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GPC/SEC & OMNISEC

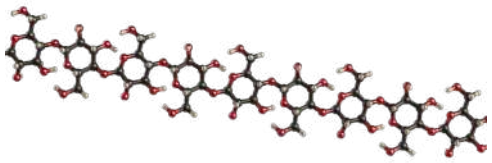
18 August 2020

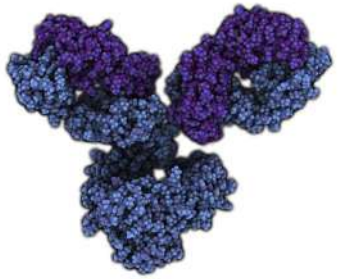
Kyle Williams, Ph.D.
Applications Scientist

Overview of GPC/SEC

- **GPC**: **G**el **P**ermeation **C**hromatography
- **SEC**: **S**ize **E**xclusion **C**hromatography

- GPC/SEC is used to characterize macromolecules
  - Natural polymers: dextran, chitosan, cellulose
  - Synthetic polymers: polystyrene, PET, Nylon
  - Proteins: oligopeptides, antibodies, membrane proteins

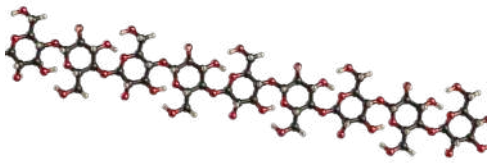


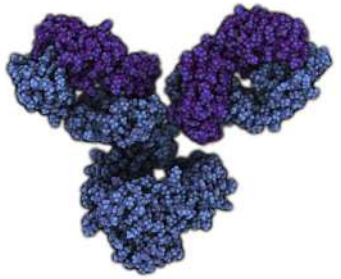



Overview of GPC/SEC

- **GPC**: **G**el **P**ermeation **C**hromatography
- **SEC**: **S**ize **E**xclusion **C**hromatography

- Data available:
  - Molecular weight (Mn, Mw, Mz) & Dispersity (*Đ* or Mw/Mn)
  - Molecular size: hydrodynamic size (Rh) & radius of gyration (Rg)
  - Intrinsic viscosity (IV or [η])
  - Concentration, % recovery, & compositional analysis
  - Mark-Houwink parameters & branching information



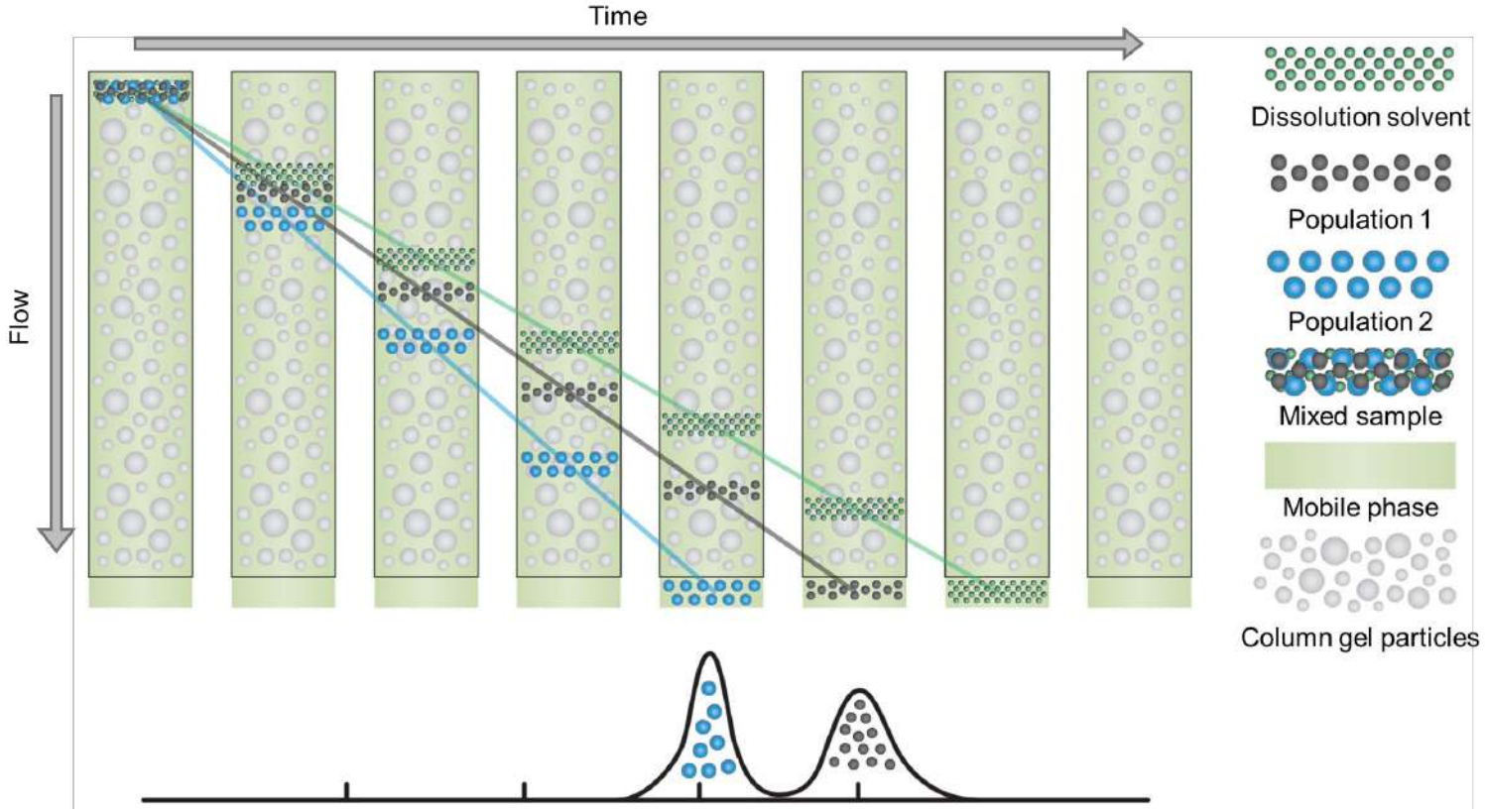



What is chromatography?

- From Greek χρώμα:*chroma*, color and γραφειν:*graphein* to write
- The collective term for a set of laboratory techniques for the **separation of mixtures**
- It involves passing a mixture dissolved in a *mobile phase* through a *stationary phase*
- This separates the analyte to be measured from other molecules in the mixture based on differential partitioning between the mobile and stationary phases.

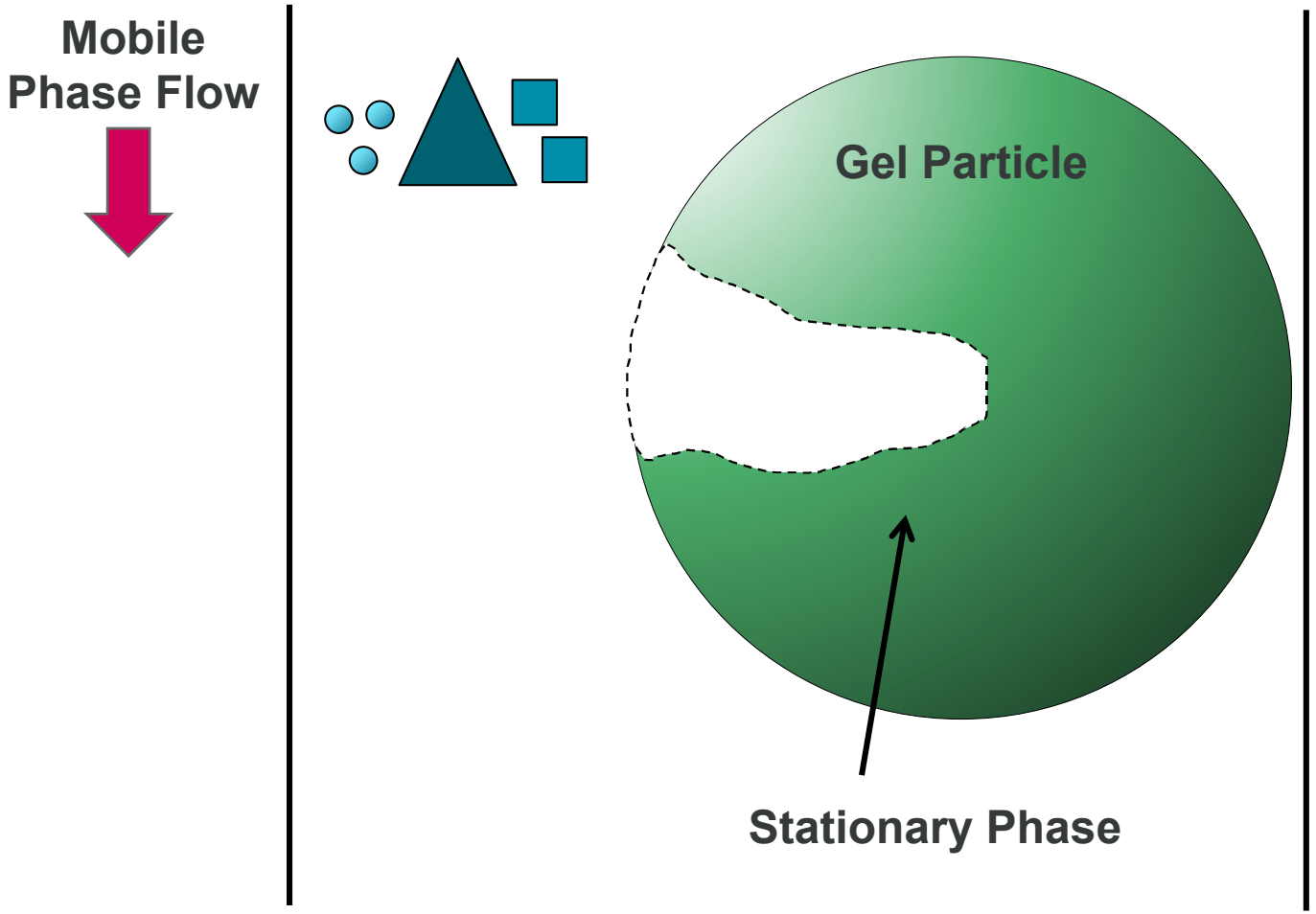
Size Exclusion / Gel Permeation Chromatography

- Separates based on molecular size
- Solution-based technique (liquid mobile phase; sample **MUST** be soluble)

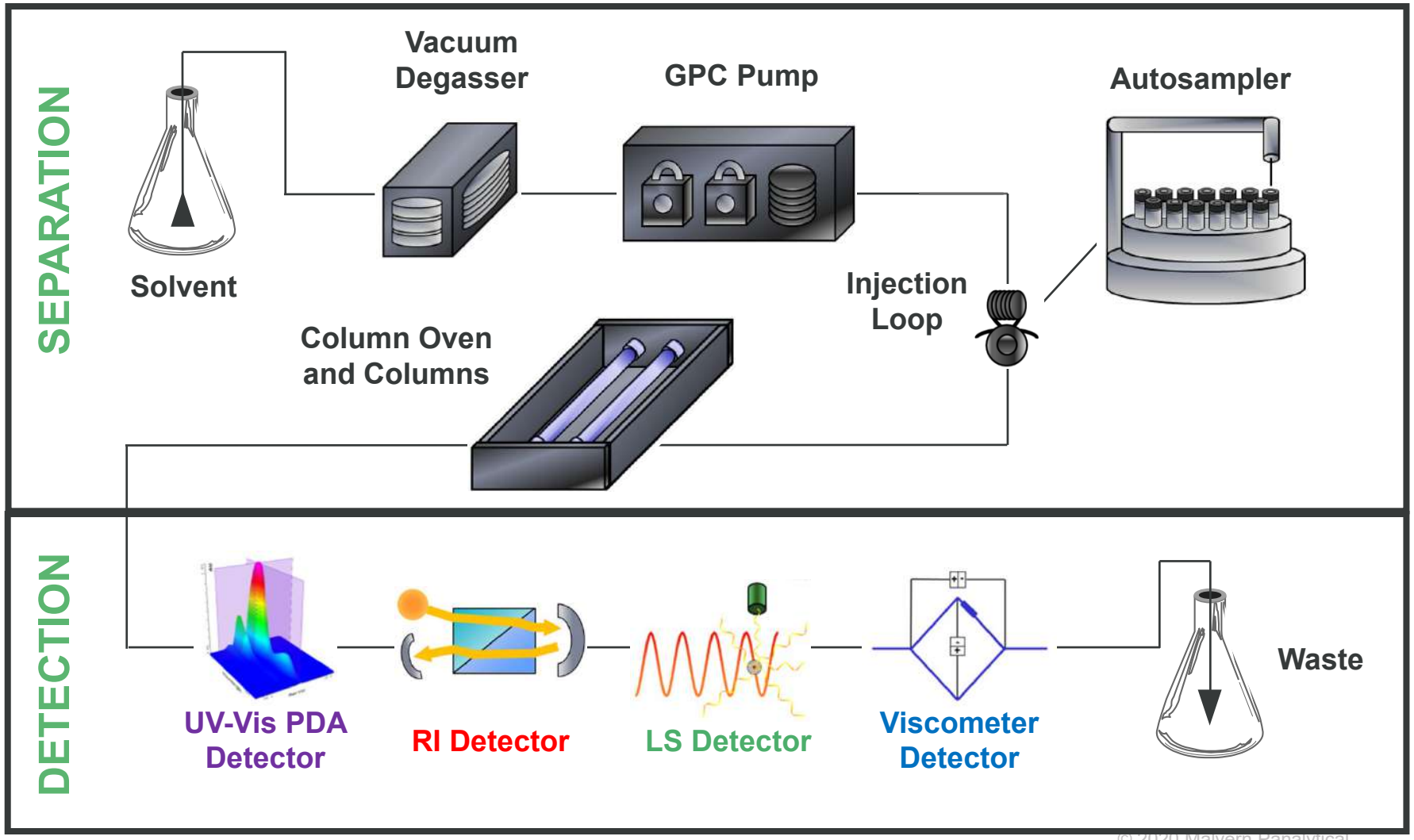


Separation is based on hydrodynamic size, NOT molecular weight!

