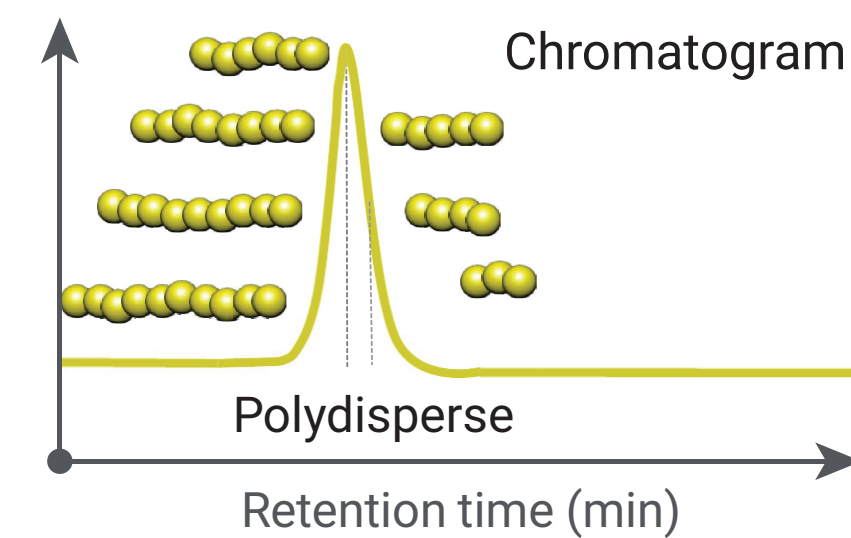
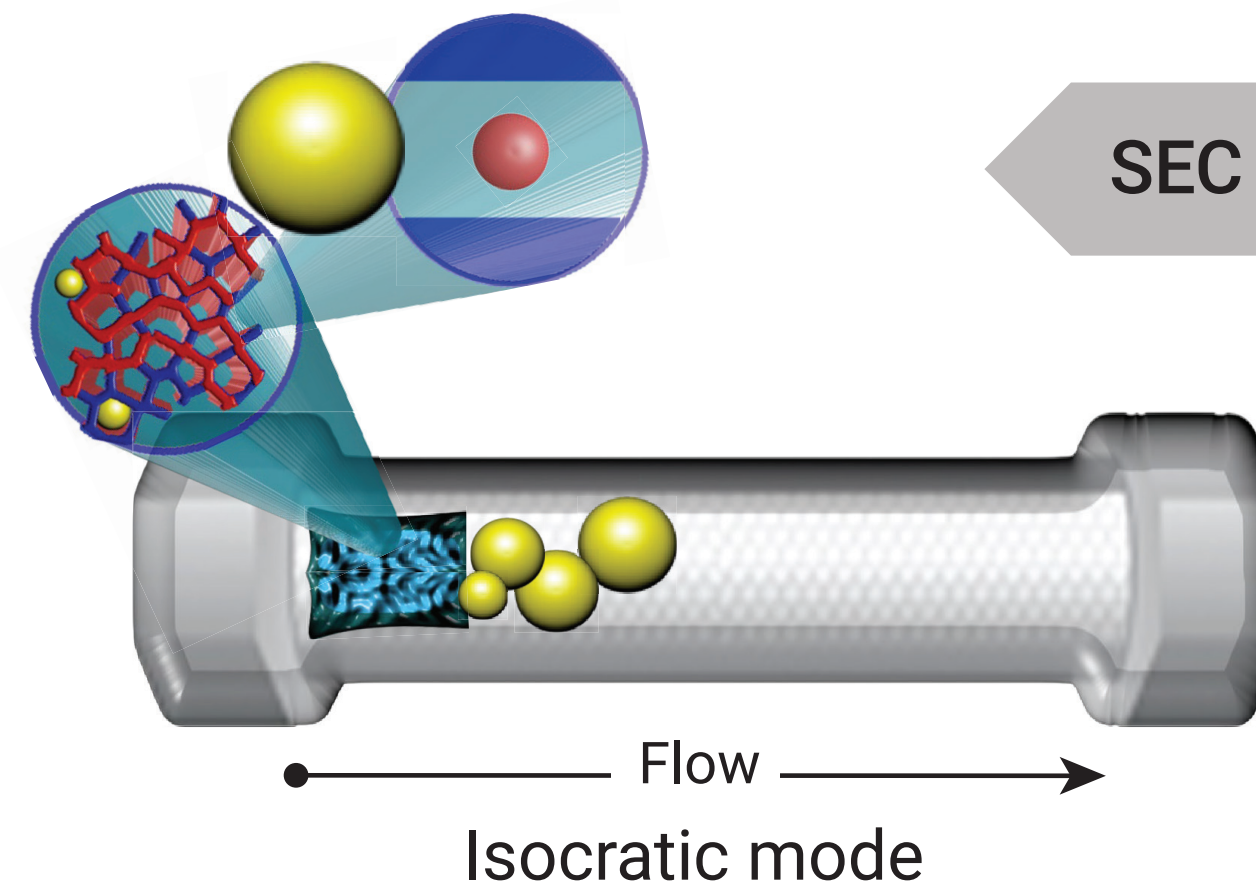


GPC/SEC eBook Series

GPC/SEC Theory and Background

What you should know when
you need to analyze polymers,
biopolymers, and proteins



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About this eBook series

GPC/SEC *Tips & Tricks* articles have been published in more than 60 editions of LC/GC's digital magazine *The Column* over the course of 10 years. These *Tips & Tricks* are designed to support GPC/SEC users in their daily work, providing comprehensive overviews on different aspects of this powerful technique.

To have all published topics at a glance, we created this series of eBooks.

The topics of these eBooks will cover:

- GPC/SEC theory and background
- GPC/SEC columns
- GPC/SEC detection
- GPC/SEC troubleshooting
- GPC/SEC applications

Each eBook contains 5 to 8 different *Tips & Tricks* publications that have been updated with the latest information, new examples, and figures.

To allow new users to GPC/SEC a continued reading experience, content has been edited, resulting in some differences compared to the original publications.

Nevertheless, the original spirit is maintained. So, the publications are independent references that allow users to read only the dedicated publication of interest.

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Introduction to GPC/SEC Theory and Background

Gel permeation chromatography (GPC), size exclusion chromatography (SEC), and gel filtration chromatography (GFC) are interchangeable synonyms used for a liquid chromatography (LC) technique to separate macromolecules based on their size in solution.

GPC/SEC is the method of choice to determine important molecular parameters of polymers, biopolymers, and proteins. For example, molar mass averages and the complete molar mass distribution can be determined with just one injection using standard LC equipment readily available in many analytical labs.

As these molecular parameters influence the macroscopic properties of materials, GPC/SEC is performed in QC and R&D alike. Applications can be found in all areas where macromolecules are used – from plastic products in the chemical industry, to proteins in the pharmaceutical industry, to biopolymers in the food industry.

One aspect of GPC/SEC is performing the actual analysis, while another is understanding and interpreting the results correctly. Even experienced scientists struggle to deal with long chains and molar mass averages instead of a defined molar mass.

The first two sections of this eBook will help you understand the meaning of molar mass averages and how the molar mass distribution is obtained from GPC/SEC raw data.

The third section elucidates calibration options in GPC/SEC and summarizes common techniques.

Sections four and five deal with accuracy, precision, and result uncertainty in GPC/SEC. The determination of result uncertainty will enhance the analytical quality substantially, so that results of sample comparisons can be interpreted more accurately.

1.1. A detailed look at molar mass averages

Why does a macromolecule have several molar masses and what do they indicate?

Unlike low molecular organic compounds, proteins, and DNA, macromolecules do not exhibit a definite molar mass. As they are composed of homologous chains differing in the number of repeat units, they exhibit molar mass distributions.

Molecular weights for macromolecules are generally described by statistical molar mass averages such as M_n , M_w , M_z . These averages and the molar mass distribution are required to describe a polymer.

The ratio of M_w and M_n yields the polydispersity index ($PDI = M_w/M_n$).

The molar mass averages M_n , M_w , and M_z are calculated by averaging over the number n ($\rightarrow M_n$) of polymer chains with a defined molar mass, or over their weight w ($\rightarrow M_w$). Thus, the averages are referred to as number-average molar mass (M_n), weight-average molar mass (M_w), or z-average molar mass (M_z) (where z refers to the German word “Zentrifuge”). This average can be determined by ultracentrifugation.

$$\bar{M}_n = \frac{\sum M_i \cdot n_i}{\sum n_i}$$

$$\bar{M}_w = \frac{\sum M_i \cdot w_i}{\sum w_i} \quad \text{with } w_i = M_i \cdot n_i \quad \rightarrow \quad \bar{M}_w = \frac{\sum M_i^2 \cdot n_i}{\sum M_i \cdot n_i}$$

$$\bar{M}_z = \frac{\sum M_i^3 \cdot n_i}{\sum M_i^2 \cdot n_i}$$

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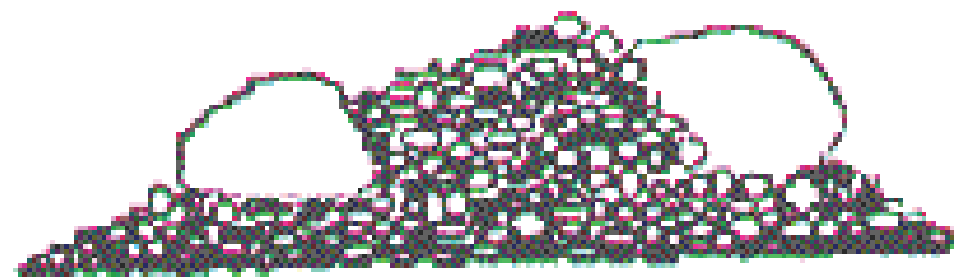
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A simple example with stones of different weights can help to understand molar mass averages¹:

Example 1

500 Stones of 1 kg = 500 kg
2 Stones of 250 kg = 500 kg
Total = 1,000 kg



Number-average molar mass (M_n):

$$\bar{M}_n = \frac{\sum M_i \cdot n_i}{\sum n_i} = \frac{1 \cdot 500 + 2 \cdot 250}{500 + 2} = 1.99$$

Weight-average molar mass (M_w):

$$\bar{M}_w = \frac{\sum M_i \cdot w_i}{\sum w_i} = \frac{1 \cdot 500 + 250 \cdot 500}{500 + 500} = 125.5$$

Polydispersity index (PDI):

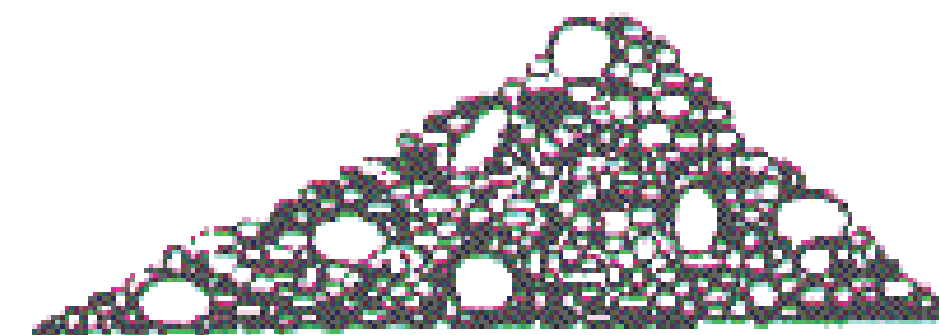
$$PDI = \frac{\bar{M}_w}{\bar{M}_n} = \frac{125.5}{1.99} = 63$$

¹Example taken from: Schlegel, A. Kunststoffe-Plastics (1957), p. 7

M_n , M_w , and PDI are important parameters that can be related to macroscopic properties of polymers such as solubility, rigidity, hardness, and viscosity. Polymers can have similar M_w values but display different M_n values. Their properties vary because they exhibit different

Example 2

400 Stones of 1 kg = 400 kg
100 Stones of 6 kg = 600 kg
Total = 1,000 kg



Number-average molar mass (M_n):

$$\bar{M}_n = \frac{\sum M_i \cdot n_i}{\sum n_i} = \frac{1 \cdot 400 + 6 \cdot 100}{400 + 100} = 2.00$$

Weight-average molar mass (M_w):

$$\bar{M}_w = \frac{\sum M_i \cdot w_i}{\sum w_i} = \frac{1 \cdot 400 + 6 \cdot 600}{400 + 600} = 4.00$$

Polydispersity index (PDI):

$$PDI = \frac{\bar{M}_w}{\bar{M}_n} = \frac{4.00}{2.00} = 2.0$$

molar mass distributions. These differences in average values and distribution are revealed by the PDI. Molar mass averages can be determined from the molar mass distribution, but reconstructing a molar mass distribution from the averages is not feasible.

