Towards small and fast size-exclusion chromatography

Popovici, S.T.

Publication date
2004

Citation for published version (APA):

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: https://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.
Chapter 4
Fast Size-Exclusion Chromatography
-Theoretical and Practical Considerations-

Abstract
Fast-SEC is a very interesting modification of conventional SEC. The need for it emerges from combinatorial chemistry and high-throughput experimentation, where high-speed analyses are required and from comprehensive two-dimensional liquid chromatography, when SEC can be used as a fast second-separation separation. The different approaches to change the speed of analysis are extensively described in this chapter. Special attention is paid to the trade-off between analysis time and resolution, and to the selection of optimal column lengths and flow rates. Simulations are used to design and to understand experiments. Integrity plots are constructed to judge the quality of various SEC systems. Fast separations in size-exclusion chromatography are found to be more favorable than suggested by conventional theory. The results are based on experimental data obtained for polystyrene using THF as mobile phase.

4.1. Introduction
Size-exclusion (SEC) is a form of liquid chromatography (LC) that separates the analytes according to their hydrodynamic volumes. SEC is the outstanding technique to measure molar-mass distributions (MMD) of natural and synthetic macromolecules. The analytes with the highest molar masses (MM) are eluting first from the separation column, while the smallest molecules are eluting last, being retained for a longer time inside the column. Conventionally, SEC analyses require times up to several hours and not less than 10 to 15 minutes. One of the most important parameters in any kind of chromatography is the resolution. Good resolution is required to adequately characterize the sample. Because in case of polymers we invariable work above the optimal flow rate [1], the best resolution is usually obtained at the lowest flow rate, which is equivalent to the longest analysis time. However, there is a definite trend towards fast size-exclusion-chromatographic separations.
Chapter 4

Fast SEC chromatography has been used by several polymer-science groups [2,3] and industrial laboratories [4,5,6,7] and dedicated columns for the purpose are available from several manufacturers [8,9]. The field is strongly application driven. Very little fundamental work has yet been devoted to the subject. The great interest in Fast SEC separations arises from two sources. First, the emergence of combinatorial approaches to (industrial) research and development and the associated need for high-throughput experimentation [7]. Second, in polymer analysis two-dimensional techniques have been developing rapidly during the last years. This requires fast second-dimension separations. Other perceived advantages of Fast SEC are the consumption of less eluent and the use of smaller columns. We believe neither of these later two arguments to be truly relevant. Reduced consumption of solvents (and stationary phase) can much better be achieved by reducing the column diameter. The prevailing reason to pursue Fast SEC is speed.

Traditionally SEC of polymers has been performed on columns packed with relatively large particles (e.g. 10-20 μm). Columns with such large particles can provide high-resolution separations, but they do require long analysis times [28]. Large (high MM) polymers are thought to require large particles to avoid shear degradation and recovery problems. To reduce the analysis times different approaches are possible (Table 1). The first one is to decrease the particle diameter of the stationary phase. The second possibility is to reduce the column length and the third to increase the flow rate.

The packing of SEC columns with smaller particles is the most attractive option for reducing the analysis times. When the length of the column is kept constant the efficiency will increase (Table 1). To reduce the separation time, the column length can be decreased or the flow rate increased. The efficiency can be (at least) maintained at the original level. The major disadvantage of this approach is the increased pressure drop across the separation column [10]. In order to overcome this mechanical limitation, McNair et al. [11,12] introduced ultra-high-pressure liquid chromatography (UHPLC) techniques. Small particles inherently produce very low column permeability and, therefore considerable heat is generated. Wu et al. [13,14] concluded that capillary columns (e.g. 30-150 μm internal diameter) are required to facilitate heat dissipation. Another solution to overcome the high pressure drop could be the use of monolithic columns. Due to their structure, silica-based monoliths can also offer an enhanced chromatographic performance [15,16,17]. They provide a unique combination of low-pressure drop and high separation efficiency.
Unfortunately, the selectivity offered by monolithic columns for SEC is still much inferior to that of typical columns packed with porous particles.

Table 1: Different approaches to change the speed of analysis in SEC. $d_p$ is the particle diameter, $L$ is the column length, $F$ is the flow rate, $N$ is the plate number, $t_R$ is the retention time and $\Delta p$ is the pressure drop across the separation column. Potential approaches to Fast SEC are printed in bold.

