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Band broadening in size-exclusion chromatography of polydisperse samples

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Abstract

Understanding and controlling the band broadening is essential to obtain accurate molar-mass distributions by size-exclusion chromatography (SEC). In this paper, band broadening in SEC is reviewed from a contemporary perspective. The observed band broadening is due to dispersion inside and outside the chromatographic column (undesirable band broadening) and to the polydispersity of the sample (desirable SEC selectivity). The various contributors to band broadening are discussed. Integrity plots are introduced as a tool to evaluate the performance of specific SEC columns at given experimental conditions. For narrow polymer standards on single SEC columns the observed peak width is dominated by the chromatographic dispersion. MALDI-ToF-MS is demonstrated as an alternative to determine the PDI of narrowly distributed samples. The plate heights encountered at very high reduced velocities are found to be lower than expected. This is advantageous for fast separations by SEC.

Keywords: Band broadening; Polydispersity; Size-exclusion chromatography

1. Introduction

Size-exclusion chromatography (SEC) is a mature form of liquid chromatography (LC). In 1979, the book *Modern Size-Exclusion Liquid Chromatography*, by Yau et al. [1] appeared. Twenty-five years later, this is still an eminently useful and astonishingly up-to-date treatise of the field. However, it is inevitable that size-exclusion chromatography has changed in a number of ways, viscometric [2] and light-scattering detectors [3] have become much more prominent [4]. Lately SEC has been coupled on-line and off-line with contemporary mass-spectrometric techniques, such as electrospray (ESI) [5] and matrix-assisted laser-desorption ionization (MALDI) [6,7]. Especially the (off-line) coupling with MALDI is expected to have a great impact on the practice of SEC [8]. Unlike other forms of LC, miniaturization has attracted only marginal interest [9]. In contrast, fast separations by SEC have drawn a great deal of interest in the last few years [10–12]. A number of important aspects associated with the trends towards small (miniaturized) and Fast SEC are summarized in Table 1.

Band broadening is a very important topic within any chromatographic technique. The provenance of the chromatographic bandwidth and the peak shape in SEC are, however, different from those in other forms of chromatography. Although the application of SEC to monodisperse analytes, such as proteins, is certainly not unimportant, the technique is most commonly applied to polydisperse samples. The discussion in this paper will be limited to the latter kind of samples. Literally, a polydisperse sample contains many different kinds of molecules. The individual molecules in a sample of a synthetic polymer can vary in many ways: molecular weight, branching, end groups and functional groups, chemical composition, block length, stereo-regularity (tacticity), etc. Any of these properties can be characterized by a distribution. Although not all distributions are relevant for all polymers (e.g. chemical composition and block length are relevant for copolymers, but not for homopolymers), it is clear that synthetic polymers consist of very complex mixtures of molecules.

SEC is mainly concerned with the determination of molecular-weight distributions (MWD) or, equivalently, molar-mass distributions (MMD). In combination with viscometric or light-scattering detection SEC can also be used for characterizing degree-of-branching distributions (DBD).

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Table 1
Effect of a decrease in the column length or the column diameter on various parameters in SEC

(a) Effect of decreasing → on ↓	The column length[a] (L) (Fast SEC)	The column diameter[b] (d_c) (micro-SEC)
The analysis time	Decreasing ($\div L$)	Not affected[a]
The retention volume	Decreasing ($\div L$)	Decreasing ($\div d_c^2$)
Elution window (time units)	Decreasing ($\div L$)	Not affected
Elution window (volume units)	Decreasing ($\div L$)	Decreasing ($\div d_c^2$)
Volume of eluent required	Decreasing ($\div L$)	Decreasing ($\div d_c^2$)
Permissible extra-column volume	Decreasing ($\div L$)	Decreasing ($\div d_c^2$)
Chromatographic resolution	Decreasing ($\div L$)	not affected
Sensitivity (peak height)	Increasing ($\div\sqrt{L}$)	Increasing ($\div d_c^2$) in case of constant injected amount Not affected in case of constant column loading
Detector compatibility	Not affected[c]	Better: MS Worse: RI, viscometry, light scattering
(b) Effect of decreasing → on ↓	The column length (L) (Fast SEC)	The column diameter (d_c) (micro-SEC)
σ_{column}	Decreases ($\div \sqrt{L}$)	Decreases ($\div d_c^2$)
σ_{PDI}	Decreases ($\div L$)	Decreases ($\div d_c^2$)
$\sigma^2_{extra\text{-}column}$	Not affected	Not affected
$\sigma^2_{column}/\sigma^2_{extra\text{-}column}$	Decreases ($\div L$)	Decreases ($\div d_c^4$)
Chromatographic-integrity index (II_{Chrom}), Eq. (13)	Decreases	Decreases (possibly strongly)
$\sigma^2_{PDI}/\sigma^2_{column}$	Decreases ($\div L$)	Not affected
Theoretical SEC-integrity index ($^{th}II_{SEC}$, Eq. (14))	Decreases	Not affected
Practical SEC-integrity index ($^{exp}II_{SEC}$, Eq. (15))	Decreases	Decreases

[a] The effect of increasing the flow rate is similar to that of decreasing the column length.
[b] The flow rate is supposed to decrease with decreasing column diameter ($F \div d_c^2$), so as to keep the linear velocity constant.
[c] If (very) high flow rates are used in Fast SEC, then this will complicate the use of various detectors (especially MS and viscometry).

In combination with other separation techniques SEC is of increasing importance for determining other, more-complex distributions. An important example is the combination of ("interactive") liquid chromatography and SEC in comprehensive two-dimensional liquid chromatography (LC × SEC) [13]. This allows the characterization of two mutually dependent distributions simultaneously. One way to describe these is as comprehensive two-dimensional distributions, representing, for example, functionality type and molecular weight (FTD × MMD) or chemical composition and molecular weight (CCD × MMD).

A distribution of a property of the molecules of a synthetic polymer can be described as a plot of the number of molecules or the weight fraction of the sample versus the value of the property. Although the complete picture is needed to fully characterize the distribution, polymer chemists usually work with characteristic averages. For example, for the molecular-weight distribution these are defined in reference 1. A key role is played by the polydispersity index (PDI or D), which is defined as the ratio of the weight-average molecular weight (M_w) and the number-average molecular weight (M_n): PDI $= M_w/M_n$. This ratio, which is equal to 1 for monodisperse samples and always greater than unity for polydisperse samples, is indicative for the width of the molecular-weight distribution and, therefore, for the polydispersity of the sample. For a monodisperse sample PDI $= 1$. For narrowly distributed polymers (standards) it is typically around 1.05 and for broadly distributed synthetic polymers the PDI can easily exceed a value of 2.

It is relevant in the context of the present paper to indicate the direct relation between the PDI value and the standard deviation of a distribution [14], i.e.

$$\sigma = M_n\sqrt{\mathrm{PDI} - 1} \tag{1}$$

This equation implies that a polydispersity of 1.05 corresponds to a relative standard deviation (i.e. relative to M_n) of more than 20%. If this were a peak in a chromatogram, then the equivalent number of plates would be

$$N_{pol} = \left(\frac{M_n}{\sigma}\right)^2 = \frac{1}{\mathrm{PDI} - 1} \tag{2}$$

For a narrow polymer standard with PDI $= 1.05$, we find $N_{pol} = 20$. Thus, what is perceived as "narrow" by polymer chemists is awfully broad from the perspective of a chromatographer.

In SEC of polydisperse samples band broadening is an ambivalent issue. In this paper different contributions to band broadening are discussed, viz. chromatographic dispersion, extra-column dispersion, and chromatographic selectivity. A clear distinction must be made between them. The former two contributions are undesirable, whereas the latter is a desirable effect. In this paper the three individual contributions are studied separately as much as possible. However, for synthetic polymers they cannot be measured independently, because monodisperse samples do not exist. Previously, Dawkins and Yeadon [15] have studied band broadening in SEC for monodisperse proteins, but they did not correlate the data obtained for monodisperse proteins with those obtained for polydisperse (but narrow) polystyrene samples.

The theory of Knox et al. [16] is applied to estimate the contribution of polydispersity (PDI) to the band broadening (chromatographic-selectivity contribution). The Knox theory relates the observed band width to the selectivity of the system (expressed in terms of the slope of the SEC calibration curve) and the homogeneity of the sample (expressed in terms of its polydispersity). In order to apply Knox' theory, the exact value of the polydispersity must be known. However, it turns out to be difficult to obtain independent measures of the PDI with sufficient accuracy and precision. It has been demonstrated [17] that the PDI of a polymeric standard specified by the manufacturer is an upper limit, while the Poisson theory (which is thought to describe the molecular-weight distribution of a polymer synthesized by anionic polymerization) yields a lower limit. In recent years matrix-assisted laser-desorption ionization (MALDI) time-of-flight (ToF) mass spectrometry (MS, together MALDI-ToF-MS) has emerged as an independent method for measuring the PDI. Such new possibilities provide new impetus for the study of band broadening in SEC.

Both chromatographic dispersion and extra-column dispersion are undesirable in SEC. The former can be minimized by operating columns that are well packed with small particles at a low flow rate. However, chromatographic dispersion is inevitable and it can never be reduced to zero. In contrast, the extra-column band-broadening contribution can (and should) be reduced to negligible values by minimizing the length and diameter of connecting tubing, by optimizing connections, minimizing injection and detector volumes, and by optimizing the flow geometries in all components of the system.

SEC can be used successfully to characterize molecular-weight distributions if the chromatographic dispersion and the extra-column dispersion are negligible in comparison with the chromatographic selectivity. If the dispersion contributions are not negligible, then it is – in principle – possible to carry out a mathematical correction [18,19], provided that samples or standards of negligible or known dispersity are available. Such mathematical corrections are beyond the scope of the present treatment. Under ideal conditions, SEC provides a direct estimate of the MMD and of the characteristic averages of the sample. However, in references [20,21] it was demonstrated that the PDI values obtained for narrowly distributed samples using temperature-gradient interaction chromatography (TGIC) approached those estimated from the theoretical Poisson distribution, while the values derived from SEC (using calibration relative to PS standards) were reported to be considerably higher. Thus, there is some doubt as to the applicability of SEC for correctly measuring polydispersities.