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Figure 1 depicts an overlay of the molar mass distribution of two different samples with almost identical M_w . The molar mass distribution of one sample is called “narrow”, since the PDI is below 1.1. The other sample has a higher PDI of around 2, and is usually regarded as a broad distribution sample.

Figure 1 displays the molar mass range of both samples. Even for the low PDI sample, the molar mass ranges from 58,000 to 230,000 Da. The broad distribution sample spans a molar mass range from 4,000 to 800,000 Da, demonstrating that samples with a M_w of 124,000 Da can contain high molecular tails.

Also note that neither M_n nor M_w can be assigned to the peak maximum or the distribution.

M_w and M_n as average values cannot describe the whole polymer or indicate any of its limits.

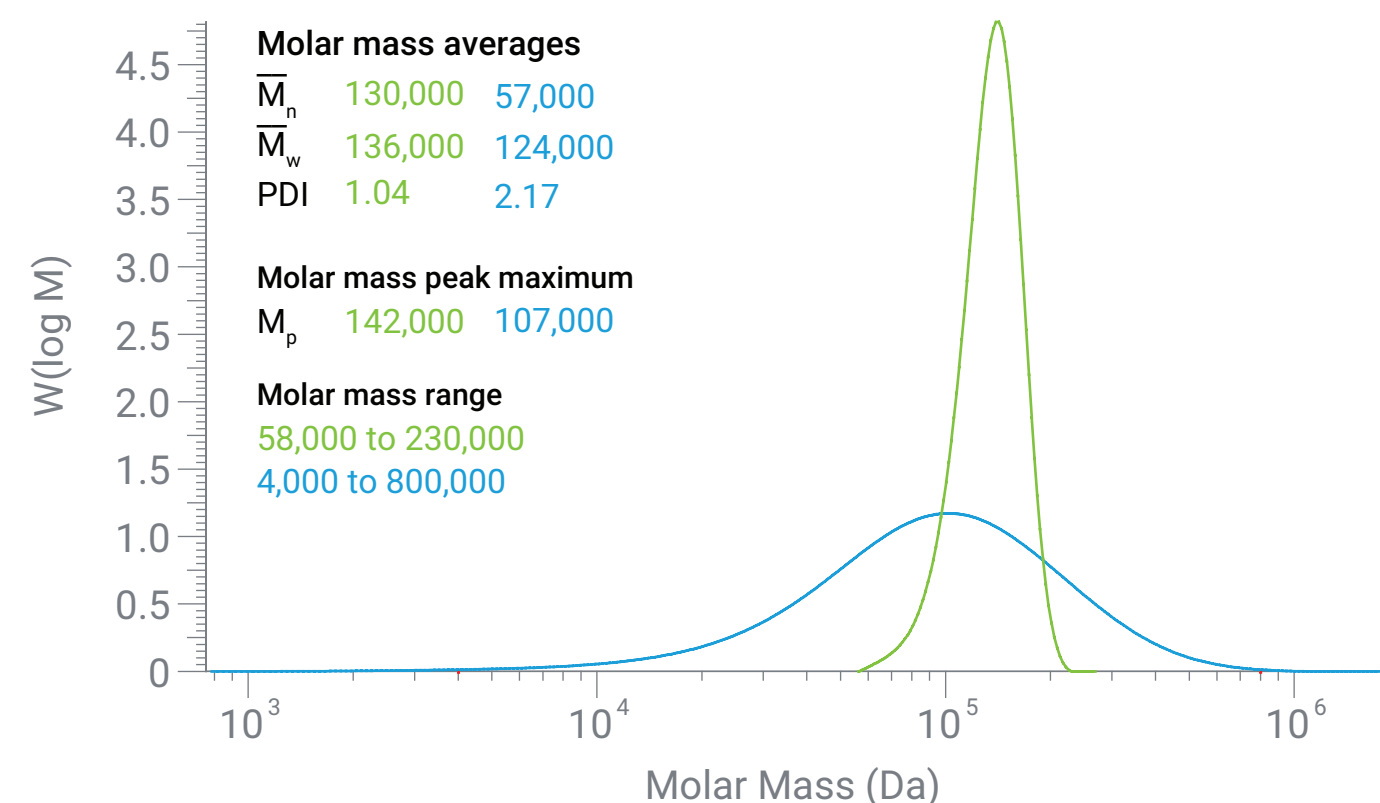


Figure 1. Comparison of samples with similar M_w but broad and narrow molar mass distributions.

How are molar mass averages measured?

Several characterization techniques are available to determine molar mass averages, based on two principles: nonfractionating and fractionating. Depending on the technique, M_n , M_w , or both can be determined.

Table 1 displays an overview of nonfractionating techniques that allow determination of a single average value.

Table 1. Techniques to determine bulk properties.

M_n	M_w
Osmometry (membrane, vapor pressure)	Static light scattering
Cryoscopy, Ebullioscopy	Dynamic light scattering
End-group analysis	Turbidimetry
NMR	Small angle x-ray scattering
	Small angle neutron scattering

There are only a few fractionation techniques that allow determination of molar mass distributions and both averages:

- GPC/SEC
- Ultracentrifugation
- Mass spectrometry/MALDI-TOF
- Field-flow fractionation

Due to its ease-of-use, GPC/SEC is the most used technique. It can be applied to a wide molar mass range, including higher molar masses (greater than 50,000 Da, the approximate limitation of MALDI-TOF) and broad distribution samples.

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Requirements for reliable GPC/SEC measurements

Dissolution

GPC/SEC requires complete dissolution. Ultrasonification should never be considered a means of sample preparation. Molar masses of polymers are frequently underestimated. Broad distribution samples with a low molar mass of 124,000 Da can easily encompass fractions up to 1,000,000 Da. GPC/SEC users should never underestimate the presence of high molar mass tails in the sample. This has an impact on dissolution time, which can require several hours. If ample dissolution time is not allowed, high molar mass fractions will not be completely dissolved, resulting in too low molar mass averages and PDIs.

Resolution

Insufficient separation occurs with columns of poor resolution or low exclusion limit. For efficient separation of all molar mass fractions and for best GPC/SEC results, high resolution columns covering a wide molar mass range are required. Combining several single porosity columns of different molar mass ranges or multiple mixed-bed columns of the same type is favorable over one single linear or mixed-bed column. An optimized column set enhances the separation capability of the polymeric analyte. Efficient separation is also required when molar mass sensitive detection, such as light scattering, viscosity, or triple detection is applied.

Detection

Every setup needs at least one concentration detector. Due to their universal applicability and ease-of-use, refractive index (RI) detectors are often favored over UV detectors or evaporative light scattering detectors (ELSD). GPC/SEC is often performed in multidetection mode.

Calibration

The values M_w and M_n are not related to a specific point in the chromatogram or the molar mass distribution. Therefore, these values should not be used to establish a calibration curve. The best value to build a calibration curve is M_p , the molar mass at peak maximum. Note that M_p is a distinct molar mass, and not a molar mass average. M_p can only be determined by GPC/SEC, and should be included in the certificate of every GPC/SEC calibration standard.

Originally published in *The Column*, October **2007**, by author Daniela Held.

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1.2. From a chromatogram to the molar mass distribution

Many macroscopic properties of macromolecules can be derived from their molar mass distribution (MMD). In contrast to molar mass averages such as M_n or M_w , which provide reduced information, the MMD describes the complete sample characteristics. Two samples can have the same molar mass averages, but have very different molar mass distributions, and therefore, macroscopic behavior.

What is the difference between a GPC/SEC chromatogram and an MMD?

The difference between an MMD and a chromatogram can be easily understood using the following example:

Two laboratories inject the same sample on different instruments. They have a different number of columns with different lengths and inner diameter, different tubing, and different detectors. So they obtain two different chromatograms as primary information. Without additional information, it is not possible to decide whether or not these chromatograms result from the same sample. It is not even possible to tell from the chromatogram if two peaks in the sample correspond to a species with a narrow or a broad molar mass distribution. A broader-looking peak can have a narrower molar mass distribution than a smaller peak, if the broad peak elutes in a column region with high resolution.

However, interlaboratory comparison and distribution information is easy to achieve if samples are evaluated, and MMDs are compared. This process eliminates the experimental conditions. Ultimately, only correctly calculated molar mass distributions allow the direct interlaboratory and long-term comparison of samples and sample properties.

How are chromatograms transformed into molar mass distributions?

The primary information of GPC/SEC measurements is a convolution of sample-related parameters and experimental conditions, called the apparent concentration distribution (chromatogram, $h(V)$).

The MMD can be calculated from the signal heights in the chromatogram by the slice method. This way, the eluted peak is separated into equidistant time, or more properly, volume slices.^{1,2}

Step 1:

First, change the retention axis (x-axis, elution volume) into a molar mass axis by using the information from the GPC/SEC calibration. There are several options to determine the molar mass/elution volume relation. From a practical point of view, the methods can be distinguished by either methods that use reference materials, or setups using static light scattering detectors to measure online molar masses for every sample.

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The most common technique when reference materials are used is to calibrate the system with polymer reference materials which have a narrow molar distribution.³

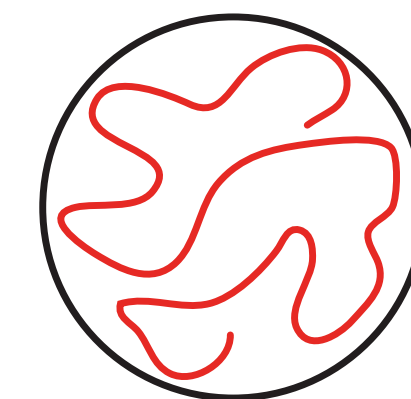
As GPC/SEC separates according to hydrodynamic volume and not by molar mass, only apparent molar masses (related to the calibration standards) are obtained if the calibration standards and the samples are chemically and/or structurally different. The deviation of the molar mass averages and the molar mass distribution can easily be in the range of 20% up to 100%. Since the results for different samples can still be compared to each other, and the method is robust and easy-to-use, many laboratories apply such procedures for quality control and sample comparison, as well as for applications where the absolute molar mass is not required.

