

# CHARACTERIZATION OF MACROMOLECULES BY TWO-DIMENSIONAL CHROMATOGRAPHY



**PRIMER FOR 2-DIMENSIONAL LIQUID CHROMATOGRAPHY (2D-LC)**



**DETERMINE THE MOLECULAR WEIGHT AND COMPOSITIONAL DISTRIBUTIONS OF COMPLEX POLYMERS**

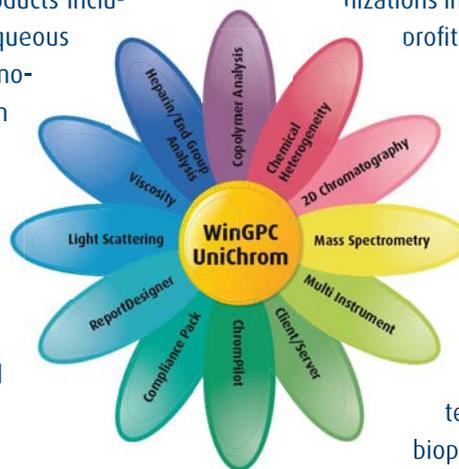
## About PSS

### Driving GPC/SEC Forward

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

PSS is fully dedicated to the advancement of macromolecular liquid chromatography, by means of materials design, synthesis, manufacturing, consulting, service, and innovative research, applying the highest standard of expertise and reliability.

Our close relationship with our customers has helped us to continuously improve the quality of our products and services. Our high caliber staff, mostly chemists, is experienced, creative and trained in problem solving. Corporations, universities, and organizations in more than 60 countries use our products and profit from our outstanding service and know-how.



### Certified DIN ISO EN 9001

PSS is certified (DIN ISO EN 9001:2008) to produce high quality reference polymers, GPC/SEC columns and software for the characterization of polymers by their molecular weight and their structural characteristics. PSS employs the latest findings in polymer science for the synthesis and characterization of polymers, block copolymers and biopolymers. PSS operates a manufacturing facility equipped with a complete state-of-the-art characterization laboratory at the headquarters in Mainz, Germany, fully supporting customers working under stringent requirements i.e., GLP, DIN, ISO certifications.

## Content

1. Why is Liquid Chromatography important for the Analysis of Macromolecules?	Page 4
2. Why is 2-dimensional Chromatography important?	Page 8
3. How does it work?	Page 12
4. PSS Products and Services for 2D Analysis	Page 18
5. Further Reading	Page 22
6. Contacts	Page 26



WHY IS LIQUID  
CHROMATOGRAPHY  
IMPORTANT FOR  
THE ANALYSIS OF  
MACROMOLECULES?

**A** large variety of different polymeric materials with tailored properties can be created by copolymerization, end-group functionalization, blending, introduction of branches and variation of the microstructure. One feature of all polymers is heterogeneity, which arises from the statistics involved in every polymerization process. Even homopolymers prepared by (ideal) living polymerization techniques, which are often incorrectly referred to as being monodisperse, contain chains of different molar masses, giving rise to a molar mass distribution (MMD).

Additionally, in copolymerizations the different comonomers are distributed over the different polymer chains, resulting in individual chains, which will vary in composition (chemical composition distribution). Other heterogeneities arise from differences in end-group functionalization, branching, microstructures etc.

Analytical methods like light scattering for molar mass determination, spectroscopic techniques for characterization of the chemical composition or end-group functionality yield average information on the complete sample, but cannot provide informa-

tion on how the monomer units or end-groups are distributed among the different molecules. Thus, a single value describing a specific molecular property is not sufficient to properly describe a polymer sample, since the same value can result from different distributions of the property. Therefore efficient separation methods are required before further characterization.

### Chromatographic modes for polymer separations

#### Size exclusion chromatography (SEC)

The most common separation technique for polymers is size exclusion chromatography (SEC) often also referred to as gel permeation chromatography (GPC). As indicated by the name size exclusion chromatography separates by the size of the molecule in solution and is commonly used to derive the molar mass distributions and molar masses averages of polymeric samples. In SEC a size based separation occurs within a column or column set filled with porous beads.

As the sample molecules migrate through the column, they diffuse into the pores of the beads thereby increasing their residence

## WHY IS LIQUID CHROMATOGRAPHY IMPORTANT FOR THE ANALYSIS OF MACROMOLECULES?

time within the column. Large molecules cannot explore the complete pore volume as they are excluded from some pores due to their large size. Smaller molecules can access more pores and will thus elute later than the larger ones.