In this paper, we will reconsider the various factors that determine the bandwidth in SEC. Extra-column contributions are measured and minimized. Various ways are explored to characterize the polydispersity of polymer standards and to establish the polydispersity contribution to chromatographic band broadening. In case of synthetic polymers it is impossible to measure the chromatographic-dispersion contribution to the observed bandwidth independently. Consequently, the chromatographic contribution is estimated from

$$\sigma^2_{\text{column}} = \sigma^2_{\text{observed}} - \sigma^2_{\text{PDI}} - \sigma^2_{\text{extra-column}} \tag{3}$$

Experimental results are compared with simulations based on conventional chromatographic theory. It is demonstrated that band broadening in SEC deviates from theory in some cases, especially around the total-exclusion limit of the column, where bands are broader than expected, and at high flow rates, where bands are narrower than expected.

2. Theory

2.1. Band broadening in size-exclusion chromatography

In chromatography band broadening is a collective term used for all the unwanted dispersion phenomena that occur during a separation. Due to dispersion and due to chromatographic separation (selectivity) of polydisperse samples the chromatographic peaks that are detected at the end of the column are broader than the initial injection profiles. Dispersion phenomena can be due to fundamental effects, such as molecular diffusion or different path lengths in a packed bed, or to experimental irregularities, such as imperfectly packed columns or poor connections. Band-broadening effects may occur inside the chromatographic column or in the injector, detector and tubing. Therefore, we distinguish between column band broadening and extra-column band broadening.

As in all forms of chromatography, band broadening in SEC is one of the factors that determine the eventual resolution. However, in SEC of synthetic polymers the peaks of individual analytes within the distribution cannot be discerned, apart from the smallest oligomers (often referred to as fingers or fingering in the SEC of low-MM standards). The peaks obtained in SEC are envelopes representing (large) series of convoluted peaks. The apparent efficiency or plate count of the separation (N_{obs}) or, equivalently, the plate height (H_{obs}) can be measured in the same way as in other forms of chromatography, viz.

$$N_{\text{obs}} = \left(\frac{V_{\text{R}}}{\sigma_{\text{V}}}\right)^2 = 5.54 \times \left(\frac{V_{\text{R}}}{W_{1/2}}\right)^2 \tag{4}$$

$$H_{\text{obs}} = \left(\frac{L}{N_{\text{obs}}}\right) \tag{5}$$

where V_{R} is the retention volume, σ_{V} the standard deviation of the peak expressed in volume units, $W_{1/2}$ the peak width at half height in volume units and L the column length. However, we should realize that N_{obs} and H_{obs} have a different meaning in the SEC of polydisperse samples.

If the eluted peak does not have a Gaussian profile, a better way to express the plate number and the plate height

is by using the statistical moments. In that case Eqs. (1) and (2) become

$$N_{obs} = \frac{{\mu_1}^2}{\mu_2} \tag{6}$$

$$H_{obs} = L\left(\frac{\mu_2}{{\mu_1}^2}\right) \tag{7}$$

where μ_1 and μ_2 are the first and second normalized central moments, respectively.

Band broadening can be discussed either in terms of peak moments or, equivalently, in terms of variances. The latter is common in chromatography and in the following discussion we will therefore use this terminology. The total observed variance for a peak of a polydisperse analyte is

$$\sigma^2_{observed} = \sigma^2_{PDI} + \sigma^2_{column} + \sigma^2_{extra\text{-}column} \tag{8}$$

Note that Eq. (8) requires that all variances are expressed in the same units, e.g. μl^2. Several different situations can be distinguished.

2.1.1. Ideal chromatography

$$\begin{aligned} &\sigma^2_{PDI} = 0 \\ &\sigma^2_{column} \gg \sigma^2_{extra\text{-}column} \\ &\sigma^2_{observed} = \sigma^2_{column} \end{aligned} \tag{9}$$

Ideally, in chromatography all individual sample components are separated (so that $\sigma^2_{PDI} = 0$), and ideally ideal chromatography is achieved by minimizing $\sigma^2_{extra\text{-}column}$, rather than by maximizing σ^2_{column}. The latter seems rather obvious, but it is not in the context of size-exclusion chromatography.

2.1.2. Ideal-SEC

$$\begin{aligned} &\sigma^2_{PDI} \gg \sigma^2_{column} \\ &\sigma^2_{column} \gg \sigma^2_{extra\text{-}column} \\ &\sigma^2_{observed} \approx \sigma^2_{PDI} \end{aligned} \tag{10}$$

In the most typical application of SEC, the objective is to determine the characteristics of the MMD (e.g. M_n, M_w and PDI), which is equivalent to determining the peak position (and converting this to a peak molecular weight by so-called calibration) and the peak width (σ^2_{PDI}). Ideally, to determine σ^2_{PDI} accurately by SEC, all other contributions to the observed band width should be negligible.

As mentioned above, the usual chromatographic practice to minimize $\sigma^2_{extra\text{-}column}$, rather than to maximize σ^2_{column} does not apply as much in SEC as in other forms of LC. There has been a sustained and undeniable trend towards miniaturization in LC. Slowly, but definitely, columns of conventional diameter (4.6 mm i.d.) are making way for narrower columns (often 1 or 2 mm i.d.). This is not true for SEC. In SEC 4.6 mm i.d. columns are being used, but more commonly column diameters are still larger (typically 7 or 8 mm i.d.). For several reasons, it is difficult to minimize the extra-column band broadening in SEC. Polymers are big and slow. Dispersion in open tubes (and in other parts of the instruments) is much greater if the molecular diffusion coefficients are smaller. Because diffusion coefficients decrease with increasing molecular weight, this implies that the extra-column band broadening is greater on the high-molecular-weight side of a SEC peak than on the low-molecular-weight side. This implies that extra-column band broadening is not only difficult to suppress, but also difficult to account for quantitatively. Therefore, the common approach in SEC is to maintain a high value for σ^2_{column}, thus reducing the necessity of paying serious attention to $\sigma^2_{extra\text{-}column}$.

Whether or not σ^2_{column} can be kept much smaller than σ^2_{PDI} depends to a large extent on the value of the latter. Clearly, it is much easier to approach "ideal-SEC" conditions for broadly distributed polymers, than it is for narrowly distributed samples ("standards").

2.1.3. Sample-challenged SEC

$$\begin{aligned} &\sigma^2_{column} \gg \sigma^2_{extra\text{-}column} \\ &\frac{\sigma^2_{column}}{\sigma^2_{PDI}} > 0.1 \\ &\sigma^2_{observed} = \sigma^2_{column} + \sigma^2_{PDI} \end{aligned} \tag{11}$$

If the PDI of the sample is low (e.g. $1 \leq PDI \leq 1.1$) it is quite difficult to achieve "ideal-SEC" conditions. The chromatographic dispersion is then of the same order as the sample polydispersity. When such narrow samples or standards are being analyzed by SEC, the observed chromatographic peak is not representative of the MMD. The conditions of Eq. (11) are typically unacceptable for (polydisperse) samples, but acceptable for standards.

2.1.4. Experimentally challenged SEC

$$\begin{aligned} &\sigma^2_{extra\text{-}column} \geq \beta^2(\sigma^2_{column} + \sigma^2_{PDI}) \\ &\sigma^2_{observed} = \sigma^2_{column} + \sigma^2_{extra\text{-}column} + \sigma^2_{PDI} \\ &\qquad = (1+\beta^2)(\sigma^2_{column} + \sigma^2_{PDI}) \end{aligned} \tag{12}$$

In this case the extra-column band broadening plays a significant role. The factor β in Eq. (12) can be used to illustrate that there is a good deal of tolerance in chromatography. For example, if the extra-column standard deviation were half as large as the combined standard deviation for column and sample effects (i.e. $\sigma^2_{extra\text{-}column} = 0.5 = 0.5\sqrt{(\sigma^2_{column} + \sigma^2_{PDI})}$), then the value of β^2 would be 0.25. Relative to the situation in which no extra-column band broadening were present, the observed dispersion ($\sigma^2_{observed}$) would increase by 25% and the observed plate count ($N_{observed}$, see Eq. (4)) would decrease by 25%. However, the observed peak width ($\sigma^2_{observed}$) would still only increase by a factor $\sqrt{(1+\beta^2)}$, i.e. by about 12%. If the purpose of a SEC separation is

to determine the sample MMD, then an increase in the observed dispersion (i.e. in the factor (PDI-1), see Eq. (2)) by 25% is arguably too large. A value of 10% ($\beta^2 = 0.1$) seems a more reasonable upper limit.

There are several different possibilities within case 4, depending on the relative magnitudes of σ^2_{column} and σ^2_{PDI}. However, we do not need to elaborate on these separately. The best advice to chromatographers is to do anything they can to change a case-4 situation into one of the three other ones by minimizing the extra-column band broadening. One way of studying the extra-column band broadening is to remove the column from the instrumental set-up. In principle, this reduces both σ^2_{column} and σ^2_{PDI} to zero, so that $\sigma^2_{\text{column}} = \sigma^2_{\text{extra-column}}$.

