Resolution increase in GPC/SEC/GFC by decreasing particle size and increasing the number of columns

White Paper

Scope

This white paper discusses practical strategies in GPC/SEC to increase the resolution. With a strong focus on **particle size** and the **number of columns** applied, it shows examples for organic and aqueous GPC/SEC separations and gives practical advice for increasing resolution and decreasing eluent consumption and time.

For a theoretical discussion of resolution or specific resolution in GPC/SEC please consult the literature. A very comprehensive overview is given in:
Modern Size-Exclusion Liquid Chromatography: Practice of Gel Permeation and Gel Filtration Chromatography, A. Striegel, W.W.Yau, J.J.Kirkland, D.D. Bly, John Wiley & Sons, New York, (2009).

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Content

1) Introduction

Size exclusion chromatography (SEC), also known as gel permeation chromatography (GPC), is a pore size limited, diffusion controlled, liquid chromatography technique that separates macromolecules based on their size in solution.

It is standard technology to measure molar mass, size, composition, purity and structure of all soluble synthetic polymers, biopolymers and proteins.

GPC/SEC Separation Mechanism

The separation in GPC/SEC is achieved in columns packed with porous particles with a narrow particle size distribution and very defined pore sizes.

Figure 1 illustrates the separation. While larger sizes cannot penetrate any of the pores and elute first, smaller sizes, diffuse into pores, are retarded and elute later.

Amongst other parameters, the size of a molecule in solution is determined by the molar mass. After calibration, GPC/SEC/GFC can therefore be used to measure the molar mass averages and the complete molar mass distribution.

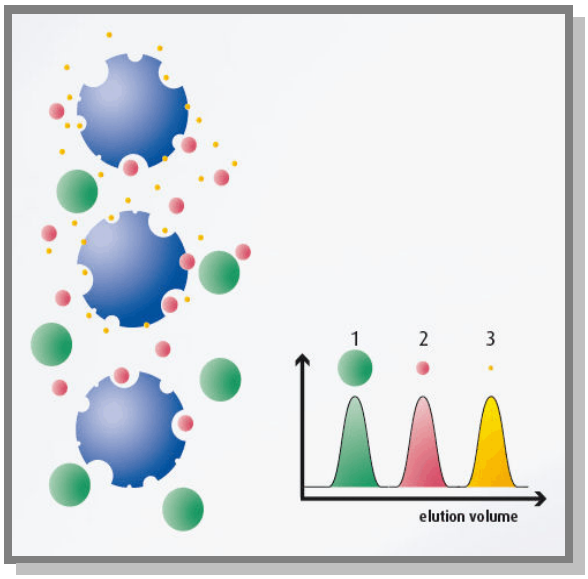

Figure 1:
GPC/SEC separation mechanism: larger sizes elute first, smaller sizes are retarded in the pores.

Pore Size/Molar Mass Separation Range

The distribution of the pore sizes in the column determines the size separation range. Since molar mass is one of the key factors that is measured with GPC/SEC, the pore size information is often accompanied with a molar mass separation range information.

GPC/SEC columns are available as *single pore size columns* with a narrow pore size distribution (very often reported in Angstrom (Å)) or as *mixed bed/linear columns* with a broader pore size distribution to cover a wider molar mass range with just one column.

To increase the molar mass separation range of single pore size columns often several columns are combined in a column bank (also called column set or column combination). Very often the materials for mixed bed/linear columns are blended of different single pore size materials to cover a broad molar mass separation range in one column.

For both, manufacturer pre-blended linear/mixed bed columns and combinations of single pore size columns, there is the inherent risk of pore size mismatch. A pore size mismatch creates dislocations resulting in chromatographic artifacts, such as shoulders, that cannot be overcome by calibration or even the use of advanced detection such as light scattering.

The use of recommended column combinations and avoiding combinations of linear and single pore size columns minimizes the risk of pore size mismatch.

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Resolution

Compared to other chromatographic techniques, GPC/SEC suffers from a limited resolution. While it is possible to separate single oligomers up to molar masses of around 1 000 Da using columns with the matching pore sizes (but often very limited molar mass separation range), it is never possible to resolve single chains of higher molar masses. Therefore, although many different molar masses are present even within the most narrow molar mass distribution, only single, but broader peaks, are observed for higher molar masses (compare e.g. Figure 2a, page 6).

The dependence of GPC/SEC column characteristics and experimental parameters on the resolution is quite complex. Column material particle size and packing quality, pore size and pore size distribution, solvent viscosity, temperature and flow rate, as well as sample concentration, injected mass and other factors influence the mass transfer and therefore the resolution.

There are two options to increase the resolution:

a) Resolution increase by adding columns of the same type

The far most commonly used practical approach to increase resolution is by combining columns with the same pore sizes to a column combination. By doubling the column length the resolution can be increased by a factor of 1.4.

