The Influence of Silica Pore Size on Efficiency, Resolution and Loading in Reversed-Phase HPLC

Application Note

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Introduction

There are a number of factors which must be considered when choosing a stationary phase for a prep or process HPLC separation. Some relate specifically to the process and others refer to the target compound and impurity profile. At the process development stage the focus is on the target compound and achieving the required purity and recovery to make the process economically viable. There are two dominant factors, the first is the selectivity of the HPLC media, maximizing the resolution between the target compound and the impurities and the second is the capacity of the media – both of these will contribute to the efficiency of the purification and the throughput of the process.

The work presented in this note evaluates the importance of the media pore size on the the separation, efficiency and capacity of a separation using a number of standard compounds.

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Experimental

A prep to process material was used for this study, SepTech, which uses ultra pure silica as the base particle and a bonding chemistry to give a high carbon load C18 material. Two pore sizes, a 60Å (ST60 10-C18) and a 150Å (ST150 10-C18) were used. The specifications for these two materials is given in Table 1.

Table 1. Specifications of the two HPLC materials used in this study.

	SepTech ST60 10-C18	SepTech ST150 10-C18
Normal Particle Size (µm)	10	10
Distribution d90/d10	<2.0	<2.0
Nominal Pore Size (Å)	60	150
Carbon Load (%)	25	15
Ligand Coverage (µmole/m^2)	3.5	3.8
Operating pH Range	1.5-10	1.5-10
Packed Bed Density (g/mL)	0.59	0.49

Determination of Pore Size (BET)

A nominal pore size is always quoted for an HPLC media, 60Å, 100Å, 120Å, 300Å etc and is used as one of the criteria for media selection. However, the pore size quoted may not be the "actual" pore size of the material in its chromatographic form.

For silica HPLC materials the method most commonly used for pore size and surface area determination is from the BET (Brunauer, Emmett and Teller) nitrogen adsorption/desorption equation. This method was used to determine pore size, pore size distribution and surface area of the base silica particles, Figure 1, and also for the media after bonding and end-capping, Figure 2. The physical parameters of the four materials are summarized in Table 2.

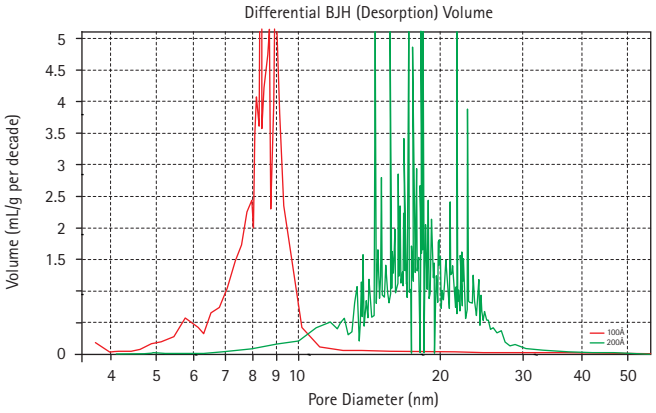

Figure 1. BET pore size data for the two base silica materials.

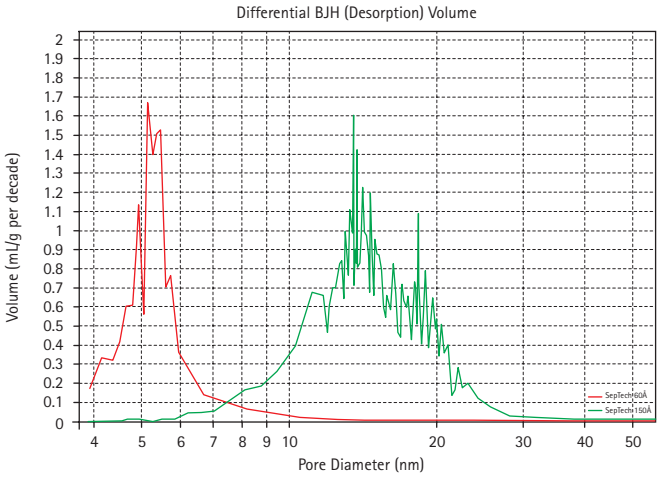

Figure 2. BET pore size data for the two products after bonding and end-capping SepTech ST60 10-C18 and SepTech ST150 10-C18.

