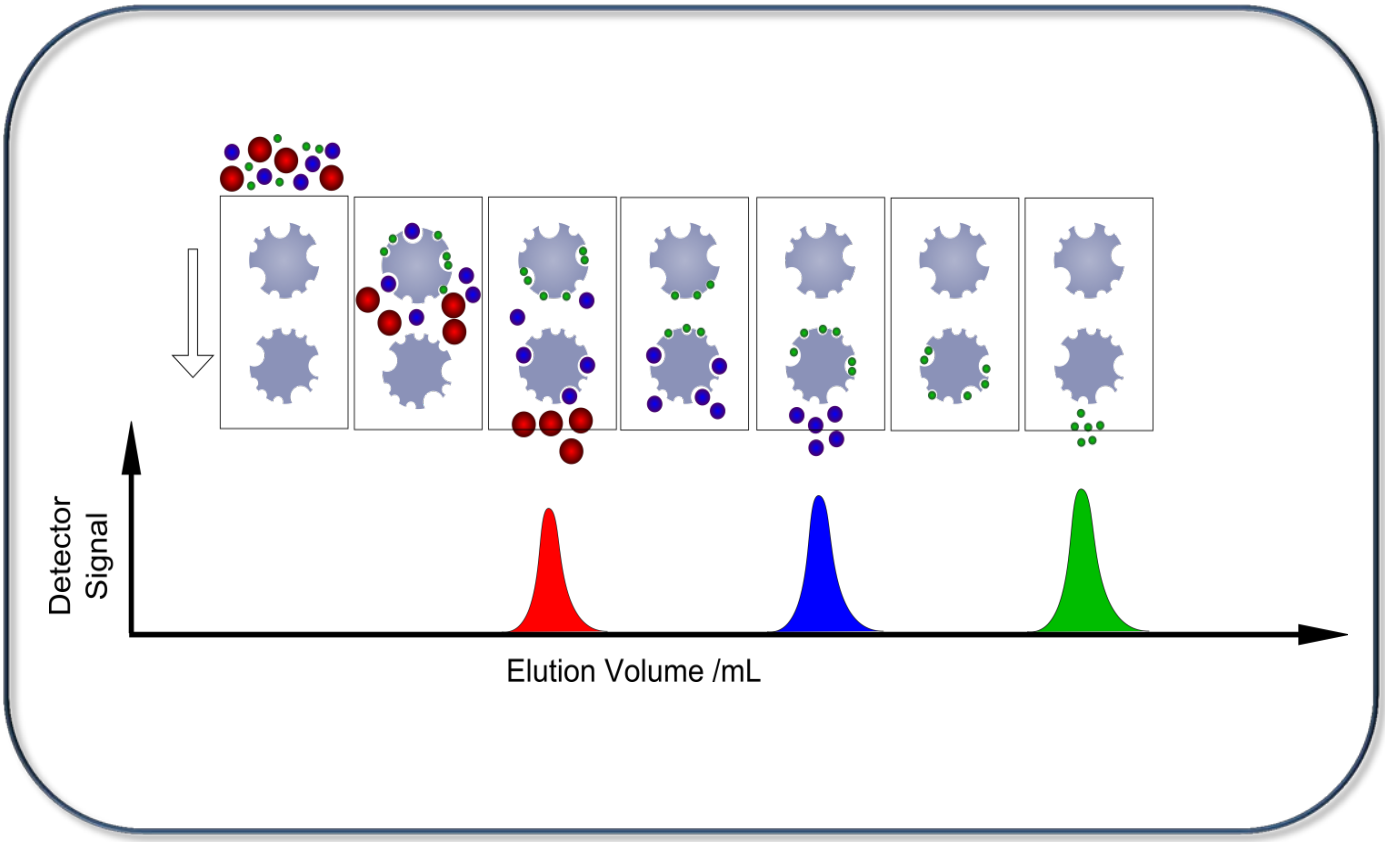
PSS e-book Series part 1

GPC/SEC Theory and Background

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



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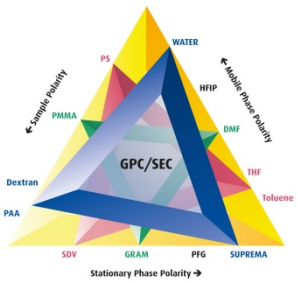

About this e-book series

Since 2007 PSS publishes GPC/SEC Tips&Tricks in LC/GCs digital magazine “The Column”. These Tips&Tricks are designed to support users of GPC/SEC in their daily work and to provide comprehensive overviews on different aspects of this powerful technique.

Many readers collect these publications and have approached us to create a compendium with all the information published in more than 60 editions in over 10 years.

When working on this project, we realized that a compendium does not reflect the true value of the compiled information. To have all published topics at-a-glance, we decided to create a series of five different e-books instead.

The topics of these e-books will reflect the different sections “GPC/SEC Theory and Background”, “GPC/SEC Columns”, “GPC/SEC Detection”, “GPC/SEC Troubleshooting” and “GPC/SEC Applications”.

Each book will be based on 5-7 different Tips&Tricks publications updated with the latest information, new examples and figures. In addition links to other PSS resources, which provide more information to this aspect, were added.

To allow novice users a continued reading experience, content has sometimes been edited so that there are differences to the original publications.

Nevertheless we maintained the original spirit so that the publications are independent references allowing advanced users to read only the dedicated publication of interest.

We hope you enjoy reading this e-book series and want to encourage you to contact us directly if you would like to discuss aspects in this book in detail.

Yours

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Introduction to part 1 “GPC/SEC Theory and Background”

GPC (Gel Permeation chromatography), SEC (Size Exclusion Chromatography) and GFC (Gel Filtration Chromatography) are interchangeably 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 or biopolymers in food industry.

