There is More to HPLC Than Reverse Phase

Column choices: Have you thought about the rest?

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Application Engineer
December 12, 2019

Why Not to Use Reverse Phase

Some reasons for trying an alternative

C18 columns are a very common ‘go to’ column for HPLC methods and may be a suitable column choice for simple methods. **But** the C18 chemistry may not be the optimal choice.

For analysis of polar analytes, it is typically suggested that these sample types can be better separated on chemistries that that have a greater polarity than C18.

Reasons to **try** another chemistry:

- Too much retention or selectivity with C18 for desired analysis time.
- Polar analytes are not well retained with low or no organic modifier.
- Polar analytes not well resolved even if retained.
- A C18 method already in use is not rugged enough (revalidate).

- Screening different column chemistries is commonly advised when sample mixtures are complex.

Why is Changing the Bonded Phase Effective?

- Differences in interactions between polar and nonpolar compounds
- Other types of interactions with a bonded phase can be exploited (for example, pi-pi interactions)
- These all change with the bonded phase
- Changing the bonded phase can improve selectivity/resolution
- Reduce analysis time

Orthogonal: Orthogonality in chromatography refers to alternative selectivity between separations.

What is Supercritical Fluid Chromatography

Definition:

Supercritical fluid chromatography (SFC) is a form of normal phase chromatography used for the analysis of low to moderate molecular weight molecules. Principles are similar to those in HPLC;

However, SFC typically uses supercritical CO_2 as the mobile phase.

It requires the entire chromatographic flow path to be pressurized.

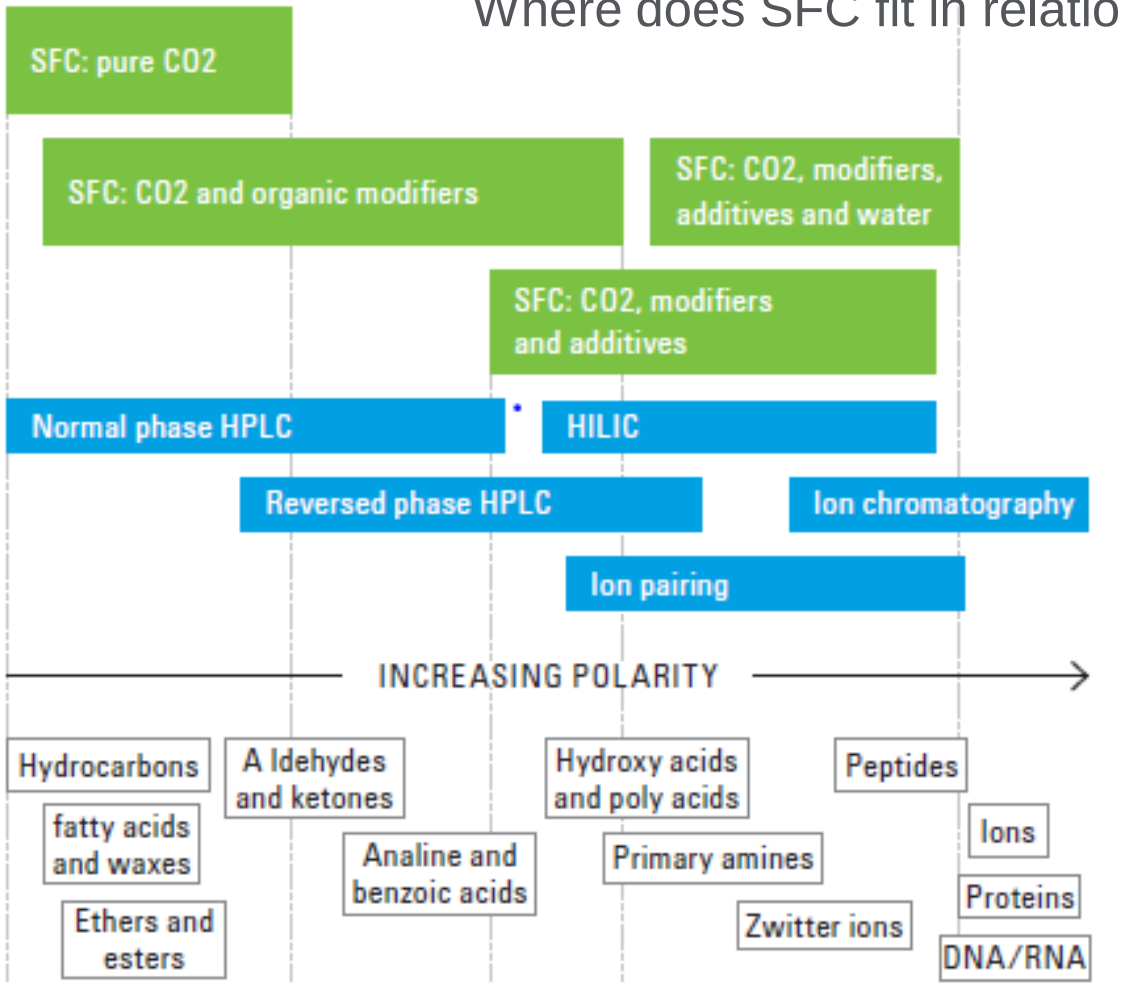
SFC can be performed at 3x higher speed compared to LC without losing separation efficiency. Solvent viscosity is lower and diffusivity higher than in LC.