The columns typically employed in SEC are not only broad, but also long. Commonly, several columns of 300–500 mm length are connected in series, to reach total column lengths (L) up to several meters. Increasing the column length does help to improve the ratio between σ^2_{column} (increasing proportionally with L) and $\sigma^2_{\text{extra-column}}$ (independent of L), so as to minimize the effect of the latter. We can define a chromatographic-integrity index (II_{Chrom}) as follows:

$$\text{II}_{\text{Chrom}} = \frac{\sigma_{\text{column}}}{\sqrt{\sigma^2_{\text{column}} + \sigma^2_{\text{extra-column}}}} \tag{13}$$

II_{Chrom}, takes on values between 0 (totally unacceptable) and 1 (100% chromatographic integrity). Both longer and broader columns will lead to an enhanced chromatographic integrity (higher value of II_{Chrom}). The effect of the column diameter on II_{Chrom} is expected to be much greater. The effects are significant when II_{Chrom} is much smaller than unity, but they diminish when complete integrity is approached ($\text{II}_{\text{Chrom}} \approx 1$).

Likewise, we can define a theoretical SEC-integrity index ($^{\text{th}}\text{II}_{\text{SEC}}$) as follows

$$^{\text{th}}\text{II}_{\text{SEC}} = \frac{\sigma_{\text{PDI}}}{\sqrt{\sigma^2_{\text{PDI}} + \sigma^2_{\text{column}}}} \tag{14}$$

which takes on a perfect value ($^{\text{th}}\text{II}_{\text{SEC}} = 1$) if the only factor affecting the observed peak shape and width is the polydispersity of the sample. $^{\text{th}}\text{II}_{\text{SEC}}$ increases when the column length is increased. However, the increase is slow. Starting from a situation in which σ^2_{column} is dominant (bad conditions for SEC), $^{\text{th}}\text{II}_{\text{SEC}}$ may increase by a factor up to $\sqrt{L}$. Under better conditions ($\sigma^2_{\text{PDI}} > \sigma^2_{\text{column}}$) the effect is much smaller. Another "golden truth" of chromatography is worth remembering. Better columns (i.e. a lower plate height, achieved by using smaller, more-homogenous particles, better packing, etc.) are a much better investment than longer columns.

Finally, we can define a practical (experimental) SEC-integrity index ($^{\text{exp}}\text{II}_{\text{SEC}}$)

$$^{\text{exp}}\text{II}_{\text{SEC}} = \frac{\sigma_{\text{PDI}}}{\sqrt{\sigma^2_{\text{PDI}} + \sigma^2_{\text{column}} + \sigma^2_{\text{extra-column}}}} \tag{15}$$

Like $^{\text{th}}\text{II}_{\text{SEC}}$, $^{\text{exp}}\text{II}_{\text{SEC}}$ only takes on a perfect value of unity if the MMD of the sample is the only factor affecting the observed peak. Unlike the theoretical index, $^{\text{exp}}\text{II}_{\text{SEC}}$ may also be affected by the column diameter. The SEC-integrity indices are defined such that they directly reflect variations in the width of the observed MMD. If $^{\text{exp}}\text{II}_{\text{SEC}} = 1$ the observed chromatographic bandwidth can be converted without correction to the sample polydispersity. If $^{\text{exp}}\text{II}_{\text{SEC}} = 0.9$ only 90% of the observed bandwidth is due to the polydispersity (and the calculated PDI will be approximately 20% higher than the true value, see Eq. (2)).

The anticipated effects of reductions in the column length (Fast SEC) or the column diameter (micro-SEC) on the chromatographic-integrity indices are summarized in Table 1b. In the case of Fast SEC the chromatographic resolution is the main point of concern. Reducing the column length (keeping other parameters constant) leads to a reduced resolution, as does an increase in flow rate. In the case of miniaturized SEC extra-column band broadening is the main threat. The theoretical SEC-integrity index is not affected, but the practical index is.

Table 2 summarizes our definitions of different types of chromatography in terms of the chromatographic-integrity indices. In ideal chromatography, extra-column band broadening is negligible. This is also the case in ideal-SEC. In addition, in ideal-SEC the chromatographic dispersion is negligible in comparison with the dispersion due to the sample PDI. If the latter is not the case, SEC will not be ideal, even if the experimental (chromatographic) factors are controlled adequately ($\text{II}_{\text{Chrom}} = 1$). If the chromatographic factors are not under control, SEC may be a good technique in theory, but not in practice.

The column variance is also affected by the flow rate (F). Because the chromatographic efficiency increases (and σ^2_{column} decreases) with decreasing flow rate, the effect of decreasing F resembles the effect of increasing L. In this paper, we will investigate some of the effects associated with variations in the column diameter, the column length and the flow rate in SEC.

If we want to control the individual contributions to the total band broadening and to achieve maximum chromatographic and size-exclusion-chromatographic integrity, we must be able

- to distinguish between the three different contributions to the total band broadening;
- to measure them independently or to otherwise obtain estimates of their respective magnitudes.

2.2. Column band broadening

Although chromatographers tend to speak of the column dispersion and, more frequently, of the column plate count (the two being related by Eq. (4)), the value of σ^2_{column} is strongly affected by the analyte and by the chromatographic conditions (mobile phase, flow rate). The process of

Table 2
Integrity indices for different kinds of chromatography

	Chromatographic-integrity index	SEC-integrity index	
	II_{Chrom} Eq. (13)	Theoretical ($^{th}II_{SEC}$ Eq. (14))	Experimental ($^{exp}II_{SEC}$ Eq. (15))
Ideal chromatography	1	0 (or N/A)	0 (or N/A)
Non-ideal chromatography	<1	0 (or N/A)	0 (or N/A)
Ideal-SEC	1	1	1
Sample-challenged SEC[a]	1	<1	$=^{th}II_{SEC}$
Experimentally-challenged SEC[b]	<1	≤1	$<^{th}II_{SEC}$

[a] Sample-challenged conditions mainly occur when characterizing narrowly distributed samples and/or when using short columns ("Fast SEC").
[b] Experimentally-challenged conditions mainly occur when using columns with narrow diameters ("MicroSEC").

peak dispersion in the column is generally considered to be governed by three phenomena: diffusion in the axial direction, flow pattern effects (consisting of eddy-diffusion and mass-transfer in the mobile phase), and resistance to mass transfer in the stationary phase (or stagnant mobile phase). In order to compare different systems, the chromatographic efficiency is often expressed in terms of the reduced (dimensionless) plate height (h), which is defined as the plate height divided by the particle diameter of the stationary phase. From Eqs. (4) and (5) we obtain

$$h = \frac{H}{d_p} = \left(\frac{L}{d_p}\right) \times \left(\frac{\sigma_V}{V_R}\right)^2 = \frac{L}{d_p} \times \frac{(W_{1/2})^2}{5.54 \times {V_R}^2} \tag{16}$$

or

$$h = \left(\frac{L}{d_p}\right)\left(\frac{\mu_2}{{\mu_1}^2}\right) \tag{16a}$$

Because the reduced plate height is proportional to the variance, Eq. (8) can also be written in terms of the reduced plate height as follows:

$$h_{observed} = h_{PDI} + h_{column} + h_{extra\text{-}column} \tag{17}$$

The concept of reduced plate heights suggests that all similar columns (e.g. all columns packed with uniform spherical particles) should perform identically when compared at identical conditions, specifically at the same reduced velocity (ν)

$$\nu = \frac{u d_p}{D_m} \tag{18}$$

where u is the average linear velocity (of a fully excluded compound), d_p the particle size and D_m the diffusion coefficient of the analyte (polymer) in the mobile phase. Diffusion coefficients depend strongly on the mobile phase (typically decreasing with increasing mobile-phase viscosity) and on the analyte (typically decreasing with increasing molecular weight). For example, the following equation [22] is commonly used to describe the diffusion coefficient (in mm^2/s) of polystyrene in THF

$$D_m = 0.0386 M^{-0.57} \tag{19}$$

This equation implies that if the molecular weight of a polystyrene sample is a factor 10 higher, the diffusion coefficient is reduced by 73%. The range of polystyrenes typically encountered in SEC studies covers four orders of magnitude, from oligomers with molecular weights of a few hundreds to large polymers with molecular weights in the millions. Across this range the diffusion coefficient decreases by a factor of about 200. In order to achieve truly comparable conditions, very large polymers should be chromatographed at a 200 times lower flow rate than oligomers. This is not realistic. In practice, SEC is performed at a constant flow rate and at a linear velocity that is a factor 2–5 lower than that typically used for eluting small analyte molecules.

The simplest way to describe the effect of the (reduced) flow rate or (reduced) velocity on the chromatographic plate height is

$$h = A + \frac{B}{\nu} + C\nu \tag{20}$$

This equation is commonly referred to as the "van-Deemter" equation (in the H versus u form), but also frequently ascribed to Giddings (in the reduced, h versus ν form). The applicability of this equation for the size-exclusion chromatography of large molecules will be investigated in this paper.

Band broadening in SEC has been studied by Busnel et al. [23], using very narrow polystyrene standards (PDI < 1.01). They neglected the contribution of the polydispersity to the total band width, which may be justified by the theory of Knox et al. [16].

2.3. Extra-column band broadening

Significant extra-column band broadening arises from different sources, such as long and wide connection tubes and inappropriately large injection or detection volumes, or from poorly designed injectors or detectors or poor connections that induce stagnant volumes. All of these result in unwanted band-broadening contributions and therefore need to be avoided as much as possible. The sample should be introduced onto the column in a sufficiently narrow band, so that peak broadening caused by injection is negligible. All fittings and connectors, anywhere in the flow path between the sample injector and the detector, should be designed to introduce a minimum dead volume. Sample detectability is limited by the noise of the detector arising from instrument

electronics, temperature fluctuations, flow changes due to pump pulsation and similar effects [24].