<table>
<thead>
<tr>
<th>$d_p$ (µm)</th>
<th>$L$ (mm)</th>
<th>$F$ (ml/min)</th>
<th>$N$</th>
<th>$t_R$ (min)</th>
<th>$\Delta p$ (bar)</th>
</tr>
</thead>
<tbody>
<tr>
<td>× 2</td>
<td>× 2</td>
<td>(&gt;)(&lt;) × 1/2</td>
<td>Cst.</td>
<td>× 2</td>
<td>× 1/2</td>
</tr>
<tr>
<td>× 2</td>
<td>× 1/2</td>
<td>(&lt;)× 2</td>
<td>Cst.</td>
<td>× 4</td>
<td>× 1/4</td>
</tr>
<tr>
<td>Cst.</td>
<td>× 2</td>
<td>(&gt;)(&lt;) × 1/4</td>
<td>Cst.</td>
<td>× 1/2</td>
<td>× 1/4</td>
</tr>
<tr>
<td>× 1/2</td>
<td>× 2</td>
<td>(&gt;)(&lt;) × 1/8</td>
<td>Cst.</td>
<td>× 1/4</td>
<td>× 1/8</td>
</tr>
<tr>
<td>× 1/2</td>
<td>× 1/2</td>
<td>(&lt;)× 1/4</td>
<td>Cst.</td>
<td>× 1/2</td>
<td>× 1/4</td>
</tr>
<tr>
<td>× 2</td>
<td>× 2</td>
<td>(&gt;)(&lt;) × 1/2</td>
<td>Cst.</td>
<td>× 2</td>
<td>× 2</td>
</tr>
<tr>
<td>× 1/2</td>
<td>× 2</td>
<td>(&lt;)× 4</td>
<td>Cst.</td>
<td>× 4</td>
<td>Cst.</td>
</tr>
<tr>
<td>Cst.</td>
<td>× 2</td>
<td>(&gt;)(&lt;) × 1/4</td>
<td>Cst.</td>
<td>× 1/2</td>
<td>× 1/2</td>
</tr>
<tr>
<td>× 1/2</td>
<td>× 2</td>
<td>(&lt;)× 1/4</td>
<td>Cst.</td>
<td>× 1/4</td>
<td>× 1/4</td>
</tr>
<tr>
<td>× 2</td>
<td>× 2</td>
<td>(&gt;)(&lt;) × 1/2</td>
<td>Cst.</td>
<td>× 2</td>
<td>× 2</td>
</tr>
<tr>
<td>× 1/2</td>
<td>× 2</td>
<td>(&lt;)× 4</td>
<td>Cst.</td>
<td>× 2</td>
<td>× 2</td>
</tr>
<tr>
<td>× 1/2</td>
<td>× 2</td>
<td>(&gt;)(&lt;) × 1/2</td>
<td>Cst.</td>
<td>× 1/2</td>
<td>× 4</td>
</tr>
<tr>
<td>× 1/2</td>
<td>× 1/2</td>
<td>(&lt;)× 2</td>
<td>Cst.</td>
<td>× 2</td>
<td>× 2</td>
</tr>
</tbody>
</table>

85
So far, monoliths have a smaller volume of pores that contain stagnant mobile phase during analysis. Other alternatives to avoid working at high-pressure are size-exclusion electrochromatography (SEEC) [18] and open-tubular or open-channel hydrodynamic chromatography (HDC) [19]. Neither of these latter options is thought to be practical at this stage.

The third option for speeding up the SEC analysis is to increase the flow rate. This can be done either on a conventional column, or while concomitantly using a shorter column (i.e. the second option). In the first case the efficiency and resolution decrease while the pressure drop will be higher. When increasing the flow rate in combination with a shorter column, the pressure drop may not increase, while the time of analysis will be shorter. However, the plate number will also decrease dramatically. Therefore, a compromise must be found between the gain in speed and the loss in efficiency (resolution).

The factors listed in the plate-count column are based on the classical van-Deemter equation, which suggests that at high flow rates $N$ is approximately proportional to $1/F$. However in reality the situation is more favorable. When doubling $F$, $N$ is expected to decrease by (much) less than a factor of two (see [1] and theory section below). This is indicated by the < and > signs in Table 1 for situations in which the flow rate is altered.

Commercial Fast SEC columns are much shorter than conventional SEC or LC columns (Table 2). Conventional LC columns have a length of 150-300 mm. The Fast-SEC columns commercialized by Polymer Laboratories (PL) are 50 mm long and have an internal diameter (i.d.) of 4.6 or 7.5 mm, while those manufactured by Polymer Standard Service (PSS) have the same length, but a larger internal diameter of 20 mm.

<table>
<thead>
<tr>
<th>Column dimensions (mm)</th>
<th>Manufacturer</th>
<th>Column volume (ml)</th>
<th>Volume eluent for LC×SEC run (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50×4.6</td>
<td>Polymer Laboratories</td>
<td>0.5</td>
<td>50</td>
</tr>
<tr>
<td>50×7.5</td>
<td>Polymer Laboratories</td>
<td>1.3</td>
<td>130</td>
</tr>
<tr>
<td>50×20</td>
<td>Polymer Standard Service</td>
<td>10</td>
<td>1000</td>
</tr>
</tbody>
</table>

1 Assuming 100 second-dimension runs
Table 2b: Required amounts of eluent for a typical LC×SEC run and suggested flow rates and column diameters for the first dimension.

