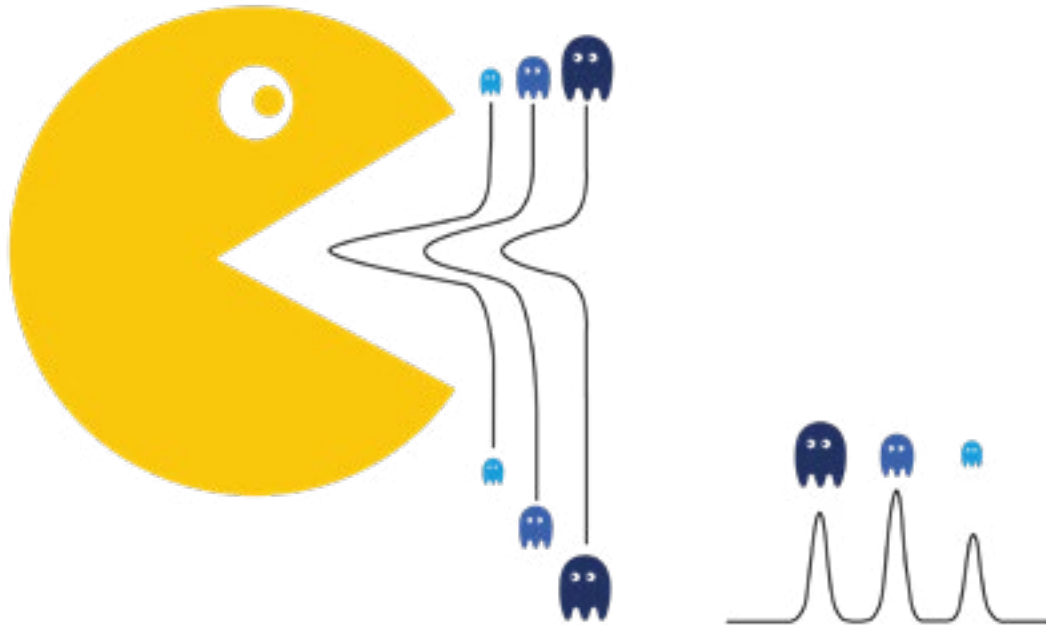


Dr. Maisch

Any Column, Any Size, Any Media



SEC Columns

for aqueous analysis of biomolecules

MADE BY DR. MAISCH

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**SEC COLUMNS
MADE BY DR. MAISCH**

From one of the biggest
**High-Performance Liquid Chromatography (HPLC) and
Ultra High-Performance Liquid Chromatography (UHPLC)**
Column Manufacturers in Europe.

Key features of ReproSil SEC columns

- **Stability**

Packing pressure of 7,000 psi (~480 bar) allows high flow rates, reduced equilibration times and faster analysis time.

- **High Efficiency**

Typically > 100,000 plates/m for 5 µm.

- **Flexibility**

From capillary to prep dimensions with different hardware options.

- **Recovery**

No evidence of protein loss. Low adsorption of basic proteins.

- **Price**

Reasonably priced SEC column series with excellent quality.

Using Size Exclusion Chromatography (SEC) means separating molecules based on their size by filtration through a gel. In the following we use the term "retention volume". Since no chemical modification (bonding) occurs on the surface of the silica particles, the separation of the molecules depends on the pore size. However the term "elution volume" would be more precise.

The gel consists of spherical beads containing pores of a specific size.

SEC is often divided by users in the following terms:

Gel Filtration Chromatography (GFC) for separations in aqueous solvents.

Gel Permeation Chromatography (GPC) for separations in organic solvents.

Recently, it was shown that ReproSil SEC columns can be used for the SFC separation and purification of Polyethylene Glycol (PEG) in analytical and prep scale. In this case the mobile phase was supercritical CO₂ (Dr. Maisch HPLC Application 0007).

The well-defined **pore size** is the key criteria of SEC columns. As the mobile phase flows over and through these particles it carries along its solutes. Solutes may flow into and out of the pores depending on size.

These molecules will be separated by differences in their sizes, or more precisely: by differences of their hydrodynamic volumes (Stokes-Einstein radius). The hydrodynamic volume is influenced by the shape of the molecules and their molecular weight.

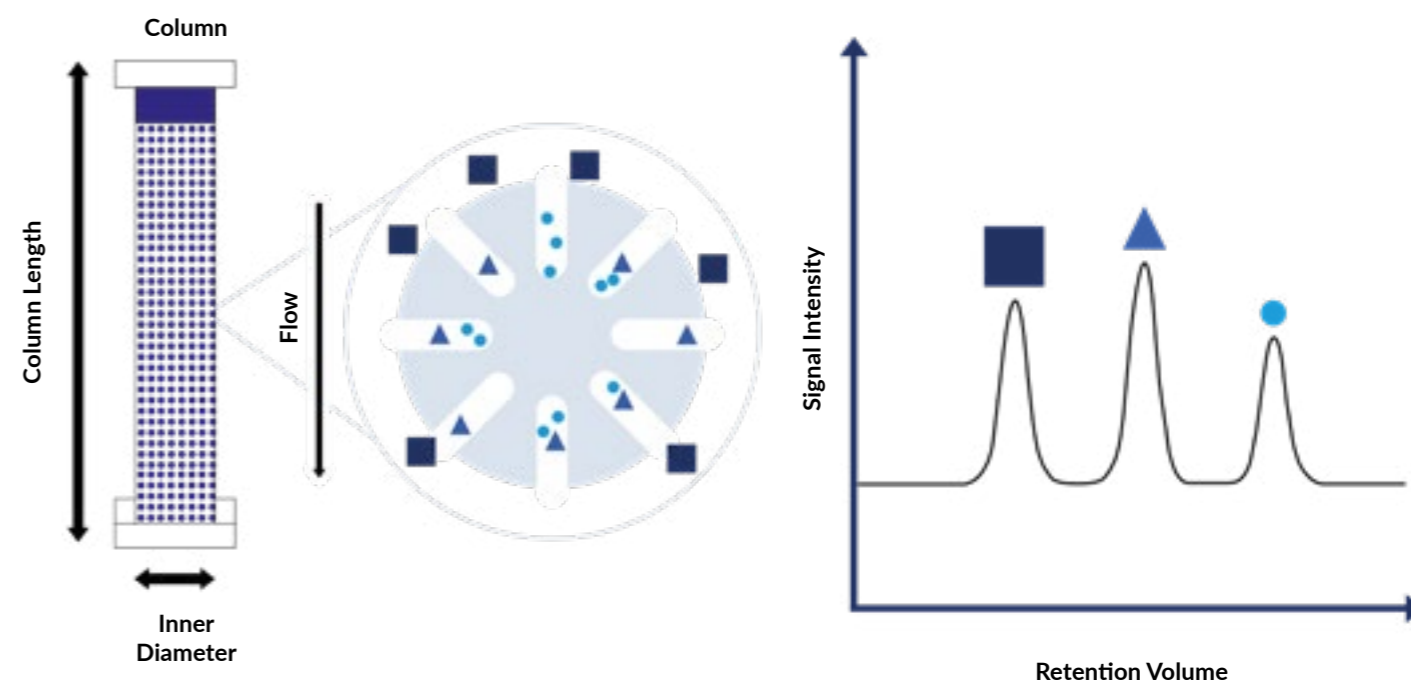


Figure 1: Illustration how molecules are separated on a SEC column relative to their hydrodynamic volume.

