Review article

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Size-exclusion chromatography as a useful tool for the assessment of polymer quality and determination of macromolecular properties

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Abstract: Polymers, macromolecules in nature and bio-polymers like proteins and antibodies are present in our every-day life and play an important role in our economy. They are obviously present as plastic products (e.g., containers, clothing, car tires) or components, e.g., as expedients in pharmaceutical formulations, additives in food and feed, stabilizers in cosmetics, used in construction materials, ceramics and are key in microchip production. Size-exclusion chromatography is the most important characterization technique for macromolecules. This review covers applications, instrumental setup, step-by-step guides for performing experiments and covers theoretical background as well as troubleshooting and tips and tricks.

Keywords: applications; best practices; macromolecules; theory; troubleshooting; size-exclusion chromatography.

This text intends to introduce readers to performing successful size-exclusion chromatography (SEC) experiments and covers basic information and guidelines in *Section A*. After a general introduction in Chapter "*Introduction*" the wide range of SEC applications are summarized to familiarize readers with the technique and its scope (Chapter "*Scope of technique*"). Requirements and experimental details are discussed in Chapter "*Experimental requirements for size-exclusion chromatography*", which also covers best practices and guidance on avoiding pitfalls. This section is concluded by step-by-step instructions on how to perform successful GPC/SEC (Chapter "*Step-by-step guide to a first SEC analysis*").

Additional information on SEC advanced methodologies (Chapter "*Optimization of SEC experiments*") and theoretical background (Chapter "*Theoretical aspects of SEC separations*") are summarized in *Section B* for readers which require knowledge about SEC theory and information about adaption and optimization of SEC for specific applications.

Troubleshooting references, additional resources and further reading are covered in *Section C* at the end of this paper (Chapter "*Further reading and resources*" and following chapters). Supplementary information is available as a slide deck which can be used in teaching this topic (Chapter "*Supplementary information*").

Section A

Introduction

SEC (Size Exclusion Chromatography), GPC (Gel Permeation Chromatography), and GFC (Gel Filtration Chromatography) are all synonyms for the most successfully applied separation technique for the characterization of

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macromolecules, polysaccharides, (bio)polymers and proteins (Kilz & Pasch, 2000). Although the term SEC is often applied to separations in aqueous solutions and GPC for separations in organic eluents, the same theoretical background, setup, advantages and limitations are valid. However, with respect to the separation mechanism SEC (size exclusion chromatography) is the most descriptive name (Striegel, Yau, Kirkland, & Bly, 2009).

SEC is a liquid chromatography technique which allows to separate molecules based on their size in solution. The size separation step occurs in a column filled with porous particles (stationary phase) and is an entropy-driven process. The sample is dissolved in a solvent, which is also used as a mobile phase in the chromatographic process, and injected onto the column. Larger sizes are excluded from penetrating the pores in the stationary phase while smaller sizes can enter them and thus are retarded on their path through the column. Enthalpic interactions of the sample with the stationary phase surface, as required for HPLC-type separations, have to be strictly avoided in SEC.

SEC allows the measurement of the molecular weights *and* their distribution of macromolecules. These are in most cases polydisperse, i.e. a physical mixture of different molecules having various chain lengths (molecular weight) in the simplest case. This is the major advantage when selecting an analytical technique for the characterization of macromolecules as competing techniques allow the measurement of bulk properties only (Kilz, 2006).

Although both, SEC and HPLC, are used for chromatographic separation and require similar instrumentation, the separation mechanism, calibration methodologies and result calculation of both techniques is distinctively different. The major differences in the practice of SEC compared to HPLC are outlined in Table 1.

Table 1: Important differences between interaction chromatography (HPLC) and size exclusion chromatography (SEC).

	HPLC	**SEC**
Sample preparation	a) Fast dissolution, b) Sample degradation unlikely	a) Slow dissolution b) Avoid shear, microwave, etc.
Chromatogram appearance	Many narrow peaks	(One) broad peak (Plus trash peaks in RID)
Results	a) Qualitative analysis b) Quantitative analysis	a) Molar mass averages b) Molar mass distribution
Information derived from	a) Peak sequence b) Peak area	a) Absolute peak position b) Peak shape
Calibration	Detector response->amount	Retention->molar mass
Detection	Single detector (UVD, DAD) (MS)	Multiple detectors (UVD, RID, LS, viscometry, FTIR, MS, etc.)

In nearly all cases, a macromolecular solution consists of chains with different chain lengths (molecular weights), and, in case of copolymers, additionally of chains with different composition of co-monomers. Each molecule occupies a certain volume in solution, its hydrodynamic volume, that defines the size of the chain in this particular solvent at given physical conditions (temperature, pressure, pH, etc.). If such a polydisperse mixture is injected onto a size-exclusion chromatography column with a matching surface polarity/chemistry (refer to chapter "*SEC column selection*"), a chromatographic separation according to the molecules' size in solution will occur.

The fundamental importance of comprehensive macromolecular characterization can be demonstrated by the product release and certification of Heparin, an active pharmaceutical ingredient (API), which is applied as an antithrombic agent in medical care to prevent the formation and extension of clots, e.g., after surgery. Heparin binds to both Antithrombin III (AT) and Thrombin (T) in a specific steric conformation which causes Antithrombin III to complex thrombin, leading to the deactivation of thrombin and thus preventing blood clogging. Several requirements on a molecular level are crucial for the efficacy and efficiency of heparin as illustrated in Figure 1 (Laposata, 2013):

(1) well-defined chain length (molar mass)
(2) specific pentasaccharide sequence (composition)
(3) amount of sulfate groups in the chain (functionality)

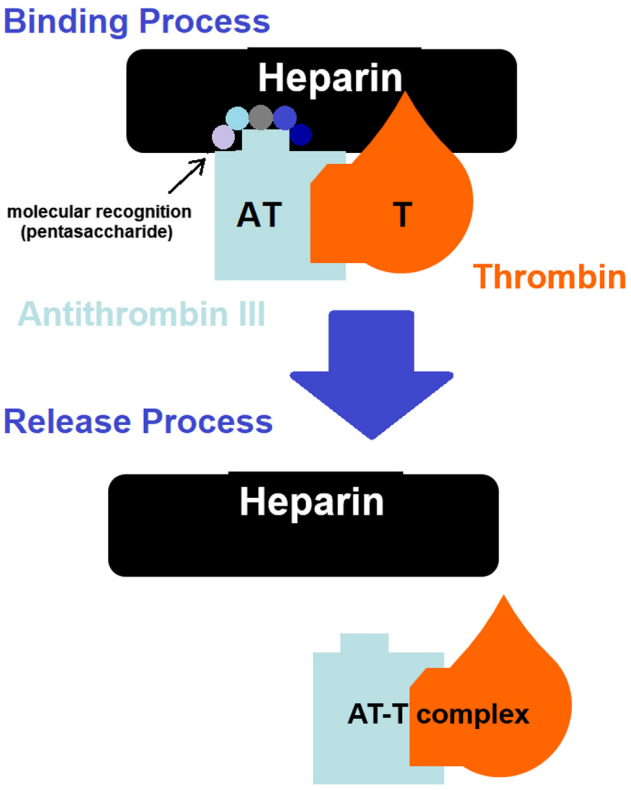


Figure 1: Heparin pathway to prevent thrombosis with main active ingredient requirements shown in schematic.

Natural heparin is a glycosaminoglycan-based polysaccharide extracted from cow and pig intestines for medical applications. Low molecular weight heparin (LMWH) is a more efficient active ingredient as compared to unfractionated heparin. LMWH formulations possess predictable anticoagulant effects and pharmacokinetics as well as fewer and less severe side effects (Lever and Page, 2002). Heparin quality is assessed regularly by SEC and strict product release criteria have been set in the respective pharmacopeia:

(1) weight-average molar mass < 8000 g/mol, and
(2) mass fraction > 60% with a molar mass < 8000 g/mol.

Figure 2 shows the results for two Heparin batches which have been obtained by SEC analysis in approx. 90 min. The product sample A (depicted in green) meets both quality requirements containing more than 60% of its mass in molecules with a molar mass of less than 8000 g/mol as compared to sample B (depicted in red), which only meets the M_w < 8000 g/mol requirement by the pharmacopeia for product release.

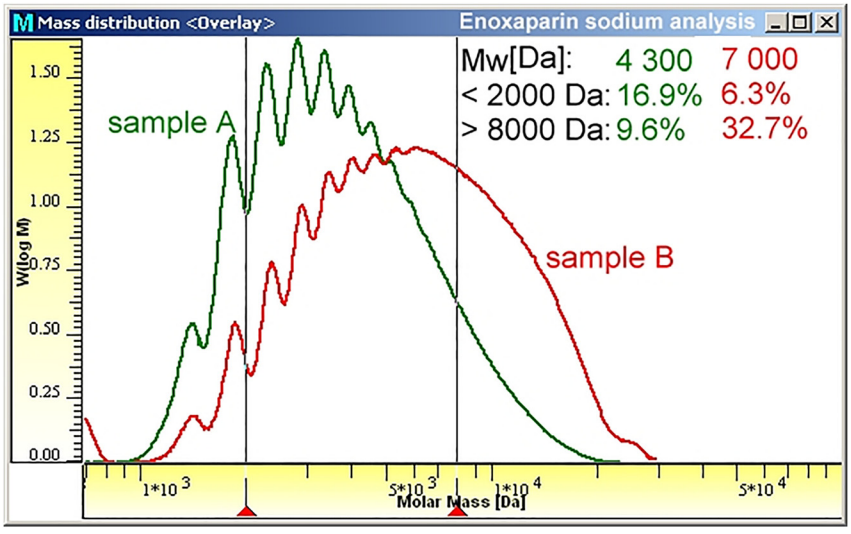


Figure 2: SEC results of two Heparin batches: sample A passes quality criteria, while sample B fails and is not certified for medical use (courtesy: PSS [Held and Gores, 2010]).