The Agilent 1260 Infinity hybrid SFC/UHPLC system combined with single quadrupole mass spectrometry detection is capable of performing both supercritical fluid chromatography (SFC) and ultrahigh performance liquid chromatography (UHPLC) by switching automatically between the two techniques.

Supercritical Fluid Chromatography

Agilent InfinityLab

Where does SFC fit in relation to HPLC?

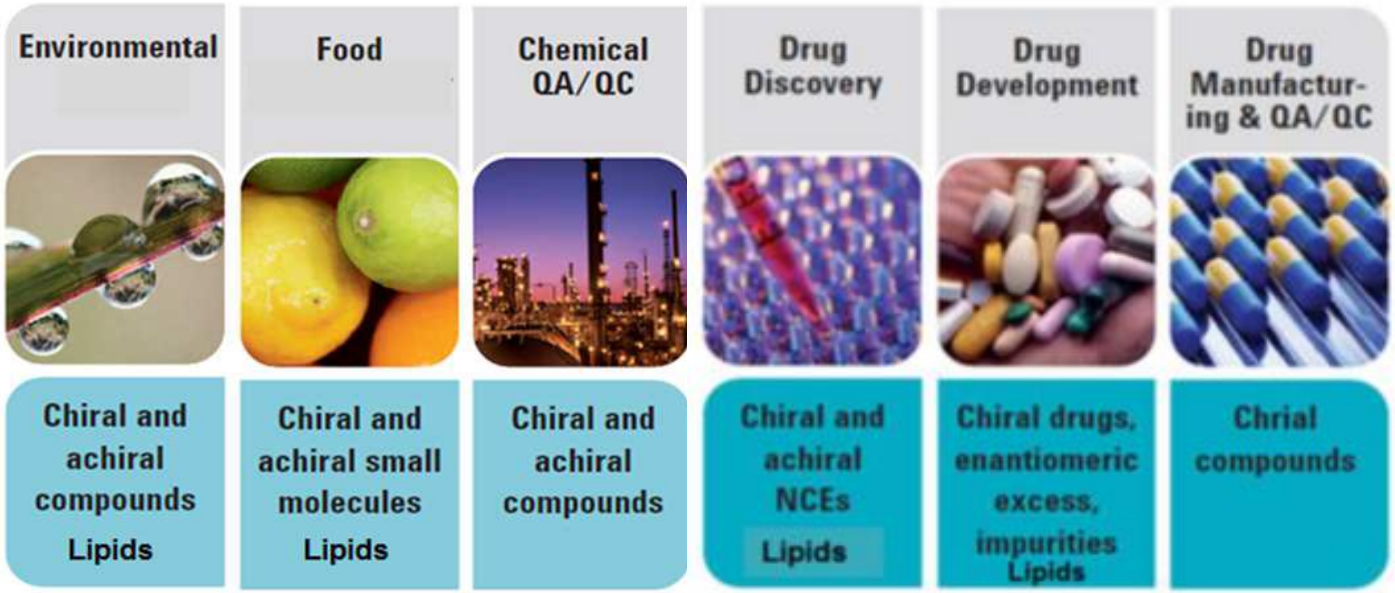


Ref: Agilent presentation: The SFC system as a routine instrument, October 12, 2015

Supercritical Fluid Chromatography

What people use SFC

Wide application range in many industries



Chiral analysis
- Chiral purity analysis (qualitative) of API
- Chiral method development for Prep SFC
- Quantitation of enantiomeric purity of starting materials, intermediates, and bulk drugs (EE)

Achiral analysis
- Normal Phase or RP phase small molecule applications
- Drug development, library screening (lead generation)
- Preprep analysis, method development for Prep SFC
- Lipids, fatty acids, vitamins
- Natural product separations
- Petrochemical, Environmental, Food and Industrial applications

Supercritical Fluid Chromatography

Agilent InfinityLab

Why use CO_2?

Today, almost all applications in SFC use CO_2, modified with an organic solvent, and sometimes a highly polar additive. CO_2 is the preferred fluid because it is:

- Readily available
- Inexpensive
- Has an accessible critical point
- Relatively safe
- Considered *green* since it has been recycled, and
- Miscible with a wide range of highly polar modifiers

Methanol is by far the most widely used modifier and among the most polar modifiers completely miscible with CO_2.