<table>
<thead>
<tr>
<th>Column dimensions (mm)</th>
<th>( t^2F ) (ml/min)</th>
<th>( t^2t_R ) (min)</th>
<th>( V_{inj}^2 ) (µl)</th>
<th>( t^1F ) (µl/min)</th>
<th>( t^1d_c ) (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50×4.6</td>
<td>0.5</td>
<td>1</td>
<td>20</td>
<td>20</td>
<td>1</td>
</tr>
<tr>
<td>50×7.5</td>
<td>1.3</td>
<td>1</td>
<td>50</td>
<td>50</td>
<td>1.5 or 2</td>
</tr>
<tr>
<td>50×20</td>
<td>10</td>
<td>1</td>
<td>400</td>
<td>400</td>
<td>4.6</td>
</tr>
</tbody>
</table>

Kilz et al. [9] have investigated options for Fast-SEC analysis. They compared different approaches, such as increasing the flow rate on a conventional SEC column (300×8 mm), shortening the length to 50 mm decreasing the column internal diameter to 4 mm or increasing the diameter to 20 mm. In a study by Pasch et al. [3] conventional styrene/divinylbenzene SEC columns (300×8 mm) and Fast-SEC columns of 50×20 mm were compared. The accuracy and the precision of Fast SEC columns were investigated using a broad reference standard. The analysis time was reduced from 10 minutes down to 2 minutes.

Applying the concept of integrity plots, which was introduced elsewhere [1] it was possible to quantitatively investigate the influence of the particle size, pore-size distribution, flow rate, and column length on the quality of separation in Fast SEC, based on a limited number of simple experiments. The aim of the present chapter is to investigate whether Fast SEC is a useful technique and, if so, for which types of analytes and under which conditions. Explicitly the scope of this chapter is to establish the range of MM and polydispersities (PDI) for which an accurate MMD can be obtained. The MMD of polymers is characterized by their average molecular weights, such as \( M_n \) (number-average molecular weight) and \( M_w \) (weight-average molecular weight). The PDI of a polymer is defined as PDI=\( M_w/M_n \).

The main fundamental obstacle to the chromatography of polymers is the slow diffusion of the analytes. As a consequence effect we have to deal with poor chromatographic efficiency and with extra-column dispersion. The molecular diffusion of polymers is strongly dependent on their MM and on the mobile phase [20]. Typically, the diffusion coefficient decreases with increasing mobile-phase viscosity and it typically decreases with increasing MM.

A SEC peak can be broadened due to the PDI of the sample and due to extra-column and column dispersion. Since in SEC the aim is to measure the MMD of the sample, the
broadening due to the PDI \(i.e.\) the chromatographic selectivity) must be as high as possible, while the other two contributions to the total band broadening must be minimized. The exact value of the polydispersity index (PDI) is very difficult to establish. The widths of molar-mass distributions (which are directly related to the PDI) have been directly estimated from SEC coupled with concentration and light-scattering detectors [21] and can also be derived from mass-spectrometric measurements using soft ionization techniques [22, 23]. In the case of commercial standards the manufacturer specifies a value, which should be seen as an upper limit. Some researchers concluded that the real PDI values are somewhat smaller than those specified by the supplier [24,25,26]. Others suggested that the true values are much smaller [27]. Strong evidence that the latter presumption is correct has been provided by Lee et al. [25] using temperature-gradient interaction chromatography (TGIC).

Two-dimensional chromatography progressed considerably in the last decade. This method involves the coupling of two different separation mechanisms on-line, exploiting the potential of both of them in order to obtain, so-called ‘comprehensive’ information. The first dimension is a slow separation, while the second one is fast. The fractions of the sample eluting from the first dimension are collected by the modulation valve and re-injected in the second dimension. The faster the second dimension, the shorter can be the total analysis time (or the higher the overall resolution). The most common combination in comprehensive two-dimensional chromatography of polymers is interactive liquid chromatography (LC) with SEC.

4.2. Theory

4.2.1. General aspects and overview

In chromatography, the degree of separation of two components \(i\) and \(j\) is given by the resolution \(R\) [28], which usually is defined as:

\[
R_s = \frac{(t_{R,j} - t_{R,i})}{1/2(w_i + w_j)} \tag{1}
\]

where \(t_{R,i}\) and \(t_{R,j}\) represents the retention times and \(w_i\) and \(w_j\) are the peak widths (in time units) at the base-line of the second (\(j\)) and the first (\(i\)) analyte, respectively. Alternatively,
Fast-Size-Exclusion Chromatography – Theoretical and Practical Considerations

Retention volumes and peak widths in volume units may be used. In SEC a different resolution concept ($R_{SEC}$) is used, which is correlated to the calibration curve as follows:

$$R_{SEC} = \frac{\Delta V_R}{4\sigma} \times \frac{1}{\log(M_j / M_i)}$$  \hspace{1cm} (2)

where $\Delta V_R=(V_{R,j}-V_{R,i})$, represents the difference in retention volumes between the second ($j$) and the first ($i$) analyte. $\sigma$ is the peak standard deviation in volume units (proportional to the peak width), and $M_i$ and $M_j$ are the molar masses of the analytes where $M_i < M_j$ [29]. Note that $R_{SEC}$ is equal to the conventional resolution ($R_S$) if we consider two peaks that differ in molar mass by an order of magnitude ($M_j=10\times M_i$).