Table 2. Pore size and surface area data of the two HPLC materials used in this study

	Silica		C18 modification	
Normal Pore Size (Å)	100	200	60	150
BET (m^2/g)	517	194	172	126
Pore Volume Desorption (mL/g)	1.18	0.99	0.37	0.61
Median Pore Size Desorption (Å)	78	176	53	141

From the data it is clear that this bonding chemistry reduces the pore size by approximately 30%.

Determination of Pore Size (GPC)

An alternative method of measuring the pore size and pore size distribution which is used with polymeric HPLC materials is gel permeation chromatography (GPC). The GPC calibration curve is generated on a packed HPLC column and shows the permeability of the material in relation to the size of the solutes being chromatographed. Figure 3 shows the GPC calibration of the of two SepTech materials. The difference in pore size is clearly evident from these curves – the ST150 10-C18 shows increased permeability for the larger molecules.

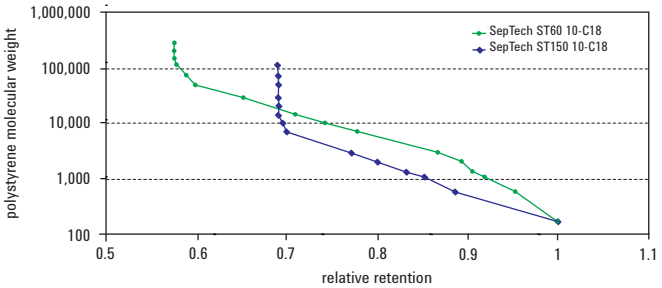

Figure 3. The GPC calibration curves of the two products after bonding and end-capping SepTech ST60 10-C18 and SepTech ST150 10-C18 250 x 4.6 mm id columns. Eluent: THF (tetrahydrofuran) and polystyrene calibrants.

Separations

The ligand and end-capping is the same for both the SepTech ST60 10-C18 and the SepTech ST150 10-C18 which results in comparable ligand coverage. Therefore, any changes in performance will be due primarily to differences in the pore size and surface area of the two materials.

An isocratic separation of a mixture of uracil and a series of tricyclic antidepressants was carried out using the two materials, Figure 4. Both columns show good symmetrical peaks but the smaller pore size 60Å material is more retentive – has the higher available surface area.

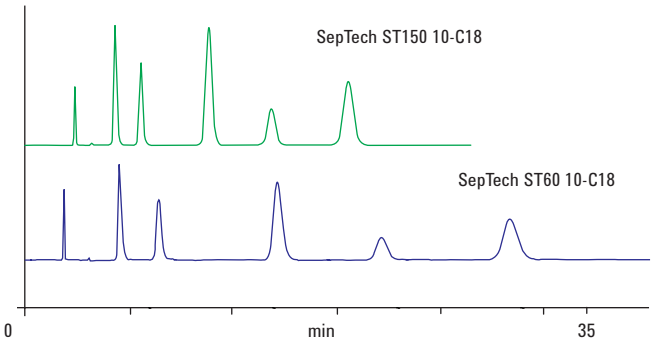

Figure 4. Separation of 1. Uracil, 2. Protriptyline, 3. Nortriptyline, 4. Doxepin, 5. Imipramine, 6. Amitriptyline on the SepTech ST60 10-C18 and SepTech ST150 10-C18 250 x 4.6 mm id columns. Eluent: methanol:20mM phosphate buffer pH 7 (70:30).

As the size of the solutes increases so issues relating to restricted diffusion into the particle become more important. This will influence not only the peak width but also the available surface area for interaction.

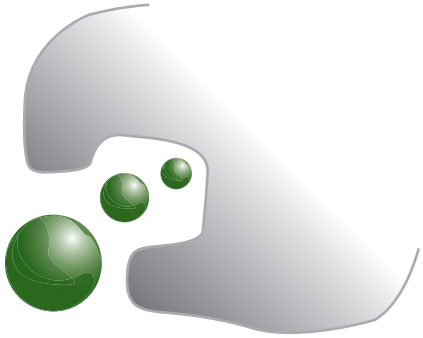

Figure 5. Solute permeability.

In Figure 6 a clear difference can be seen in the separation achieved with the 60Å and 150Å pore size materials – resolution is improved by using the larger pore size material.