Advantages of methanol include:

- Availability
- Inexpensive
- Complete miscibility with CO_2
- Low UV cut-off (about 205 nm)
- Relatively low toxicity

SFC primer
Agilent publication number 5991-5509EN

SFC Supercritical Fluid Chromatography
Agilent column options

Agilent InfinityLab

Offering column chemistries for your separation challenge:

Stationary Phase	Fully Porous (ZORBAX)	Superficially Porous (Poroshell)
Conventional RP	Eclipse Plus C8 Eclipse Plus C18	EC-C8 EC-C18 Bonus RP Phenyl-Hexyl
Pure Silica	Rx-SIL	--
HILIC	HILIC Plus	HILIC
Cyano	Eclipse XDB-CN	EC-CN

Columns can be critical for your SFC application

Supercritical Fluid Chromatography

Uses of different chemistries

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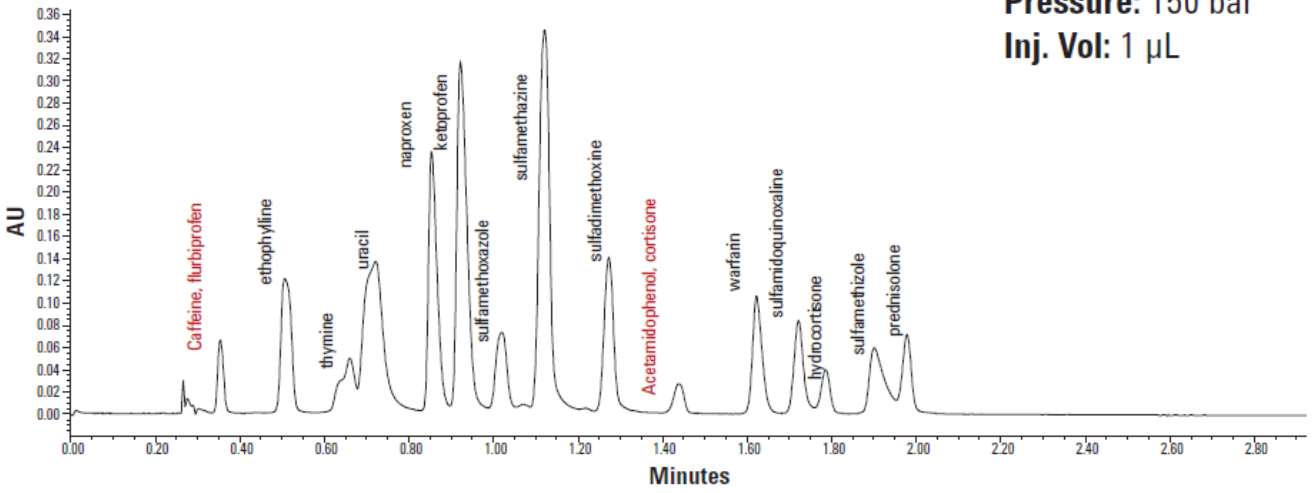
5-20% in 4 mins