The importance of proper heparin quality assurance became obvious when more than 100 people died and several thousand patients fell ill in 2008 after several pharmaceutical companies failed to discover fraudulent heparin materials used in their medical grade production (Usdin, 2009).

Scope of technique

Analyzing macromolecules by SEC has many advantages. First of all, it is a fractionating technique providing access to distribution information as well as to property averages, e.g., all molar mass averages, in a single measurement. Since in most cases nondestructive detectors are used, it is possible to re-collect the sample and/or to investigate the fractions with other analytical methods. Basic laboratory equipment can be used and it is easy to run the samples. Furthermore, a SEC system can be fully adapted to the characterization needs. Different kinds of columns are available enabling goals such as high sample throughput/fast analysis, high information depth/high resolution, saving solvent/green chemistry or high sample loading/small scale clean-up to be met (see Table 2). The use of hyphenated techniques (Kilz & Pasch, 2000) and the addition of specialty detectors increase the information content and measurement of different results and distributions simultaneously with just one injection. This short summary explains why SEC is considered an indispensable tool when working with macromolecules.

Table 2: SEC analysis tasks identifying instrument requirements and typical examples.

Task	What do i need	Application examples
Sample preparation (often on PSS micro SEC columns)	SEC instrument, SEC columns, Reference standards	– Serum proteins from drugs (then GC or HPLC) – Highly toxic residual monomers in polymers (SEC-GC) – Pesticides in contaminated soils (SEC-GC) – Triglycerides from fat (subsequent GC analysis) – Removal of high molar mass species from flavor mixtures
Sample fractionation (often on PSS prep SEC columns)	SEC instrument, SEC columns, Model compounds, Fraction collection device (no software)	– Fullerenes: C_{60}, C_{70}, … – Odors for cosmetics industry – Toxic or non-active by products – Compounds from extracts
Sample identification (on-line or off-line)	SEC instrument, SEC columns (analytical to prep), Fraction collection device (no software)	– Fourier-transform infrared spectroscopy – Mass spectrometry (MS) – MALDI – Nuclear magnetic resonance (NMR)
Good/Bad sample comparison	SEC instrument, SEC columns, SEC software	– QA in production – QC in polymer processing – Failure testing
Molecular characterization	SEC instrument, SEC columns, SEC standards, SEC software (additional detectors)	– Molar mass averages – Molar mass distribution (MMD) – Chemical composition distribution (CCD) – Functional-type distribution (FTD) – Polymer architecture (MAD)
Property correlations	SEC instrument, SEC columns, SEC standards, SEC software (additional detectors)	– Tensile strength (correlating to M_z) – Polymer flow (correlating to M_n)
Degradation studies	SEC instrument, SEC columns, SEC software	– Wear and tear inspection – Stability of biodegradable polymers

Table 2: (continued)

Task	What do i need	Application examples
Kinetics and migration studies	SEC instrument, SEC columns, SEC standards, SEC software (additional detectors)	– Reactor modeling – Complex/conjugate formation – By-product formation – Metabolization – Polymer-polymer interface – Additives, stabilizers
Inverse SEC	SEC instrument, SEC columns, Size calibration	– Membrane characterization – Porous solids – Adsorbers/adsorbents
2-Dimensional chromatography	2 instruments, 2 separation methods, transfer valve, SEC columns, SEC standards, 2D-software	– Copolymer characterization (CCD) – Polymer deformulation – Architecture elucidation (MAD)

Experimental requirements for size-exclusion chromatography

Figure 3 shows schematically a simple lab setup consisting of instrumentation, consumables and computer software for data acquisition and processing.

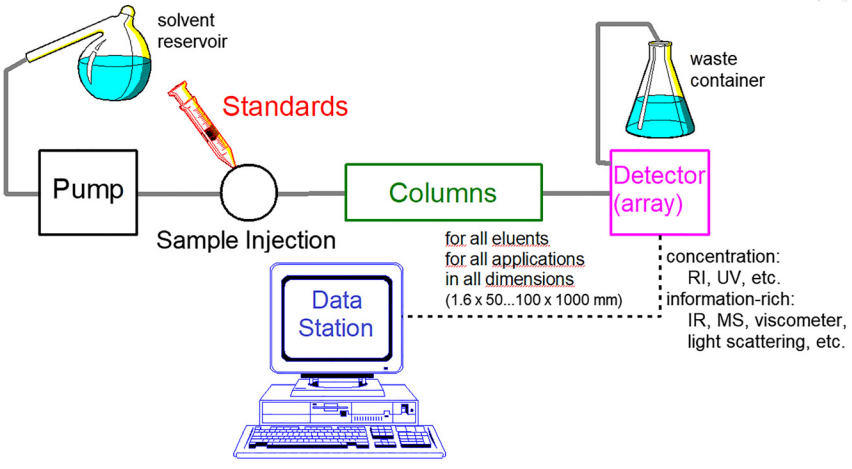


Figure 3: Schematic setup of SEC instrument including consumables, detection options and MCDS software.

Instrumentation

Minimum requirements are a SEC instrument with a solvent delivery pump (with high flow-accuracy), high-pressure injection system (manual or automated), one or more SEC column(s), one or more detector(s) and a computer with a software that allows acquiring, calibrating and analyzing data. Other components such as degasser or column heater/thermostat are optional and depend on the application as well as on the lab environment and conditions. Solvent reservoir, waste bottle and solvent retainer (for the glass bottles) are required to manage eluents safely.

Consumables

High purity solvents (typically > 99%), reference materials for calibration/verification/checkout, syringes, membrane filters.

Software

As size-exclusion, data processing and calibration are unique and distinctively different from conventional HPLC or GC chromatographic data systems (CDS), a macromolecular CDS (MCDS) especially designed for GPC/SEC work is a good investment as it saves time, energy and meets requirements which are special to GPC/SEC. The software is used to control instrumentation, acquire real-time raw data from one or more systems, perform molar mass calibrations, allows to set baselines, integration limits, calculates results and result uncertainties and creates reports.

SEC column selection

The major parameter for column selection is the intended application (Kilz, 1999). A balance of mobile phase polarity in comparison with the polarity of the stationary phase and sample polarity is important for pure SEC separations. In general, users will select their columns according to the mobile phase they need to use. Stationary phase materials can be either silica or polymeric based. Table 3 shows an overview of stationary phases with different polarities typically used in SEC.

Table 3: Typical stationary phase packings for SEC columns.

	Polymer packing			Inorganic packing	
Polarity	**Non**	**Medium**	**Polar**	**Medium**	**Polar**
Chemical nature	Styrene-divinylbenzene	Acrylic, polyester	OH-acrylic	Diol-modified silica	Silica
Typical eluents	THF, TCM, toluene	DMF, NMP, DMAc, DMSO	Water (pH 1.5 to 13)	Aqueous (pH < 9)	THF
Use for	PS, PMMA PVC, PC, Resins, PE, PP, etc.	PU, starch, cellulose, polyimide etc.	PEG/PEO, pullulan, dextran, PAA, etc.	Proteins, peptides, PLA, polyester, POM, etc.	PS, PB, PIB

After selecting the best matching column material for the analyte, other column parameters have to be taken into account to meet the analysis goals. Here column dimensions, the particle size, and the porosity are of importance.

Column dimensions

Different column dimensions are available. Most common are analytical SEC columns with an inner diameter between 0.7 and 0.8 cm and a length of 25–30 cm. Sixty centimeter columns are still available but rarely used. Preparative columns with the same length but a larger diameter (up to several cm) allow to fractionate larger sample amounts. (Semi)micro columns with approximately the same length and a smaller inner diameter are used with dedicated instrumentation for saving solvent and if only small sample amounts are available.

Particle size

The smaller the particle, the better the chromatographic resolution as dispersion effects are minimized (Kilz, 2006). However, high molar masses and high viscous solvents require larger particle sizes because the injection solution cannot be diluted by the eluent flow through the column. So there is an optimum particle size depending on the application (Kilz, 2006; Striegel et al., 2009).

Pore size

Another important parameter for column selection is the proper choice of sorbent porosity (Kilz, 2006). The molar mass range of the samples to be investigated determines the column porosity. The larger the pores, the higher molar mass samples can be characterized. Unfortunately, there is no general nomenclature, which will allow easy selection of column pore sizes. Each manufacturer has its own system for pore size designation. The easiest method to find out which columns will be useful for a selected task uses the calibration curve which every manufacturer shows in their literature.

In general, SEC columns can be either single porosity columns with narrow pore size distribution or linear (also called mixed-bed) or multipore columns with a very broad pore size distribution. SEC separation capacity is limited by the available pore volume and depends on sorbent type, column dimensions and the slope of the calibration curve. The highest selectivity for a separation is determined by the lowest slope of the calibration curve.

For single porosity columns the separation capacity is concentrated in a narrow molar mass range. This yields a calibration curve with a flat or shallow slope in this region. Therefore, single porosity columns have a limited molar mass separation range, but a high resolution in that range. In contrast to that, columns with a broad pore size distribution provide a larger separation range and the calibration curve has a steeper slope and therefore less resolution.

Often linear or mixed-bed columns are either used in QC for fast screening experiments or to identify the molar mass range of a sample, so that it can be investigated on a matching single porosity column bank with higher precision.

SEC detector selection

There is a wide range of chromatography detectors available on the market. However, not all are useful in macromolecular analysis and several “specialty” detectors are specific to macromolecular analytes. An in-depth discussion of detector technology for SEC use can be found in References (Kilz & Pasch, 2000; Striegel et al., 2009).

As each chromatography technique requires the online determination of concentrations of each separated (analytical) fraction such detectors are required in every instrumental setup.

Concentration detectors

Refractive index detectors (RID)

The RID measures the change in refractive index of the column effluent passing through the flow cell compared to that of the pure eluent stored in the reference cell.

This is the most useful and generally applicable concentration detector in SEC as it detects most analytes independent of, e.g., solvent and wavelength (as in the case of an ultraviolet spectroscopy detector (UVD)). Only isorefractive samples cannot be detected. An example for this is poly(dimethylsiloxane) in tetrahydrofurane (THF). Such samples should be analyzed, e.g., in toluene to ensure proper concentration determination.

