GPC/SEC eBook Series

GPC/SEC Troubleshooting and Good Practice

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

Agilent

Trusted Answers

Contents

Contents

About this eBook series

Introduction to GPC/SEC troubleshooting/good practice

1.1. What you need to know for efficient troubleshooting

1.2. Mobile phase considerations

1.3. GPC/SEC Columns – do's and dont's

1.4. Frequently asked questions: GPC/SEC columns and good practices

1.5. How to get a stable baseline

1.6. Do's and dont's of data analysis

1.7. Do's and dont's in GPC/SEC light scattering

Glossary

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.

This eBook series was created to have all published topics at a glance.

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.

Introduction to GPC/SEC troubleshooting/good practice

Liquid chromatography (LC) instruments are multi-component systems comprising at least one pump, an injector (automated or manual), one or more separation column(s), and one or more detector(s). To obtain reliable and accurate results, all components within such a system must work properly. Troubleshooting of such multi-component systems can be quite challenging.

In addition to the challenges that all analytical scientists face, scientists who work with GPC/SEC have to deal with even more tasks.

Hazardous solvents and mobile phase additives or modifiers, like salts (required to suppress undesired interactions or sample aggregation) are very common in GPC/SEC, and increase the complexity of the system.

Sample preparation for macromolecular samples can be very demanding due to solubility issues. Data analysis is very different compared to other LC methods.

Scientists who operate GPC/SEC systems need advanced troubleshooting strategies.

The first section of this eBook provides some general advice on how to develop a thorough understanding of a GPC/SEC system to allow for efficient troubleshooting.

Sections two to four deal with mobile phases and stationary phases, while section five discusses typical issues from a detection point of view.

Finally, the last two sections of this eBook review data analysis for conventional GPC/SEC and GPC/SEC-light scattering, and provide some information for obtaining reproducible results.

Last but not least, this eBook also includes a few links to request additional resources.

1.1. What you need to know for efficient GPC/SEC troubleshooting

As with LC users, GPC/SEC users can also face unexpected problems in their daily work. Typical issues with GPC/SEC systems include:

- Pressure increase
- Loss of column resolution
- Drifting baselines, specifically when using refractive index (RI) detection

If a problem occurs, an efficient troubleshooting strategy can help minimize instrument downtime. This requires scientists to be familiar with their setup. Knowing a few important parameters helps to identify the root cause of a problem quickly.

Pressure issues

An isocratic pump is used to deliver the LC mobile phase through the autosampler, the GPC/SEC columns, and the detectors. The inner diameter and length of the tubing used to connect the components (inside the components) needs to be optimized for the system requirements. Narrow bore (ID 0.17 mm and less) tubing is a requirement for semimicro and analytical setups, to avoid band broadening. Highly viscous solvents or preparative instrument setups often require tubing with a larger inner diameter (e.g., 0.25 mm or 0.5 mm).

Both columns and tubing generate backpressure. The actual pressure value depends on the viscosity of the mobile phase, the flow rate set at the pump, the number and type of columns, and the inner diameter and length of all tubing (inside and outside the detectors). Each setup has a unique pressure value that should only vary slightly over time. A pressure change can be an indicator of a problem.

It is highly recommended to monitor the pressure during analysis. In addition, users should know the typical pressure value for their setup conditions (solvent, flow rate, temperature) with and without columns installed.

Before installing new columns[1], users should first document the pressure value for the pump, autosampler, and tubing to the precolumn.

- First, set the desired flow rate at the pump. Collect the waste at the tubing end, and write down the system pressure.
- Second, install the precolumn according to the user documentation, setting the desired flow rate. Write down the value for the system with the precolumn.
- Add the first analytical column, write down the pressure value, and continue until all columns are in place.

If the column manufacturer provides a pressure value on the column certificate, this can be compared to the observed value, keeping in mind that different flow rates, different solvents, and different temperatures (as temperature influences the solvent viscosity) will generate a different pressure. Some deviations can be expected. Table 1 shows an example of two well-documented setups.

As operating at too high a pressure can damage the column packing, destroying the column, it is good practice to set an upper pressure limit for the system. If the limit is exceeded, this setting will cause the pump to stop. Typically, the normal total pressure (plus 20 to 30 bar) is used as an upper limit. However, check the column user documentation (or directly with the column supplier) for the maximum pressure the columns can withstand, and make sure this value is not exceeded.

Table 1 can help to find the root cause in case of a pressure increase. Open the tubing at the entry port of the precolumn and check if the system pressure is too high. If this is the case, either the pump, the autosampler, or the connecting tubing is causing the problem. If this is not the case, the columns are likely responsible.

Please note that the detector(s) and tubing that come after the columns rarely cause the pressure increase. This can be double-checked by reconnecting the columns, and by opening the connection at the exit of the last column. If the pressure drops back to the value with all analytical columns installed, the detector (or one of the tubes after the columns) is the cause of the issue.

After identifying the block of components causing the problem (pump/autosampler, columns – or, less commonly – detectors) each component in this block needs to be checked separately. For this, remove piece-by-piece, starting from the end. Loosen each connection in turn, while the flow rate is set as usual and check if an unusually high pressure drop occurs. It is good practice to replace any filters while the instrument is down. In the case of a blocked tubing or needle, replace and dispose of this part immediately.

If the precolumn is the root cause of the problem, it has done its job and should be replaced. In the case of an analytical column, applying a cleaning procedure or replacing the column frits can be an option. For this, please refer to the column user documentation.

If a detector is the cause of the problem, refer to the manual for instructions on cleaning the cell.

Table 1. Example for two well-documented GPC/SEC setups after column installation.

System	Conditions	System Pressure (No Detectors)	Precolumn Installed	Analytical Column 1 Installed	Analytical Column 2 Installed	Total Pressure
GPC 1	THF, 1 mL/min, 35 °C	5 bar	11 bar	31 bar	53 bar	57 bar
GPC 2	Water, 1 mL/min, 35 °C	7 bar	10 bar	18 bar	25 bar	29 bar

Column performance

The columns in the setup are required to separate the molecules by size. A properly working GPC/SEC column has high resolution power and generates narrow, symmetric peaks for monodisperse samples. If the resolution decreases for each of the columns in a set, the precolumn could be the sole source of this behavior. In case of distorted chromatograms (broad peaks, double peaks, peaks with tailing or fronting) there is a realistic chance that only one of the columns in a set is malfunctioning.

Good measurable values that describe column performance are the plate count, the asymmetry, and the specific resolution.[2] These parameters should be determined for the complete column set immediately after installation. National and international standards provide acceptance limits for columns, often specifically for the mobile phase used. It is good practice to write down the parameters after installation and to compare these values with the data given on the column certificate. As the plate count depends on various parameters, it is individualized for each setup. If available, document the values of each of the single columns installed.

The plate count test should be repeated regularly, but at a minimum of when a problem is suspected. If the column set no longer passes the acceptance limits, or if the deviations from previous measurements are too high, each column needs to be tested individually. Figure 1 shows an example where the asymmetry of a column set is out of specification due to peak fronting. The plate count test should be repeated for each of the three columns individually, to verify if only one column needs to be replaced, or if the complete set is damaged.

After identifying the faulty column, it is good practice to review the samples previously analyzed to verify if one of them is causing the issue.

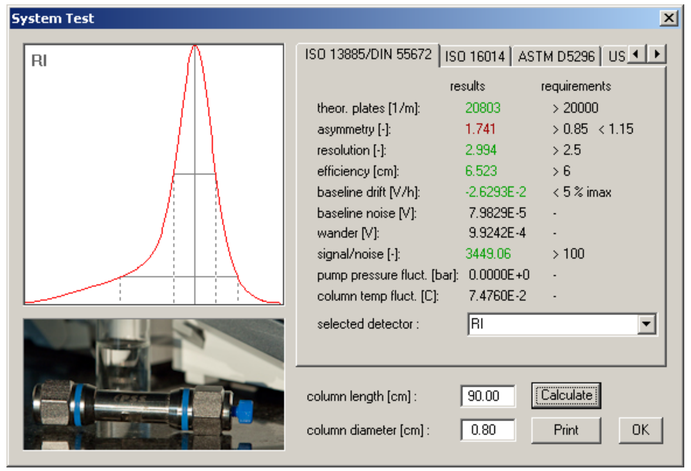

Figure 1. Complete system test with one injection (results for column set).

Note that the behavior shown in the example can sometimes be attributed to a wrong tubing connection. Each time new columns are installed (especially when the supplier has been changed) the fittings and ferrules should be replaced to ensure low dead-volume connections with matching stop depth.