Calibration methods to overcome this limitation involve the use of matching reference materials, or the use of any of the following techniques:

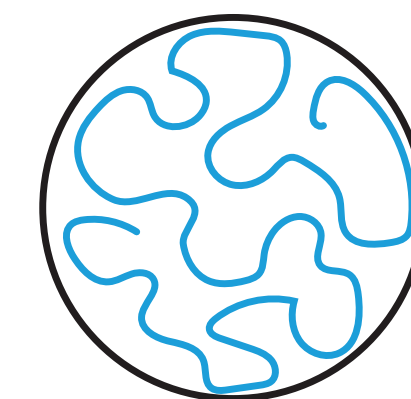
- Universal calibration using Mark-Houwink coefficients
- Broad standard calibration⁷
- Cumulative match calibration⁴
- Calibration using an online viscometer⁵

Another popular approach is the use of online static light scattering detectors, such as MALS, RALS, or LALS for suitable samples.⁶

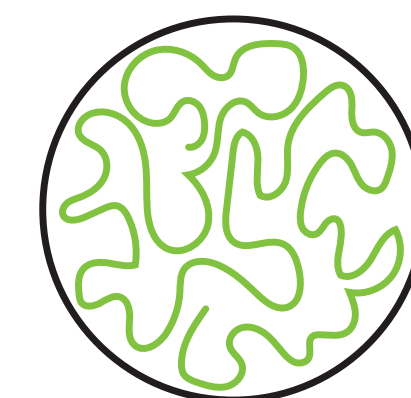
Different polymer types in the same solvent



Polyisoprene (PI)
M = 15,000 Da



Polystyrene (PS)
M = 20,000 Da



PMMA
M = 24,000 Da

$$V_h(\text{PI}) = V_h(\text{PS}) = V_h(\text{PMMA})$$

$$M(\text{PI}) < M(\text{PS}) < M(\text{PMMA})$$

Figure 1. GPC/SEC separates based on hydrodynamic volume V_h ; all sizes will elute at the same volume.

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Step 2:

Secondly, convert the Y-axis into mass fractions $w(\lg M)$ (one molar mass increment). This is necessary because detector signals in a chromatogram are recorded at a constant time interval. However, a molar mass distribution necessitates a constant concentration in a molar mass interval.

When determining the correct molar mass distribution, the normalized signal height, h_i , must be corrected with the slope of the calibration curve. This correction can only be neglected in the case of strictly linear calibration curves over the complete separation range, a feature which most commercial linear mixed-bed columns usually do not exhibit. As soon as a typical GPC/SEC fit function (e.g., cubic fit, polynomial 3, polynomial 5, etc.) is used to achieve higher result accuracy, the correction is necessary because the data recording occurs linear in time, while the molar mass change is not linear. For the same measured height, h_i , the number of polymer chains on the high molar mass fraction of the elugram is much smaller than on the low molar mass fraction.

The differential distribution, $w(M)$, of the molar mass M , is defined as $w(M) = dm/dM$, the mass fraction (m) of the molecule in a dM interval (molar mass).

By simple transformation, $w(M)$ can be expressed by measured quantities, with $h(V)$, detector signal and $\sigma(V)$, slope of the calibration curve.

$$w(M) = \frac{h(V)}{M(V)\sigma(V)} \quad ; \quad \sigma(V) = \frac{d \lg M}{dV}$$

The molar mass averages can be calculated from the moments, μ_i , of the molar mass distribution.

Number-average molar mass:

$$M_n = \frac{\sum h(M)}{\sum h(M)/M}$$

Weight-average molar mass:

$$M_w = \frac{\sum h(M)M}{\sum h(M)}$$

Note that GPC software modules of many HPLC chromatography software programs do not perform the correction with the slope of the calibration curve. This results in wrong molar mass distributions for all setups, with typical nonlinear GPC/SEC calibration behavior. The errors that occur as a result will increase with the width of the sample, and decrease with the data recording

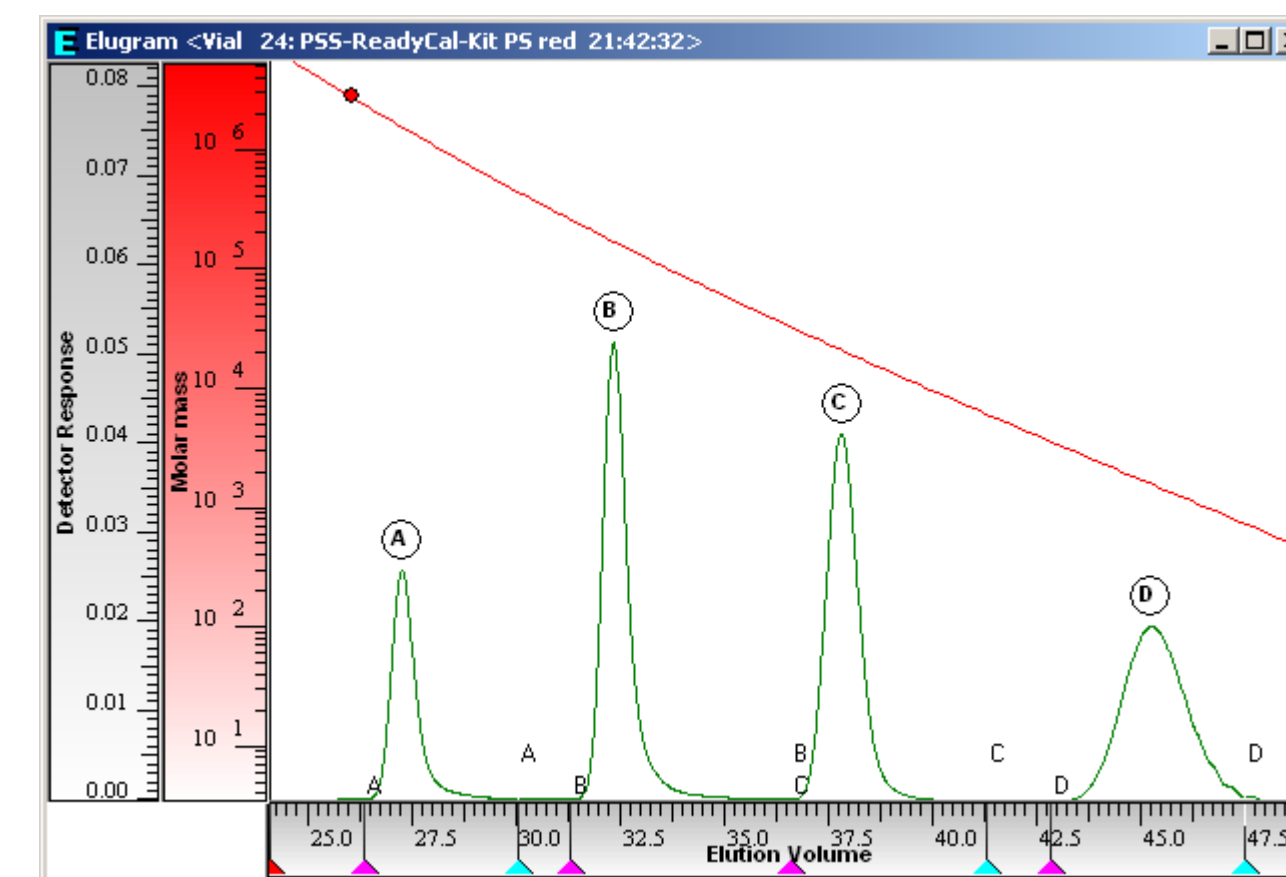


Figure 2. While a chromatogram shows the detector signal height (Y-axis) versus the elution volume (X-axis), an MMD displays $w(\lg M)$ on the Y-axis versus $\lg M$ on the X-axis. Peak shape and breadth can change during transformation depending on the slope of the nonlinear calibration curve (displayed in red).

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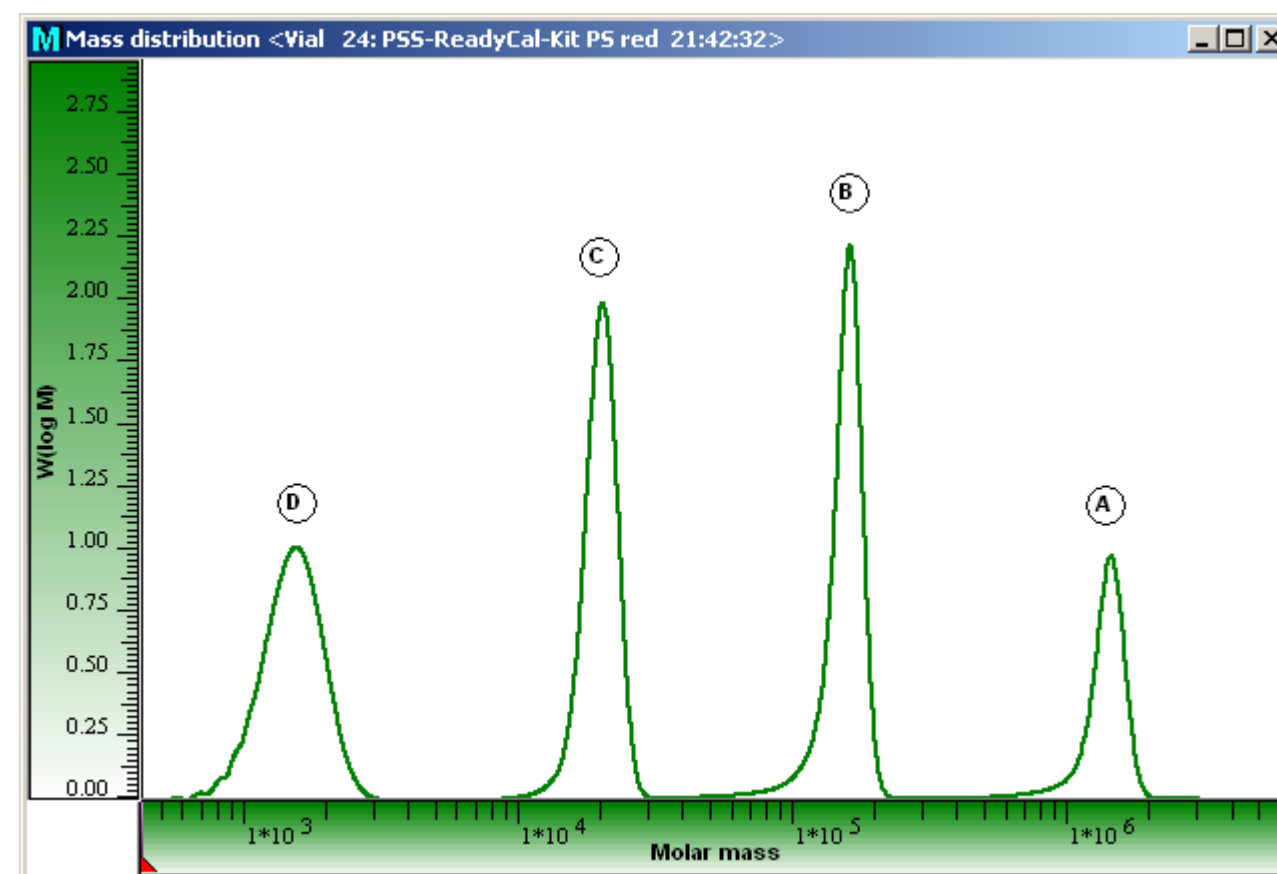


Figure 3. Chromatogram versus molar mass distribution. An MMD displays $w(\log M)$ on the Y-axis versus $\log M$ on the X-axis. Graphs that display the signal height or similar on the Y-axis probably do not show true molar mass distributions.)

frequency. This is dangerous when submitting GPC/SEC results to regulatory organizations such as FDA or EMEA, or for REACH registration.

