Towards small and fast size-exclusion chromatography
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Chapter 6
Two-Dimensional Chromatography as a Tool for Studying Band Broadening in Size-Exclusion Chromatography

Abstract
Comprehensive two-dimensional Size-Exclusion Chromatography (SEC×SEC) has being realized. SEC×SEC is not a useful technique for characterizing complex polymers. However, it is shown to be an elegant tool to study the band broadening phenomena. The fractions collected from the first dimension are shown to be narrow, so that the band broadening in the second dimension is due only to chromatographic band broadening. This allows a clear distinction to be made between chromatographic dispersion (column and extra-column) and the SEC selectivity (the band broadening due to sample polydispersity).

6.1. Introduction
Polymers are complex chemical compounds, the molecules of which can vary in many different ways: molecular weight, branching, end-groups, functional groups, chemical composition, block length, tacticity, etc. Any of these properties can be characterized by a distribution. When all monomers forming the macromolecule, as well as the end-groups, are identical, the homopolymer can be characterized by only a molar-mass distribution (MMD). When more than one type of repetitive unit is used to create a copolymer then also a chemical-composition distribution (CCD) exists. When functional groups are present on the polymer, either as end-groups or along the backbone, a functionality-type distribution (FTD) will be present. Some other possible distributions are the degree-of-branching distribution (DBD), block-length distribution (BLD), and tacticity distribution (TcD). In order to establish relationships between the molecular structure and the material properties of polymers, we need to obtain information about the relevant distributions. Different distributions can be independent or mutually dependent. For example, if a copolymer has the same chemical-composition distribution for any molar-mass fraction (and the same molar-mass distribution for any chemical-composition fraction), then the
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CCD and the MMD are independent. If, however, the high-MM part of the copolymer exhibits a CCD different from that of the low-MM part, the MMD and CCD are mutually dependent. In general, we may not assume that the different distributions featured by complex polymers are independent.

In order to characterize a distribution, we must be able to separate the polymer according to the specific molecular property (mass or size, chemical composition, functionality, etc.). For any molar-mass distribution the standard deviation of the chromatographic peak is given by \( \sigma = M_n \sqrt{\text{PDI} - 1} \). [1], where PDI is the polydispersity (PDI=\( M_n/M_w \)), \( M_n \) the number-average molecular weight and \( M_w \) is the weight-average molecular weight. Without a separation, only information on specific averages can be obtained. For example, \( M_n \) may be obtained from colligative properties (e.g. osmotic pressure) or from quantitative end-group analysis (by titration, NMR, etc.). \( M_n \) may be obtained from light-scattering experiments. However, it is difficult to determine both \( M_n \) and \( M_w \) accurately for any one polymer. A separation method, such as size-exclusion chromatography (SEC), yields the complete MMD and all characteristic averages and indices much-more rapidly and much-more conveniently.

If more than one distribution exists, more than one separation will be needed. In order to obtain information on the different distributions, these separations should follow different mechanisms, corresponding to the relevant properties of the molecules [2]. To characterize two (or multiple) mutually dependent distributions, two-dimensional (or multi-dimensional) separations are required. When the sample of interest features, for example, an MMD and a CCD then we need two-dimensional separations in which one mechanism distinguishes molecules according to differences in molar mass and the second mechanism according to differences in chemical composition. In this chapter we apply comprehensive two-dimensional SEC (SEC×SEC) not to measure two different distributions, but to study band-broadening phenomena.

6.2. Theory

Size-exclusion chromatography (SEC) separates the analytes according to their hydrodynamic volumes. It is the outstanding technique to measure the MMD of natural and synthetic polymers. It can be coupled to a variety of detectors to yield information on the molecular-size distribution (MSD). UV detectors [3,4,5,6], refractive-index detectors (RI)
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[3,7], and evaporative light-scattering detectors (ELSD) [7] are commonly used for this purpose. In addition, SEC can yield information on the intrinsic viscosity [3,8] and the degree of branching by coupling it to viscometric (Vis) or light-scattering detectors (LS) [9,10,11]. SEC can also be combined (on-line or off-line) with NMR or IR spectroscopy or with mass spectrometry (MS) to yield information on the (average) chemical composition and functionality in each fraction of the effluent. In the present context, some combinations are especially relevant, as they yield information on the molecular size or weight of the polymers in the SEC effluent (see Table 1).