As described in the introduction of this paper, an important reason for our interest in Fast SEC is its use as the second dimension in two-dimensional chromatography. In order to obtain comprehensive two-dimensional information, the most commonly employed coupling is liquid chromatography (LC) with size-exclusion chromatography (SEC), abbreviated to LC×SEC [30]. Reducing the column length of the second dimension and increasing the flow rate would greatly decrease the run time in SEC analysis (Table 1). As a result, the total analysis time in LC×SEC would become shorter. In Table 2 commercial short columns are listed. A smaller i.d. implies a smaller column volume. The PL columns have volumes of 0.5 ml and 1.3 ml while the PSS column has a volume of 10 ml. Typically, in LC×SEC 100 fractions from the first dimension may be taken to characterize the sample. Because in SEC the volume of eluent required for one run is approximately equal to the column volume, the total volume required for a two-dimensional analysis assuming 100 fractions is 50 ml for the 4.6-mm i.d. column, 130 ml for 7.5-mm i.d. column, and 1000 ml when the 20-mm i.d. column is used. The smallest column is most attractive from the points of view of toxic waste and cost of analysis. Also, the flow rate of 10 ml/min associated with the 20-mm i.d. column is not compatible with many common SEC detectors.

In truly comprehensive two-dimensional liquid chromatography the maximum injection volume in the second dimension and the second dimension analysis time determine the maximum flow rate in the first-dimension (eq. 3) [31].

$$F_{max} = \frac{V_{m, max}}{t_R}$$  \hspace{1cm} (3)
where, $F_{\text{max}}$ is the maximum flow rate in the first dimension, expressed in $\mu l/min$, $V_{i,j,\text{max}}$ the maximum injection volume in the second dimension, expressed in $\mu l$ and $t_{R}$ is the retention time in the second dimension, expressed in min.

The maximum flow rate ($F_{\text{max}}$) in turn suggests a suitable internal diameter for the first-dimension column ($d_i$). The advantage of using a 20-mm i.d. column in the second dimension is that a conventional column can be used in the first dimension. Choosing a 4.6 mm or a 7.5-mm i.d. second-dimension column necessitates the use of a narrow-bore column in the first dimension. Alternatively, the effluent can be split after the first column, but this may impart quantitative applications of LC\times SEC. The effluent viscosity varies greatly when polymeric samples are eluted and this would inevitably cause variations in the split ratio.

Optimizing the column configuration, flow rate and injection volume in conjunction with the mobile phase conditions can lead to an enhanced resolution [32]. Ricker et al. have investigated the influence of the flow rate on the column efficiency and on the resolution using a Zorbax GF-250 column (250×9.4 mm i.d.). By decreasing the sample volume from 200 $\mu L$ to 2 $\mu L$ the resolution was improved from 0.7 to 1.3. The authors stressed that sample volume is the limiting factor in SEC and not sample concentration. However, above concentrations of roughly 5-10 mg/ml the sample becomes very viscous and then a loss in resolution can also be due to high concentrations.

The main objective in Fast SEC is to characterize the sample at the optimum experimental conditions representing the best compromise between speed and resolution. The efficiency in chromatography is commonly expressed in terms of the plate height.

$$h = H / d_p$$

$$v = ud_p / D_m$$

where $h$ is the reduced plate height, $v$ is the reduced velocity, $H$ is the plate height expressed in mm, $d_p$ represents the particle diameter (mm), $u$ is the interstitial velocity (mm/s) and $D_m$ is the diffusion coefficient of the sample, expressed in mm²/s.

Giddings [33] has introduced plots of the reduced plate height ($h$) versus the reduced velocity ($v$). The reduced plate height reflects all dispersion terms in the conventional van-
Deemter (eq. 4), *i.e.* eddy diffusion (*A* term), molecular diffusion (*B* term), and resistance to mass transfer (*C* term).

\[ h = A + B / \nu + C \nu \] (4)

Later, Knox and Giddings [34] have modified this by introducing a *D* term, which represents the combined effects of the *A* and *C* terms ('coupling term').

\[ h = \frac{B}{\nu} + D \nu^a \] (4a)

The difference between equations 4 and 4a is especially relevant at high reduced-velocities (see Figure 1a). These are encountered most of all in the case of fast separations (high *u*) of large molecules (low *D_m*).

---

**Figure 1:** Reduced-plate-height vs. reduced-velocity plot according to conventional theory and according to eq. 4a.
In Figure 1 the conventional plate-height curve (eq. 4) is compared to the plate-height curve obtained using eq. 4a. The value of the terms $B$ and $D$ are obtained from the best fit describing the experimental data (Figure 2). The results were reported elsewhere [1]. At lower reduced velocities the two plate-height curves, conventional and coupled, are almost identical (Figure 1a). At higher values of the reduced velocities (Figure 1b) the plate-height curve obtained from eq. 4a shows much lower values than the conventional eq. 4.

