The Role of Pore Size in Reversed Phase HPLC

SGE is excited to launch a new HPLC product line under the ProteCol™ brand.

Fundamental to the new ProteCol™ line of columns is the continued focus on inert column design that was first created with the ProteCol™ PEEKsil™ offering (polymer - sheathed fused silica tubing). SGE is excited to offer a new range of ultra pure reversed phase silicas in both the unique GLT™ (glass lined tubing) column format as well as a new PEEK™ lined stainless steel format. The benefit is the most comprehensive inert HPLC reversed phase column offering from the 150 micron ID PEEKsil™ format through to the new 4.6 mm ID PEEK™ lined stainless steel columns.

Why is Inert HPLC Column Design Important?

Non-specific interactions between the target analyte and the silica particles in the HPLC column are now well controlled with the availability of ultrapure silicas. Today, most chromatographers expect that the silica sourced by manufacturers is of the highest purity and SGE confirms we have rigorously researched the quality of silicas using the standard reference material (SRM) provided by the National Institute of Standards & Technology. Testing of silicas using SRM 870 (NIST) identifies non-specific interactions associated with metal contamination as well as non-end capped silanols (see Figure 1).

What is often not considered is the role column hardware may play in non-specific interactions – the frit and internal column hardware can both influence the behavior of analytes with known metal chelating activity. Most pharmaceutically active compounds and natural products have the potential to interact with metals. Coordination between the metal ion and the analyte is facilitated by a lone electron pair on the analyte molecule.

If two electron donor groups (either oxygen or nitrogen) are located in a favorable position, a chelate can be formed and while the enthalpy of the complex formation for two monodentate ligands and a bidentate ligand is similar, the chelate is entropically favoured and leads to a stronger interaction. For this reason molecules like quinizarin,

