CALIBRATING GPC COLUMNS

A Guide to Best Practice

The Measure of Confidence

Primer

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Introduction

Calibration of GPC/SEC column sets using narrow molecular weight standards is a well-established and common technique for the molecular weight distribution of samples. However, analysts sometimes underutilize this technique or do not get the best from it. This paper describes best practice in the calibration of GPC columns, to ensure superior results are achieved in any laboratory. By incorporating the following information into standard operating procedures, every analyst can achieve the many advantages offered when the calibration curve is viewed as an integral component of method development.

Rethinking how and how often we use **standards in GPC applications can yield significant improvements** to the overall quality of results, including:

- Improved reproducibility
- Improved resolution, leading to better accuracy
- Earlier detection of problems
- Reduced trouble-shooting and system downtime
- Statistically significant analysis of the system

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Table 1. *GPC/SEC solvents and appropriate calibration standards.*

Eluent Type	Solvent	Standard
Organic	Tetrahydrofuran (THF), chloroform, toluene	Polystyrene (alternatively polymethylmethacrylates)
Polar organic or organic/water mixtures	Dimethylformamide (DMF), *N*-methyl-2-pyrolidone (NMP)	Polyethylene glycols/oxides (alternatively polymethylmethacrylates)
Aqueous	Water	Polyethylene glycols/oxides

What standards to use?

Your choice of calibration standards depends on the solvent you are using. There is a wide variety of standards available but typically four common standards have universal appeal and cover most applications, as shown in Table 1.

For column sets operating over a large MW range, prepackaged calibration kits containing 0.2 g of ten different molecular weights are available. If operating over a narrower MW range then the analyst should select individual standards to focus around the operating limitations of the column. To provide the greatest value in this scenario, individual molecular weight standards can be purchased in 1, 5, and 10 g quantities.

Regardless of how molecular weight standards are supplied, when sourcing standards it is critical to select the narrowest polydispersity possible. A poly-dispersity index (D) of 1.10 should be the minimum requirement. If a larger polydispersity standard is used, reproducibility will be compromised as the Mp will be less defined from injection to injection.

How are standards prepared?

It is important that calibration standards are correctly prepared to avoid spurious peak shapes, inaccurate calibration curves, and potential blockages in the filters and column frits.

Standards should always be dissolved with the same solvent being used as the mobile phase; this should be high purity grade solvent and filtered, typically through a 0.2 to 0.45 µm filter. It should not be necessary to filter the standard solutions providing they are fully dissolved. Vortexing, sonicating, and shaking of standard solutions should also be avoided. All of these high-shear activities will inevitably result in a change of peak shape, retention time and MW.

The concentration of standard solutions is also of critical importance. Too high a concentration leads to reduced mass transfer and band broadening due to excessive sample viscosity; too low and the signal-to-noise ratio will be too small to reproducibly integrate. In some instances viscosity effects can even change the retention time of the individual standards. Figure 1 shows the effects of using different concentrations of standards. Considering that viscosity varies with MW concentration, the optimal concentration for all polymer solutions is dependent upon the MW of the standard/sample being used (Table 2).

Table 2. *Standard molecular weight and corresponding concentration.*

Molecular weight (g/mol)	Concentration (weight to volume)
<5,000	<1.0
5,000 to 25,000	<0.5
200 to 400,000	<0.25
200 to 2,000,000	<0.20
10,000,000	<0.05

Sample solubility is a complex subject, and as such, simply because a standard is not visible in the presence of solvent does not guarantee it is fully in solution. True sample solubility only exists when equilibrium of solvent inside and outside the random coil polymer chain is achieved. Until this point the hydrodynamic volume of a particular standard is dynamic and hence reproducibility of retention time and molecular weight will also be dynamic. Higher MW standards will take longer to fully dissolve and some solvent/standard pairings may also require temperature to assist the dissolution process. When using temperature to aid in dissolution it is important to keep the temperature 10 °C below the boiling point of the solvent and to be sure thermal degradation is not observed in the form of tailing peak shapes.

To simplify the effects of concentration, preweighed prepared kits are available, for example Agilent's EasiVial or EasiCal ranges.