![Figure 2: Dimensionless plate-height curve plotted on a logarithmic scale for polystyrene standards (Table 3) on the PL-Gel-Mixed-C 50×7.5 mm i.d. column. See text for description of experimental data.](image)

In SEC the samples of interest are synthetic and natural macromolecules. The diffusion coefficients of polymers depend on their molar mass (MM) and on the solvent. For polystyrene in tetrahydrofuran (THF) at room temperature the diffusion coefficient is given by the following empirical equation [20]:

$$
D_m = 0.0386 \times (\text{MM})^{-0.57}
$$

(7)

where $D_m$ is in mm²/s and MM is in g/mol.

The diffusion coefficient is thought to have a large effect on the chromatographic dispersion of polymers. The plate height for high-MM polymers is thought to reach very large values indicating great losses in efficiency. Indeed, the main obstacle to fast polymer SEC is thought to be the slow diffusion of polymers. Another consequence of the slow diffusion is an increased risk of extra-column dispersion.
4.2.2. Integrity plots

A quantitative tool to investigate the potential of Fast SEC is provided by the concept of integrity plots [1]. Integrity plots portray the SEC resolving power as a function of the sample MM and polydispersity. In SEC we are aiming to evaluate the MMD and to measure the PDI value of the sample. As in all chromatographic techniques, the contributions to the band broadening arising from the column and from the auxiliary flow path must be kept minimal. In SEC the PDI contribution should be dominant.

Previously, [1] the practical SEC integrity index, $H_{SEC}$, was defined as:

$$ H_{SEC} = \frac{\sigma_{PD}}{\sqrt{\sigma_{PDI}^2 + \sigma_{col}^2 + \sigma_{extra-col}^2}} $$

(8)

where, $\sigma_{PDI}^2$ is the contribution of the sample PDI to the variance of the peak, while $\sigma_{col}^2$ and $\sigma_{extra-col}^2$ are the contributions of the column and extra-column dispersion to the variance of the peak, respectively. Two important cases can be recognized:

1) If $\sigma_{PDI}^2 > \sigma_{col}^2 + \sigma_{extra-col}^2$ (0.8 < $H_{SEC}$ ≤ 1), the SEC elution profile reflects the MMD and not the broadening due to the chromatographic dispersion. In ideal SEC, $H_{SEC}$ is equal to unity.

2) If $\sigma_{PDI}^2 < \sigma_{col}^2 + \sigma_{extra-col}^2$ (0 ≤ $H_{SEC}$ < 0.2), SEC cannot be used to measure the MMD. Only the location of the peak maximum, corresponding to the peak molar mass ($M_p$) is meaningful.

The SEC integrity index is defined such that it directly reflects variations in the width of the observed MMD. If $H_{SEC} = 1$ the observed chromatographic bandwidth can be converted without correction to the sample polydispersity. If $H_{SEC} = 0.9$ only 90% of the observed bandwidth is due to the polydispersity (and the calculated PDI will be approximately 20% higher than the true value) [1].

The manner used to calculate the SEC integrity index was described in detail elsewhere [1].

The three terms affecting the SEC resolving power are the PDI contribution, column dispersion, and extra-column band broadening.
4.3. Experimental

4.3.1. Chemicals and procedures

Five separation columns from Polymer Laboratories (Church Stretton, Shropshire, UK) were used, all packed with 5-μm Mixed-C stationary phase. were used. The reported data concern a 50×7.5 mm *i.d.* column, unless specified otherwise. Comparable results were obtained on a 50×4.6 mm *i.d.* column packed with the same material. The effects of column length and flow rate on resolving power in Fast SEC were studied by comparing the 50×4.6 mm *i.d.* column with 100×4.6 mm and 150×4.6 mm *i.d.* columns. The specified effective range of the Mixed-C stationary phase is from 200 to 2,000,000 Da. The Fast-SEC columns were compared with a PL-Gel column with dimensions 300×6.8 mm *i.d.* (5-μm particles; effective MM range: 500-60,000 Da). The samples studied were polystyrene standards (PS) obtained from various manufacturers (Table 3). Standard solutions were prepared in tetrahydrofuran (THF) at concentrations of 1 mg/ml.