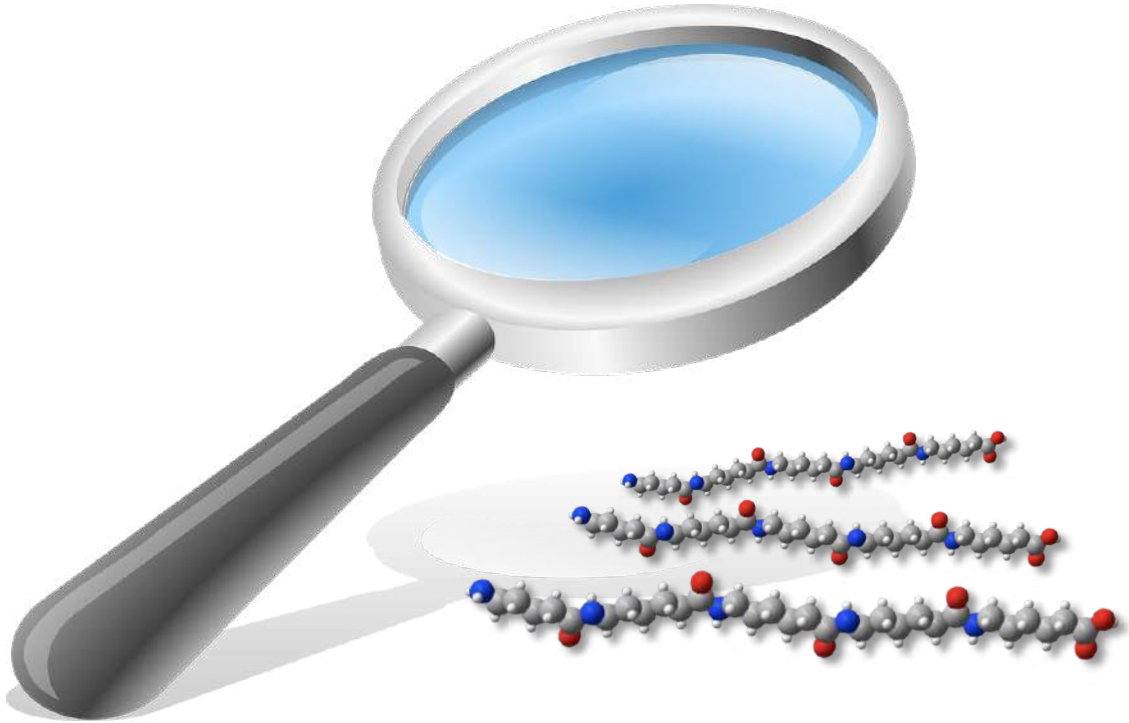
The separation process



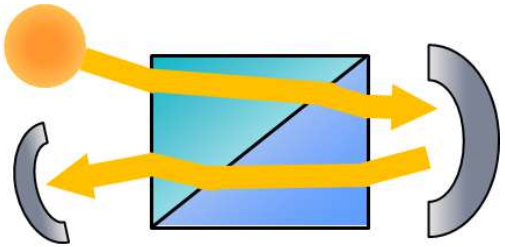
Before the Measurement: GPC / SEC Setup



The detectors: how we see our samples

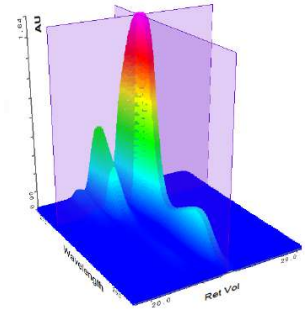


The detectors: how we see our samples



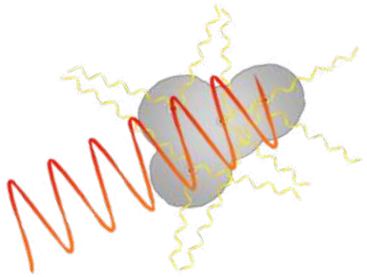
Refractive index

Concentration



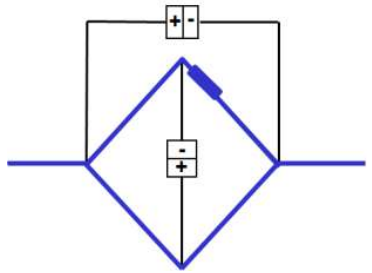
UV/Vis-PDA

Concentration
& Absorption



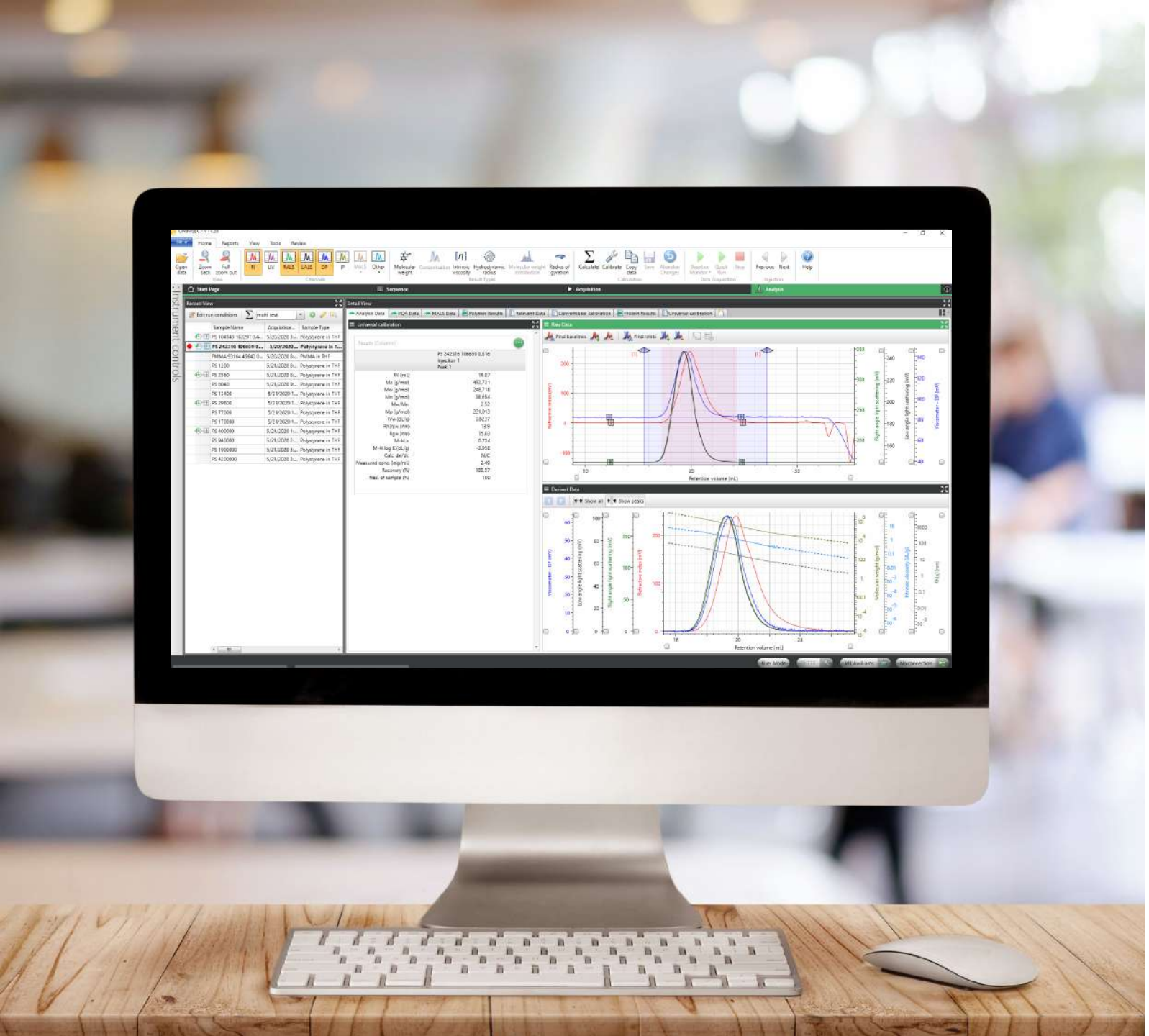
Light scattering

Molecular Weight



Viscometer

Intrinsic Viscosity



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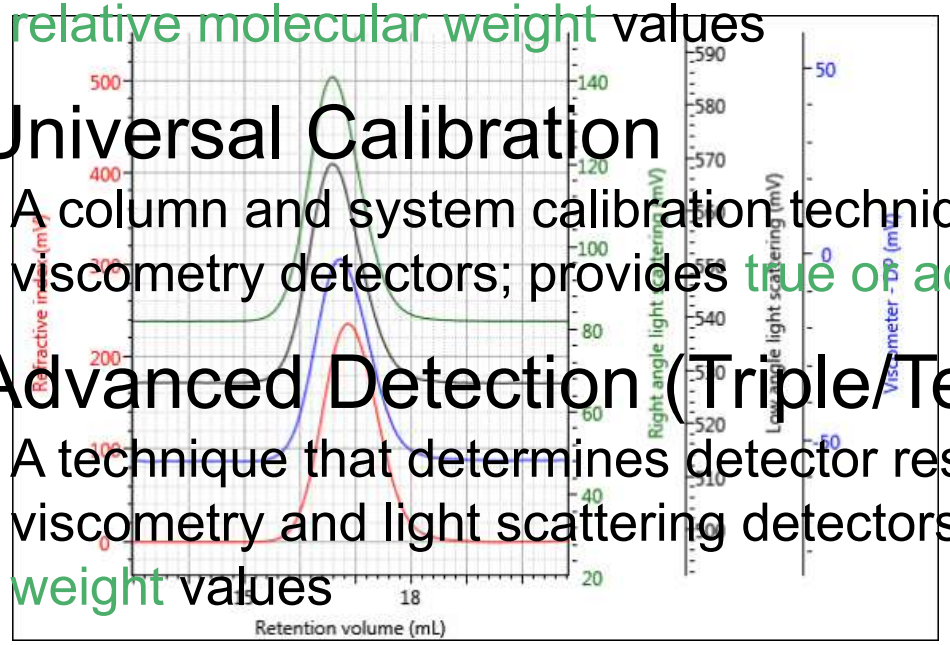
Analysis Methods

How to calculate data

- Conventional calibration
- Universal calibration
- Light scattering

Analysis methods: how data is calculated

- Conventional Calibration
  - A column and system calibration technique employing a concentration detector; provides relative molecular weight values
- Universal Calibration
  - A column and system calibration technique employing a concentration AND solution viscometry detectors; provides true or accurate molecular weight values
- Advanced Detection (Triple/Tetra Detection)
  - A technique that determines detector response factors by employing concentration, solution viscometry and light scattering detectors; provides intrinsic viscosity and absolute molecular weight values



?

Results by sample and peak

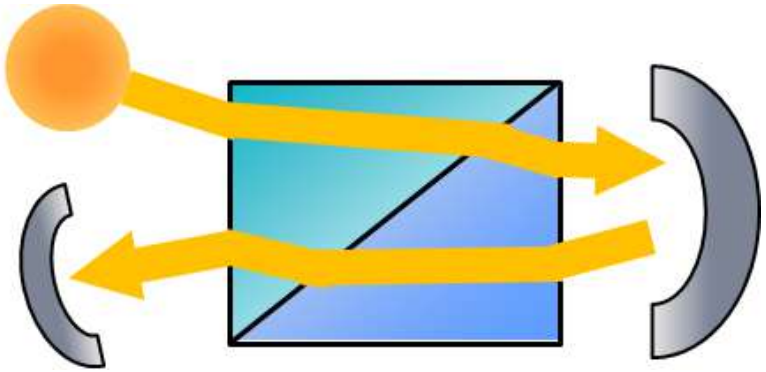
Parameter	Inj. 1 Dextran70k 8/3/201...
	Peak 1
Mz (g/mol)	90,960
Mw (g/mol)	63,230
Mn (g/mol)	36,800
Mw/Mn	1.718
IVw (dL/g)	0.2416
Mark-Houwink a	6.02
Rgw (nm)	N/C
Calc. dn/dC (mL/g)	N/C
Recovery (%)	96.99
Frac. of sample (%)	100

Sample Info

Parameter	Dextran70k Injection 1
Sample name	Dextran70k
Injection No.	1
Sample type name	Dextran in aqueous

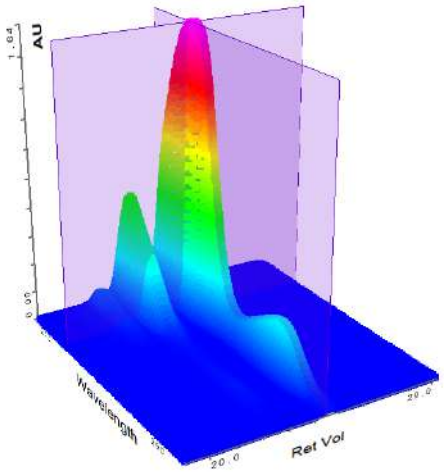
Raw data **Results**

Conventional calibration



Refractive index detector

or

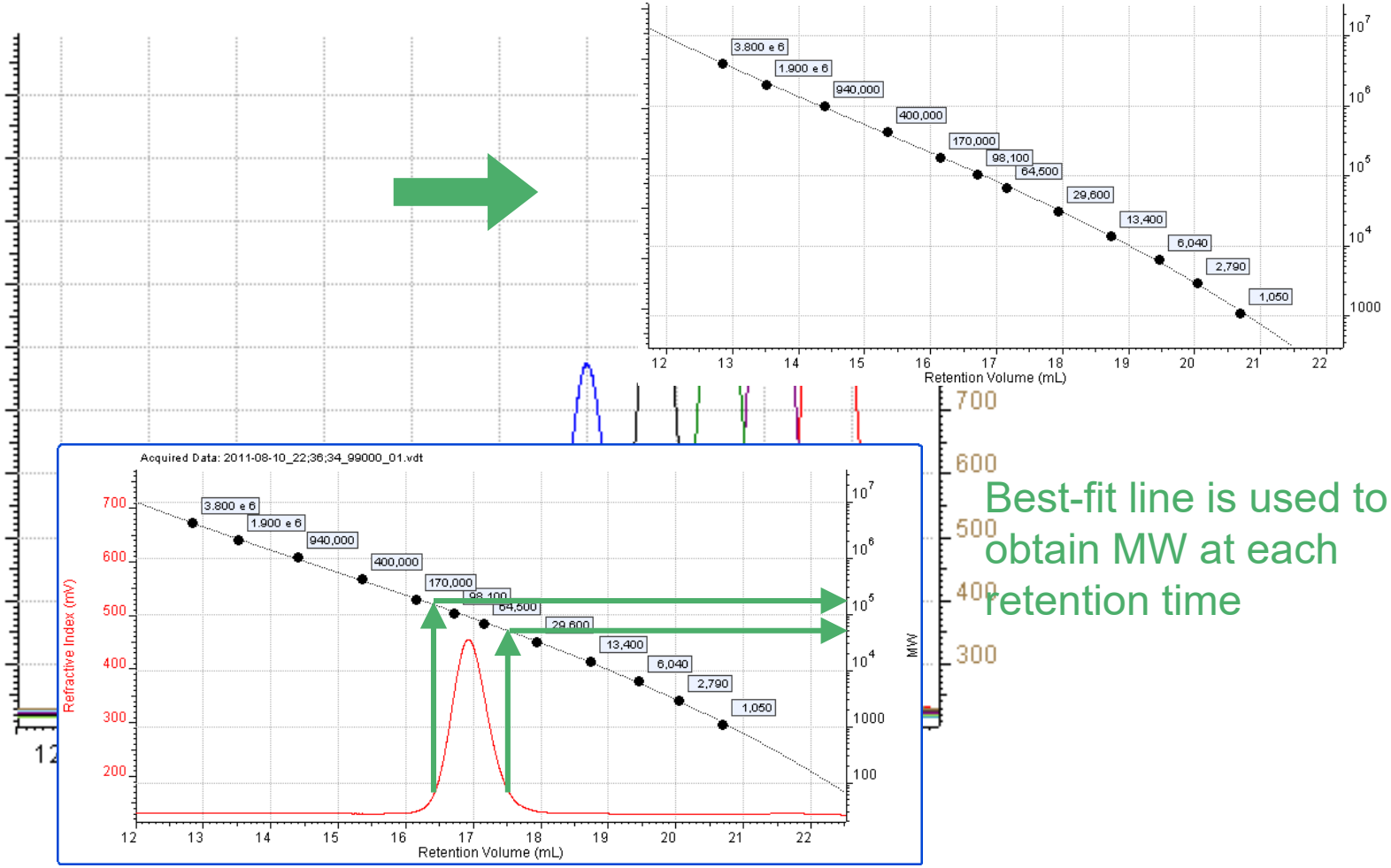


UV/Vis-PDA detector

Conventional calibration

A series of standards

Determine the best-fit line



Best-fit line is used to obtain MW at each retention time

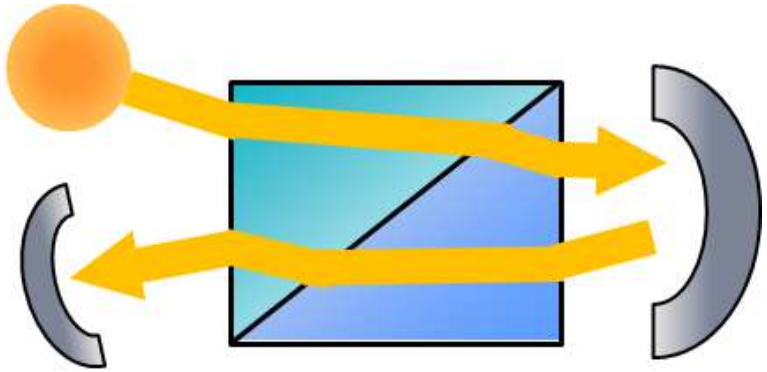
Conventional calibration

Results by sample and peak.	
Parameter	Inj. 1 Dextran70k 8/3/201...
	Peak 1
Mz (g/mol)	90,960
Mw (g/mol)	63,230
Mn (g/mol)	36,800
Mw/Mn	1.718
Frac. of sample (%)	100