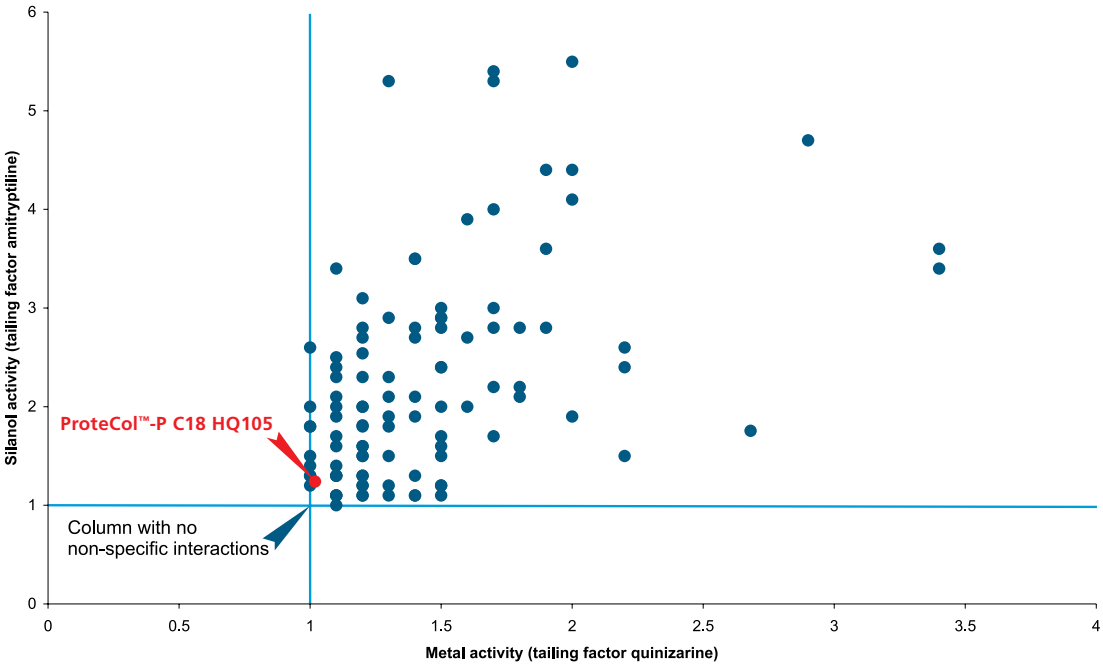
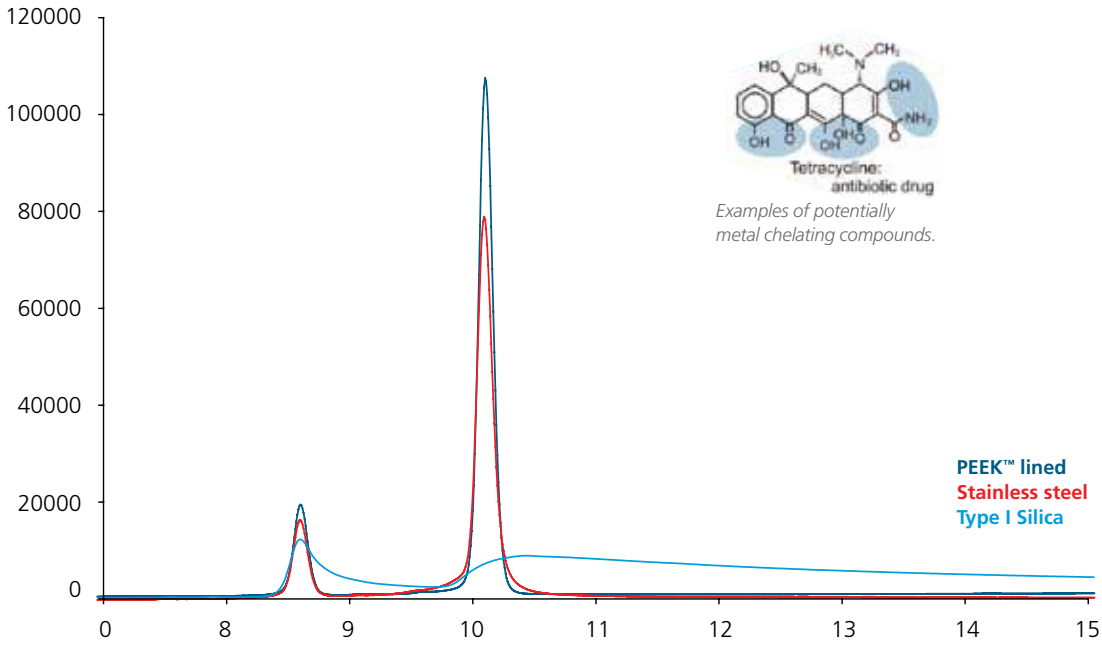


Figure 1: Non-specific interactions of the NIST SRM870 probe molecules on commercially available C18 columns. (Comparison data available at: www.usp.org/USPNF/columns.html)



Examples of potentially metal chelating compounds.

Figure 2: Chromatogram of tetracycline (antibiotic) and its major degradation product. Note the peak broadening on the base of the peak run through the stainless steel column leading to a 25% decrease in sensitivity. Inset: the tetracycline molecule depicting the three potential chelating groups.

tetracycline or ciclopirox form tailing peaks in the presence of metal in the column/ system (see Figure 2).

To address this potential risk, SGE's ProteCol™ column development has focused exclusively on the most inert C8 and C18 phases in a variety of pore and particle sizes in the most inert column hardwares – glass lined, PEEK™ lined stainless steel (see Figure 3) and PEEKsil™.

Why Focus on Reversed Phase and Pore Size?

Reversed phase chromatography is by far the most commonly used form of liquid chromatography and most chromatographers prefer to stay in the reversed phase environment rather than move into other less conventional buffer systems.

Alkane modified silicas were developed in the 1970s and because of the better resolution and higher reproducibility, quickly became the most popular separation technique in liquid chromatography. Since the elution conditions and elution order are opposite to what was "normal" chromatography, the term "reversed" phase was coined and has remained the general term describing a hydrophobic bonded stationary phase. When it comes to considering the optimal reversed phase column for the separation of a target analyte, the majority of liquid chromatographers base their selection on the type of bonded phase (C18, C8, C4, Phenyl etc), whether the column is end capped and the overall carbon loading.