One aspect of GPC/SEC is to perform the actual analysis, a different aspect is to understand and interpret the results correctly. Even many experienced scientists struggle with the fact that they are now dealing with long chains and molar mass averages instead of a defined molar mass.

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PSS Webcast:
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Therefore the first two sections will help you to understand (or explain) 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 and results of sample comparisons can be interpreted more accurately.

1.1. A detailed look at molar mass averages

Originally published in: The Column 10/2007, Author: Daniela Held

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

Unlike low molecular organic compounds and some 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 requisite to describe a polymer.
The ratio of M_w and M_n yields the polydispersity index (PDI; PDI = M_w/M_n).

The molar mass averages M_n, M_w and M_z are calculated by averaging over the number *n* (→ M_n) of polymer chains with a defined molar mass or their weight *w* (→ 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). z refers to the German word 'Zentrifuge'. This average can be determined by ultracentrifugation.

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

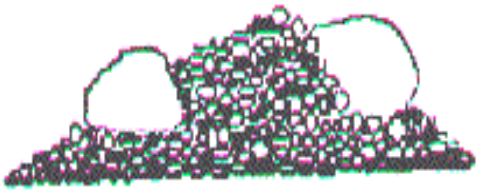
$$\bar{M}_w = \frac{\sum M_i \cdot w_i}{\sum w_i} \quad \text{with} = w_i \cdot M_i \cdot n \quad \rightarrow \quad \bar{M}_w = \frac{\sum M_i^2 \cdot n_i}{\sum M_i \cdot n_i}$$

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

A simple example with stones of different weights can help to understand molar mass averages[1]:

Example 1

500 Stones of 1kg = 500kg
2 Stones of 250kg = 500kg
Total = 1000kg



Example 1

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):

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

Example 2

400 Stones of 1kg = 400kg
100 Stones of 6kg = 600kg
Total = 1000kg

Example 2

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

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

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

[1]Example taken from: A. Schlegel, 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 numbers but display different M_n numbers. Their properties vary, because they exhibit different molar mass distributions. These differences in average numbers and distribution are revealed by the PDI. Molar mass averages can be determined from the molar mass distribution, but reconstructing a molar mass distribution of the averages is not feasible.

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 so called "narrow", since the PDI is below 1.1. The other sample has a higher PDI of around 2 and is usually regarded as broad distribution sample.

Figure 1 clearly displays the molar mass range of both samples. Even for the low PDI sample, the molar mass range is from 58 000 Da 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.

Figure 1: Comparison MMD distribution narrow and broad

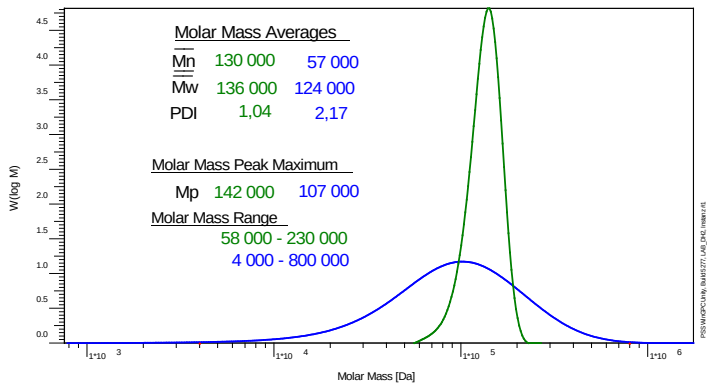


Figure 1: Comparison of samples with similar M_w's but broad and narrow molar mass distributions.

Please 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 numbers cannot describe the whole polymer or indicate any of its limits.

How are molar mass averages measured?

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

Table I displays an overview of non-fractionating techniques that allow determination of a single average number.

Table I: 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	SAXS (Small angle x-ray scattering)
	SANS (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 (FFF)

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

Requirements for reliable GPC/SEC measurements

a) Dissolution

GPC/SEC requires complete dissolution. Ultrasonification should never be considered as 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 also an impact on dissolution time which can take up 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.

b) Resolution

Insufficient separation occurs with columns of poor resolution or low exclusion limit. For efficient separation of all molar mass fractions and best GPC/SEC results, high resolution columns covering a wide molar mass range are required. Oftentimes combining several single porosity columns of different molar mass ranges is favorable over one single linear or mixed-bed column. An optimized column set enhances 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.

c) 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's. GPC/SEC is often performed in multi detection mode.

d) Calibration

M_w and M_n are not related to a specific point in the chromatogram or the molar mass distribution. Therefore these should not be used to establish a calibration curve. The best number to build a calibration curve is M_p, the molar mass at peak maximum. Please 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.

1.2. From a chromatogram to the molar mass distribution

Originally published in: The Column 8/2007and 12/2014, Authors: Daniela Held, Peter Kilz

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 a 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 tubings and different detectors. So they obtain two different chromatograms as primary information. Without additional information it is not possible to decide, if these chromatograms result from the same sample or not. 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, inter-laboratory 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 inter-laboratory and long-term comparison of samples and sample properties.

How are chromatograms transformed into molar mass distributions?

As discussed above the primary information of GPC/SEC measurements is a convolution of sample related parameters and experimental conditions, the apparent concentration distribution (chromatogram, h*(V)*).