The dispersion caused by (long) capillaries can be estimated from the Taylor equation

$$\sigma^2_{\text{tube}} = \frac{u_{\text{tube}} d^2_{\text{tube}}}{96(\pi/4) L_{\text{tube}} D_{\text{m}}} t^2_{\text{tube}} = \frac{F}{96 L_{\text{tube}} D_{\text{m}}} \left(\frac{(\pi/4) d^2_{\text{tube}} L_{\text{tube}}}{F} \right)^2 = \frac{(\pi/4) d^4_{\text{tube}} L_{\text{tube}}}{96 D_{\text{m}} F} \quad (21)$$

where u_{tube} is the mean linear velocity in the tube, d_{tube} and L_{tube} are the tube internal diameter and length, respectively, and t_{tube} the mean residence time in the tube. Eq. (21) suggests that the dispersion due to connection tubing is increasing linearly with the tube length and dramatically with the tube diameter. Although this conclusion may be qualitatively correct, the quantitative application of Eq. (21) is highly questionable, especially for (slowly diffusing) polymers. Several equations have been suggested in the literature, which predict σ^2_{tube} to be much lower than predicted by Eq. (21) [25,26]. Recent experimental results obtained with dextrans yield dispersion values that are up to a thousand times lower than predicted by the Taylor equation [27].

Nevertheless, large diameters of the connecting tubing will lead to increased dispersion, because the distance across which the analyte molecules need to diffuse in order to sample all the different regimes in the parabolic flow profile increases. Longer tubing also results in an increased variance of the chromatographic peak. Other factors, such as non-ideal flow profiles in connections, cannot easily be estimated. The many influencing factors and uncertainties make extra-column band-broadening a very complex phenomenon. In this paper we will evaluate how much of the total variance of the peak is due to the extra-column band broadening, and we will try to minimize these effects. We compare the experimental results obtained on a conventional SEC system with runs of the same samples with the SEC column being replaced by tubing, directly connecting the injector to the detector.

2.4. Band broadening due to polydispersity

In SEC of synthetic polymers band broadening results in distortion of the calculated MMD, as well as in errors in the average molar-mass values obtained [28,29]. To achieve ideal-SEC conditions (Eq. (10)) and high SEC-integrity (Eq. (15)) we need the band dispersion due to extra- and intra-column effects to be minimized, while the band dispersion due to the sample PDI (viz. the selectivity of the separation) should be maximized. To obtain good estimates of the MMD of polymers, they should be measured under conditions at which $^{\text{exp}}\text{II}_{\text{SEC}}$ approaches unity, viz. the chromatographic peak width is completely determined by the polydispersity contribution and other band-broadening effects can be considered negligible.

Knox et al. [16] proposed an equation to estimate the contribution of the polydispersity to the observed variance

$$\sigma^2_{\text{PDI}} = S^2(\text{PDI} - 1)(\alpha + 1) \quad (22)$$

or to the total (apparent) plate height

$$h_{\text{PDI}} = \left(\frac{L}{d_{\text{p}}} \right) \left(\frac{S}{V_{\text{R}}} \right)^2 (\text{PDI} - 1)(\alpha + 1) \quad (22a)$$

where, S is the negative inverse slope of the SEC calibration curve ($-\text{d}V_{\text{R}}/\text{d}(\ln M)$) and α a correction factor that depends on the polydispersity of the polymer

$$\alpha = \frac{11}{4}(\text{PDI} - 1) + \frac{137}{12}(\text{PDI} - 1)^2 \quad (23)$$

In order to compute the PDI contribution to the (reduced) plate height, accurate knowledge of the polydispersity of the narrow standards used is essential. The widths of molar-mass distributions (which are directly related to the PDI, Eq. (2)) have been estimated from size-exclusion chromatography with concentration and light-scattering detection [24]. Also, the PDI can be obtained from mass-spectrometric measurements using soft ionization techniques [30,31].

In case of commercial standards, the manufacturer specifies a value. Usually, an upper limit is specified (e.g. PDI < 1.05). Some researchers have suggested that the real PDI values are much smaller than those specified by the suppliers [28,29,32]. Stegeman et al. [28] claimed that h_{PDI} is overestimated, probably due to an overestimation of the PDI reported by the manufacturer of the standards. Also other authors [29,32] claim that the real PDI is considerably smaller than the nominal values reported, because SEC, which is used for their estimation, is significantly affected by band-broadening effects.

Temperature-gradient interaction chromatography (TGIC) has been found to give much narrower peaks than SEC and thus leads to much lower PDI estimates [20,21]. For polymers (e.g. polystyrenes) made by anionic polymerisation the TGIC peaks observed approached a Poisson distribution and the estimated PDI values were close to those derived from the Poisson distribution. Interactive liquid chromatography offers another possible way to study the MMD of very narrowly distributed polymer samples [33].

Knox et al. [16] demonstrated the implications of Eq. (8) using simulations. In this study we want to evaluate the contribution of polydispersity to the total peak width in practical situations, on different SEC columns and applying different flow rates.

3. Experimental

In this paper we report on a number of interesting observations in relation to band broadening in size-exclusion chro-

matography a more-elaborate discussion involving larger experimental data sets will be presented elsewhere [34].

3.1. Instrumentation and chemicals

Estimates of the extra-column band broadening were obtained by connecting the injector to the detector using a 50 cm long connecting tube, with an internal diameter of 0.0254 cm, manufactured by UPCHURCH Scientific, INC (Oak Harbour, WA, USA). The solvent delivery module used was a LC-10AD VP pump, from Shimadzu (Kyoto, Japan). The Rheodyne (Bensheim, Germany) injection valve had a fixed loop of 40 μl. Detection was performed with an Applied Biosystems (Ramsey, NJ, USA) UV detector at a wavelength of 254 nm and with a detector cell of 8 μl. The peaks were recorded and examined using a routine written in our department in Matlab 5.2 (MathWorks, Natick, MA, USA).

In order to get an indication of the percentage of extra-column band broadening in a conventional system, we used the same system as earlier, but we included a separation column. We used three different PL-Gel Individual-Pore-Size GPC/SEC columns from Polymer Laboratories (Church Stretton, Shropshire, UK), with dimensions 300 mm × 6.8 mm i.d. and packed with 5 μm particles. The columns had different pore sizes: (i) 10^3 Å (effective MM range: 500–60,000 Da), (ii) 10^4 Å (effective MM range: 10,000–600,000 Da), and (iii) 10^5 Å (effective MM range: 60,000–2,000,000 Da). For the Fast SEC experiments we used a 50 mm × 7.5 mm i.d. column packed with PL-Gel 5 μm MIXED-C stationary phase (effective MM range: 200–2,000,000 Da). The system temperature was maintained at 30 °C.

Data were recorded using the Waters (Milford, MA, USA) Millennium32 software. Calculations and data treatment on the chromatographic peaks were performed using software written in-house on a Matlab-5.2 platform. The eluent was non-stabililized tetrahydrofurane (THF) from Biosolve (Valkenswaard, The Netherlands). The standards used were polystyrenes (PS) from Polymer Labs or Pressure Chemical (Pittsburgh, PA). Their properties are shown in Table 3. The concentration of all standard solutions was 1 mg/ml in non-stabilized THF. The marker, toluene, was obtained from Merck (Darmstadt, Germany). It was used in a concentration of 0.1 mg/ml. The experiments shown in this paper were performed at a flow rate of 0.7 ml/min.

Table 3
Peak molar mass (M_p), manufacturer and specified polydispersity of the polystyrene standards used in this study

M_p (Da)	Manufacturer	PDI
1,700	Polymer Labs	1.06
2,450	Polymer Labs	1.05
3,250	Polymer Labs	1.04
5,050	Polymer Labs	1.05
7,000	Polymer Labs	1.01
11,600	Polymer Labs	1.03
22,000	Polymer Labs	1.03
76,600	Polymer Labs	1.03
200,000	Pressure Chemical	1.03
475,000	Polymer Labs	1.03
675,000	Polymer Labs	1.07
900,000	Pressure Chemical	1.07
2,000,000	Pressure Chemical	1.03
2,200,000	Polymer Labs	1.04

Table 4
Elution times and peak standard deviations (in time and volume units) obtained for standards of different molar weights in experiments on the extra-column band broadening using a 500 mm × 0.25 mm piece of connection tubing instead of the separation column, eluent THF at 1 ml/min, UV detector at 254 nm

M_p (Da)	t_R (s)	σ_t (s)	σ_V (μl)
Toluene	1.1	1.77	29.50
1,700	1.3	2.13	35.50
2,450	1.9	2.03	33.83
3,250	2.0	2.19	36.50
5,050	2.0	2.21	36.83
7,000	1.8	2.12	35.33
11,600	1.5	1.94	32.33
76,600	1.3	2.48	41.33
200,000	1.9	2.84	47.33
271,000	1.2	2.74	45.67
675,000	1.5	2.02	33.67
900,000	1.5	2.90	48.33
2,000,000	1.5	2.84	47.33
2,200,000	1.9	2.84	47.33

For the validation of the Knox equation (Eq. (22), [16]), we used measurements performed on a Waters Alliance SEC system, equipped with a Waters 410 refractive-index (RI) detector. The system temperature was maintained at 30 °C. Data were recorded using the Waters Millennium32 software. Calculations and data treatment on the chromatographic peaks were performed using in-house Matlab-5.2 software.

3.2. Procedures

The polystyrene standards and the toluene (Table 4) were injected on the various SEC columns. For the determination of extra-column band broadening we compared the peak width of the conventional SEC system with the peak width observed in the experiments with a connector tube installed between the pump and the detector. Because the peaks were typically not Gaussian in shape, we used statistical moments to determine the retention volumes and the peak standard deviations [35].