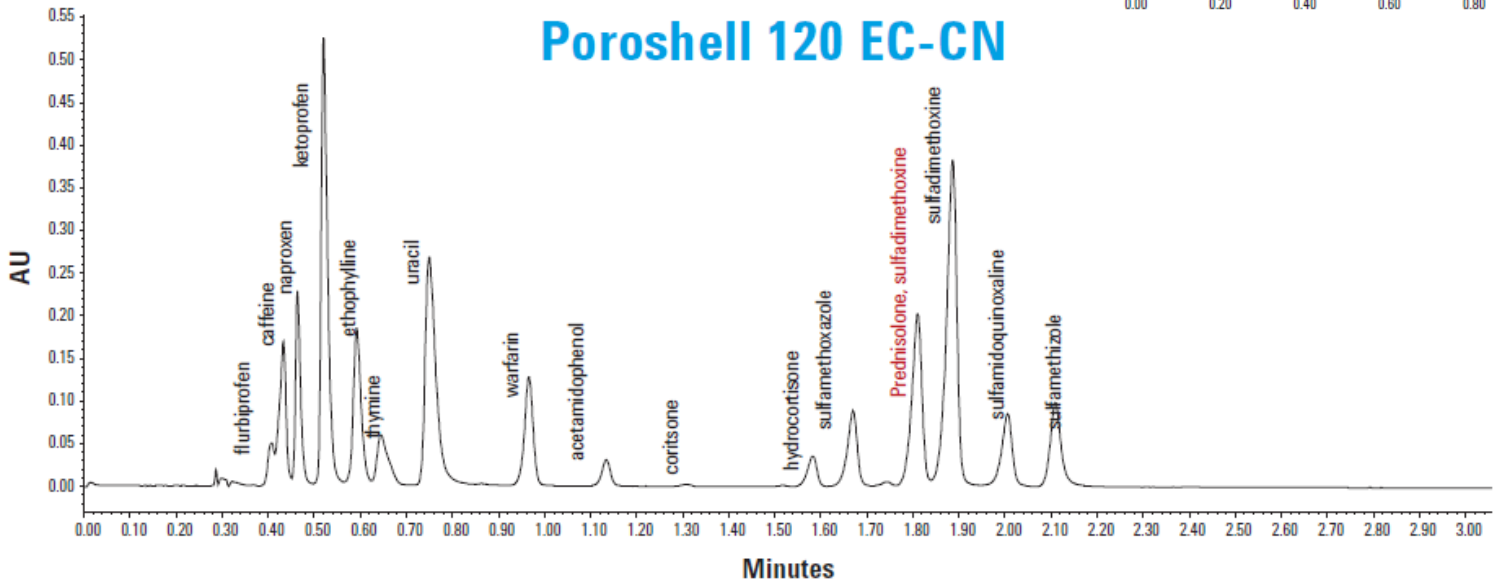
Poroshell 120 Bonus-RP

Column temp: 55 °C
Flow rate: 2.5 mL/min
Pressure: 150 bar
Inj. Vol: 1 µL



Column temp: 55 °C
Flow rate: 2.5 mL/min
Pressure: 150 bar
Inj. Vol: 1 µL

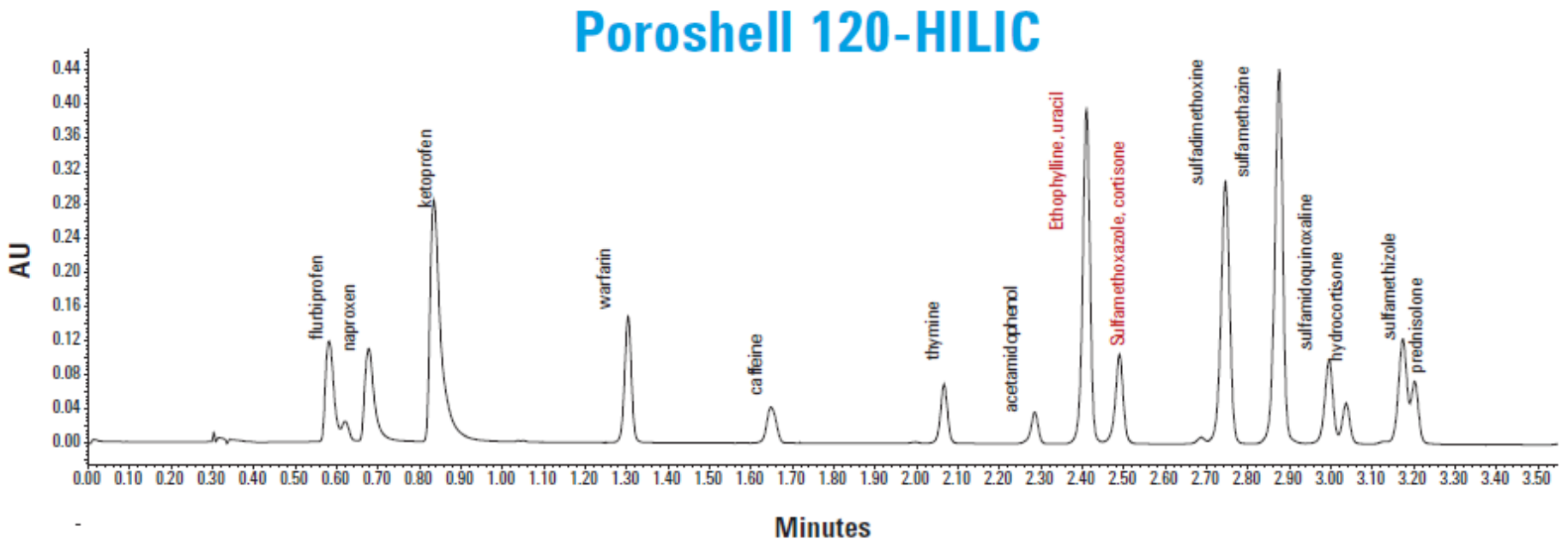
Poroshell 120 EC-CN



Supercritical Fluid Chromatography

Agilent InfinityLab

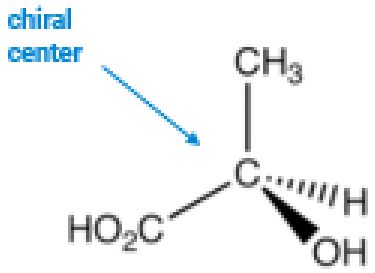
Column temp: 55 °C
Flow rate: 2.5 mL/min
Pressure: 150 bar
Inj. Vol: 1 µL



Chiral Chromatography

Chiral compounds are stereoisomers with a mirror image that is nonsuperimposable

The compound will have a chiral center



Chiral centers

- Stereo-center attached to four different substituents
- The two mirror image forms are called **enantiomers**
- Are not superimposable on their mirror image
  - For example: Think of hands, feet
- Enantiomers are optically active and rotate polarized light (+ or -)
- A **racemic mixture (racemate)** is a mixture of enantiomers

Chiral Separations

- Chiral stationary phases are bonded with ‘chiral selectors’ – compounds that can selectively target +/- enantiomers.
- Chiral stationary phases have varying affinities and interactions specific to each enantiomer.
- Three simultaneous, different modes of interactions are necessary for chiral separations.
- Typical interactions can include:
    - H-bonding
    - π-π interactions
    - Dipole stacking
    - Steric interactions
    - Ionic interactions

Why Do Chiral Separations

- Most small molecule drugs on the market today are either racemates or enantiomerically pure.
- Enantiomers: Same chemical and physical properties, but can have **very different behavorial properties.**
- It is important to characterize each enantiomer.