The MMD can be calculated from the signal heights in the chromatogram by the slice method. Hereby, the eluted peak is separated into equidistant time, or more properly, volume slices.[1, 2]

Step 1:

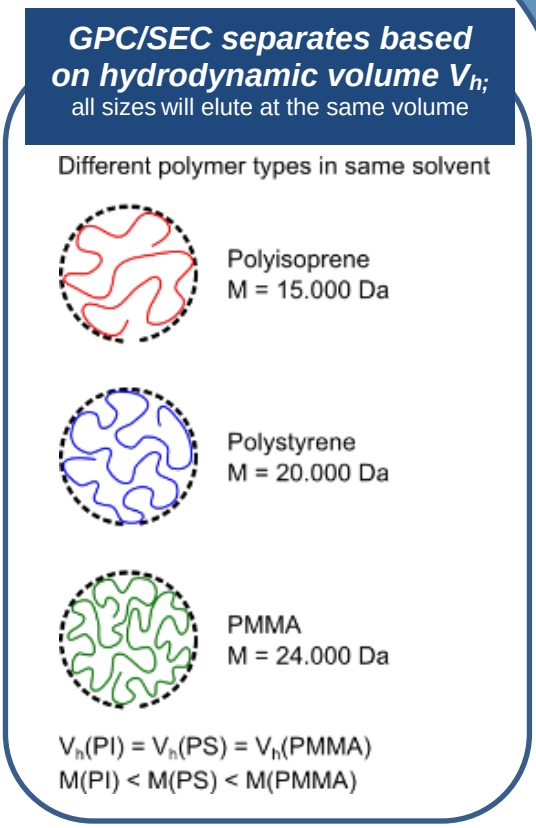
First, the retention axis (x-axis, elution volume) will be changed into a molar mass axis by using the information of the GPC/SEC calibration. There are several options to determine the molar mass / elution volume relation. From a practical point of view, the methods can be distinguished between methods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

The most common technique when reference materials are used is to calibrate the system with polymer reference materials with a narrow molar distribtution.[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 be easily in the range of 20% up to several 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 are the use of matching reference materials or the use of any of the following techniques

- universal calibration using Mark-Houwink coefficients
- broad standard calibration[7]
- cumulative match calibration[4] or
- calibration using an online viscometer[5]



Another popular approach is the use of online static light scattering detectors, such as MALLS, RALLS, LALLS for suitable samples. [6]

Step 2:

Secondly, the y-axis is converted 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 lag. 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, due to the fact that the data recording occurs linear in the time, while the molar mass change is not linear. In practical terms this means that 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 σ(V) slope of the calibration curve.

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

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

number-average molar mass:

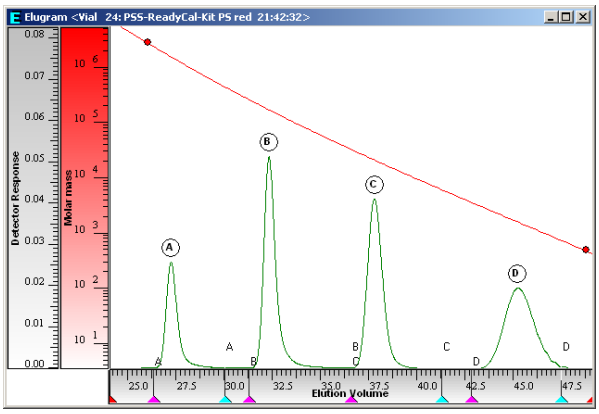
$$M_n \frac{\sum h(M)}{\sum h(M)/M}$$

weight-average molar mass:

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

Figure 1 & 2: Comparison chromatogram and resulting molar mass distribution

Figure 1: While a chromatogram shows the detector signal height (y-axis) versus the elution volume (x-axis), a MMD displays *w(log M)*on the y-axis versus *log M* on the x-axis. Peak shape and breadth can change during transformation depending on the slope of the non-linear calibration curve (displayed in red)



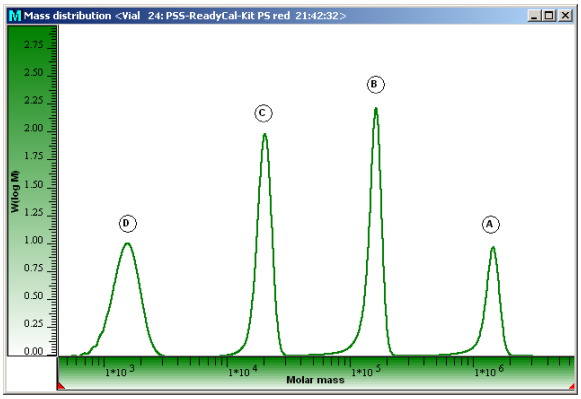
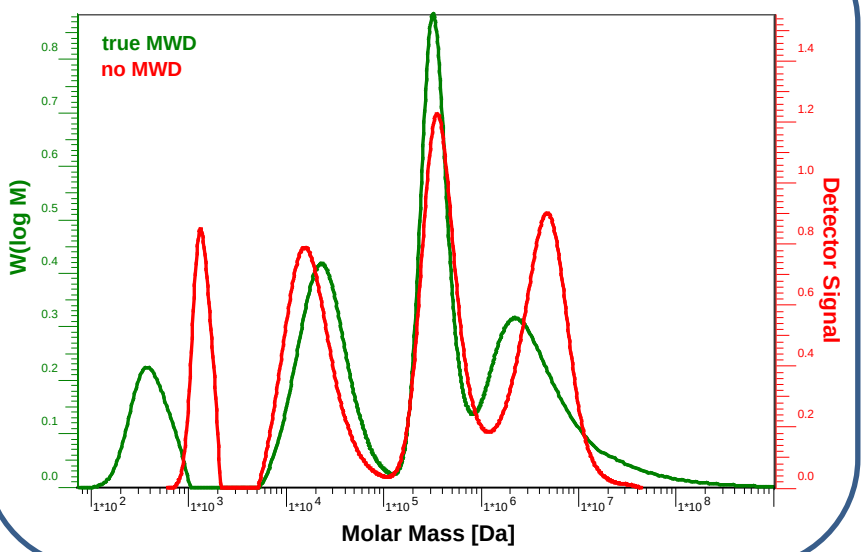


Figure 2: Chromatogram versus molar mass distribution. A MMD displays *w(log M)* on the y-axis versus *log M* on the x-axis. Graphs that display the signal height or similar on the y-axis probably do not show true molar mass distributions.