4. Results and discussion

4.1. Extra-column band broadening versus observed dispersion

We injected the toluene and the PS standards individually. We used the first normalized central moment to determine

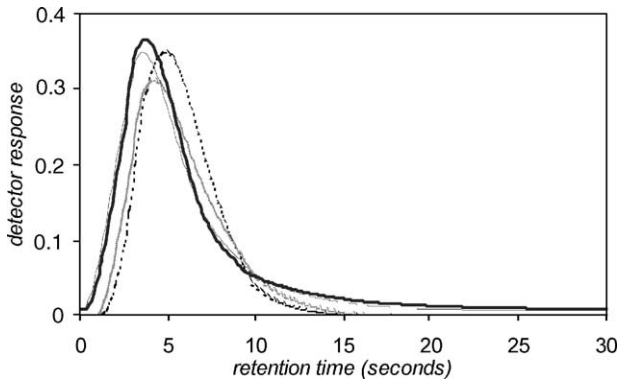

Fig. 1. Indication on the contribution to the variance of the peak due to extra-column band broadening (solutes, toluene; polystyrene standards of 7000, 76,600, and 675,000 Da) at 0.7 ml/min flow rate.

the retention time and the second normalized central moment to measure the peak variance (Table 4). Some of the observed profiles are shown in Fig. 1. The asymmetry (tailing) of the peaks seems to increase with increasing molar mass. This can be explained by the facts that higher molar-mass polymer sample solutions have a higher viscosity and that the diffusion coefficients strongly decrease with increasing molecular weight (Eq. (19)). However, the increase in the variance is not dramatic and much smaller than would be expected from Eq. (21). A faster injection may help to reduce the tail of the high molar-mass peaks, by introducing a narrow injection profile, which will implicitly result in a narrower analyte peak. We have performed experiments with a fast-switching valve and time-split injections. Indeed, some improvements can be obtained and the tailing of the profiles can be reduced [34].

With the current set-up, the extra-column band broadening ($\sigma_{\text{extra-column}}$) is in the range of 30–50 μl and the variance ($\sigma^2_{\text{extra-column}}$) is approximately in the range of 1000–2000 μl^2 (0.001–0.002 ml^2). To discuss the integrity of the size-exclusion chromatographic system, we must compare this value with the column variance (σ^2_{column}) and the variance due to the sample polydispersity (σ^2_{PDI}). For these experiments we installed three different individual-pore-size separation columns (PL-Gel 10^3 Å, PL-Gel 10^4 Å or PL-Gel 10^5 Å; one column at a time) of conventional SEC size (300 mm × 6.8 mm) in the system. From each experiment with a column installed we obtained the observed variance of the peak (σ_{observed}). To obtain the relative (percentage) contribution ($\text{rc}_{\text{extra-column}}$) of extra-column band broadening we divided the variance of the peak obtained in the experiment without the column installed by the variance of the same molar-mass standard analyzed in the conventional SEC system, as follows

$$\text{rc}_{\text{extra-column}} = \left(\frac{\sigma^2_{\text{extra-column}}}{\sigma^2_{\text{observed}}} \right) \times 100 \quad (24)$$

In Table 5, we present an example of peak variances obtained using the PL-Gel 10^3 Å column, from which the relative contributions of extra-column band broadening ($\text{rc}_{\text{extra-column}}$) were calculated. From Table 5, it appears that only a few percent of the total band broadening originated from the extra-column dispersion in the case of low MM standards. Here the extra-column dispersion was relatively small and the observed dispersion relatively large. For the highest MM standards which were totally excluded, the observed dispersion decreased, while the extra-column dispersion increased. As a result, the average relative contribution of the extra-column dispersion typically exceeded 10%, for the totally excluded standards. In Table 6, we list some $\text{rc}_{\text{extra-column}}$ values obtained with three different columns for relatively small standards. It appears that also in case of total permeation the relative contribution of the extra-column dispersion increases. The low-MM standards are totally permeating on the 10^5 Å column.

In chromatography 20% extra-column dispersion can be allowed without influencing the final peak width significantly [36] (see discussion in Section 2.1). However, the PDI obtained from SEC experiments is affected by variations in the variance, rather than the peak width and an extra-column contribution of 10% to the dispersion seems only marginally acceptable. Thus the band broadening arising from tubing, connectors, injector and detector is acceptably low in this experiment only within the effective working range of the column. For totally excluded compounds the

Table 5
Peak variances obtained on a conventional system having a PL-Gel 10^3 Å separation column (300 mm × 6.8 mm), peak variances estimated from the experiment without column installed and extra-column band-broadening contribution to the total band-broadening at 1.0 ml/min flow rate

MM (Da)	σ_{observed} (μl)	$\sigma_{\text{extra-column}}$ (μl)	$\text{rc}_{\text{extra-column}}$ (%)
Toluene	216.67	29.50	1.85
1,700	225.00	35.50	2.49
2,450	213.33	33.83	2.52
3,250	193.33	36.50	3.56
5,050	200.00	36.83	3.39
7,000	180.00	35.33	3.85
11,600	143.33	32.33	5.09
76,600	136.67	41.33	9.15
200,000	131.67	47.33	12.92
675,000	125.00	33.67	7.25
2,000,000	133.33	47.33	12.60
2,200,000	133.33	47.33	12.60

Table 6
Percentages of extra-column band broadening ($\text{rc}_{\text{extra-column}}$) as contribution to the total band dispersion, obtained with three different separation columns, PL-Gel 10^3 Å, PL-Gel 10^4 Å and PL-Gel 10^5 Å (300 mm × 6.8 mm)

Column (Å)	Standard M_p (Da)					
	Toluene	1,700	7,000	11,600	22,000	76,600
10^3	1.85	2.49	3.85	5.09	6.14	9.15
10^4	1.50	2.99	2.95	2.61	2.96	5.51
10^5	4.81	6.89	12.00	8.43	5.61	11.31

Table 7
Effect of the concentration on the extra-column band-broadening contribution, RI detector, tubing (1500 mm × 0.25 mm), at 1 ml/min flow rate

M_P (Da)	Concentration (mg/ml)	σ_t (s)	σ_V (μl)
7,000	0.70	2.02	35.17
	0.50	2.00	33.27
	0.35	2.11	33.62
76,000	0.70	2.25	37.55
	0.50	2.14	35.59
	0.35	2.08	34.59
900,000	0.70	2.76	45.97
	0.50	2.52	41.99
	0.35	2.23	37.24

extra-column dispersion may be quite significant. However, in this case SEC cannot be used to obtain PDI values in any case. In some commercial SEC systems we found considerably higher dispersion values, probably due to long pieces of connection tubing. These systems are typically used with several columns connected in series. To make them suitable for single-column SEC experiments, the extra-column dispersion must be reduced. These systems – and the system used for the present experiments – are not suitable for micro-SEC experiments involving columns with diameters (much) smaller than the 6.8 mm used in the present case.

The effect of the polymer concentration on the extra-column band broadening was found to be very small. Some examples are given in Table 7. For the PS 7000 and PS 76,600 standards doubling the concentration changes the observed standard deviation only by a few percent. For very large polymers such as the PS 900,000 standard, the effect is greater, but certainly not dramatic.

4.2. Sample polydispersity versus observed dispersion

4.2.1. Estimating h_{PDI}

The variance due to the sample dispersion can be estimated from Eq. (22), while the contribution of the sample polydispersity to the observed plate height can be calculated from Eq. (22a). The Knox equations can be theoretically derived [34] and we have previously verified them by numerical calculations of h_{PDI} [17]. The negative, inverse slope of the calibration curve ($S = -dV_R/d(\ln M)$) in Eq. (22), was estimated in two different ways [37]. One value was derived from the calibration models best describing the entire range and encompassing all standards; The second method employed the slope of local straight parts of the calibration curve. Based on all these efforts, we are confident that the Knox equations do provide a good estimate of h_{PDI}.

In Fig. 2, we show the estimated values of h_{PDI} as a function of the elution volume for a 300 mm × 6.8 mm PS 10^3 Å column at 0.7 ml/min. The shape of the calibration curve is indicated in the figure (thin line; not scaled). The bell-shaped curves are calculated for PDI values of 1.05, 1.03, 1.02, and 1.01 (from top to bottom). The curves demonstrate that by

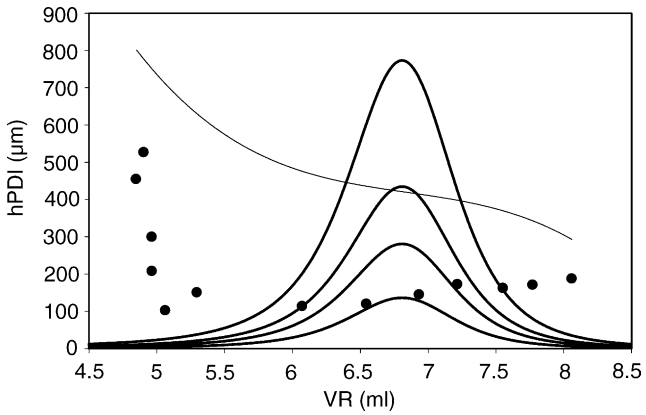

Fig. 2. Calculated contributions of the sample polydispersity to the observed plate height using the Knox equation. Column, PL-Gel 10^3 Å; flow rate, 0.7 ml/min; calibration curve $\ln MM = -0.4673V_R{}^3 + 9.5946V_R{}^2 - 66.368V_R + 163.52$. Drawn lines (top to bottom) PDI = 1.05, 1.03, 1.02, 1.01. Dots represent experimental data for the total (observed) reduced plate height. The thin line illustrates the shape of the calibration curve (not matching the vertical axis).

far the greatest contribution from the sample polydispersity to the predicted peak width is observed in the region where the calibration curve is nearly horizontal. The inverse slope of the calibration curve is very much higher in this region (more than 10 times higher than on the left-hand-side of the figure, so that h_{PDI} is more than 100 times higher). This once again lays emphasis on the need to work well within the effective range of SEC columns if at all possible.

Also included in Fig. 2 are the experimentally observed plate heights for a number of standards run under the specified conditions. This leads to several striking observations.