Detector performance

The detector(s) are used to detect any sample fractions eluting from the column. When pure eluent is passing through the detector, a baseline is obtained. The quality of detector signals is generally described by reviewing baseline drift and wander, and the signal-to-noise ratio (S/N). National and international standards provide acceptance limits for drift and S/N. The influence of these values on GPC/SEC results has been investigated intensively.[3]

To identify if a problem is related to chromatographic or detection issues, it is best to measure a simple sample. Plate count test substances (usually small, low molecular weight materials) can be used to determine drift, wander, and S/N for concentration detectors, such as RI, UV/DAD, or ELSD. If S/N, drift, and wander for this injection are documented after system installation, troubleshooting is easier.

Figure 1 shows the results for drift, wander, and S/N, determined using the injection of the plate count test substance for a refractive index detector. All detection requirements, as specified by ISO13885 are met. Out-of-specification results for baseline drift can often attributed to temperature fluctuations. Here the conditions in the lab need to be reviewed (influence of draught from air conditioning or column compartment). A low S/N for concentration detectors is often an indicator of a dirty flow cell. Refer to the user documentation for cleaning procedures.

Conclusion

Knowing the pressure of the system with and without the separation columns installed helps to quickly identify if the columns or the instrument are responsible for a pressure increase.

Single column testing helps to identify a blocked (too high pressure) or malfunctioning column (low plate count, asymmetry, resolution).

The sample used for plate count determination can also be used to verify detector performance (drift, S/N).

References

1. Held, D. How to Install GPC/SEC Columns. *The Column* **2014**, (2).
2. Gores, F.; Held, D. How to Test GPC/SEC Columns. *The Column* **2014**, (8).
3. Kilz, P.; Held, D. Quantification in LC and GC - A Practical Guide to Good Chromatographic Data. Wiley-VCH, **2008**.

Originally published in *The Column*, December **2018**, by author Daniela Held.

1.2. Mobile phase considerations

There are many sources of advice for selecting the most appropriate column stationary phase for sample analysis, but the mobile phase is often disregarded. On the contrary, the mobile phase is an essential and integral part of an analytical system, affecting the outcome of the analysis, and quality of data and results. Correct mobile phase selection and its use in sample preparation can save time that would otherwise be needed for troubleshooting.

The choice of mobile phases available for GPC/SEC is limited to those that can dissolve the sample, and are compatible with the stationary phase. Typically, GPC/SEC mobile phases are either:

- Aqueous (pure water or solutions with salts and/or buffers added) or
- Organic (e.g., tetrahydrofuran (THF), toluene, dimethylacetamide (DMAc), dimethylformamide (DMF), chloroform, hexafluoroisopropanol (HFIP), trichlorobenzene (TCB)).

The wrong mobile phase can irreversibly damage or destroy the stationary phase. For example, columns packed with a highly cross-linked hydrophobic polymeric stationary phase (such as styrene-divinlybenzene) swollen in an appropriate organic mobile phase will shrink and lose all performance if used with an aqueous mobile phase.

However, even if a mobile phase is compatible with the stationary phase, it may still not be effective when used on its own. The suppression of sample-stationary phase interactions often requires the addition of low molecular weight modifiers or salts. Lithium bromide (LiBr) or lithium chloride (LiCl) is often added to polar organic solvents such as DMAc or DMF. Aqueous systems require the addition of additives to prevent algae growth and salts to suppress interactions.

Sometimes, even with additives, a true size-separation cannot be achieved if the polarity of the three involved components (sample – mobile phase – stationary phase) does not match. An example is the separation of polystyrene (PS) on styrene-divinlybenzene material in medium polar organic solvents such as DMF or DMAc. In this case, PS oligomers will coelute with or after the salt peak (Figure 1). Therefore, either a stationary phase with matched polarity should be used (e.g., PSS GRAM) or polymethyl methacrylate (PMMA) is required to fully calibrate the system.[1]

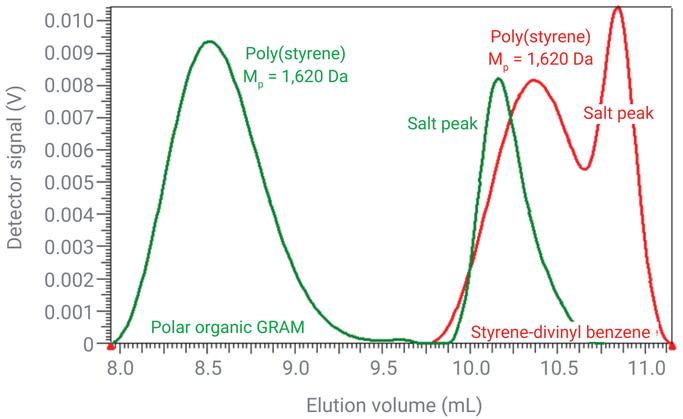

Figure 1. The same polystyrene run in DMAc with salt on a GRAM stationary phase (green) and on a styrene-divinylbenzene phase (red) where it coelutes with the salt peak.

It is also important to ensure that the mobile phase does not mask the detection of the samples. In the case of refractive index (RI) detection, the refractive indices of the mobile phase and sample need to differ as much as possible. If the difference is too small, the signal intensity will be low. A prominent example of a mismatched mobile phase/sample pairing is polydimethylsiloxane dissolved in THF. Both have the same refractive index (they are isorefractive) therefore, the refractive index increment dn/dc = 0. So, although the sample is fully dissolved, it cannot be analyzed because there is no RI detector response. It is necessary to change the mobile phase to something with a different refractive index, for example, toluene (dn/dc <0), or to use another detector such as an evaporative light scattering detector (ELSD).

Another factor to consider is mobile phase viscosity. Low viscosity improves resolution. Elevated temperatures (60 to 80 °C) should be used with highly viscous mobile phases (such as DMAc and DMF) to help reduce the viscosity and increase the resolution. Additionally, lower flow rates for highly viscous mobile phases enhance mass transport.

Mobile phase quality and solvent preparation

Different solvent grades vary in purity. Although more expensive, solvents should be HPLC-grade (high purity). All mobile phases and buffers should be prepared on the day they are used. This will ensure that the buffer pH is stable and no microbial growth is triggered. Both could affect chromatographic results.

Isocratic GPC/SEC necessitates a homogeneous mobile phase. Special attention must be given to mixed mobile phases or mobile phases containing modifiers, especially when using an RI detector. If there is a big density difference within a mixture, or if a salt is partially dissolved, chromatograms will display baseline shifts at the beginning and the end of a run due to changing mobile phase composition. This applies especially to polar organic mobile phases.

Careful preparation of the mobile phase is required when using DMAc or DMF. Lithium salts are often added as modifiers, but these salts have low solubility in polar organics at ambient temperature. LiBr dissolves more easily than LiCl. Raising the temperature to the range of 50 to 80 °C enhances solubility and decreases viscosity. The mobile phase should be stirred for at least 2 hours.

A shift might also occur in chromatograms if the organic mobile phase is hygroscopic and water content increases over time, or if the mobile phase composition changes due to oxidation. THF should be stabilized with, for example, a small amount of butylated hydroxytoluene (BHT) and chloroform with, for example, ethanol. Sodium azide (NaN_3) or similar should be added to aqueous mobile phases to prevent algae and bacterial growth.

Prior to using the freshly prepared mobile phase, it should be thoroughly degassed to eliminate dissolved air and avoid baseline problems. The most convenient way to degas mobile phases is using an inline degasser. It is important to install it before the pump, so that dissolved air-related pressure fluctuations can be avoided.

Consult with the manufacturer if HPLC degassers are used with organic GPC/SEC solvents. Some degassers have membranes that are not compatible with regular GPC/SEC or aggressive solvents.

Filtration of the mobile phase is strongly recommended to remove small particulates and dust that could clog tubing and/or columns. In general, mobile phases should be replaced regularly. Performing an analysis with aged mobile phases that have been in recycle mode for some time will most probably yield low quality data with drifting and wavy baselines (Figure 2).

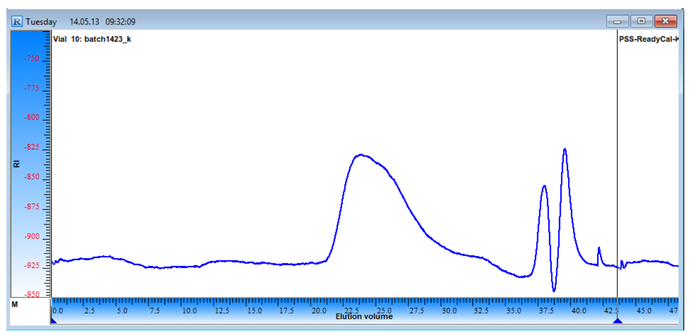

Figure 2. Baseline response to low solvent quality.