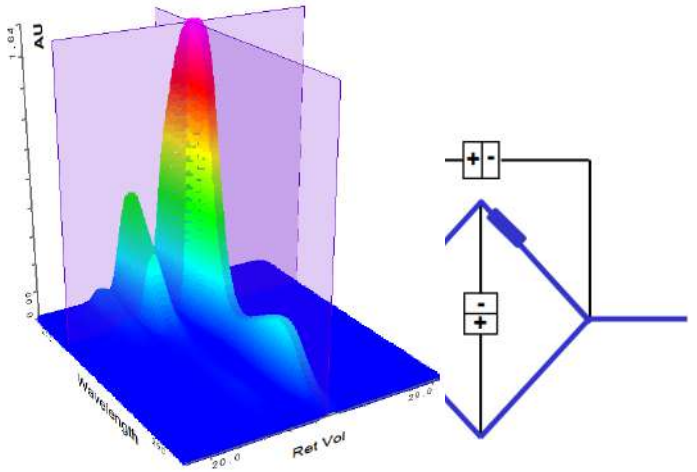
Sample Info	
Parameter	Dextran70k Injection 1
Sample name	Dextran70k
Injection No.	1
Sample type name	Dextran in aqueous

- Molecular weight values obtained are **relative** to the standards used
- Every GPC system (columns, mobile phase, flow rate, temperature) has its own calibration curve
- Every polymer type has its own calibration line based on unique molecular shape
- Accuracy of data depends on how similar the molecular structure of the sample is to the standards
- Data is limited to molecular weight; no viscosity, size, or structural information available

Universal calibration



or

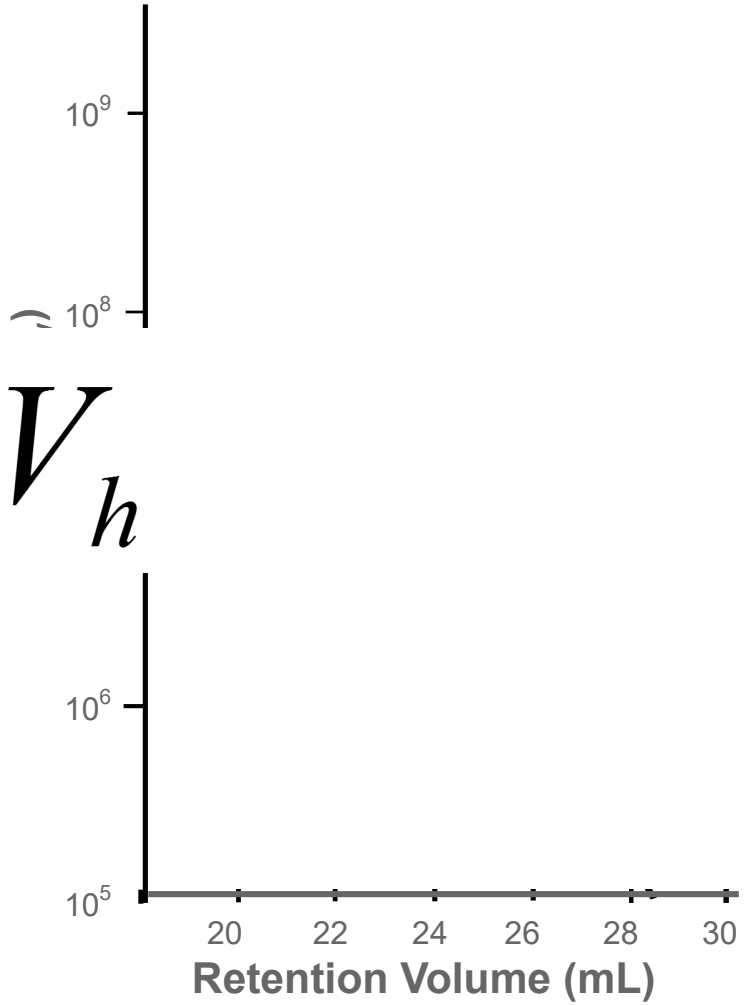


Refractive index detector

UV/Vis-PDA detector

Viscometer

Universal calibration



One calibration curve means the identity of standards is irrelevant

Benoit, M., Grubisic, F., and Rempp, R. *J. Polym. Sci. B.* 5, 753 (1967)

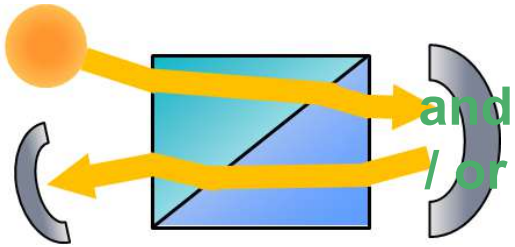
Universal calibration

Results by sample and peak.	
Parameter	Inj. 1 Dextran70k 8/3/201...
	Peak 1
Mz (g/mol)	90,960
Mw (g/mol)	63,230
Mn (g/mol)	36,800
Mw/Mn	1.718
IVw (dL/g)	0.2416
Rh(η)w (nm)	6.02
Recovery (%)	96.99
Frac. of sample (%)	100

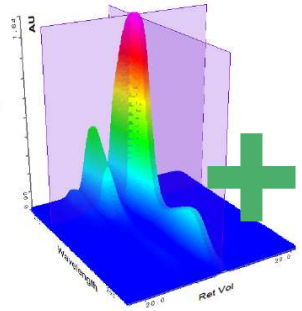
Sample Info	
Parameter	Dextran70k Injection 1
Sample name	Dextran70k
Injection No.	1
Sample type name	Dextran in aqueous

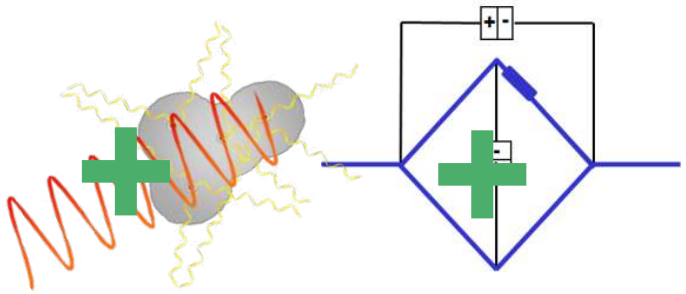
- Molecular weight values obtained are **true or accurate**, regardless of the standards used
- Every polymer type ***falls on the one universal calibration line***
- Accuracy of data is independent of standards
- Every GPC system (columns, mobile phase, flow rate, temperature) has its own calibration curve
- Data available includes molecular weight moments, intrinsic viscosity, hydrodynamic radius, and Mark-Houwink parameters

Advanced detection (triple/tetra detection)



or





Refractive index **UV/Vis-PDA** **Light scattering** **Viscometer**

Advanced detection (triple/tetra detection)

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Results by sample and peak.	
Parameter	Inj. 1 Dextran70k 8/3/201...
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Rgw (nm)	N/C
Calc. dn/dC (mL/g)	N/C
Recovery (%)	96.99
Frac. of sample (%)	100

Sample Info	
Parameter	Dextran70k Injection 1
Sample name	Dextran70k
Injection No.	1
Sample type name	Dextran in aqueous

- Advanced detection provides **absolute** molecular weight values; no calibration curve necessary
- A single narrow standard is used to determine instrument constants and detector offsets
- Accuracy of data is independent on standard
- Each detector tells different piece of story
- Data available from single injection: molecular weight moments, intrinsic viscosity, hydrodynamic radius, radius of gyration, structural information, concentration, recovery, and potentially absorption profile and compositional analysis

GPC/SEC at Malvern Panalytical

How we provide macromolecular characterization solutions

OMNISEC

RESOLVE & REVEAL

- Complete GPC/SEC system
- Detectors include:
  - Refractive index (RI)
  - UV-Vis photodiode array
  - Right & Low Angle Light Scattering
  - Viscometer

SEC-MALS 20

Multi-Angle Light Scattering

- Light Scattering detector only
- Add-on detector for systems with existing concentration (RI &/or UV detectors)
- Includes 20 angles

UPLC REVEAL

Paired with Waters's APC front end

- MP has collaboration with Waters
- Variety of REVEAL detector configurations
- UPLC affords:
  - Shorter analysis times
  - Less sample and solvent required
  - Higher resolution in low MW range

OMNISEC Hardware Overview

Complete GPC/SEC system

- OMNISEC RESOLVE
- OMNISEC REVEAL
- Multi-Angle Light Scattering (MALS) detector

OMNISEC Hardware Overview

Pump & degasser compartment

- OMNISEC RESOLVE
  - Degasser
  - Pump
  - Autosampler
  - Column compartment
- OMNISEC REVEAL
  - Refractive index (RI) detector
  - UV-Vis photodiode array detector
  - Right angle & Low angle light scattering detector
  - Viscometer detector
- Multi-Angle Light Scattering (MALS) detector

OMNISEC Hardware Overview

Autosampler

- OMNISEC RESOLVE
  - Degasser
  - Pump
  - Autosampler
  - Column compartment
- OMNISEC REVEAL
  - Refractive index (RI) detector
  - UV-Vis photodiode array detector
  - Right angle & Low angle light scattering detector
  - Viscometer detector
- Multi-Angle Light Scattering (MALS) detector

OMNISEC Hardware Overview

Column oven

- OMNISEC RESOLVE
  - Degasser
  - Pump
  - Autosampler
  - Column compartment
- OMNISEC REVEAL
  - Refractive index (RI) detector
  - UV-Vis photodiode array detector
  - Right angle & Low angle light scattering detector
  - Viscometer detector
- Multi-Angle Light Scattering (MALS) detector

OMNISEC Hardware Overview

REVEAL detector unit

- OMNISEC RESOLVE
  - Degasser
  - Pump
  - Autosampler
  - Column compartment
- OMNISEC REVEAL
  - Refractive index (RI) detector
  - UV-Vis photodiode array detector
  - Right angle & Low angle light scattering detector
  - Viscometer detector
- Multi-Angle Light Scattering (MALS) detector

Multi-Angle Light Scattering

SEC-MALS 20 detector

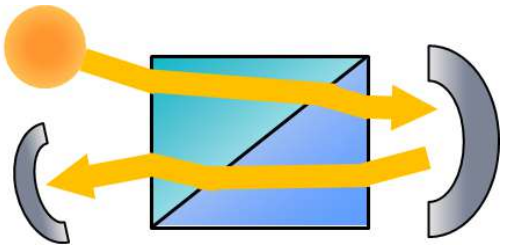
- A modular multi-angle light scattering system with 20 measurement angles
- Works with other Malvern systems
- Interfaces with 3rd party SEC systems

Info on MALS & RALS/LALS detectors

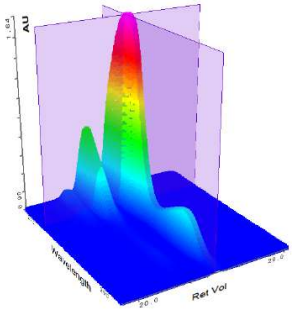
Detectors available with OMNISEC

Various configurations available

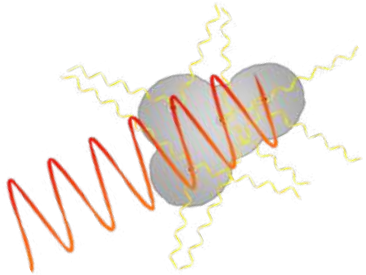
- Refractive Index – concentration
- UV-Vis Photodiode Array – concentration and absorption
- Right Angle & Low Angle Light Scattering – molecular weight
- Viscometer – intrinsic viscosity
- Multi-Angle Light Scattering – molecular weight (alternative to RALS/LALS)



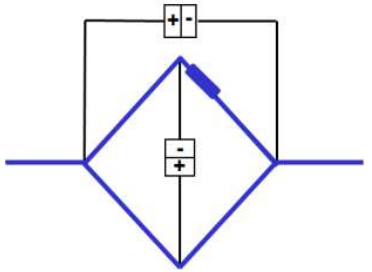
Refractive index



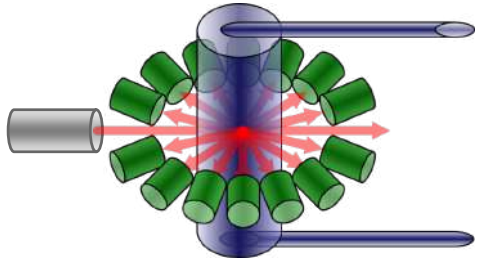
UV/Vis-PDA



Light scattering



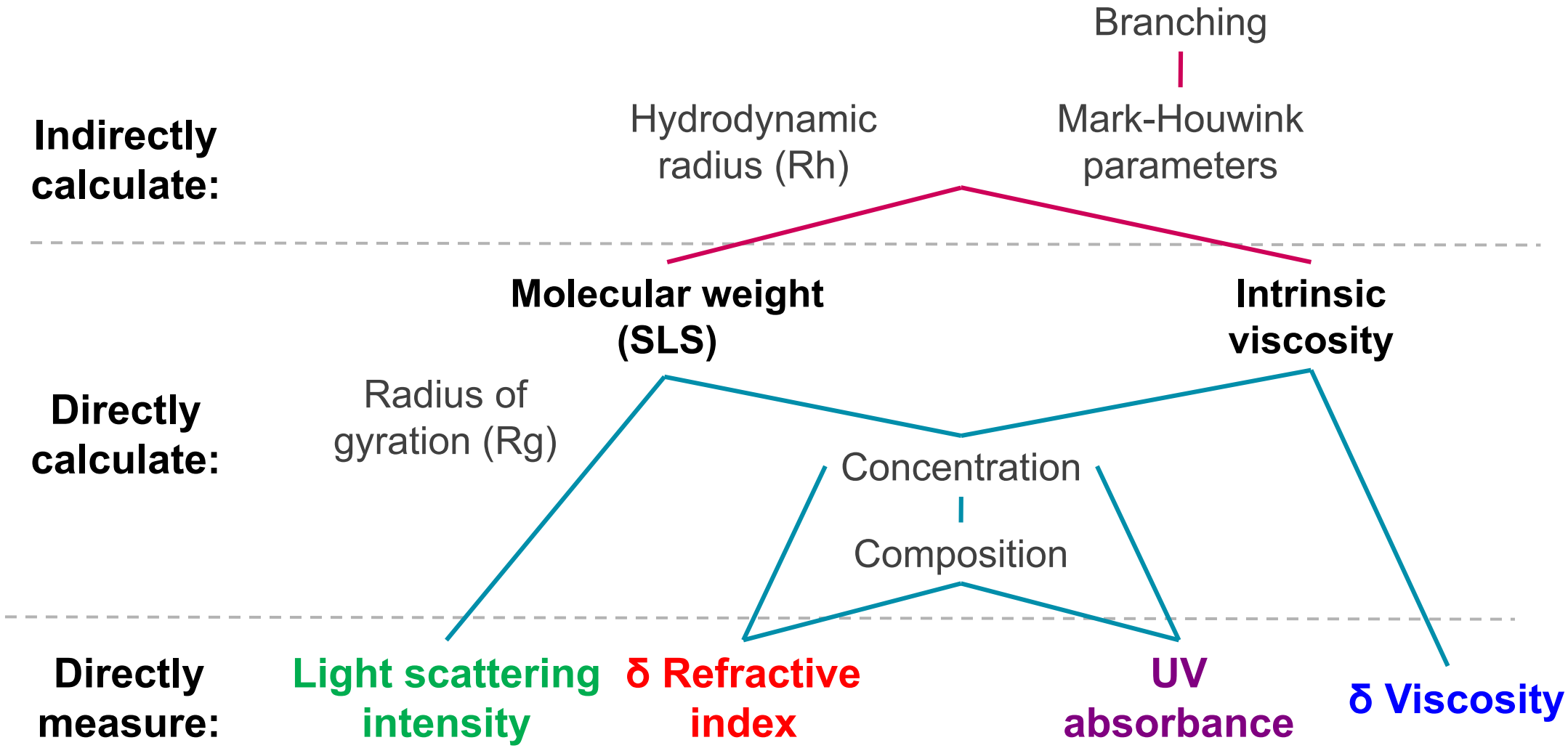
Viscometer



MALS

Multi-Detector Pyramid

How the detectors work together



[Info on how Multi-Detector GPC/SEC works]