Figure 4 compares a true MMD with a molar mass diagram obtained when neglecting the correct transfer to $w(\log M)$. It clearly depicts that peak position (molar mass) and peak width (PDI) can differ. Molar mass averages are often not affected by this phenomenon, because these are usually calculated separately from distribution curves. Thus, molar mass distributions yield unmodified information and allow direct comparison of product specifications.

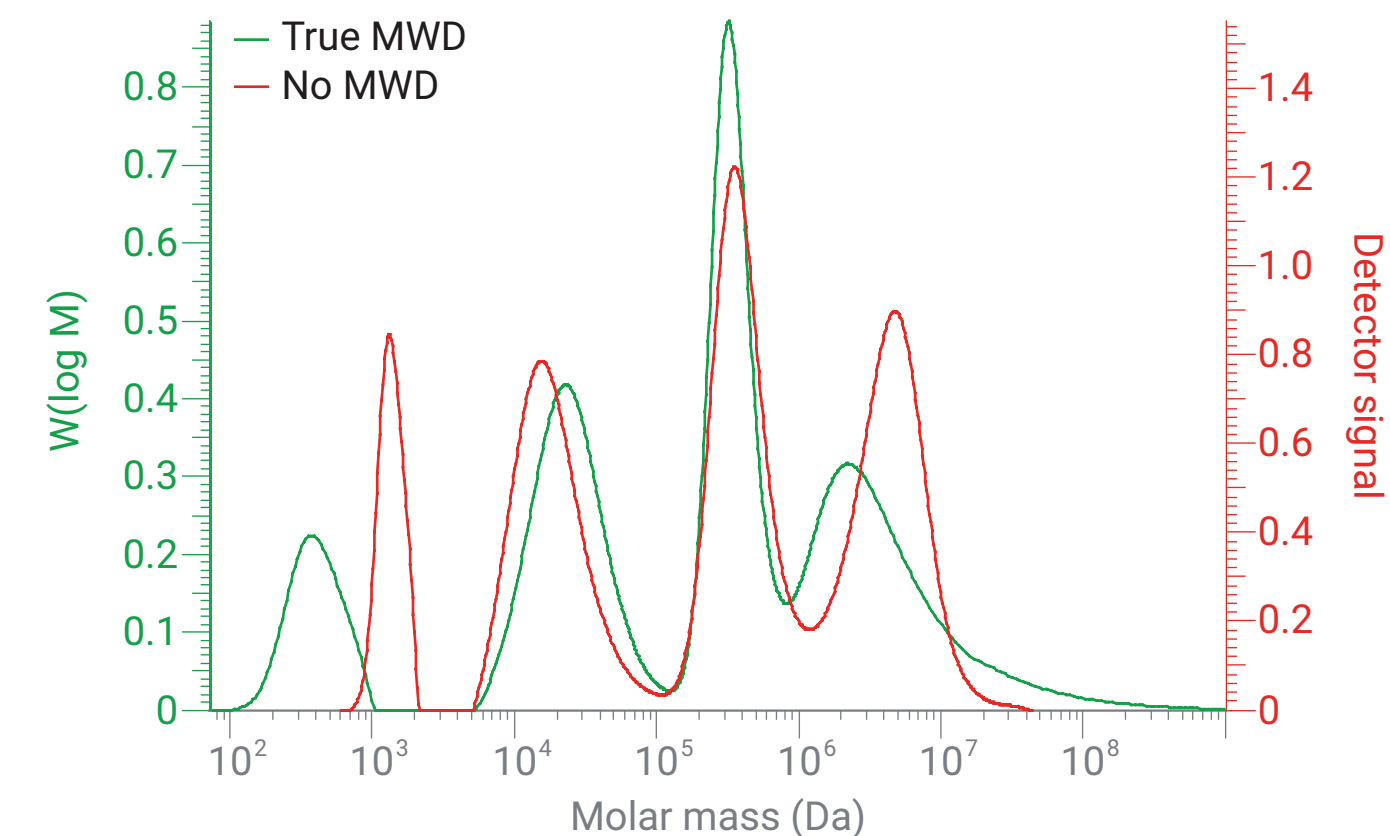


Figure 4. Overlay of an MMD (green) with a molar mass diagram falsely processed by HPLC software (red).

An easy test can show if molar mass distributions or molar mass diagrams are displayed. Inject a polymer standard mixture at the same concentration onto a GPC/SEC column (not a linear or mixed-bed column) and generate a nonlinear molar mass calibration using any polynomial fit function (e.g., cubic fit, 3rd polynomial). Analyze a standard mixture and plot molar mass distribution. If peak heights and peak widths do not vary, the software has not calculated molar mass distributions, just molar mass-scaled chromatograms (compare with Figure 2).

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1.3. How do I calibrate a GPC/SEC system?

GPC/SEC is the method of choice for characterizing polymers by determining their molar mass averages, as well as their distributions (MMD). This easy-to-use technique can be performed on standard LC equipment using proper high-resolution GPC/SEC columns and dedicated GPC/SEC software for data acquisition and analysis.

Why does GPC/SEC require a calibration?

The primary information obtained by standard GPC/SEC detectors (UV, ELSD, or RI) is not molar mass, but apparent concentration at a certain elution volume. A combination of calibration curve and concentration profile from a concentration detector is required to calculate all molar mass averages and MMD.^{1,2} GPC/SEC is therefore regarded as a relative technique. Calibration of columns is based on assigning an elution volume to a molar mass. This is the opposite of HPLC procedures, which rely on calibrating and assigning a concentration to a detector response (signal intensity, peak area).

What are the general characteristics of a GPC/SEC calibration curve?

For a GPC/SEC calibration curve, the logarithm of molar mass is plotted against elution volume. Most calibration curves, including those for linear or mixed-bed columns, have a sigmoidal shape that agrees with the fundamental separation characteristics. This contrasts with other calibrations in chromatography, where linear calibration curves are established by plotting peak area versus concentration.

GPC/SEC calibration curves can be divided into three distinct regions, as shown in Figure 1. Region one is the region where insufficient separation of large-sized polymeric species takes place. The pores are too small to separate these according to their hydrodynamic volume. This region is referred to as the exclusion limit of the column set, where large species, independent of their size, elute at the same volume. Region two reflects the optimum separation range of a column set. Polymers are separated according to their size in solution. Larger fractions elute first, and fractions of smaller hydrodynamic volume elute at higher elution volumes. In region three (total permeation volume), separation can be retarded due to temporary interaction. The determined elution volume is not only related to the molar mass of the polymer, but also to its solution structure and chemistry.

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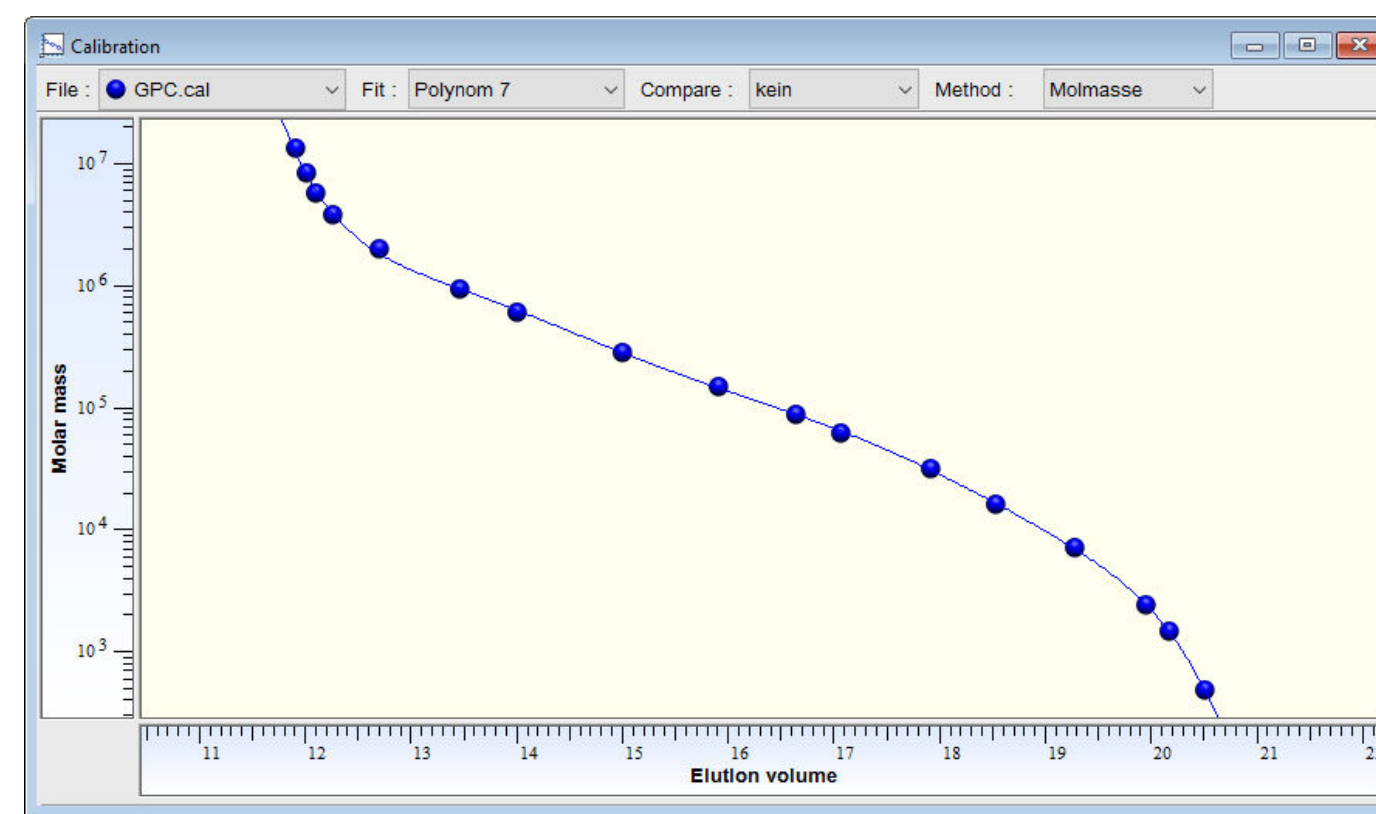


Figure 1. Typical GPC/SEC calibration curve with three distinct regions. The recommended sample concentration for calibrating depends on the molar mass (shown on the Y-axis).

How are GPC/SEC calibration curves created?

There are several methods for establishing a calibration curve. Table 1 gives an overview of the different methods, including their advantages and disadvantages. All methods described in Table 1 are based on the use of molar mass calibration standards.