RID detectors are primarily used to measure concentration profiles. In addition, they are used to measure the fraction concentration when working with online light scattering detectors, viscometers or online mass spectrometry.

In combination with other concentration detectors they are used to measure the comonomer distribution and molar mass in copolymers or end group distributions.

Disadvantages of this detector are its low sensitivity, relatively long times to stabilize, their tendency to drift (especially with poor thermostatization and in solvent mixtures) and their large cell volume.

Variable wavelength (UVD) and diode-array (DAD) detectors

They measure the UV adsorption at a fixed (user-selectable) wavelength for samples with chromophores. Since SEC is used to measure the molar masses and the distribution, it is sufficient to measure at one or two fixed wavelengths where the sample shows absorption. Spectra from diode array detectors (typically used for substance identification in HPLC) are only rarely needed, e.g., for the analysis and identification of oligomers with special properties.

UVD detectors are used to measure concentration profiles and (if used alone) molar masses based on a calibration curve. In addition they are used to measure the fraction concentration required when working with online light scattering detectors, viscometers or mass spectrometers. They are the most common detectors for protein characterization.

In combination with other concentration detectors they are used to measure the comonomer distribution and molar mass in copolymers or end group distributions.

UV detectors possess small cell volumes, are easy to use and have a good sensitivity (compared to a RID). A disadvantage of this detector is that it can only be applied for a limited number of polymeric samples due to missing chromophores (e.g., in PDMS, PVC, PE, PEG/PEO) and potentially strong changes of response factors with minor chemical or oligomer molar mass variation.

Evaporative light-scattering detector (ELSD)

This detector destroys (evaporates) the mobile phase to create a particle stream with the number of particles changing with the analyte concentration. However, the relationship between sample concentration and response (peak area) is not linear and therefore this detector is not suitable for quantitative analysis. The main advantage of this detector is the high sensitivity, suitability for solvent gradients and that it can detect samples which do not possess chromophores.

This detector cannot be used with high flow rates, samples which evaporate and eluents which contain non-volatile components (e.g., salts).

Note that this is not a detector to measure molar masses (weight-average molecular weight) in solution, since it uses light scattering to determine the number of particles in non-condensed phase.

Non-concentration detectors

Online light scattering detectors

They measure at one or more fixed detection angle(s) the time-averaged intensity of light scattered by macromolecules in solution. Low-angle laser light scattering (LALLS) detectors measure scattered light intensity at 5–7° and right-angle laser light scattering (RALLS) detectors at 90°. Multiangle laser light scattering (MALLS) detectors measure light intensity at multiple angles simultaneously. They are used to determine absolute molar masses for homopolymers and proteins and polymer structures in solution (MALLS only).

Online viscosity detectors

Online viscometers come in a range of different configurations (single, dual, or four-capillary type viscometers with a symmetrical or asymmetrical bridge). The most suitable type is a 4-capillary bridge viscosity detector which combines high sensitivity with flow independence.

They measure the pressure difference between a sample path and a reference path filled with pure solvent. Viscometers are used to measure specific and intrinsic viscosity, molar masses based on Benoit's universal calibration approach (Grubisic, Rempp, & Benoit, 1967) and Mark-Houwink coefficients.

Mass spectrometry detectors

Different mass spectrometric methods have been used in macromolecular analysis. They are used to determine absolute molar masses for homopolymers and copolymers and to detect polymer structures. Matrix Assisted Laser Desorption Ionization–Time of Flight (MALDI-ToF) and Electrospray Ionization (ESI) are the most common instruments used in combination with SEC. A recent application summary can be found in (Barner-Kowollik, 2011).

IR detectors

Online detection with an IR detector is mainly used in high temperature SEC (HT SEC) for the characterization of polyolefins. FTIR signals provide information, e.g., on short chain branching, if the ratio of different wavelengths is compared. For many other SEC applications online IR detection cannot be used due to the fact that the typical solvents absorb in the same region as the investigated polymers. Far more important are offline techniques (Kilz & Pasch, 2000). The SEC effluent is directed to a heated nozzle for evaporation of the solvent followed by sample deposition on a germanium disc. The disc is then scanned in an offline step in standard FTIR spectrometers. This elegant technique allows to separate and to detect without the influence of the solvent and is often used in additive analysis to identify unknowns, e.g., in master batches.

Modern SEC instruments in R&D are today often equipped with 3 or 4 detectors in series. However, this does not minimize the user input required and the knowledge of each method applied (SEC, light scattering, viscometry) is indispensable. Additional system parameters, such as the inter detector delay, need to be determined carefully as they influence the results (Held & Kilz, 2009). In addition band broadening due to use of several detectors can be a problem (Gaborieau, Gilbert, Gray-Weale, Hernandez, & Castignolles, 2007; Mader and Schnöll-Bitai, 2005; Meira, Netopilík, Potschka, Schnöll-Bitai, & Vega, 2007).

Best practices and avoiding experimental pitfalls

Despite general knowledge about chromatography instrumentation, successful macromolecular characterization by SEC/GPC/GPC techniques require special attention to instrument modules, sample preparation, eluent preparation and column use. The following chapters present best practices and how to avoid common pitfalls and oversights. Additional information and details on working with advanced detection systems can be found in (PSS Polymer Standards Service, 2019).

Instrument readiness testing

Table 4 summarizes important experimental component features and provides a test description with pass criteria for successful operation of SEC instrumentation (PSS Polymer Standards Service, 2019).

It also lists items which are often overlooked and can make lab life difficult or render analytical results ambiguous.

Table 4: Hardware readiness test.

Module	Important feature(s)	Precautions	Suitability Test
Eluent reservoir	Solvent purity Degassed solvent Solvent composition	Remove particulates by filtering Degas and reservoir position higher than pump Homogeneous composition	Filter through 10 µm membrane before use No bubbles in solvent line to pump Avoid mixed solvents, stir eluent
Pump	Constant flow	Do not use gradient pumps Alternative: bypass proportioning valve	>4 repeat injections of test molecule, e.g., BHT, acetone, ethylene glycol; Pass at retention time deviation < 0.1%
Manual injector/ Autosampler	Constant injection volume delivery	Manual loop fill; Sample drain to waste (hydrostatic syphoning effect)	>4 repeat injections of test molecule, e.g., BHT, acetone, ethylene glycol; Pass at peak area deviation < 5%

Table 4: (continued)

Module	Important feature(s)	Precautions	Suitability Test
Column	Column readiness	Equilibrate column with current eluent, temperature, system pressure	>2 repeat injections of polymer calibration standard: Pass at -retention time deviation < 0.1%, *and*—signal shape is identical overlay signals), *and* - peak area deviation < 5%.
Detector(s)	Plumbing sequence	Back pressure issues	UVD/DAD: Typical first detector after column(s) RID: last module downstream
Detector(s)	Stable baseline	Signal drift Signal noise	Inject polymer calibration standard: Pass if signal drift/h < 5% of peak height. Inject eluent measure: Pass if signal noise <2% of peak height
Detector(s)	Detector readiness	Equilibrate detector	UVD/DAD: > 0.5 h lamp burn time. RID: Purge reference; wait >4 h to stabilize.

Sample preparation

Analysis of synthetic polymers require often aggressive solvents and long dissolution times are often required for sample preparation together with additional considerations:

- Allow samples to be dissolved on a molecular level in order to prevent aggregation/agglomeration and thus abnormal chromatograms.
- Ensure eluent and sample compatibility with installed separation columns.
- Verify stability (degradation, reactivity with samples/sample constituents, …).
- Consider environmental, health and safety aspects (e.g., toxicity, elevated temperature, etc.).

General sample dissolution aspects:

- In order to minimize so-called system peaks, especially for RID, use a portion of solvent directly from the eluent reservoir for sample preparation and minimize injection volume (if possible).
- Avoid high shear, stirring, sonication during dissolution.

The optimal sample concentration depends on molecular weight and sample polydispersity (local concentration in the column during elution); refer to Table 5 for guidance.

Recommended sample concentrations and dissolution times for typical samples analyzed on conventional SEC columns (typical dimension: 300 × 8 mm ID) in good solvents are presented in Table 5.

Table 5: Recommended sample related parameters.

M_w range [kg/mole]	Concentration[a] [mg/mL]	Dissolution Time[b] [h]	Flow rate [mL/min]
0.1–10	20–3	0.5	2–1
10–1000	2	1–4	1–0.5
1000–3000	0.5 mg/ml	14–24	0.5–0.2
>3000	<0.2 mg/ml	24–72	0.1

[a]sample concentration also depends on sample polydispersity PDI (higher PDI allows higher conc.). [b]dissolution time depends also on temperature and crystallinity of the sample.

Sample filtration:

Samples containing particles and/or (micro) gel contents can cause overpressure issues and column blocking directly after injection.

- Filter off insoluble residues through 0.2–1.0 µm membrane filters (e.g., use disposable syringes with suitable filters, consider solvent and sample compatibility).
- Some samples (e.g., natural rubber, native starch samples, …) might be difficult to filter. Then, centrifugation is the method of choice.

Eluent recommendations

General eluent requirements for GPC/SEC applications are:

- eluent and solvent for sample preparation must be filtered through <10 µm membrane filters after additives, salts, etc. have been dissolved
- eluent temperature should be < 30 °C below its boiling point
- eluent must be free of visible bubbles before being fed into the pump
- replace eluent regularly (typically every two weeks)
- eluent should be kept in brown glass bottles and not be exposed to direct sunlight, aqueous mobile phases need additives (e.g., 0.05 M sodium azide) to prevent algae growth
- eluent bottle must be capped with ability for atmospheric pressure exchange
- system effluent must not be fed back to the eluent reservoir on a regular basis
- flush all new tubing with eluent before connecting to a module/column
- flush columns with eluent (approx. 3x column volume) before connecting to a detector
- eluents containing salts are not suitable for storing columns and should not be left in in detectors or in systems without mobile phase flow

Step-by-step guide to a first SEC analysis

This guided SEC analysis assumes that the SEC system as outlined in chapter "*Experimental requirements for size-exclusion chromatography*" is equipped with a (minimum) RID detector and operating in THF as the eluent and a SEC column packed with styrene-divinylbenzene (SDV) packing material. Each injection requires approx. 15 mL of eluent and sample elution will take 15 min to complete (at a pump flow rate of 1 mL/min).