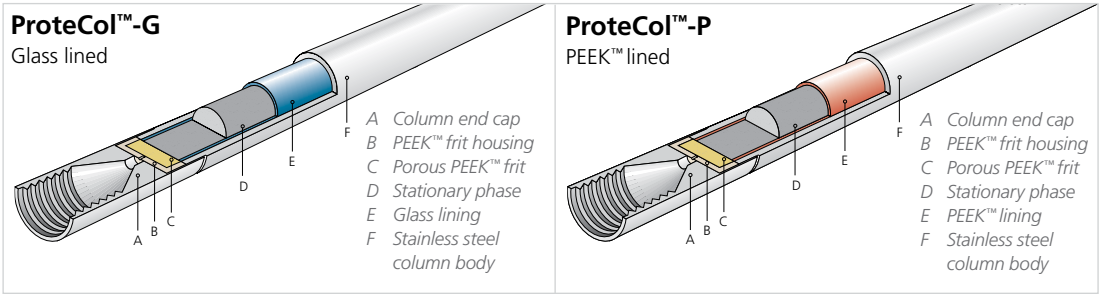


Figure 3. SGE's inert column hardware includes glass and PEEK™ lined stainless steel.

Don't Forget to Choose the Appropriate Pore Size of Your Reversed Phase Column!

What is often ignored by chromatographers is the choice of optimal pore size of the silica for the appropriate application. In liquid chromatography virtually all interactions (and therefore retention) takes place inside the pore system. The exterior surface of common porous silica makes up less than 1 % of the total surface area. In order to use the available interactive surface, the analyte molecule needs unrestricted access to the particle interior. In most chromatographic applications pore diffusion is the time limiting step (the slowest step which therefore governs the overall kinetics). After overcoming the film mass transfer resistance, the solute has to diffuse into the pore system in order to bind to the surface since most of the surface is inside a porous particle (>99 %). For larger molecules such as proteins, pore diffusion becomes a crucial factor.

A number of models have been derived to describe the effect of the pore diameter on the diffusion constant of a solute molecule. These models range from the Fickian diffusion where the diffusion rate is purely concentration driven (large pores – small solute molecules – the mean free path of the Brownian motion is small compared to the pore diameter); to the Knudsen diffusion, where the mean path of the Brownian motion is equal or larger than the pore diameter (collisions with the wall play a major role in the determination of the diffusion rate). An extreme case is the single file diffusion, where the diameter of the solute solute molecule is larger than the radius of the pore. In this case molecules are unable to pass each other. In addition, an estimation for the steric hindrance at the pore entrance and the frictional resistance within the pore system was provided by Renkin.

$$D_p = D_f \left(1-\frac{r_s}{r_p}\right)^2 \left[1-2.104\left(\frac{r_s}{r_p}\right)+2.09\left(\frac{r_s}{r_p}\right)^3-0.956\left(\frac{r_s}{r_p}\right)^5\right]$$

D_f = Free molecular diffusion coefficient.
D_p = Diffusion as the coefficient inside the pore.
r_s, r_p = Radii of the solute and the pore respectively.

A graphical representation of the Renkin model and its implications are shown in Figure 4.