The disadvantage of this concept is that analysis time, pressure, solvent consumption and waste increase when more columns are used. Nevertheless typical setups in GPC/SEC consist of 2 - 4 columns plus a precolumn.

b) Resolution increase by particle size decrease

An additional approach to increase the resolution is to decrease the particle size. Plate height and column permeability decrease with the particle diameter. Smaller particle size columns provide therefore a better resolution.

A disadvantage of smaller particles is that the pressure increases with decreasing particle size.

A potential threat when using smaller particle sizes, especially when discussing larger macromolecules, is shear degradation. According to current scientific investigation small particle sizes down to 3µm can be applied for oligomers in low viscous solvents and for proteins. It is still under investigation if higher molar masses or more rigid structures can be measured on small particle size columns with narrow frits without the danger of chain scission and without chromatographic artifacts.

Additional options:
All other parameters that can affect resolution are summarized in the PSS recommendations for method optimization, where flow rates, temperatures, concentrations etc. are discussed. Please check our catalog, the website, the column user documentation or our GPC/SEC Tips&Tricks.

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Silica vrs Polymer-based Columns

In general, there are two types of column materials used in GPC/SEC: silica based materials and copolymer network based materials.

Silica packings are popular for aqueous separations, that do not require pH extremes. e.g. for proteins and peptides or sometimes for certain polar polymers using polar organic solvents.

Silica materials are mechanical rigid, have well defined pore sizes and do not swell significantly when changing solvent. For this reason, plate counts on silica columns tend to be higher than for polymer columns.

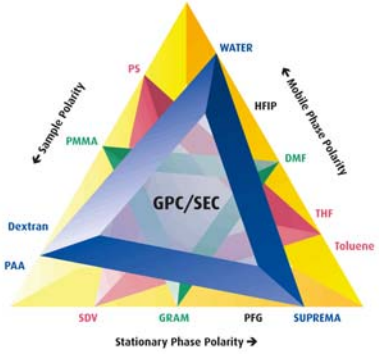
The main weaknesses of silica based columns are the chemical stability, particularly at the extremes of the pH scale, and the fact that the fundamental chemistry of the particle cannot be easily altered to be made compatible with popular solute\eluent combinations and surface modifications often lead to undesired solute adsorption problems.
In addition, while silica based materials often have a very high resolution in a small molar mass region, the resolution decreases strongly above and below this region. This increases the danger of pore size mismatch when columns are combined to cover a wider molar mass range.

Polymer packings are popular for most organic separations, e.g. polymers and also for aqueous separations that require extreme pH or have other compatibility criteria.e.g. aqueous soluble synthetic polymers, starches, polysaccharides.

Polymer materials offer much better chemical stability and a far wider range of available chemistries for different solute\eluent combinations. This often results in longer lifetime compared to silica columns due to the reduced danger of interaction. In addition their pores can be designed to have calibration curves with less steep exclusion limits to reduce the risk of pore size mismatch for column combinations and to increase the molar mass separation range within one column.

The main weakness of polymer materials is that they are less rigid and they swell in different solvents. This results in less pressure stability and more required care when changing between solvents with different polarity.

One of the key initial factors when choosing a column packing material for an application is to match the polarity of the packing material with that of the solute and the eluent to reduce the danger of interactions. This choice should be made before choosing the pore size or the particle size. The PSS Magic Triangle is an excellent visual tool, to support choosing the right polarity for a given sample.



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Summary of the Key Points

The ideal GPC/SEC column would have pores of all sizes to be able to separate over a very wide molar mass separation range (e.g. 100 to 30 000 000 Da) plus a large pore volume to provide constant and high resolution.

In reality, this goal is not fully achievable, especially in a short analysis time.

The molar mass separation range depends on the size of the pores. The most common approach to increase the **molar mass separation range** is therefore combining columns with different pore sizes.

An easy approach to increase the **resolution** is to combine columns with the same pore sizes to increase the pore volume.

The Specific Resolution per decade is defined as :

$$\mathbf{R_{sp} = 0.25 / D\,\sigma}$$

where:
D = Slope of the calibration curve
σ = Dispersion caused by the system.

For these reasons many GPC/SEC applications use 2,3 or even 4 columns plus a precolumn to optimize the molar mass separation range and/or increase the resolution.

Adding columns of the same pore size decreases the slope D of the calibration curve and increases the resolution.

Similarly *decreasing the particle size*, reduces the dispersion in the system and increases the resolution.

Care needs to be taken when combining different pore size columns as sudden changes in the calibration slope can cause artifacts in chromatograms.

GPC/SEC column manufacturers like PSS have applied the concept of mismatch free column combinations and smaller particle sizes already by offering the best column combination for a defined molar mass separation range.

E.g the PSS SDV combination low (100 - 60 kDa) comes with 3µm particles, the combinations medium (100 - 1 MDa) and high (100 - 3 MDa) come with 5 µm and the ultrahigh combination (100 - 30 MDa) comes with 10 µm particles.