Please refer to the manufacturer documentation for details.

1. Ensure that the SEC system is operational and ready to run (see chapter "*SEC detector selection*" and Reference (PSS Polymer Standards Service, 2019) for details).
2. Attach a linear (mixed bed) analytical column (with approx. molar mass range 1–3000 kg/mol) to the SEC system. Start the pump to deliver eluent through the column for a minimum of 4 column volumes (approximately 1 h at 1.0 mL/min). After column conditioning is complete, stop the pump flow, connect the column outlet to the detector(s) and start the pump up again.
   Note: If the column is new or has been stored for a longer time, do not attach the column outlet to the detector(s) but initially direct the effluent to a waste container to purge out aged components. This will prevent contaminating the detector with species trapped in the column.
3. Dissolve polystyrene molar mass calibration standards for the calibration of your SEC system by adding the required eluent volume from the reservoir bottle to each of the calibration vials (refer to Table 5 for details). Vials should sit a minimum of 2 h on the lab bench without agitation (vortex, ultrasound, etc.). The most easy-to-use reference standards are pre-mixed so-called ReadyCal standards which just require mobile phase addition to get ready (PSS Polymer Standards Service, 2016).
4. Start your chromatography data system (preferably a dedicated (optimized) SEC MCDS software package (Kilz, 2019) and record the baseline.
5. Perform a column plate count and asymmetry test as described in the column user documentation or on the certificate of analysis.
6. Perform a test injection with one of the calibration standard solutions and determine the detector signal drift (which should be < 5% of the maximum peak height) and signal noise (<2% of peak maximum height). Repeat injection if pass criteria are not met (see Table 4 for remedies). Continue to step 7 if signal drift and baseline noise pass test criteria. If the test fails, replace the column with approximately 3 m of *new* stainless steel capillary (0.1 mm internal diameter) and rerun the test. If the test now passes, replace the SEC column with a new one. If the signal test still fails, follow the troubleshooting hints in the detector user documentation.

7. Inject 20 µL of each of the calibration standard solutions, create a molar mass calibration table, and graph in the chromatography data system as shown in Figure 4.

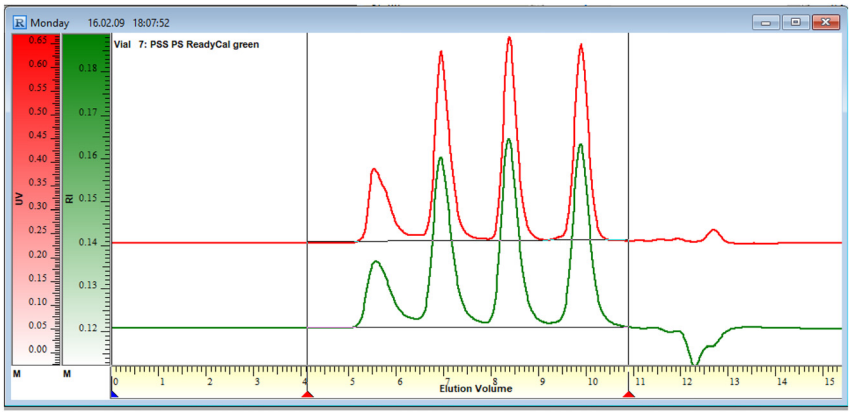
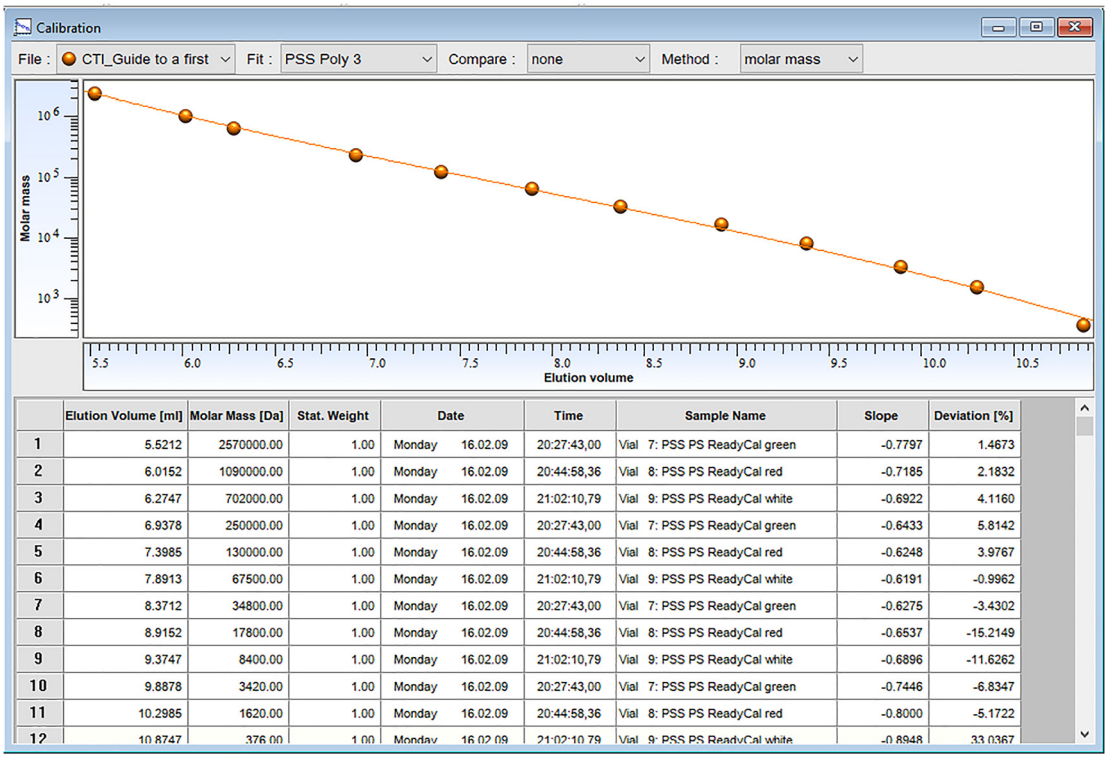


Figure 4: a) SEC chromatogram of a Polystyrene PSS ReadyCal reference material with UVD (red) and RID (green) detector traces.



	Elution Volume [ml]	Molar Mass [Da]	Stat. Weight	Date	Time	Sample Name	Slope	Deviation [%]
1	5.5212	2570000.00	1.00	Monday 16.02.09	20:27:43,00	Vial 7: PSS PS ReadyCal green	-0.7797	1.4673
2	6.0152	1090000.00	1.00	Monday 16.02.09	20:44:58,36	Vial 8: PSS PS ReadyCal red	-0.7185	2.1832
3	6.2747	702000.00	1.00	Monday 16.02.09	21:02:10,79	Vial 9: PSS PS ReadyCal white	-0.6922	4.1160
4	6.9378	250000.00	1.00	Monday 16.02.09	20:27:43,00	Vial 7: PSS PS ReadyCal green	-0.6433	5.8142
5	7.3985	130000.00	1.00	Monday 16.02.09	20:44:58,36	Vial 8: PSS PS ReadyCal red	-0.6248	3.9767
6	7.8913	67500.00	1.00	Monday 16.02.09	21:02:10,79	Vial 9: PSS PS ReadyCal white	-0.6191	-0.9962
7	8.3712	34800.00	1.00	Monday 16.02.09	20:27:43,00	Vial 7: PSS PS ReadyCal green	-0.6275	-3.4302
8	8.9152	17800.00	1.00	Monday 16.02.09	20:44:58,36	Vial 8: PSS PS ReadyCal red	-0.6537	-15.2149
9	9.3747	8400.00	1.00	Monday 16.02.09	21:02:10,79	Vial 9: PSS PS ReadyCal white	-0.6896	-11.6262
10	9.8878	3420.00	1.00	Monday 16.02.09	20:27:43,00	Vial 7: PSS PS ReadyCal green	-0.7446	-6.8347
11	10.2985	1620.00	1.00	Monday 16.02.09	20:44:58,36	Vial 8: PSS PS ReadyCal red	-0.8000	-5.1722
12	10.8747	376.00	1.00	Monday 16.02.09	21:02:10,79	Vial 9: PSS PS ReadyCal white	-0.8948	33.0367

Figure 4: b) Molar mass calibration graph (top) and numeric table with details for each of the 12 calibration reference materials.

8. Save the calibration and assign it to the current sample sequence.
9. Create or modify an existing data acquisition method with the calibration established in step 8.
10. Prepare a broad Polystyrene reference material (e.g., available from www.pss-shop.com) or a commercial polystyrene sample (e.g., Styrofoam, plastic cup) by dissolving the sample in the eluent over night without agitation. After >12 h swirl the sample solution slightly and filter it into a glass vial/bottle.
11. Load the data acquisition method prepared in step 9 and inject 50 µL of sample solution in triplicate. Process all 3 injections, calculate the molar mass results and molar mass distribution. Molar mass averages of all three repeat injections should deviate <5% for M_w and <10% for M_n and M_z. Overlay the 3 chromatograms (elugrams) and molar mass distribution curves for review as shown in Figure 5.