Table 1: Techniques that may be coupled to SEC to yield information on the molecular size or weight of polymers in the effluent.

<table>
<thead>
<tr>
<th>Technique coupled to SEC</th>
<th>Information obtained</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Viscometry</td>
<td>Intrinsic viscosity</td>
<td>MM &gt; ca. 5,000</td>
</tr>
<tr>
<td></td>
<td>Viscosity-average</td>
<td>Requires calibration with Mark-</td>
</tr>
<tr>
<td></td>
<td>molecular weight ($M_c$)</td>
<td>Houwink constants</td>
</tr>
<tr>
<td>Light scattering</td>
<td>Weight-average molecular</td>
<td>MM &gt; ca. 10,000;</td>
</tr>
<tr>
<td></td>
<td>weight ($M_w$)</td>
<td>Calibration with RI increment (dn/dc)</td>
</tr>
<tr>
<td></td>
<td>Root-mean-square radius</td>
<td>MM &gt; ca. 50,000</td>
</tr>
<tr>
<td>Mass spectrometry</td>
<td>Complete (accurate) MMD</td>
<td>If fraction is narrow in terms of molecular weight and composition and functionality</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Applicable to ionizable (sufficiently polar) polymers</td>
</tr>
<tr>
<td>SEC</td>
<td>Complete (precise) MMD</td>
<td>Applicable to all soluble polymers across a very broad MM range</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Same mechanisms help avoid confounding effects (due to, e.g., branching)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Information on dispersion of narrow fractions limited by chromatographic band broadening</td>
</tr>
</tbody>
</table>
One of the objectives of the present chapter is to study the origin and quality of separation in SEC. All the techniques listed in Table 1 allow us to decide whether the SEC elution profile is dominated by selective separations (molecular weight, size, and intrinsic viscosity decreasing with time) or by chromatographic band broadening (average molecular properties invariable with time). Viscometry and light-scattering work best for relatively large (high-MM) polymers. In combination with SEC the most suitable and versatile ionization technique for mass spectrometry (MS) is matrix-assisted laser desorption/ionization (MALDI) [12,13,14]. MALDI is usually performed off-line and it works well on narrowly distributed polymers. Its combination with SEC is very attractive, at least in case where only the MMD (and not, for example, an FTD and/or CCD) is relevant. MALDI is only applicable to ionizable (i.e. sufficiently polar) polymers. SEC, as a second-dimension separation technique, is (by default) applicable to all polymers that have been separated by SEC as a first-dimension separation technique. Also, by exploiting the same mechanism, confounding effects can be avoided. For example, if branching occurs in the sample, then molecules of equal hydrodynamic volume, but with different molecular weights would co-elute from the first dimension SEC column. In MALDI this would (correctly) result in a broadening of the MMD, but this result does not properly reflect the performance (separation efficiency) of the first dimension SEC column. SEC does, in principle, yield precise information on the MMD. Accurate ("absolute") data can be obtained after proper calibration. However, for very narrow fractions SEC only yields information on the most-common (peak) molecular weight ($M_p$) [32].