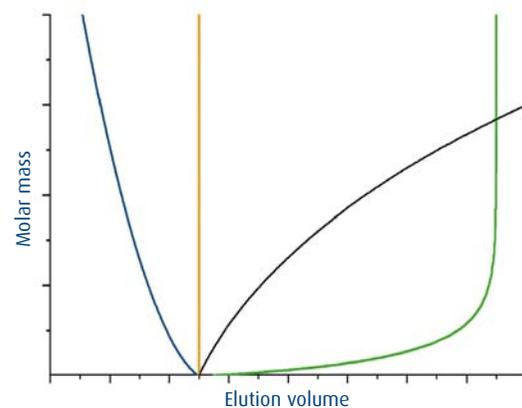
Since the separation is governed by molecular size, the column set needs to be calibrated. The most commonly applied calibration technique uses standards having a narrow molar mass distribution and known molar mass in order to relate the elution volume to the molar mass of the polymer. Suitable standards are commercially available for a large number of different polymers, e.g. from PSS Polymer Standards Service. Alternative but more labor intense methods for SEC column calibration involve calibration using broad standards with precisely known molar mass averages or of known molar mass distribution, universal calibration or the application of molar mass sensitive detection systems, such as light scattering detectors or viscometers.

### Liquid adsorption chromatography (LAC)

SEC is a powerful technique but due to its size based separation mechanism it lacks information on chemical composition or end-group functionality. To separate blends or copolymers according to chemical composition methods of interaction chromatography (IC) methods have to be applied. If attractive interactions between the stationary phase and the repeating units are present, the retention will be influenced by the molar mass and the chemical structure of the repeating units. Since polymer retention increases rapidly in isocratic interaction chromatography, solvent gradients are usually required in order to completely elute polymeric samples in reasonable times.

At lower molar masses the retention in gradient chromatography is influenced by molar mass and the structure of the repeating units. However, for higher molar masses the molar mass dependence of elution volume vanishes (Figure 1) and elution volume is mainly governed by the chemical structure of the repeating units. This allows separating polymer blends or copolymers according to chemical composition.

FIGURE 1



Schematic representation of the molar mass dependences on elution volume in SEC (blue), isocratic adsorption chromatography (black), liquid chromatography at critical conditions (orange) and in gradient chromatography (green).

#### Liquid adsorption chromatography at critical conditions (LACCC)

If a separation according to the type or number of functionalized end-groups is aimed for, liquid chromatography at critical conditions (critical chromatography, LACCC) is the method of choice. In LACCC the mobile phase is adjusted such that steric exclusion and effects of interaction chromatography completely balance each other. Under these conditions, non-functionalized homopolymers elute independent of chain length at the void volume of the column. If at critical conditions a functional end-group gives rise to additional retention, a separation according to the type and number of functional end-groups can be achieved.

Other methods of interaction chromatography involve the use of barrier methods (chromatography at limiting conditions), SEC-gradients or temperature gradient chromatography (TGIC), which have their distinct advantages for certain separation problems.

A complete discussion on the various modes of interaction chromatography, the pros and cons, its potential and limitations is beyond the scope of this primer. We therefore refer to recent reviews.



WHY IS 2-DIMENSIONAL  
CHROMATOGRAPHY  
IMPORTANT?

Since in polymer samples the number of different structures easily reaches a few hundreds or even thousands, a high peak capacity would be beneficial for a separation. Technically speaking two-dimensional chromatography increases the peak capacity, i.e. the number of peaks that can be resolved within a given time. At optimized conditions, the peak capacity in a two dimensional separation is the product of the peak capacities of the individual separations.

EQUATION 1

$$n_{2D} = n_1 \times n_2$$

However, does the above technical statement on peak capacity help in separating complex polymer samples?

Since the peak capacity in a single chromatographic experiment is insufficient to resolve all molecular structures of a complex polymer, the conventional approaches of polymer liquid chromatography aim for separating according to one structural feature only.

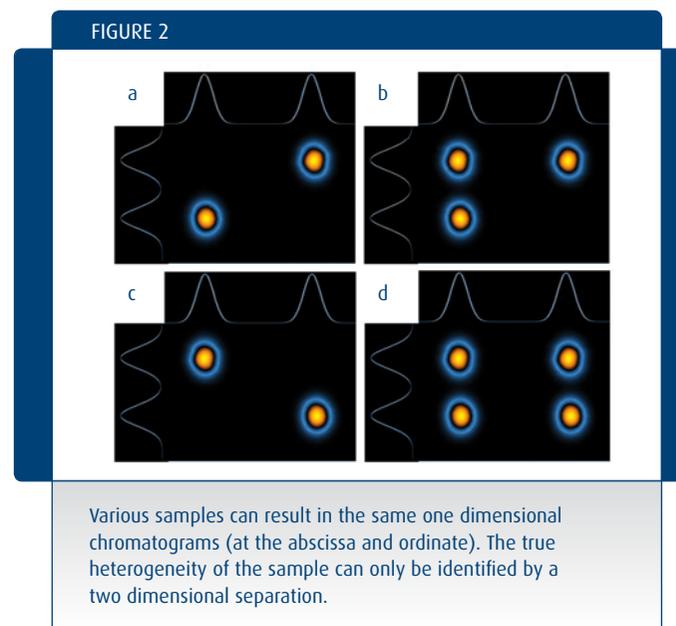
Such features can be end-group functionality, copolymer composition, topology or molar mass / molecular size. However, if a separation by a single structural feature is realized, information on the distribution of the other structural features is lost. For example, an ideal separation with respect to chemical composition will result in coelution of macromolecules having identical composition but different molar masses.