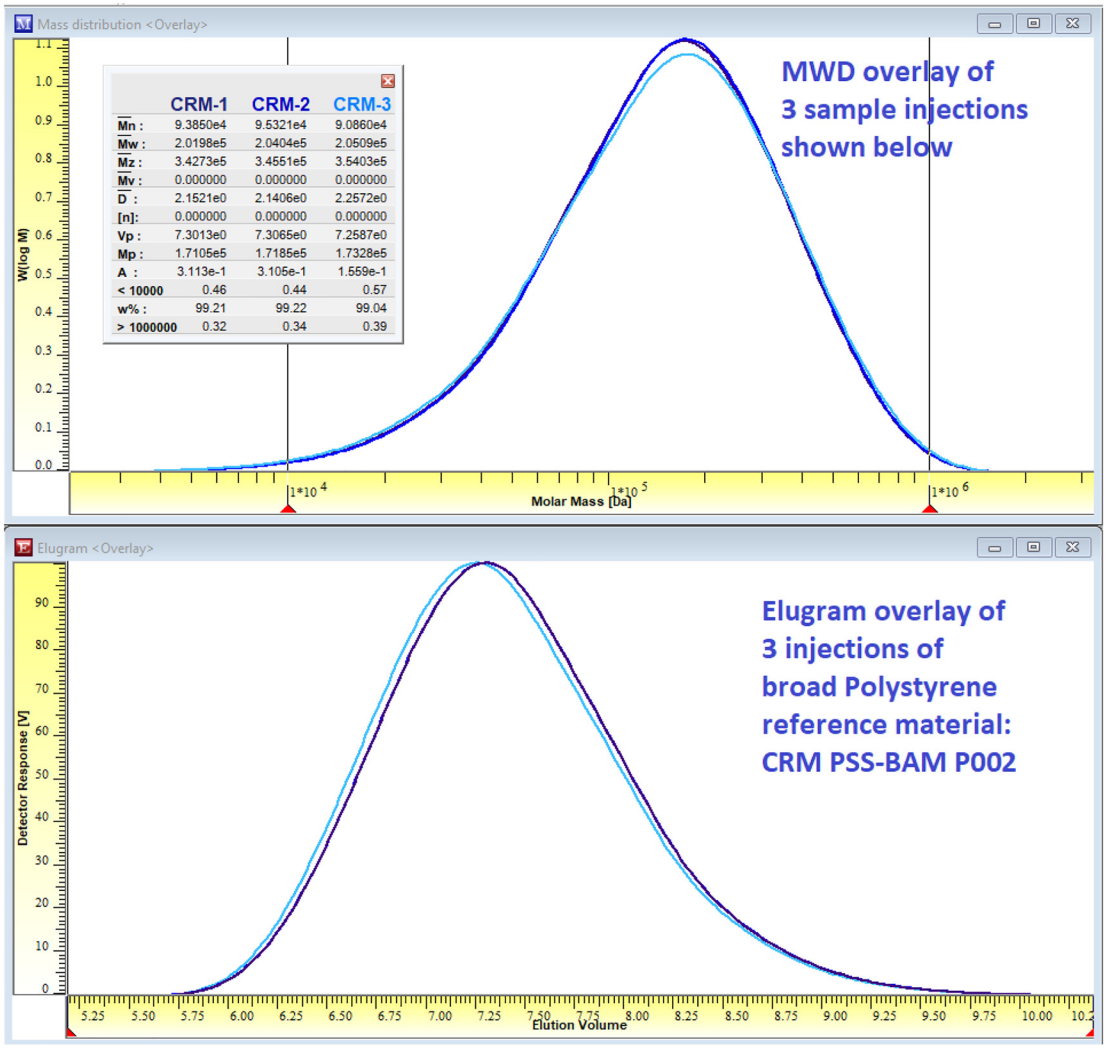


Figure 5: Triplicate SEC analysis of broad Polystyrene CRM (PSS-BAM P002): molar mass distributions and results overlay shown in top graph, raw signal overlay shown below.

Section B

Optimization of SEC experiments

SEC separations require interaction-free diffusion of the sample molecules into and out of the pores of the stationary phase. In general, this goal is easier to achieve in organic eluents than in aqueous solutions. In aqueous mobile phases more parameters (e.g., type of salt, salt concentration, pH, addition of organic modifier, concentration of co-solvent) have to be adjusted correctly. In addition, due to the presence of charged functional groups, hydrophobic, and/or hydrophilic regions in the molecule, water soluble macromolecules have more possibilities to interfere with the stationary phase.

A proper SEC experiment has to be balanced with respect to polarities. In order to obtain a true and pure SEC separation, the polarity of stationary phase (column material), the polarity of eluent and the polarity of sample have to be matched. This is visualized by the magic triangle (see Figure 6). Dominance of size separation is only maintained in the center of the triangle (bright area). where the overall system is balanced. Otherwise specific interactions will occur, which will overlay with the normal SEC elution behavior.

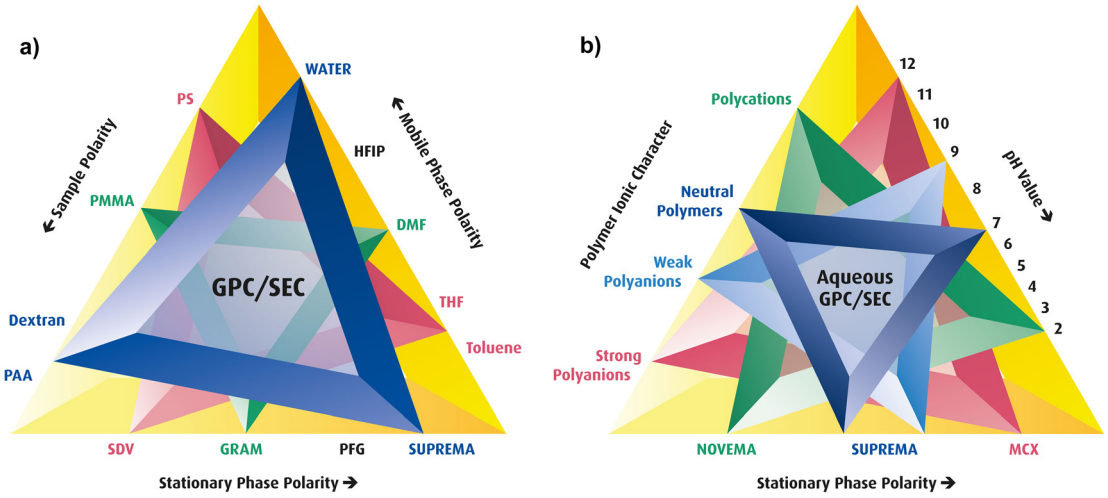


Figure 6: Balancing the polarities of the phase system in SEC applications for interaction-free separations; optimization of sample, eluent and column for a) organic eluents and b) aqueous mobile phases (courtesy: PSS Polymer Standards Service, pss-polymer.com).

SEC method optimization

To increase the resolution and/or the separation range a very simple approach can be applied. Instead of just using one column, multiple columns are combined to a column combination or a column bank; 2 to 4 columns (plus a guard column) are typical in SEC. A column combination or column bank provides more available pore volume for more efficient separations. If two columns with the same pore sizes (single porosity or linear/mixed bed/multipore) are combined, the slope of the calibration curve will be smaller and the resolution increases by a factor of 1.4 (Kilz, 2006). However, the separation time increases by a factor of 2 (refer to Figure 7 for a visual impression). If columns with different porosities are combined the molar mass separation range increases (Kilz, 2006).

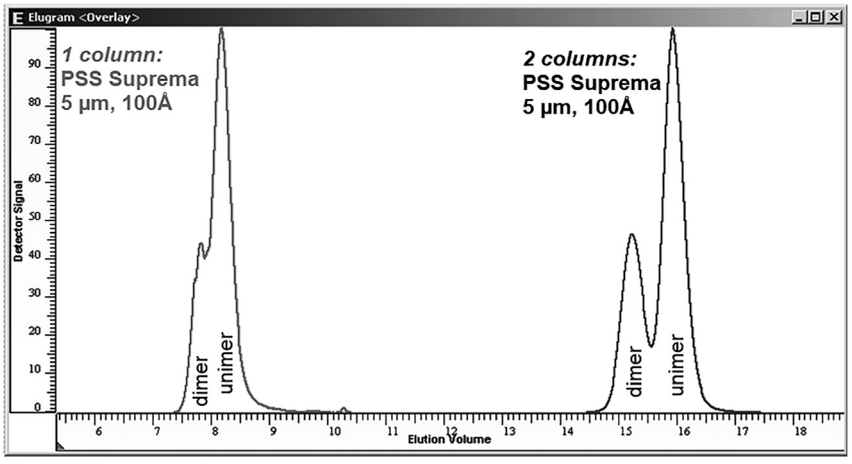


Figure 7: Comparison of SEC resolution enhancement of myoglobin unimer-dimer pairs by increasing the column length by a factor of 2.

Figures 8 and 9 show a comparison of the same sample mixture analyzed on two different column banks. In Figure 8 the columns are optimized for the characterization of low molar masses while the column bank in Figure 9 is optimized for the separation of medium molar masses. This example illustrates also the influence of the slope of the calibration curve on the resolution, as well as the difficulty for inter-laboratory comparison of chromatograms: the look of chromatograms or the raw data depends of the columns used. Therefore it is always recommended to compare the molar mass distribution instead of the chromatograms or raw data.

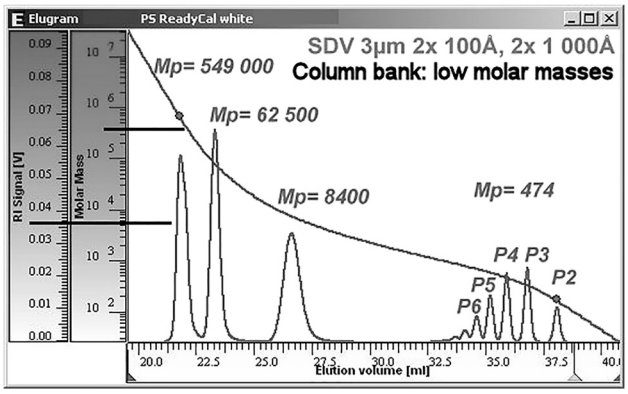


Figure 8: Separation of a poly(styrene) standards cocktail on a SEC column bank optimized for oligomer separation by combining narrow pore-size columns; the flat calibration curve indicates best resolution at low molar mass.

