Improved Protein and Peptide Analysis Using Wide-Pore HPLC Columns

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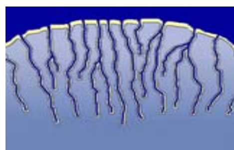
Introduction

Reversed phase HPLC is an important technique for analysis and purification of proteins and peptides. Limitations are often encountered when analyzing samples containing complex mixtures of closely-related analytes. Wide-pore silica (e.g., 300Å) columns are designed specifically for protein and peptide separations, addressing this need for more resolving power. Using a 300Å silica enhances resolution of similar and related analytes for several reasons. For analytes of molecular weight greater than ~3000Da, silica materials with the majority of pore diameters in the 250-350Å range are ideal. Large pore materials provide greater retention because higher molecular weight analytes are able to enter the pores to gain access to more surface area. Theoretically, the more access the analyte has to available surface area, the greater the retention. Additionally, a narrow distribution around the mean pore diameter aids in separating closely related analytes that differ only slightly in hydrodynamic size, or molecule size in solution. Keeping these principles in mind, we have developed a 300Å silica that offers the best protein separation among the commercial phases evaluated.

Figure 1 depicts a typical porous silica particle. In general, as the total number of pores in a silica particle is increased, surface area increases proportionately. Typically, pore volume increases with pore diameter. While smaller pores (e.g., 60Å) maximize retention of small molecules, larger pores are necessary when analyzing higher molecular weight analytes such as proteins and peptides.

Figure 1. A typical porous silica substrate: as number of pores increase, total surface area and pore volume increase.



Purpose

In the following studies, we evaluated surface properties of several wide-pore silica materials using Barrett-Joyner-Halenda (BJH) nitrogen desorption and compared chromatographic retention properties of Restek's Viva™ 300Å C18 with 300Å C18 columns from four other manufacturers using three different test mixes. In our surface area analyses, we discovered that some stationary phases advertised as "wide pore" materials do not possess sufficiently large pore volume within the pore diameter range required to most effectively separate large molecules. In the comparison of retention behavior, we found Viva™ 300Å C18 column to outperform the other wide pore materials, some of which were found to be poorly base deactivated.

Surface Area Measurements

To determine surface area, BJH nitrogen desorption was performed on bulk 300Å silica material. Pore volume was then calculated using the BJH method of determining pore size distribution[1];

$$\ln \frac{P}{P^o} \geq \frac{-2\gamma V_L}{RT} \frac{1}{r - t(P)} \quad \text{Equation 1}$$

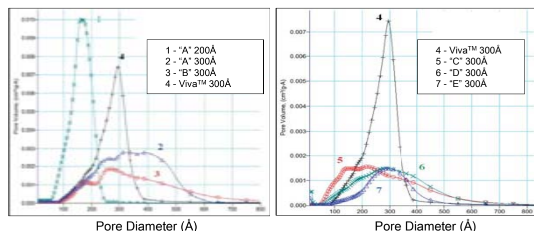
where γ is surface tension, V_L is molar volume of liquid or gas, $t(P)$ is surface adsorption layer thickness, P^o is vapor pressure, and r is pore radius. Table I shows the calculated total surface area and the percentage of total surface area attributable to each range of pore diameters.

Table I. Surface area versus pore diameter range using BJH calculations based on nitrogen desorption data.

Silica	Total Surface Area (m²/g)	% of Total Surface Area <200Å	200-300Å	>300Å
Viva™ 300Å	128.0	2.5	67.3	30.2
(7) 300Å	51.8	65.6	18.5	15.9
(6) 300Å	87.2	53.6	22.2	24.2
(5) 300Å	105.8	56.3	22.3	21.4
(3) 300Å	83.5	40.5	24.5	35.0
("B") 200Å	231.5	66.1	33.1	0.8
("B") 300Å	118.1	8.3	34.3	57.4

Figure 2 shows plots of pore volume versus pore diameter for each wic pore silica material evaluated. Viva™ silica has the most narro distribution around 300Å, in addition to a majority of pores over 200Å diameter. The other silicas have either broad pore diameter distribution or a majority of pores under 200Å in diameter.

Figure 2. Pore volume vs. pore diameter for commercial wide pore silicas (BJH calculations). Note change in scale for plots at right.



Column Evaluation

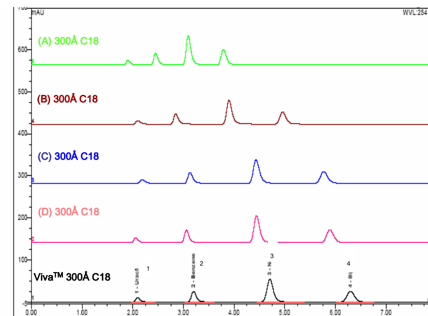
Using a reversed phase test mix, we compared column efficiency, peak asymmetry, and retention for Viva™ C18 columns and four other commercially available C18 wide pore HPLC columns. Test mix components were uracil (for determination of elution time for an unretained compound), benzene and napthalene (for measurement of hydrophobic retention), and biphenyl (for measurement of asymmetry and column efficiency). The Viva™ C18 column provided the highest degree of retention, best peak symmetry, and lowest column backpressure, favorable for maximizing column lifetime (Figure 3 and Table 2).