Conditions

Column: PLgel 10 µm MIXED-B, 7.5 x 300 mm (p/n PL1110-6100)
Standards: Polystyrene
Eluent: THF
Flow rate: 1.0 mL/min
Detection: UV

Key:
1. 8,500,000
2. 1,130,000
3. 170,000
4. 34,500
5. 5,100
6. 580

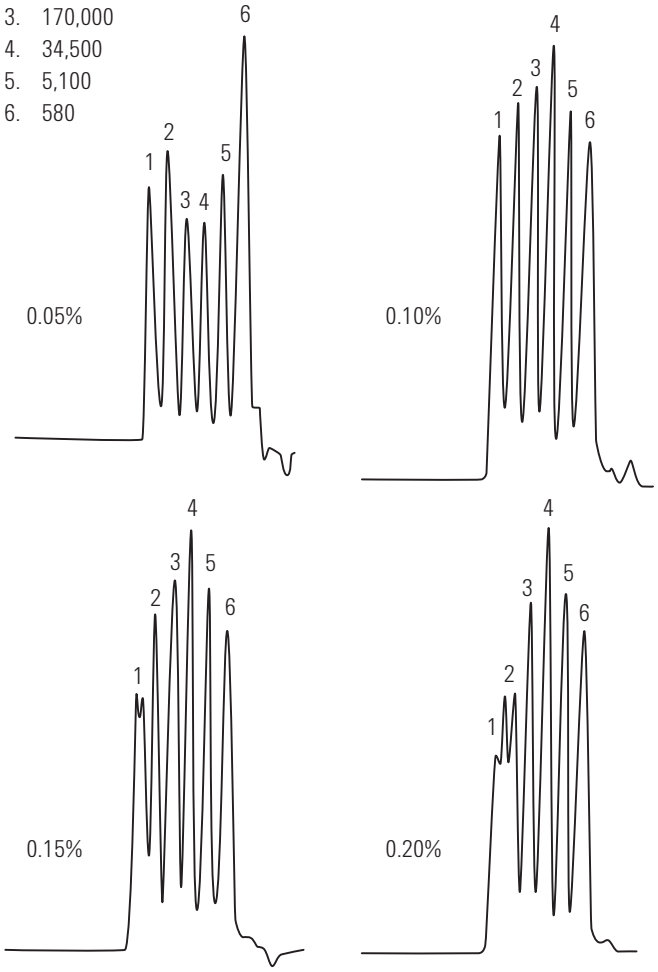

Figure 1. *Standard molecular weight and corresponding concentration.*

How many standards are required for successful calibration?

A calibration curve is typically constructed from ten to twelve data points. This number of data pointes is universally accepted as sufficient to provide an accurate polynomial regression while limiting the amount of time required to generate the standard curve. The standards should ideally cover the entire resolving range of the column set to reduce extrapolation errors during MW calculations. By calibrating the full range of the column, reproducibility will be increased when comparing calibrations and unknown samples over time. If it is not possible to calibrate the full molecular weight range of the selected columns, it is suggested that a linear extended range column such as PLgel MIXED be employed. All GPC software will yield greater reproducibility when extrapolating a linear regression. At minimum it is suggested that the selection of standards brackets the full molecular weight distribution of the unknown.

To eliminate the analysis time required for twelve data points, molecular weight standards are often injected as cocktails consisting of three to four standards each. To assure the most reproducible results when preparing cocktails, it is recommended that baseline separation of all components is achieved, when possible. This will avoid shifting retention times attributed to artificial band broadening. To help achieve this result, it is recommended that the individual molecular weight standards present in a cocktail be separated by a 10x difference. To simplify this procedure Agilent offers two forms of prefabricated molecular weight standard cocktails in multiple standard types and MW ranges:

- EasiVial is the quickest and most convenient method to deliver an accurate column calibration for organic and aqueous GPC/SEC. The preprepared kit contains three color coded 2 or 4 mL autosampler vials, each with a mixture of four accurately weighed polymer standards, providing a twelve-point calibration in just three injections.
- The EasiCal system for organic solvents consists of two different combs, each with ten detachable spatulas supporting a mixture of five polymer standards. The thin film of polymer (approximately 5 mg) on the tip of the PTFE spatulas rapidly dissolves when immersed in eluent to provide two GPC/SEC calibration solutions.

How frequently should new standards be made up?