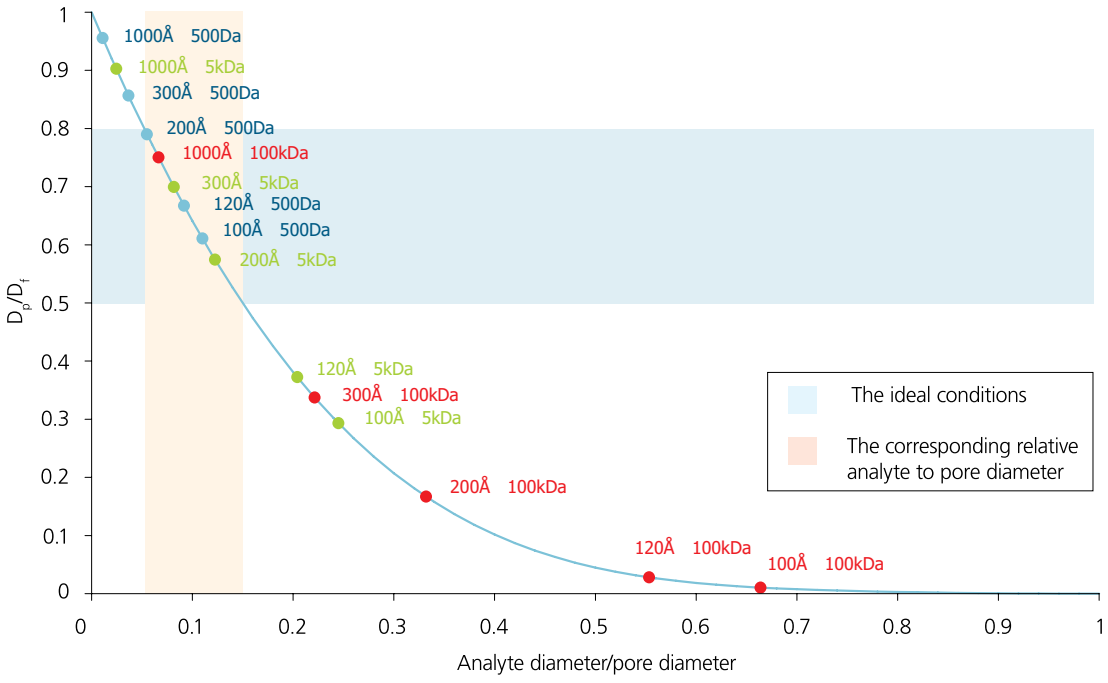


Figure 4: Relative pore diffusion coefficients of large molecules in different pore size stationary phases

The drawback of large pore sizes in HPLC columns is the reduction in specific surface area. As the pore size increases, the accessible surface area for the solute to bind to decreases and with it the capacity of the column. Figure 5 shows the specific surface area of commercially available packing material versus the pore diameter.

The choice of pore size for a given separation is a compromise between resolution on the high pore diameter end and load-ability (capacity) on the low pore diameter end. A rough guide for suitable pore diameter ranges (where the pore diffusion coefficient is between 50 and 80 % of the diffusion rate in free liquid) is shown in Figure 6.

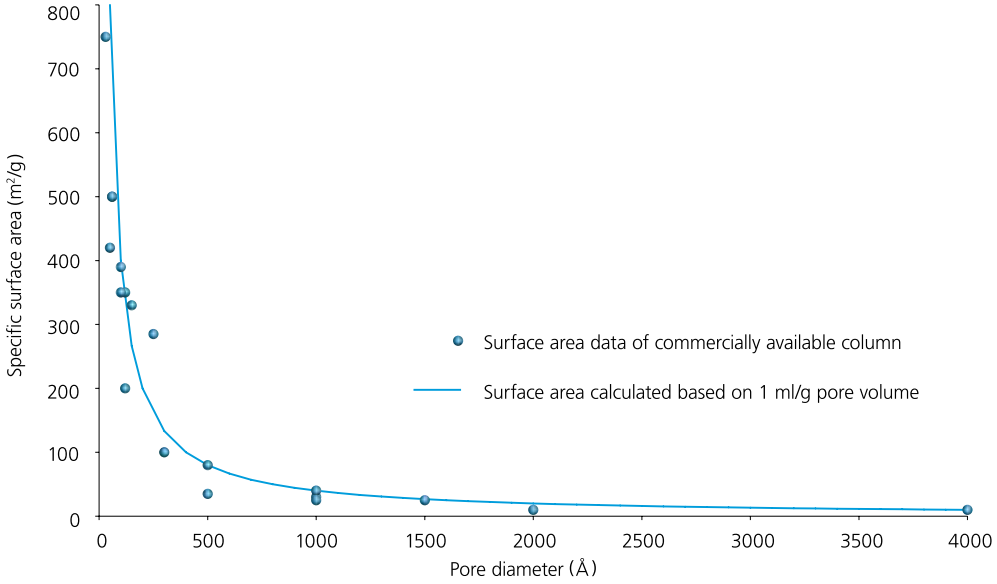


Figure 5. Reduction in column capacity due to increased pore size (blue dots represent published data by various column suppliers)