In addition, separating macromolecules with respect to only one structural feature using a single separation technique is often not possible, since separation methods are influenced by more than a single molecular property. For example size exclusion chromatography (SEC) separates by molecular size. Since the size of the macromolecule is influenced by molar mass as well as by chemical composition, molecules differing in composition and molar mass might coelute at a given elution volume (see x-axis Figure 3). Even application of highly sophisticated molar mass sensitive detectors, which are valuable tools in polymer characterization, cannot overcome this dilemma. In contrast, heterogeneity at a given SEC volume can even create more problems for molar mass sensitive detection.

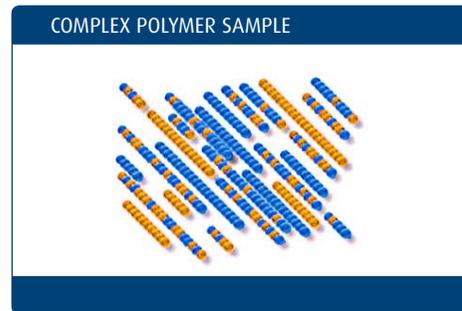
## WHY IS 2-DIMENSIONAL CHROMATOGRAPHY IMPORTANT?

If a separation with respect to composition is performed and another independent separation is performed purely based on molar mass, it is not possible to gain the complete information on the composition of the sample, as various products can reveal the same one-dimensional chromatograms, as is shown in Figure 2. Although all samples exhibit two peaks in HPLC (ordinate) and SEC (abscissa), it is not possible to identify the number of peaks in the samples simply from the two one-dimensional chromatograms. Thus, other approaches are required to gain information on the complex distribution of modern polymer samples.

In order to separate such complex polymers two-dimensional chromatography is the method of choice. In two-dimensional chromatography the sample is fractionated in a 1st dimension by one structural parameter and each fraction is subsequently separated in a second chromatographic experiment according to a second structural feature. This is schematically illustrated in Figure 3 on a copolymer sample being composed of chains differing in molar mass (as indicated by the different number of pearls) and chemical composition (indicated by the differently colors of the pearls). A pure size-based separation by SEC will result in the

