size were found to give adequate resolution of a protein test mixture in most cases. Two columns (E and M) failed to elute three of the five proteins under any eluent conditions, but one of these (E) was a nominally wide pore material sold specifically for the separation of proteins. The phrase "wide pore" is not clearly associated with the ability of a particular column to separate a protein mixture. Neither resolution of proteins nor efficiency was clearly correlated with MPD. In contrast to small molecule separations, highly efficient RPLC columns do not appear to offer a significant advantage for the separation of proteins. Specifically, column efficiency was not found to cause a significant reduction in peak width beyond a value of about 1,000 plates. Our findings do suggest that one advantage that wide pore packings may offer is higher sample capacity. However, this is a tentative conclusion based upon the comparison of columns which differ in variables other than MPD. A controlled study is needed to clarify this issue, based on a set of materials having identical silica, bonded phase, and chemistry as well as carbon loading and coating level. Additionally, the concern for "ghosting" of proteins was not addressed in this study and is another area where an evaluation is needed.

ACKNOWLEDGEMENT

The authors thank S. Cohen for helpful discussions, J. Newman for preparation of the manuscript, and G. Witham for assistance with the artwork.

REFERENCES

1) Regnier, F.E., Science, 222, 245 (1983).

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

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

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

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

6) Pearson, J.D., Lin, N.T., and Regnier, F.G., Anal. Biochem., 124, 217 (1982).

7) Wilson, K.J., Van Wieringen. E., Klauser, S., and Berchtold, M.W., J. Chromatogr., 237, 407 (1982).

8) Walters, R.R., J. Chromatogr., 249, 19 (1982).

9) Klee, C.B., Oldervartel, M.D., Williams, J.F., and Lee, J.W., Biochem. Int., 2, 485 (1981).

10) Strickler, M.P., Gemski, M.J., and Doctor, B.P., J. Liq. Chromatogr., 4, 1765 (1981).

11) Titani, K., Sasagawa, T., Resing, K., and Walsh, K.A., Anal. Biochem., 123, 408 (1982).

12) Kayakawa, K., Okada, E., Higashikuze, H., and Kawanisto, T., J. Chromatogr., 256, 171 (1983).

13) Unger, K.K. "Porous Silica", Elsevier, Amsterdam, (1979).

14) Engelhardt, H., Universitat des Saarlandes, Saarbrucken (G.F.R.) personal communication (1982).

15) Warren, F.V. and Bidlingmeyer, B.A. Paper presented at Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy, Atlantic City, N.J., March 7-12, 1982 Abstract No. 136

16) Warren, F.V. and Bidlingmeyer, B.A., Anal. Chem., 56, 950 (1984).

17) Halasz, I., Ber. Bunsenges Phys. Chem., 79, 731 (1975).

18) Halasz, I. and Martin, K., Angew. Chem.; Int. Ed. Engl., 17, 901 (1978).

19) Halasz, I., Vogtel, P. Angew, Chem. Int. Ed. Engl. (1980), 19, 24.

20) Werner, W., Halasz, I. Chromatographia (1980), 13, 271.

21) Nikolov, R., Werner, W., Halasz, I. J. Chromatog. Sci. (1980), 18, 207.

22) Werner, W., Halasz, R. J. Chromatog. Sci. (1980), 18, 277.

23) Groh, R., Halasz, I. Anal. Chem., (1981), 53, 1325.

24) Crispin, T., Halasz, I. , J. Chromatogr., (1982), 239, 351.

25) Freeman, D.H., Poinesca, I.C. Anal. Chem., (1977), 49, 1183.

26) Schram, S.B., Freeman, D.H., J. Liq. Chromatogr., (1980), 3, 403.

27) Freeman, D.H., Schram, S.B. Anal. Chem., (1981), 53, 1235.

28) Kuga, S. , J. Chromatogr., (1981), 206, 449.

29) Warren, F.V., Bidlingmeyer, B.A., Richardson, H., Ekmanis, J. In "Recent Advances in Size Exclusion Chromatography", T. Provder, Ed., ACS Symposium Series, Volume 245, Chapter 11.