As a rule of thumb, the pores should be 2-3 times larger than the largest analyte. Molecules are separated based on their size as they pass through the column and are eluted in order of decreasing hydrodynamic volume.

Common types of separations performed by SEC are determination of the Molecular Weight (MW), impurity control (e.g. agglomeration), fractionation, desalting or buffer exchange.

Calibration curves of SEC columns are used to illustrate for which MW range a SEC column is suitable.

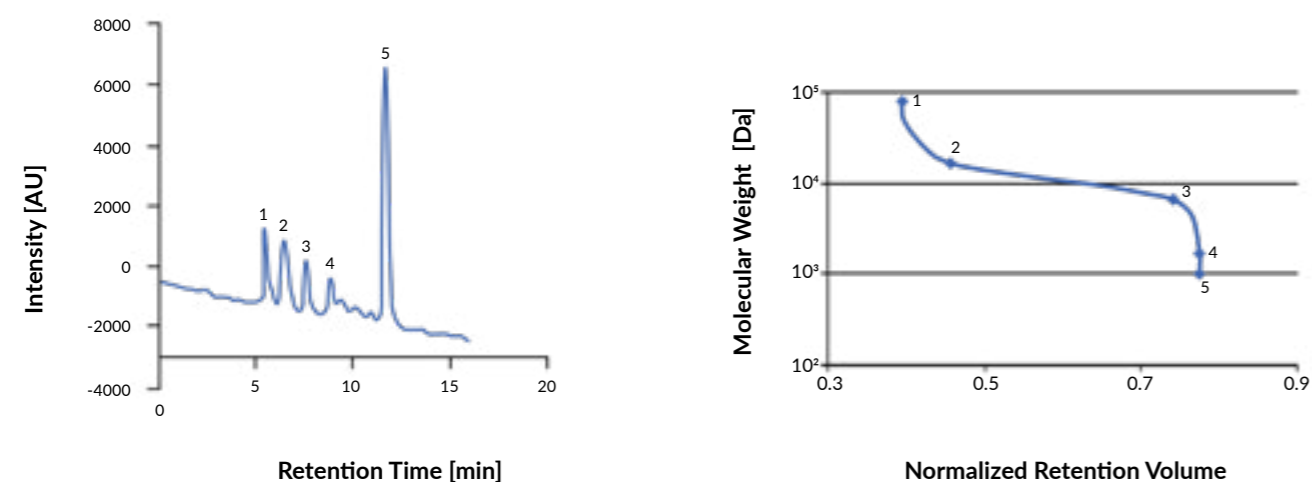


Figure 2: ReproSil SEC 200, 5 µm, 300 x 4.6 mm - Mobile Phase: 100 mM Na-Phosphate, pH 6.8, Flow Rate: 0.35 ml/min, Detection: UV 254 nm.

Analytes	Retention Time [min]	Molecular Weight [Da]
1. Thyroglobulin bovine	~12	670,000 Da
2. Gamma-Globuline	~8	150,000 Da
3. Albumine chicken egg	~7	44,300 Da
4. Ribonuclease A	~6	13,700 Da
5. 4-Aminobenzoic acid	~5	137 Da

In **Figure 2** molecular weight is plotted against the associated retention time (or normalized retention volume) for differently sized standards.

The resulting curves mostly have a **steep part** for very high exclusion volume and very low molecular weights. That means that there is no change in the retention time/volume in these regions and consequently no separation despite differences in the molecular weight.

The **shallow part** of the calibration curve shows a nearly linear correlation between the retention time¹ and the molecular weight. Thus, the column can be used for separating molecules in this MW range.

¹ General comment: Elution time or elution volume would be the correct term to use because there is no retention in SEC separations. But nowadays retention time or volume is used in most publications dealing with SEC. For easier understanding we use on the following pages in these brochure also retention.

The **calibration standards** should be identical or at least similar to the analyte:

- For protein analysis the calibration curve should be performed with proteins, (ideally with proteins of a similar shape or surface structure).
- For polysaccharide analysis the calibration curve should be performed with dextran.

Common **column dimensions** for SEC applications:

Table 1: Selection of dimension depending on type of analysis.

Type of Analysis	Length [mm]	Inner Diameter [mm]
HPLC	300	7.8 - 8.0
UHPLC	300	2.0 - 4.6
Fast Analysis High Throughput	≤ 150	≤ 4.6
Very Low Concentrated Samples	≤ 150	≤ 2.0
Prep Applications	300	≥ 20

Size exclusion separations benefit from long columns as the number of theoretical plates and the separation efficiency are directly proportional to the column length.

However, long columns also lead to long analysis times. Therefore, a compromise between run time and separation performance results in the optimal column length.

Low **inner diameters** of a SEC column reduce the solvent consumption, the total applicable injection volume and increases peak heights which is beneficial for low sample amounts.

The columns can also be used in **tandem** for increased resolution or as **coupled SEC columns** with different pore sizes. The limiting factor will be the allowed maximum back pressure of the LC system / LC column.

Many biomolecules (e.g. proteins, peptides, oligonucleotides etc.) show secondary interactions that have a negative impact on the performance of the chromatographic separation and recovery of these molecules.

SIZE EXCLUSION CHROMATOGRAPHY (SEC)

To avoid such undesired interactions Dr. Maisch offers 2 **bioinert hardware** options to the standard stainless steel hardware:

1. Polyether Ether Ketone (PEEK) hardware.
2. Bioinert coated hardware (surface coating on the column body and frit).

Bioinert hardware is preferred and recommended for challenging substances such as oligonucleotides, phospholipids and metal-chelating small molecules.

The higher cost of this hardware is justified by improved peak shape, sensitivity and reproducibility. Sample carry-over is eliminated and sample-conditioning is not necessary anymore.

GFC columns from Dr. Maisch are offered with 2 different **base materials**:

Table 2: Base materials for GFC columns from Dr. Maisch.