Please note that GPC software modules of many HPLC chromatography software programs do only perform the correction with the slope of the calibration curve. This results in wrong molar mass distributions for all setups with typical non-linear GPC/SEC calibration behavior. The errors caused by this will increase with the width of the sample and decrease with the data recording frequency. This is dangerous when submitting GPC/SEC results to regulatory organizations such as FDA or EMEA or for REACH registration.

Figure 3 compares a true MMD with a molar mass diagram obtained when neglecting the correct transfer to w(logM). 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 3: Overlay of a MMD (green) with a molar mass diagram falsely processed by HPLC software (red)



An easy test shows, if molar mass distributions or just molar mass diagrams are displayed. Inject a polymer standard mixture at the same concentration onto a GPC/SEC column (no linear or mixed bed column) and generate a non-linear 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 Figure 1).

Literature

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

Originally published in: The Column 6/2008, Author: Daniela Held

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 utilizing proper high resolution GPC/SEC columns and dedicated GPC/SEC software for data acquisition and analysis.

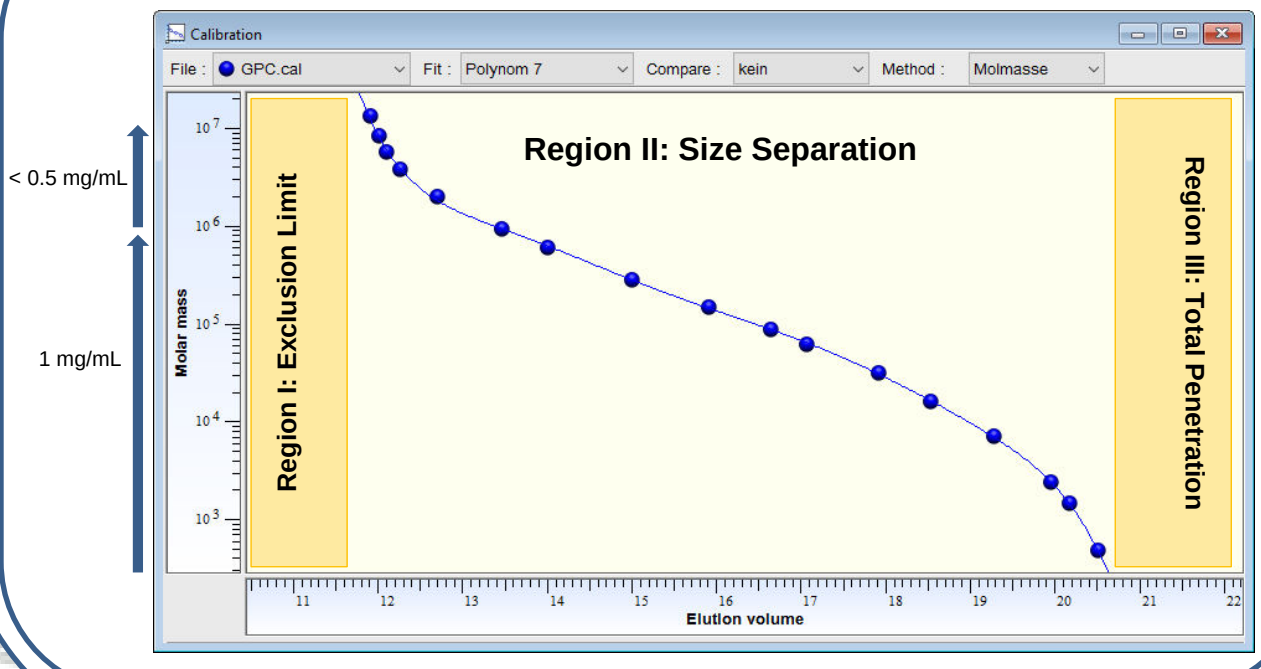
Why does GPC/SEC require a calibration?

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 opposite to 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, 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 is in agreement with the fundamental separation characteristics. This is in contrast to other calibrations in chromatography where linear calibration curves are established by plotting peak area versus concentration.

Figure 1: Typical GPC/SEC calibration curve with 3 distinct regions. The recommended sample concentration for calibrating depends on the molar mass and is also shown (y-axis).



GPC/SEC calibration curves can be divided into three distinct regions as shown in figure 1. Region I 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 resembles the exclusion limit of the column set where large species independent of their size elute at the same volume. Region II reflects the optimum separation range of a column set. Polymers are separated according to their size in solution. Larger fractions elute first, fractions of smaller hydrodynamic volume elute at higher elution volumes. In Region III (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.

How are GPC/SEC calibration curves created?

There are several ways and methods how to establish a calibration curve. Table I shows an overview of the different methods including their advantages and disadvantages. All the methods described in table I are based on the use of molar mass calibration standards.

The most popular method for calibration is the method that utilizes 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.