Operation with a mobile phase that has not been optimized for the analysis and prepared correctly can impede pump, injector, and detector functions. Special care is required when running instrumentation with mobile phases with a high salt content. Always run the system at a low flow rate to prevent corrosion of instruments or columns. Flush the entire system with a pure solvent to remove mobile phase-containing salts before exchanging solvent, turning off the pump, or storing a column.

Conclusion

- The mobile phase is an integral part of the system and needs to be selected carefully.
- In the case of polymeric (cross-linked) stationary phases, the wrong type of solvent can destroy the column (organic on aqueous gels or water on organic gels). The gel structure collapses.
- The use of high purity (HPLC-grade) solvents, freshly prepared and degassed, prevents instrumental and chromatographic problems, and can save time.

Reference

1. Hofe, T.; Reinhold, G. How to Find the Ideal Stationary GPC/SEC Separation Phase. *The Column* **2007**, (12).

Originally published in *The Column*, April **2014**, by author Daniela Held.

1.3. GPC/SEC columns – do's and dont's

The GPC/SEC column is a key component for the separation of macromolecules by hydrodynamic volume or size. Generally, GPC/SEC columns are very stable - mechanically and chemically. They are packed with either porous, highly cross-linked polymer particles, or porous silica particles. GPC/SEC columns, like all LC columns, have a finite lifetime. In order to get the best performance and a long lifetime out of the columns, they must be treated in an appropriate manner.

There are many ways and reasons for a column to become damaged (or even destroyed), or have its properties and performance altered:

- The polymer network can break down
- Pores can collapse partially or entirely
- The polarity of the column surface can be modified
- Column hardware (metal of the column housing) can be damaged or oxidized
- Frits can be damaged or blocked

Column performance can be determined by plate count, separation power, and resolution measurements. Pore volume and pore size distribution can impact separation power and resolution. The plate count depends mainly on particle size, packed bed density, and interstitial volume. The overall column performance increases by optimizing the value of these three parameters.

Column backpressure also has to be taken into account. The backpressure is mainly affected by particle size and solvent viscosity. As the particle size decreases and the solvent viscosity increases, the detected backpressure on a column will increase.

The major question is: how should a column be correctly operated?

Solvent considerations

In GPC/SEC, the solvent plays a key role in enabling successful analysis. The solvent must not only dissolve the sample, but must also be compatible with the column packing material. The compatibility of the solvent and the stationary phase of the column is optimized when the polarity of both is similar, as is described by the Magic Triangle (see Columns eBook).

Refrain from using a nonsolvent for analysis. A nonsolvent prevents the packing material from interacting with the solvent, especially for the polymeric-type packings. This causes the pores to collapse and causes the column material to become irreversibly damaged.

How should the column be properly stored?

If columns will not be used for weeks or months, it is good practice to store them in a pure, stable, salt-free solvent. Check the miscibility, boiling and freezing point temperatures, and vapor pressure prior to using a storage solvent. Columns filled with high-boiling solvents can be stored at room temperature. Store columns placed in volatile solvents (high vapor pressure) in a refrigerator (4 °C) to prevent evaporation and solvent degradation.[1]

Do not store a column in salt solution. A salt solution is a potential source of corrosion, and it can precipitate or crystallize in the column if the solvent evaporates as a result of poor storage. Precipitated salt could damage the packing material upon reinstallation. The only exception is for storage of aqueous columns, which require the addition of a small amount of sodium azide (NaN_3) to prevent algae or bacterial growth.

If a column has been stored in a refrigerator, do not install the column right away. Allow sufficient time for the column to return to ambient temperature before installation, and (if necessary) after installation, before bringing it up to operating temperature.

Solvent exchange

The porosity of packing particles is in the nanometer range, up to several hundred nanometers. The particle size itself is in the range of micrometers (see Figure 1). An efficiently packed GPC/SEC column has a very large total surface area. The solvent not only covers the external surface of each particle (small surface area), but the internal surface as well (large surface area). This fact has to be taken into account when installing a new or stored column. The solvent in the pores impedes complete solvent exchange.

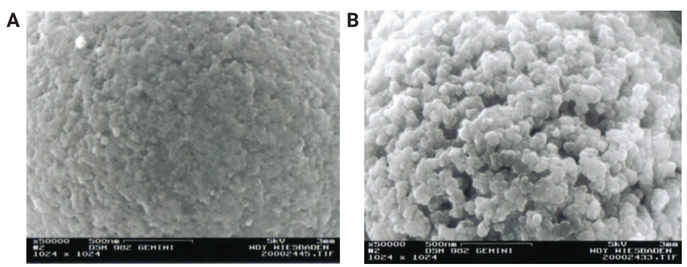

Figure 1. Electron microscopy picture of various pore sizes of PSS SDV 5 µm particles, with pores of 100 (A) and 10^5 (B); amplification: 50,000.

Solvent miscibility is a major requirement for efficient solvent exchange. The exchange solvent has to be a good choice for the stationary phase, to wet the particles properly. When exchanging solvents, the solvent should always go directly to waste. Detectors should not be connected.

As a general rule for complete solvent exchange, the amount of solvent needed should be 10 times the total amount of solvent contained in the column (often referred to as column volume). The solvent viscosity can also influence the progression of the exchange. As the viscosity of the exchange solvent increases, the longer it takes for a complete exchange. The recommended flow rate for the exchange process should be set to approximately 10 to 30% of the operating flow rate. Keep in mind that during the first phase of solvent exchange, a solvent mixture forms that can possibly lead to a short pressure increase.

Column fittings and flow direction

In regard to the do's and don'ts of fittings and tubing, consult the eBook on columns.

Flow direction along a column axis is usually marked by an arrow on the column body. Always install the column in the correct flow direction. The flow direction also indicates the direction of the column packing. Due to high pressure and flow rate during the packing process, the particles and packed column bed will be aligned along the flow direction. As such, the particles in a packed column have a preferential direction along the flow vector.

Reversing the flow direction of a column can create a disruption of the packed particles, increasing the interstitial volume and causing band broadening.

Monitoring backpressure

The pressure applied while packing a column is much higher than will be experienced when operating the column, and can be considered the upper limit of the column operating pressure. Generally, a single column will operate at approximately 10 to 20% of the original packing pressure (depending on the packing particle size), which allows for both single-column and multicolumn operation. It is recommended to operate a GPC/SEC column/column set at no more than 70 to 80% of the given pressure limit on the column certificate (if this information is supplied). This will ensure stability of the packing material and pore structure, and extend column life. If the backpressure increases significantly for any extended period of time and approaches the packing pressure, stop using the column immediately. The packing pressure should never be exceeded, even for short periods of time. If operation continues, the preferential orientation will break down, and the pore structure tends to collapse. As the pore size increases, the pore structure becomes more fragile, tending to fracture more easily. Hence, a 10 nm pore on a 5 µm particle is far more stable than a 10^4 nm pore on a 5 µm particle.

For a very short period of time, column pressure can be increased to the given limit. When the pressure is increased slowly, a brief column operation close to the limit is possible.

Reference

1. Reinhold, G. How to Expand the Lifetime of Columns. *The Column* **2009**, (12).

Originally published in *The Column*, July **2011**, by author Thorsten Hofe.

1.4. Frequently asked questions about GPC/SEC columns and good practices

Developing a robust and high resolution GPC/SEC method that delivers accurate and long-term reproducible results is a challenging task. It is therefore not surprising that many users require expert advice when choosing the optimal column/column set.

Users have asked a variety of good questions that others may also have. Here is a selection of these frequently asked questions, with answers provided based on scientific experience.

Q: What advice can you give about the pros and cons of mixed bed/linear columns versus individual pore size columns?

A: There are many aspects to consider. Let's review the general expectation of a linear calibration curve. Unfortunately, for a single GPC/SEC column, the relation between the logarithm of molar mass and elution volume is not linear, due to the exclusion and total permeation of the individual pore size of the packing material. Thus, GPC/SEC calibration curves commonly have a sigmoidal shape. Most of the time, third-order (cubic) polynomial functions will fit the calibration data, so this approach also enables full use of the entire separation range of the column. There are also fifth- and seventh-order polynomial fits available, if necessary, to fit data. However, care should be taken to ensure the slope of the calibration curve is not over-fitted.[1] Note that linear columns can display nonlinear behavior at the upper and lower ends. If samples elute in that region, a different fit is required.

Linear or mixed-bed columns are the outcome of intense work by column manufacturers. The production involves either a special synthesis route, or more commonly, careful blending of individual pore sizes. The separation capability of linear columns over a wide molar mass range with a constant resolution has a major advantage. These are ideal for routine QC or as screening columns, if users have to deal with a variety of molar masses. Resolution can easily be increased by adding other linear columns of the same type.