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2) Visualizing resolution - Understanding chromatograms

The GPC/SEC resolution is often visualized by showing the separation of oligomers, since here it is in general possible to resolve single molar masses when columns with small pore sizes are used. However, please note that when discussing resolution the molar mass separation range, that is covered by the column, plays an important role.

The influence of pore size and particle size on chromatograms is nicely visualized in figure 2 a and b, where a mixture of of 4 different polystyrene molar mass reference materials is separated on a column set with best performance in oligomer to medium separation (a) and a column set with optimized performance in the medium to high molar mass range (b).

Figure 2 a:
Separation of 4 different molar mass reference standards. The column combination is designed to have the best separation in the oligomeric and medium molar mass region (100 - 60 000 Da). Therefore it is possible to separate the single chains of the oligomer Mp= 474 into single peaks corresponding to chains with 2 (P2), 3 (P3), 4 (P4) etc. repetition units.

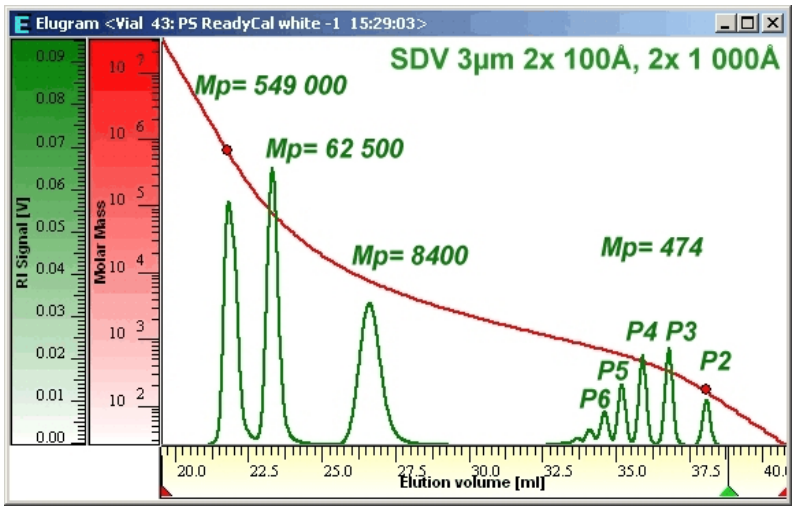
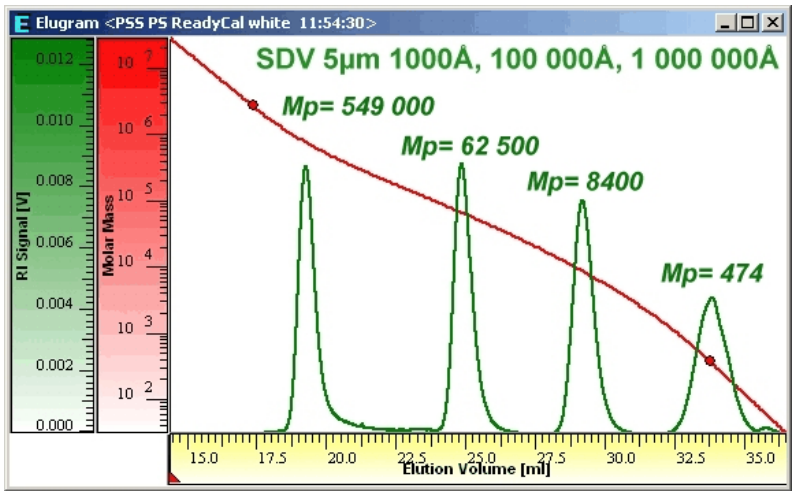


Figure 2 b:
The same mixture measured on a column combination optimized for medium to high molar mass range (100 to 3 000 000 Da). The resolution in the low molar mass region is not sufficient to resolve the oligomer Mp=474 into single peaks. It elutes, like the higher molar masses too, in just one peak. However, this set allows to analyze medium to higher molar masses with a much better resolution meeting national and international GPC/SEC standards. Molar mass results for oligomers will nevertheless be correct.



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2) Examples for reducing particle size and increasing the number of columns - effect on separation

• Organic separations - Example PSS SDV columns

The most traditional stationary phase material for unpolar and medium polar organic solvents, such as Tetrahydrofuran, Toluene or Chloroform, is a copolymer network made of styrene and divinylbenzene. Very often this material is also used for polar organic solvents, such as DMF, DMAc or DMSO. However with modern, more polar stationary phases, such as PSS GRAM or PolarSil, a better solution with less interactions is available.

The PSS brand name for this material is SDV. It is offered in 3 different particle sizes (3µm, 5µm and 10 µm), other particle sizes can be produced on request.

The material is packed in
- semi-micro columns (dimensions 4.6 x 250 mm)
- analytical columns (dimensions 8 x 300 mm)
- HighSpeed columns (dimensions 20 x 50 mm)
- or preparative columns (dimensions 20 x 300 mm)

Other dimensions can be offered on request.

a) Smaller particles effect

Figure 3 shows a comparison of separations of an polystyrene oligomer on an analytical GPC/SEC column for an separation range of 100 to 60 000 Da filled with 10µm, 5µm or 3µm respectively.