<table>
<thead>
<tr>
<th>Molecular weight (Da)</th>
<th>Manufacturer</th>
<th>Polydispersity index</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,700</td>
<td>Polymer Laboratories</td>
<td>1.06</td>
</tr>
<tr>
<td>3,250</td>
<td>Polymer Laboratories</td>
<td>1.04</td>
</tr>
<tr>
<td>10,900</td>
<td>Polymer Standard Service</td>
<td>1.03</td>
</tr>
<tr>
<td>39,200</td>
<td>Polymer Standard Service</td>
<td>1.03</td>
</tr>
<tr>
<td>117,000</td>
<td>Polymer Standard Service</td>
<td>1.03</td>
</tr>
<tr>
<td>325,000</td>
<td>Polymer Standard Service</td>
<td>1.03</td>
</tr>
<tr>
<td>1,260,000</td>
<td>Polymer Standard Service</td>
<td>1.05</td>
</tr>
<tr>
<td>2,200,000</td>
<td>Macherey-Nagel</td>
<td>1.04</td>
</tr>
<tr>
<td>3,530,000</td>
<td>Polymer Standard Service</td>
<td>1.13</td>
</tr>
</tbody>
</table>

A Shimadzu (Kyoto, Japan) LC-10ADVP solvent-delivery module was used. The automated injection valve from VICI (Valco Instruments, Ontario, Ca) was equipped with a 0.5 μL loop. The analytes were detected with a UV detector, model 200 from Linear Instruments (Reno, Nevada, USA). The detector-cell volume and band broadening were miniaturized by
installing a fused-silica capillary (Polymicro Technologies, Phoenix, Arizona, USA) with an internal diameter of 250 μm. The UV detector was operated at a wavelength of 260 nm. The applied flow rate was varied from 0.3 ml/min to 0.5, 0.7, 1.0, 1.2, 1.4, 1.6, and 1.8 ml/min, until the pressure limit of the column was reached.

4.3.2. Simulation program

The polymeric sample is assumed to feature a log normal MMD, the standard deviation of which is adjusted to match the specified PDI. This distribution is divided in one hundred equidistant fractions between $M_p-3\sigma$ and $M_p+3\sigma$. The concentrations of the fractions are calculated to follow the Gaussian profile. Each fraction is then considered as an individual sample to which conventional chromatographic theory applies. The retention time is obtained from an experimental SEC calibration curve and the peak width from the empirical equation 4a, obtained based on actual experiments. The efficiency of the 50×7.5 mm i.d. column is presented in Figure 2. From the knowledge gained from Knox et al. [34] the reduced plate height was plotted versus the reduced velocity on a logarithmic scale. In this way the reduced plate heights obtained at high reduced-velocities can be examined without extrapolation. A line can be fitted through all experimental data, and thus is used to obtain the $D$ and $n$ coefficients that best describe the experimental results, as in eq. 4a. Thus, in this paper the following coefficients will be used: $B=1.50$; $D=1.12$ and $n=0.21$

$$h=\frac{1.50}{v} + 1.12\times v^{0.21}$$ (9)

The hundred peak profiles are then added to obtain the SEC envelope profile. In general, the polymer distribution covers a much broader range than 100 members of the polymeric series (individual masses). Thus, the fractions used in the calculation are ‘pseudo-oligomers’ and not real oligomers. If any resolution between the separate fractions (‘fingering’) is observed in the SEC profile, then this is usually an artifact, that can be removed by increasing the number of pseudo-oligomers (e.g. from 100 to 500) at the expense of larger computations times.
4.4. Results and discussion

4.4.1. Influence of the polydispersity on the peak shape in SEC - results of simulations

The influence of the PDI on the peak shape in SEC was investigated using the simulation program described above.

To investigate how much of the broadening of the peak is due to the chromatographic dispersion and how much to the sample polydispersity, several profiles were simulated, i.e. a monodisperse PS standard (PDI=1) and polydisperse standards (PDI > 1). The differences between the various profiles indicate to what extend the observed peak profile is due to the MMD of the sample. Peaks are simulated for a conventional (300×7.8 mm i.d., Figure 3) and a short (50×7.5 mm i.d., Figure 4) SEC column, both packed with Mixed-C-material.

![Figure 3: Simulated chromatograms for a standard of 117,000 Da on a 300×7.8 mm i.d column, flow rate 1.0 ml/min comparison between PDI=1.00 (---) and a) PDI=1.01 (—), b) PDI=1.03 (——), c) PDI=1.06 (-----) and PDI=1.2 (——).](image)

The flow rate is 1 ml/min and the sample of interest has an $M_p$ of 117,000 Da. The dashed profiles in figures 3 and 4 represent the corresponding monodisperse polymer (PDI=1). This signal reflects the chromatographic band broadening. Only in case of Figure 4a does...
the observed band broadening seem to be equal to the chromatographic band broadening. This is also approximately the case for Figures 3a and 4b. Thus, for very narrow standards (PDI=1.01) on short (50 mm long) or conventional (300 mm long) columns and for narrow standards (PDI=1.03) the chromatographic band broadening truly dominates only on the short column. In other cases the band broadening due to separation selectivity (PDI) plays a significant role. In broad samples (PDI ≥ 1.2, Figures 3d and 4d) the polydispersity contribution is dominant and SEC is an excellent tool for measuring MMDs.