Care has to be taken in picking the right pore sizes to cover the required separation range, especially if higher or lower molar masses need to be separated in the same sample. Risk of porosity mismatch is very high, if (for example) linear columns are combined with individual columns that are ideally suited for oligomer separations.[2]

Despite a limited molar mass range, the high separation efficiency of individual pore size columns is a major advantage. Individual pore size columns are frequently combined in column sets. In order to tailor the molar mass range for an application, columns can be added and removed to adjust the range.

Q: To avoid high backpressure and shear for high molar mass macromolecules, is it sufficient to run GPC/SEC at a reduced flow rate, or is the use of a larger particle size column the better option?

A: This question addresses the problem that macromolecules can be very sensitive. Forcing high molar mass macromolecules or stiff polymer chains through a GPC/SEC system at high backpressure can result in extensional flow. This can induce chain scission causing shear degradation, yielding molar mass values based on artifact fragments.

The backpressure in a system depends mainly on flow rate, mobile phase viscosity, temperature, inner diameter, packing particle size, number of columns used, and all connecting tubing. A reduction of the flow rate to reduce the backpressure is unfortunately often not sufficient when analyzing high-molar-mass samples. It is recommended to use larger particle sizes to eliminate or minimize the effects of shear degradation.

Columns packed with small particles (e.g., 5 µm or fewer) should not be used for high and ultrahigh molar mass (UHMW) samples.[3] Also note that columns packed with large pore size and large particle size require the use of bigger porosity frits, which further reduces shear stress. For UHMW samples, a combination of large particle sizes, low flow rate, and reduced concentration and injection volume is recommended. When using column sets with a range of pore sizes, the column with the largest porosity can be put first in line, so that "viscous fingering" can be avoided when high molar mass samples with viscous chains are separated. To further reduce viscosity effects, the use of a column oven to operate at higher than ambient temperatures is recommended.

Q: Dimethylformamide (DMF) with lithium bromide (LiBr) is rough on the instrument, but yields good results. Are some salts more/less corrosive for equipment than others?

A: Halides are usually more corrosive than other salts, and chlorides are more aggressive than bromides. Fortunately, LiCl and LiBr have better solubility in common organic solvents than other halide salts. Lithium ions are superior in breaking up aggregates compared to other counter ions. In aqueous solvents, less aggressive salts, such as nitrates or sulfates, can be used instead of NaCl to minimize corrosion.

The main reasons for adding salt to a mobile phase are to shield electrostatic interaction and reduce the formation of aggregates. When using salt solutions for analysis, it is important to ensure the system is always run with freshly prepared solutions. The GPC/SEC system should not be left idle for a long time. In order to avoid salt crystallizing, keep the system at a low flow rate to prevent corrosion. If the system is not going to be used for a long time, or will be switched off, it should flushed with a pure, modifier-free solvent.

Q: Are polymeric columns stable enough to tolerate solvent exchanges? Is it recommended to have a column set devoted to a particular solvent, rather than one column set for multiple solvents? What if you repeatedly change between pure tetrahydrofuran (THF) and THF with small amounts of additives (acids or amines)?

A: In general, solvents with similar polarities, such as THF, chloroform, dichloromethane, or toluene should not harm columns during a solvent exchange, as columns are packed with a physically and chemically robust styrene-divinylbenzene packing material. It is recommended to exchange solvents slowly, at reduced flow rates of 0.3 to 0.5 mL/min. The column effluent should go directly to waste, and the detectors should be disconnected. So operationally, there is no reason not to exchange solvents. However, it is a time-consuming procedure. It often takes longer to completely re-establish the swelling equilibrium after the solvent exchange than to reach a stable RI-baseline. Frequent solvent exchanges also reduce the column lifetime.

If solvents differ substantially in polarity, compared to the column material, it is best to use columns with a different packing. Not only will there be a different degree of swelling of the gel, but there is also a distinct possibility of nonsteric interactions.[4] Another option is to order columns in the solvent to be used, as solvent exchange is more risky.

For an exchange of pure solvents and solvent with additives (amines, acids, salts, etc.), frequent exchanges should not cause problems. However, keep in mind that columns should always be stored in pure solvents without modifiers. The only exceptions are columns for aqueous applications, where a small amount of methanol or azide (0.05 g/L) should be added to avoid algal or bacterial growth.

Q: Do detectors require recalibration if the solvent composition is changed by adding salts or other cosolvents? Should the standards be run with the same modifier as for the samples?

A: It is necessary (and considered good laboratory practice) to recalibrate columns or detectors when even minor changes are made to a system setup. A solvent change will cause a change in the hydrodynamic volume of a polymer in solution, invalidating an existing calibration.

There are two types of calibrations:

- The calibration most frequently performed is column calibration. A calibration curve is constructed by plotting the logarithm of the molar mass of calibration standards against their elution volume at peak maximum. However, a solvent change will cause a change in the hydrodynamic volume of the calibration standards (and the macromolecule to be analyzed) in solution, invalidating an existing calibration.
- In the case of multidetection GPC/SEC, or concentration determination for copolymer analysis, users calibrate their detectors in a fashion similar to HPLC. Different concentrations are measured to determine detector responses.

For both types, it is always recommended to apply the same conditions for calibration as for sample analysis. Standards and samples should be prepared with, and run in the same batch as, the mobile phase. It is best to take off a portion from the mobile phase reservoir that feeds the system.

Conclusion

As with other analytical techniques, minor details can have a big impact in GPC/SEC.

Selecting an optimal column or column set and conducting proper method development can be time-consuming, but can save time and money in the future. Guess work and using the wrong settings at the beginning is to no one's benefit. It never hurts to ask questions, so we encourage all readers to share their questions and concerns with us, because there are more answers that can help users make educated choices.

References

1. Held, D. How Do I Calibrate a GPC/SEC System? *The Column* **2008**, (6).
2. Hofe, T. Beware of Mismatch. *The Column* **2008**, (4).
3. Held, D. The Art of Analyzing High Molar Mass Samples. *The Column* **2014**, (6).
4. Hofe, T. ; Reinhold, G. How to Find the Ideal Stationary GPC/SEC Separation Phase. *The Column* **2007**, (12).

Originally published in *The Column*, June **2015**, by authors Daniela Held and Wolfgang Radke.

1.5. How to get a stable baseline

A stable baseline is a prerequisite for precise, accurate, and reproducible GPC/SEC analysis, especially for broad distribution samples. Baseline problems can include drift, wander (long-term noise), noise/spikes (short-term noise), as well as positive or negative system peaks.

- Drift is a steady shift of the baseline either up or down the detector response scale.
- Wander or long-term noise is a detector signal variation with frequencies between 6 and 60 cycles/hour.
- Random detector signal variation with a frequency above one cycle per second is called short-term noise.

Figure 1 illustrates a baseline with a small drift, with wander and detector noise.

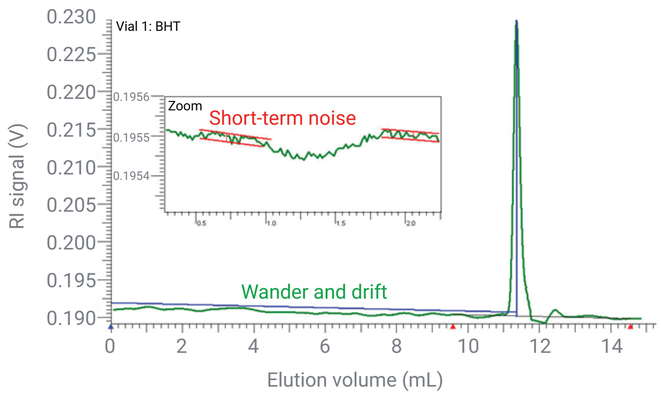

Figure 1. Baseline drift, long-term noise (wander), and short-term noise. The blue line visualizes the small negative baseline drift.

Several international standards describe testing procedures, and provide specifications for drift, long-term noise, and short-term noise.[1]

Potential sources of wander

Most of the time, baseline wander is caused by pump pressure pulsations. An overlay of a pressure trace and the baseline of a blank injection can indicate if pressure pulsations are causing the problem. Leaking seals, worn or scratched pistons, and/or faulty check-valves can cause pulsations. Other causes can be incorrect compressibility settings on the pump, poorly degassed solvents, or air bubbles trapped in the pump. Shifting elution volumes or observing generally poor reproducibility hint at pump problems. Table 1 shows solutions to some potential problems that may be causing baseline wander.