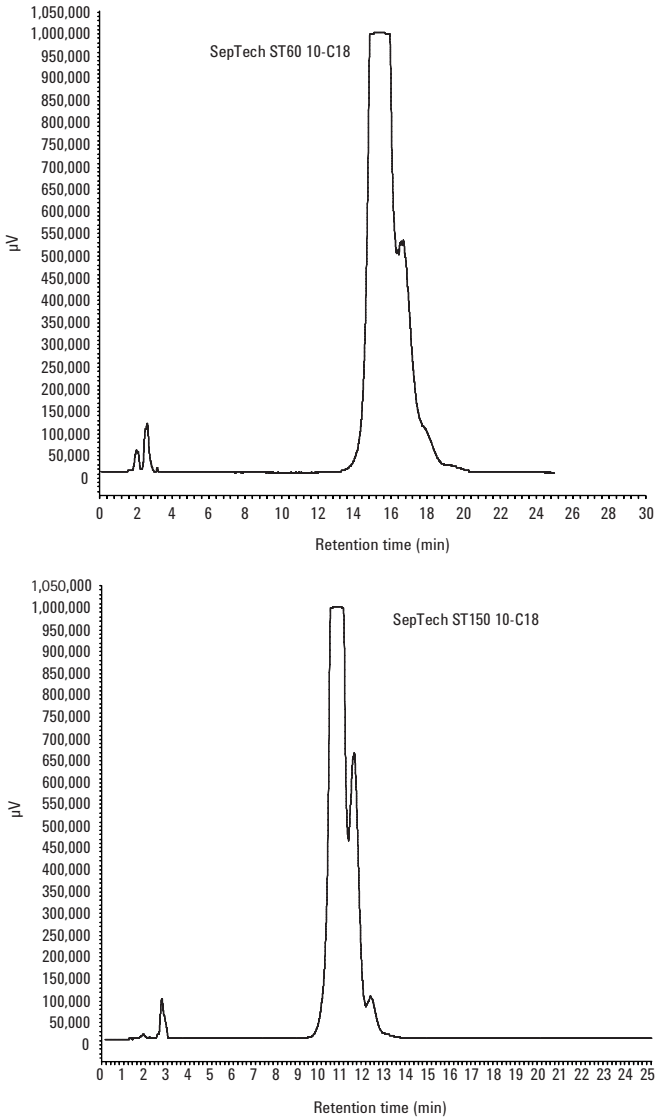

Figure 6. Comparison of the separation of a saponin sample using the SepTech ST60 10-C18 and SepTech ST150 10-C18 media. Eluent: acetone:water (30:70), Detector: Agilent ELSD.

Peptides can range in size from two amino acids up to several hundred amino acids but the most common range for synthetic peptides is approximately 5 to 40, although HPLC is rarely used for purification of the smaller peptides. A mixture of peptides, ranging in size from oxytocin with a molecular weight of 1007 to insulin with a molecular weight of 5700 Daltons, was used to investigate the influence of pore size on the efficiency, resolution and capacity of peptide separations.

A comparison of the separation of the peptide mixture at an analytical column load, 100 µg, was carried out. The two chromatograms are shown in Figure 7 and the efficiency and resolution values shown in the bar charts in Figure 8. It is clear that even at analytical load the 60Å pore size is too small for efficient separations.