The most popular method for calibration uses narrow distribution standards. Elution volumes of standard peaks are determined at peak maximum, and plotted against the logarithm of their molar mass. Good GPC/SEC practices recommend at least three standards per molar mass decade.⁴ Although concentration is not needed to obtain accurate GPC/SEC results, it is important to inject narrow distribution standards at a reasonable concentration.⁵ Figure 1 also displays the recommended concentration range (Y-axis) that is dependent on the molar mass of the standard.

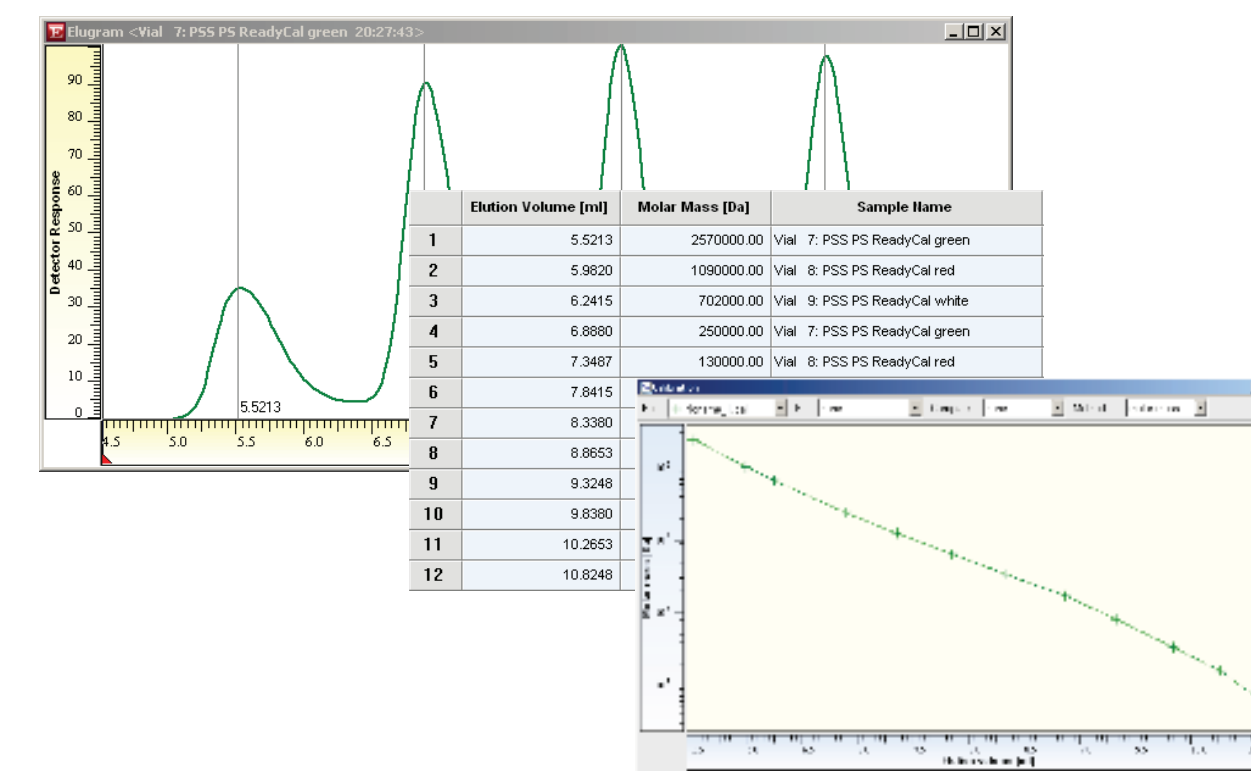


Figure 2. Calibration with narrow distribution standards.

After measuring elution volumes and plotting these against the logarithm of molar mass (usually peak molar masses²), a fit function describing the shape of the calibration curve has to be selected. Unfortunately, there is no recommendation for an optimal fit function for a given column set, so users must select a suitable fit function based on multiple factors.

Fit functions generally will not be linear. These fits are calculated based on standard polynomial functions from third (cubic) to seventh order. There are also fit functions available based on modified polynomial functions so that typical pitfalls are avoided (calibration functions).

Another possibility to measure the relationship between molar mass and elution volume is by combining a concentration detector (RI, UV, ELSD) with an online light scattering detector (LALS, RALS, or MALS, but not ELSD). With a single standard, the light scattering detector constant (calibration constant) and the concentration

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Table 1. GPC/SEC calibration methods and their advantages/disadvantages.

Calibration With	Advantages	Disadvantages
Narrow GPC/SEC standards	<ul style="list-style-type: none">– Easy and straight forward– Precise method³– Accurate method³ for samples and standards of the same structural and chemical nature– Accuracy based on the average deviation of all calibrants	<ul style="list-style-type: none">– Results only accurate for compounds of the same structural and chemical nature– Narrow distribution standards not available for all polymer types
Well-characterized broad distribution standards	<ul style="list-style-type: none">– Easy and accurate– Ideal for internal referencing of analytical conditions as internal standard	<ul style="list-style-type: none">– Calibration only accurate for compounds of the same structural and chemical nature– Only limited amount of standards commercially available– Single broad standard covers only a limited molar mass range– Not all software is capable of applying this method
Integral calibration/cumulative calibration	<ul style="list-style-type: none">– Easy and accurate– Calibration accurate for compounds of the same structural and chemical nature	<ul style="list-style-type: none">– Only a limited amount of standards commercially available– Accuracy is limited by quality of standard– High and low molecular regions are less accurate (requires extrapolation)
Narrow distribution standards and Mark-Houwink coefficients (universal calibration I)	<ul style="list-style-type: none">– Easy and accurate– Matching calibration curves for different polymer types can be established from a single curve	<ul style="list-style-type: none">– Calibration is precise, but only accurate for a particular polymer type– Precision depends on the accuracy of the Mark-Houwink coefficients
Narrow distribution standards and additional online viscometer detection (universal calibration II)	<ul style="list-style-type: none">– Easy and accurate– One calibration curve valid for all types of polymers	<ul style="list-style-type: none">– Increased experimental complexity and cost– Additional experimental error from concentration dependence, band broadening, and interdetector delay

detector constant (response factor) are determined. Based on the constants, true molar mass averages are calculated. The resulting curve reflects the molar mass plot. Light scattering software often tends to show fitted data rather than raw data. For smooth molar mass plots, a fit must be applied.

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How can I decide if the best GPC/SEC calibration fit has been selected?

There are three factors at hand that help the user decide if a suitable function has been selected:

- Regression coefficient, R^2
- Deviation of the calibration point from the fitted value (average deviation)
- Slope of the calibration curve

These choices are illustrated in Table 2, which shows regression coefficients for identical calibration data with different fit functions, and average deviation for all data points. When choosing an optimal calibration fit function, the regression coefficient is not the best parameter to consider because large average deviations are observed, even for a regression coefficient very close to unity. If the data processing software provides the regression coefficient as the only selection criteria for the fit function, a value of >0.999 should be achieved for GPC/SEC results with highest precision.

Table 2. Influence of the calibration fit function on regression coefficients.

Fit Function	R^2	Average Deviation (%)
Linear (square)	0.9925	30.2
Polynomial 3 (cubic)	0.9986	10.4
Polynomial 5	0.9995	7.35
Polynomial 7*	0.9999	3.57
PSS Polynomial 7	0.9998	4.92

* First derivative is intermittent; this function should not be used.

Table 2 also shows that the regression coefficient increases and the average deviation becomes smaller when selecting a polynomial function of higher degree. However, it is not recommended to use the highest order function that generates the lowest average deviation. The shape of the calibration curve is more important than small deviations, which should be in general agreement with the separation mechanism. Therefore, a good measure is the first derivative of the calibration curve (slope).

Figure 4 depicts an ideal first derivative for a calibration curve. The slope only changes close to exclusion limit as well as total permeation volume and is constant for the optimum separation range. If a 7th order polynomial fit function is chosen (see Figure 3), the slope is not constant and local maxima and minima appear. This fit function should therefore be avoided, since it can generate artifacts such as shoulders in the MMD that are not related to sample characteristics.⁵

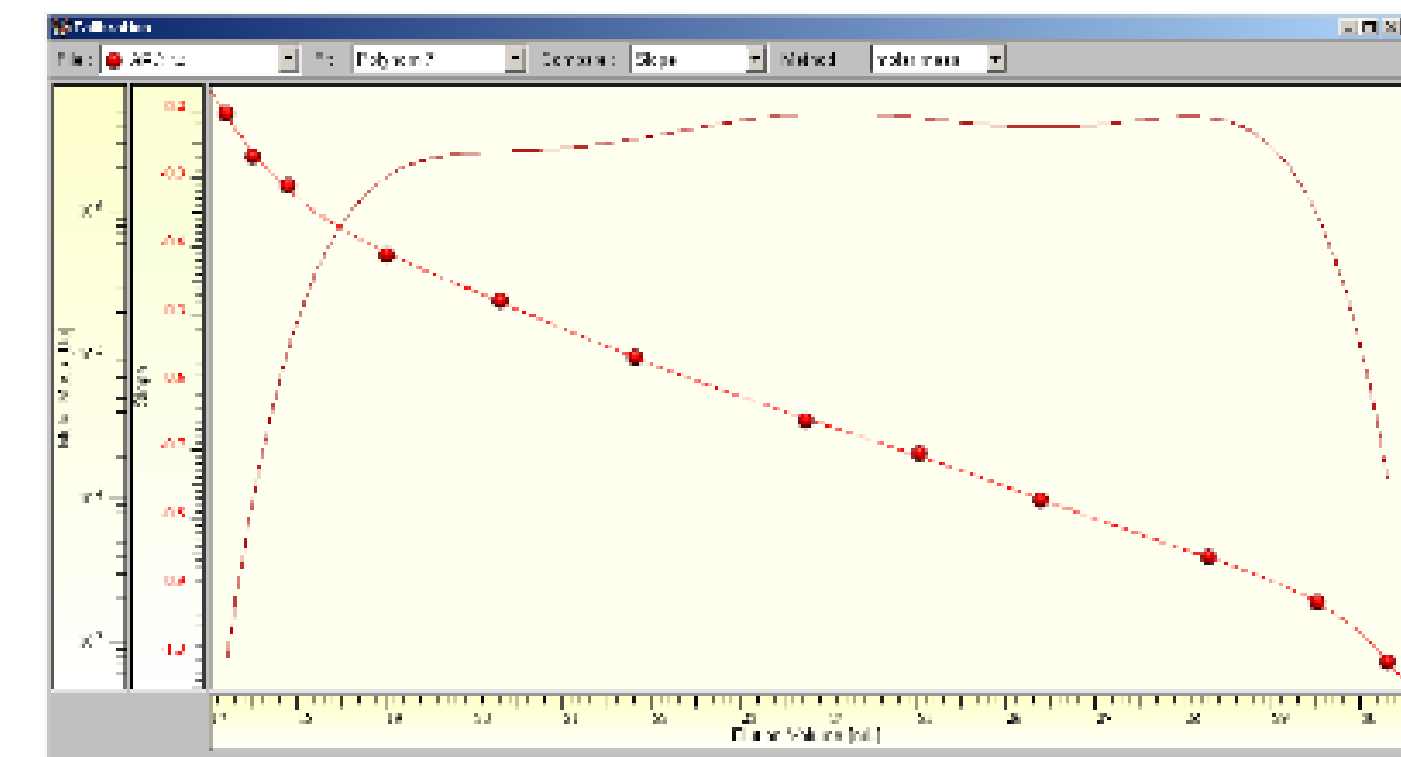


Figure 3. Calibration curve with seventh-order polynomial fit function. The first derivative shows locally false maxima and minima. This function is not suitable.