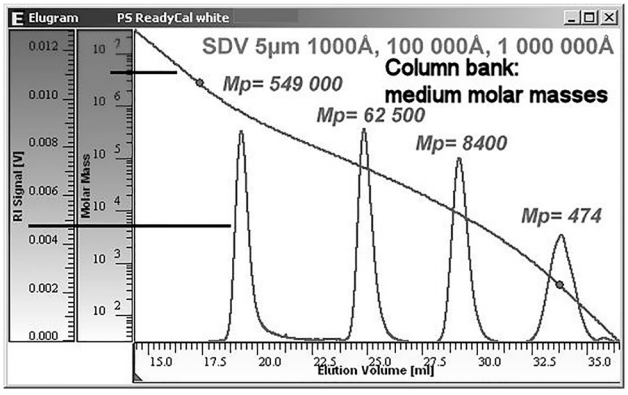


Figure 9: Separation of the same poly(styrene) standards cocktail as in previous Figure on a SEC column bank optimized for medium molar masses. The lower resolution in the low molar mass region results in a single peak instead of multiple peaks for each single oligomer.

Disadvantages of column banks are that price, pressure, analysis time and eluent consumption increase. An increased pressure might result in the need to reduce the flow-rate and/or to increase the temperature to have better chromatographic conditions, especially for high molar mass macromolecules. In addition, there is the potential danger of porosity mismatch for all column types, linear/mixed bed or single porosity alike. Porosity mismatch often shows itself in peak shoulders which might be misinterpreted as better resolution, but are artifacts of a column bank due to nonmatching porosities. This phenomenon can also be observed if non-matching porosities are mixed in one linear/mixed bed column to provide a wide linear separation range.

Method optimization with respect to a better resolution includes also to adjust all parameters that improve the mass transfer. The following parameters can be used to optimize the separation:

Particle size

Theoretical plate height and column permeability decrease with the particle diameter (Kilz, 2006). Smaller particle size columns provide therefore a better resolution. This concept, that led to the development of UHPLC, can also be adapted to SEC taking some peculiarities into account. Figure 10 shows a comparison of a protein mixture measured on the same column material with different particle sizes. The mass transfer for the 5 µm material is much better resulting in an increased resolution. Therefore, if the molar mass and rigidness of the macromolecules permit and no shear degradation occurs, the higher prices for small particle columns are a good investment in higher resolution.

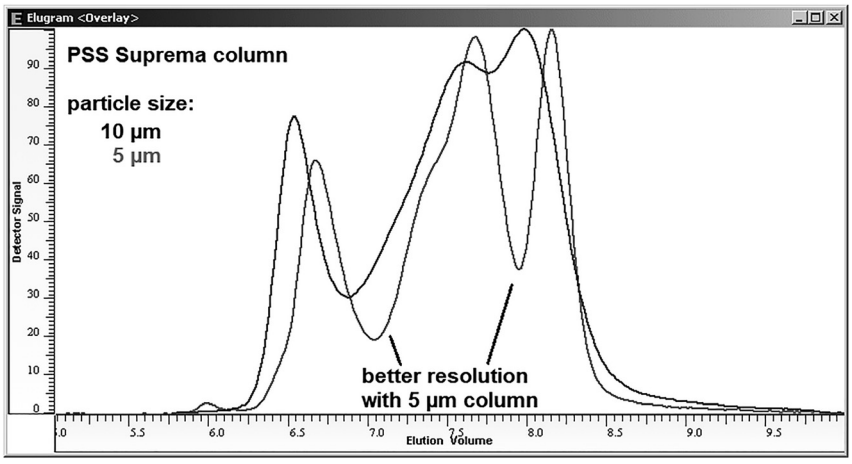


Figure 10: Influence of particle size of column packing material on the resolution of a protein mixture under otherwise identical conditions.

The general rule of thumb is that oligomers in low viscous solvents and proteins allow columns packed with 3 µm particles, for medium molar masses 5–10 µm particles are recommended and for high molar masses and high viscous solvents 10–20 µm particles sizes are used as column packing materials.

Flow-rate

1 mL/min flow-rate is often applied for analytical SEC columns with an inner diameter between 7 and 8 mm as the flow-rate with the best compromise between resolution and analysis time. Especially for higher molar masses a decrease of the flow-rate results in a higher resolution. Columns with larger inner diameter are best operated with higher flow-rates while columns with smaller inner diameter are used with lower flow-rates to maintain the same linear flow velocity.

Temperature

A temperature increase will in general also result in a better resolution due to the enhanced mass transfer. However, this is not applicable for all macromolecules. For example, polyethylene glycol (PEG) in aqueous solution shows a better resolution at lower temperatures.

High temperature SEC systems, where the complete system is heated, is needed for macromolecules that are only soluble at elevated temperatures, e.g., polyethylene (PE) or polypropylene (PP).

Table 6 summarizes various ways to optimize SEC separations by proper selection and combination of SEC columns.

Table 6: Optimization of SEC separations.

Task	Optimized by
Better peak separation	Addition of similar columns; use of 3 µm columns, if M < 100 kg/mol; use of 2D chromatography
Better separation of high molar mass fractions	Addition of column(s) with large pore width/large porosity
Better separation of small molecules, additives, solvents, eluent, etc.	Addition of column(s) with small pore width/small porosity
Avoiding exclusion peaks	Addition of column with large pore width/large porosity
Increase selectivity	Change phase system (column, eluent) use universal detector (RID)
Faster analyses	Use of high speed columns
Faster calibrations	Use of premixed calibration cocktails (ReadyCal)
Fast screening of unknown samples	Use of (less) linear/mixed-bed columns
Better reproducibility	Use of internal standard correction (flow marker)

Optimizing detection in SEC

At least one detector in SEC is required to detect the eluting sample. In many cases, SEC instruments for research are fitted with 3 or 4 detectors in series. However, advanced detection hardware capabilities come at a price as they require additional care and operator knowledge. Moreover, additional instrument parameters, e.g., the inter detector delay volumes, are required as they may bias the results significantly (Held & Kilz, 2009). Special attention has to be taken on band broadening caused by the multiple cell volumes and connection capillaries when several detectors are incorporated into a SEC instrument (Gaborieau et al., 2007; Mader and Schnöll-Bitai, 2005; Meira et al., 2007).

The molar mass distribution is then derived from the measured concentration of the separated fraction, its molar mass (from a calibration curve or measured directly using additional detectors as light scattering detectors or mass spectrometers), and the slope of the calibration curve.

The requirements for detectors in SEC are the same as for detectors in other methods: first of all, the detector must be able to detect the sample in the desired application, while the ability to detect a broad range of samples is a definite plus. Additional requirements, e.g., sensitivity, detection limit, linearity, baseline drift, noise, cell volume, and ease-of-use have to be taken into account. As detector design is discussed in many chromatography books (e.g., Kilz & Pasch, 2000; Striegel et al., 2009; Meyer 2010), this text will focus on the applicability and usability of detectors in polymer analysis.

In contrast to HPLC, the combined parallel or in series use of detectors with different principles is one of the major advantages in modern SEC experiments. It allows access to more detailed sample information, sometimes to absolute molar masses, sometimes to other types of distributions, that can be present in complex polymeric samples (e.g., chemical composition distribution, end group distribution, structural distributions, etc.). Table 7 presents an overview of typical detector combinations used to investigate specific sample properties. Additional information can be found in review papers (Kilz & Pasch, 2000; Striegel et al., 2009) and in the Supplementary Information.

Table 7: Summary of SEC applications with different detector combinations.

Detector combination	Applicable for
UVD with dual wavelength or any other concentration detector combination, e.g., UVD/RID/IR	a) Copolymer characterization: b) Copolymer composition distribution, copolymer molar mass. c) Simultaneous spectral identification of species with IR.
UVD with RID	As above plus: a) Absolute molar mass determination by UV selective chain end b) End group analysis.
RALLS/LALLS/MALLS with RID/UVD	a) Homopolymer characterization: b) Absolute molar masses and molar mass distribution. c) MALLS only: Radius of gyration averages and size distribution, polymer structure, branching.
Viscometer with RID/UVD	Specific and intrinsic viscosity, molar masses and molar mass distribution based on Benoit's universal calibration hydrodynamic radius, branching, polymer structure and size distribution.
RALLS with viscometer and RID/UVD (triple detection)	Homopolymer characterization: a) Absolute molar masses and molar mass distribution. b) Specific and intrinsic viscosity, molar masses and molar mass distribution based on Benoit's universal calibration hydrodynamic radius, branching, polymer structure and size distribution.
MALLS with viscometer and RID/UVD	a) Homopolymer characterization: absolute molar masses and molar mass distribution. b) Radius of gyration (averages and distribution), polymer structure, branching. c) Specific and intrinsic viscosity, molar masses and molar mass distribution based on Benoit's universal calibration hydrodynamic radius, branching, polymer structure and size distribution.

Table 7: (continued)

Detector combination	Applicable for
UVD/RID with FTIR/MALDI sample collector (off-line FTIR/MALDI)	a) Molar masses and molar mass distribution. b) Additive identification and quantification. c) Tacticity, copolymer composition.
MS with RID/UVD/ELSD	Low to medium molar mass samples: absolute molar masses and molar mass distribution, end groups

Theoretical aspects of SEC separations

The basic principle of chromatography separation can be described by simple thermodynamic principles applying the thermodynamic distribution coefficient, K:

$$K = a_s/a_m = \exp(-\Delta G/RT) = \exp((-\Delta H + T\Delta S)/RT) \tag{1}$$

with

a activity (concentration) of the molecule in the stationary phase (indexed s) and the mobile phase (indexed m).

ΔG free energy change between the species in the stationary phase and the mobile phase.

R universal Gas constant.

T absolute temperature.

ΔH enthalpy difference between the species in the stationary phase and the mobile phase.

ΔS entropy difference between species in the stationary phase and the mobile phase.

In SEC separations, the enthalpic contribution, ΔH, to the free energy term is negligible, assuming no energetic interaction between analyte and sorbent as expressed by the size-exclusion distribution coefficient, K_{SEC}:

$$K_{SEC} = \exp(\Delta S/R), \quad 0 < K_{SEC} \leq 1, \quad \Delta H \approx 0 \tag{2}$$

ΔS entropy loss when a molecule enters the pore of the stationary phase.