Product Name	Base Material	Advantages	Disadvantages	Applications
ReproSil-SEC	Silica	High pressure stability. High flow rates. Highest efficiency & resolution. < 2 µm porous particles available.	pH stability: 2-7	mAbs proteins peptides at physiological pH
Repromer-OH	Polymethacrylate	High pH stability.	Secondary interactions (Hydrophobicity)	SEC at high pH

The particle size impacts separation efficiency: smaller particles are more efficient (better resolution) due to a smaller mass transfer resistance than larger particles.

However, a small particle size increases the column backpressure. Sub-2 µm columns require UHPLC instrumentation (LC system back pressures up to 1000 bar).

GEL FILTRATION CHROMATOGRAPHY (GFC) COLUMNS FOR AQUEOUS CONDITIONS – REPROSIL SEC COLUMNS

Dr. Maisch offers a variety of pore sizes (50 Å – 800 Å) for SEC separations which allow separations in the range from 500 Da – 1,250,000 Da.

Silica-based SEC columns are modified by a hydrophilic surface bonding in order to minimize electrostatic interactions of positively charged moieties on proteins and other analytes.

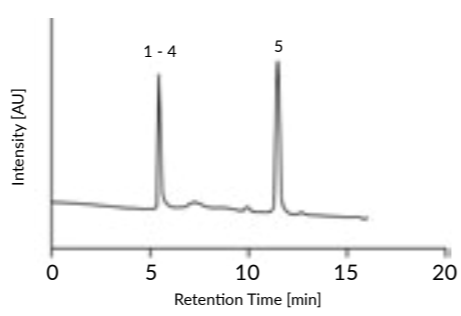
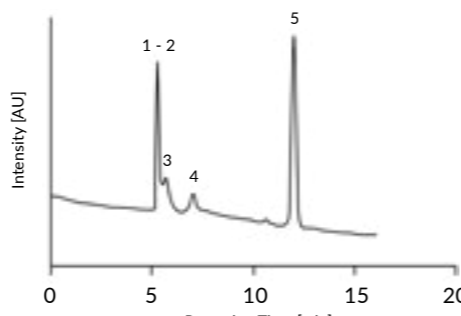
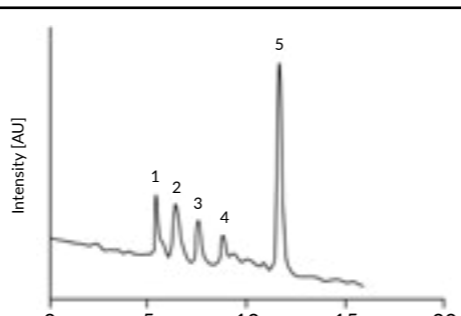
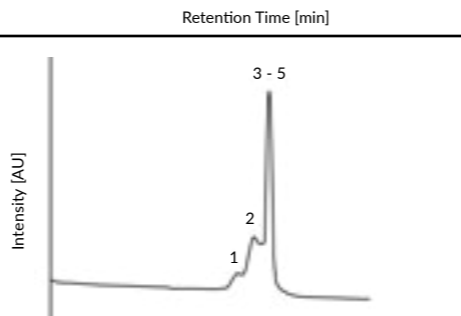
Dr. Maisch offers two different SEC column versions (PEG and Diol) that results in significantly different separations.

Table 3: Silica-based GFC media from Dr. Maisch.

Product Name	Pore Size [Å]	Hydrophilic Modification	MW Range [Da] ¹		Part Number (PN)			
			Minimum	Maximum	1.9 µm	3 µm	5 µm	10 µm
ReproSil 50 SEC	50	DIOL	500	10,000	N/A	N/A	r05.sec	r00.sec
ReproSil 125 SEC	125	DIOL	5000	100,000	N/A	r13.sec	r15.sec	r10.sec
ReproSil 200 SEC	250	PEG	10,000	500,000	r219.sec	on request	r25.sec	on request
ReproSil 200 SEC-2	200	DIOL	10,000	500,000	r219.sec2	r23.sec2	r25.sec2	r20.sec2
ReproSil 300 SEC	300	PEG	10,000	1,000,000	N/A	r33.sec	r35.sec	on request
ReproSil 400 SEC	400	PEG	20,000	500,000	N/A	N/A	r45.sec	on request
ReproSil 500 SEC	800	PEG	150,000	1,250,000	N/A	N/A	r55.sec	on request

1) MW range is based on "linear" molecules.

Table 4: Influence of pore size on SEC separation of a standard protein-mix.

Product Name	Pore Size [Å]	Chromatogram
ReproSil SEC 50	50	
ReproSil SEC 100	100	
ReproSil SEC 200	250	
ReproSil SEC 5000	800	

Analytes	1.	Thyroglobulin bovine	670,000 Da	Column Dimension:	300 x 4.6 mm
	2.	Gamma-Globuline	150,000 Da	Particle Size:	5 µm
	3.	Albumine chicken egg	44,300 Da	Mobile Phase:	100 mM Na-Phosphate pH 6.8
	4.	Ribonuclease A	13,700 Da	Flow Rate:	0.35 ml/min
	5.	4-Aminobenzoic acid	137 Da	Detection:	UV 254 nm

ReproSil 50 SEC

Suitable for SEC analysis of very small peptides and proteins (MW: 500 Da – 10,000 Da).

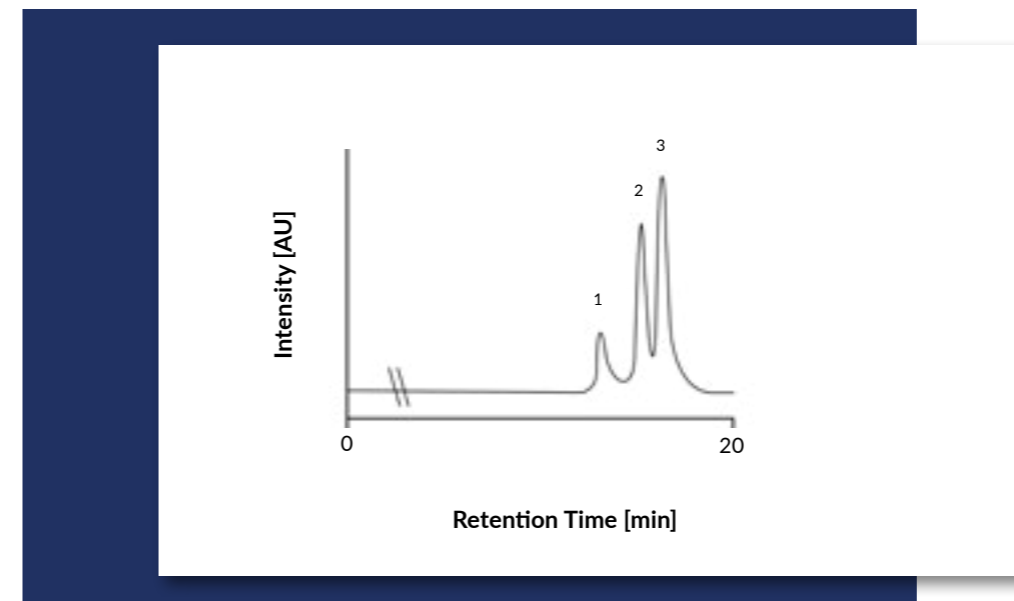


Figure 3: ReproSil SEC 50, 5 µm, 300 x 8 mm (PN: r05.sec.s3008). Eluent: 50 mM KH₂PO₄ + 100 mM KCl (pH 6.5), Flow Rate: 0.5 ml/min, Sample: Peptide-Standard: 1. Peptide MW 3.894 Da, 2. Peptide MW 1.593 Da, 3. Peptide MW 826 Da.