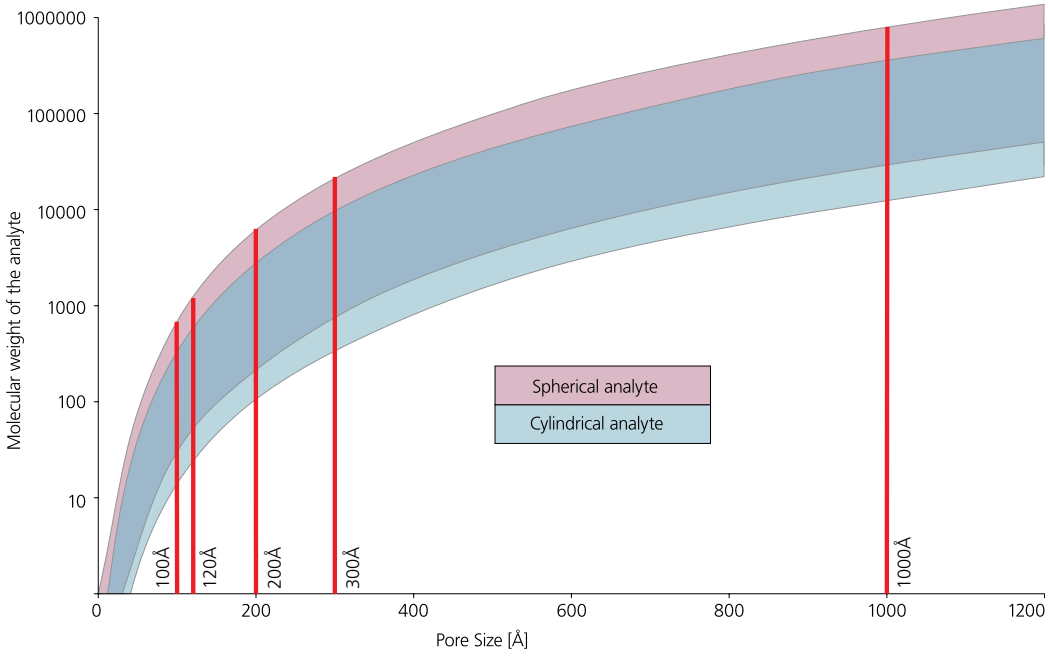


Figure 6: Acceptable molecular weight range of spherical & cylindrical shaped analytes in relation to pore size