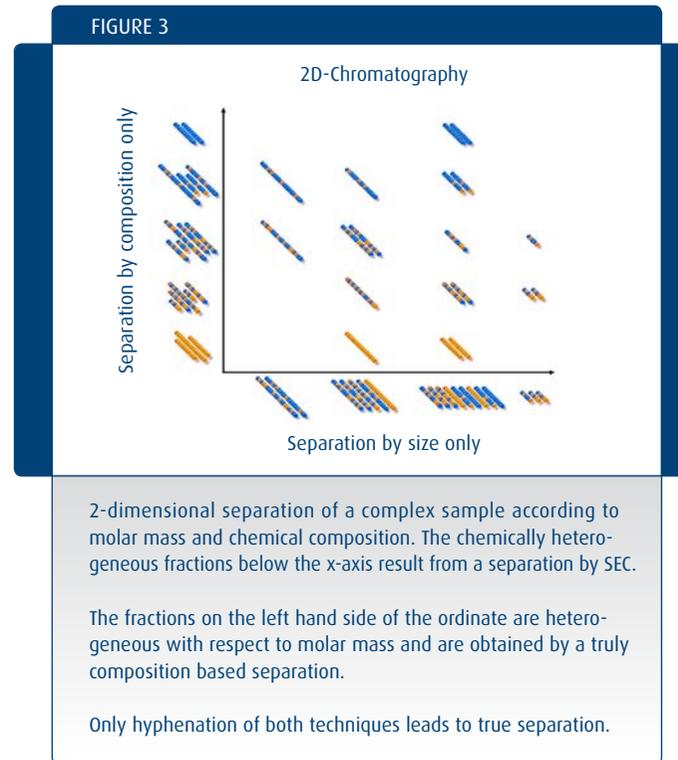


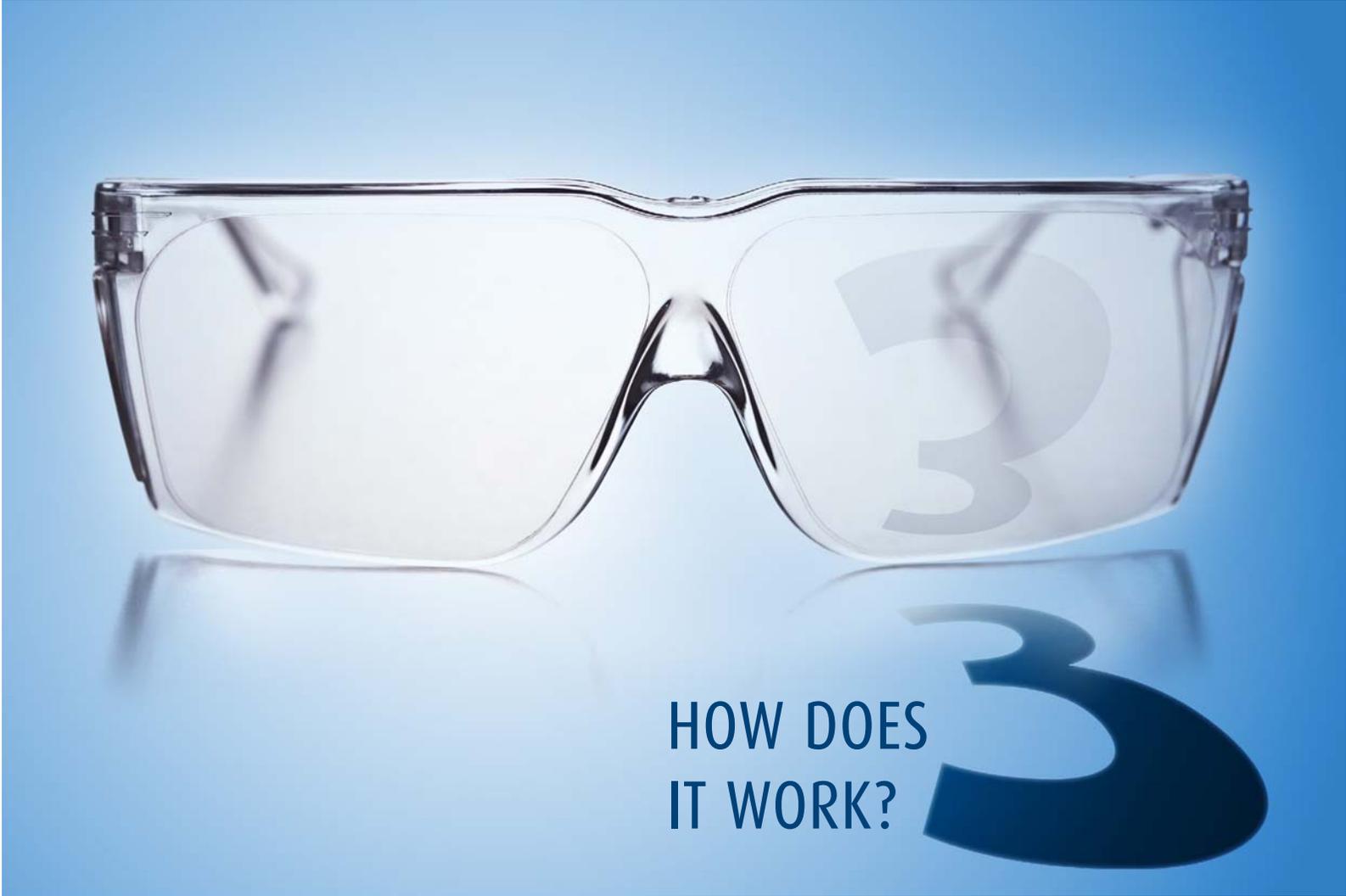
fractions shown below the x-axis of the figure. The chains are separated with respect to the chain length, but the fractions contain molecules of different composition.



On the contrary an ideal separation with respect to composition would reveal the fractions on the left side of the y-axis. These fractions are homogenous with respect to compositions, but contain molecules differing in molar mass. However, if these fractions will be subjected to a size based separation, all different species are clearly separated.

It should be noted that orthogonality, i.e. completely independent retention mechanisms for both separations, is not required for useful two-dimensional separations. A substantial increase in information is gained even if retention in both dimensions depends on the same structural features, but to different degrees.





HOW DOES  
IT WORK?



The first two-dimensional separations were performed manually by tedious manual fractionation and reinjection of the fractions into the 2nd dimension. Today fully automated equipment is commercially available, allowing for unattended operation. This reduces the possibility of human errors, increases repeatability, reduces work-load and allows for high sample throughput resulting in cost effective analysis.