ReproSil 125 SEC

Suitable for SEC analysis of intermediate proteins (MW: 5,000 Da – 100,000 Da).

GEL FILTRATION CHROMATOGRAPHY (GFC) COLUMNS FOR AQUEOUS CONDITIONS – REPROSIL SEC COLUMNS

GEL FILTRATION CHROMATOGRAPHY (GFC) COLUMNS FOR AQUEOUS CONDITIONS – REPROSIL SEC COLUMNS

ReproSil 200 SEC & ReproSil 200 SEC-2

Suitable for SEC analysis of larger proteins (MW: 10,000 Da – 500,000 Da).

Most popular pore size in SEC for analysis of large biomolecules with two different hydrophilic modifications (200 SEC = PEG and 200 SEC-2 = Diol) available.

Sub-2 μm media available for use in UHPLC-SEC-applications.

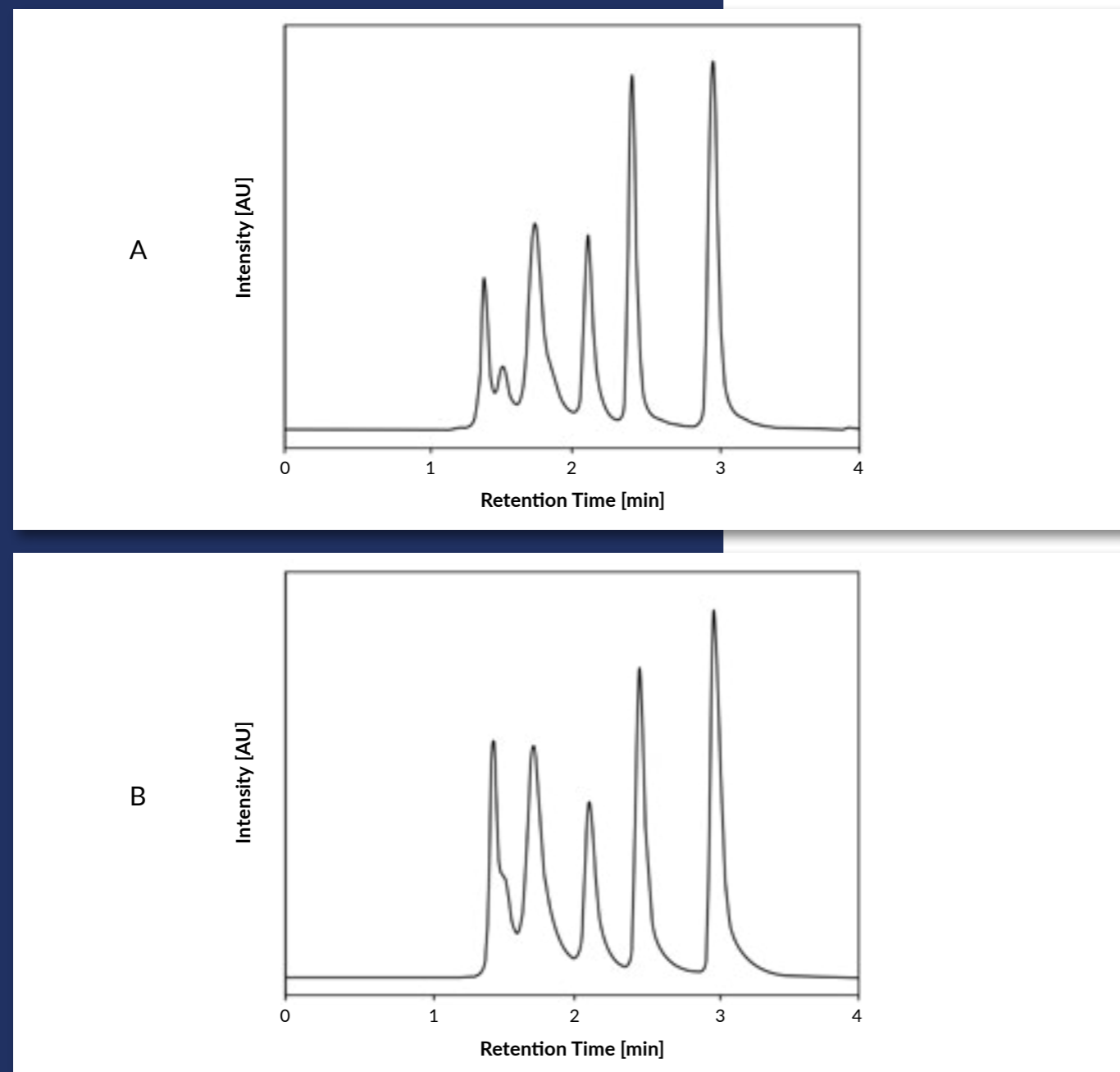


Figure 4: Comparison of 75 x 4.6 mm UHPLC-SEC columns. Both packed by Dr. Maisch.
 A: ACQUITY¹ UHPLC Protein BEH SEC column, 200 Å, 1.7 μm , 75 x 4.6 mm.
 B: ReproSil 200 SEC-2, 1.9 μm , 75 x 4.6 mm (r219.sec2.s0746).