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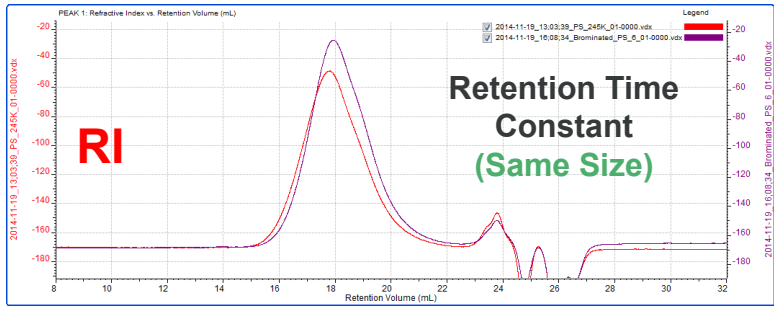
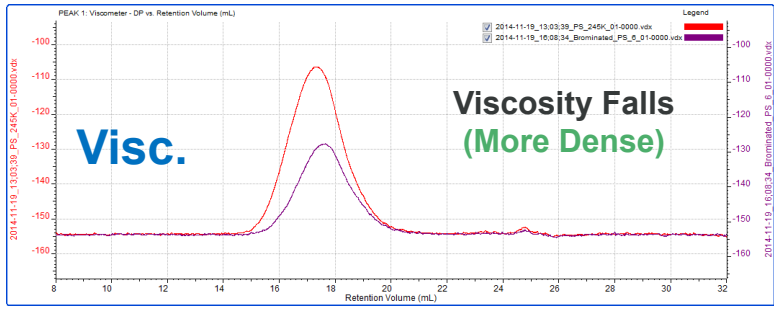
Applications

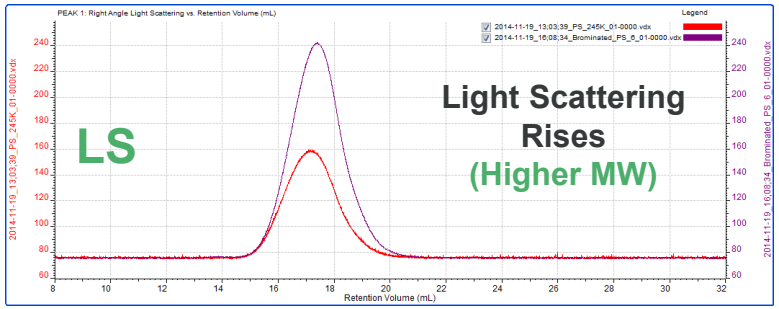
A variety of sample types

- Plain vs. Brominated PS
- Linear vs. branched samples
- PLA-PLGA copolymers
- Antibodies of different sizes
- Beta-amylase

Detectors Respond Differently:
PS & Brominated PS





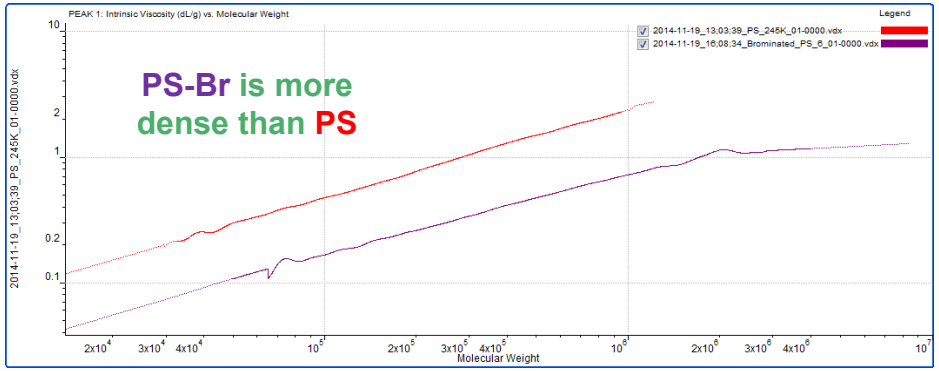


RI signals show similar retention volume = same molecular size

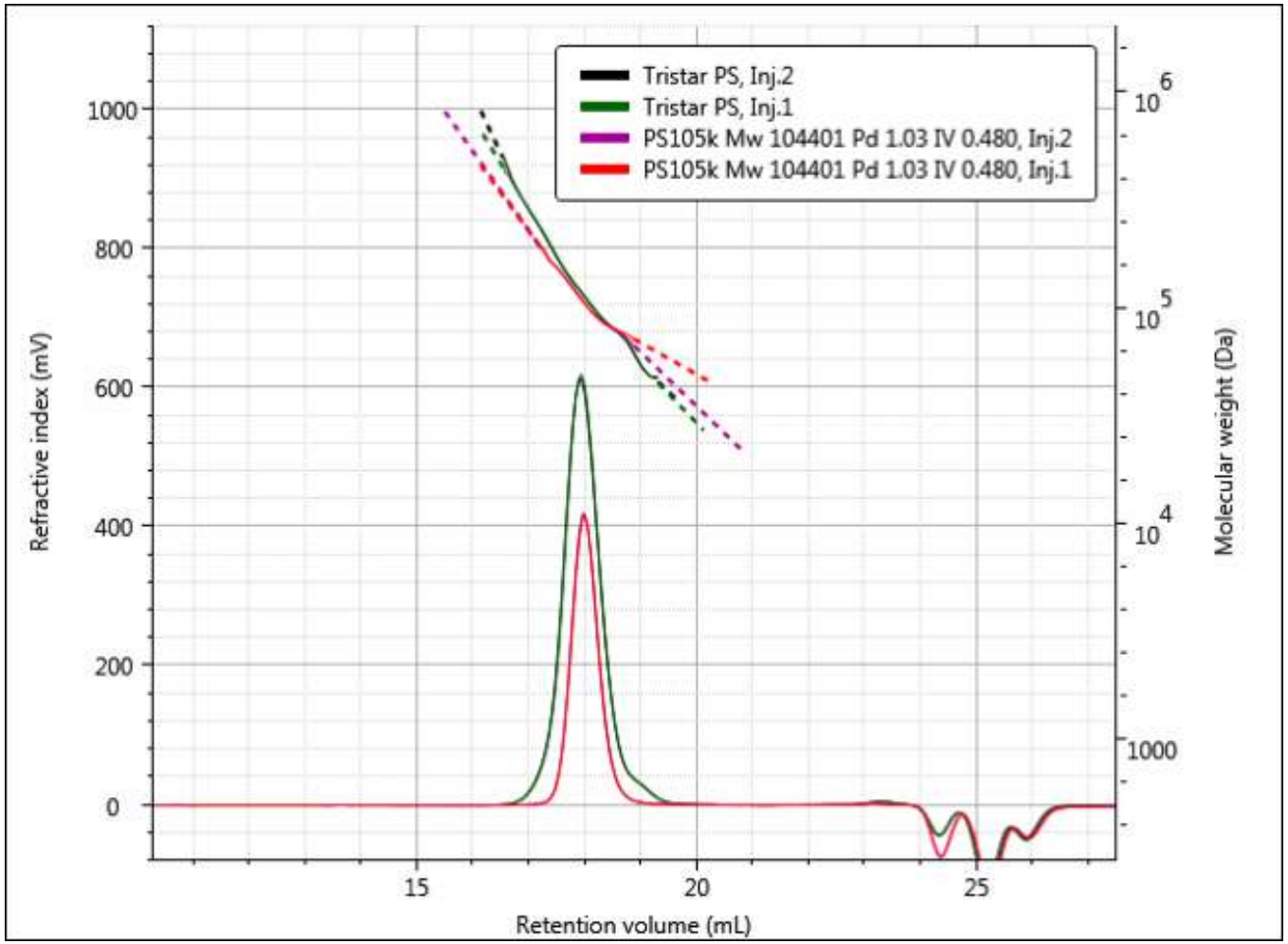
Visc. shows **PS-Br** has weaker signal = **PS-Br** is more dense (i.e. has a lower viscosity)

LS shows **PS-Br** has stronger signal = **PS-Br** has higher MW

	PS	PS-Br
Mw	246 kDa	429 kDa
IV	0.835 dL/g	0.389 dL/g
Rh	13.95 nm	13.01 nm



3-arm **PS star** overlaid with **linear PS**



Results by sample and peak		
Parameter	Inj. 2 PS105k Mw 104401...	Inj. 1 Tristar PS 11/12/20...
RV (mL)	18.00	17.95
Mn (g/mol)	102,500	111,800
Mw (g/mol)	106,400	122,600
Mz (g/mol)	110,100	135,400
Mw/Mn	1.038	1.096
IVw (dL/g)	0.4766	0.4432
Rh(η)w (nm)	9.275	9.439
Rgw (nm)	N/C	N/C
M-H a	0.6778	0.5397
M-H log K (dL/g)	-3.731	-3.094
RI peak (mV·mL)	245.8	496.1
RALS peak (mV·mL)	102.6	236.7
LALS peak (mV·mL)	58.00	133.3
DP peak (mV·mL)	37.37	69.84
Calc. dn/dC (mL/g)	N/C	N/C
Recovery (%)	100.7	94.16

3-arm **PS star** overlaid with **linear PS**

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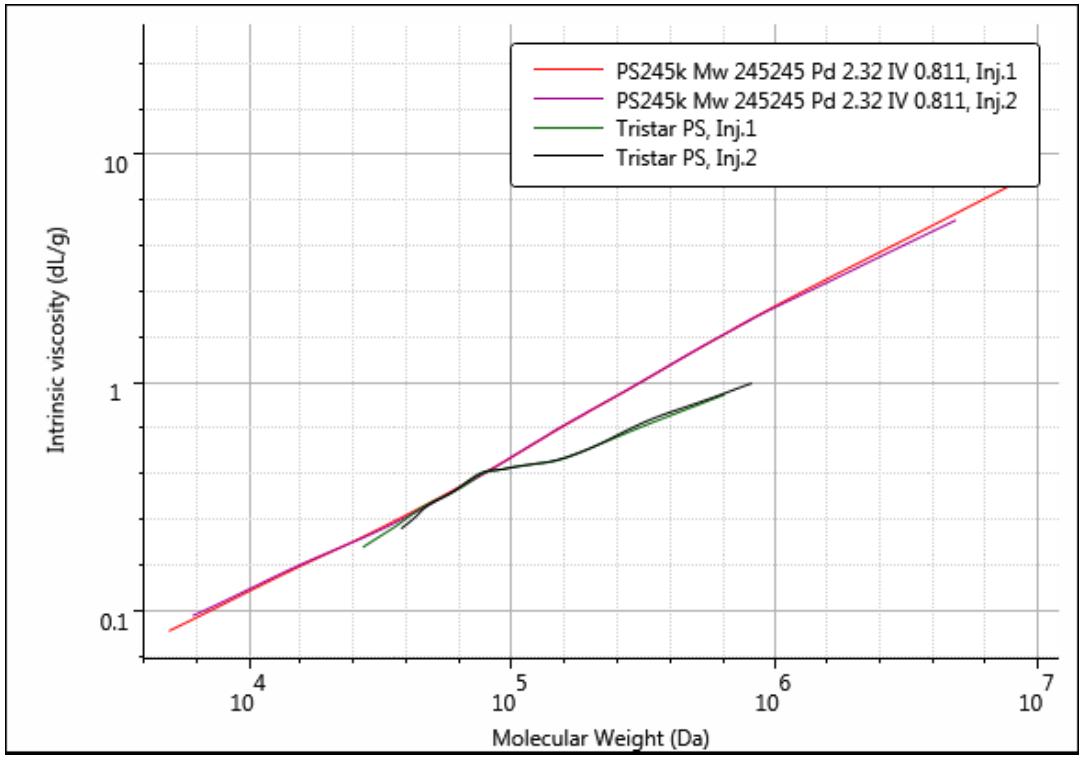
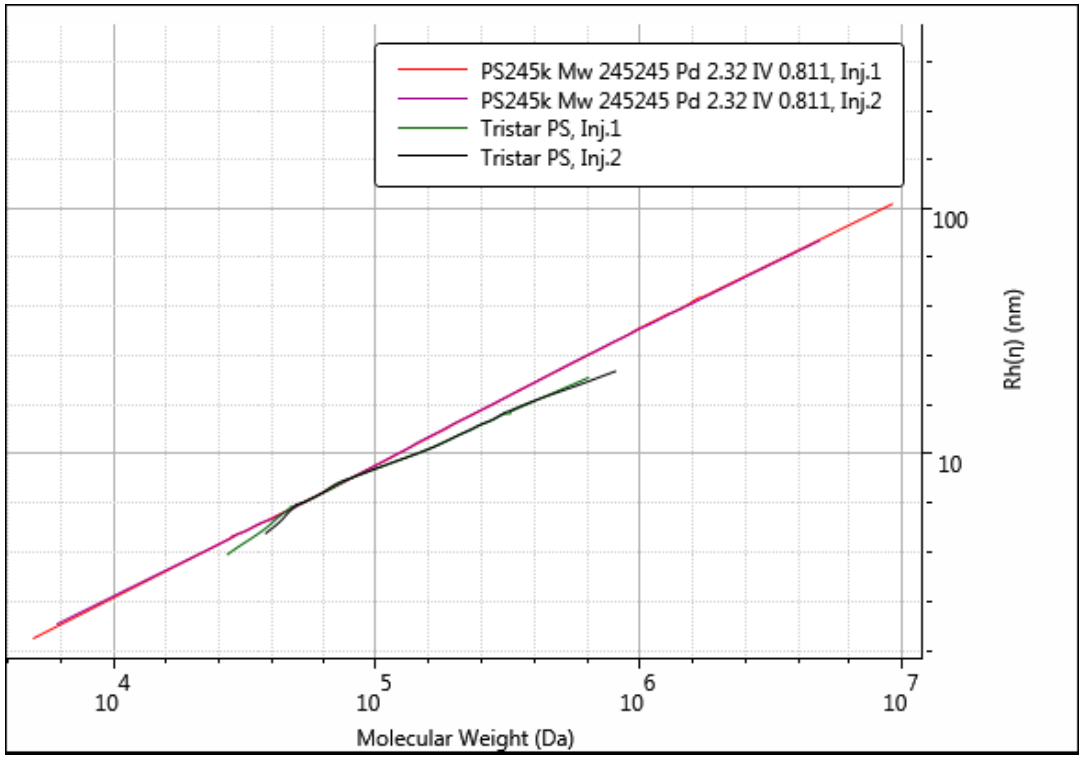
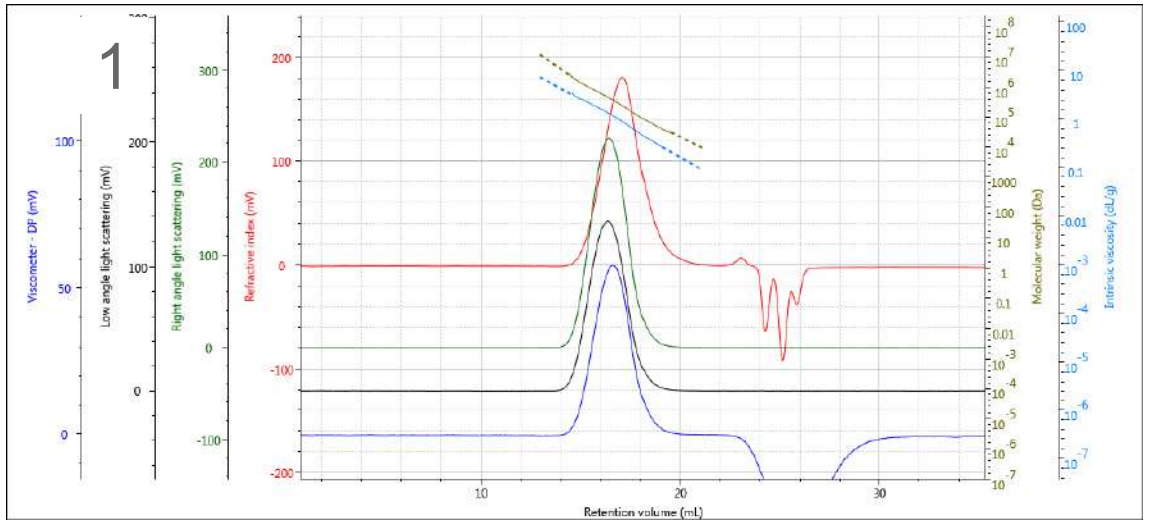
Mark-Houwink