In order to get accurate information from SEC on the sample of interest, it is very important to distinguish between the different contributions to the observed peak profile. In polymer analysis we want the peak profile to accurately reflect the MMD of the sample. However, this information can be confounded with column- and extra-column-dispersion contributions. Thus, the contribution from the sample polydispersity should be maximized, while dispersion contributions need to be minimized. In terms of peak variances we can write

$$
\sigma^2_{\text{observed}} = \sigma^2_{\text{extra-col}} + \sigma^2_{\text{col}} + \sigma^2_{\text{PDI}}
$$

(1)

where $\sigma_{\text{observed}}$ is the observed peak-width, $\sigma_{\text{extra-col}}$ and $\sigma_{\text{col}}$ are the contributions due to extra-column and column band broadening, and $\sigma_{\text{PDI}}$ represents the peak variance due to the polydispersity of the sample.
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Various researchers have made efforts to distinguish between the different contributions in eq. 1. Dawkins and Yeadon [15] studied the band broadening in SEC for monodisperse proteins, but were not able to correlate the data obtained for monodisperse proteins to those for polydisperse polystyrene samples. Busnel et al. [4] used very narrow polydisperse standards (PDI<1.01) to characterize the band broadening in SEC, but the contribution to the total peak dispersion was not evaluated. In 1977 Knox and McLennan proposed a theory to predict the contribution of the sample PDI to the total band broadening [16]. To apply this theory, knowledge of the exact value of the PDI is essential. Manufacturers specify values for commercial standards, which are usually upper limits. Several research groups have demonstrated that these specifications overestimate the true PDI values [9,10,17]. Other authors [9,10,18] have claimed that the PDI obtained by SEC is also an upper limit, because it is significantly affected by band-broadening phenomena.

Temperature-gradient-interaction chromatography (TGIC) was found to give much narrower peaks than SEC [9,18]. This leads to lower PDI values, closer to the values predicted to result from anionic polymerization under ideal conditions (Poisson statistics). However, to evaluate the band-broadening contributions separately, knowing the exact value of the PDI is essential. Vander Heyden et al. [5] calculated the maximum PDI value in case \( \sigma_{PDI} \) dominates the chromatographic band-width. These values were found to be somewhat smaller than those specified by the manufacturers and larger than the minimum values estimated from the Poisson distribution. An intermediate PDI value was obtained from MALDI-Time-of-Flight (ToF)-MS experiments. MALDI-ToF-MS spectra are directly indicative for the molecular-weight distribution of the polymer. Unfortunately this technique has its limitations when applied to polymers. It requires chemical homogeneity and some polarity. It works best for polymers with a relatively low molar-mass (MM) [13] and it requires a low sample polydispersity (PDI<1.2 or preferably lower) [14,19].

Experimental results from previous work [20] indicated that in SEC of narrow polymer standards band broadening is dominated by chromatographic dispersion. This implies that \( \sigma^2_{PDI} < \sigma^2_{\text{extra-col}} + \sigma^2_{\text{col}} \). Since extra-column band broadening was found to account for about 10% from the total dispersion, it was concluded that column dispersion dominates the observed bandwidth.
The concept of the SEC integrity index ($II_{SEC}$) was introduced to quantitatively assess the performance of a SEC system.

$$II_{SEC} = \frac{\sigma_{PDI}}{\sqrt{\sigma^2_{PDI} + \sigma^2_{col} + \sigma^2_{extra-col}}} \quad (2)$$

As indicated by eq. 2, $II_{SEC}$ compares the dispersion arising from the PDI to the total band broadening. $II_{SEC}$ approaches a maximum value of unity, when $\sigma^2_{PDI} >> \sigma^2_{extra-col} + \sigma^2_{col}$, i.e. when the chromatographic system performs very well, and a minimum value of zero, when $\sigma^2_{PDI} < \sigma^2_{extra-col} + \sigma^2_{col}$. i.e. when the chromatographic band broadening is dominating the separation. In reference [20], $II_{SEC}$ was plotted (for specific columns under specific conditions) as a function of the sample PDI and MM. From such figures (called integrity plots) quantitative information can be obtained on the performance of SEC systems for samples with different MM and PDI values. These plots can be applied to any type of column at different experimental conditions (e.g. flow rates), facilitating a judicious choice of the most-important experimental SEC parameters.