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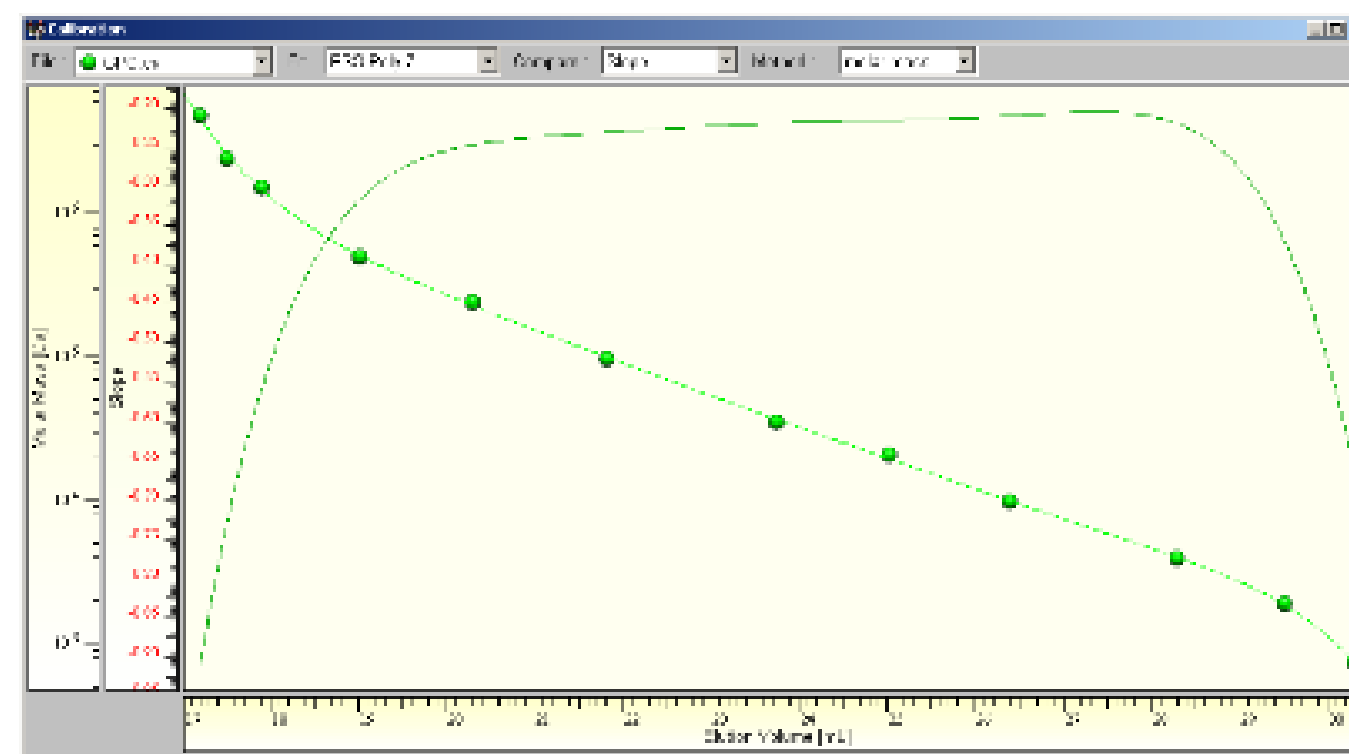


Figure 4. Calibration curve with PSS fit function Poly 7: The maxima and minima are eliminated, so this function is recommended. The selected fit function can be used.

How often should I recalibrate ?

It depends. Many analytical laboratories calibrate their column sets before and after the samples have been run. This is a fast way to assure columns are still separating properly after sample analysis. The time and effort required for this quality assurance approach has been significantly reduced by modern GPC/SEC software that offers automated calibration/recalibration routines in combination with convenient single-use calibration sets in autosampler vials, such as EasiVial and ReadyCal kits, to which solvent alone must be added.

Another approach is to run a validation sample with every sample sequence. If the results of the validation sample fall within a defined range, recalibration is not required. The use of an internal flow marker/standard when calibrants and samples are run at different times marks good practice for providing long-term reproducibility. This is

a low molar mass compound eluting at the end of the chromatogram.⁶ The highest accuracy and precision can be achieved with minimum effort, if calibration and sample runs are correlated with this internal reference.

A good rule to follow is to increase the recalibration frequency when many different samples are run on the same columns, and/or if sample purity is questionable (e.g. when the sample might contain low molecular impurities from manufacturing processes).

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1.4. Accuracy and precision in GPC/SEC

Any analytical technique has intrinsic inaccuracy due to a variety of factors. To interpret results correctly, it is important to know the inherent precision and accuracy of the analytical technique. Furthermore, it is necessary to know how these factors can be influenced and improved with simple tools, or proper experimental setup.

Before discussing the tools, generic definitions of factors are as follows:

- The accuracy of an analytical procedure expresses the closeness of the agreement between the value accepted (either as a conventional (true) value, or a generally accepted reference value), and the value found.
- The precision of an analytical procedure expresses the closeness of the agreement (degree of scatter) between a series of measurements obtained from multiple samplings of the same homogeneous sample under the same conditions.

In more detail, precision can be discussed in terms of short-term and long-term precision:

- Repeatability describes short-term intralaboratory empirical variance of results of multiple measurements of a sample.
- Intermediate precision expresses long-term intralaboratory variations.

Another important term is reproducibility, assessed by means of interlaboratory deviations.

Accuracy and precision

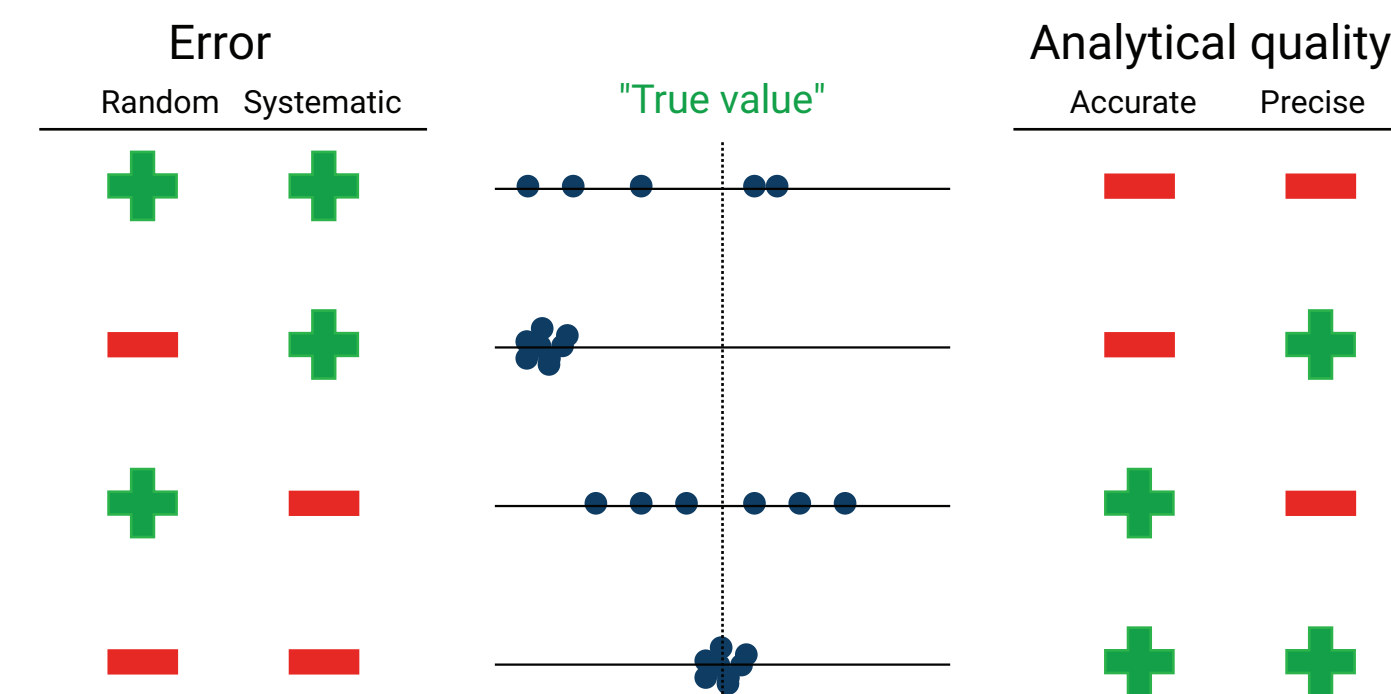


Figure 1. Difference between accuracy and precision.

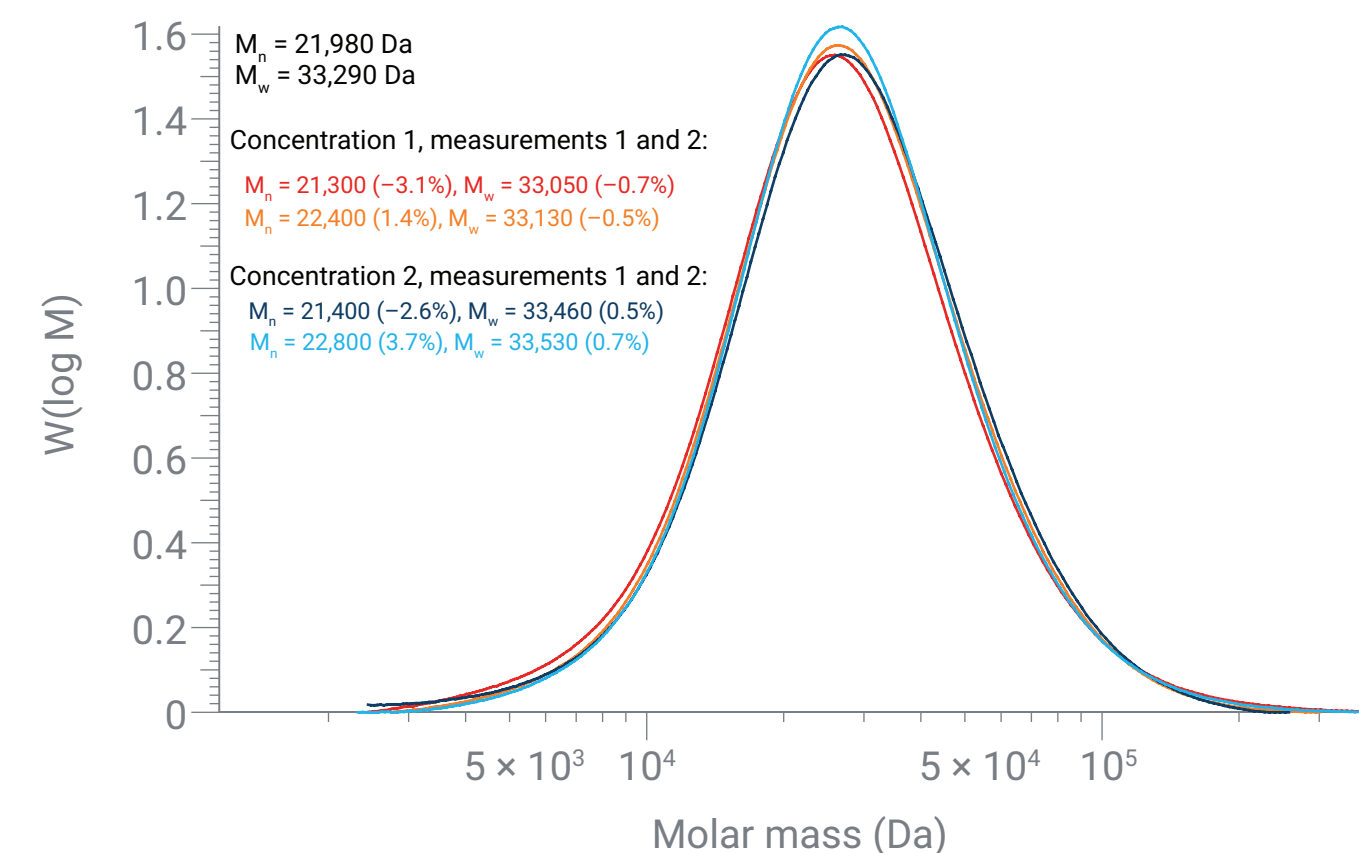


Figure 2. Repeatability of an aqueous GPC/SEC run (two concentrations, duplicate injections).