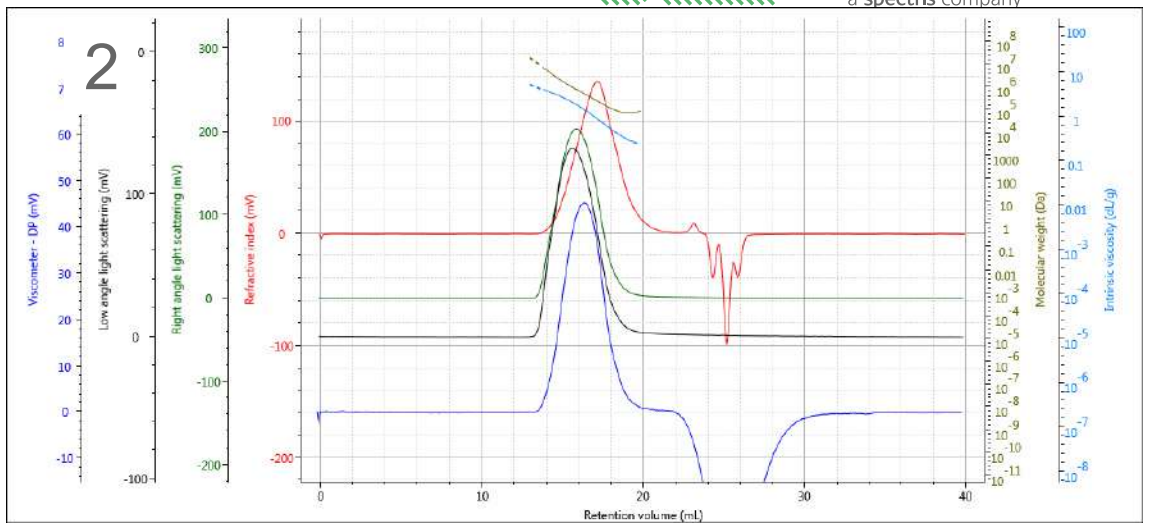


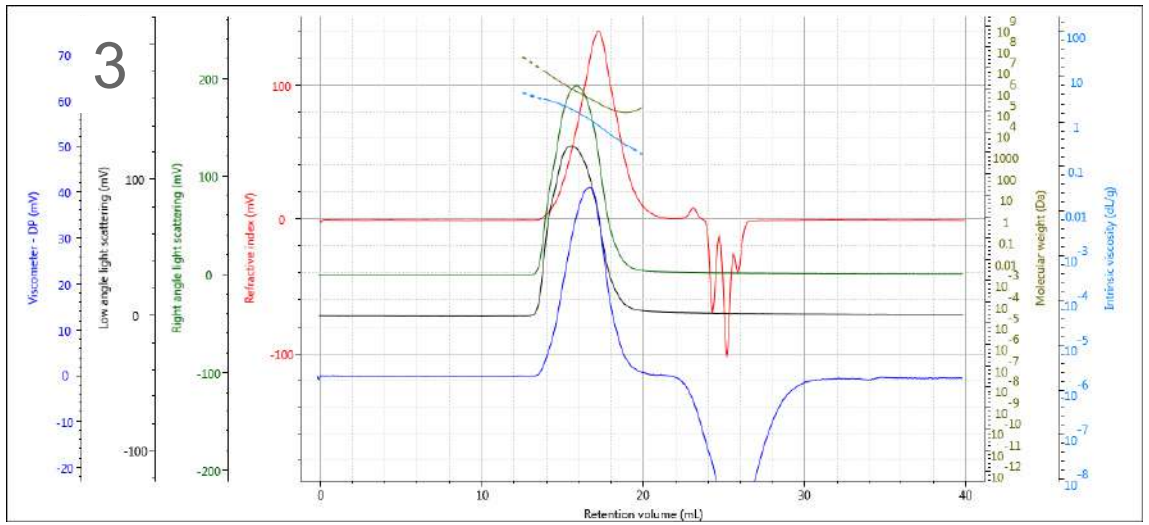
Rh vs MW

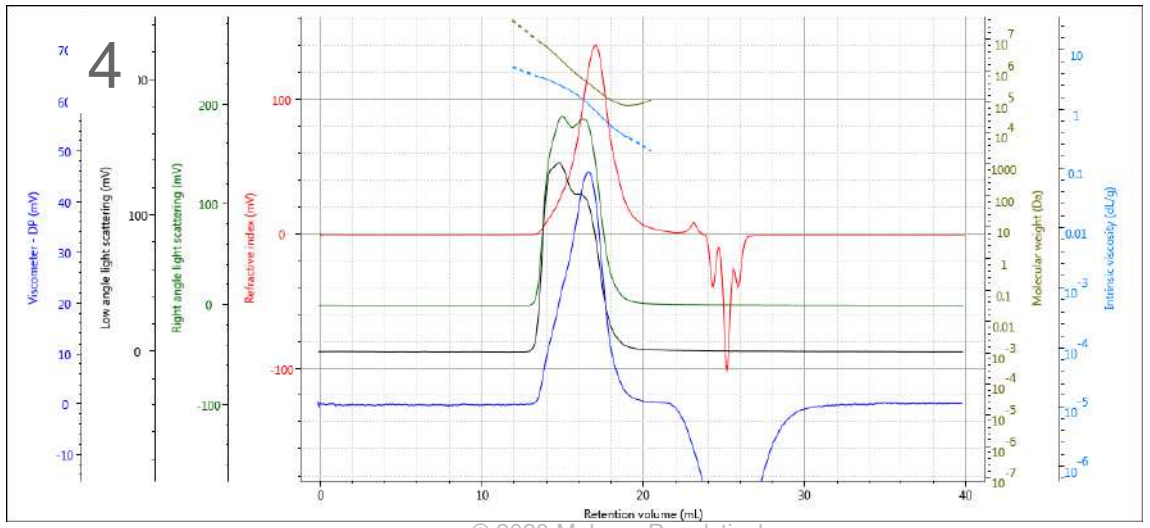


Branched series of polystyrenes

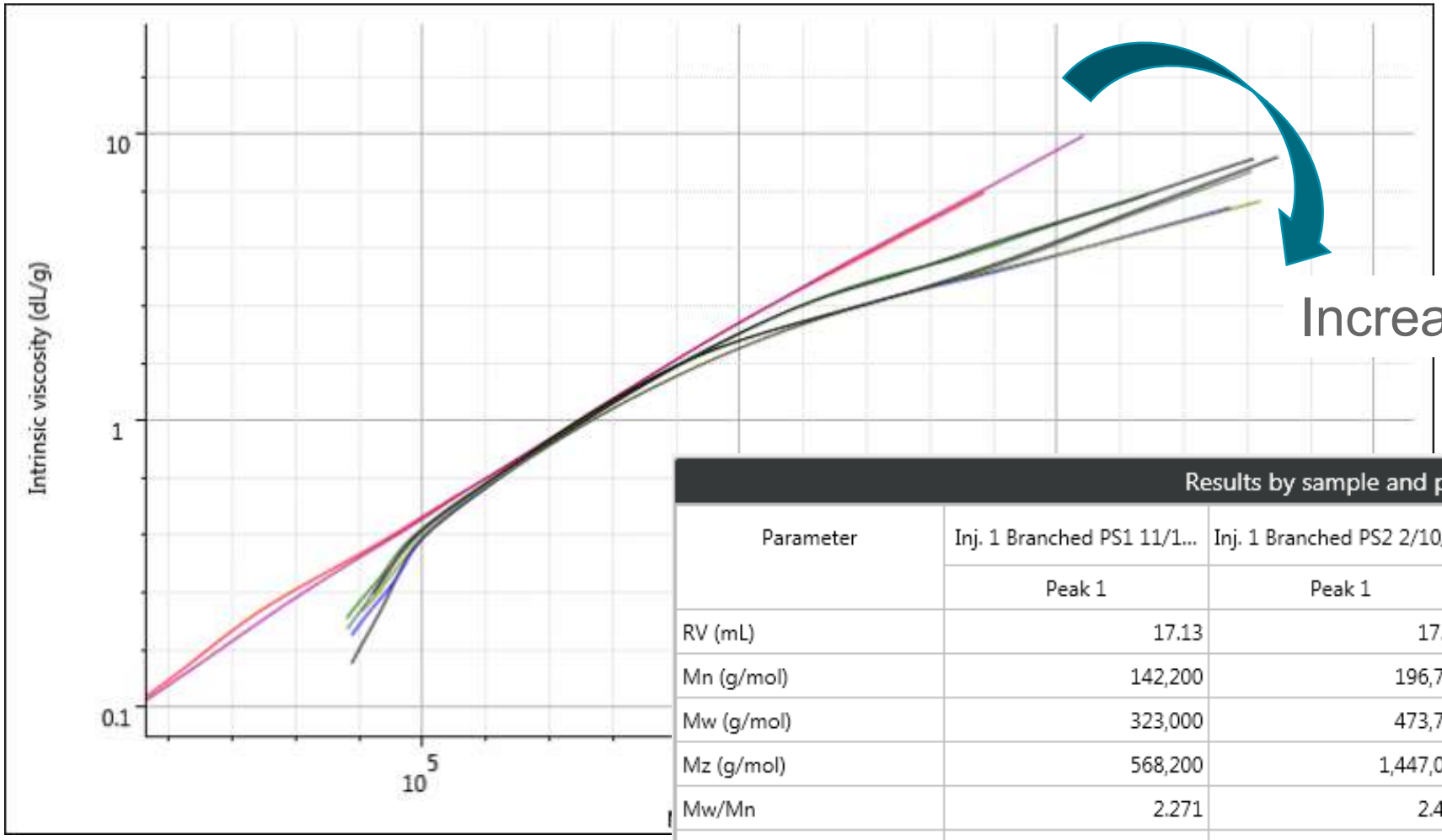








Mark-Houwink Comparison of branched polystyrenes

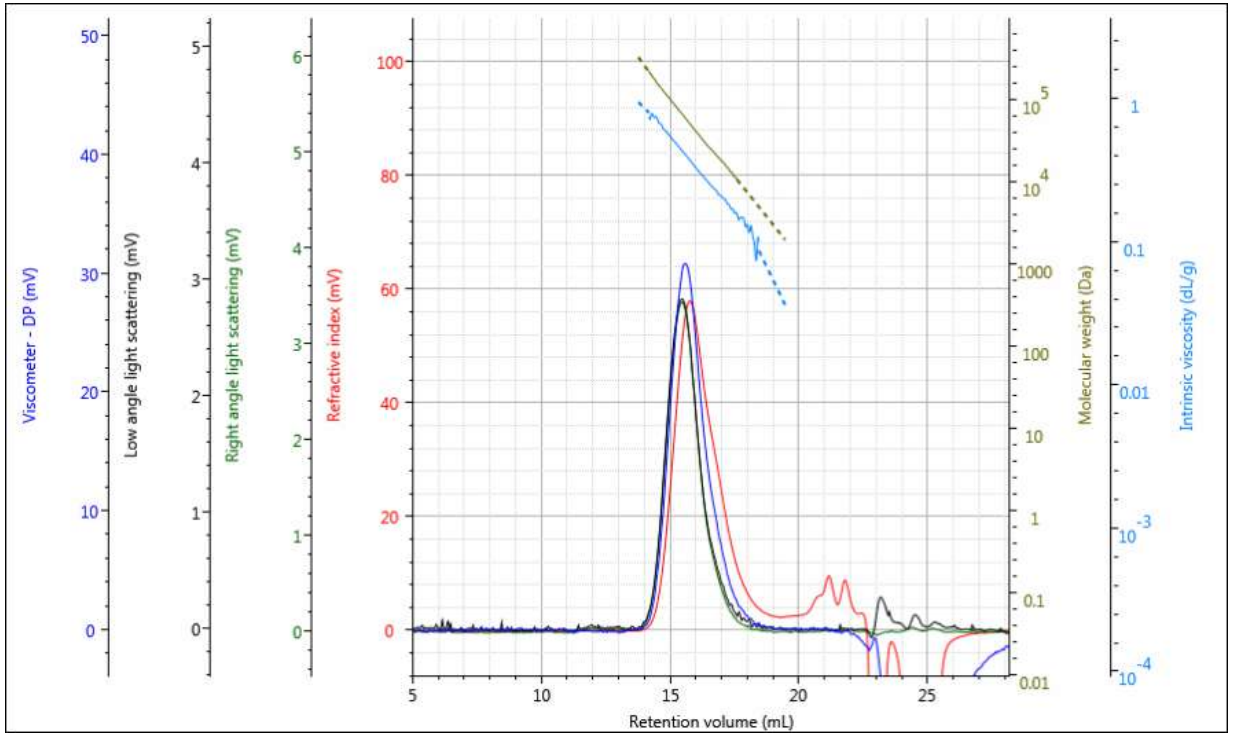


Increasing branching

Results by sample and peak.				
Parameter	Inj. 1 Branched PS1 11/1...	Inj. 1 Branched PS2 2/10/...	Inj. 1 Branched PS3 2/10/...	Inj. 1 Branched PS4 11/1...
	Peak 1	Peak 1	Peak 1	Peak 1
RV (mL)	17.13	17.21	17.28	17.10
Mn (g/mol)	142,200	196,700	205,000	228,200
Mw (g/mol)	323,000	473,700	492,200	603,700
Mz (g/mol)	568,200	1,447,000	1,788,000	2,187,000
Mw/Mn	2.271	2.408	2.400	2.645
IVw (dL/g)	0.9590	1.062	0.9698	1.127
Rh(η)w (nm)	16.05	18.22	17.74	19.76

PLGA 50:50

- PLGA copolymer
- Low dn/dc sample – historically difficult to measure
- 3.028 mg/ml; 100 µl



Results by sample and peak.	
Parameter	Inj. 4 PLGA 5050 mid M...
	Peak 1
RV (mL)	15.79
Mn (g/mol)	24,950
Mw (g/mol)	48,860
Mz (g/mol)	72,280
Mw/Mn	1.958
IVw (dL/g)	0.3386
Rh(η)w (nm)	6.151
Calc. dn/dC (mL/g)	0.04933
M-H a	0.5900
M-H log K (dL/g)	-3.210

PLA/PLGA

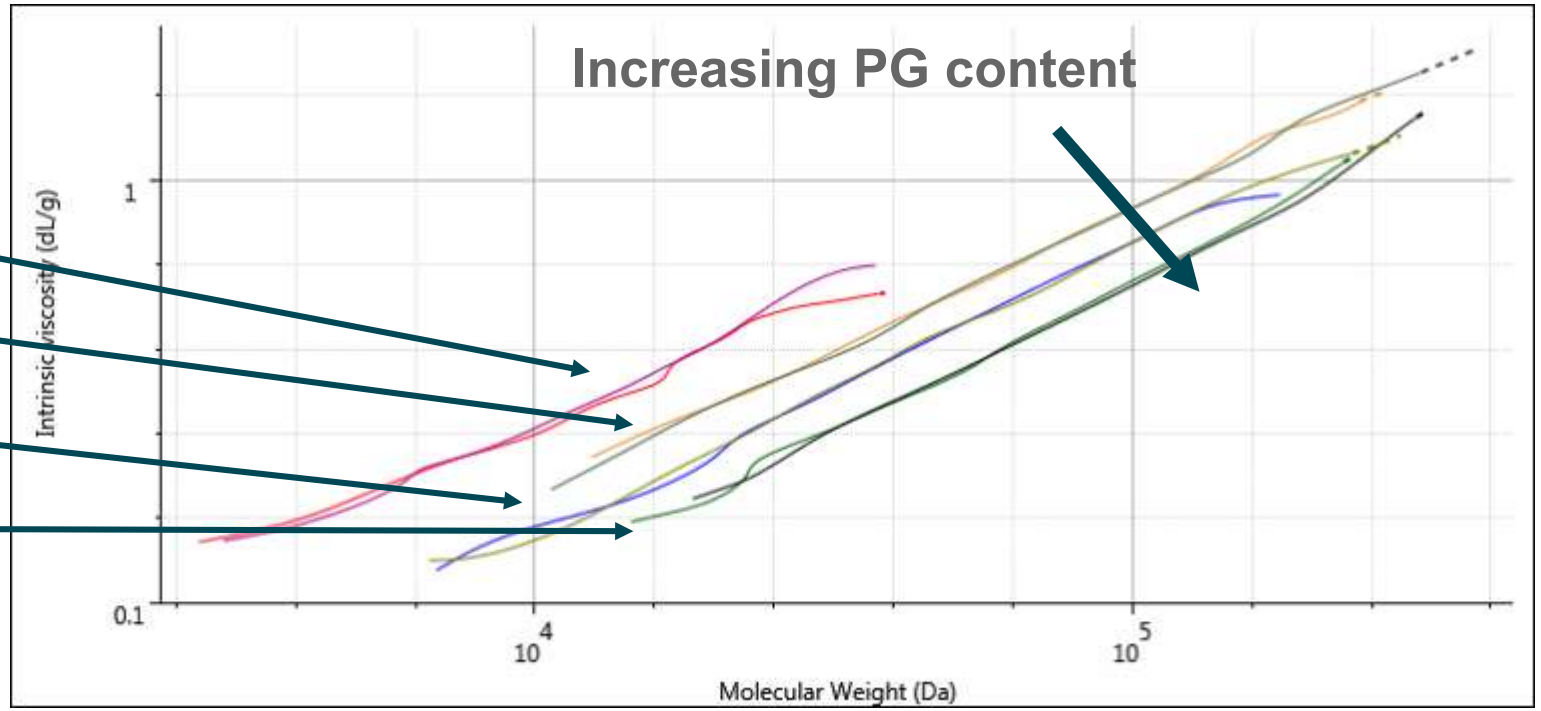
- Different copolymer compositions can be distinguished using the Mark-Houwink plot
- The greater the proportion of PG, the more dense/compact the molecule