Calibration with narrow distribution standards

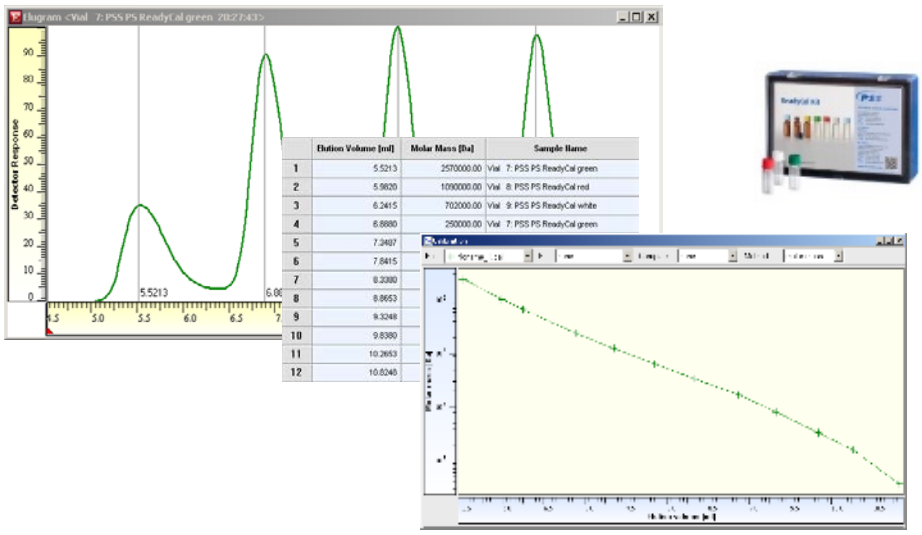


Table I: GPC/SEC calibration methods and their advantages/disadvantages

Calibration with	Advantages	Disadvantages
narrow GPC/SEC standards	• Easy and straight forward • Precise method[3] • Accurate method[3] for samples and standards of the same structural and chemical nature • Accuracy is based on the average deviation of all calibrants	• Results only accurate for compounds of the same structural and chemical nature • Narrow distribution standards are not available for all polymer types
well characterized broad distribution standards	• Easy and accurate • Ideal for internal referencing of analytical conditions as internal standard	• 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 are capable to apply this method
Integral calibration/ Cumulative calibration	• Easy and accurate • Calibration accurate for compounds of the same structural and chemical nature	• 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)	• Easy and accurate • Matching calibration curves for different polymer types can be established from a single curve	• 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)	• Easy and accurate • One calibration curve valid for all types of polymers	• Increased experimental complexity and cost • Additional experimental error from concentration dependence, band broadening and inter detector delay

After measuring elution volumes and plotting these versus the logarithm of molar mass (usually peak molar masses[2]), a fit function describing 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 have to select a suitable fit function based on multiple factors. As discussed before, fit functions generally will not be linear. These fits are calculated based on polynomial functions from third (cubic) to 7th order. There are also modified fit functions based on polynomial functions available, so that typical pitfalls are avoided (PSS calibration functions).

Another possibility to measure the relationship between molar mass and elution volume is combining a concentration detector (RI, UV, ELSD) with an online light scattering detector (LALLS, RALLS, MALLS but not ELSD). With a single standard the light scattering detector constant (calibration constant) and the concentration detector constant (response factor) is 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 has to be applied.

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 II; it 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 take into account, 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 II: 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.

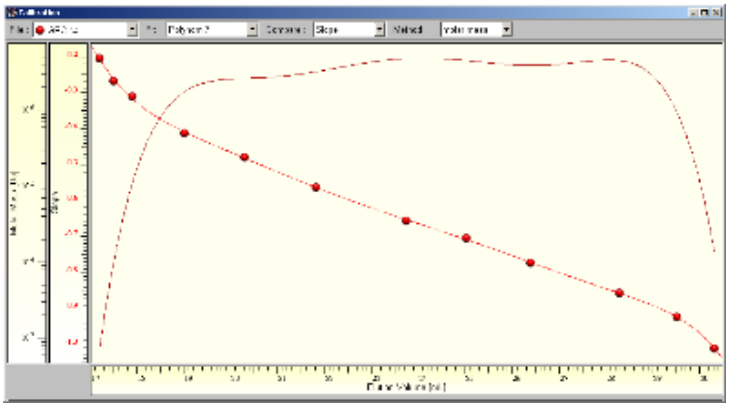
Table II also shows that the regression coefficient and the average deviation become smaller when selecting a polynomial function of higher degree. However, it is not recommended to use the highest order function that generates the lowest average deviation. The shape of the calibration curve is more important than small deviations

which should be in general agreement with the separation mechanism. Hence a good measure is the first derivative of the calibration curve (slope).

Figure 3 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 2), 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 2 & 3: Comparison first derivative of two different fit functions

Figure 2: Calibration curve with 7th order polynomial fit function. First derivative shows locally false maxima and minima. This function is not suitable.



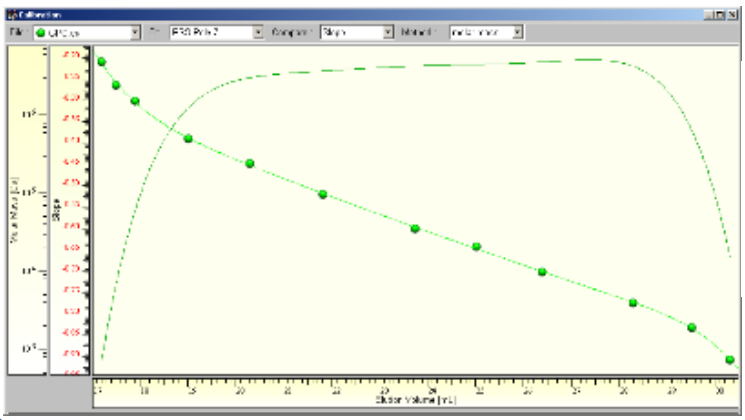


Figure 3: Calibration curve with PSS fit function Poly 7: maxima and minima are eliminated, this function is recommended. The selected fit function can be used.

How often should I (re)calibrate?