(1) The observed dispersion (in terms of h_{PDI}) depends only slightly on the molecular weight and thus on the elution volume, except for the largest standards (675,000–2,200,000 Da), which elute near the exclusion limit of the column. For these latter standards the band broadening is much greater than for the other standards. This has been observed before and the phenomenon is not completely understood. Pasti et al. [38] have connected it to the small number of times that the largest molecules enter a pore during their passage through the column.
(2) The observed peak width is not much greater in the middle of the calibration curve. This seems to indicate that high-integrity SEC (where h_{PDI} dominates h_{column} and $h_{extra\text{-}column}$, so that ${}^{exp}II_{SEC} \approx 1$) is not possible for narrow standards, at least not on a single (300 mm long) SEC column.
(3) Around the centre of the curves, the observed plate height is much smaller than could be expected based on the specified PDI values.

For reasons specified above, we believe that the Knox equation is essentially correct. Therefore, the PS 7000 standard (V_R = 6.93 ml) and the PS 11,600 standard (V_R

= 6.54 ml) cannot have polydispersities much greater than 1.01, because they fall just above this line in Fig. 2. This is an upper limit (PDI_{max}), because the observed band width is likely to include a significant contribution from chromatographic band broadening. The PS 7000 standard has a specified polydispersity of 1.01 or less (see Table 3), which is justified based on Fig. 2. However, the PS 11,600 standard has a specified polydispersity 1.03 or less. This is a technically correct, but extremely careful specification. Likewise, the PS 5050 standard (V_R = 7.21 ml), with a specified polydispersity of 1.05 can be assigned a PDI_{max} value of about 1.02 based on Fig. 2. The points towards the edges of Fig. 2 show an observed band broadening that is much higher than the predicted contribution from polydispersity (h_{PDI}). However, this is not because these standards have a greater polydispersity, but because the present SEC column shows a limited selectivity in their molecular-weight range. A greater range can be studied by using coupled columns or mixed-bed ("linear") columns, but narrow standards can best be studied on columns with a narrow pore-size distribution in the appropriate range [1]. In other words, the SEC-integrity index will not be improved by using these other column configurations. By studying many different standards on many different columns, we have shown that the PDI values of PS standards specified by the manufacturer are usually rather conservative upper limits [17].

4.2.2. Sample polydispersity

4.2.2.1. Poisson distributions. Narrow polystyrene standards can be prepared by ("living") anionic polymerization typically using butyl lithium as the initiator. Under ideal conditions (perfect mixing, absence of scavengers such as oxygen, absence of branching reactions, etc.) such reactions are expected to result in Poisson distributions for the degree of polymerization. In Table 8, the theoretical polydispersities (PDI_{th}) are given for polystyrene standards prepared under ideal conditions. It is seen that the theoretical values are much lower than the specified values, especially for high molecular weights. However, in the latter case ideal conditions are hard to maintain (mixing problems, long reaction times, etc.). Nevertheless, this calculation demonstrates that lower PDI values than those specified by the manufacturer are not unrealistic.

Table 8
Theoretical PDI values obtained for the different polystyrene standards by assuming a Poisson distribution

M_p	n	PDI_{th}	PDI-1	Manufacturer-specified PDI
1,700	16	1.0625	6.25E−02	1.06
2,450	23	1.0435	4.35E−02	1.05
3,250	31	1.0323	3.23E−02	1.04
5,050	48	1.0208	2.08E−02	1.05
7,000	67	1.0149	1.49E−02	1.04
11,600	111	1.00901	9.01E−03	1.03
22,000	211	1.00474	4.74E−03	1.03
76,600	735	1.00136	1.36E−03	1.03
200,000	1920	1.000520	5.20E−04	1.03
475,000	4561	1.000219	2.19E−04	1.03
675,000	6481	1.000154	1.54E−04	1.07
900,000	8641	1.000116	1.16E−04	1.07
2,000,000	19202	1.0000520	5.20E−05	1.03
2,200,000	21123	1.0000473	4.73E−05	1.04

Chang et al. [20,21] used temperature-gradient interaction chromatography (TGIC) to achieve a greater selectivity for polystyrene standards than commonly achieved with SEC. They found PDI values that approached the theoretical (Poisson) values listed in Table 8. The PDI values obtained from SEC were reported to be considerably higher. Fitzpatrick et al. [33] reached similar conclusions using gradient-elution liquid chromatography instead of TGIC.

4.2.2.2. Matrix-assisted laser-desorption ionization (MALDI) mass spectrometry. In principle, mass spectrometry offers a direct way to measure the polydispersity of narrowly distributed polymers (standards). In practice, however, a number of stringent requirements must be met. The sample must be representatively ionized, i.e. the ionization efficiency should be the same for all molecules (big and small, functionalized or non-functionalized, etc.); All ions must be analyzed and detected with the same sensitivity; All ions must be singly charged (or it must be possible to correct for multiple ionization through software, which is difficult for complex samples of large molecules); The entire distribution must be clearly discernable from the noise and from the baseline. In practice (MALDI-ToF-MS) comes close to meeting these requirements. MALDI is a very soft ionisation technique, which yields large, non-fragmented ions. However, the technique should preferably only be applied to samples that are quite homogeneous in terms of size (i.e. a narrow MMD or low PDI) and very homogeneous in terms of chemical composition and functionality. Thus, MALDI should only be applied to study distributions in combination with a liquid-phase separation method [8]. If all the above conditions are met, the MALDI-MS spectrum is directly indicative of the molecular-weight distribution of a polymer. Because the signal is proportional to the number of ions of a certain mass, the centre of gravity of the MS signal is the number average molecular weight (M_n). This parameter can be determined most accurately using MS. Calculations also allow M_w and the PDI to be derived from the spectrum. The latter two parameters tend to be less accurate [39]. Large ions are more likely to be obscured by noise and baseline problems are aggravated. As a result, MALDI has become a pretty reliable technique for measuring M_n, but its merits as a tool for accurately determining M_w and PDI values are still the subject of debate.

We applied MALDI to three polystyrene standards with specified molecular weights of 4700, 6770, and 76,600, and with polydispersities of 1.05, 1.03, and 1.03, respectively (values specified by the manufacturers). An example of a MALDI-ToF-MS spectrum is shown as Fig. 3. Two correc-

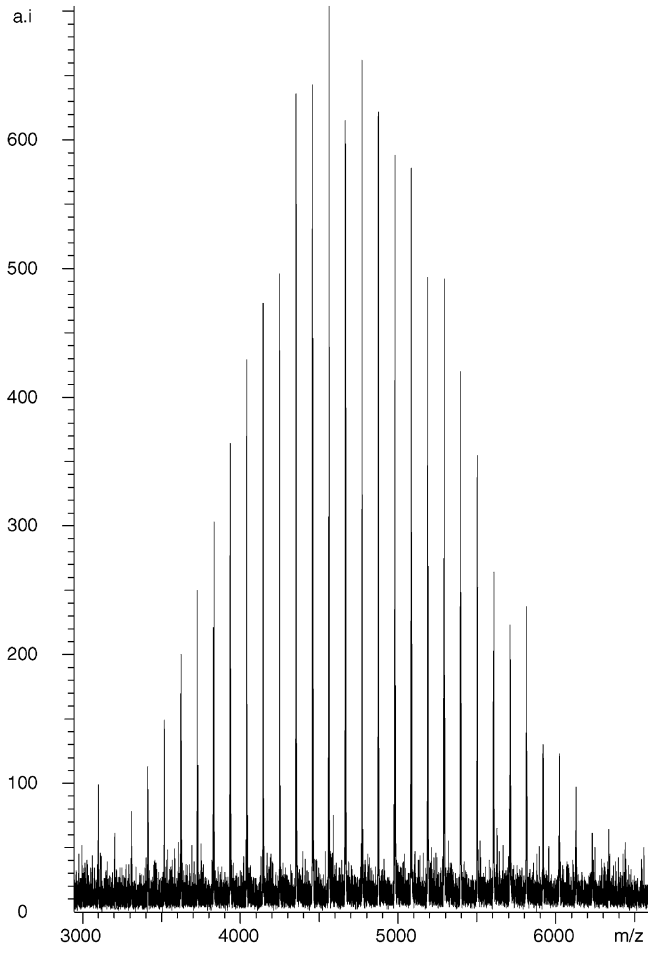

Fig. 3. MALDI-ToF-MS spectrum for the 5050 M_p polystyrene standard with a manufacturer-specified value of 1.05 for the PDI.

tions were performed on the raw MS data. Firstly, the signal intensity was changed from number fractions to weight fractions by multiplying the intensity with the relevant molecular weight. Secondly, a correction was introduced to account for the isotope pattern, based on the natural abundance of the ^{12}C and ^{13}C isotopes. The measured intensity (signal height) around a given *m*/*z* value arises from the molecules with the most frequently occurring ^{12}C–^{13}C isotope composition. If the number of carbon atoms is known, then a correction can be made to obtain the total signal intensity. For instance, it can be calculated from a binomial distribution that for a peak with 272 carbon atoms (peak around *m*/*z* of 3500 in Fig. 3) the most probably combination is three ^{13}C and 269 ^{12}C atoms. It can also be calculated that this peak represents 22.5% of the polymer molecules with 272 carbons, so that the height of this peak will be multiplied with a factor of $100/22.5 = 4.4$. The highest signal around *m*/*z* 6000 is due to the polymer with 464 C atoms, five of which are ^{13}C atoms. The highest signal represents 17.6% of this polymer and the correction factor becomes 5.7. Applying the correction factor to the highest peak is advantageous, because it will be more easily discernable from the noise than the entire isotope pattern. However, because the magnitude of the correction factor increases with increasing molecular weight, this is yet another reason why the high molecular-weight end of the mass spectrum is susceptible to noise.