Standards are typically stable in the form they are supplied in, but once made up can degrade, expedited by UV and heat. It is good practice to make up new standards on a weekly basis. We recommend that PEOs and PEGs are refrigerated for storage but brought to ambient temperature before use.

How can molecular weight standards be used in optimizing the LC system?

Because GPC molecular weight standards have a narrow polydispersity they allow the analyst to accomplish many statistical measurements as they apply to the overall health of the GPC instruments such as:

- Specific resolution per MW decade
- Resolution factor
- Plate count
- Peak symmetry
- Band broadening (effects of dead volume)

Even a simple calibration overlay before and after a sample set will bring confidence to the results achieved and immediately notify the analyst if the system is providing inconsistent results. Incorporating any and all of these measurements into a SOP will dramatically increase the relative standard deviation of results over time and reduce instrument downtime by allowing the analyst to decide when it is time to replace columns or schedule a service call.

Calculating specific resolution per MW decade across all MW regions

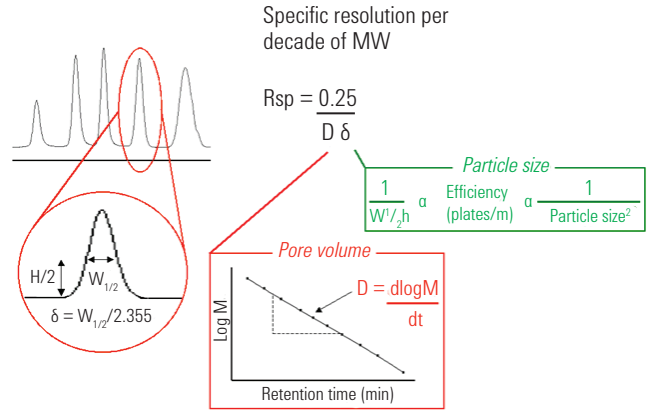

Figure 2. *Calculating specific resolution per MW decade across all MW regions.*

Calculating the resolution factor

$$Rs = 2\ \frac{(V_1 - V_2)}{(W_1 + W_2)}$$

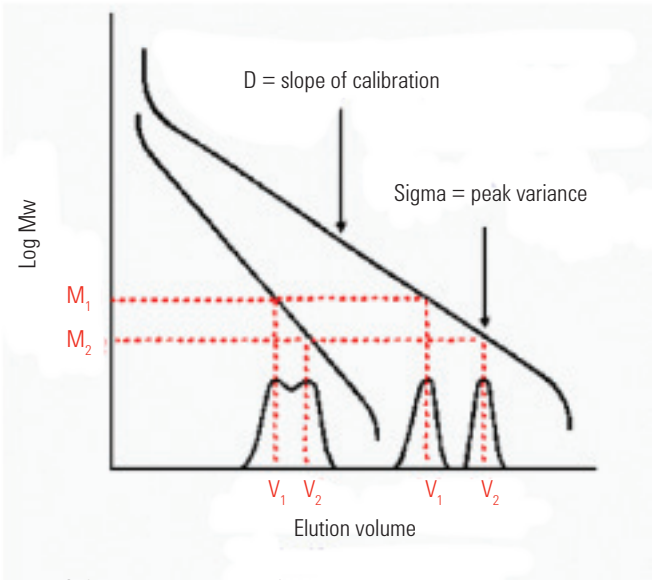

Figure 3. *Calculating the resolution factor.*

Calculating the plate count

$$N = 5.545 \left(\frac{t_R}{w_h} \right)^2$$

Where N is number of theoretical plates, ^{t}R is retention time, and ^{w}H is peak width at half height.