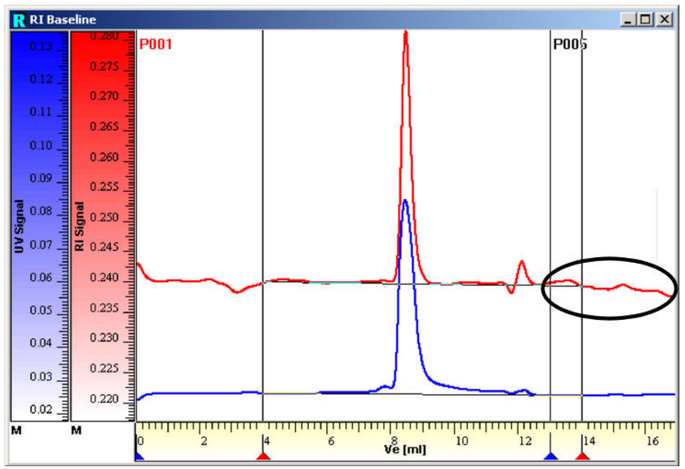

Figure 2. Sample with a narrow molar mass distribution. The RI especially shows strong detector wander.

Table 1. Troubleshooting baseline wander.

Observation	Potential Problem	Solutions
Wander	Pump pressure pulsations	Check for loose fittings or (detector cell) leaks. Service the pump and exchange seals. Clean or replace check valves.
Wander (cyclic baseline rise and fall, wavy, RI)	Degasser does not work properly	Remove degasser to see if waves disappear. Only use degassers suitable for GPC/SEC solvents. Adjust the vacuum level for highly volatile solvents.
Wander (cyclic baseline rise and fall, wavy, RI)	Draft (air conditioners), temperature changes in lab	Turn off air conditioning, stabilize room temperature, and move instrument out of draft zone and away from windows. Insulate tubing, or use column oven.
Wander (UV/DAD/PDA)	Weak UV lamp intensity (short-term noise and wander)	Perform a lamp intensity test following manufacturer instructions. Replace lamp(s), if necessary.

Potential sources of baseline drift

A drifting RI signal is one of the most frequently observed problems in GPC/SEC. To locate the source of the drift, compare the baseline with and without pump flow to check if the pump or other system components (e.g., detectors) are contributing to the drift.

In practice, the two main sources of RI baseline drift are:

- Large temperature difference between columns and detector cells
- Insufficient system equilibration, for example, after solvent exchange

The temperature problem can be overcome by using RI detectors with a well-thermostatted cell.

The temperature difference between the columns and the cell should be kept as small as possible. When exchanging mobile phases, system equilibration takes at least 5 to 10 column (or column set) volumes. Flushing or purging of the RI detector reference cell (lasting a few minutes) should be conducted several times.

Usually, baseline wander affects an analysis more strongly than drift. A continuous drift can often be corrected if baseline limits and integration limits are determined and referenced against internationally recognized GPC/SEC standards. This is not possible for baseline wander. While the baseline drift in Figure 3 can be corrected, the baseline wander shown in Figure 2 does not allow calculation of precise results (data taken from the EasyValid validation kit). Table 2 shows solutions to some potential problems that may be causing baseline drift.

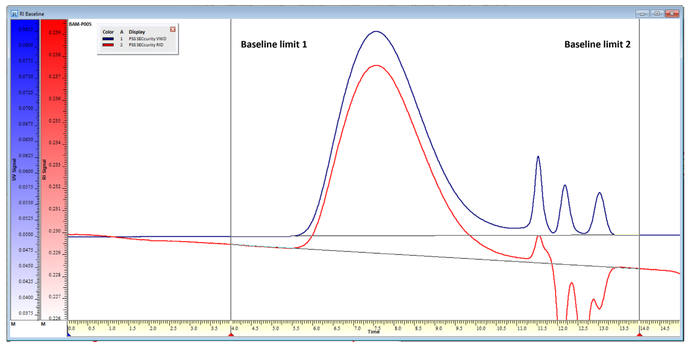

Figure 3. Sample with a broad molar mass distribution. The RI signal shows a constant drift, whereas the UV signal is stable.

Table 2. Troubleshooting continuous baseline drift.

Potential Problem Continuous Baseline Drift	Solutions
Large temperature difference between columns and cell	Use an RI detector with a thermostatted cell.
Insufficient column equilibration, especially after solvent exchange	– Wait at least 5 to 10 column (set) volumes after solvent exchange. Flush RI detector cell thoroughly. In case of immiscible solvents, use an intermediate solvent first. Do some blank injections before starting a new sequence.
Mobile phase contaminated, inhomogeneous or poor quality	– Use high purity, freshly prepared mobile phases. – If mixed solvents are used, stir mobile phase thoroughly prior to use. – Discard any buffer solutions that are stored at room temperature for more than two days. Avoid microbial growth in aqueous solvents with near-neutral pH.
Contaminant or air in detector cell	– Clean detector cell as per the manufacturer's instructions. Remove columns and flush with different flow rates to remove air bubbles. – Make sure that all detector parts (including all seals) are compatible with common GPC/SEC solvents.

Potential sources of system peaks

Several positive and negative signals at the end of a chromatogram are normal for refractive index detectors. These ghost or system peaks mark the end of size separation, and are most likely not eluting in GPC/SEC mode, but in HPLC/LAC mode.

Ghost or system peaks can be identified by making a blank injection with neat mobile phase. It is important to treat samples and blank injection in the same manner, for example, use the same syringes, filter types, flask, vials, caps, etc. to prepare the blank injection.

Additional peaks in oligomer analysis can cause a problem. Adding a small-porosity column sometimes helps to improve resolution on the low molecular end. However, a combination of linear/mixed bed columns with a single porosity column of small porosity is generally not recommended due to porosity mismatch.[2] The use of high purity, freshly prepared mobile phases reduces the intensity and amount of system peaks.

Potential sources of noise/spikes

A potential source for regular spikes is air in the mobile phase or in the system, e.g., the detector cell. Stop the pump to see if the spikes disappear. If so, it is most likely an air bubble in the detector cell. Trapped air bubbles are best removed by applying different flow rates (columns not installed).

To avoid introduction of air bubbles, it is important to degas both aqueous and organic mobile phases. High pressure keeps bubbles dissolved in the eluent contained in columns, and releases them as they exit the columns and enter the flow cell. Thus, inadequate degassing leads to air bubbles in the flow cell, resulting in a noisy signal. An inline degasser can resolve the problems. Additionally, applying low backpressure on the detector cell can help. Table 3 shows solutions to some potential problems that may be causing spikes.

Table 3. Troubleshooting spikes.

Observation	Potential Problem	Solutions
Spikes	Air bubbles	– Degas mobile phases. In the case of online degassers, make sure that they are compatible with typical GPC/SEC solvents, especially HFIP. – Position the waste bottle above the detector outlet to generate a small backpressure on the detector cell. If this is insufficient, use a backpressure regulator at the detector outlet. Do not exceed the maximum pressure of cells. RI detectors especially cannot withstand elevated pressure.
Spikes	Column temperature above boiling point of mobile phase	Use lower column temperature.
Short-term noise, spikes (UV/DAD/PDA)	Weak UV lamp intensity	A weak lamp can be a source of short-term noise and wandering baselines. For UV detectors, perform a lamp intensity test.
Short-term noise, spikes	Dirty cell	Clean cell as per manufacturer's instructions.

Conclusion

- Use an RI with a thermostatted cell. If a column oven is used, set temperature of the RI detector cell as close as possible to the column oven temperature, and insulate the connecting tubing from the oven to the detector.
- Degas the mobile phase. Use high purity, freshly prepared mobile phases, to avoid degradation product buildup over time. Stir well if mixed solvents are used.
- Perform preventive maintenance on the system and check (e.g., pump performance) regularly. After exchanging equipment, check thoroughly for leaks.
- Use the same mobile phase for sample preparation. Reduce injection volume if possible. Use a blank injection to identify ghost/system peaks.

References

1. ASTM International. Standard Practice for Testing Variable Wavelength Photometric Detectors Used in Liquid Chromatography; *ASTM E* 1657-98.
2. ASTM International. Test Method for Molecular Weight Averages and Molecular Weight Distribution of Polystyrene by High Performance Size-Exclusion Chromatography; *ASTM D* 5296-05.
3. Hofe, T. Beware of Mismatch. *The Column* **2008**, (4).

Originally published in *The Column*, April **2012**, by author Daniela Held.

1.6. Do's and dont's of data analysis

GPC/SEC data processing requirements are fundamentally different from other liquid chromatographic techniques such as HPLC.[1] Figure 1 displays a flow scheme for GPC/SEC data analysis, starting with raw data produced by one or more detectors.