A flow-rate of 1 mL/min has been applied, temperature was ambient, no column compartment was used.

Please note that further method optimization could include working at a different flow-rate or (in case of more viscous solvents) working at elevated temperatures. This would further increase the resolution for all sizes.

Also using smaller pore sizes or a column with a narrower pore size distribution optimized only for oligomers would allow a better separation for this particular sample.

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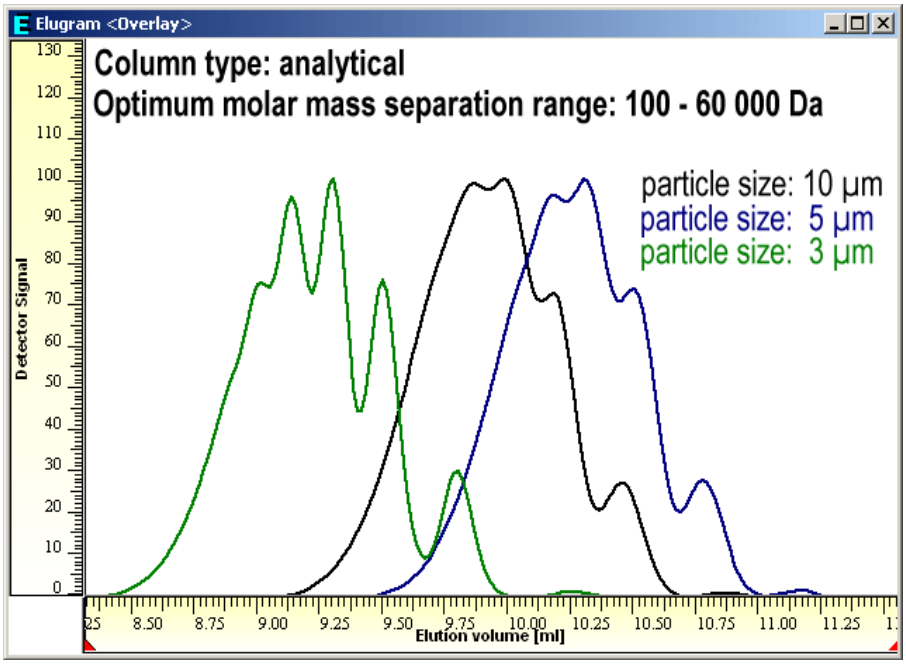

Figure 3:
Influence of particle size: Here an oligomer has been separated on one analytical column with oligomer to medium molar mass separation range. It is clearly visible that the resolution increases with decreasing pore size.

Another interesting comparison for different particle sizes is when reviewing the results, the molar mass distribution. Figure 4 shows a comparison of the separation of a polystyrene mixture of four different molar masses including the oligomer shown in Figure 3. As expected the measured molar mass distributions are the same, independent on particle size. However the resolution power of smaller particle sizes for the oligomers is clearly visible.

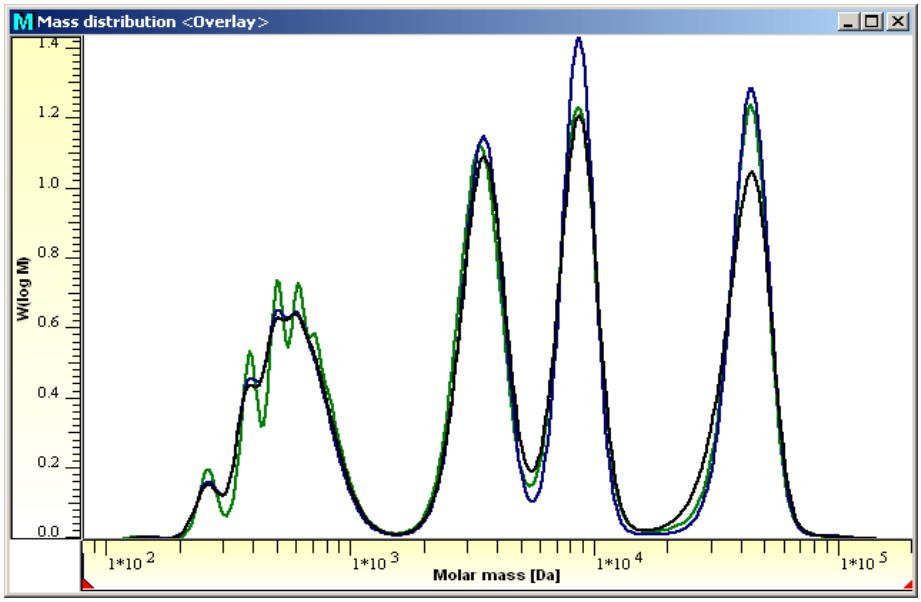

Figure 4:
Mass distribution results for the separations shown in Figure 3 (influence of particle size on separations using SDV columns in THF). The results are identical within the method error, however the resolution power for the 3µm particles (green) is higher revealing the single chains better.