![Simulated chromatograms for a standard of 117,000 Da on a 50x7.5 mm i.d column, flow rate 1.0 ml/min. Comparison between PDI=1.00 (----) and a) PDI=1.01 (---), b) PDI=1.03 (-----), c) PDI=1.06 (-----), d) PDI=1.2 (-----)](image)

Figure 4: Simulated chromatograms for a standard of 117,000 Da on a 50x7.5 mm i.d column, flow rate 1.0 ml/min. Comparison between PDI=1.00 (----) and a) PDI=1.01 (---), b) PDI=1.03 (-----), c) PDI=1.06 (-----), d) PDI=1.2 (-----)

In Figure 5 the simulated chromatogram is compared with the experimental results for the same mixture. The PDI values as specified by the manufacturers (see Table 3) are used in the simulation. In the experiment a flow rate of 0.3 ml/min was used. The similarity between the experimental and simulated figures is demonstrating the accuracy of the simulation program. From the knowledge gained in figures 3 and 4, we can conclude that the loss in resolution between the MM 1,700 Da (PDI=1.06) and MM 10,900 Da (PDI=1.03) peaks is due to sample polydispersity and thus reflects the properties of the sample.
4.4.2. Experimental results and discussion

4.4.2.1. Analysis time vs. resolution

For this experimental study two different mixtures were prepared. Because the results were equivalent, only one mixture is presented in this chapter. The polymer-standard mixture was injected on the Fast-SEC column at different flow rates, varying from 0.3 ml/min to 1.8 ml/min (Figure 6). 1.0 ml/min was found to be the apparent optimum flow rate, in agreement with the specification of the manufacturer of this column. At the lower flow rates (0.3 and 0.5 ml/min) the chromatograms show a good resolution. When increasing the flow rate, the analysis time becomes shorter, but resolution starts to be diminished. At a flow rate of 0.3 ml/min (Figure 6) the total analysis time is 5 min, decreasing to 3.1 min (at 0.5 ml/min), 2.1 min (at 0.7 ml/min), 1.5 min (at 1.0 ml/min), 1.3 min (at 1.2 ml/min), and finally 0.8 min (at 1.8 ml/min). At flow rates higher than 1.0 ml/min the resolution starts to
decrease more quickly. At 1.8 ml/min the gain in analysis time no longer seems to outweigh the loss in resolution.

![Flow rate 0.5 ml/min](image1)

![Flow rate 1.0 ml/min](image2)

![Flow rate 1.2 ml/min](image3)

![Flow rate 1.8 ml/min](image4)

Figure 6: Mixture of 1,700; 10,900; 117,000; 2,200,000 Da polystyrene standards analyzed on the 50x7.5 mm i.d. columns at different flow rates.

4.4.2.2. Calibration curves

To create integrity plots, calibration curves must be measured. We have found small, but systematic effects of the flow rate on the calibration curve, especially in the region of high MM (Figure 7a). In this range (Figure 7b) the slope of the calibration curve increases systematically with increasing flow rate. Therefore, the selectivity of the separation is decreasing somewhat at higher flow rates. As a consequence, the PDI contribution to the total peak width is also decreasing at higher flow rates. Tentatively the largest molecules, around the exclusion limit, may experience shear deformation. As a result, these analytes may be slightly retained (less excluded). It has been noted before [24, 35] that close to the exclusion limit of the column not only the SEC mechanism prevails, but also hydrodynamic chromatography (HDC) effects play a role.
4.4.2.3. Integrity plots

Integrity plots are evaluating the quality of SEC information obtained with a certain column at a chosen flow rate. They display the SEC integrity index, $H_{SEC}$ (eq. 8), as a function of the MM and the PDI of the sample. Using integrity plots it can be decide in which range of MM and PDI a given column at given conditions can provide reliable information on the MMD. First the influence of the flow rate on the performance of the Fast-SEC column is evaluated (Figure 8). Then, examples will be shown to illustrate the effects of the length of the column and the pore-size distribution of the packing material.
4.4.2.3.1. Influence of the flow rate on the integrity plot

Figure 8a shows the integrity plot of the 50×7.5 mm i.d. Fast-SEC column obtained at 0.5 ml/min. At 0.5 ml/min this particular column can be used to characterize the MMD of broad polymers (the right-hand side, Figure 8). If the sample of interest has a narrow distribution (PDI < 1.1), then another (longer) column must be used. For (ionizable) narrowly distributed polymers MALDI-MS offers a viable alternative [36]. The sample MM has little effect on these conclusions across the range from 10,000 to 1,000,000 Da. The column truly behaves as a ‘linear column’.