In the HPLC case of non-steric interaction of the molecule with the stationary phase, the retention can be described by the enthalpic term alone, as expressed by the HPLC distribution coefficient, K_{HPLC}:

$$K_{HPLC} = \exp(-\Delta H/RT), \quad K_{HPLC} \geq 1 \quad T\Delta S \approx 0 \tag{3}$$

ΔH enthalpy change when a molecule is adsorbed by the stationary phase.

Equations (2) and (3) describe the two ideal extremes of chromatography (SEC and HPLC), when there is no contribution of entropy or enthalpy, respectively.

Calculation of molar mass averages

SEC separates based on the hydrodynamic volume and the molar mass information is only available when a correlation between molar mass and elution volume has been established by a calibration or absolute detection by molar mass sensitive detectors.

The calculation of the molecular weight averages uses the so-called slice method (Held & Kilz, 2009; Kilz, 2006; Schröder, Müller, & Arndt, 1998). The eluted concentration profile is cut into equidistant volume slices and the elution volume domain is transformed to the molar mass domain.

The molecular weight averages are defined and calculated by:

$$\text{number average molecular weight, } M_n\text{: } \overline{M}_n = \frac{\mu_0}{\mu_{-1}} = \frac{\sum_i h_i}{\sum_i \frac{h_i}{M_i}} \tag{4}$$

$$\text{weight average molecular weight, } M_w\text{: } \overline{M}_w = \frac{\mu_1}{\mu_0} = \frac{\sum_i h_i \cdot M_i}{\sum_i h_i} \tag{5}$$

$$z - \text{average molecular weight, } M_z\text{: } \overline{M}_z = \frac{\mu_2}{\mu_1} = \frac{\sum_i h_i \cdot M_i^2}{\sum_i h_i \cdot M_i} \tag{6}$$

with h the signal height and M the molar mass of the slice (analytical fraction) i.

μ represents the moment of the distribution function and are a more general method to calculate different averages of distributions. The molecular weight averages can then be calculated from the moments, μ, of the molar mass distribution, $w(M)$, as described above (Held & Kilz, 2009; International Organization for Standardization, 2008; Schröder et al., 1998):

$$\mu_i = \int_0^\infty M^i w(M) dM \tag{7}$$

The width of the molar mass distribution can be described by the polydispersity, D, also called polydispersity index, PDI:

$$D = \frac{\overline{M}_w}{\overline{M}_n} \tag{8}$$

However, molar mass averages are reduced information only and do not describe a polydisperse sample comprehensively. The macroscopic properties of macromolecules can better be derived from their molar mass distribution, $w(M)$. Two samples can have the same molar mass averages but still have very different molar mass distributions and therefore macroscopic properties. It is possible to derive the molar mass averages from the molar mass distribution but not vice versa.

The molar mass distribution can be calculated from the signal heights, h(V).

The differential distribution, $w(M)$, of the molar mass M is defined as

$$w(M) = \left(\frac{dm}{dM}\right) \tag{9}$$

where: dm/dM is the mass fraction of polymer in a dM interval

By simple transformations, $w(M)$ can be expressed by quantities measured by SEC instrumentation directly:

$$w(M) = \frac{h(V)}{M(V)\sigma(V)}; \sigma(V) = \frac{dlgM}{dV}; \tag{10}$$

with: $h(V)$ detector signal with elution volume, V, as observed in the chromatogram.

σ(V) slope of the calibration curve.

$M(V)$ molar mass change with elution volume, V.

The correction with the slope of the calibration curve is necessary, because the data recording is linear in the time domain while the molar mass does not increase linearly due to the separation process (calibration influence). This means, that the number of polymer chains with the same concentration on the high molecular weight part of the chromatogram is much smaller than on the low molecular weight part. Only with strictly linear calibration curves, a behavior observed only for a very limited number of setups, the correction is not needed.

Fundamentals of SEC calibration

The primary information obtained from SEC is not the molar mass, but the apparent concentration at an elution volume. Only by matching SEC calibration and the concentration profile from the concentration detector can the molar mass averages and the molar mass distribution be obtained. SEC is therefore a relative method if no

absolute detection is employed (Striegel et al., 2009). The SEC calibration is based on assigning a molar mass to an elution volume (calibration of *x*-axis). This is in contrast to HPLC, where the detector response (signal intensity, peak area) is calibrated and assigned to a concentration (calibration of *y*-axis).

The relation molar mass to elution volume can be determined using one of the following methods or setups:

- SEC calibration with molar mass reference materials, e.g., with
  - narrow molar mass distribution
  - broad molar mass distribution
- use of an online viscometer and a concentration detector and universal calibration of the SEC system,
- use of a light scattering and a concentration detector,
- use of a mass spectrometer and a concentration detector.

SEC calibration with reference materials with narrow molar mass distribution is by far the most commonly used and most accurate method for calibration (International Organization for Standardization, 2008; Schröder et al., 1998). The standards come with certificates showing at least the molar mass averages. They are used for conventional calibration or for universal calibration with or without an online viscometer. The calibration curve is created by measuring the elution volumes of the reference materials and by plotting them versus the logarithm of the molar masses (in general the molar mass at the peak maximum). Then a fit function, that describes the shape of the calibration curve, has to be chosen. Unfortunately, there is no general fit function that can be used for all columns/column banks, but users have to select a proper fit function based on multiple criteria.

Most calibration curves, often even the ones for linear or mixed-bed columns, have a sigmoidal shape. This is in agreement with the fundamental separation characteristics and in contrast to other calibrations in chromatography, where linear calibration curves for the peak area plotted versus the concentration are obtained.

A SEC calibration curve can be divided into three distinct regions, as shown in Figure 11. Domain I represents the exclusion limit of the column(s), where no separation occurs as species larger than the largest pores in the column packing elute at this position in the chromatogram. Domain II is the optimum size separation range for macromolecular characterization. Molecules are separated according to their hydrodynamic volume in solution. Large molecules with high molar masses elute first; molecules with lower molar masses and smaller hydrodynamic volumes elute later. Domain III represents sample interaction with the stationary phase (HPLC mode). This limit is called total penetration volume, where all molecules elute that are smaller than the smallest pores in the column packing.

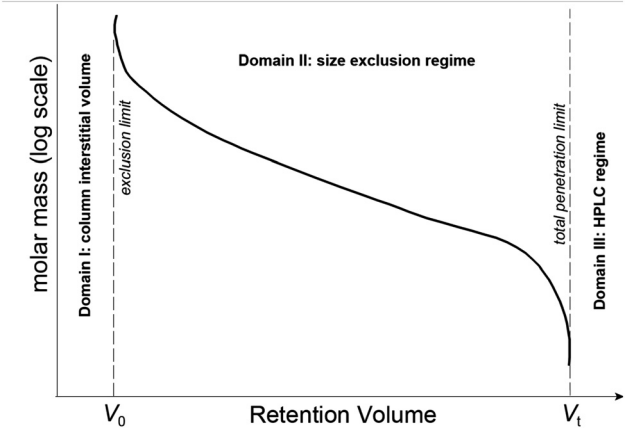


Figure 11: Generalized shape of SEC calibration curves covering the complete separation range (V_0 represents complete exclusion from pores, V_t represents total penetration into pores).

In most cases SEC fit functions are based on polynomial functions with a degree of 3 (cubic) or higher (4–7). If a linear (mixed-bed) SEC column is used in the separation, a linear calibration fit can be applied. There are also special dedicated fit functions (e.g., PSS calibration functions) available that are based on polynomial functions, but optimized for the SEC separation behavior to avoid typical pitfalls (refer to the calibration curve shown in Figure 11 which must be represented by the user-selected mathematical equation).

Three decision criteria can help to decide if the proper function has been selected: These are the regression coefficient, R^2, the deviation of the calibration point from the fitted value (e.g., average deviation), and the slope of the calibration curve.

Table 8 illustrates this decision making process. It shows the regression coefficients and the average deviation for all data points for identical calibration data fitted with different functions. It is obvious that the regression coefficient alone is not a proper parameter to select the best fit function. Large average deviations are observed even for a regression coefficient very close to unity. If the SEC software provides the regression coefficient as the only selection criterion, a value of $R^2 > 0.999$ should be achieved.

Table 8: Influence of the calibration fit function on the regression coefficient and the average relative deviation.

Calibration Fit Model	R^2	Average deviation [%]	Comment
Linear	0.9925	30.2	use for “mixed bed” columns
Polynomial 3 (cubic)	0.9986	10.4	Most often recommended in GPC/SEC standards (International Organization for Standardization, 2008)
Polynomial 5	0.9995	7.35	Check if first derivative is discontinuous
Polynomial 7	0.9999	3.57	First derivative is often discontinuous; then this fit should not be used despite lower molar mass deviation
PSS polynomial 7	0.9998	4.92	Better than “polynomial 7” fit as it has fewer degrees of freedom

In addition, this table shows that when polynomial functions with a higher degree are selected, the regression coefficient and the average deviation become smaller. However, it is not physically meaningful to use the function with the highest degree, despite that this will always generate the lowest average deviation. More important than small deviations is that the shape of the calibration curve is in general agreement with the separation mechanism. A good measure for a physically meaningful fit is the first derivative of the calibration curve, the slope of the calibration curve.

Figure 12 shows an ideal first derivative for a calibration curve. The slope is constant for the optimum separation range and changes only close to the exclusion limit and the total penetration volume. If a higher polynomial fit function (e.g., seventh degree used in Figure 13) is chosen, local maxima and minima, lacking any physical significance, will appear. Over-fitting should be avoided, since it can produce artifacts in the molar mass distribution (e.g., shoulders), that are not related to the sample characteristics.