Metadata originates from several sources, such as automatically created process parameters, user input, and user default settings. These are necessary to calculate results. They contain sample-related information, method parameters, and all evaluation parameters required for proper evaluation, consistent with GPC/SEC standards. This includes, for example, baseline limits, integration limits, and parameters for mass fraction calculation, as required by GPC/SEC guidelines.

GPC/SEC calibration data are a special type of data, containing a combination of raw data and metadata. Calibration data consist of:

- Retention volume (column calibration)
- Intensity calibration (for some applications only, optional)
- Parameters documenting type of calibration, reason for calibration, and used reference values

GPC/SEC users should follow some basic rules when applying metadata and calibration data to calculate sample results. The following tips help to obtain accurate and reproducible results.

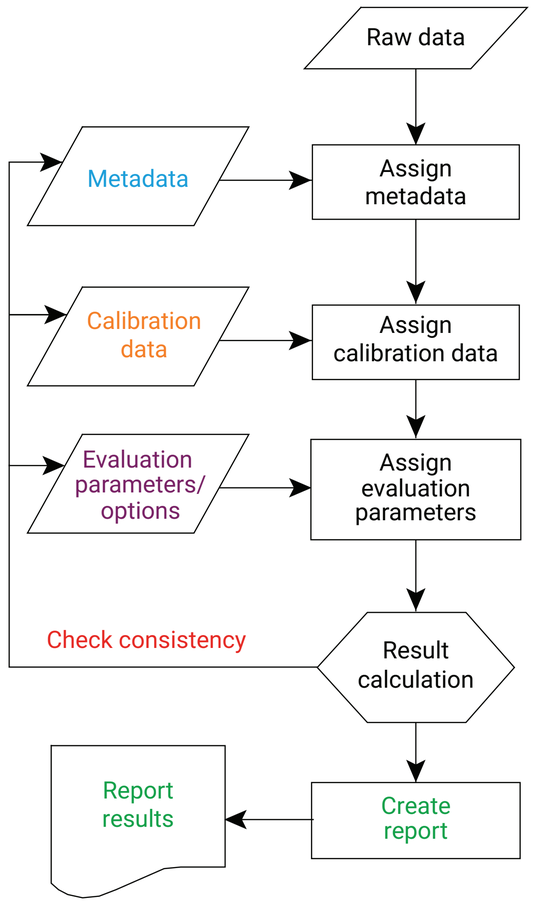

Figure 1. Flowchart of how GPC/SEC results are obtained from raw data.

Selecting peaks for result calculation

In GPC/SEC, it is quite common for broad distribution macromolecular material that the detector signal does not fully return to the original baseline. Thus, a two-step evaluation procedure is recommended.

- Baseline limits are first defined so that the baseline starts at the exclusion limit and ends behind the last system peak (usually a negative peak in the RI signal). In most cases, the signal has returned to the initial baseline value.
- Integration limits are then defined around the sample peak. It is important to exclude any ghost or system peaks that may be present. Ghost peaks or system peaks can be easily identified by injecting neat mobile phase.

For correct GPC/SEC data evaluation, the baseline is subtracted according to the limits from the first step, whereas molar mass averages and distribution are determined within the integration limits from the second step.

This two-step procedure allows the user independent and robust evaluation over the entire sample range. Figure 2 depicts the two-step procedure demonstrated on a certified reference material. The vertical lines denote integration limits. The baseline limits from the two-step procedure are marked in green. The superimposed red trace shows a single step evaluation procedure, where baseline and integration limits are not independent. The red area designates part of the sample that is excluded by the baseline limits-only procedure. In that case, the molar mass distribution is offset, and the molar mass averages are overestimated as is reported in Table 1.

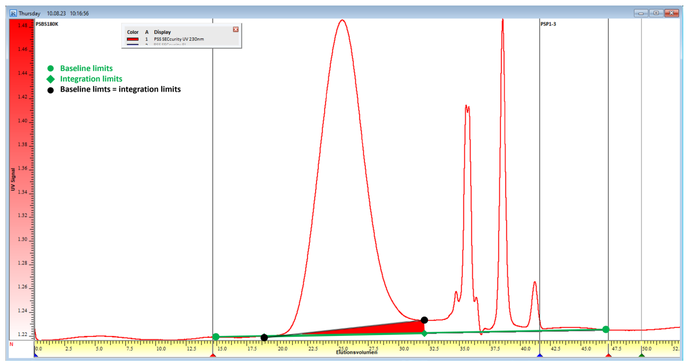

Figure 2. Comparison of two-step evaluation procedure with independent settings for baseline limits, and integration limits with the one-step method with only one pair of limits.

Table 1. Results for a broad distribution sample, demonstrating the wrong one-step procedure and the correct two-step procedure.

Result	One-step procedure	Two-step procedure
M_n (Da)	86,400	66,700
M_w (Da)	181,300	178,400
M_z (Da)	327,200	351,600
PDI	2.10	2.68
<10,000 Da (%)	0.52	1.87
>1,000,000 Da (%)	0.38	0.44

Applying a matching calibration

GPC/SEC (with concentration detectors such as RI, ELSD, or UV) is a relative method for producing MW data, not an absolute method. Measured retention volumes (V_e) have no direct relation to the molar masses of the analyzed sample, and instead depend on analysis conditions (type of polymer, columns, solvents, etc.). This necessitates a calibration performed under the same analytical conditions.

The use of narrow-distribution polymeric standards is the simplest and most precise method for calibration (assigning molar mass to elution volume).[2] Accuracy depends on the calibration standards used. True molar masses are only obtained for chemically identical compounds. A wide range of reference materials are available, so that for many homopolymers (polystyrene, polyethylenoxides, polyacrylates, and polymethyl methacrylates), matching calibration curves can be established.

If narrow distribution standards are not available, other methods can be used. These include:

- Broad calibration[3]
- Cumulative calibration[4]
- Universal calibration based on Mark-Houwink coefficients or universal calibration with online viscometry[5]
- Online light scattering detectors[6] (in combination with a concentration detector) can be used to determine sample molar mass at every elution volume slice

Determination of a correct molar mass distribution requires fitting the measured data points with a mathematical function so that the molecular weight at any elution volume can be calculated. Keep in mind that even when using online light scattering, data are often fitted. The quality of the fit function directly influences the quality of the result. The best fit function is the one that generates the smallest deviations, and where the first derivative yields no inflection points.[2]

GPC/SEC data analysis for sample comparison and quality control only requires correct concentrations and injection volumes for measurements of standards, and the choice of an optimal fit function for the calibration curve. Measurement conditions for running reference materials depend on molar mass, polydispersity, and the number of columns used for separation.

If accurate molar masses are required, it is recommended to use chemically identical compounds for calibration, or to use molar-mass-sensitive detectors. If molar mass sensitive detectors are applied, the results depend on evaluation parameters, such as the refractive index increment, dn/dc, or the method used for determining slice concentration. For interlaboratory comparison, metadata should be documented extensively.[7]

Flow correction with an internal standard/flow marker

Reproducibility of analysis conditions and the relation to the calibration impacts GPC/SEC data analysis. GPC/SEC results are obtained from an accurate and reproducible peak position. Thus, all parameters influencing peak position will affect the accuracy of the final results.

One key necessity for GPC/SEC is to have reproducible and stable flow rates. Although modern LC pumps provide stable flow rates, their influence should not be neglected, as wear and tear on the pump piston and valve seals can change the delivered elution volume over time. Also, incorrectly equilibrated columns can shift elution volumes.

Both factors can easily be monitored by using an internal standard/flow rate correction marker (FRCM). A typical FRCM is a low molecular weight compound, added to all standard and sample solutions. An ideal FRCM is long-term stable, nontoxic, and generates a strong signal at the detector. It does not react with the solvent, the stationary phase, or the sample. It elutes without interfering with oligomers or residual initiator, so that quantification is still feasible.

BHT, toluene, acetone, or sulfur are typical FRCMs for organic solvents. Ethylene glycol, acetone, or glucose can be used in aqueous mobile phases. Figure 3 shows an example of methylbenzoate used as a flow marker in DMAc with salt.

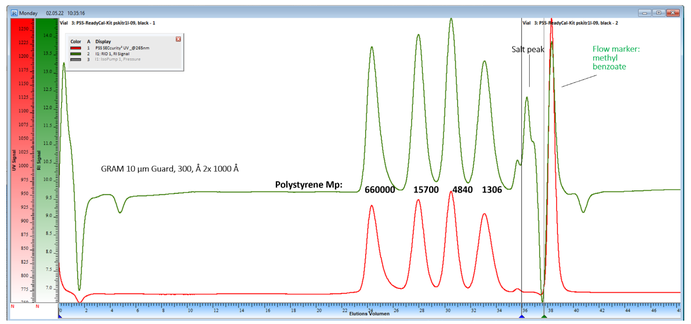

Figure 3. Raw data (RI (green), and UV 280 nm (red)) for a mixture of four different molar masses of polystyrene plus the flow marker methylbenzoate in DMAc and 5 g/L LiBr.