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b) Increased column number effect

A common next step to increase the resolution is to increase the number of separation columns.

As analysis time and the amount of consumed solvent increase linearly with the number of columns, a direct visual comparison of chromatograms is often difficult due to scaling issues. Here the molar mass distribution results can provide further insight.

Figure 5 compares the distribution results and the molar mass averages for a separation on 3µm SDV particles using one analytical column (dark green) and three analytical columns (light green). It is clearly visible that the resolution in the oligomeric region increases significantly.

While the numerical results for the molar mass averages are the same within the method error, the results uncertainty for the data obtained with one column is slightly higher. The use of the column set is advantageous when one of the analysis goals is to characterize the oligomers precisely also with the weight % determination below 500 and 1 000 Dalton.

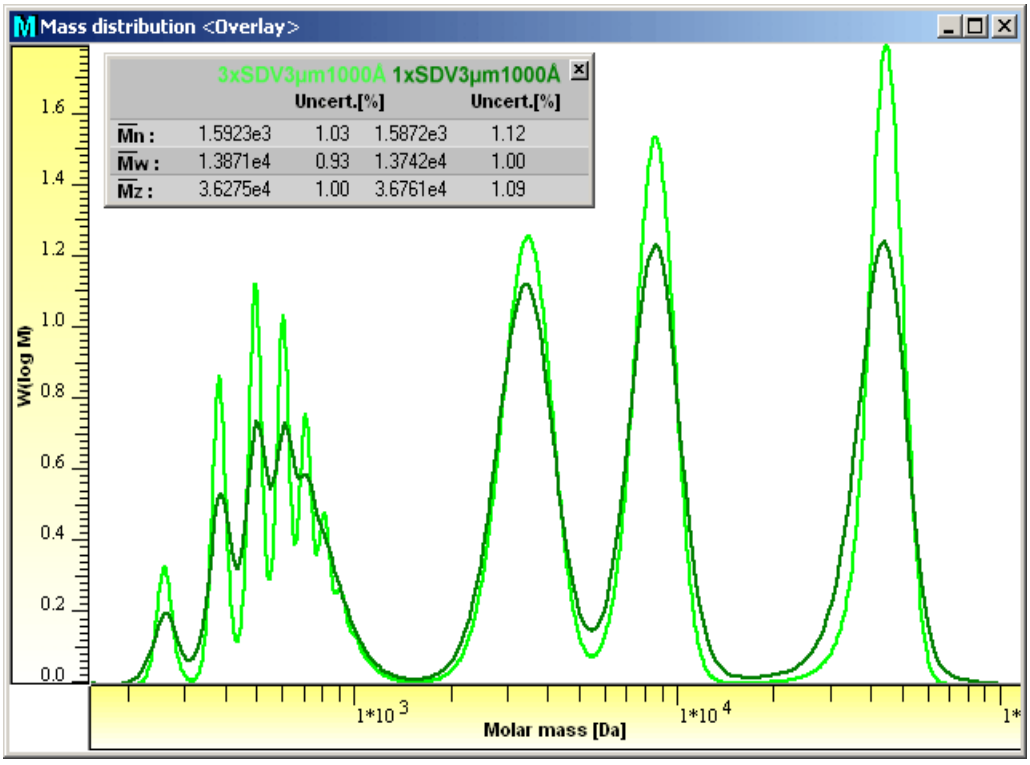

Figure 5:
Influence of the number of columns on separations using SDV columns filled with 3µm particle size in THF. Here the results are shown for a combination of three columns (light green) compared to the results obtained with one analytical column (dark green).

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• ***Aqueous separations - Example PSS SUPREMA columns***

The most traditional polymer stationary phase materials for aqueous separation are copolymer networks based on acrylate monomers. The PSS brand here is the SUPREMA material, a very universal material for all neutral and anionic macromolecules. It is applicable over a wide pH range from 1.5 to 13.
SUPREMA is offered in 2 different particle sizes (5µm and 10 µm), other particle sizes can be produced on request.
The material is packed in

- semi-micro columns (dimensions 4.6 x 250 mm),
- analytical columns (dimensions 8 x 300 mm),
- HighSpeed columns (dimensions 20 x 50 mm)
- or preparative columns (dimensions 20 x 300 mm)

Other dimensions can be offered on request.

All resolution optimization strategies as discussed in detail for SDV are also applicable for SUPREMA. Many people expect that separations in water have a low resolution, since water has a higher viscosity as typical organic GPC/SEC elutents and the mass transfer is less efficient. Therefore historically larger particle sizes have been used to avoid high pressure. To increase the mass transfer it is recommended for nearly all applications to work at elevated temperatures (e.g. 45°C) and, if required, at lower flow-rates (e.g. 0.5 mL/min).
Figure 6 summarizes the comparison of the chromatograms using **different particle sizes** and **a different number of columns** for PSS SUPREMA molar mass range 100 - 100 000 Da. Here it is possible to resolve an oligomer into single chains using two columns filled with particles of 5µm particle size.