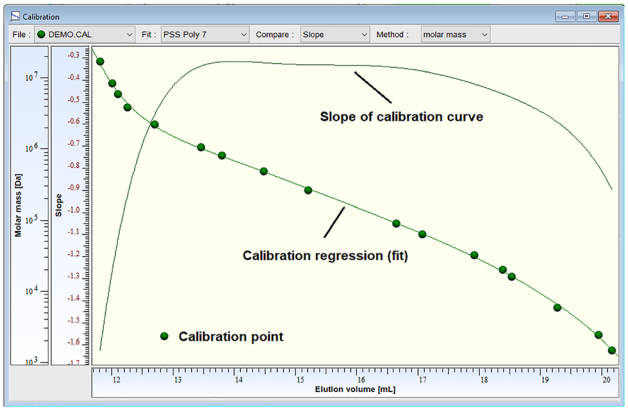


Figure 12: Example of a good SEC calibration with small deviations and continuous first derivative which covers the complete column pore volume.

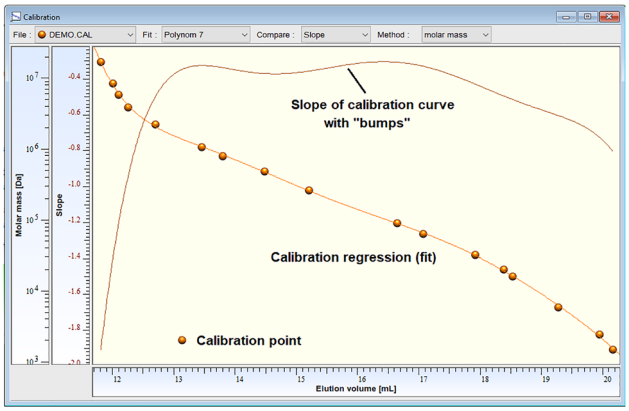


Figure 13: Poor SEC calibration fit (same data points as in Figure 12) with small deviations but discontinuous first derivative which will lead to artifacts (e.g., shoulders) in the molar mass distribution.

Therefore the optimum fit function is the one with the lowest deviations that still has a constant slope without maxima or minima.

However, one of the major limitations in SEC is that the separation is based on the hydrodynamic volume. This does not only depend on the molar mass of the molecule but also on its chemical nature and topology. Therefore a calibration curve created from reference materials is strictly only valid for samples with the same chemistry and topology. For other samples apparent molar masses will be obtained. It is still possible to compare the samples, but it is not possible to measure accurate molar masses.

Several solutions are available to overcome this limitation:

a) There are many different types of reference materials available to create the matching calibration curve for many samples. In addition universal calibration with Mark-Houwink coefficients and broad calibration methods are available.

b) Online viscometers can be used to measure a universal calibration curve (Grubisic, Rempp, & Benoit, 1967). Here the logarithm of the hydrodynamic volume, the molar mass multiplied by the intrinsic viscosity, is plotted against the elution volume. Universal calibration curves are valid for all types of polymers and copolymers independent on the topology. For creating a universal curve and choosing a fit function the same rules apply as for a conventional calibration curve. The only difference is that the intrinsic viscosity measured using the viscosity detector is additionally used.

c) Molar mass sensitive detectors such as online light scattering detectors and MS detectors allow to measure the molar mass at every elution volume directly.

The calibration options mentioned in b) and c) are beyond this introductory text; more information can be found in (Kilz & Pasch, 2000; Striegel et al., 2009).

Section C

Further reading and resources

It is impossible to cover all aspects of size-exclusion chromatography, its application to various macromolecules and how to troubleshoot instrumental or separation method in a single paper. Instead, this chapter summarizes important target industries with their respective SEC applications and lists resources for further reading. Relevant resources listed below are collected to the authors' best knowledge, but might change as Internet offerings are very dynamic.

Markets and applications for size-exclusion chromatography

Table 9 summarizes important markets for macromolecular products and SEC application fields (non-exclusive listing; many others are known). Application resources are presented in chapter "*Where to find SEC applications?*".

Table 9: Commercial use of SEC analysis and important application fields.

Industry	Application examples
Adhesives	Compositional drift
	Product deformulation
	Surface activity
Coatings	Binders/resins
	Chemical composition
	Aging
Construction	Concrete additives
	Hydrophobization agents
	Emulsifiers
	Micro-capillary drainage
Cosmetic	Viscosity enhancers
	Stabilizers
	Surfactants
Electronic	Lithography resins
	Molar mass dependence
	Dimensional stability
Food	Rheological behavior
	Structure-property-function relationship
	Stabilizers
	Aging studies
Forensic	Finger printing
	Molar mass determination
	Identification
	2D chromatography mapping
Imaging	Support stability (PET, gelatin)
	Toner technology
	Recycling
Medical devices	QC
	Aging
	Composition analysis
	Identification of components
	Additives
Oil	Product deformulation
	Additives
	Polymer architecture
	Degradation studies
Paint	Binders/resins
	Dispersing agents
	Product deformulation
Plastics	Property optimization
	Product properties
	Copolymer composition
	Additives
	Stabilizers
	Recycling

Table 9: (continued)

Industry	Application examples
Pharmaceutical	Quality assurance
	Stability/aggregation
	Drug delivery systems
	Pharmaceutical technology
	Metabolization
Processing	Good/bad comparison
	Quality assurance
Rubber	Blending
	Mechanical properties
	Degradation studies
	Product deformulation

Where to find SEC applications?

Internet search is certainly a first resources especially if the reader is working on a current topic probably covered in primary literature. However, do not search for the term SEC but for GPC. Otherwise, search results for SEC will most certainly obstruct relevant information behind a huge number of references to publications related to the United States "Securities and Exchange Commission" also known as "SEC".

A good resource for meaningful application notes and compendia are the major vendors for SEC instrumentation and consumables listed in Table 10.

Table 10: Major chromatography vendors, their SEC product, and service offering.

Vendor	Product Focus	URL
Agilent Technologies	Instrumentation, columns, reference materials, software	www.agilent.com
Malvern Panalytical	Instrumentation, software	www.malvernpanalytical.com
PSS Polymer Standards Service	Instrumentation, columns, reference materials, software, method development, analytical services, training classes	www.pss-polymer.com
Shimadzu	Instrumentation	www.shimadzu.com
Shodex	Instrumentation, columns, reference materials	www.shodex.com/en
Thermo Fisher and Thermo Scientific	Consumables Instrumentation	www.thermofisher.com
Tosoh Bioscience	Instrumentation, columns, reference materials, software	www.tosohbioscience.com
Waters	Instrumentation, columns, reference materials, software, training classes	www.waters.com

How to find SEC troubleshooting information

The first stop for finding valuable information about a product is the product documentation itself. In most cases the user documentation will cover installation and use and also contain general chromatography troubleshooting information which might not address a specific SEC aspect, however. Most vendors listed in Table 10 will offer specific guidance on their SEC products.

Separation Science Lab Journal offers a series of "GPC/SEC Good Practice & Troubleshooting Tutorials" (https://learning.sepscience.com/form/gpc-sec-good-practice-troubleshooting-tutorials).

SEC tips and tricks for meaningful results

Most vendors listed in Table 10 will offer comprehensive documentation and optimization for their SEC products.

Practical knowledge and how to make SEC painless and avoid non-obvious traps can be found in the following resources:
LCGC and PSS: collection of GPC/SEC tips&tricks written by PSS scientists
https://www.pss-polymer.com/en/support/librarypss-publications/gpcsec-tipstricks.html.

Chromatography online: collection of GPC/SEC advice written by PSS scientists
https://www.chromatographyonline.com/search?searchTerm=sec%20tips.

LaborPraxis and PSS: collection of more than 70 GPC/SEC tips written by PSS practitioners (in German)
https://www.pss-polymer.com/de/support/bibliothek-pss-publikationen/gpc-tipps-und-tricks-deutsch.html.

PSS tips on software best practices as well as tips and tricks:
https://www.pss-polymer.com/en/support/software-support/wingpc-newsletter.html.

Conclusions

SEC characterization of macromolecular products with various compositions and architectures deliver deep insight into molecular design and allow the establishment of structure–property–function correlations. The results are robust, repeatable and dependable and are indispensable in product control and QA release when SEC specific procedures are adhered to as outlines in this paper.

Supplementary information

Lecture notes on SEC from the Characterization Workshop during the IUPAC MACRO Congress 2016 in Turkey are available as supplementary information. The slides focus on size-exclusion methodology and expand the scope of this introductory text for advanced detection and separation techniques. Additional applications demonstrate the use of these techniques. PowerPoint slides may be obtained by the author for educational use.

Abbreviations and acronyms

BHT	butylated hydroxytoluene (2,6-Di-tert-butyl-4-methylphenol)
CCD	chemical composition distribution
DAD	diode-array detector
DMAc	dimethyl acetamide
DMF	dimethyl formamide
DMSO	dimethyl sulfoxide
ELSD	evaporative light-scattering detector
FTD	functional-type distribution
FTIR	Fourier-transform infrared spectroscopy
GC	gas chromatography
GFC	gel filtration chromatography
GPC	gel permeation chromatography
HPLC	high performance liquid chromatography
K	distribution coefficient
LC	liquid chromatography

MAD	molecular architecture distribution
MALDI	matrix-assisted laser-desorption ionization mass spectrometry
MMD	molar mass distribution
MS	mass spectrometry
NMP	*N*-methyl pyrrolidone
NMR	nuclear magnetic resonance
PAA	poly(acrylic acid)
PB	poly(butadiene)
PC	poly(carbonate)
PDMS	poly(dimethyl siloxane)
PE	poly(ethylene)
PEG	poly(ethylene glycol)
PEO	poly(ethylene oxide)
PET	poly(ethylene terephthalate)
PIB	poly(iso-butylene)
PLA	poly(lactic acid)
PMMA	poly(methyl methacrylate)
POM	poly(oxymethylene)
PP	poly(propylene)
PS	poly(styrene)
PU	polyurethane
PVC	poly(vinyl chloride)
QA	quality assurance
QC	quality control
RID	refractive index detector
SEC	size-exclusion chromatography
TCM	trichloromethane
THF	tetrahydrofurane
UVD	ultra-violet detector

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