In its early ages, two-dimensional chromatography was carried out in an off-line mode. Fractions collected from the first dimension were re-injected in the second dimension [21,22,23]. Disadvantages with respect to the analytical repeatability included contamination of the fractions and losses or degradation during solvent evaporation. To overcome these problems and to make the method less laborious, on-line two-dimensional chromatography was introduced [24,25]. Successive fractions from the first dimension were injected directly in the second dimension, with the help of an automated switching valve [26,27,28]. In this way no sample goes to waste, allowing quantitative information to be obtained. Taking a large number of fractions from the first dimension enhances the resolving power of the technique.

In order to gain information on the sample from two-dimensional separations, different retention mechanisms should be used in the two dimensions. When coupling liquid chromatography (LC) to LC, numerous separation mechanisms can be exploited. When an MMD and a CCD are required, one dimension can be ‘interactive’ LC (separating according to chemical composition) and the other one SEC (separating according to hydrodynamic volume). The most commonly employed coupling is LC followed by SEC, abbreviated as LC×SEC [29], which is more attractive than SEC×LC for a number of reasons [30].
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The first dimension must be a slow separation, in order to allow several fractions to be collected per first-dimension peak. If we are to transfer all of the first-dimension eluent to the second-dimension column, narrow-bore columns are preferred. Low first-dimension flow rates may reduce the band broadening in the second dimension [31]. The second dimension is always a fast separation, while the efficiency should be as high as possible. Some important rules in two-dimensional chromatography are the following.

1) The maximum injection volume in the second dimension and the second dimension analysis time determine the maximum flow rate in the first dimension

$$1F_{\text{max}} = \frac{2V_{\text{inj,max}}}{2t_R}$$ (3)

Where $1F_{\text{max}}$ is the maximum flow rate in the first dimension, $2V_{\text{inj,max}}$ is the maximum injection volume in the second dimension and $2t_R$ is the retention time in the second dimension.

2) The total analysis time is determined by the number of first-dimension fractions (directly affecting the resolution) and the second-dimension analysis time.

When studying band broadening in SEC, the exact value of the polydispersity of a standard is usually not known. SEC×SEC allows us to reduce the PDI of the sample injected in the first dimension and to obtain a large number of narrow fractions that are injected into the second dimension. We will demonstrate that SEC×SEC is an elegant tool to study band broadening in SEC.

6.3. Experimental

6.3.1. Instrumentation for two-dimensional chromatography

The experiments were carried out using a Shimadzu LC-10ADVP solvent-delivery pump (Shimadzu, 's Hertogenbosch, The Netherlands) at a flow rate of 10 μl/min (for a 250×4.6 mm i.d. column) or 4 μl/min (for 250×1.0 mm i.d.) for the first dimension ($1D$) separation. A Rheodyne (Berkeley, CA, USA) two-position six-port valve, equipped with a 2-μl loop, was used as injection valve for the $1D$ separation. The second-dimension ($2D$) SEC separation system consisted of a Kratos Spectroflow 400 pump (ABI, Ramsey, NJ, USA) equipped with either a 50×4.6 mm i.d. or a 150×4.6 mm i.d column. All columns
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were from Polymer Laboratories (Church Stretton, Shropshire, UK) and packed with Mixed-C material. The detector used in the second dimension was a UV spectrometer (Kratos Spectroflow 757, ABI, Ramsey, NY, USA), used at a wavelength of 260 nm. The flow rate in the second dimension was 0.6 ml/min (for the 50×4.6 mm i.d. column) or 1.5 ml/min (for the 150×4.6 mm i.d. column). To obtain a comprehensive SEC×SEC set-up, the two SEC dimensions were coupled by an air-actuated VICI two-position ten-port injection valve (Valco, Schenken, Switzerland). This valve was operated using a high-speed switching accessory (switching-time of 20 ms using 5 bar N₂) and dual injection loops of equal volume (10 μl). Data were collected using a Keithley KNM-DCV 12 Smartlink interface (Cleveland, OH, USA). An in-house program written in a Matlab (Natick, MA, USA) was used to control the valve switching and data acquisition and to perform calculations on the data.