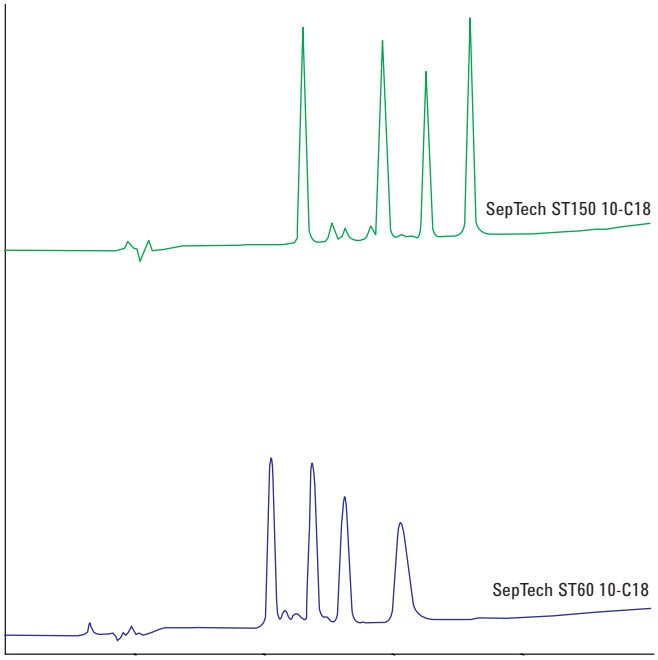

Figure 7. Separation of mixture of peptide standards, oxytocin, angiotensin I, angiotensin II and insulin Eluent A: 0.1% TFA in 20% ACN:80% water, Eluent B: 0.1% TFA in 50% ACN:50%, Gradient: 0-100% B in 15 min.

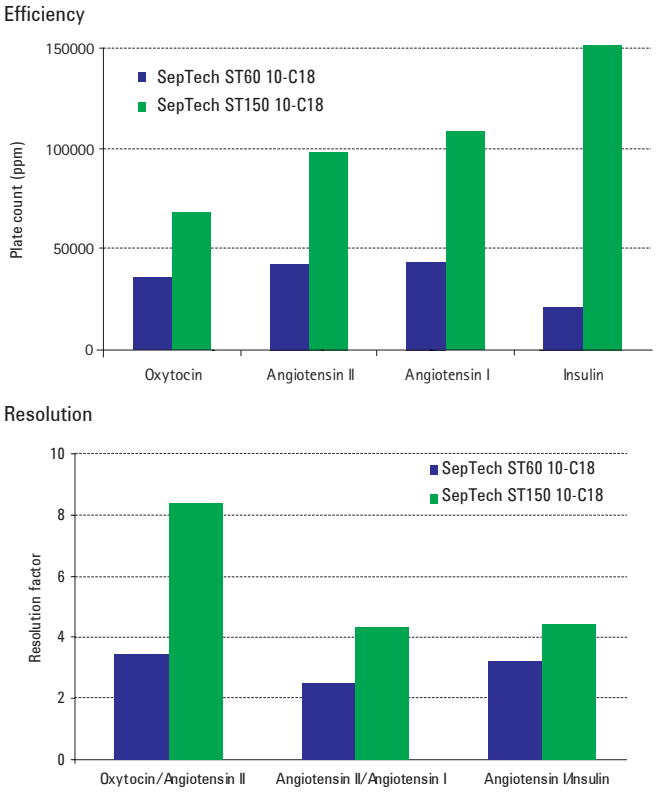

Figure 8. Efficiency and resolution data for the peptide separations on the SepTech ST60 10-C18 and SepTech ST150 10-C18 250 x 4.6 mm id columns.

For a purification process to be viable, the media used must have a high capacity for the target compound. Frontal loading experiments were performed to determine the dynamic binding capacity of the SepTech ST60 10-C18 and SepTech ST150 10-C18 media for a small synthetic peptide and insulin, two loading conditions were used for the insulin. Figure 9 shows the capacities obtained. It is clear from this that the pore size of SepTech ST60 10-C18 is too small for peptides to access the internal pore surface area.

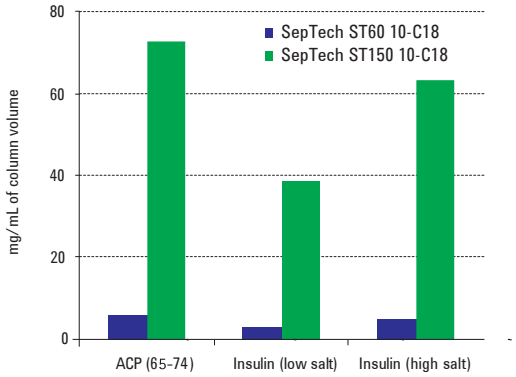

Figure 9. Dynamic capacity data.

Conclusion

For a viable prep or process purification, efficiency, resolution and capacity are required. The work presented in this note demonstrates that the pore size of the HPLC media affects all three of these parameters.

Although the small pore materials will have the highest surface area, as determined by BET, most is located in the internal pore structure of the particles. If the size of the compound to be separated is such that diffusion into the pore structure is restricted, then the efficiency, resolution and capacity are affected (reduced).

Small molecules are best purified using a small pore material such as the SepTech ST60 10-C18, but larger natural compound and peptides require a larger pore size material, SepTech ST150 10-C18, for a viable process.

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