Calculating peak symmetry

Symmetry = W_1/W_2

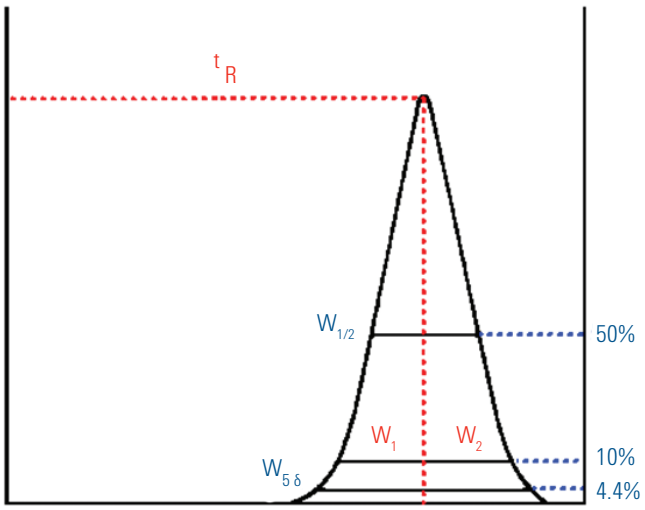

Figure 4. *Calculating peak symmetry.*

Measuring band broadening

$$H = Au^{1/3} + B/u + Cu$$

Where H is proportional to efficiency, Au is width of the exclusion peak, Cu is the width of the total permeation peak and B/u is the longitudinal diffusion or band broadening.

What components of an LC system cause dead volume and hence band broadening?

All parts of the system can contribute dead volume, from the injector through to detection. Common sources include the sample loop or injection rotor, column fittings and connectors, detector plumbing, and detector cells.

Reducing dead volume

The first step is to ensure that connectors and plumbing are as short, and the id as narrow, as possible. Check these are fitted correctly to the mating parts. Particular care should be taken to make sure that column connections are assembled correctly as dimensions can vary from vendor to vendor.

Secondly, use the smallest feasible sample loop. Sample loops should not be partially filled; it is not good practice to inject 20 µL of solution into a 100 µL sample loop, for example. Figure 5 compares the use of wide-bore and narrow-bore tubing. The benefit of narrow-bore tubing is clear.

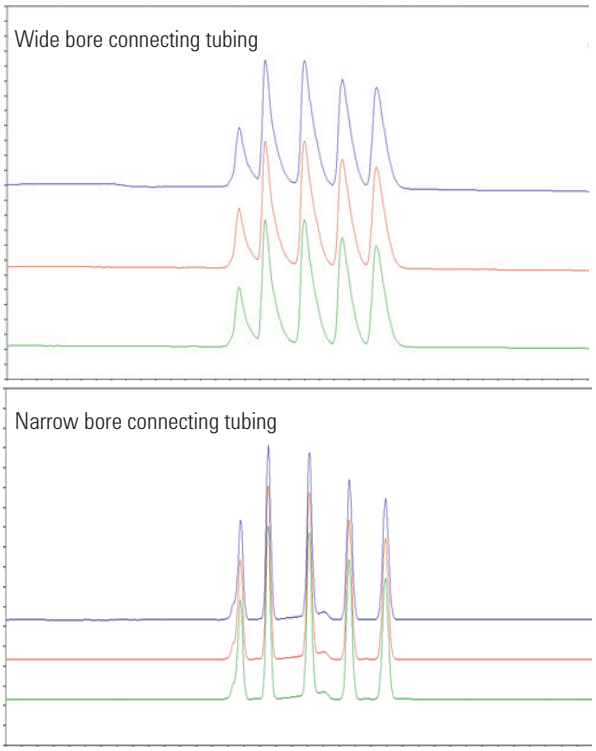

Figure 5. *Narrow bore tubing improves peak shape in GPC/SEC, compared to wide bore tubing.*

What else improves resolution?

There are a number of elements that can be modified to further improve resolution, such as increasing temperature, using columns with smaller particle sizes, changing flow rate, and increasing the number of columns in the column set.

Increase temperature

Temperature depends on the solvents, samples, and column types. Increasing temperature generally results in a reduction in viscosity of the mobile phase. This may improve resolution or allow additional columns to be connected in series. Maximum operating temperature is dependent on eluent boiling point, sample stability, and other factors including column stability.

Smaller particles

Columns with smaller particle size have increased efficiency. However, this option is dependent on the MW of the sample being analyzed; as the MW increases shear can occur when using very small-particle-size columns.

Change the flow rate

Optimal flow rates can differ with the particle size of your column, as shown in Figure 6.