The FRCM can also be used to compensate for small deviations in the flow rate or equilibrium conditions. Such effects can be balanced by comparing the experimental elution volume of the FRCM with the elution volume when the original calibration curve was produced, and calculating a ratio.[8]

All chromatograms are then corrected using the ratio in the same manner, so that the conditions for calibration and sample runs are identical. Should the flow marker elute slightly earlier or later at higher elution volumes, sample peak elution volumes are shifted slice-by-slice to compensate for flow rate fluctuations or equilibration deficiency.

Using this procedure simplifies the reliable use of calibration libraries, and potentially eliminates calibrations prior to each sample set. Use of an internal standard/flow marker reflects continuous change of the packing and/or the flow. However, drastic changes cannot be corrected with this approach, and will require recalibration.

References

1. Held, D.; Kilz, P. Qualification of GPC/GFC/SEC Data and Results in Quantification in LC and GC, A Practical Guide to Good Chromatographic Data; Wiley-VCH, **2009**.
2. Held, D. How Do I Calibrate a GPC/SEC System? *The Column* **2008**, *(6)*.
3. Radke, W.; Held, D. Calibration Using Broad Standards. *The Column* **2015**, *(2)*.
4. Held, D.; Kilz, P. An Alternative to Calibration with Narrow Standards. *The Column* **2013**, *(8)*.
5. Held, D. GPC/SEC-Viscometry – a Versatile Tool for Structure Determination and More. *The Column* **2012**, *(2)*.
6. Held, D.; Kilz, P. How to Choose a Static Light-Scattering Technique for Molar Mass Determination. *The Column* **2009**, *(4)*.
7. Held, D.; Kilz,P. Improving the Reliability of GPC/SEC Light Scattering and Viscometry Results. *The Column* **2011**, *(10)*.
8. Held, D. ; Radke, W. Flow Marker - An Easy Concept to Increase Reproducibility, LCGC *The Column* **2016**, *(4)*.

Originally published in *The Column*, February **2013**, by author Daniela Held.

1.7. Do's and dont's in GPC/SEC-light scattering

Light scattering (LS) is one of the few absolute techniques for determination of molar mass and molecular size of polymers and biopolymers. LS instruments became commercially available in the late 1950s. For GPC/SEC, LS detectors appeared in the mid-1970s. Since then, these detectors have become an important tool for the analysis of macromolecules.

Absolute does not mean parameter-free. Sometimes, even an absolute technique is not suitable for specific types of macromolecules, as it requires knowledge of calculation parameters and a thorough setup of measurements.

The equation required to analyze polydisperse, nonisotropic scatterers in nonideal solutions shows that accurate concentration plays an important role.

$$\frac{K \cdot c}{R_\theta} = \frac{1}{M_w P(\theta)} + 2A_2 c + 3A_3 c^2 + \ldots$$

with:
c = concentration
θ = scattering angle
R_θ = Rayleigh ratio
M_w = weight average molecular weight
A_2 = second virial coefficient

and K, the optical constant,

$$K = \frac{4\pi^2}{\lambda_0^4 N_A}\left(n_0 \frac{dn}{dc}\right)^2$$

with:
λ_0 = laser wavelength
N_A = Avogadro's number
n_0 = refractive index of solvent
dn/dc = refractive index increment

and

$$\frac{1}{P_\theta} = 1 + \frac{16\pi^2 n_0^2 R_g^2}{3\lambda_0^2} Sin^2\left(\frac{\Theta}{2}\right)$$

where R_g is the molecule's radius of gyration.

Weigh your samples precisely

Although GPC/SEC does not require knowledge of the accurate concentration in a chromatographic slice, GPC/SEC-LS does. In order to calculate the molar mass from scattering intensity, sample concentration or injected mass in a chromatographic slice must be known.[1] An error in the slice concentration leads to wrong molar mass determination.

The slice concentration can be determined by a concentration detector (RI, UV detector).

Two different methods can be used:

- Method 1 requires accurate knowledge of the bulk sample concentration. The slice concentration is calculated from the batch concentration, assuming that 100% of the sample elutes from the column and that the injection system works properly. The sample needs to be weighed out accurately on an analytical balance.
- For method 2, the calibration constant of the concentration detector must be known, as well as the specific response of the sample. In the case of RI detectors, the refractive index increment (dn/dc) is the specific response. Standards such as polystyrene or pullulan, with accurately known dn/dc and concentrations, can be used to determine the calibration constant of the concentration detector using method 1.

In general, method 2 generates more accurate results, as errors resulting from incorrect injection volumes, on-column adsorption, or insoluble sample components do not affect the slice concentration, and therefore the sample molar mass.[2]

Nonetheless, it is recommended to weigh the sample precisely, because it is possible to calculate sample recovery. The recovery helps to identify systematic errors or malfunctioning system components early in the process.

Influence of dn/dc

The dn/dc of the sample has to be known to calculate molar mass from the scattering intensity. The dn/dc marks the change, dn, of the refractive index n of the solution, with the change of sample concentration, dc. The dn/dc of a sample depends on parameters such as sample composition, solvent, temperature, and wavelength of the light source used. Usually, the dn/dc can be regarded as constant above approximately 10,000 Da, however, for low molar masses, end group effects are observed.[3] Any error in dn/dc impacts molar mass determination.

Table 1 shows how deviations in method parameters can influence GPC/SEC-LS results. Data from a theoretical Schulz-Flory distribution with known molar mass averages[4] have been evaluated using inaccurate method parameters.[5]

Table 1. Influence of evaluation parameters on the accuracy of molar mass averages for GPC/SEC-light scattering.

	Variation	M_w (Da)	Deviation (%)	M_n (Da)	Deviation (%)
Reference value	–	300,000	–	150,000	–
dn/dc	5%	331,000	10.3	165,600	10.4
Concentration	5%	315,800	5.27	158,000	5.33
Interdetector delay	5%	295,100	1.63	159,500	6.33

Precise and accurate dn/dc values can be determined in batch mode using specific dn/dc instrumentation. However, it is common practice to determine dn/dc from the GPC/SEC-LS experiment using concentration data (usually RI). If this method is used, method 2 (as outlined) cannot be applied for determination of the slice concentration. For both batch mode and online dn/dc determination, precise knowledge of sample concentration is required. Insoluble sample components, water, and/or salt content must be corrected to achieve reliable results. Measurements should be performed at the same temperature and wavelength of the light scattering detector to minimize errors.

Use check out samples and validate your system

Even when working with an absolute detector, system validation is a requirement. Light scattering validation standards or certified reference materials can be used to test detector performance, identify systematic errors, and verify system integrity.

Knowledge of a number of system-related parameters is required for GPC/SEC-LS data evaluation. Proper data analysis can only be conducted if the following are known:

- Interdetector delay between LS and concentration detector
- Detector constant of the LS detector
- Detector constant of the concentration detector
- Normalization coefficients (MALS detector only)

If any of these parameters are wrong or have changed, the results will be incorrect. Using a check out sample helps to identify unintended or unidentified changes in the setup. Moreover, a wisely selected check out sample can be used to determine the actual system-related parameters.

Don't expect accurate results...

1) For copolymers

Calculating accurate molar mass values using light scattering detectors is governed by dn/dc accuracy. The dn/dc for a macromolecular material depends on its composition. For a homopolymer, the dn/dc value can be accurately determined. For a copolymer with a heterogeneous distribution, the dn/dc will vary unpredictably across the chromatogram, making it impossible to determine accurate and absolute molar mass values.