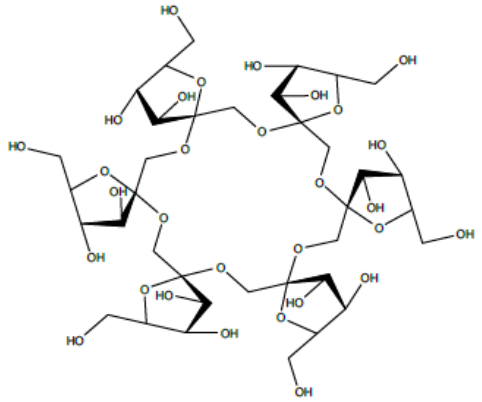
Agilent InfinityLab Chromatography Columns

Agilent InfinityLab Chromatography Column Offerings

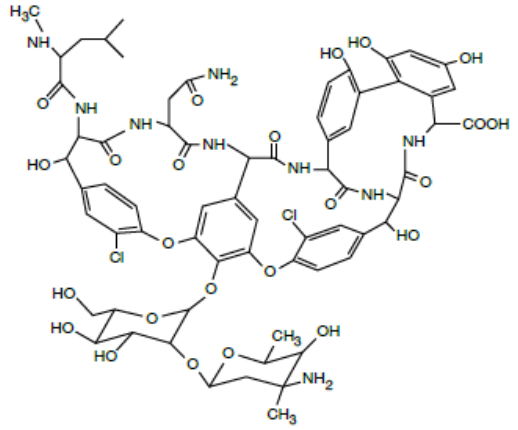
Affinity	Ion Exchange	HIC	Size Exclusion	Chiral
Selective affinity between phase and molecule	*Complementary attraction between opposite charges*	*Hydrophobic interactions between phase and protein*	*Noninteractive with stationary phase – size in solution*	*Multiple types of interactions between phase and molecule*
Bio-Monolith Protein A	**Bio IEX (SCX, WCX, SAX, WAX)**	**AdvanceBio HIC**	**AdvanceBio SEC**	**Poroshell Chiral CF (Derivatised cyclofructan)**
Bio-Monolith Protein G	**Bio MAb (WCX)**		**Bio SEC-3**	**Poroshell Chiral CD (Hydroxypropylated b cyclodextrin)**
	PL-SAX, PL-SCX		**Bio SEC-5**	**Poroshell Chiral V (Vancomycin)**
	Zorbax IEX (SCX, SAX)		ProSEC 300S	**Poroshell Chiral T (Teicoplanin)**
	Bio-Monolith **(QA, DEAE, SO_3^-)**		Zorbax GF250 and GF450	

InfinityLab Poroshell 120 Chiral Chemistries

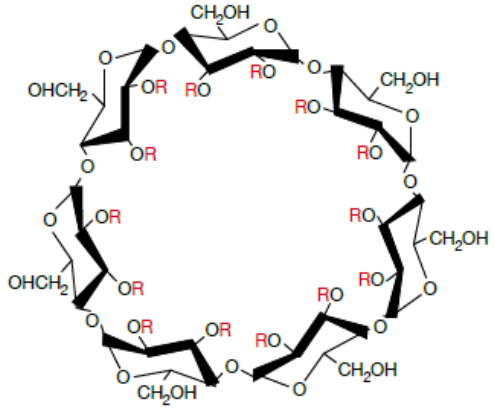
InfinityLab Poroshell 120 Chiral-CF
(Cyclofructan CF-6)



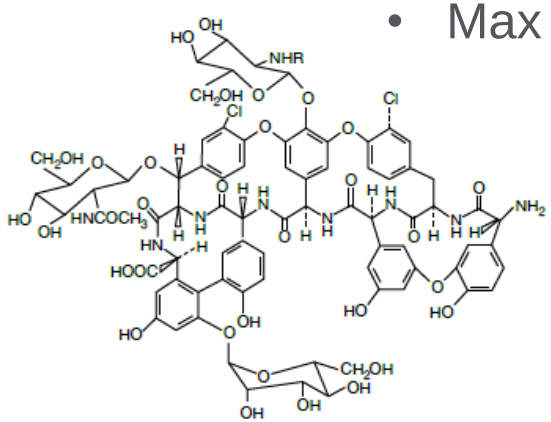
InfinityLab Poroshell 120 Chiral-V
(Vancomycin)



InfinityLab Poroshell 120 Chiral-CD
(Hydroxypropylated beta-cyclodextrin)



InfinityLab Poroshell 120 Chiral-T
(Teicoplanin)