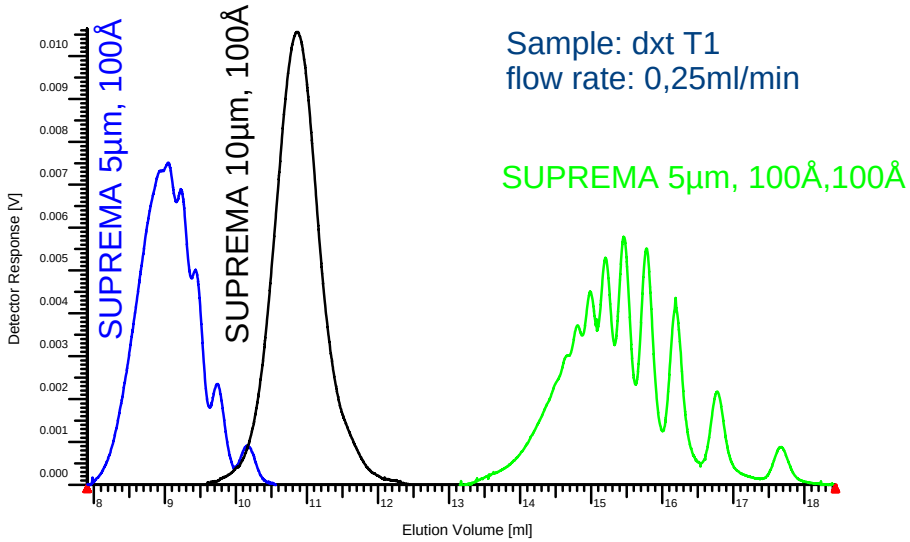

Figure 6:
Comparison of the separation of a dextran oligomer on particle size 10µm (1 column, black) and on 1 (blue) or 2 (green) 5µm columns (Range 100 - 100 000 Da)

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A special application of growing importance is the separation of proteins using aqueous GPC/SEC. A major analysis goal for many proteins is to determine the protein purity and to calculate the amount of dimer, trimer or other oligomers present.

Since the molar mass separation range that needs to be covered for purity determination is very narrow, often silica based materials, such as PSS PROTEEMA, are used. However there are some applications where the use of PSS SUPREMA is recommended due to either to avoid interactions or to the wider pH-applicability of SUPREMA.

Figure 7 shows a comparison on SUPREMA 5µm particles for a separation on one analytical column (red) and 2 analytical columns (blue).

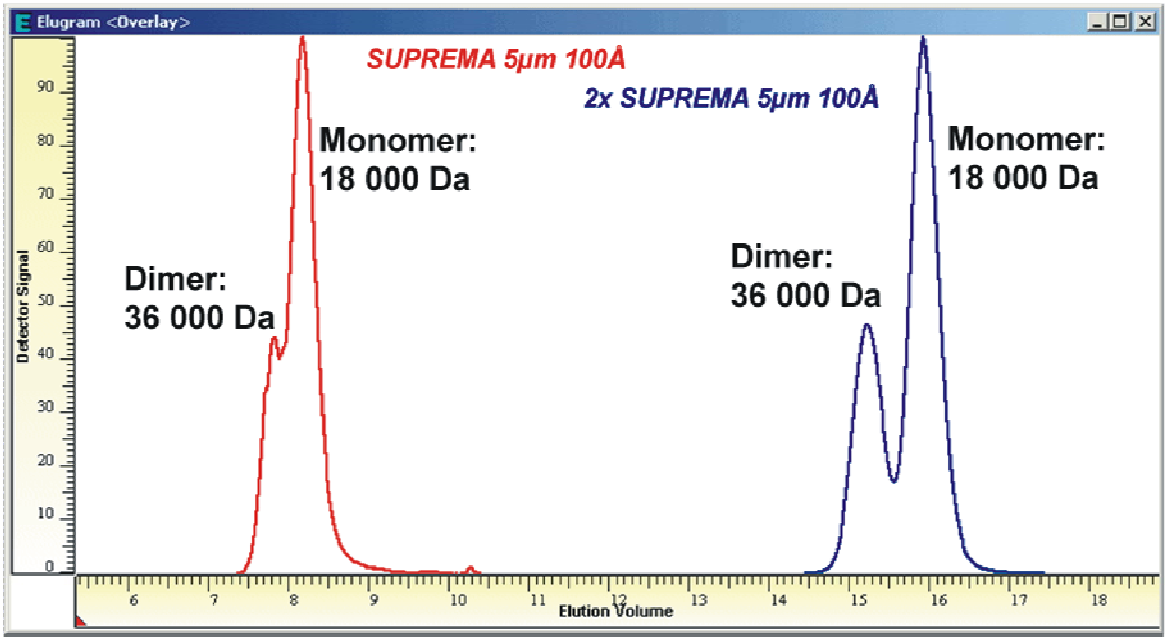

Figure 7:
Protein purity test on one SUPREMA 5µm column compared to the separation on two analytical SUPREMA 5µm columns. The use of two columns increases the resolution by a factor of 1.4. Please note that standard material for proteins is PSS PROTEEMA; that is also available as 3µm particle size. This special application required SUPREMA due to interaction on silica based materials.