ReproSil 300 SEC, ReproSil 4000 SEC, ReproSil 5000 SEC

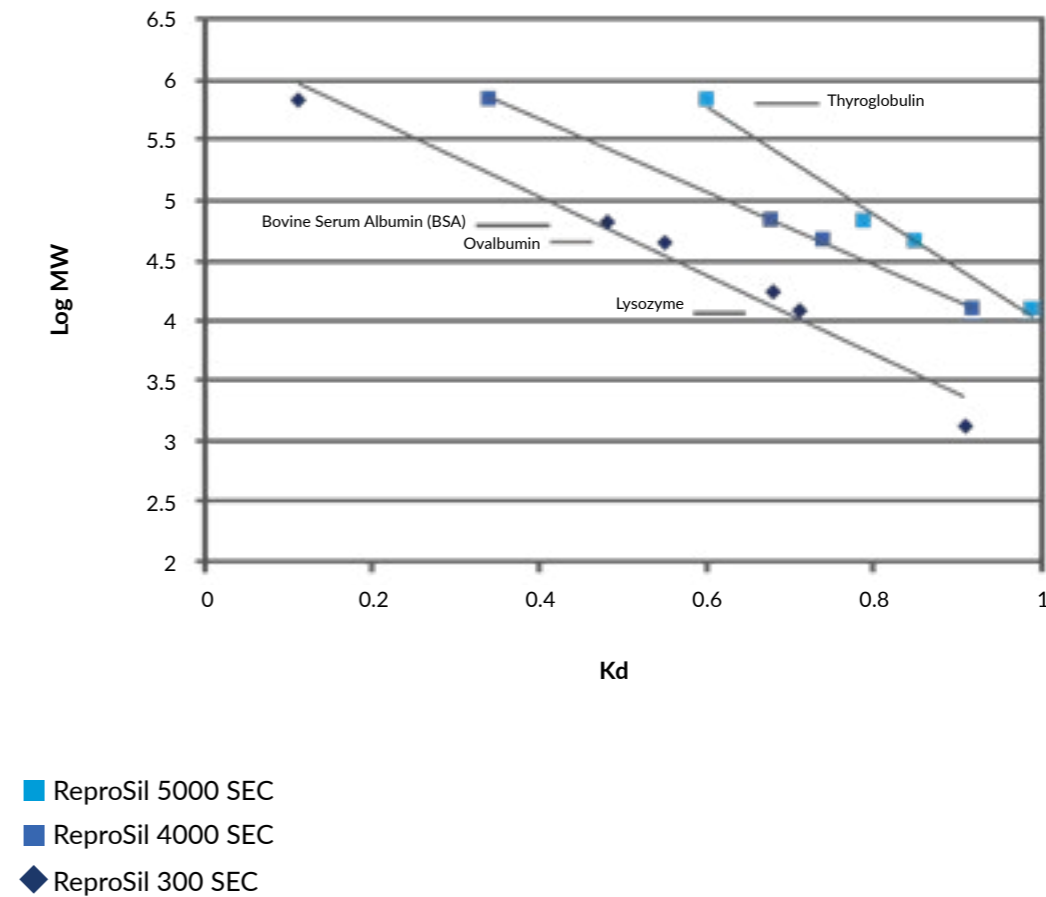


Figure 5: Gel Filtration Chromatography (GFC) of proteins shows that the ReproSil 5000 SEC phase shifts the retention of proteins to higher Kd values compared to the ReproSil 300 SEC and ReproSil 4000 SEC.

1) ACQUITY is a registered trademark® of Waters Technologies Corporation.

GEL FILTRATION CHROMATOGRAPHY (GFC) COLUMNS FOR AQUEOUS CONDITIONS – REPROSIL SEC COLUMNS

GEL FILTRATION CHROMATOGRAPHY (GFC) COLUMNS FOR AQUEOUS CONDITIONS – REPROSIL SEC COLUMNS

ReproSil 300 SEC

Suitable for SEC analysis of large proteins (MW: 10,000 Da– 1,000,000 Da).

Excellent TSK G3000SWxl¹ alternative.

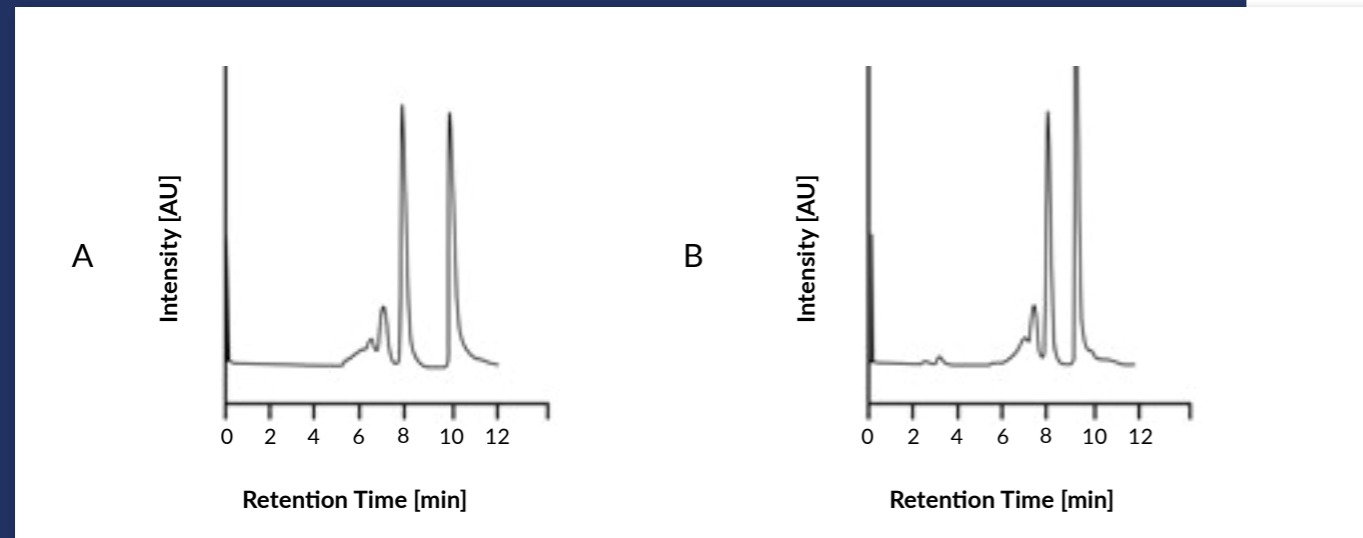


Figure 6: Separation of Bovine Serum Albumin and Cytochrome C on A) 300 x 7.8 mm TSK G3000SWxl¹ and B) 300 x 8 mm Reprosil SEC 300 columns using 50mM Potassium Phosphate pH 7/150 mM Sodium Chloride eluent 1.0 ml/min.

ReproSil 5000 SEC

High performance size exclusion separation of very high molecular weight proteins.