- Four different Chiral phases
- 2.7 µm Poroshell 120 particles

- 2.1 mm and 4.6 mm id
- 50 mm, 100 mm and 150 mm lengths

- 400 bar max pressure limit
- Max pH 7
- Max temp 45 °C

InfinityLab Poroshell 120 Chiral Chemistries

Column Chemistry	Chiral Selector (bonded chemistry)	Typical LC Mode	Typical Applications
InfinityLab Poroshell 120 Chiral-CF	Derivatized cyclofructan (CF6)	Polar Organic (PO)	Primary amines
		Normal Phase (NP)	Primary amines
InfinityLab Poroshell 120 Chiral-CD	Hydroxypropylated-β-cyclodextrin	Reversed Phase (RP)	Stimulants, fungicides, t-boc amino acids
		Polar Organic (PO)	Complex molecules
InfinityLab Poroshell 120 Chiral-V	Vancomycin (macrolide antibiotic)	Polar Ionic (PI)	Basic pharmaceuticals (various)
		Reversed Phase (RP)	Amines, profens
		Polar Organic (PO)	Complex neutral molecules
InfinityLab Poroshell 120 Chiral-T	Teicoplanin (macrolide antibiotic)	Polar Ionic (PI)	Beta blockers, hydroxyl acids
		Reversed Phase (RP)	Amino acids, hydroxyl acids, profens
		Polar Organic (PO)	Hydantoins, benzodiazepines

Modes of Separation Used with Infinity Lab Chiral Columns

Agilent InfinityLab

Polar ionic mode

- Methanol with acid or base or volatile salt <0.2 % wt. (MeOH + HOAc + TEA)
- Nonaqueous mobile phase; fast, MS detection; for ionizable molecules – any acid or base
- Dominant interactions: Ionic interaction, hydrogen bonding
- Example: MeOH with 0.2 wt% ammonium formate

Reversed-phase mode

- Methanol/Water/Buffer,
- MS compatible, ideal for manufacturing QC, bio-analysis for all types of molecules
- Example: 30/70 MeOH/20 mM ammonium formate (pH 4)

Polar organic mode

- Acetonitrile/Methanol/Ethanol/Isopropanol+ HOAc + TEA
- Dominant interactions: Hydrogen bonding, dipole-dipole
- Example: 60/40/0.3/0.2 ACN/MeOH/acetic acid/TEA

Normal phase

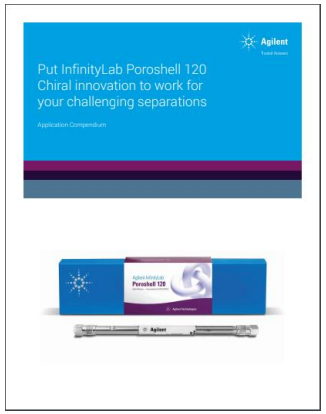
- Heptane (or hexane)/methanol or ethanol
- Example: 60/40/0.3/0.2 ACN/MeOH/acetic acid/TEA

Recommendations for Chiral Method Development

Agilent InfinityLab

Priority of mobile phases for screening

Column Chemistry	First choice	Second choice	Third choice
InfinityLab Poroshell Chiral-T	#5 (PI)	#2 (RP)	#1 (RP)
InfinityLab Poroshell Chiral-V	#5 (PI)	#3 (RP)	#1 (RP)
InfinityLab Poroshell Chiral-CD	#3 (RP)	#6 (PO)	#4 (RP)
InfinityLab Poroshell Chiral-CF	#6 (PO)	#7 (NP)	-



Ref: InfinityLab Chiral Applications Compendium
Publication number: 5991-8450EN

Chiral screening protocol

#	Mobile Phase	Composition (% v)	Type
1	MeOH/20 mM ammonium formate, pH 4.0	90/10	RP
2	MeOH/20 mM ammonium formate, pH 4.0	30/70	RP
3	ACN/20 mM ammonium formate, pH 4.0	30/70	RP
4	ACN/20 mM ammonium formate, pH 4.0	10/90	RP
5	MeOH/ammonium formate	100/0.2wt%	PI
6	ACN/MeOH/Acetic Acid/TEA	60/40/0.3/0.2	PO
7	EtOH/Heptane/Acetic Acid/TEA	20/80/0.3/0.2	NP

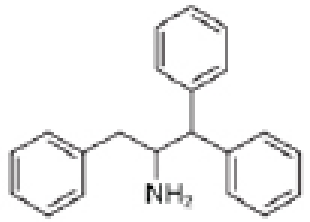
RP = Reversed phase PI = Polar iconic PO = Polar organic NP = Normal phase