PLA:PG 100:0

PLA:PG 75:25

PLA:PG 65:35

PLA:PG 50:50

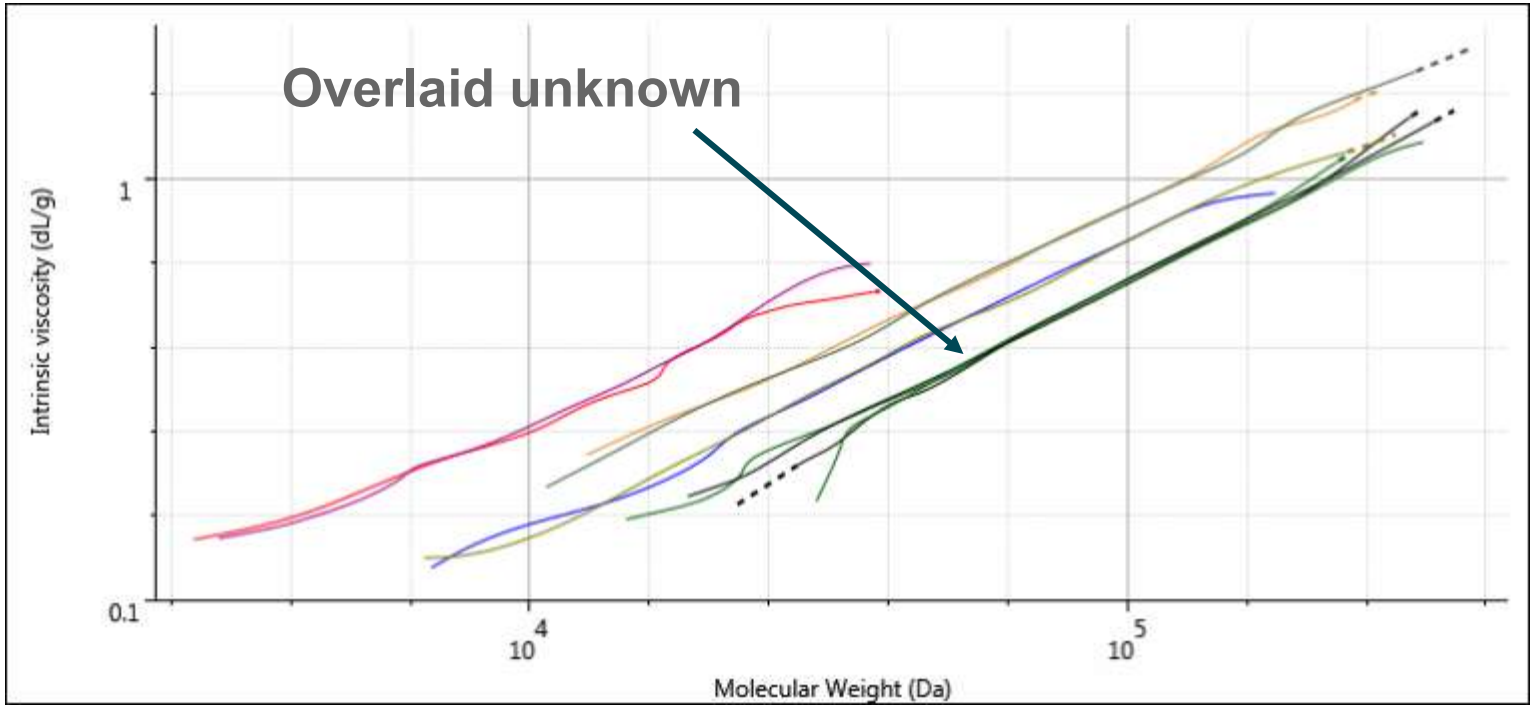


PLA/PLGA

- This unknown sample overlays perfectly with 50:50 copolymer, therefore must have the same composition

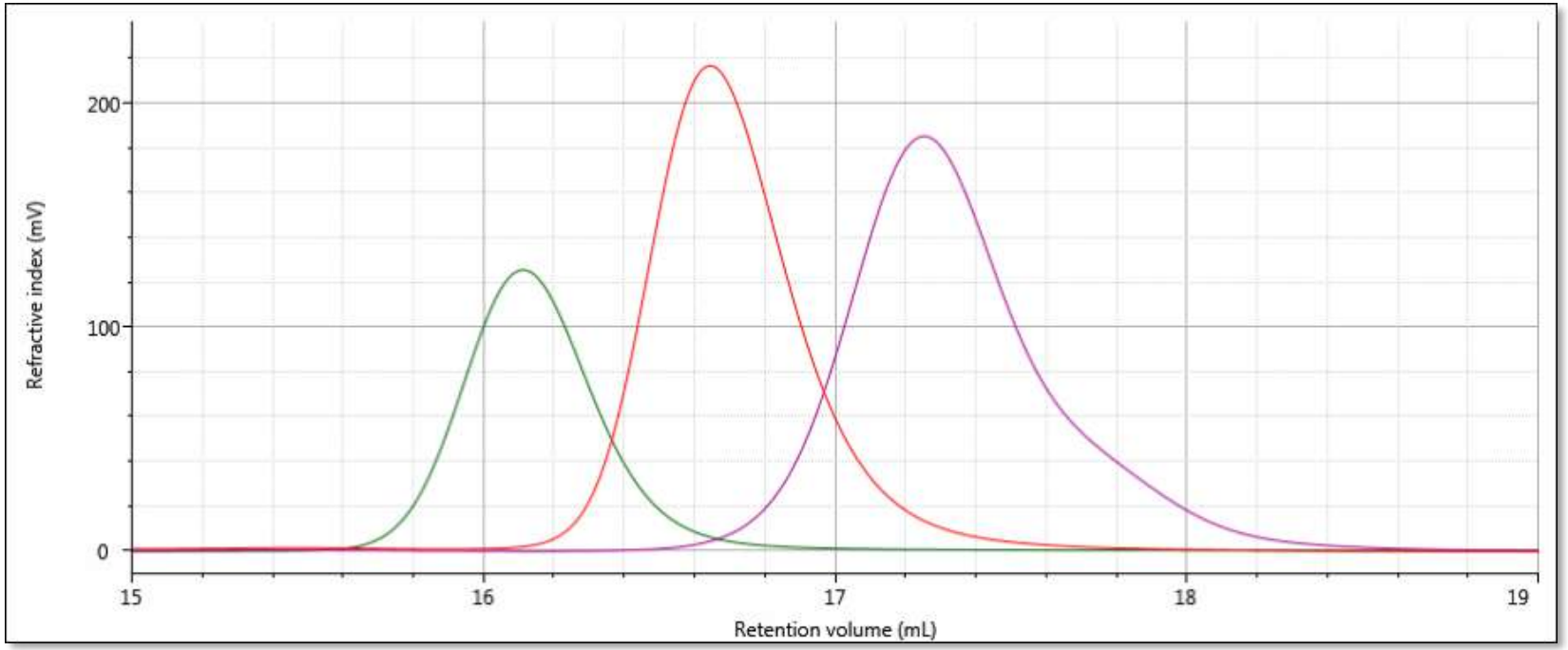
Unknown sample

	Mean	% RSD
RV (mL)	18.24	0.1679
Mn (g/mol)	63,870	2.073
Mw (g/mol)	86,510	2.483
Mw/Mn	1.355	0.4105
IVw (dL/g)	0.4955	1.257
Rh (nm)	8.533	1.337
M-H a	0.762	0.7376
M-H log K (dL/g)	-4.053	-0.6432



Analysis of three antibodies

- Three antibodies eluting at different times in the chromatogram
- They have different molecular sizes but do they have different molecular weights?



Column calibration vs. advanced detection

Column calibration

Sample ID	Mw (Da)	Mw/Mn
1	143,209	1.021
2	96,863	1.035
3	201,996	1.029

Advanced detection

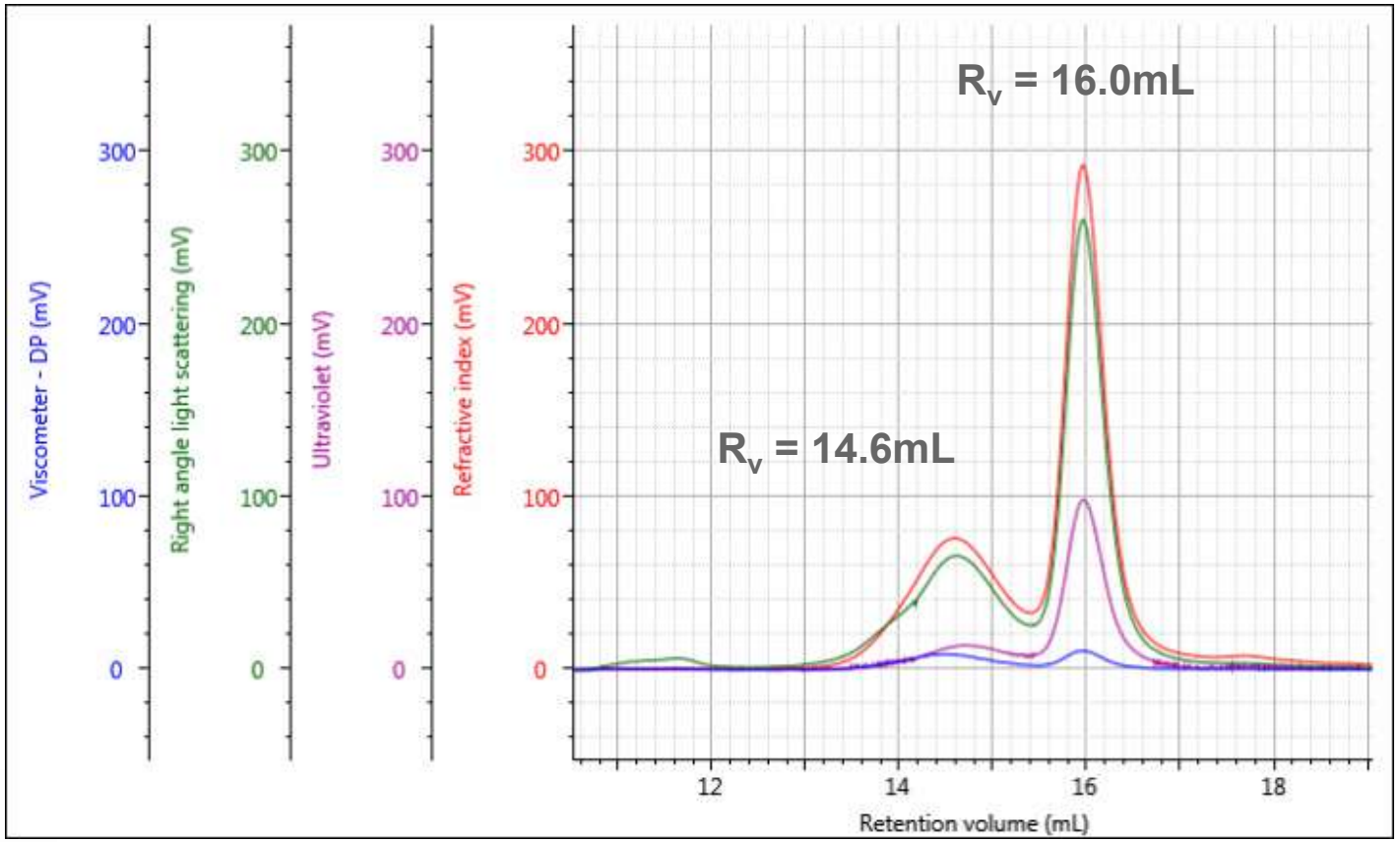
Sample ID	Mw (Da)	Mw/Mn	IV (dL/g)	Rh (nm)
1	149,300	1.000	0.065	5.37
2	151,100	1.000	0.062	5.29
3	150,000	1.002	0.070	5.49

- Column calibration data ties molecular weight to retention volume → samples that elute earlier have higher molecular weight
- Advanced detection uses light scattering to measure molecular weight independently of retention volume → absolute molecular weight
- Even though the three antibody samples have different molecular sizes, the molecular weight of all three samples is 150 kDa

β-Amylase

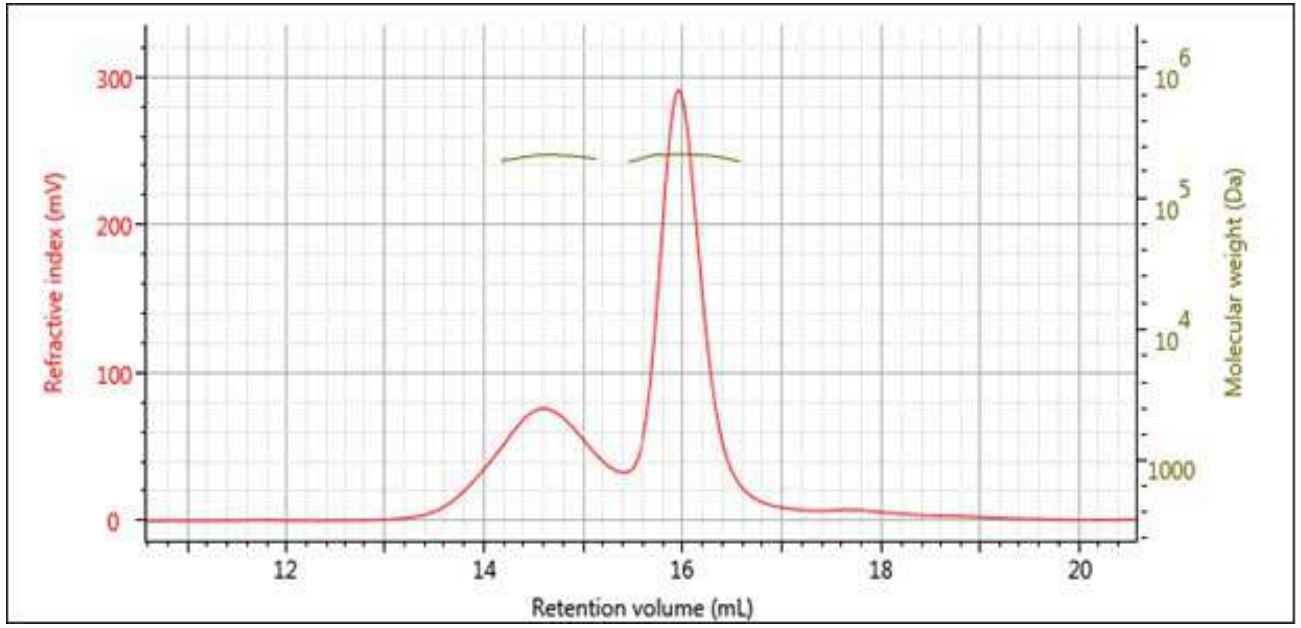
- Beta amylase from sweet potato
- Theoretical Mw ~ 223kDa
- Native structure is a homo-tetramer

- β-amylase elutes as two peaks
- The peak used for the conventional calibration is the one at 16 ml, which is assumed to be the monomer
- The earlier eluting peak is broader and would generally be assumed to be some aggregated material



β-Amylase

- Similar Mw
- Small increase in Mw doesn't explain the significant increase in IV
- Larger IV indicative of decrease in density
- Peak 1 has more open structure – supported by increase in Rh
- However – they have different levels of UV absorbance! – likely a contaminating protein



Results by sample and peak.		
Parameter	Inj. 1 Beta amylase 24/08/2015 13:37:51	
	Peak 1	Peak 2
RV (mL)	14.60	15.97
Mw (g/mol)	208,500	212,800
Mw/Mn	1.001	1.001
IVw (dL/g)	0.1728	0.06318
Rh(η)w (nm)	8.283	5.957
Frac. of sample (%)	28.29	71.71
RI peak (mV·mL)	62.07	157.3
RALS peak (mV·mL)	52.49	136.8

Columns

Which ones to use?