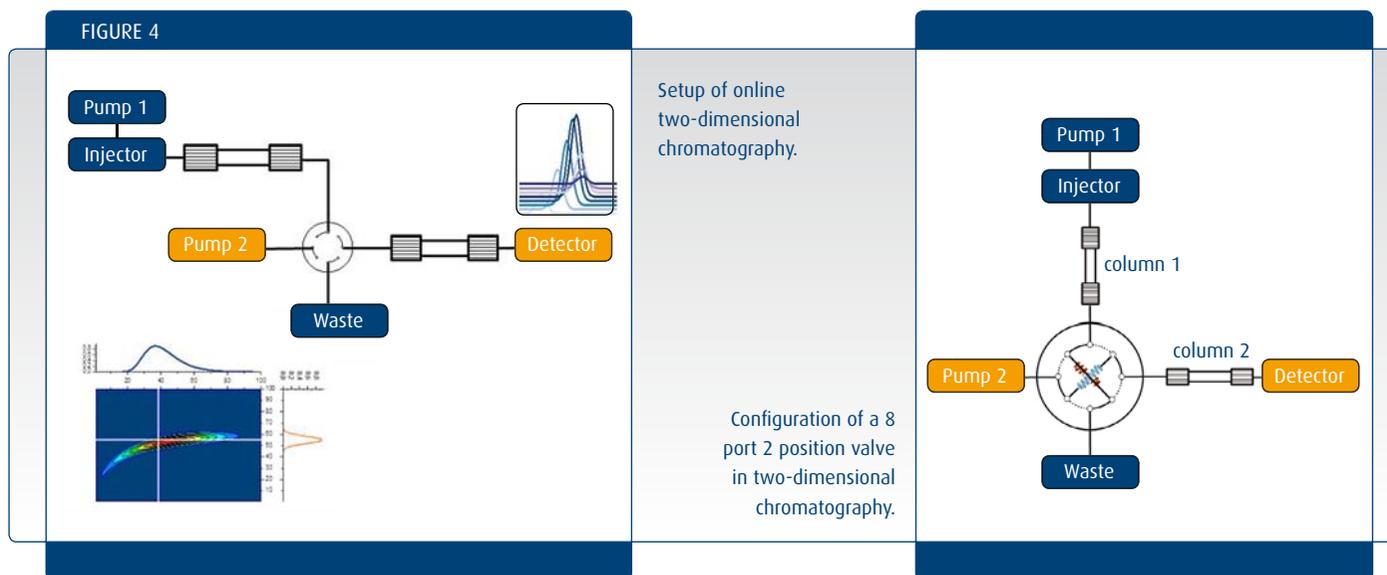
The principle setup of a two-dimensional separation system consists of a typical HPLC-system for the 1st dimension (Figure 4 left). A two position transfer valve is installed after the column. This acts as injector for the 2nd dimension, which consists of a pump, the valve, the 2nd dimension column and finally a detector.

The central part of the two-dimensional setup is the two position transfer valve, which is equipped with two loops of identical size (Figure 4 right). The effluent from the 1st dimension fills one of the loops, while the other loop is analyzed in the 2nd dimension. At the end of the 2nd dimension analysis, the valve is actuated such that the loop filled with the 1st dimension effluent is injected

into the second dimension, while the previously analyzed loop is now filled by the effluent from the 1st dimension. This operation principle requires low flow rates in the 1st and high flow rates in the 2nd dimension separation for comprehensive sample analysis.

By this procedure a series of 2nd dimension chromatograms is obtained, which are related via their injection times to the 1st dimension analysis time. Suitable software allows visualizing the two-dimensional chromatograms as images, where the x- and y-axis represent the retention times of the individual dimensions and the z-axis indicates the detector signal. Other representations are waterfall or contour plots. By evaluating the peak areas or peak volumes quantitative information can be extracted. If one of the chromatographic separations is performed using SEC, molar mass information can be obtained upon suitable calibration. To properly account for the variation of chemical composition with 1st dimension elution volume, the software must be able to apply composition dependent calibration curves. Calibrating the first dimension with samples of different chemical composition allows obtaining the chemical heterogeneity distribution.

HOW DOES  
IT WORK?

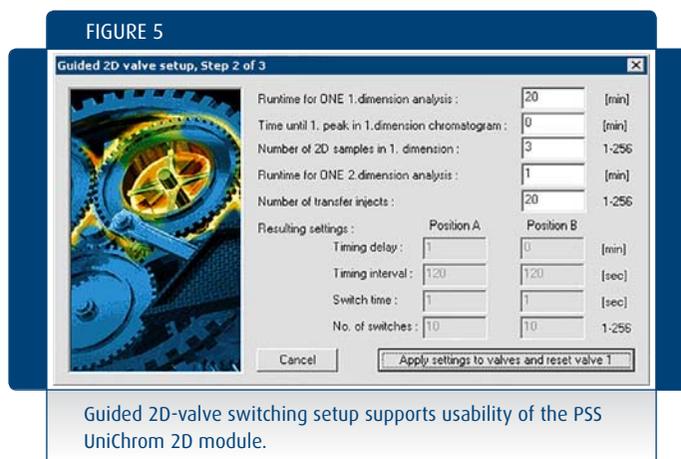


Although online two-dimensional chromatography reduces sources of human error, offline two-dimensional chromatography might be required if solvent exchange or manual fractionation for

increased sample concentration in 2nd dimension are necessary. PSS UniChrom Software allows constructing two-dimensional chromatograms even from offline recorded data.

## Data Acquisition

The 2D module of the PSS UniChrom Software allows data acquisition and evaluation of two-dimensional separations. For comprehensive two-dimensional chromatography, i.e. complete sample transfer from the 1st into the 2nd dimension without significant loss of 1st dimension resolution, the flow rates for both dimensions, the transfer volumes and injection times must be carefully matched. PSS UniChrom's user friendly guided 2D-valve