For both corrections the change is larger for higher molar masses, which suggests that the calculated M_w and PDI values will increase. However, for low-molecular-weight standards almost equal values are obtained with and without the corrections, For example, for the standard with $M_p = 6770$, PDI values of 1.020, 1.018 and 1.018 were found for the measured, the molecular-weight corrected and the isotope-pattern corrected spectrum, respectively. MALDI is still a much-more-limited technique for characterizing high-molecular-weight polymers. PDI values of 1.021, 1.020, and 1.003 were estimated for the respective standards. The resulting values for the PDI indicate that for the first two samples estimates were found comparable with those found with SEC and TGIC for similar polymers. The accuracy of the PDI values obtained with MALDI will be extensively discussed elsewhere [40].

4.3. Total observed band dispersion

4.3.1. Relative contributions to band broadening and integrity indices

In previous sections, we have discussed extra-column band broadening and sample dispersion in relation to the total observed band broadening. We have concluded that neither contribution is dominant. Extra-column band broadening can be estimated independently and it can be kept sufficiently small in most practical situations. The effect of the sample dispersion on the observed bandwidth can be predicted with good accuracy, but the total bandwidth does not follow the predicted pattern. Thus, column band broadening is a significant contribution in SEC of narrow standards. However, we cannot measure the column band broadening independently. Eq. (3) can be used to obtain estimated values, but the accuracy of such estimates is low, unless accurate values of the sample polydispersity are available. As discussed above, there is ample evidence to conclude that the values specified by the suppliers are conservative upper limits. For low-MM standards, we believe that MALDI provides the best estimates. However, at this point in time MALDI results may not yet be rigorously correct. For very high-MM standards there is some evidence [20,21] that the Poisson limit may be approached. Thus, for the high-MM standards this may be our best current estimate. Still, our best current estimates are not good enough to use Eq. (3) with any kind of confidence. Therefore, we are taking a different approach in this study.

We concluded from Fig. 2 that the chromatographic band broadening forms a large, if not dominant contribution to the observed band widths. In the worst case, all of the observed band broadening can be ascribed to intra-column and extra-column dispersion and none of it is due to the

sample polydispersity. Thus, the observed peak variance corresponds to the maximum possible contribution from chromatographic dispersion. If we then calculate the contributions from the sample polydispersity to the peak width, which can be done with some confidence using the Knox equation (Eq. (22)), we can evaluate the relative contributions and predict (minimum) values of the SEC-integrity indices. This is done in Fig. 4 for three different SEC columns. The chromatographic variance ($\sigma^2_{\text{column}} + \sigma^2_{\text{extra-column}}$) is assumed to be equal to the variance observed using narrow standards and it is assumed to be independent of the sample polydispersity. The contribution from the latter is calculated using Eq. (22). For samples of different molecular weight and different polydispersity, the SEC-integrity indices ($^{\text{exp}}\text{II}_{\text{SEC}}$) can then be calculated using Eq. (15). Fig. 4 shows contour plots, in which the value of $^{\text{exp}}\text{II}_{\text{SEC}}$ is plotted as a function of the sample PDI (horizontal axis; proportional to log(PDI-1)) and molecular weight (vertical axis; log MM). Generally and according to expectation, the SEC-integrity is low on the left-hand side of the plots (narrow samples) and high on the right-hand side (broad samples). The rate of changes from low to high values depends on the sample MM in relation to the selectivity (calibration curve) of the column. Thus, on the PL-Gel 10^3 Å column (Fig. 4a) samples with a molecular weight of about 10,000 yield the highest values for $^{\text{exp}}\text{II}_{\text{SEC}}$. Samples with a PDI larger than about 1.02 will give rise to an $^{\text{exp}}\text{II}_{\text{SEC}}$ value in excess of 0.8 (dark area). On this single column, samples with PDI = 1.1 can be analyzed with good integrity ($^{\text{exp}}\text{II}_{\text{SEC}}$ >0.8) in the approximate range 2500 < MM < 30,000. The corresponding ranges for the 10^4 Å (Fig. 4b) and 10^5 Å (Fig. 4c) columns are 15,000 < MM < 300,000 and 20,000 < MM < 500,000, respectively. Based on observed peak widths for a series of standards and the SEC calibration curves, SEC-integrity plots as the ones shown in Fig. 4 can easily be calculated for any kind of column or column configuration. This provides a clear and objective indication for the selection of suitable SEC columns and it can provide guidelines in the study of various types of SEC columns (e.g. miniaturized SEC, Fast SEC).

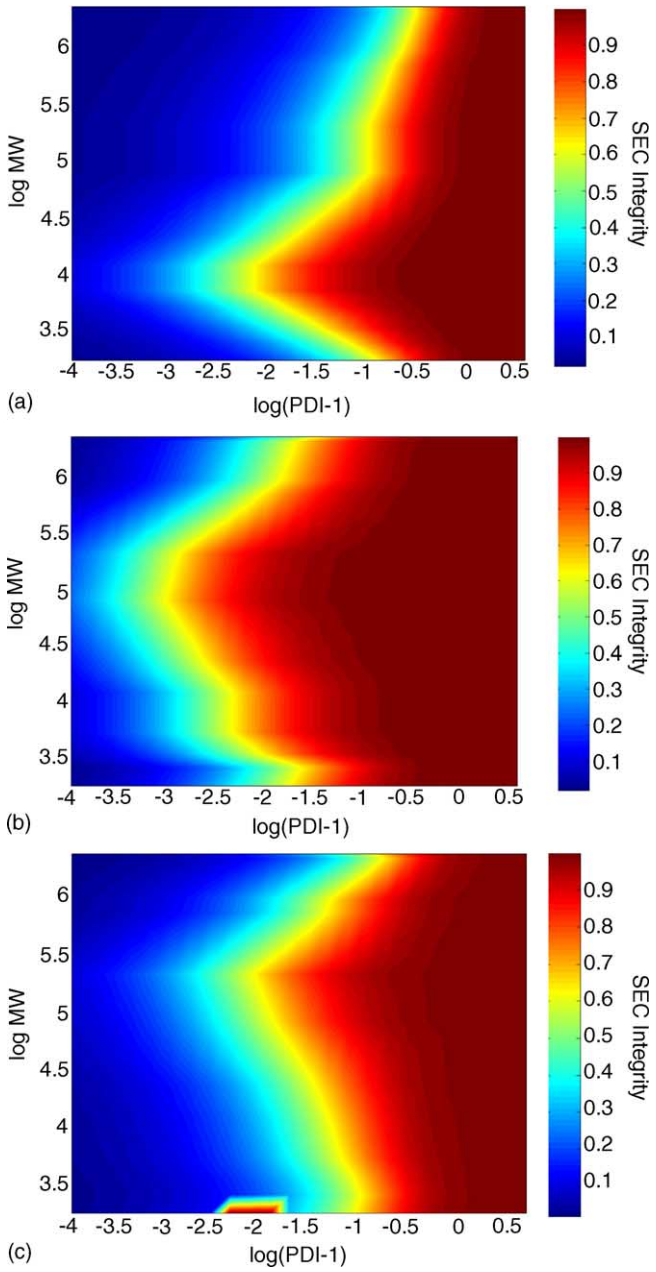

Fig. 4. (a) SEC-integrity plot, showing the experimental SEC-integrity index (Eq. (15)) as a function of the sample PDI (horizontal axis; proportional to log(PDI-1)) and molecular weight (vertical axis; log MM) for a single (300 mm × 6.8 mm) PL-Gel 10^3 Å column. (b) As (a), except for a PL-Gel 10^4 Å column. (c) As (a), except for a PL-Gel 10^5 Å column.

4.3.2. SEC at high flow rates

Eq. (20) describes the conventional relationship between chromatographic plate height and mobile-phase velocity according to Giddings and Knox. The reduced velocity (ν) in this equation is inversely proportional to the molecular diffusion coefficient of the analyte in the mobile phase (Eq. (18)). The very slow molecular diffusion of high-MM polymers (Eq. (19)) results in very large ν values. If Eq. (20) is valid and the coefficient C is constant (i.e. if Giddings' principle of reduced plate heights applies), then we must anticipate very high values of h (very low plate numbers) for SEC at high flow rates. Indeed, SEC (of polymers) is usually performed at considerably lower flow rates than is HPLC (of low-MM analytes). To draw an h versus ν curve, some reasonable assumptions have to be made for the parameters A, B and C in Eq. (20). In HPLC, typical values may be $A = 2$, $B = 1$ and $C = 0.05$ [44]. However, the validity of such a general curve in SEC is questionable. The very high reduced velocities encountered in SEC imply that the application of a reduced-plate-height plot obtained from HPLC requires massive extrapolation.