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4) Outlook: how can these facts help to save time and reduce solvent while keeping the resolution?

There are many reasons why a reduction of solvent and time is always an issue in analytical laboratories. Considered are

• Health aspects:
the Committee for Risk Assessment (RAC) has agreed to a proposal to classify THF, a widely used solvent in GPC/SEC, which cannot be substituted easily, as carcinogenic.

• Environmental aspects:
reducing solvent consumption and waste helps to protect the environment.

• Economical aspects:
more samples need to be analyzed which requires a decrease of the ratio required time per sample

A solution to save time and solvent would be to switch from traditional analytical columns with dimensions 8 x 300 mm to **semi-micro columns** with dimensions 4.6 x 250 mm.
It is possible to save 2/3 of the solvent with this approach.
The reduction in time is less pronounced when the traditionally recommended flow-rate of 0.33 mL/min (or 1/3 of the actual application flow-rate) is applied. However, modern stationary phases can be easily run at a flow-rate of 0.6 mL/min allowing also to save significantly more time.

Unfortunately the consequence of using shorter semi-micro columns is that the pore volume is reduced and therefore a reduction in resolution is observed even if the other operational parameters such as flow rate and injection volume are scaled accordingly. A direct 1 by 1 replacement would result in less resolution. Fortunately for many applications the resolution loss can be alleviated by changing also to a smaller particle size. E.g. applications that now use 5µ analytical columns might be changed to 3µ semi-micro columns.

The huge advantage of this approach is that the chemistry of the stationary phase material can be kept avoiding potential reproducibility issues.

Figure 8 shows the comparison of the analysis of a polystyrene oligomer on a SDV 5µ analytical column and on a SDV 3µm semi-micro column. Analytical conditions (injected mass, flow-rate, etc.) and instrumentation (RI-detector) have been set to recommended standard conditions.

To allow an easier visual comparison the large figure uses a time based axis. The flow rates have been set to traditional recommendations, so there is only limited time saving and the chromatograms can be nicely compared. The inset shows the consumed solvent on the axis as x-axis demonstrating the significant amount of savings here, while the resolution is even slightly increased.

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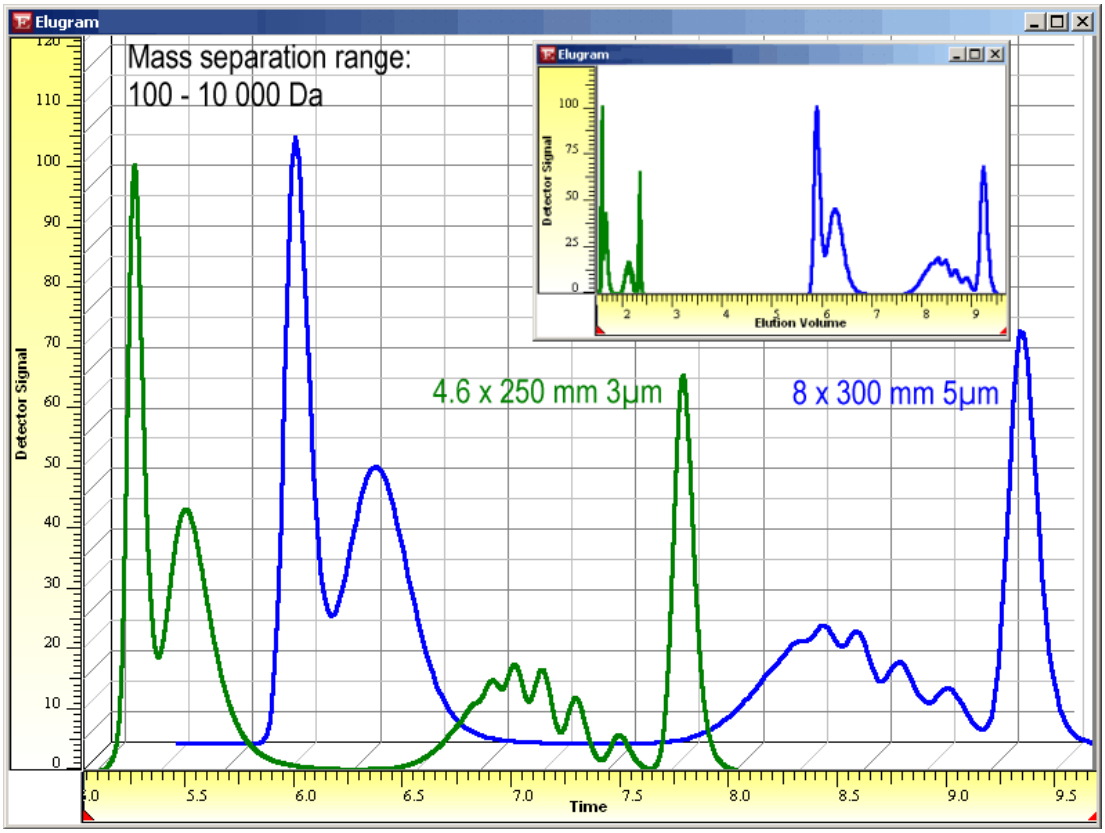