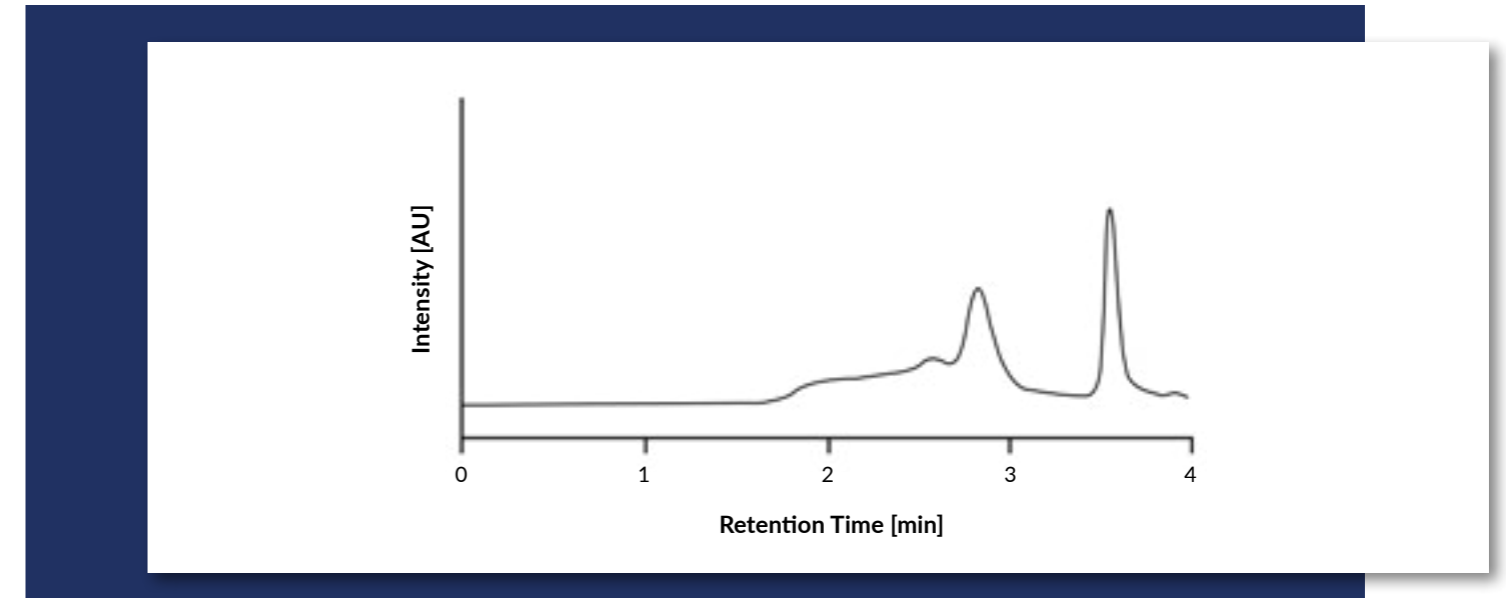


Figure 8: Separation of Thyroglobulin and Riboflavin on 250 x 4.6 mm Reprosil SEC 5000 at 1 ml/min using 50 mM Potassium Phosphate pH 7 /150 mM Sodium Chloride eluent.

ReproSil 4000 SEC

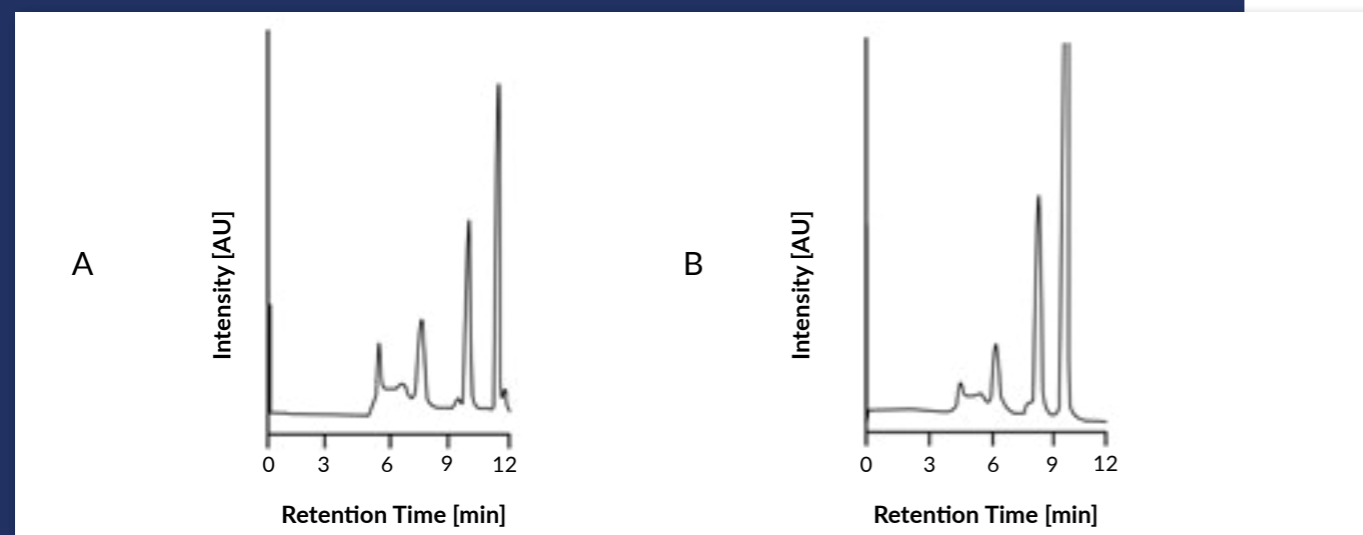


Figure 7: Separation of Thyroglobulin, Ovalbumin and Phenylalanine on Reprosil 4000 SEC columns: A) 300 x 8 mm and B) 250 x 4.6 mm using 50 mM Potassium Phosphate pH 7/150 mM Sodium Chloride eluent 1 ml/min (8 mm) and 0.33 ml/min (4.6 mm).

1) TSKgel is a registered trademark® of Tosoh Corporation.

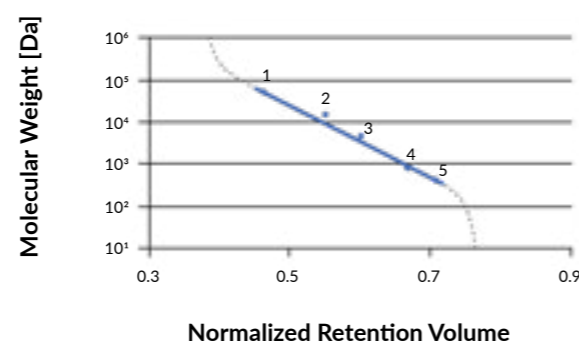
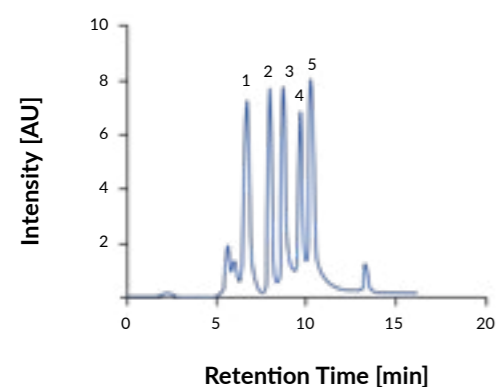
The **eluent plays** an important role in GFC separations. Proper selection of eluting conditions is necessary to maximize the molecular sieving mechanism and to minimize secondary effects, such as ionic and hydrophobic interactions between the sample and the stationary phase.