Figures 8 b, c, d, and e show the integrity plots at 1.0, 1.2, 1.4, and 1.6 ml/min. In general, the conclusions for experiments at 1.0 ml/min are identical to those drawn for experiments at 0.5 ml/min. Raising the flow rate to 1.0 ml/min does not significantly affect the performance of the SEC system. If the samples of interest have a PDI of about 1.1, then the highest flow rate at which reliable information on the MMD can be obtained is around 1 ml/min. This flow rate seems to offer the best compromise between speed of analysis and resolution. When the flow rate reaches 1.2 ml/min, a significant loss in resolution starts to be observed (Figure 8c). This continues further at higher flow rates (Figure 8 d, e). Samples of high MM may deform at such flow rates and different separation mechanisms may start to play a role. The calibration curves around the exclusion limit are shifting to the right, so that selectivity is lost (Figure 7-a and b). The integrity plots in figures 8c-e illustrate this effect. At the highest flow rates, the MMD of samples of relatively low MM can only be characterized on this particular column if their PDI is very large (e.g. at 1.6 ml/min PDI>1.5 for MM<10^4, see Figure 8c). At higher MM the situation is a bit more favorable (at 1.6 ml/min PDI>1.3 for MM ≈10^5 and PDI>1.2 for MM ≈10^6).

The variations in the flow rate affect most strongly the separation of samples with an MM lower than 40,000 Da.
Figure 8: Integrity plots for the Fast-SEC column of 50×7.5 mm i.d. at a) 0.5 ml/min, b) 1.0 ml/min, c) 1.2 ml/min d) 1.4 ml/min, e) 1.6 ml/min.
4.4.2.3.2. Effect of the pore-size distribution

In Figure 9 the SEC integrity index is plotted for a PLgel $10^3$ Å column at 0.7 ml/min. A different trend in the curvature of the integrity plots is observed compared to the plots obtained for the ‘mixed-bed’ columns. The shape of the curvature is dependent on the pore-size distribution. The calibration curve of this column shows an inflexion point around an MM of 10,000. This MM and this flow rate, we can use this column to characterize very narrowly distributed polymers, possible down to PDI=1.02. To measure reliable MMDs for low-PDI samples long columns with a narrow pore-size distribution are recommended.
4.4.2.4. Effect of length and flow rates

When increasing the flow rate the analysis times become shorter while the resolution diminishes. Earlier in this chapter, the approach of using short columns at high flow rates was studied. In Table I several approaches for increasing the separation speed are highlighted. The way to increase the resolution, while realizing fast separations is to double the length of the column and the flow rate. This is expected to result in an increased efficiency at constant analysis time.

Three separation columns were studied 50×4.6 mm i.d. 100×4.6 mm i.d. and 150×4.6 mm i.d. The same mixture as before (1,700, 10,900, 117,000, 1,260,000 Da) was injected in all columns at the corresponding flow rate (Table 1), 0.3 ml/min, 0.6 ml/min and 0.9 ml/min respectively. The results are shown in Figure 10.

Figure 9: Integrity plot for the PLgel 10^5 Å column at 0.7 ml/min.
Figure 10: The effect of concomitant changes in the column length and flow rate on the resolving power of Fast SEC-columns (a: 50×4.6 mm i.d. at 0.3 ml/min.; b: 100×4.6 mm i.d. at 0.6 ml/min; c: 150×4.6 mm i.d. at 0.9 ml/min.

From this result we can conclude that higher flow rates and longer columns result in better resolution. Similar results were obtained at 0.5 ml/min on a column 50×4.6 mm i.d. compared with 1.0 ml/min (100×4.6× mm i.d.) and 1.5 ml/min (150×4.6 mm i.d.).

The integrity plots of the 50×4.6 mm i.d. column (at 0.5 ml/min), compared to 100×4.6 mm i.d. (at 1.0 ml/min) and 150×4.6 mm i.d. (at 1.5 ml/min) show (as described in Table 1) that longer columns and higher flow rates yields more-efficient separations (Figure 11). Thus, the concomitant change of length and flow rate will enhance the capability of the column towards characterizing MMDs of narrower samples.
Figure 11: Integrity plots illustrating the combined effects of concomitant changes in the length and the higher flow rate on the resolving power of Fast SEC columns.
4.5. Conclusions

Fast SEC fills a need in combinatorial chemistry and high-throughput experimentation. Several approaches to change the speed of analysis have been presented highlighting the ones corresponding to the interest of this subject. Fast SEC is a very interesting modification of conventional SEC, due to its advantages in terms of the speed of analysis and the production of toxic waste. In this chapter it has been demonstrated that fast size-exclusion chromatography can be performed in practice. The main limitation remains the loss in resolution. A compromise must be found between the loss in resolution and the gain in speed. Fast SEC can only be used to measure the MMD of broadly distributed polymers. An increase in the length of the column combined with higher flow rates will improve the resolution. The main limitation is then the pressure drop across the column. Based on the observed peak widths for a series of standards and the SEC calibration curves, SEC integrity plots can easily be constructed for any kind of column or column configuration. The influence of the pore-size distribution, flow rate and column length have been discussed. These plots provide clear and objective information for the selection of suitable SEC columns and they can provide guidelines for a study of various types of SEC columns (e.g. miniaturized SEC, Fast SEC).

4.6. Acknowledgements

The author thanks Aschwin van der Horst for his technical support, Wim Decrop and Marcel van Engelen for their help in creating the integrity plots.

References