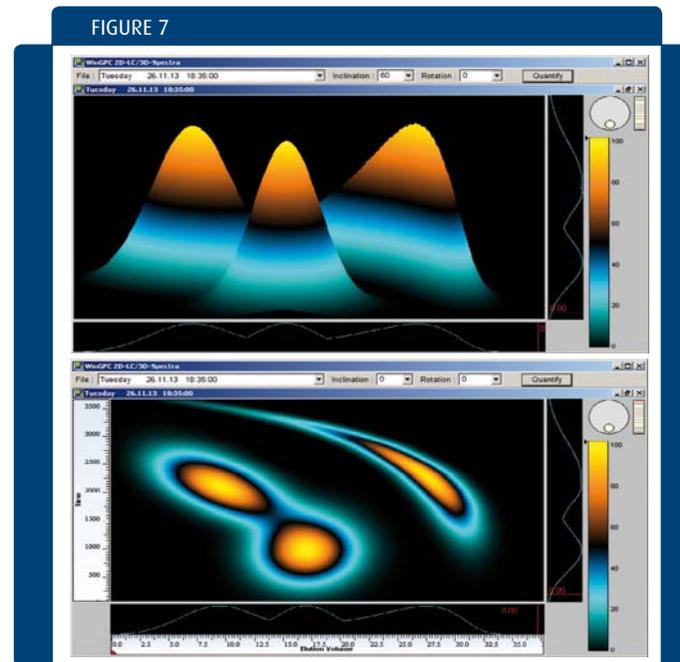
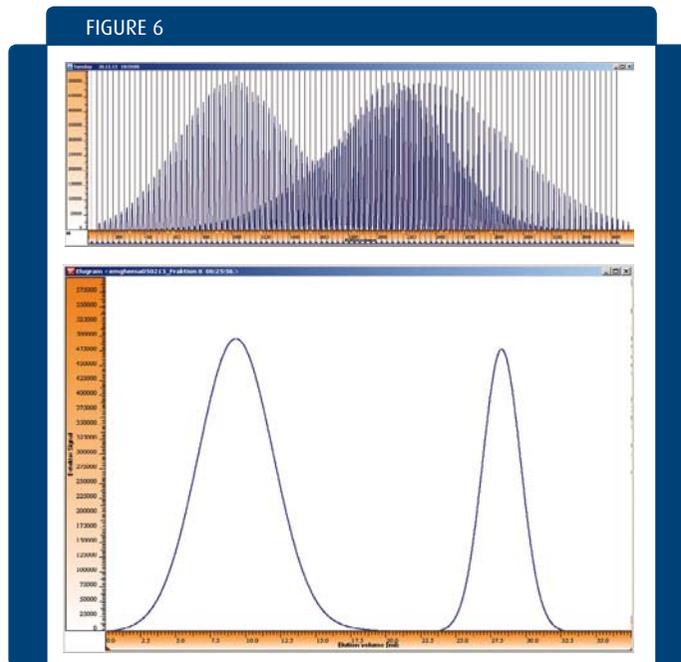


setup automatically calculates the required switching times based on chromatographic run times in both dimensions (Figure 5). This allows for easy data acquisition, reducing the risk of human error.

## Data Representation

Having acquired the series of 2nd dimension chromatograms (Figure 6), the data are sent to the 2D window for further visualization as 3D (Figure 7), contour (Figure 7) or height line diagrams (Figure 8) and for further data treatment. The 3D diagram in Figure 7 reveals 3 distinct peaks, which would not be resolved by either the 1st dimension separation (along the vertical axis, nor by SEC (separation along the horizontal axis). Any cut along the vertical axis in Figure 7, bottom, corresponds to the HPLC (1st dimension) chromatogram at the cursor position, while any cut along the horizontal axis indicates the 2nd dimension (SEC) chromatogram at the cursor position. Cuts can be sent to the overlay module for detailed comparison of the chromatograms obtained at different 1st or 2nd dimension retention times. Self-evident color palettes and other options are available to adjust the plots to the preferences.

HOW DOES  
IT WORK?

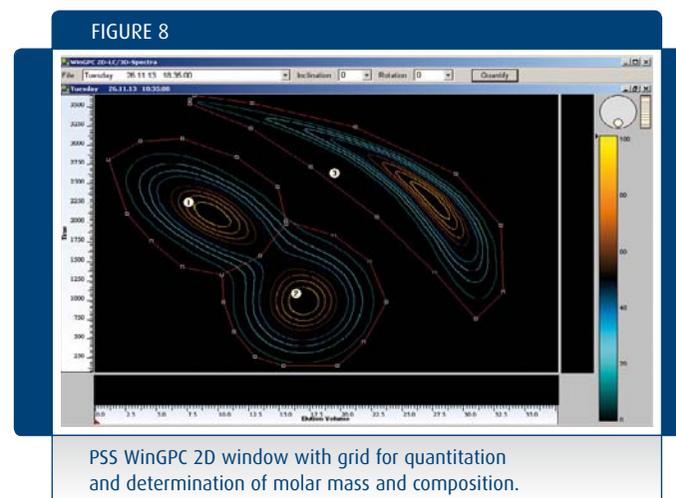


## Data Analysis

3D surface plots (Figure 7, top) are very good for visualizing results, but not suitable for quantification. However contour plots (or contour maps), which are 2-dimensional representations of the 3D surface plots that use different colours to represent different concentrations, are ideal for such quantification purposes.