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How accurate is GPC/SEC?

GPC/SEC is a relative method - the obtained molar masses can only be accurate if the calibration standards match the chemical composition of the analyzed samples.

Standards like ISO 13885 for GPC/SEC do not give references for accuracy, but many analytical labs report an inaccuracy of <5% for M_w , and 10 to 15% for M_n , depending on the complexity of the samples. In cases where no matching calibration standards are available, deviations of 100% are possible.

Fortunately, many GPC/SEC users focus only on repeatable and precise measurements, such as when quality control of products/product comparison is required. However, if accurate results of true molar masses are necessary, several options are available to overcome the limitation of unavailability of matching calibration standards:

- Universal calibration with Mark-Houwink coefficients
- Broad calibration
- Integral calibration
- Use of molar mass-sensitive detectors, such as online viscometers or light scattering detectors

For all options, reference values are required. Accuracy of results for an unknown sample depends strongly on the accuracy of reference values. This is also true for GPC/SEC runs with light scattering detection (usually referred to as an absolute method). Here, accuracy of evaluation parameters and constants also influence the accuracy of the results.

To achieve the highest accuracy, careful calibration of the system and precise evaluation is required. National and international guidelines such as ISO EN 13885 for GPC/SEC¹ provide valuable information and describe correct evaluation, with separate baseline and integration limits and proper calibration procedures. From an instrument point of view, pumps with a high flow precision are needed, along with sensitive detectors. The columns used should be in good condition, and should be suited for the molar mass range in which the samples are expected. Column sets (e.g., a combination of columns with different porosities) can provide more accurate results than single (linear or mixed-bed) columns, due to better resolution and efficiency.

How precise is GPC/SEC?

Precision can be discussed as short-term precision (repeatability) and as long-term precision (intermediate precision). Several round-robin tests provide results for repeatability and interlaboratory reproducibility, as shown in Table 1. These results were obtained from complex samples with broad molar mass distributions.

Table 1. Precision and reproducibility for selected solvents.

	THF	DMA	H ₂ O	THF	DMA	H ₂ O
	Precision/Repeatability			Reproducibility		
M_n	3%	2%	2%	15%	15%	15%
M_w	2%	2%	2%	10%	15%	15%
M_z	3%	3%	3%	15%	24%	24%
M_n/M_w	3%	3%	3%	15%	24%	24%

Repeatability is an important element of method validation. The repeatability can be improved when working with standardized calculation algorithms.

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Separate baseline and integration limits increase the repeatability, especially when broad distribution samples with a high amount of low molecular species and oligomers are investigated.⁴ The use of a low molar mass internal standard as a flow marker is also recommended.^{2,5}

Typical variations that influence the intermediate precision include different days, different equipment, and different operators. Separation columns also play an important role. It is essential that suitable column material is used that allows interaction-free, size-based separation.³ Without suitable material, slight recipe changes by the column manufacturer, or new columns made from a different batch, might lead to different interactions. This would then lead to systematic deviations, and therefore, low intermediate precision.

The intermediate precision can be improved by establishing stringent workflows for system setup, sample preparation, calibration, and data processing. It is recommended to allocate a column set for each product group and avoid running different applications on the same column. This practice should be considered when running samples with reactive groups (e.g., isocyanate, amine, polyol). Aqueous applications with polyelectrolytes (e.g., polyanions, polycations) can lead to interactions with the packing surface. Rigorous quality control (as in pharmaceutical applications) could eliminate potential

problems by reserving column batches.

Typical deviations for reproducibility are reported in Table 1. Other critical applications, such as GPC/SEC on polyelectrolytes or GPC/SEC for light scattering couplings could cause higher deviations. However, deviations can be substantially reduced by using the same equipment and the same data analysis software. Column sets should be produced from the same packing batch, using the same calibration standards and fit. This way, reproducibility deviations fall into the range of repeatability.

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1.5. Result uncertainty – how reliable are results?

GPC/SEC results and their validity are crucial in many applications such as QC/QA testing for product release, registration and accreditation of polymer-based products, or formulations at regulatory agencies such as FDA and REACH. Accuracy and precision of GPC/SEC results are key factors in this regard.

Determination of result uncertainty can help to select tolerance criteria during method development and validation. This eliminates time-consuming work and the high costs involved when tolerance limits of a validated method can no longer be met.

What does result uncertainty mean?

Many methodological aspects and experimental details can influence the results and quality of an analytical experiment.¹ Various systematic and random contributions impact the accuracy and precision of the final results. High analytical quality is only achieved if both systematic and random errors are eliminated.

Since many error sources contribute to overall deviation of the result from its true value, advanced error propagation calculations must be performed to get a reliable estimate of the final result uncertainty. While software can be used to do these calculations, it cannot control systematic errors that are specific to the user's environment. Software can only assess result uncertainty that contributes to random errors.

Definition of result uncertainty

GPC/SEC results with uncertainty numbers are reported in the following form:

result \pm result uncertainty (at a confidence level of one standard deviation)

This means that the result (G) of an analysis falls within $G - \Delta G$ and $G + \Delta G$ with a probability of 68%. Higher result uncertainties can easily be acquired by applying higher orders of significance, which can be obtained by using a factor greater than 1 for the uncertainty value. Generally, results are reported as:

$G \pm k \Delta G$ with $k = 1, 2, 3...$

The commonly accepted default value for result uncertainty is $k = 1$, which corresponds to a confidence level of 68% based on Gaussian statistics. Higher significance numbers can be obtained by using higher k-factors; e.g. a confidence level of 96% is obtained for $k = 2$, and a confidence level of 99.7% is achieved for $k = 3$.

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Figure 1 displays a molar mass distribution, including a typical result table (columns 1 and 2) and respective relative result uncertainty in percent (column 3).

In this example, the weight-average molar mass (M_w) is 4,304 Da, with an uncertainty of 3.84% (that translates to 165 Da). Consequently, the true M_w value of this sample will be between 4,139 Da and 4,469 Da, with a confidence level of about 68%. To achieve (practically) 100% confidence in a result, the error must be multiplied by 3 ($k = 3$), which means true M_w falls into the 3,808 to 4,800 Da range. These result uncertainties exemplify that results of independent experiments (repeats or good-bad comparisons) are identical to a validity of 68%, if individual results fall within confidence limits of 4,139 and 4,469 Da (99.7% being 3,808 Da and 4,800 Da).

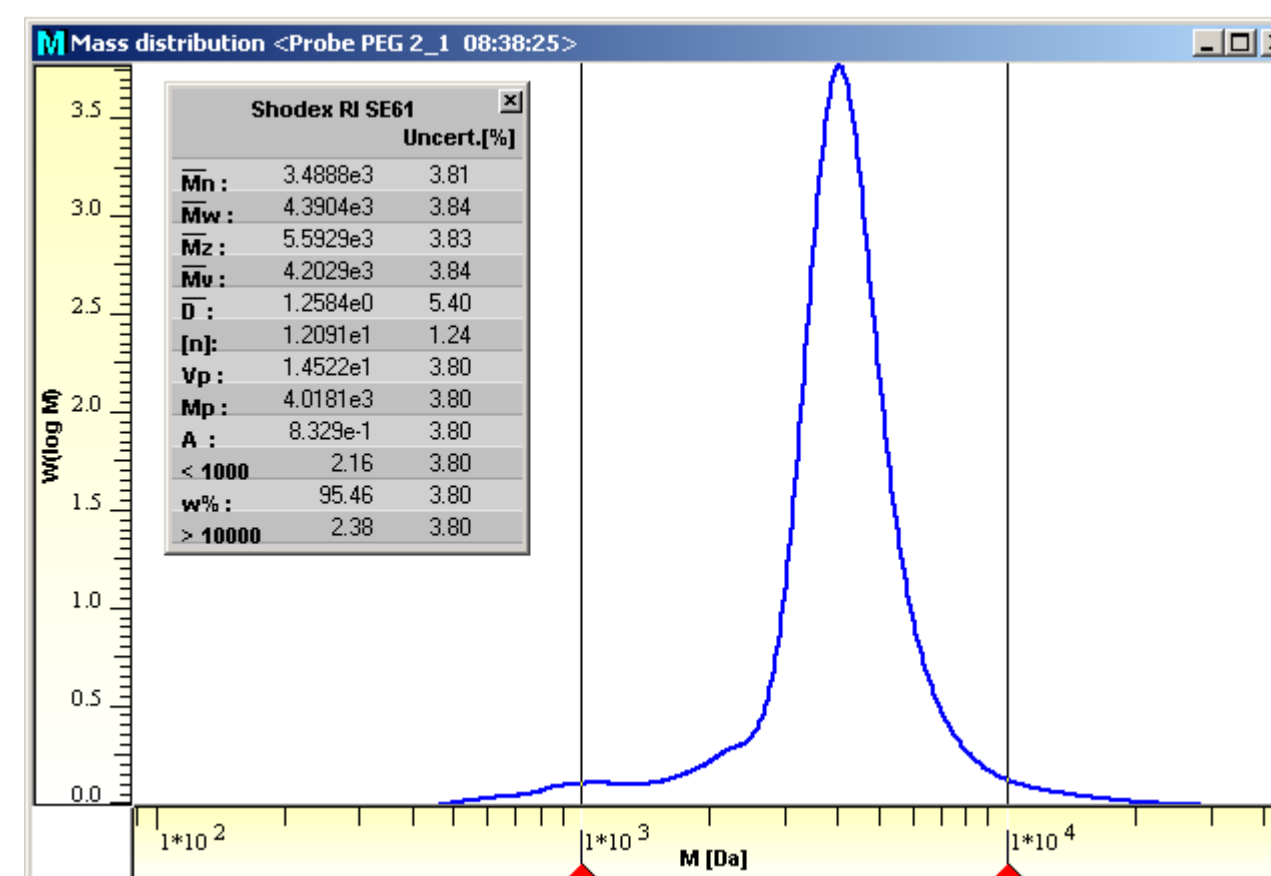


Figure 1. GPC/SEC results with molar mass distribution and respective relative result uncertainty in percent.