Figure 8:
Comparison of an analytical SDV column with 5µm particles and a semi-micro column with 3µm particles. The large figure shows the required time to obtain the chromatogram, the inset shows the amount of required solvent. A direct comparison of the oligomer resolution shows that a resolution loss can be alleviated.

All aspects for resolution (or molar mass range) increase discussed and demonstrated above for the different materials are also valid for materials in semi-micro columns

Consequences for instrumentation

An additional advantage of using semi-micro columns is less dispersion. This results in very narrow and sharp peaks which are especially beneficial for multi-detection GPC/SEC.

Unfortunately, the cell volume of a lot of traditional GPC/SEC detector cells is too large to profit from the enhanced separation power of semi-micro columns filled with small particles.

While UV/VIS detectors are, in general, ready for these applications either a modification of existing refractive index detectors (RI) or even the purchase of new µRIs, light scattering detectors of viscometers is required.

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Figure 9 shows how the excellent resolution of micro column gets lost when analytical RI detectors are used for detection.

Optimized RIs, such as the µSECcurity RI, provide much better results.

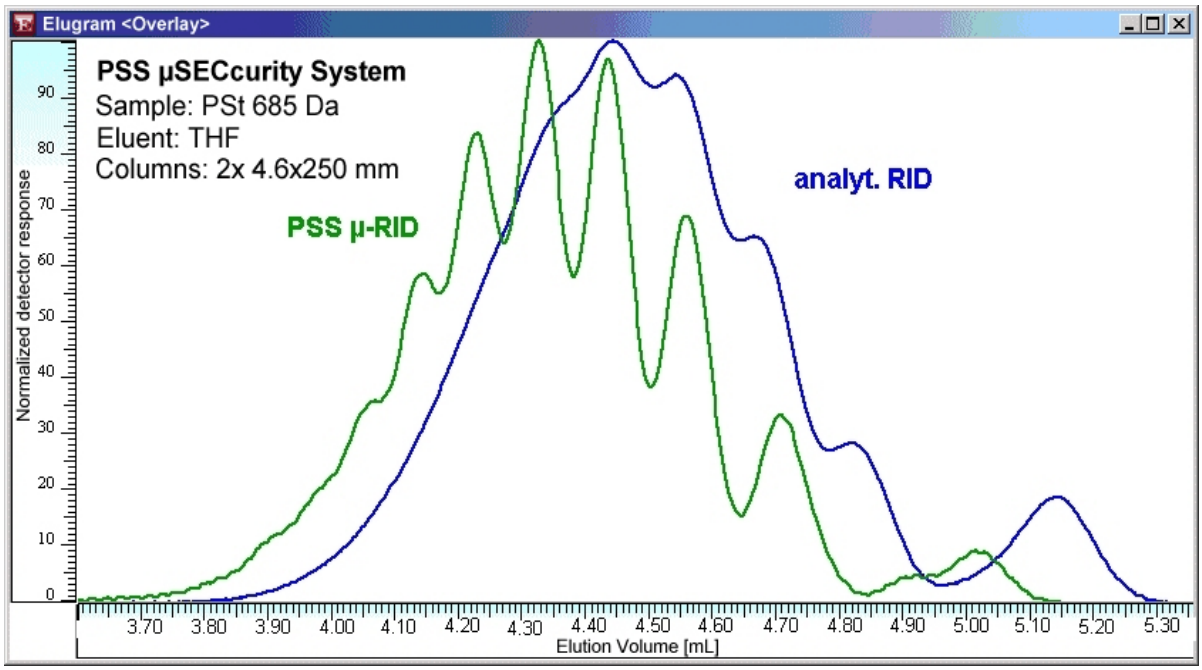

Figure 9:
Comparison of the chromatogram of a polystyrene oligomer separated in 5.5 mL using micro columns and a standards RI detector (blue trace) versus an optimized µRI detector with smaller cell volume (green trace).

Conclusions

Modern GPC/SEC column packing materials are nowadays available in small particle sizes. This offers the chance to transfer existing applications to semi-micro columns and to keep the chemistry of the stationary phase. The smaller particle sizes alleviate the resolution loss due to the decreased pore volume. The stability of the materials allow operation at flow rates that are higher than normally recommended for semi-micro columns allowing not only to save significant amounts of solvent, but also of time.

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