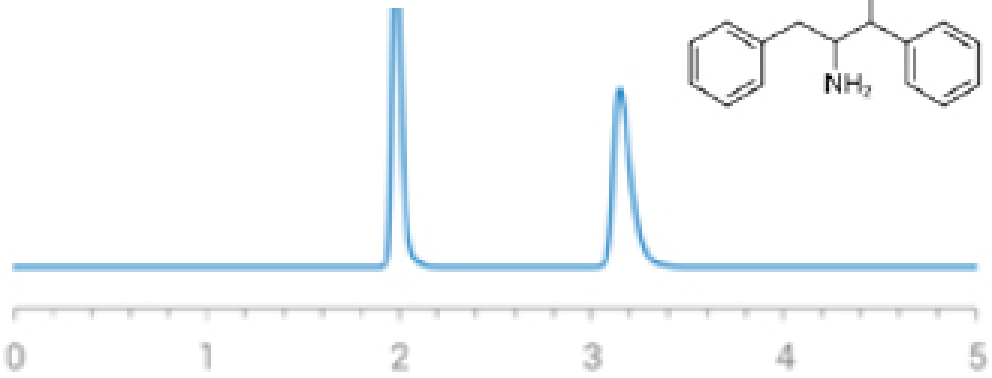
Application Examples
InfinityLab Poroshell Chiral Columns

Agilent InfinityLab

1-benzyl-2,2,diphenylethylamine

Retention (min): 1.98/3.15 (Peak1/Peak 2)
Resolution: 9.47



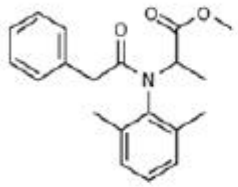


Method Conditions

Column:	InfinityLab Poroshell 120 Chiral-V (10 cm x 4.6 mm, 2.7 µm)
Mobile phase:	100/0.1 wt %: Methanol/Ammonium Trifluoroacetate
Flow Rate:	1.0 mL/min
Temperature:	Ambient (23 °C)
Injection Volume:	1.0 µL
Detection:	UV 220 nm

Benalaxyl – fungicide

Retention (min): 1.64/1.87 (Peak1/Peak 2)
Resolution: 3.13



Method Conditions

Column:	InfinityLab Poroshell 120 Chiral-CD (10 cm x 4.6 mm, 2.7 µm)
Mobile phase:	30/70: Acetonitrile/15 mM Ammonium Formate (pH 3.6)
Flow Rate:	1.0 mL/min
Temperature:	Ambient (23 °C)
Injection Volume:	1.0 µL
Detection:	UV 220 nm

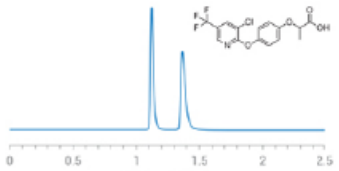
InfinityLab Chiral Applications Compendium

Publication number: 5991-8450EN

Agilent InfinityLab

Fungicides

Haloxyfop

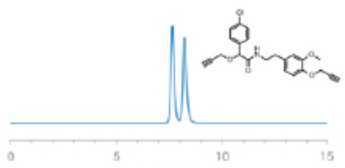


Retention (min): 1.12/1.37 (Peak1/Peak 2)
Resolution: 4.48

Method Conditions

Column: InfinityLab Poroshell 120 Chiral-T (10 cm x 4.6 mm, 2.7 µm)
Mobile phase: 100/0.3 wt %: Methanol/Ammonium Trifluoroacetate
Flow Rate: 1.0 mL/min
Temperature: Ambient (23 °C)
Injection Volume: 1.0 µL
Detection: UV 220 nm

Mandipropamid

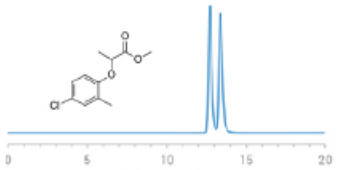


Retention (min): 7.65/8.22 (Peak1/Peak 2)
Resolution: 1.87

Method Conditions

Column: InfinityLab Poroshell 120 Chiral-V (10 cm x 4.6 mm, 2.7 µm)
Mobile phase: 30/70: Methanol/15 mM Ammonium Formate (pH 3.6)
Flow Rate: 0.5 mL/min
Temperature: 45 °C
Injection Volume: 1.0 µL
Detection: UV 230 nm

Mecoprop methyl ester

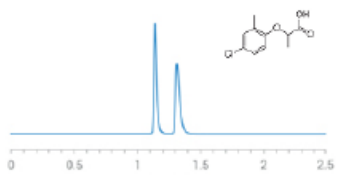


Retention (min): 12.73/13.39 (Peak1/Peak 2)
Resolution: 1.74

Method Conditions

Column: InfinityLab Poroshell 120 Chiral-T (15 cm x 4.6 mm, 2.7 µm)
Mobile phase: 30/70: Methanol/50 mM Ammonium Formate (pH 3.6)
Flow Rate: 0.5 mL/min
Temperature: 45 °C
Injection Volume: 1.0 µL
Detection: UV 230 nm

Mecoprop



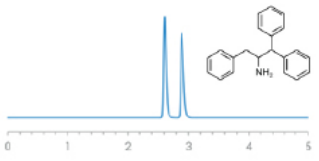
Retention (min): 1.14/1.31 (Peak1/Peak 2)
Resolution: 3.59

Method Conditions

Column: InfinityLab Poroshell 120 Chiral-T (10 cm x 4.6 mm, 2.7 µm)
Mobile phase: 100/0.3 wt %: Methanol/Ammonium Trifluoroacetate
Flow Rate: 1.0 mL/min
Temperature: Ambient (23 °C)
Injection Volume: 1.0 µL
Detection: UV 220 nm