Unfortunately, it is insufficient to measure a single batch to determine an average dn/dc and assume it is identical in every chromatographic slice. There is no singular method to determine a true dn/dc of a copolymer. Therefore, molar mass results of copolymers from GPC/SEC-LS are inaccurate. An exception to this rule is the analysis of narrow distribution block-copolymers. In this case, the calculated molar masses are similar to the true molar masses.[6] As the width of the copolymers' chemical composition distribution increases, and values of homopolymer dn/dc change, the measured apparent molar mass and true molar mass will differ significantly (Figure 1).[7]

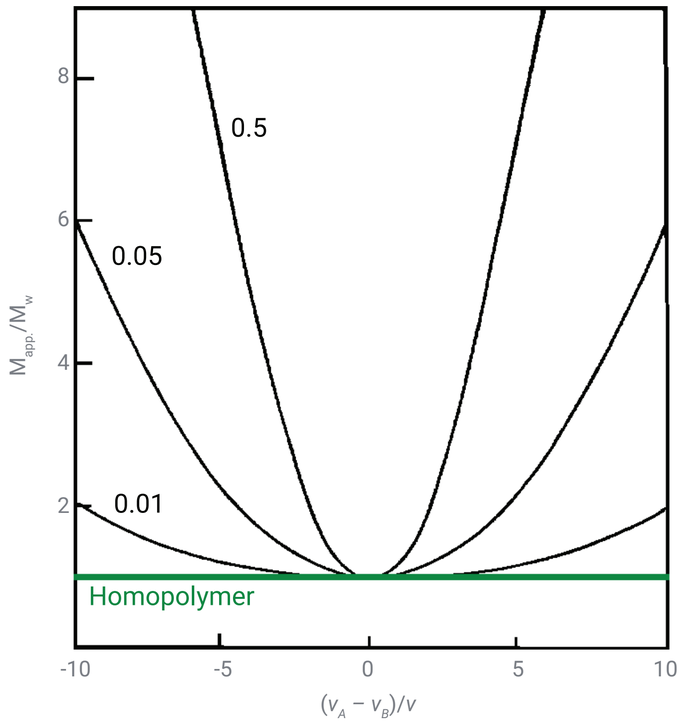

Figure 1. Variation of apparent molar mass and true molar mass for a copolymer with heterogeneous chemical composition (data from literature).[7]

Moreover, GPC/SEC-LS experiments do not reveal any information on copolymer composition or sample homogeneity. Other techniques, such as dual detection analysis, interaction polymer chromatography (IPC), or 2D chromatography on copolymers yield more insight than just an apparent molar mass.[8]

2) In mixed solvents

Selective sorption, also referred to as preferential adsorption, is a common effect in solvent mixtures. If a polymer develops a higher affinity to one solvent in a mixture, the concentration of this solvent is higher, closer to the polymer chain than in the polymer-free solvent.[7] This phenomenon impacts the dn/dc, and how it should be determined. An approach for mixed solvents is to measure dn/dc with polymer solution and mixed solvent in osmotic equilibrium. Without this procedure, the dn/dc will be inaccurate. For this reason, solvent-enhanced light scattering should be used with extreme caution.

Don't expect accurate molar mass distribution information...

1) When the column is overloaded

Light scattering (LS) in batch-mode allows only the determination of the weight-average molar mass (M_w) of a sample. LS, in combination with a fractionating system such as a GPC/SEC column, permits determination of the other molar mass averages (M_n, M_z), the polydispersity index, PDI, and molar mass distribution.

The analysis software generates chromatographic slices that are essentially monodisperse (i.e,. only one molar mass is present). Thus, the LS detector can produce a calibration curve based on the slice-by-slice M_w values of the specific sample from the sample injection. Often, this calibration curve is fitted and then used to calculate the molar mass distribution from the concentration detector signal.

If the separation is insufficient and the slices are not monodisperse, this approach will fail. The only valuable result from the GPC/SEC-LS experiment will then be the weight-average molar mass (and z-average of the radius of gyration (RG) for MALS detectors).

Distribution information is thus not available when a column is overloaded. For low molar mass samples (and low dn/dc), it is often recommended to increase concentration, so that higher signals and better signal-to-noise ratios are obtained. Should the concentration increase be too high, overloading the column, then the separation step is meaningless, leaving M_w as the only accurately measurable parameter. In this case, a better option is to perform light scattering in batch-mode.

2) Without a properly developed GPC/SEC method

All distribution information will also be lost if the GPC/SEC method is not working properly. However, the information obtained from the LS detector can give hints as to whether the GPC/SEC method is working.

Figure 2 illustrates two examples (see red curves) of separations for which a method re-evaluation is recommended, as the molar mass does not decrease with elution volume. There are even regions in which molar mass increases with elution volume, which contradicts the GPC/SEC separation mechanism. In addition, the figure shows an example where expected behavior is observed (see green curve).

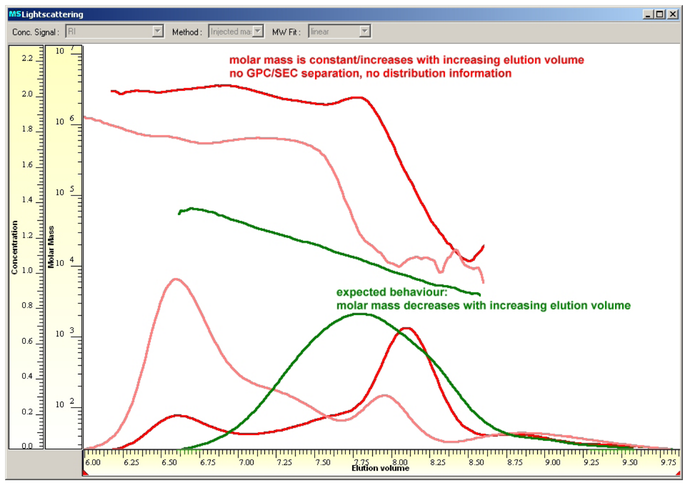

Figure 2. Red: Examples of measurements run with a wrong GPC/SEC method. Green: Expected molar mass decreases with increasing elution volume.

Conclusion

GPC/SEC-light scattering is ideal to identify high molar mass content at low concentrations and to investigate aggregates. It is a powerful, noninvasive technique to measure molar mass distributions of homopolymers, if:

- dn/dc value is known or can be measured
- The GPC/SEC method is developed properly
- Experimental parameters are determined accurately and monitored over the complete system's life cycle

References

1. Held, D. Understanding Positive and Negative Detector Signals. *The Column* **2008**, *(12)*.
2. Held, D. GPC/SEC-Light Scattering: Garbage In, Garbage Out. *The Column* **2016**, *(8)*.
3. Mori, S.; Barth, H. Size Exclusion Chromatography. *Springer*, **1999**, *(122)*.
4. Kilz, P.; Reinhold, G. Proceedings International GPC Symposium San Diego 1996, Boston, **1997**.
5. Kilz, P.; Held, D. Quantification in LC and GC - A Practical Guide to Good Chromatographic Data, Wiley-VCH, **2009**.
6. Gores, F.; Kilz, P. Copolymer Characterization Using Conventional Size-Exclusion Chromatography and Molar-Mass-Sensitive Detectors. *Chromatography of Polymers*, **1993**, ACS Symp. *Ser. 521*, 122–148.
7. Kratochvil, P. Classical Light Scattering from Polymer Solutions; *Elsevier*, New York, **1987**.
8. eBook: Practical Copolmer Analysis using GPC/SEC and Related techniques.

Originally published in *The Column*, February **2009**, by author Daniela Held.

Glossary

BHT	Butylated hydroxytoluene
Da	Dalton (g/mol)
DAD	Diode array detector
DMAc	Dimethylacetamide
DMF	Dimethylformamide
dn/dc	Refractive index increment
ELSD	Evaporative light scattering detector
FRCM	Flow rate correction marker (internal standard)
GPC	Gel permeation chromatography
HFIP	Hexafluoroisopropanol
LAC	Liquid adsorption chromatography
LC	Liquid chromatography
LiBr	Lithium bromide
LiCl	Lithium chloride
LS	Light scattering
MALS	Multi-angle laser light scattering
M_n	Number-average molar mass
Mobile phase	Liquid phase used in a chromatography system
M_w	Weight-average molar mass
M_z	z-Average molar mass

NaCl	Sodium chloride
NaN_3	Sodium azide
QC	Quality control
PDI	Polydispersity index ($D = M_w/M_n$)
PDMS	Polydimethylsiloxane
PMMA	Polymethyl methacrylate
PS	Polystyrene
RG	Radius of gyration
RI	Refractive index (detection/detector)
SDV	Styrene-divinylbenzene
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
TCB	Trichlorobenzene
THF	Tetrahydrofuran
UV	Ultraviolet (detection/detector)
V_e	Elution volume

Learn more:
www.agilent.com

Buy online:
www.agilent.com/chem/store

Get answers to your technical questions and access resources in the Agilent Community:
community.agilent.com

U.S. and Canada
1-800-227-9770
agilent_inquiries@agilent.com

Europe
info_agilent@agilent.com

Asia Pacific
inquiry_lsca@agilent.com

This information is subject to change without notice.

DE52520805

© Agilent Technologies, Inc. 2024
Published in the USA, February 13, 2024
5994-6433EN

Agilent

Trusted Answers