Systematic and random errors

Typical systematic errors in GPC/SEC experiments could be a result of:

- leaks in the GPC/SEC system
- invalid method parameters (wrong column set, eluent, or temperature)
- sample concentration resulting from incomplete dissolution
- molar mass calculation based on outdated calibration or incorrect sample parameters
- wrong injection volume
- incorrect use of DPT sensitivity factor in viscosity detection
- unknown or wrong dn/dc values from light scattering and/or triple detection
- incorrect instrument calibration factors in viscosity, triple detection and/or light scattering setups

These influences cannot be incorporated into the result uncertainty calculation by any GPC/SEC software. Fortunately, systematic errors do not occur frequently due to operator control.

Random error contributions have a graver impact, and are more difficult to control. Robust statistical models are available, and are well-suited to quantify the influence of random errors on result deviations from the true (or commonly accepted) value.²

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Typical contributions to random (statistical) error (amongst others) are:

- pump flow fluctuation
- old (noisy) UV lamps
- unpurged RI detector
- insufficient degassing of eluent and air bubbles
- inadequate calibration fit
- large variations in MALS detector normalization

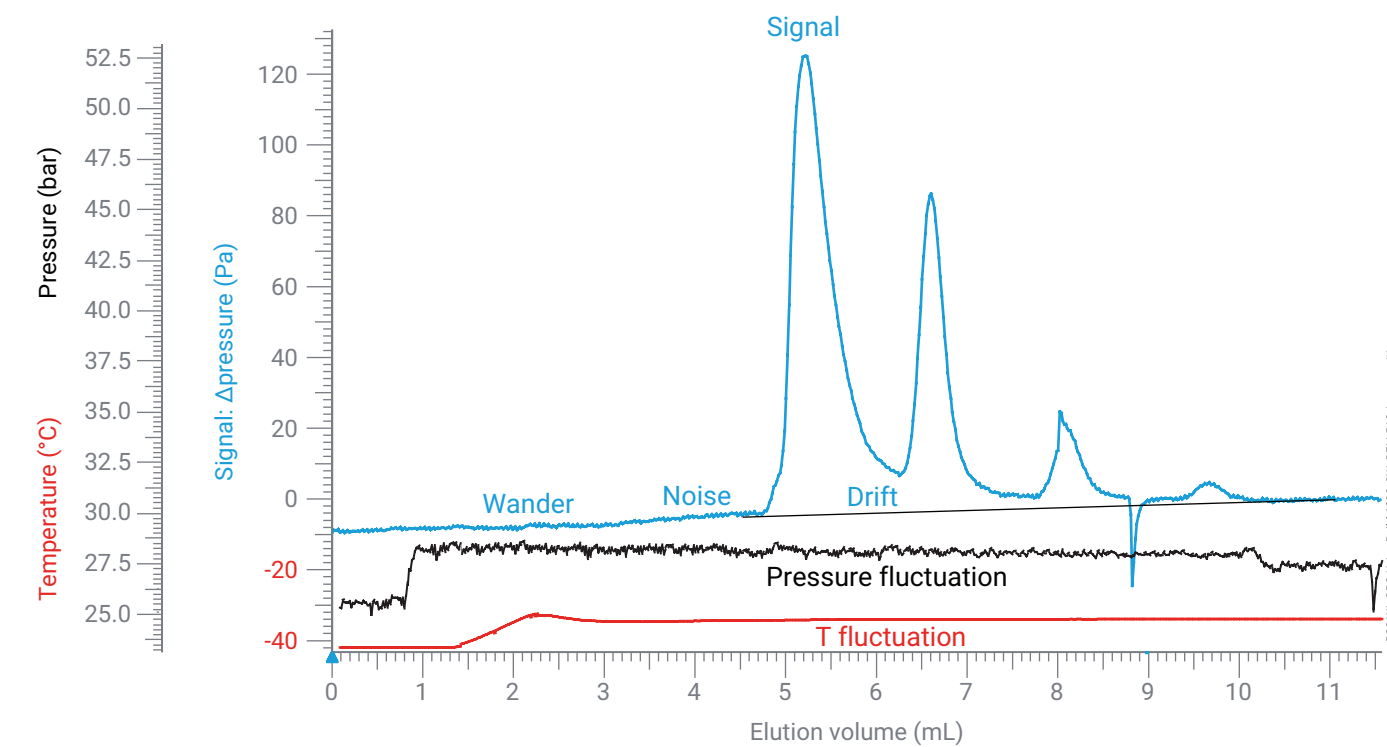


Figure 2. Different random errors contributing to result uncertainty.

Determination of result uncertainty

Final results will be affected by system properties in different ways, which must be taken into account when calculating overall result uncertainty.

For calculation, the software should therefore manage:

- flow stability
- pressure fluctuations
- temperature stability
- injection reproducibility
- signal noise, drift, and wander
- calibration range and quality
- precision of viscosity and light-scattering data

Uncertainty of a given parameter x , contributing to overall results, can be calculated from its standard deviation, σ_x . For example, temperature stability is calculated online from measured average temperature and standard deviation, according to:

$$T = \langle T \rangle \pm \Delta T$$

with: $\langle T \rangle$: average temperature

ΔT : standard deviation, σ_T

Due to the number of parameters, error propagation methods of the entire parameter set must be considered to determine the uncertainty value.³

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The analytical quality of each analysis can easily be judged by an uncertainty assessment, where contributions are summarized with respect to signal quality (e.g., detector noise), system stability (e.g., flow rate), and calibration quality (e.g., deviations).

Figure 3 shows an uncertainty assessment example of GPC/SEC results calculated from RI data. Here, the quality of the calibration is a major contributor to result precision. System stability and signal quality contribute to a much lesser extent. To improve result quality, the user can first optimize the calibration (conventional, universal, or light scattering). Additionally, results will be further improved if the user repeats the measurement with better signal quality (stabilized detector signal, optimized injection volume, and/or injection concentration).

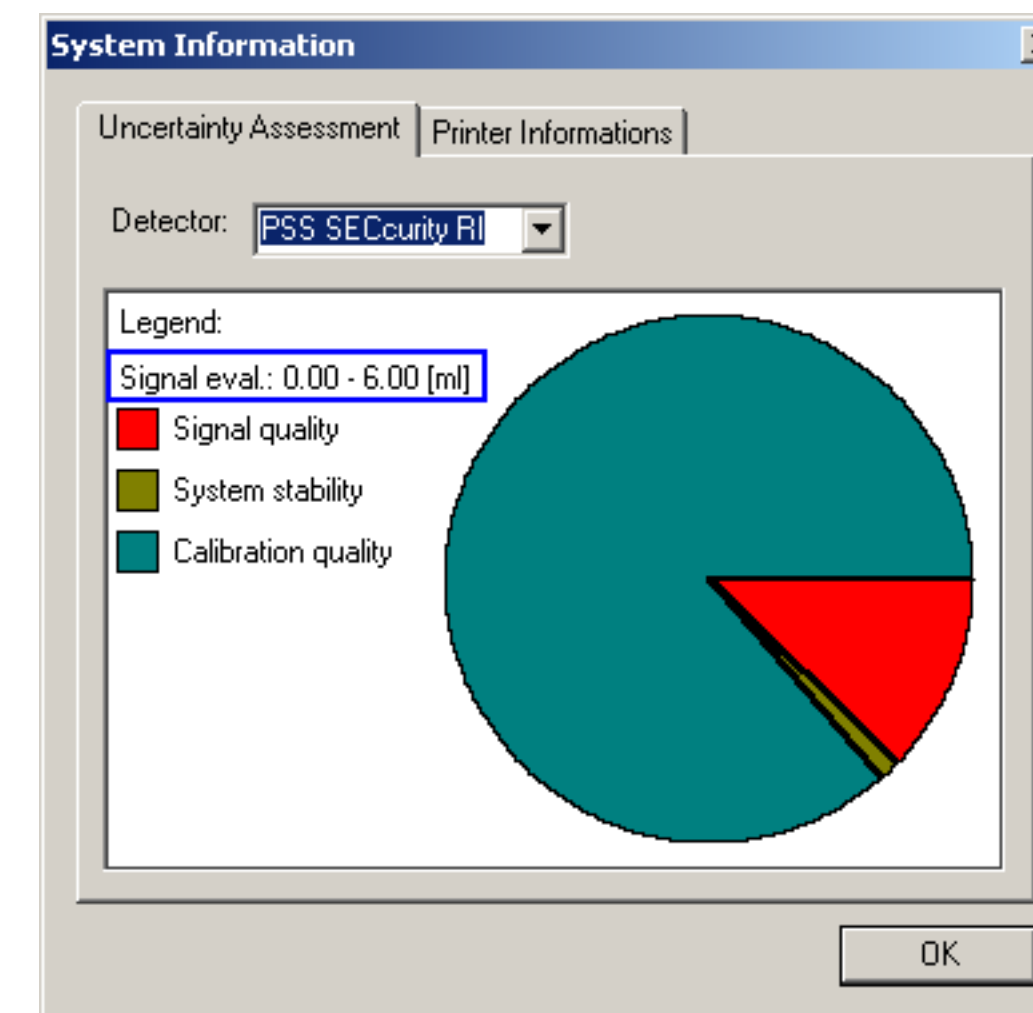


Figure 3. Error contributions of system stability, signal quality, and calibration quality are combined for the uncertainty assessment. This allows identification of the largest contributor to result uncertainty to improve the result quality.

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Conclusion

- Determination of result uncertainty will enhance analytical quality substantially.
- Results of sample comparisons can be interpreted more accurately to be identical to, or different from, within uncertainty limits.
- Standard GPC/SEC software can perform result precision without additional steps from the user.

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Da	Dalton (g/mol)	MMD	Molar mass distribution
DMA	Dimethyl amine	PDI	Polydispersity index ($D=M_w/M_n$)
DMAc	Dimethylacetamide	PMMA	Polymethyl methacrylate
dn/dc	Refractive index increment	PS	Polystyrene
ELSD	Evaporative light scattering detector	R ²	Regression coefficient
Eluent	Fluid used to elute a substance	RALS	Right angle laser light scattering
Exclusion limit	Marks the upper limit of the separation capability of a column. Large analyte species can no longer penetrate the pores of the packing.	RI	Refractive index (detection/detector)
GPC	Gel permeation chromatography	SEC	Size exclusion chromatography
H ₂ O	Water	Solvent	Liquid in which a solute is dissolved to create a solution
HPLC	High performance liquid chromatography	Stationary phase	Solid phase in a separation device on which materials will be separated
LALS	Low angle laser light scattering	THF	Tetrahydrofuran
M _n	Number-average molar mass	Total permeation limit	Also total penetration limit. Marks the lower limit of the separation capability of a column. The sizes of corresponding molecules are small enough to access all of the pores in the column packing material.
M _w	Weight-average molar mass	UV	Ultraviolet (detection/detector)
M _z	z-average molar mass		
MALS	Multi-angle laser light scattering		
Mobile phase	Liquid phase used on a chromatography system		

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