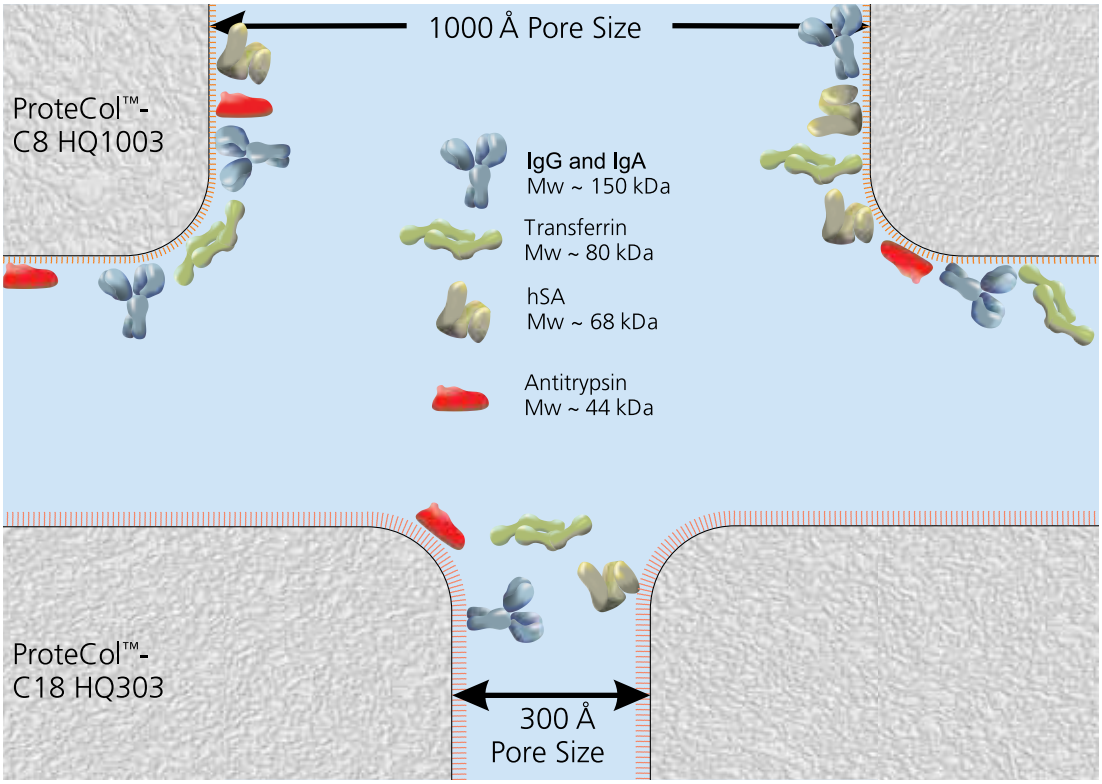


Figure 7: Schematic representation of the relative sizes of the five most abundant plasma proteins.

As Figure 7 suggests, the use of 300 Å pore size stationary phases does have a practical limit for large molecules and you should be mindful of the sample's complexity before considering the separation of large macromolecules on this type of phase. Proteins are large macro molecules and diffuse slowly (small diffusion rate constant). Proteins are also more likely to cause a steric hindrance once they bind to the pore surface.

In conclusion, SGE recommends the following guide to selecting the appropriate pore size based on your target analytes molecular weight range.

	Spherical Analyte		Cylindrical Analyte	
Pore Size	Lower	Upper	Lower	Upper
100	30	800	13	350
120	50	1,400	23	610
200	240	6,400	100	2,800
300	800	21,400	350	9,500
1000	29,400	800,000	13,000	350,000

Pore Size (Å) Lower and Upper ranges shown in Daltons (Da)

Expert Tips for Small Molecules

Simple DO's and DON'Ts to protect your HPLC Columns:

- DO use a guard cartridge.
- DO de-gas solvents.
- DO tightly cap column ends.
- DO use purified water and high purity solvents.
- DO use filters.
- Do check the purity of solvents when they arrive in the laboratory.
- DO flush several volumes of methanol (or similar) through the HPLC system prior to shutdown.
- DO flush several milliliters of solvent through new filters, tubing and fittings before connection to the column.
- DO filter both the mobile phase and sample.
- DO store column at room temperature.
- DO protect the column from knocks and bumps.
- DO worry about salt build-up at fitting connections.
- DO attempt to control the temperature of a column.
- DO rinse organic solvents from the column with 50/50 organic/aqueous solvent prior to using buffers.
- DO use PEEK™ ferrules on one piece fingertights where possible.
- DO contact SGE if in doubt.
- DON'T inject crude biological samples directly into a column.
- DON'T let the column dry out.
- DON'T use a pH outside the manufacturer's recommended range for your column.
- DON'T drop your HPLC column.
- DON'T use too much connecting tubing.
- DON'T place excessive back pressure on your column or system.
- DON'T leave a buffer in a column or HPLC system at a zero flow for extended periods of time.
- DON'T use low-grade solvents.
- DON'T encourage growth of microorganisms in aqueous buffers by preparing them days in advance.
- DON'T open the ends of the column – just to see what's inside.
- DON'T use a huge wrench to tighten tiny fittings.
- DON'T use stainless steel ferrules in connections.
- DON'T use high concentrations of aggressive mobile phases.
- DON'T top up mobile phases.