Fig. 5a shows a plot for the reduced plate height observed in the SEC of narrow PS standards. The (approximate) slopes of such plots (C-term in Eq. (20)) are often much lower than expected and great deviations from linearity are encountered. Across the very large range of ν values encountered

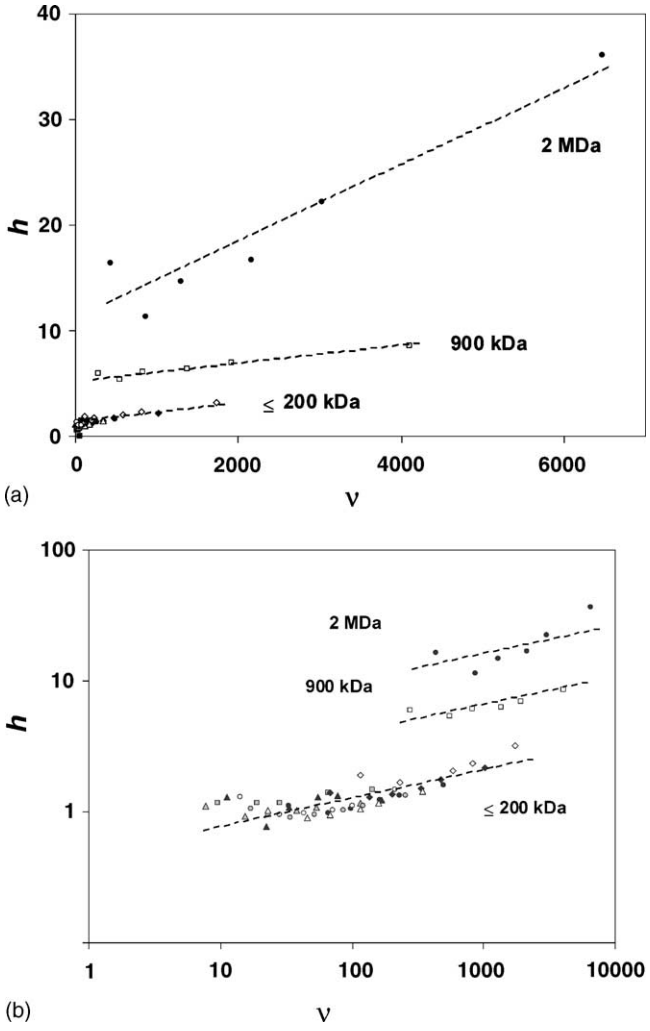

Fig. 5. (a) Calculated dimensionless plate-height curve and observed total plate heights for polystyrene standards on PL-Gel 10^5 Å column (300 mm × 6.8 mm i.d.). (b) As for the (a), but plotted on a logaritmic scale (both axes).

in Fig. 5a, it is more practical to refer to a logarithmic scale. This was also done in the extensive SEC-band-broadening studies of Knox et al. [16,43,44,45]. Fig. 5b shows the same data as Fig. 5a in a log–log format. The slopes of the lines in this figure are much smaller than unity. Because of contributions from extra-column band broadening and sample polydispersity, the total observed plate height must exceed the chromatographic band broadening. However, the experimentally observed (total) plate height is very much lower than one would expect based on Eq. (20). In Fast SEC short columns and high flow rates are typically used. Here even higher values of ν are encountered. Fig. 6 shows a $\log h$ versus $\log \nu$ curve up to reduced velocities of about 50,000, very much higher than those studied by Knox [44]. Especially for high-molecular-weight PS standards at (very) high flow rates, we observed much less dispersion than expected. Similar observations have also been reported by others [11].

One possible reason for the observed deviations from Eq. (20) has already been identified by Giddings, who included a coupling term in his plate-height equation that accounted for Eddy diffusion in the radial direction [41,42]. This results in flattening (and curvature) of the C-term (high-ν) branch of the plate-height curve (Eq. (20)). In case of high-molecular-weight polymers, where the molecular diffusion coefficient is extremely low, it is easy to envisage that the Eddy-diffusion contribution to the (favourable) radial diffusion of polymers is dominant. In any case, the effective diffusion of high-molecular-weight polymers seems to be more favourable (or less unfavourable) than predicted by Eq. (19). The latter equation only accounts for the molecular diffusion (of polystyrene samples in THF).

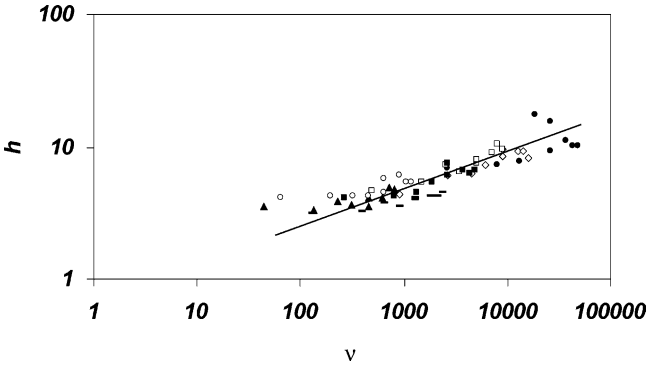

Fig. 6. Calculated dimensionless plate-height curve plotted on a logarithmic scale for polystyrene standards on a PL-Gel Mixed C 50 mm × 7.5 mm i.d. column.

Knox and Parcher [43] studied the dispersion for unretained solutes at very high reduced velocities. In a later account, Knox recalled that very high values of ν required either working at very high pressures or with very large particles [44]. In the chromatography of high-molecular-weight synthetic polymers we incidentally work at very high ν values because of a third reason, namely extremely low values of D_m (see Eq. (18)). Knox and Parcher found that, due to the coupling of the A term (Eddy diffusion) and the mobile-phase contribution to the C-term, the (reduced) plate height did not increase proportionally with ν (as suggested in Eq. (20)), but increased with $\nu^{0.33}$ [43]. For columns with relatively large diameters even more favourable results were obtained with $h \div \nu^{0.15}$. However, to profit from this "infinite-diameter" effect, samples must be introduced at the centre of the column, using a "curtain-flow system". We observe the most favourable relationships between reduced plate heights (h) and reduced velocities (ν) in Fig. 5b for samples for which total exclusion occurs, i.e. unretained solutes in the terminology of Knox and Parcher. For retained solutes Knox and Scott [45] found a combination of a (coupled) A'-term and a (stationary-phase) C'-term, i.e.

$$h = A'\nu^{0.33} + C'\nu \tag{25}$$

where the value of the coefficient C' was about 10 times lower than that of C in Eq. (20).

One obvious consequence of results as shown in Figs. 5 and 6 is the possibility to perform SEC at higher flow rates than previously thought desirable. Indeed, there is a strong trend towards the use of so-called Fast SEC techniques [10,11]. Because short and wide columns are typically used for Fast SEC, the "infinite-diameter" effect may be exploited, but this requires suitable injection devices. Some of the columns advocated for use in Fast SEC approach the infinite-diameter idea in a more literal sense [11].

5. Conclusions and outlook

In this paper we have discussed a number of aspects of band broadening in size-exclusion chromatography. We have introduced integrity indices for chromatography in general and for SEC in particular and we have demonstrated how these can be used to judge the suitability of SEC systems in various situations.

We have investigated extra-column band broadening and we have concluded that this contribution can be kept sufficiently small in most practical situations. However, problems are anticipated when SEC columns are miniaturized, without concomitant adaptation of the instrumentation. Some miniaturization of SEC is reasonably straightforward, but the use of commercial viscometric and light-scattering detectors in combination with miniaturized SEC systems is not easily possible.

We have also considered the characterization of narrow polymer standards by SEC, with special emphasis on the contribution of the sample polydispersity (PDI) to the observed peak width. If the PDI and the SEC calibration curve are accurately known, than the PDI contribution can be confidently predicted. However, accurate PDI values are not easily obtained. Matrix-assisted laser-desorption ionization (MALDI) mass spectrometry is a promising, but not yet fully matured technique, by which PDI values for narrowly distributed polymers may be measured directly. We have demonstrated that the PDI values specified by the suppliers of polymer standards are conservative upper limits. We have also demonstrated that in SEC of narrow standards the contribution of sample polydispersity is not dominant, not even under conditions where selectivity is highest (i.e. in the shallowest part of the SEC calibration curve).

Column band broadening is usually dominant in the SEC of narrow standards. Band broadening is demonstrated to be especially large around the total-exclusion limit of a SEC column. In this case the total observed band broadening is much greater than expected. Also unexpected, but much more welcome, is the relatively low band broadening observed for (very) high-molecular-weight polymeric standards at (very) high flow rates. This is especially favourable for Fast SEC separations.

To some extent, SEC-band broadening can be corrected for by mathematical deconvolution, if suitable standards are available [18,19]. The trend towards fast (low-resolution) SEC separations will increase the need for such algorithms. However, if no suitable standards are available (e.g. in case of copolymers) mathematical deconvolution is not possible. SEC-MALLS and SEC-viscosity do not alleviate the need for good SEC separations (high SEC-integrity indices). In contrast, the interpretation of the data obtained with such techniques is greatly simplified if very narrow fractions are detected at any one time. For the (off-line) coupling of SEC with matrix-assisted laser-desorption ionization mass spectrometry or for the (on-line) coupling of SEC with electrospray-ionization mass spectrometry narrow fractions are also highly desirable.

One prevailing conclusion of the present study is that it remains difficult to distinguish between the various contributions to the total observed peak width (i.e. extra-column, intra-column, and sample dispersion). An elegant technique to help in this process may be comprehensive two-dimensional liquid chromatography in a rather unconventional mode, using size-exclusion chromatography in both dimensions (SECxSEC). The fractions obtained from the first dimension will be more narrowly distributed than the initial sample. In the second dimension sample dispersion will thus play a much smaller role. If the extra-column band broadening can be kept sufficiently low, the observed dispersion after the first dimension will be determined by the column band broadening and by the sample dispersion. Ideally, the observed dispersion after the second dimension will be determined only by the column band broadening. We will report on the use of SEC × SEC for studying band broadening in SEC elsewhere [34].

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Appendix A. PDI for a Poisson distribution

A Poisson distribution depends on only one parameter, which is the mean of the distribution. In our case this is the number of monomeric units (degree of polymerization, n). The standard deviation of the Poisson distribution is the square root of n

$$\sigma_n = \sqrt{n} \tag{A.1}$$

In Eq. (2) we considered the standard deviation ($\sigma = \sigma_n M_{\mathrm{mon}}$) of the distribution in molecular-weight units (Da) and the average molecular weight ($M_n = nM_{\mathrm{mon}}$). By dividing

both properties by the molecular weight of the monomeric unit (M_{mon}) we can rewrite Eq. (2) to obtain

$$\mathrm{PDI} = 1 + \left(\frac{{\sigma_n}^2}{n^2}\right) = 1 + \frac{1}{n} = 1 + \frac{M_{mon}}{M_n} \quad (A.2)$$

For monomeric weight of the styrene unit (M_{mon}) is 104 g/mol, and therefore

$$\mathrm{PDI} = 1 + \frac{104}{M_n} \quad (A.3)$$

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