6.3.2. Chemicals

Tetrahydrofuran (THF) from Biosolve (Valkenswaard, The Netherlands) was used as mobile phase for both dimensions. Polystyrene standard solutions (1 mg/ml in THF) with different molar masses and PDI (Table 1), obtained from Pressure Chemicals (Pittsburgh, PA, USA) and Polymer Laboratories (Church Stretton, Shropshire, UK), were used for calibration and a broad PS (MM=271,000 and PDI=2.1) manufactured by BDH Chemicals (Poole, UK) was used as sample. HPLC-grade toluene from Rathburn Chemicals (Walkeburn, Scotland) was used as marker for the total permeation limit.

<table>
<thead>
<tr>
<th>MM (Da)</th>
<th>Manufacturer</th>
<th>PDI</th>
</tr>
</thead>
<tbody>
<tr>
<td>3,250</td>
<td>Polymer laboratories</td>
<td>1.03</td>
</tr>
<tr>
<td>39,200</td>
<td>Polymer laboratories</td>
<td>1.03</td>
</tr>
<tr>
<td>411,000</td>
<td>Polymer standard service</td>
<td>1.03</td>
</tr>
<tr>
<td>3,530,000</td>
<td>Polymer standard service</td>
<td>1.13</td>
</tr>
<tr>
<td>271,000</td>
<td>BDH Chemicals</td>
<td>2.1</td>
</tr>
</tbody>
</table>
6.4. Results and discussion

From the knowledge gained previously [32] it was concluded that longer SEC column operated at higher flow rates offered an improved resolution in Fast SEC. Therefore, the 150×4.6 mm *i.d.* column operated at 1.5 ml/min was preferred in the second dimension to the 50×4.6 mm *i.d.* column. Since truly monodisperse polymer samples are not available for studying SEC, the first dimension was used for sample-preparation purposes. A low flow rate and a relatively long column were used in the first dimension to obtain very narrow fractions. Therefore, the effect of the PDI on the observed peak width in the (relatively inefficient) second dimension could be neglected.

Figure 1 shows the one-dimensional projection of the entire series of SEC elution profiles obtained for a broad standard (271,000 Da, PDI=2.1). All the peaks present in the Figure represent fractions that were collected from the first dimension and re-injected in the second dimension. Clearly, large amounts of data are obtained from a single SEC×SEC experiment. Each fraction has a low polydispersity, so that only the contributions of the column and extra-column band broadening are relevant in the second dimension.

![Figure 1a](image)

*Figure 1a*: One-dimensional projection of the second-dimension SEC profiles obtained for a broad PS standard (271,000 Da, PDI=2.1). Experimental conditions PL columns packed with Mixed-C material and 250×4.6 mm *i.d.* at 10 µl/min (first dimension) and 150×4.6 mm *i.d.* at 1.5 ml/min (second dimension).
Two-dimensional contour plots provide a much clearer representation of the data. They were calculated with the Matlab software. Figure 1b shows the same data as Figure 1a in such a format. The width of the overall profile in the first dimension (horizontal direction) is seen to be much greater than the width of the profile at any point in the vertical direction. The concomitant increase of $^1t_R$ and $^2t_R$ indicates that the largest polymers elute first in the first-dimension separation, and that this separation is dominated by SEC selectivity.