To avoid ionic interactions the **choice of the pH** is supposed to be close to the isoelectric point and salt will be added traditionally at high concentrations. Based on experience, these salts are often phosphate and/or sodium chloride buffers which are not compatible with LC/MS.

For modern SEC, when **ESI-LC/MS** is required, volatile buffers are used at lower concentrations. Additionally, the use of Acetonitrile (ACN) will reduce the dissociation and will improve the spray in the interface. Since acetonitrile acts as a **denaturing agent**, it should be kept at concentrations below 30%. In case of **denaturation** the exclusion limits for proteins become smaller since they lose their folded three-dimensional structure.

The choice of buffer and buffer concentration has a huge influence on the performance of the separation.

Phosphate Buffer



Acetate Buffer

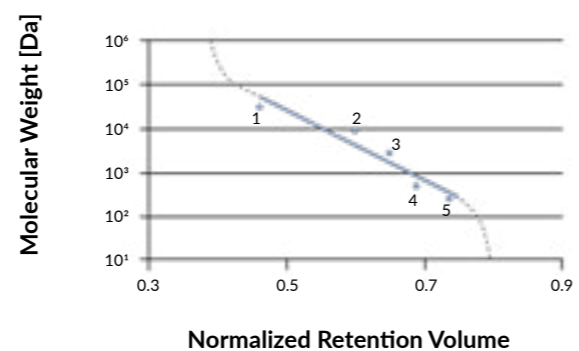
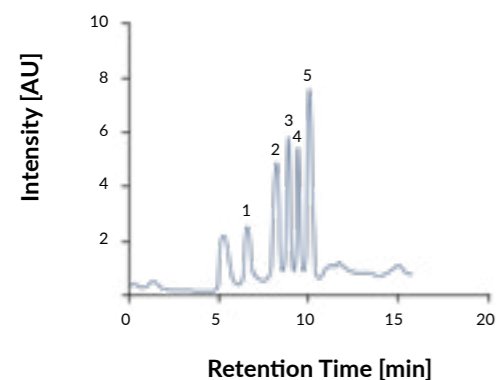


Figure 9: Modern SEC Separation, LC/MS compatible. ReproSil SEC 125, 5 μm , 250 x 4.6 mm. Mobile Phase: 25 mM NH_4 -Acetate / ACN = 80:20 v/v.

Analytes		
1.	Ovalbumin	45 kDa
2.	Myoglobin	17 kDa
3.	Aprotinin	6.7 kDa
4.	Neurotensin	1.7 kDa
5.	Angiotensin	1 kDa

Table 5: Ordering Information for ReproSil SEC columns.

ReproSil SEC	Particle Size [μm]	Dimension [mm]	Part Number (PN)	
ReproSil 50 SEC	5	300 x 4.6	r05.sec.s3046	
	5	300 x 8	r05.sec.s3008	
	Guard, 5	20 x 8	r05.sec.s0208	
ReproSil 125 SEC	3	300 x 4.6	r13.sec.s3046	
	5	300 x 4.6	r15.sec.s3046	
	3	300 x 8	r13.sec.s3008	
	5	300 x 8	r15.sec.s3008	
	Guard, 5	20 x 8	r15.sec.s0208	
	10	300 x 8	r10.sec.s3008	
	Guard, 10	20 x 8	r10.sec.s0208	
ReproSil 200 SEC	1.9	300 x 4.6	r219.sec.s3046	
	5	300 x 4.6	r25.sec.s3046	
	5	300 x 8	r25.sec.s3008	
Guard, 5		20 x 8	r25.sec.s0208	
	ReproSil 200 SEC-2	1.9	300 x 4.6	r219.sec2.s3046
		3	300 x 4.6	r23.sec2.s3046
3		300 x 8	r23.sec2.s3008	
5		300 x 4.6	r25.sec2.s3046	
5		300 x 8	r25.sec2.s3008	
Guard, 5		20 x 8	r25.sec2.s0208	
10		300 x 8	r20.sec2.s3008	
Guard, 10		20 x 8	r20.sec2.s0208	
ReproSil 300 SEC		5	300 x 4.6	r35.sec.s3046
		3	300 x 4.6	r33.sec.s3046
	5	300 x 8	r35.sec.s3008	
	3	300 x 8	r33.sec.s3008	
Guard, 5		20 x 8	r35.sec.s0208	
	ReproSil 4000 SEC	5	300 x 4.6	r45.sec.s3046
5		300 x 8	r45.sec.s3008	
Guard, 5		20 x 8	r45.sec.s0208	
ReproSil 5000 SEC	5	300 x 4.6	r55.sec.s3046	
	5	300 x 8	r55.sec.s3008	
	Guard, 5	20 x 8	r55.sec.s0208	

NOTE: Capillary (50 - 800 μm ID) and prep dimensions available on request.

Table 6: Column Care Information for ReproSil SEC columns.

ReproSil SEC	300 x 4.6 mm	300 x 8 mm
Sample Load	Max.: 100 µg Optimal: 10 - 40 µg	Max.: 200 - 300 µg Optimal: 20 - 100 µg sample
Injection Volume	< 10 µl	1-20 µl
Flow Rate (normal)	0.4 ml/min	1 ml/min
Temperature	Max.: 50 °C	
Flow Rate	Max.: 1.5 ml/min (if back pressure < packing pressure).	
pH-range	2.5 - 7.5	
Back Pressure	Column pressure must not exceed the maximum packing pressure (see QC sheet).	
Organic Modifier	Up to 100% ACN 10% DMSO 500 mM-mercaptoethanol	
Buffer Concentration	High ionic strength buffers is recommended for most protein applications. A neutral salt is often added to increase ionic strength. Maximum salt concentration: 1 M.	
Detergents	<p>In general: detergents stick on the SEC-media surface affecting column lifetime and the future use of the column.</p> <p>If SEC method requires denaturing conditions (SDS) or formulation detergents (Tween 20, Triton): ReproSil SEC columns can be operated under these conditions but with reduced life time. Use of guard columns is recommended.</p> <p>SEC columns that were in "contact" with denaturing reagents should be used later on ONLY with detergent containing eluents.</p>	
Arginine	<p>In very rare cases proteins bind to the SEC media and lead to a compromised protein elution and peak resolution which results in incorrect determination of the size of the eluted proteins and the amount of the aggregated species.</p> <p>In such cases arginine (e.g. 0.2 M Arginine-HCl) can be added to the mobile phase instead. This mobile phase reduces non-specific protein binding while not affecting the protein structure.</p>	
Cleaning Procedure	General Protein Removal.	Wash with 30 ml of 0.1 M NaH ₂ PO ₄ , pH 3.0.
	Hydrophobic Compounds.	Use acetonitrile gradient.
	Charged Contaminants.	0.5 mol/l salt (Sodium chloride or sodium sulfate).
	Strongly Absorbed Proteins.	Wash with 6 M guanidine thiocyanate or 10% DMSO.
Column Storage	Overnight: run mobile phase at 0.2 ml/min. Prolonged Storage: use 0.05% sodium azide in water or 20% methanol in water.	