Conditions

Column: PL Rapide M, 10 x 100 mm (p/n PL1013-2500)
Standards: EasiCal PS-1 polystyrene standards (p/n PL2010-0505)
Eluent: THF
Flow rate: Various
Detection: UV, 254 nm

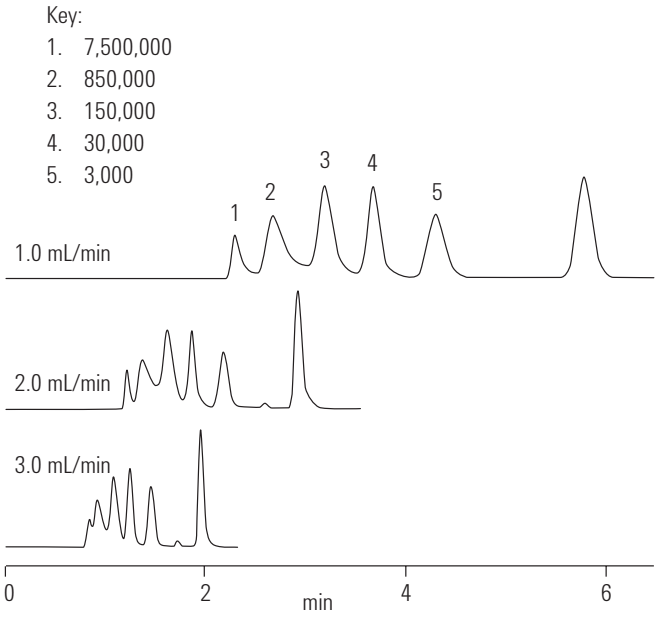

Figure 6. *Take care to choose the best flow rate, depending on the particle size of the column.*

Add more columns

Increasing the number of columns in series is possible, as shown in Figure 7, though this will be limited by pressure restrictions. Table 3 is a starting point when assessing the most appropriate column set.

Table 3. *Particle sizes and corresponding number of GPC columns.*

Particle size (µm)	Number of GPC/SEC columns
3	1 to 2
5	2 to 3
8 to 10	2 to 4
20	3 to 4

Conditions

Column: PLgel
Standards: EasiCal PS-1
Eluent: THF, stabilized
Flow rate: 1 mL/min
Detection: UV

Mp values

Injection 1	Injection 2
1. 7,500,000	6. 2,560,000
2. 841,700	7. 320,000
3. 148,000	8. 59,500
4. 28,500	9. 10,850
5. 2,930	10. 580

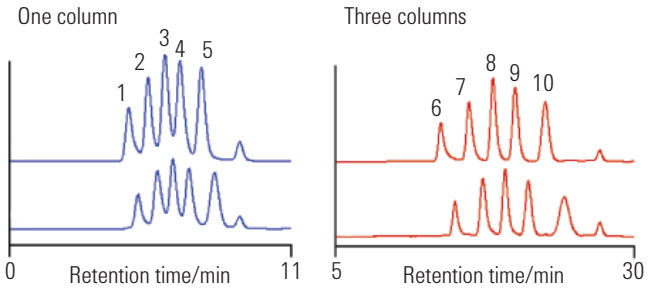

Figure 7. *Increasing the number of Agilent PLgel columns improves baseline resolution and peak height.*

How often should you calibrate?

Frequency of calibration is subjective. For continuous work it is advisable to calibrate daily, but with the use of internal verification a weekly calibration should be performed at minimum. It is essential to recalibrate whenever a component of the system is altered or there is an eluent change. However, to guarantee optimum performance we suggest performing bracketing calibrations for every column set. By doing so, confidence in the data collected will be significantly increased.

Remember an LC system is finite, with multiple components that degrade over time. Instrument issues such as blocked filters, blocked check valves, leaking injector seals, and leaking or blocked connectors will affect the calibration, and therefore the results you obtain from your sample run. In addition, any degradation to the column such as blocked or partially blocked frits, or a buildup of contamination leading to performance reduction with age, will also have significant effects. By calibrating frequently these issues can quickly and easily be highlighted and preventative steps immediately put in place.

Conclusions

GPC/SEC is the only established method for obtaining a comprehensive understanding of the molecular weight distribution of a polymer. However, it is important that proper attention is paid to regular column calibration to achieve the best results from the instruments and columns.

Polymer standards from Agilent are the ideal reference materials for generating accurate, reliable GPC/SEC column calibrations, with the assurance of the ISO 9001:2000 quality standard. Additional applications for our highly characterized homopolymers exhibiting unique characteristics are used as model polymers for research and analytical method development.

For more information on Agilent calibration standards, and to find out which is best suited for your application, refer to the GPC/SEC Standards Product Guide (5990-7996EN).

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