To further clarify the kinds of effects observed in Figure 1b, the contour plot of a mixture of four polystyrene standards (3,250, 39,200, 411,000, and 3,250,000 Da) is shown in Figure 2. The individual second dimension chromatograms were scaled and an alignment was performed, using the maximum values for the toluene peak-top used as permeation marker. The number of fractions per contour plot can be counted. Exact switching times were recorded for accurate “slicing” (obtaining vertical segments in the SEC×SEC chromatogram from the continuous detector trace). This is demonstrated in Figure 3, which shows a close up of one of the peaks and the effects of the software corrections. Figures 2 and 3a show about ten cuts across each peak. The Matlab program allows us to extract first- and second-dimension chromatograms at any position of the SEC×SEC contour plots.
The positions of the four peaks were determined by the calibration curves in the two dimensions. Because we used the same packing material (PL-Gel Mixed-C) in both dimensions, the peaks should all appear on a straight line. The slope of this line is determined by the column dimensions, flow rates, and scaling factors for the plot. A 45° diagonal will be obtained if we plot dimensionless retention times, \((\frac{t_R - t_{co}}{t_{perm} - t_{co}})\), on both axis \((i = 1 \text{ or } 2)\). If the same material is used in both columns and if extra-column resistance times can be neglected, a similar result can be achieved by plotting relative retention times \((\frac{t_R}{t_{perm}})\). The top three peaks (first three contours from right to left) are eluted in the linear range of the Mixed-C stationary phase. The highest-MM peak (3,250,000 Da) is almost completely excluded. Therefore, it appears sharper, especially on the bottom and left sides.

Most peaks appear as horizontal or vertical ellipses. Narrower peaks are usually obtained from the first dimension, due to the higher chromatographic efficiency. This implies that the peak widths in the first (and second) dimensions are completely dominated by the chromatographic band broadening. Only the peak for the lowest-MM standard (MM 3,250) seems to be slightly slanted, which indicates a non-negligible contribution from the
standard polydispersity on the $^1$D peak width. This last peak could also have been studied by (off-line) SEC//MALDI-ToF-MS. The higher-MW peaks could also have been characterized (on-line) by SEC-LS or SEC-Vis. However, only SEC×SEC allowed the study of the mechanisms underlying the band broadening across the entire range from a (few) thousand to a few million Da.

![Contour plots](image)

**Figure 3:** Enlarged contour plot of 39,200 Da PS obtained by SEC×SEC (Figure 2). (a) observed raw data (b) contour plot after software corrections. Experimental conditions as in Figure 2.

The effect of the PDI on the shape of the contour plot is obvious from comparing Figure 1b with Figures 2 and 3. The contours for the broad standard (Figure 1) are slanted, indicating that the SEC separation in the first dimension (x-axes) is dominated by the PDI of the sample. In terms of peak widths (eq. 1) the contribution of the PDI is illustrated in Figure 4. The top two figures (a and d) correspond to the narrow standard. The chromatograms on the right (b, d) show the $^1$D (continuous line) projected SEC chromatogram and a $^2$D cut
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through the of the peak (dashed line). The difference between the two profiles is indicative for the difference in plate counts between the two columns. The two bottom figures (c, d) correspond to the broad reference material. The $^1$D projection (d – continuous line) is much broader than the $^2$D cut (d-dashed line), indicating the difference in polydispersity between the broad standard and the narrow fraction.

![Two-Dimensional SEC×SEC chromatogram of (a) a narrow PS standard (MM=39,200 Da and PDI=1.03) and (c) a broad PS standard (MM=271,000 Da and PDI=2.1). The one-dimensional chromatograms show the $^1$D projection and a the highest $^2$D cut for (b) the narrow and (d) the broad standard.](image)

Figure 4: Two-dimensional SEC×SEC chromatograms of (a) a narrow PS standard (MM=39,200 Da and PDI=1.03) and (c) a broad PS standard (MM=271,000 Da and PDI=2.1). The one-dimensional chromatograms show the $^1$D projection and a the highest $^2$D cut for (b) the narrow and (d) the broad standard.

Table 2 lists the variances corresponding to the two profiles on the right-hand-side of Figure 4 (Figure 4 b, d). The difference between $^1$N and $^2$N (Figure 4b) is largely due to the difference in column length and flow rate. Although flow rates, we have found [20,32] that $H$ is proportional to $u_0^{0.21}$, when $u_0$ is the linear (interparticle) velocity. Therefore we can expect

$$\frac{^1N}{^2N} = \frac{^1L}{^2L} \times \frac{^2H}{^1H} = \frac{^1L}{^2L} \left(\frac{^2u_0}{^1u_0}\right)^{0.21} = \frac{250}{150} \left(\frac{1500}{10}\right)^{0.21} \approx 5 \quad (4)$$

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This is in good agreement with the ratio of about 6 observed in Table 2.

