EFFECTS OF PARTICLE POROSITY ON THE SEPARATION OF LARGER MOLECULES

Wirth H.J., Gooley A. - SGE Analytical Science, 7 Argent Street, Ringwood, Vic. 3134, Australia

ABSTRACT

The pore structure of a chromatographic stationary phase accounts for the vast majority of the surface area responsible for the separation. The pore diameter influences the overall surface area and with it the capacity of the column but also limits the size of the analyte the column can or should be used for. In adsorption chromatography the limiting effect of the pore diameter is further enhanced by adsorbed analyte molecules partially blocking the pore structure.

In the analysis of large molecules pore diffusion becomes a crucial parameter in the efficiency of the column. A number of models deal with hindered mass transfer in porous systems.

In this presentation effects of particle porosity on the separation of larger molecules are discussed and examples for the separation of small, medium and large analytes on various pore size stationary phases are given.

PORE AND ANALYTE SIZE IN ADSORPTION CHROMATOGRAPHY

When analyzing large molecules two effects influence the chromatographic behavior: reduced diffusion coefficients and the reduction in effective pore size by already adsorbed analyte molecules (see figure 1).

MASS TRANSFER AND CHROMATOGRAPHY

The Van Deemter Equation\(^1\) provides a correlation of various chromatographic parameters with the column performance. The “C-term” describes the effect of restricted diffusion and is the most relevant for the separation of large molecules.

\[ H = A + \frac{B}{\mu} + C \cdot \mu \]

\[ H = 2 \lambda d_p + \frac{8 \gamma D_f \mu}{\pi^2 (1 + KF_m/F_i)^2 D_p F_i} \]

For globular proteins the stokes radius is close to the molecular radius, which can be estimated by:

\[ R \approx 0.81 M^{1/3} \]

For molecular shapes other than spheres the Stokes radius can increase significantly. For example the equivalent spherical diameter of a cylinder can be calculated by:\(^2\)

\[ d = (6 \pi^2 h)^{1/3} \]

\(d\) = equivalent spherical diameter
\(r_c\) = radius of the cylinder
\(h\) = height of the cylinder

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\(1\) Wirth H.J., Gooley A. - SGE Analytical Science, 7 Argent Street, Ringwood, Vic. 3134, Australia

\(2\) For an aspect ratio of 2 it follows that \(h=4r_c\)
Assuming the same volume for the sphere and the cylinder:

\[ \frac{3}{4} \pi r_s^3 = V = \pi r_c^2 h = \pi r_c^2 \cdot 4r_c \]

\( r_s \) = radius of a spherical molecule

and combining the equations [5] and [6] the equivalent spherical radius of the cylinder turns out to be 1.31 times the radius of the sphere with the same volume. This translates in a 2.25 times increase in terms of the apparent molecular weight of a cylindrical molecule - a 100 kDa protein behaves like a 225 kDa protein.

**RENKIN MODEL FOR RESTRICTED PORE DIFFUSION**

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 Fickian diffusion where the diffusion rate is purely concentration driven to 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\(^3,4\)).

An estimation for the steric hindrance at the pore entrance and the frictional resistance within the pore system was provided by Renkin\(^5\):

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

\( D_f \) = free molecular diffusion coefficient,
\( D_p \) = diffusion coefficient inside the pore
\( r_s, r_p \) = radii of the solute and the pore respectively

The drawback of large pore size is the reduction in specific surface area and with it a reduction in the column capacity. In general silica based stationary phases have about 1 ml/g specific pore volume. The effect of the pore size on the surface area is shown in figure 3.

**Figure 2: Graphical representation of the Renkin model**

The ideal conditions

The corresponding relative analyte to pore diameter

**Figure 3: Specific surface areas of silica based stationary phases**

Choosing the right column for a certain analyte size now is a compromise between desired column capacity and the reduction in mass transfer rate one can tolerate. As a guideline we propose relative diffusion rates between the inside of the pores and the free mobile phase to be between 0.5 and 0.8. This means the analyte diameter is between 5 and 15 % of the pore diameter.

**Figure 4: Analyte size range for various pore size stationary phases**

The recommended molecular weight ranges (Da) are:

<table>
<thead>
<tr>
<th>Pore Size</th>
<th>Spherical Analyte</th>
<th>Cylindrical Analyte</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>30 800</td>
<td>13 350</td>
</tr>
<tr>
<td>120</td>
<td>50 1400</td>
<td>23 610</td>
</tr>
<tr>
<td>200</td>
<td>240 6400</td>
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<tr>
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<td>350 9500</td>
</tr>
<tr>
<td>1000</td>
<td>29400 800000</td>
<td>13000 350000</td>
</tr>
</tbody>
</table>

**Table 1: Recommended molecular weight ranges**
UNIQUE COLUMN FEATURES FOR THE ANALYSIS OF BIOLOGICAL SAMPLES

Some classes of biological samples (classical examples are phosphorylated peptides and proteins) have the ability to interact with metal ions and surfaces. The stationary phase therefore should be based on a high purity silica support material and all metal surfaces in the flow path should be avoided. ProteCol™ HQ columns for large molecules feature glass lined column hardware and porous PEEK™ frits to ensure the sample does not come into contact with any metal.

COLUMNS FOR PEPTIDE ANALYSIS: 200 Å VS 300 Å

200 Å and 300 Å stationary phases are ideal for analytes of the size of peptides commonly encountered in tryptic digests of proteins. The increased surface area of the 200 Å columns provides a higher carbon load and an increased capacity which in general gives sharper peaks. The exception is the undigested BSA peak at 45-46 minutes.

COLUMNS FOR PROTEIN ANALYSIS: 1000 Å - C8

Stationary phases with 1000 Å pore size are designed for the analysis of macromolecules. The C8 surface coverage optimizes the recovery of proteins.

The sample was prepared from the large lobe of the liver of male C57BL/6j mice. A membrane protein standard was developed in the laboratories of Bill Jordan at the Victoria University in Wellington, NZ as part of the membrane protein initiative of the Asia Oceania Human Proteome Organisation (AOHUPO).

SUMMARY

Pore size is an important parameter when analyzing larger molecules. By selecting the right pore size for a task the capacity and the mass transfer trade-off can be optimized to achieve the best possible separation. For tryptic digests a 200 Å stationary phase is superior to a 300 Å due to its two-fold increase in surface area while 1000 Å are required for the separation of proteins.

REFERENCES