WinGPC UniChrom software is the only commercial software that allows convenient peak definition and the calibration of both dimensions. WinGPC not only shows the separated components, but also determines the peak area and volume, as well as the average molar masses and the mean composition, width and skewness for each peak.

Data analysis grids can be created (Figure 8) to define the peaks of interest and stored as templates. Different calibration types, matching the separation techniques applied, can be assigned to the 1st and the 2nd dimensions. WinGPC supports options for both, molar mass and composition calibration. The flexible design even allows to use different molar mass calibrations for each of



the injections in the 2nd dimensions to account for the chemical heterogeneity of the fractionated peaks.

Just one single run provides comprehensive characterization combining efficient separation into single peaks with the simultaneous measurement of their concentration, their molar masses and their composition.



PSS PRODUCTS  
AND SERVICES  
FOR 2D ANALYSIS



can provide you either with a turn-key system for 2-dimensional chromatography or with the necessary components to upgrade existing equipment. PSS has pioneered the development of a unique software package, WinGPC, that has the capability of performing 2D-LC-analysis and of providing results for single peaks including molar mass averages and composition.

Optimized PSS columns and reference materials are available for many existing applications, for method development and for self-training. PSS Services include consultancy, method development and transfer as well as customized training.

### The PSS 2D-Analyzer

2D-chromatography has special demands on pumps and detectors resulting from the low flow rates in the 1st and high flow rates in the 2nd dimension. The PSS 2D Analyzer is a preconfigured optimized package of hard- and software components specially designed to run 2-dimensional chromatography.

FIGURE 9



The PSS 2D analyzer: A preconfigured optimized package of hard- and software specially designed to run 2-dimensional chromatography.



In the standard package the 1st dimension of the PSS 2D analyzer is composed of a binary pump giving the capabilities to perform either isocratic (SEC, LACCC, TGIC) or gradient experiments in the 1st dimension. An autosampler enables unattended overnight experiments. A column oven reduces temperature fluctuations, which are detrimental to separations in the various modes of interaction chromatography. The transfer valve injects the fractions obtained into the second dimension.

The 2nd dimension consists of an isocratic pump and PSS High-Speed columns. Furthermore the 2D analyzer contains the PSS WinGPC Unichrom Software with 2D chromatography option for

valve switching, data acquisition and processing. It should be noted that this PSS system can easily be transformed to a conventional LAC/GPC/SEC system for times when no 2D capabilities are required in the lab.

### Detection and software options

To adjust the PSS 2D analyzer to the specific needs of the customer, various options are available. These include:

- Temperature programmed column oven in the 1st dimension giving TGIC capabilities (TGIC= temperature gradient interaction chromatography).
- Various detectors for the 1st and 2nd dimension (UV, ELSD, DAD, molar mass sensitive detection) can be ordered depending on the specific customer requirements. PSS strongly recommends at least an evaporative light scattering detector (ELSD) for gradient applications and to avoid solvent signals.
- Different transfer valve options are available with either an integrated valve handling up to 52 transfer injections per sample or an external valve with an option for either up to 100 or 255 injections.

The PSS WinGPC software, which is incorporated in the 2D-Analyzer, is also available separately to upgrade systems from other vendors and provide 2D capability. Required is WinGPC with two independent time bases and the 2D software module.

Recommended options are the ChromPilot System control, the ReportDesigner and the software modules Copolymer Analysis and Chemical Heterogeneity. While the copolymer module adds calibration options for molar mass determination of copolymers and therefore sophisticated calibration of the SEC, which is typically the 2nd dimension, the chemical heterogeneity module adds calibration options for the 1st dimension.

Modules like Mass Spectrometry, 3D spectra analysis, Light scattering, Viscometry or others can be added any time if these detection options are added to the system.

### Columns and reference materials

To find the best compromise between time consumption and resolution PSS offers columns with different dimensions to match