<table>
<thead>
<tr>
<th></th>
<th>$^1D$ (reconstructed chromatogram)</th>
<th>$^2D$ (highest peak)</th>
<th>$^3D$ (projection)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Narrow standard</td>
<td>3600</td>
<td>610</td>
<td>640</td>
</tr>
<tr>
<td>39,200 Da</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Broad standard</td>
<td>170</td>
<td>670</td>
<td>140</td>
</tr>
<tr>
<td>271,000 Da</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a sum of all $^3D$ peaks. Not shown in Figure 4.

The number of plates corresponding to the projection of $^2D$ chromatograms on the vertical axis is approximately equal to the number of plates observed for the highest second-dimension peak. This confirms that the narrow standard is not fractionated further, in other words that the first-dimension separation reconstructed chromatogram for the broad standard shows a much lower apparent number of theoretical plates ($^1N_{app}=170$), demonstrating that the peak width is mainly determined by the sample polydispersity. Likewise, the second-dimension projection of the entire profile is very broad ($^2N_{prof}=140$). However, the peak count corresponding to an individual factor is similar to that observed for a fraction of the narrow standard ($^3N=670$). Thus, the fractions collected from the first dimension can be used to study the chromatographic band-broadening in the second dimension.

Finally, Figure 5 provides an overlay of the two-dimensional contour plots of the narrow and broad PS standards. The concept of slanted (broad) and vertical (narrow) contours is clearly illustrated. The polydispersity of the broad 271,000 Da standard causes this peak to elute in the first dimension from well before the narrow 411,000 Da standard to around the 39,200 Da standard. In the second dimension the cuts obtained from the narrow and broad standards are about equally broad, again indicating that the polydispersity of the fractions obtained from the first-dimension separation is similar to that of the narrow standards.
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Figure 5: Overlay of contour plots of narrow (from left to right 3,250,000; 411,000; 39,200; 3,250 Da) and broad (271,000 Da,) PS standards. Experimental conditions as in Figure 1.

SEC×SEC can also be used to diagnose the proper functioning of SEC columns and instruments. This is illustrated in Figure 6, which shows a SEC×SEC contour plot with a poor chromatographic efficiency in the first dimension. The number of fractions collected is much smaller than in previous pictures (about five cuts for each broad first-dimension peak). Therefore, strings of dots are found for each peak. The broad peaks that are obtained in the first dimension are not due to sample polydispersity, but to chromatographic band broadening, as is evident from the fact that the dots for each peak appear at the same vertical position in the SEC×SEC chromatogram.
6.5. Conclusions

Comprehensive two-dimensional size-exclusion chromatography, SEC×SEC is an elegant tool to evaluate the origin of the observed band broadening in SEC. The first dimension is used for fractionation purposes, reducing the polydispersity (PDI) of broad standards to fractions with PDI values similar to those of narrow, commercial SEC standards. These fractions are subsequently analyzed in the second dimension.

Other approaches, such as on-line coupling with viscometric or light-scattering detectors or off-line coupling with mass spectrometry also allows the band broadening in SEC to be studied. However, SEC×SEC can be used across the entire MM range of SEC (from about 1,000-5,000,000 Da), whereas other techniques apply across more-limited ranges. Also, confounding effects (e.g. due to the presence of branched molecules in the sample) are avoided in SEC×SEC. A distinct disadvantage of SEC (and SEC×SEC) is its limited resolution.

It is demonstrated in this chapter how a clear distinction can be made between chromatographic (column and extra-column) band broadening and SEC selectivity (band
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broadening due to sample polydispersity). In the studied systems (using a single 250-mm column in the first dimension) the observed bandwidths for narrow PS standards were clearly dominated by chromatographic dispersion. This vindicates assumptions made elsewhere in this thesis (chapters 1,2,4), for constructing SEC integrity plots.

Acknowledgement

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