- Solvents & applications
- Types of columns
  - Single-pore
  - General mixed bed
  - Linear mixed bed
- Building a column set

Malvern Panalytical's GPC/SEC Columns

Choose the right column for your application

- T-columns — THF (& other organic solvents)
  - D-, C- & HFIP columns for DMF, chloroform & HFIP, respectively

- A-columns — Aqueous
- P-columns — Protein
- I-columns — Inert (polar organic solvents)
- Cationic columns — Cationic

Malvern's Analytical GPC/SEC Columns

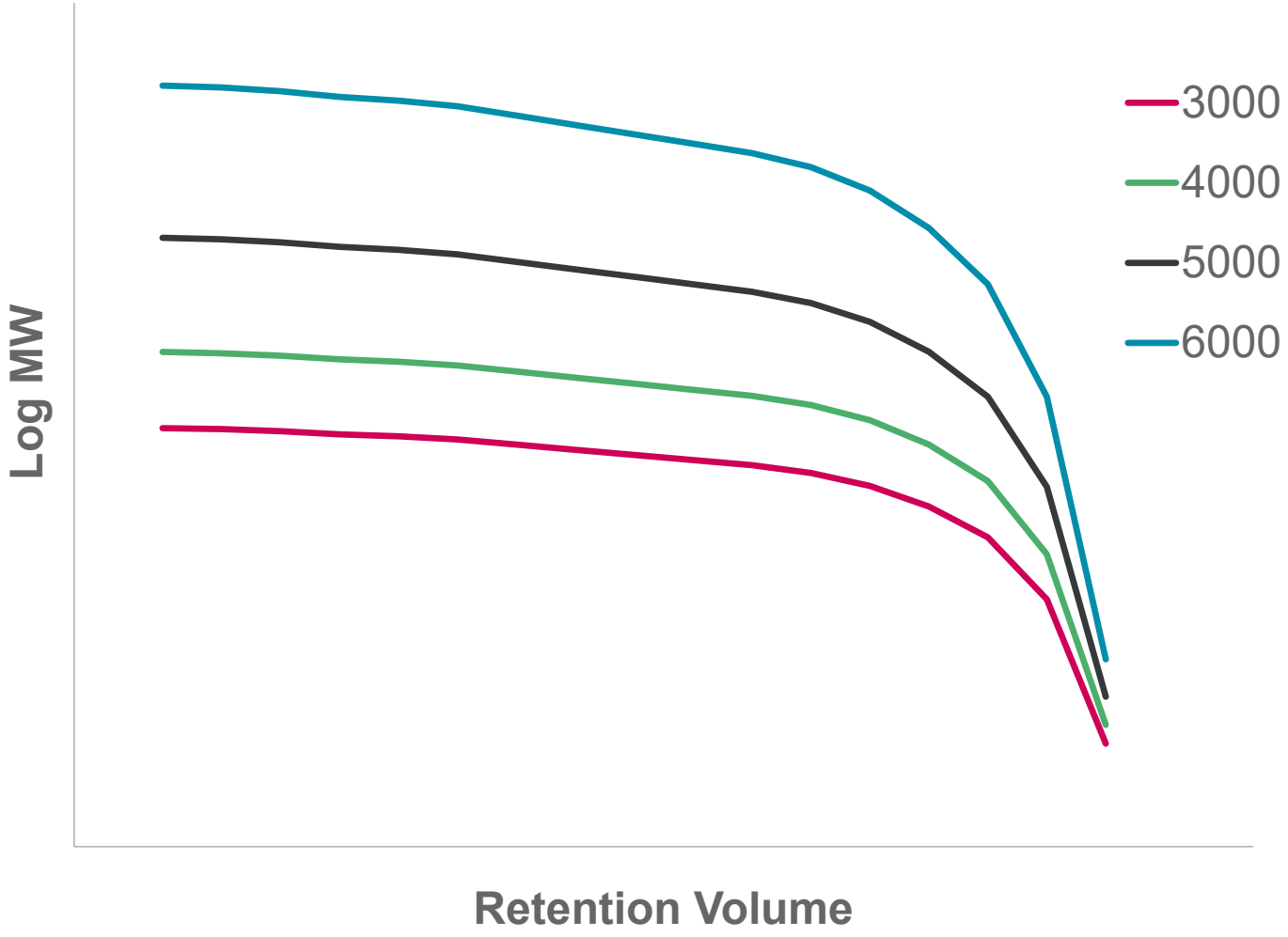
- T-columns
  - T-1000
  - T-2000
  - T-2500
  - T-3000
  - T-4000
  - T-5000
  - T-6000
  - T-7000
  - T-6000M
  - LT-3000L
  - LT-4000L
  - LT-5000L
  - LT-6000L
  - LT-7000L
- For applications using DMF, Chloroform and HFIP Malvern Panalytical sells T-columns packed in those solvents, designated as D-, C- and HFIP-columns, respectively.
- Aqueous (A-) and Protein (P-) columns follow same naming system

Types of GPC/SEC Columns

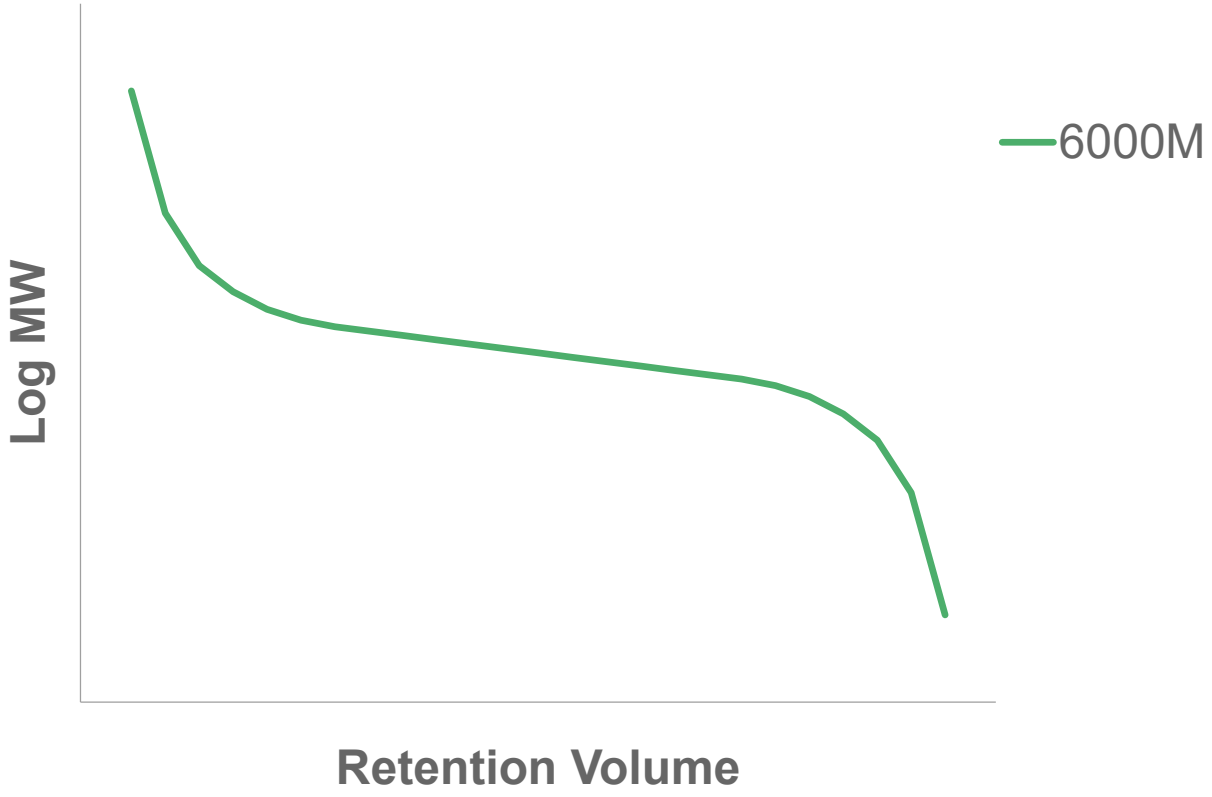
Choose the right column type for your application

- Single-Pore Size Columns
  - T-1000, T-2000, T-2500, T-3000, etc.
  - Contain one type of gel
  - Provide great resolution in limited MW range
- Mixed Bed Columns
  - T-6000M, LT-6000L, etc.
  - Contain a combination of gels with different pore sizes
  - Provide decent resolution over wider MW range
- These are general types of columns that exist in both organic and aqueous versions