Table 2. Experimentally determined retention characteristics of all chromatographically evaluated columns.

Column	Efficiency (plates/meter)	Asymmetry (biphenyl)	Retention Time (min, biphenyl)	Column Pressure (bar)
Viva™ 300Å C18	>50,000	1.16	6.30	60
Column A 300Å C18	<50,000	1.49	3.79	80
Column B 300Å C18	>50,000	1.46	4.96	102
Column C 300Å C18	~50,000	1.46	5.77	72
Column D 300Å C18	>50,000	1.30	5.89	66

Figure 3. Column comparison using reversed phase test mix. This mixture provides information regarding hydrophobic retention and resolution, as well as column efficiency. Elution order is consistent for all chromatograms.

Columns: 150x2.1mm C18, 5µm
Mobile Phase: water : methanol, 25:75
Flow Rate: 0.2mL/min.
Detector: UV @ 254nm
Sample: 10µL Reversed Phase Test Mix, methanol: water (75:25): 1. uracil;0.02mg/mL, 2. benzene; 3.00mg/mL, 3. napthalene; 0.50mg/mL, 4. biphenyl; 0.06mg/mL



Next, we evaluated surface characteristics of each material using the NIST 870 mix, a mixture of uracil (unretained peak), toluene and ethylbenzene (polar neutral compounds, for resolution and retention), quinizarin (metal chelating agent, for active metal sites on surface), and amitriptyline HCl (polar basic compound, for acidic silanols on surface). Viva™ C18 shows the most favorable retention behavior for all mixture components (Figure 4). The most extreme effects can be seen for column (B) 300Å, showing a wide quinizarin peak and peak tailing for amitriptyline. Reversed elution order for quinizarin and amitriptyline for column (C) 300Å indicates a shift in average pore size compared to the other columns.

To determine overall resolving power, degree of retention, and peak shape for proteins, we evaluated each column using a four-protein standard test mix. The Viva™ C18 column shows excellent resolution and peak shape, as shown in Figure 5.

Figure 4. Column comparison using NIST 870 mix. This mixture provides information regarding hydrophobic retention and resolution as well as peak shape for basic analytes.

Columns: 150x2.1mm C18, 5µm
Mobile Phase: A=20% 5mmol K_2HPO_4, pH = 7.0; B=80% Methanol
Flow Rate: 0.2mL/min.
Detector: UV, 254nm
Injection: 1mL NIST 870 test mix, methanol: 1. uracil; 28µg/mL, 2. toluene; 1400µg/mL, 3. ethylbenzene; 1700µg/mL, 4. quinizarin; 94µg/mL, 5. amitriptyline HCl; 2900µg/mL

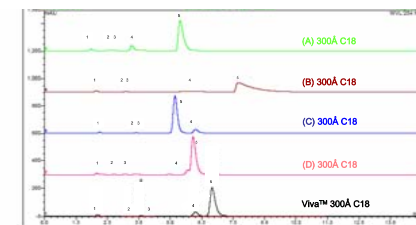
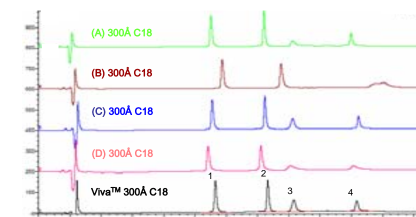


Figure 5. Column Comparison using a four-protein test mixture. Elution order is consistent for all chromatograms, although retention times and peak widths do vary. This mixture provides information regarding resolving power for biomolecules of similar molecular weight.

Columns: 150x2.1mm C18, 5µm
Mobile Phase: A: 0.1% TFA in water, pH 2.0; B=0.1% TFA in acetonitrile 20% B to 70% B in 30 min.
Flow Rate: 0.2mL/min.
Detector: UV @ 214nm
Injection: Four-Protein Standard Test Mix*, Sigma Aldrich #H2899



*Peak Identification (unconfirmed elution order – provided by Sigma Aldrich standard cat# H2899-1VL) 1. ribonuclease A (13,700 Da), 2. cytochrome C (12,327 Da), 3. holo-transferrin, human (76,000-81,000 Da), 4. apomyoglobin (16,951 Da)

Conclusions

In selecting a wide pore material, it is important to know the available surface area in relation to pore volume and pore diameter distribution, surface deactivation, and retention capabilities, as all are critical factors in large molecule analyses. The thorough base deactivation, exceptionally large available surface area, along with the highly desirable pore volume and narrow pore diameter distribution of Viva™ 300Å silica, helps to ensure effective resolution of peptides, proteins, and other large molecules.

Reference

1. Gelb, L.D. *Langmuir* **1999**, *15*, 305-308