Table 7: Cross Reference Chart for SEC columns.

Pore Size [Å]	Dr. Maisch	TSK	Phenomenex	Waters	Agilent	Sepax	YMC
50	ReproSil 50 SEC	N/A	N/A	N/A	N/A	N/A	YMC-Pack Diol-60
125	ReproSil 125 SEC	TSKgel G2000SW TSKgel G2000SWxl	BioSep SEC-s2000 Yarra 2000	BioSuite Diol (OH) 125Å	ZORBAX GF-250	SRT SEC-100 SRT SEC-150	YMC-Pack Diol-120
250	ReproSil 200 SEC	TSKgel G3000SW TSKgel G3000SWxl	BioSep SEC-s3000 Yarra 3000 Biozen dSEC-2 ¹	N/A	ZORBAX GF-450	SRT SEC-300	YMC-Pack Diol-200
200	ReproSil 200 SEC-2	TSKgel G3000SW TSKgel G3000SWxl	BioSep SEC-s3000 Yarra 3000 Biozen dSEC-2 ¹	BioSuite Diol (OH) 250Å	N/A	N/A	YMC-Pack Diol-200
300	ReproSil 300 SEC	TSKgel G3000SW TSKgel G3000SWxl	BioSep SEC-s3000 Yarra 3000 Biozen dSEC-3 ¹	N/A	ZORBAX GF-450	SRT SEC-300	YMC-Pack Diol-300
400	ReproSil 4000 SEC	TSKgel G4000SW TSKgel G4000SWxl	BioSep SEC-s4000 Yarra 4000	BioSuite Diol (OH) 450Å	N/A	SRT SEC-500	N/A
800	ReproSil 5000 SEC	N/A	Biozen dSEC-7 ¹	N/A	N/A	N/A	N/A

1) Require bioinert hardware.

Key features of Repromer OH columns

- Performance**

4 different pore sizes for the range from <20,000-1,000,000 Da.
 Large pore volume for high resolution.
 Low column bleeding for low detector noise.

- Flexibility**

From capillary to prep dimensions with different hardware options.

- Price**

Reasonably priced SEC column series with excellent quality.

The Repromer OH (SEC) series is based on methacrylate and is suitable for aqueous size exclusion chromatography of neutral or anionic polymers.

Typical samples are neutral and anionic polymers (Polyethylene oxide, polyethylene glycol, pullulan, hyaluronic acid, polyacrylic acid, dextran sulfates, heparin, pectin, polyvinyl alcohol, etc).

Table 9: Ordering Information for Repromer OH SEC columns.

Product Name	Exclusion Limit [Da]	Dimension [mm]	Part Number (PN)
Repromer OH-2500	< 20,000	300 x 8	rm0.oh25.s3008
		250 x 8	rm0.oh25.s2508
		30 x 8	rm0.oh25.s0308
Repromer OH-3000 (= Repromer Aqua 100 OH, rm10.aqua.)	< 80,000	300 x 8	rm0.oh3.s3008
		250 x 8	rm0.oh3.s2508
		30 x 8	rm0.oh3.s0308
Repromer OH-4000	1,000 - 300,000	300 x 8	rm0.oh4.s3008
		250 x 8	rm0.oh4.s2508
		30 x 8	rm0.oh4.s0308
Repromer OH-5000	2,500 - 1,000,000	300 x 8	rm0.oh5.s3008
		250 x 8	rm0.oh5.s2508
		30 x 8	rm0.oh5.s0308

NOTE: Capillary (50 - 800 µm ID) and prep dimensions available on request.

Table 8: Polymer-based media from Dr. Maisch SEC.

Product Name	Min. Molecular Weight [Da]	Max. Molecular Weight [Da]	Part Number (PN) for 10 µm Media
Repromer OH-2500	N/A	<20,000	rm0.oh25
Repromer OH-3000	N/A	<80,000	rm0.oh3
Repromer OH-4000	1,000	300,000	rm0.oh4
Repromer OH-5000	2,500	1,000,000	rm0.oh5

Table 10: Column Care Information for Repromer OH SEC column 300 x 8 mm.

Repromer OH	Dimension: 300 x 8 mm	
Injection Volume	10-20 µl (typical)	
Flow Rate	Max. 1 ml/min (if back pressure < packing pressure)	
Temperature	Max.: 80 °C	
pH-Range	2 - 10	
Max Back Pressure	50 - 80 bar	
Mobile Phase	Water with salts / buffers. Max 0.5 M buffer concentration.	
Organic Modifier	MeOH, ACN if necessary (<70%).	
Storage	For overnight, pump water at 0.2 ml/min. Longer storage: use 0.025% NaN ₃ in water or 10% methanol in water.	
Avoid	Freezing and drying.	
Column Cleaning	General.	Up to 70% ACN for cleaning procedure. Max. 45 bar during cleaning procedure.
	Strongly Absorbed Proteins.	0.5% SDS or 6 M guanidine thiocyanate.
Column Coupling	First: small-pore column. Second: big-pore column.	

Table 11: Cross Reference Chart for SEC columns.

MW Range	Dr. Maisch	TSK	Pheno-menex	Waters	Agilent	PSS (Agilent)	Shodex	Tessek
Very Low	Repromer OH-2000	TSKgel G1000PW	Polysep GFC-P2000	N/A	PL aquagel-OH 20	N/A	OHpak SB-802	N/A
Low	Repromer OH-3000	TSKgel G3000PW	Polysep-GFC-P3000	Ultrahydrogel 120	PL aquagel-OH 30	Suprema 30A	OHpak SB-802.5	HEMA-BIO 40
Middle	Repromer OH-4000	TSKgel G4000PW TSKgel G4000PWxl	Polysep-GFC-P3000 Polysep-GFC-P4000	Ultrahydrogel 250	PL-aquagel OH-40	Suprema 100A	OHpak SB-803	HEMA-BIO 100
Large	Repromer OH-5000	TSKgel G5000PWxl	Polysep GFC-4000 Polysep GFC-P5000	Ultrahydrogel 500 Ultrahydrogel 1000	PL-aquagel OH-50	Suprema 300A / Suprema 1000A	OHpak SB-804	HEMA-BIO 300

Dr. Maisch

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