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. Time and effort required for this quality assurance approach has been significantly reduced by modern GPC/SEC software that offer automated calibration/re-calibration routines in combination with convenient single-use calibration sets in autosampler vials, such as ReadyCals, to which just solvent has to 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 molecular compound eluting at the end of the chromatogram.[6] Highest accuracy and precision can be achieved with minimum effort, if calibration and sample runs are correlated to this internal reference.

Rule of thumb is to increase the recalibration frequency when many different samples are run on the same columns and/or if sample purity is questionable (sample might contain low molecular impurities from manufacturing processes).

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1.4. Accuracy and precision in GPC/SEC

Originally published in: The Column 2/2008, Authors: Peter Kilz, Friedhelm Gores

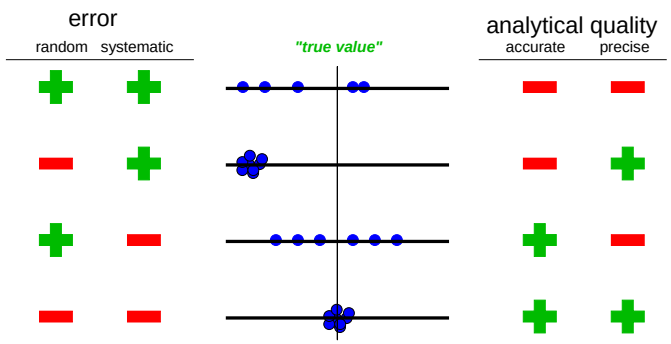
Any analytical technique has its intrinsic inaccuracy due to a variety of factors. In order to interpret results in a correct manner, it is important to know these inherent precision and accuracy of the analytical technique. Furthermore, it is necessary to know how these factor limits can be influenced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

Before discussing the tools, generic definitions of factors should be clear:

The **accuracy** of an analytical procedure expresses the closeness of the agreement between the value which is accepted, either as 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.

Figure 1: Difference between Accuracy and Precison



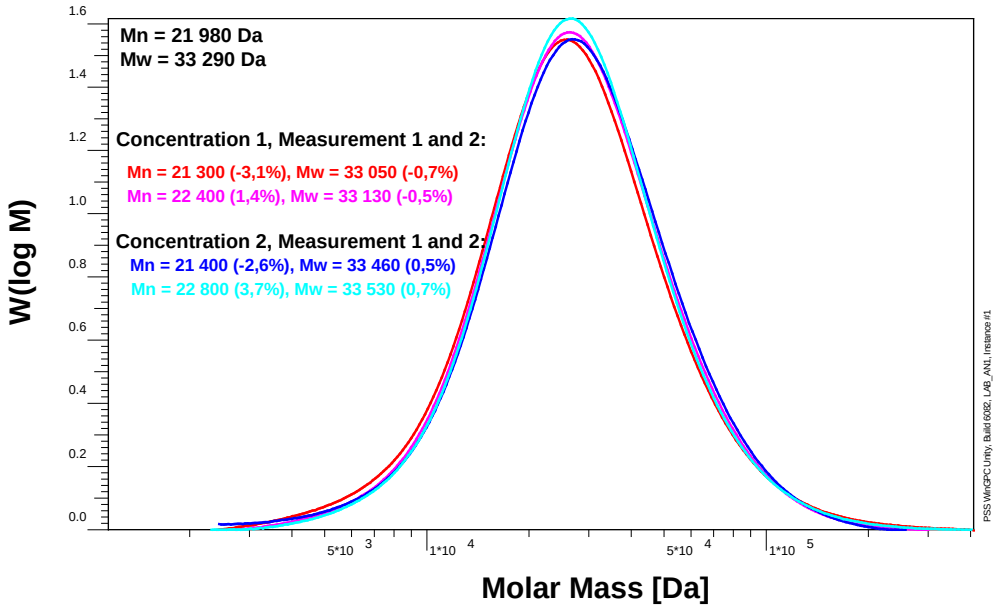
In more detail, precision can be discussed in terms of short and long-term precision:

The **repeatability** describes short-term intra-laboratory empirical variance of results of multiple measurements of a sample.

The **intermediate precision** expresses long-term intra-laboratory variations.

Another important term is the **reproducibility** that is assessed by means of inter-laboratory deviations.

Figure 2: Repeatability of an aqueous GPC/SEC run (2 concentrations, dublicate injections



How accurate is GPC/SEC?

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

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

Fortunately, many GPC/SEC users focus only on repeatable and precise measurements, e.g. when only quality control of products or product comparison is required. However, if accurate results of true molar masses are needed, 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 of these options, reference values are required. Accuracy of the results of 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 influence the accuracy of the results as well.

To achieve highest accuracy careful calibration of the system and precise evaluation is required. National and international guidelines such as ISO EN 13885 for GPC/SEC[1] 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 suited for the molar mass range in which the samples are expected. Column sets i.e. a combination of columns with different porosities can provide more accurate results than single (linear or mixed bed) columns due to the better resolution and efficiency.

How precise is GPC/SEC?

As described above 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 inter-laboratory reproducibility as shown in table I. These results were obtained from complex samples with broad molar mass distributions.

Table I: Precision and Reproducibility for selected solvents

	THF	DMA	H_2O	THF	DMA	H_2O
	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. 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 molecular internal standard as a flow marker is also recommended.[2,5]

Typical variations that influence the intermediate precision are different days, different equipment and operators. Separation columns play an important role as well. It is essential that suitable column material is used that allows interaction-free and size-based separation.[3] If that is not the case, slight recipe changes by the column manufacturer or even new columns made from a different batch might lead to different interactions. This would then lead to systematic deviations and therefore a low intermediate precision.

The intermediate precision can be improved by establishing stringent workflows for system set-ups, 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 interaction with the packing surface. Rigorous quality control applications such as pharmaceutical applications could eliminate potential problems by reserving column batches.