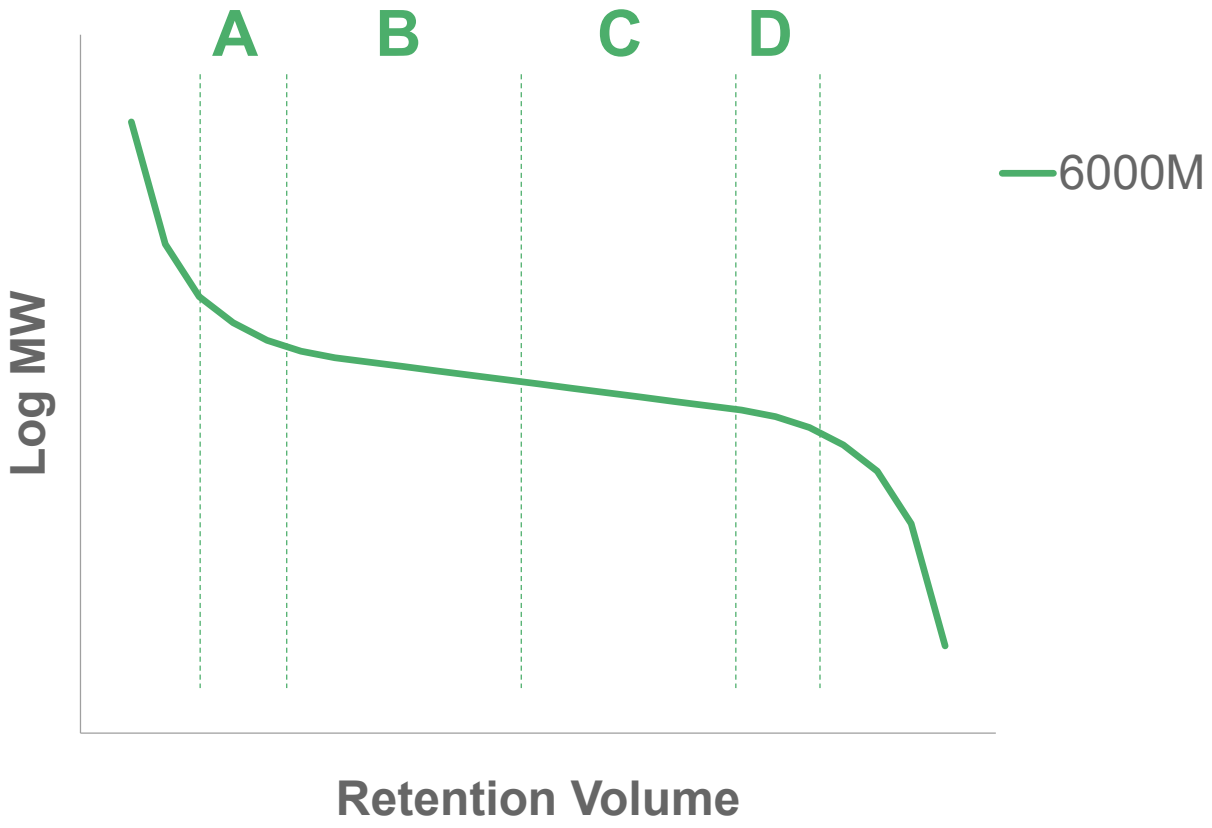
Single-Pore Size Columns



Mixed Bed Columns

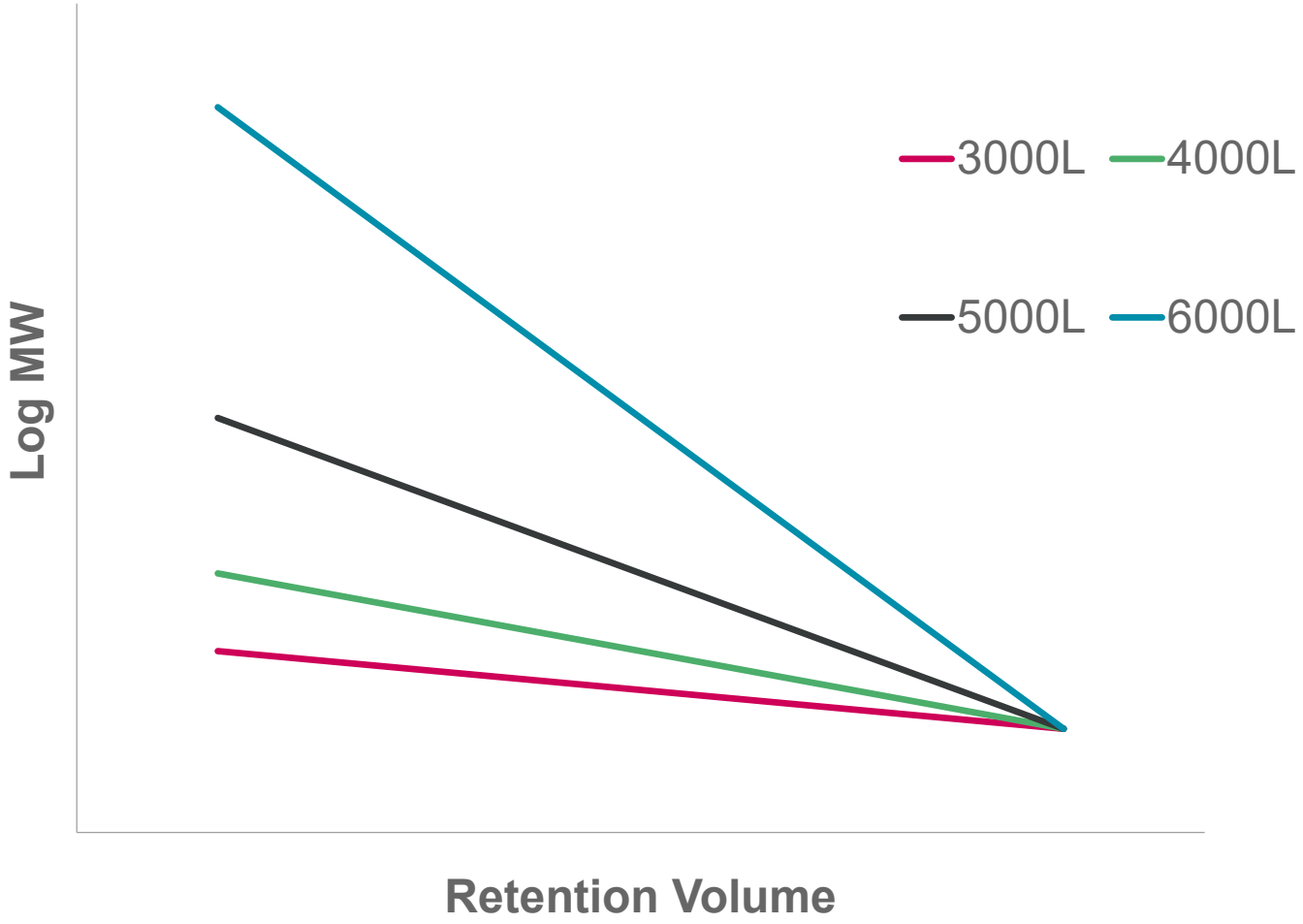


Mixed Bed Columns

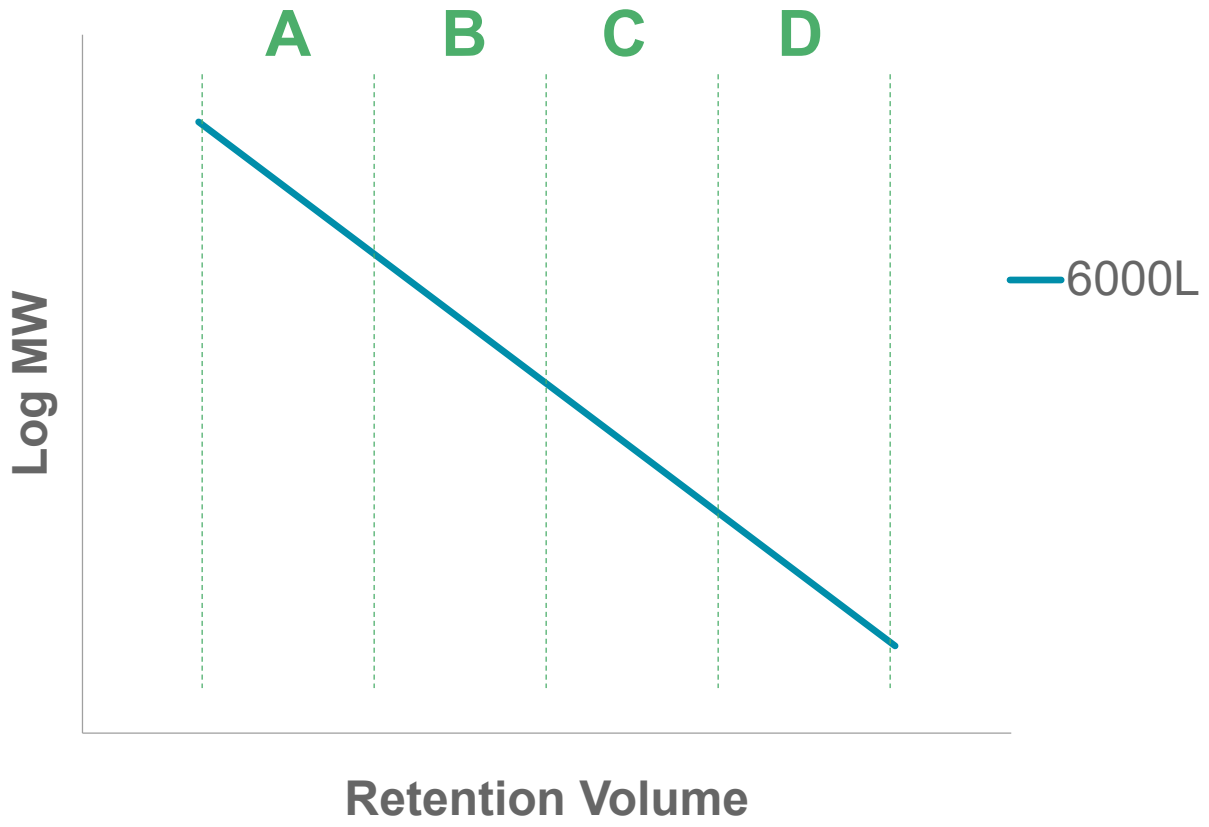


- Regions B & C are emphasized; provides higher resolution in the middle of the MW range, less resolution on extremes

Linear Mixed Bed Columns

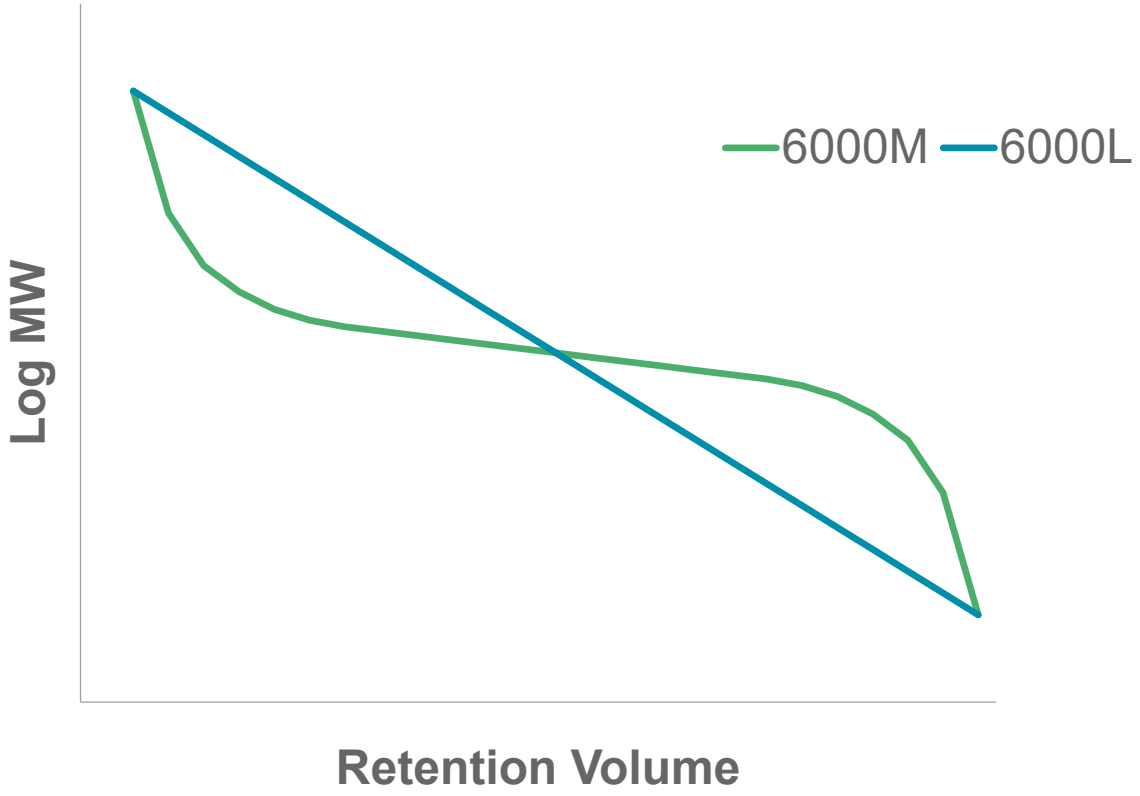


Linear Mixed Bed Columns



- Quantity of each gel is designed to provide a linear distribution over entire MW range; decent resolution over entire MW range

Mixed Bed vs. Linear Mixed Bed Columns

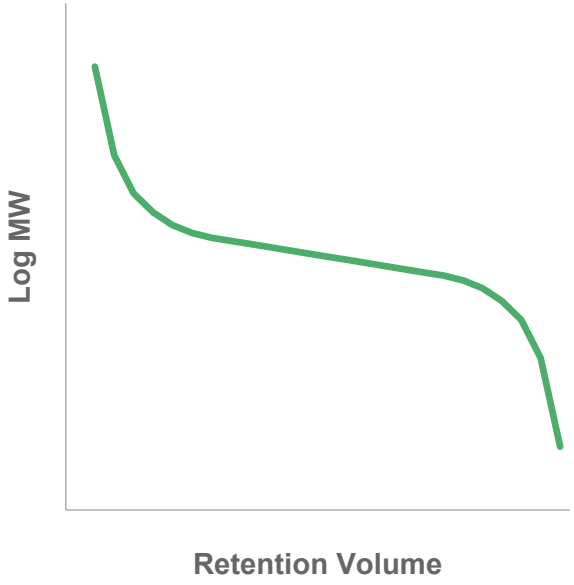


- If MW is unknown, 6000L will provide higher consistent resolution over entire MW range

Mixed Bed vs. Linear Mixed Bed Columns

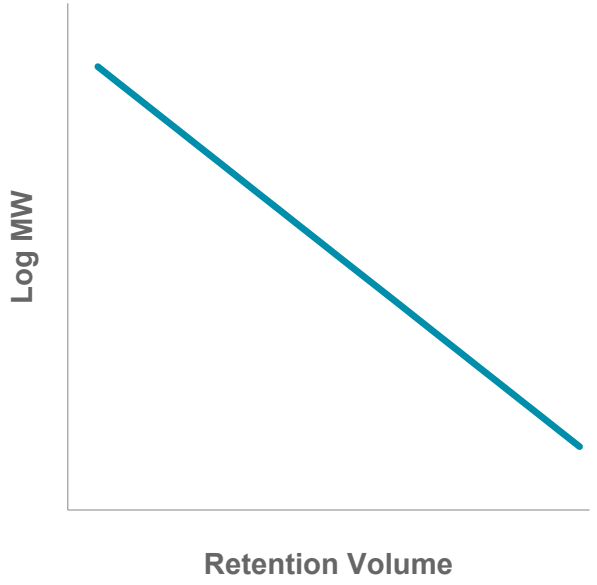
Mixed Bed (6000M)

- Contains all pore sizes
- Intermediate pore sizes emphasized for better local resolution
- Larger “sweet spot”



Linear Mixed Bed (6000L)

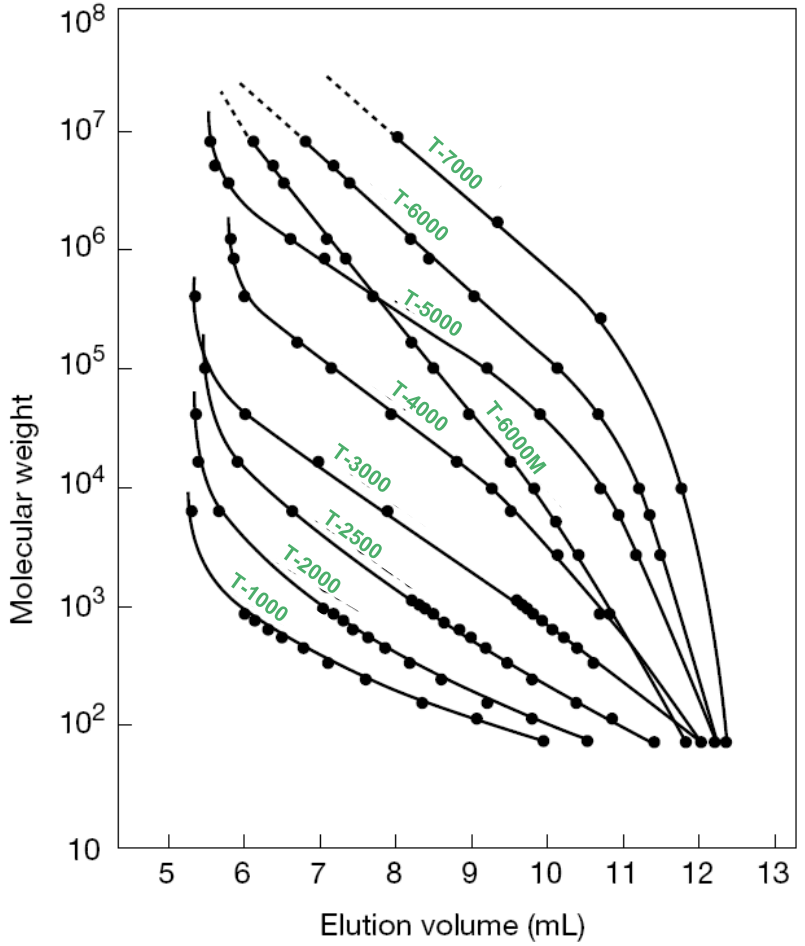
- Contains all pore sizes
- Quantity of each pore size designed to provide a linear distribution
- Provides consistent resolution across entire MW range



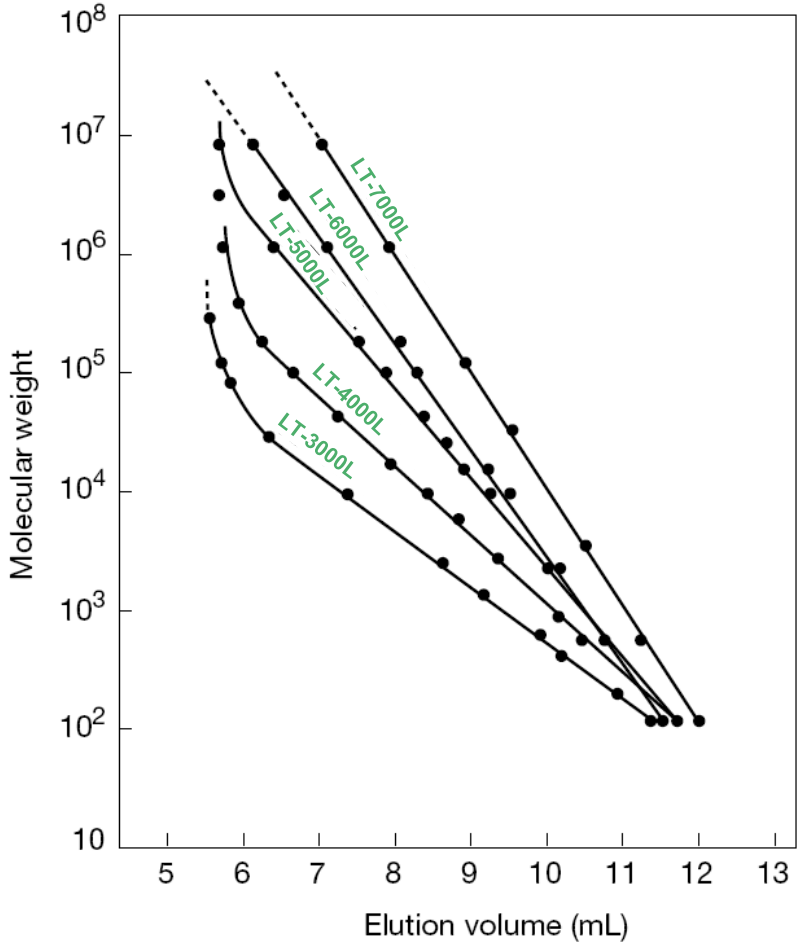
Examples of Calibration Curves

Malvern Panalytical
a spectris company

Single-Pore & Mixed Bed

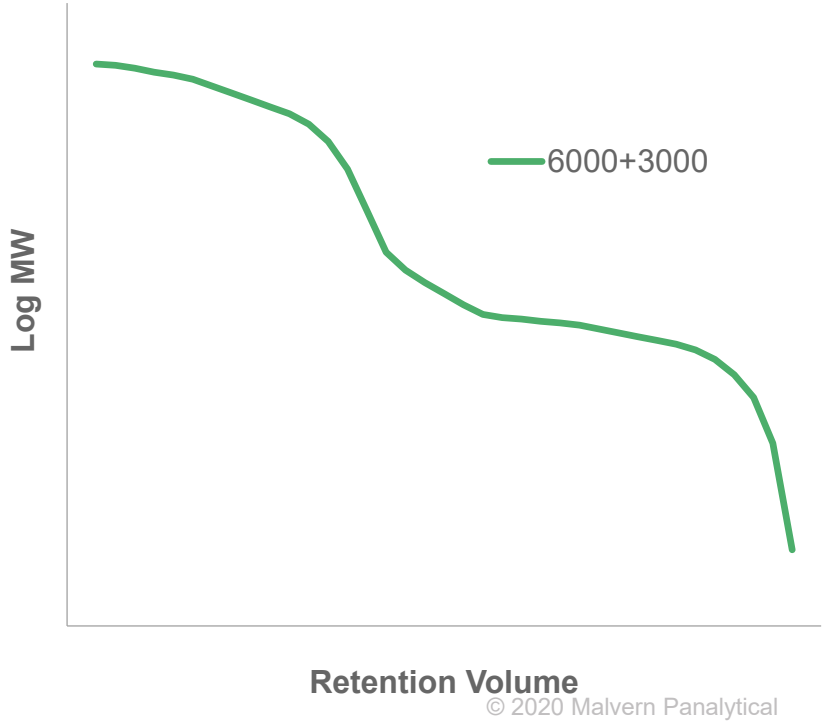


Linear Mixed Bed



Building a GPC/SEC Column Set

- Most column sets consist of 2 – 4 columns
- Ideally, multiple columns of the same type will be used
- If MW range of sample requires multiple column types, combine columns containing a mixture of pore sizes, such as the mixed bed or linear mixed bed columns
- If single-pore size columns are mixed, the resulting calibration curve may not be linear
- This can create artificial shoulders in sample peaks



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a spectris company

Conclusions

How can GPC/SEC & OMNISEC help you?

- GPC/SEC is the ideal technique for characterizing macromolecules
- Different combinations of detectors allow for different analysis methods
- Multi-detector systems offer complete characterization
- OMNISEC can be a multi-or single detector system
- Applications range from synthetic and natural polymers to proteins or other biomaterials
- Columns are designed to work with specific solvents, applications, and molecular size ranges

¡Gracias!

www.malvernpanalytical.com

MUCHAS GRACIAS!

www.cas-instrumental.com.ar

consultas@cas-instrumental.com.ar