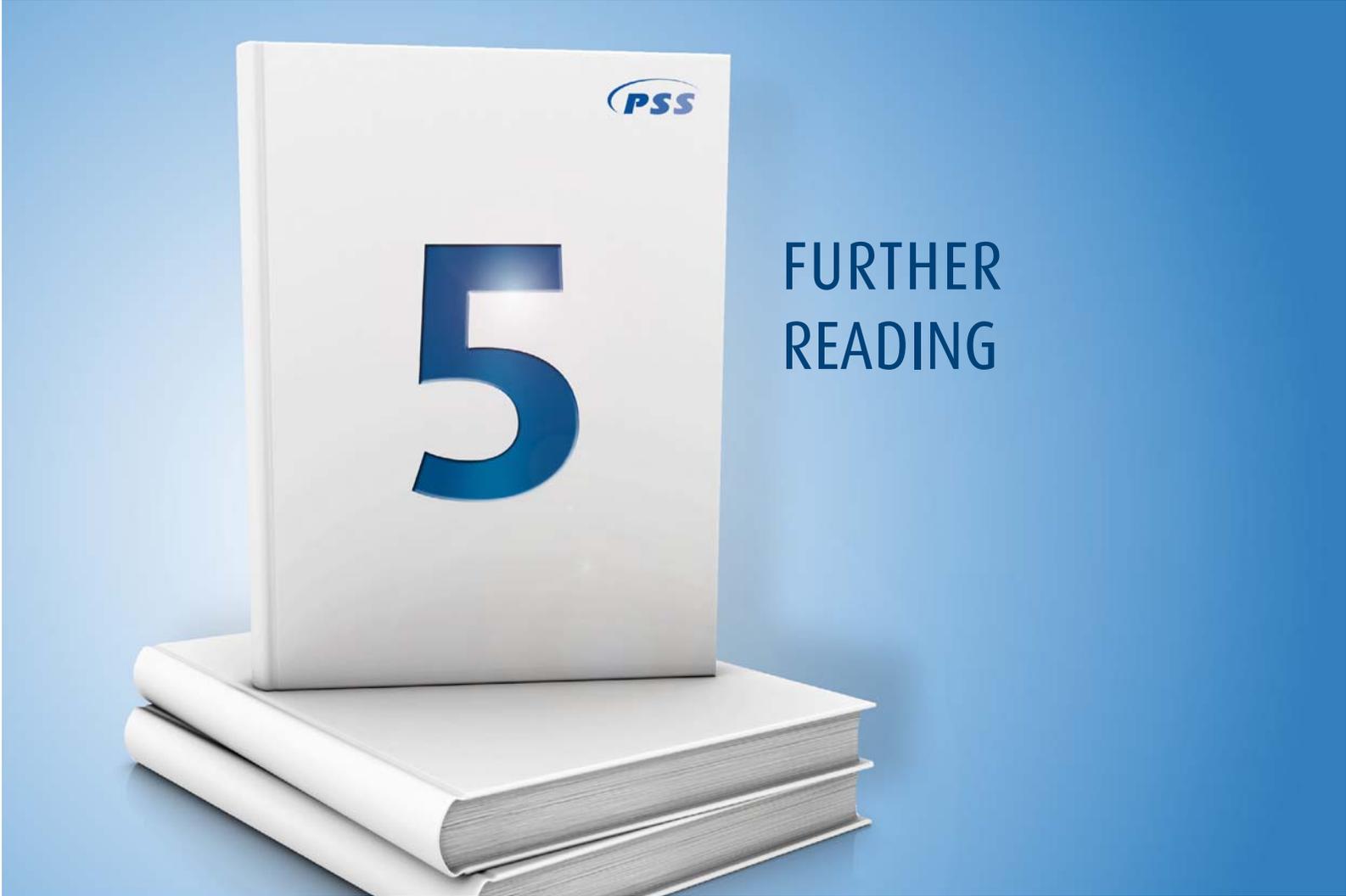
all application requirements for flow rates and time. Special High-Speed columns with a larger inner diameter can be used at higher flow rates to reduce the time for the 2nd dimension SEC runs. Shorter columns can be used for low molar mass products.

PSS not only produces (homopolymer) molar mass reference materials but also copolymers with known composition and molar mass. These copolymers can be used to calibrate the systems or for system testing and self training. PSS also offers custom synthesis.

### Service & Support

PSS uses 2D-chromatography for customer analysis and method development in our own labs. This knowledge gained from our daily usage is used beneficially for customer support and consultancy. We also offer method development services. Single dimension and complete 2D method development for particular samples is offered according to the customer needs.

**Make our long experience yours.**



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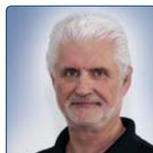
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# PSS PRODUCT OVERVIEW

## Supplies and Services for Comprehensive Characterization of Natural and Synthetic Macromolecules

### Reference Polymer Standards

- GPC/SEC Standards and Kits
- Certified Reference Materials
- MALDI Kits
- Viscosity & Light Scattering Validation Kits
- ReadyCal Kits
- Deuterated Polymers
- Tailor made Polymers and Copolymers

### GPC/SEC Columns

- For all Organic Eluents
- For all Aqueous Eluents
- For High and Low Molecular Weight
- Synthetic and Bio-Polymers
- From Micro GPC/SEC up to Preparative Scale
- HighSpeed Columns for fast Analysis

### Software

- WinGPC UniChrom MCDS
- Light Scattering Module for LALLS, RALLS, TALLS, MALLS
- Viscosity Module
- Copolymer Module
- End-group Analysis Module
- 2-dimensional Chromatography Module
- Heparin Module
- LAN/Server Solutions
- Compliance Pack

### PoroCheck

### Analytical Services

- Molar Mass Determination
- Branching/Structure Information
- Method Development and Transfer
- Complete Product Deformulation
- Consulting

### GPC/SEC Instruments

- Complete Systems and Components
- Light Scattering Detectors
- Viscosity Detectors
- dn/dc Instrumentation

### GPC/SEC Schools and Support

- Full Services from Installation to Validation, Operation and Repair
- GPC/SEC and Software Training Schools
- GPC/SEC In-house Training
- User Meetings
- NetCommunity with Application and Publication Downloads

[www.pss-polymer.com](http://www.pss-polymer.com)