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

Literature

1 ISO EN 13885: GPC in Tetrahydrofuran

2 P. Kilz, D. Held; Qualification of GPC/GFC/SEC Data and Results in Quantification in LC and GC - a practical guide to good chromatography data by S. Kromidas, H-J. Kuss (Eds.), Wiley-VCH, Weinheim, 2008

3 T. Hofe, G. Reinhold; How to find the ideal stationary GPC/SEC separation phase, The Column 12/2007

4 D. Held; GPC/SEC do's and don'ts for data analysis; The Column 02/2013

5 D. Held, W. Radke; Flow Marker - An Easy Concept to Increase Reproducibility; The Column 04/2016

1.5. Result uncertainty – how reliable are results?

Originally published in: The Column 6/2012, Authors: Peter Kilz, Daniela Held

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, REACH etc. Accuracy and precision of GPC/SEC results are a key factor in this regard.

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

What does result uncertainty mean?

Every chromatographer knows from experience that many methodological aspects and experimental details can influence results and quality of an analytical experiment.[1] Various systematic and random contributions impact accuracy and precision of 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 have to be performed to get a reliable estimate of the final result uncertainty. Software can be used to do these calculations. However, software cannot control systematic errors that are specific to user 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 ± result uncertainty (at a confidence level of 1st standard deviation)

This means that the result G of an analysis falls within G - ΔG and G + ΔG 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 >1 for the uncertainty value. Generally, results are reported as:

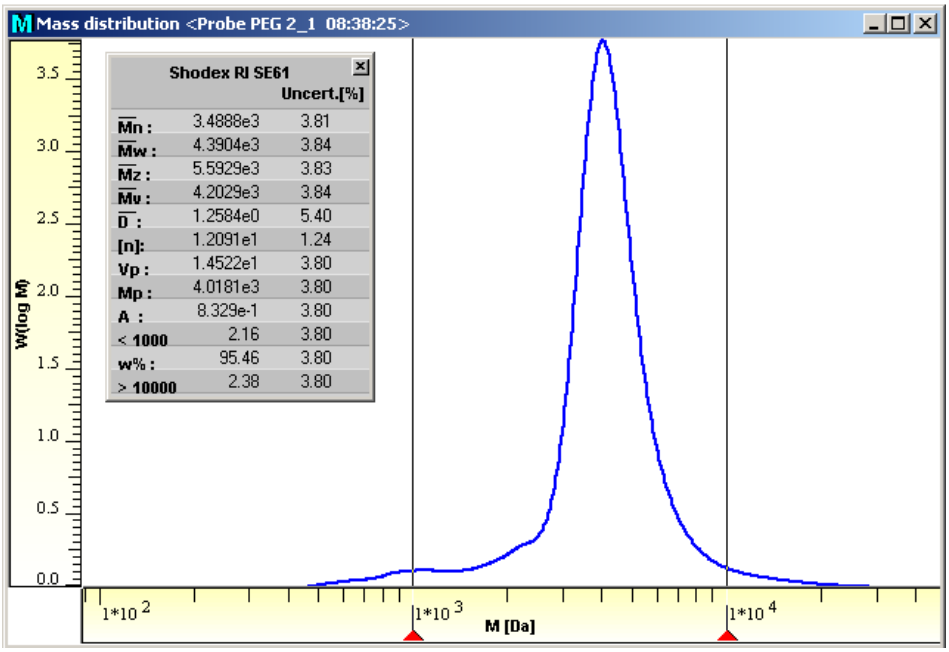
$G \pm k\,\Delta G$ with k: 1, 2, 3...

The commonly accepted default value for result uncertainty is k = 1 that 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 or confidence level of 99.7% is achieved for k = 3.

Figure 1 displays a molar mass distribution including a typical result table (column 1 and 2) and respective relative result uncertainty in per cent (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%. In order to achieve (practically) 100% confidence of a result, the error has to be multiplied by 3 (k = 3) which means true M_w falls into 3 808 Da and 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 Da and 4 469 Da (99.7% → 3 808 Da and 4 800 Da).

Figure 1: GPC/SEC results with molar mass distribution and respective relative result uncertainty in per cent



Systematic and random errors

Typical systematic errors in GPC/SEC experiments could be:

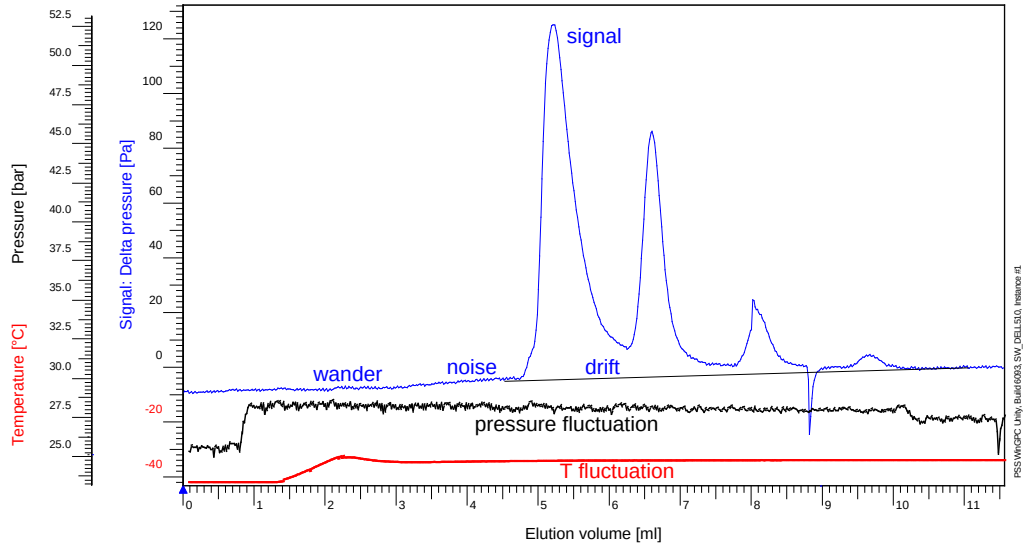
- leak in GPC/SEC system
- method parameters (wrong column set, eluent, temperature)
- sample concentration resulting from incomplete dissolution
- molar mass calculation based on outdated calibration or incorrect sample parameters
- wrong injection volume
- incorrect use of DPT sensitivity factor in 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

As mentioned above, 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 well suited to quantify influence of random errors on result deviations from the true (or commonly accepted) value.[2]
Typical contributions to random (statistical) error amongst others are:

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

Figure 2: Different random errors contributing to the result uncertainty



Determination of result uncertainty

Final results will be affected by system properties in different ways that have to 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 its 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 amount of parameters, error propagation methods of the entire parameter set have to be considered to determine the uncertainty value.[3]

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: How can results be improved?

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.

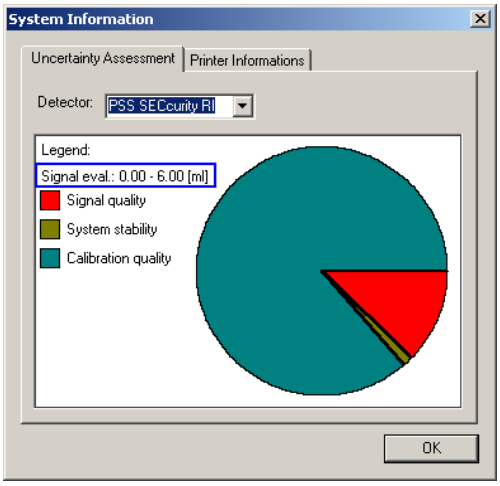


Figure 3 shows an uncertainty assessment example of GPC/SEC results calculated from RI data. Here the quality of the calibration is a major contributor to result precision. System stability and the signal quality contribute to a much lesser extent. In order to improve result quality, the user can first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

Conclusions

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

Literature

1 a) D.Held, P.Kilz; Qualification of GPC/GFC/SEC Data and Results, in: Quantification in LC and GC, S. Kromidas, H.-J. Kuss (eds.), Wiley-VCH, Weinheim, 2009

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f) EURACHEM/CITAC Guide: Traceability in Chemical Measurement; S. Ellison, M. Rosslein, A. Williams, (eds.), London, 2003

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3 See PSS WinGPC UniChrom documentation and references within

Glossary

Da	Dalton (≡ g/mol)
DMA	Dimethyl amine
DMAc	Dimethylacetamide
dn/dc	Refractive index increment
ELSD	Evaporative light scattering detector
Eluent	Fluid used to elute a substance
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.
GPC	Gel Permeation Chromatography
H_2O	Water
HPLC	High performance liquid chrmatography
LALLS	Low angle laser light scattering
M_n	Number-average molar mass
M_w	Weight-average molar mass
M_z	z-average molar mass
MALLS	Multi-angle laser light scattering
Mobile phase	Liquid phase used on a chromatography system
MMD	Molar mass distribution
PDI	Polydispersity index ($D=M_w/M_n$)
PMMA	Polymethyl methacrylate
PS	Polystyrene
R^2	Regression coefficient
RALLS	Right angle laser light scattering
RI	Refractive Index (Detection/Detector)
SEC	Size Exclusion Chromatography
Solvent	Liquid in which a solute is dissolved to create a solution
Stationary phase	Solid phase in a separation device on which materials will be separated
THF	Tetrahydrofuran
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.
UV	Ultraviolet (Detection/Detector)

About PSS

Perfect Separation Solutions

PSS GmbH was founded in 1985 by two PhD students at the University of Mainz, Germany, producing polymer standards at the University facilities. In the following years PSS expanded staff and products to include tailor-made polymers, organic and aqueous GPC/SEC columns and GPC/SEC software. In 2001, PSS moved into own facilities located in Mainz, Germany. PSS-USA opened its office in 1994, servicing North and South American customers from Amherst, Massachusetts. To date, PSS has successfully gained leadership in the GPC/SEC market, making innovative contributions not only in Germany and the USA, but around the world.

PSS is fully dedicated to the advancement of macromolecular liquid chromatography, means of materials design, synthesis, manufacturing, consulting, service and innovative research, applying the highest standards of expertise and reliability. Our close relationship with our customers has helped us to continuously improve the quality of our products and services.

Our high caliber staff, mostly chemists, is experienced, creative and trained in problem solving. Corporations, universities and organizations in more than 60 countries use our products and profit from our outstanding service and know-how.

Authors

Friedhelm Gores is the head of the contract analysis lab at the PSS headquarter in Mainz. He is responsible for method development and hyphenated techniques.

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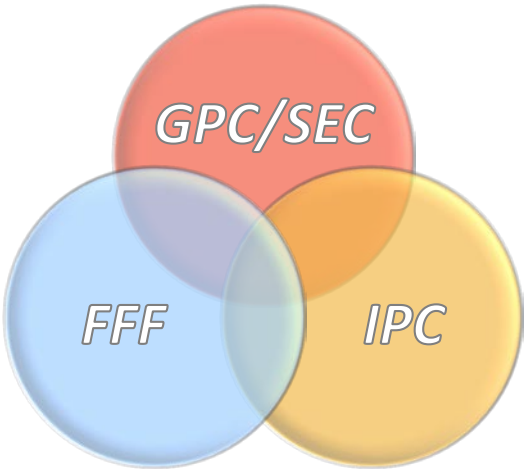

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