Amines

1-benzyl-2,2-diphenylethylamine

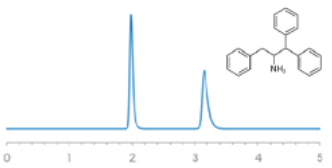


Retention (min): 2.61/2.89 (Peak1/Peak 2)
Resolution: 3.51

Method Conditions

Column: InfinityLab Poroshell 120 Chiral-CD (15 cm x 4.6 mm, 2.7 µm)
Mobile phase: 30/70: Acetonitrile/15 mM Ammonium Formate (pH 3.6)
Flow Rate: 1.0 mL/min
Temperature: Ambient (23 °C)
Injection Volume: 1.0 µL
Detection: UV 220 nm

1-benzyl-2,2-diphenylethylamine

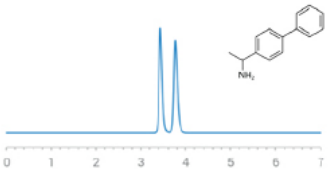


Retention (min): 1.98/3.15 (Peak1/Peak 2)
Resolution: 9.47

Method Conditions

Column: InfinityLab Poroshell 120 Chiral-V (10 cm x 4.6 mm, 2.7 µm)
Mobile phase: 100/0.1 wt %: Methanol/Ammonium Trifluoroacetate
Flow Rate: 1.0 mL/min
Temperature: Ambient (23 °C)
Injection Volume: 1.0 µL
Detection: UV 220 nm

1,1'-binaphthyl-2,2'-diamine

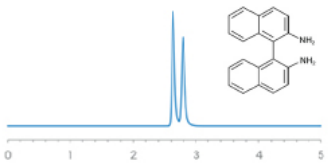


Retention (min): 3.44/3.77 (Peak1/Peak 2)
Resolution: 2.11

Method Conditions

Column: InfinityLab Poroshell 120 Chiral-CD (10 cm x 4.6 mm, 2.7 µm)
Mobile phase: 90/10/0.3/0.2: Acetonitrile/Methanol/Trifluoroacetate/TEA
Flow Rate: 1.0 mL/min
Temperature: Ambient (23 °C)
Injection Volume: 1.0 µL
Detection: UV 254 nm

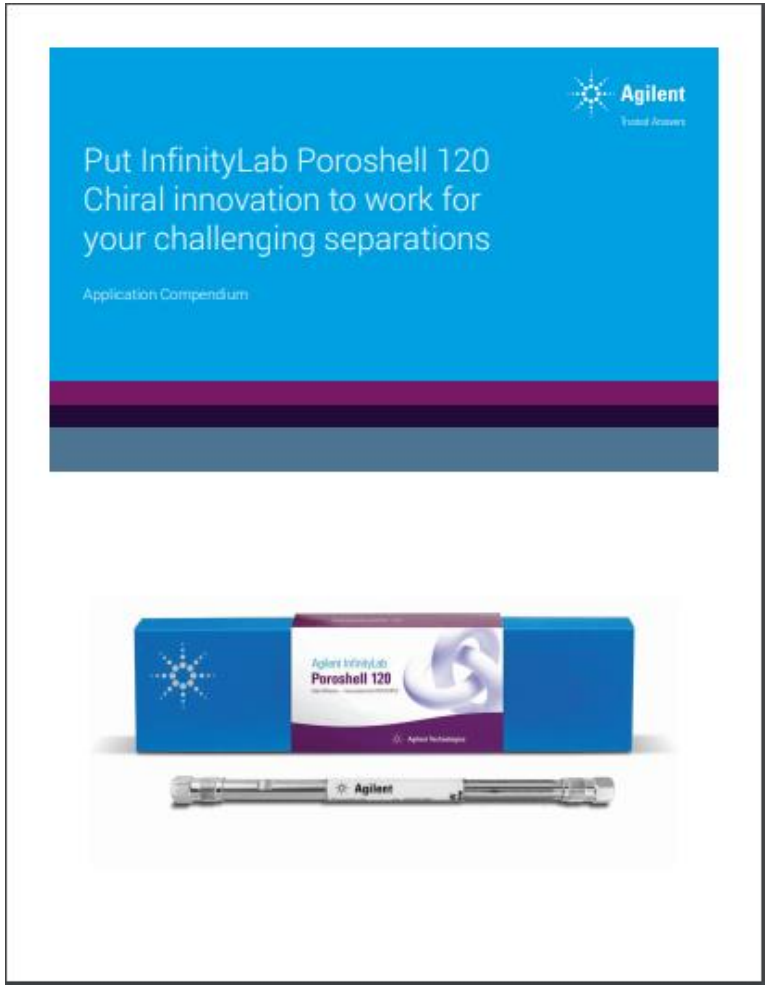
1,1'-binaphthyl-2,2'-diamine



Retention (min): 2.64/2.79 (Peak1/Peak 2)
Resolution: 1.31

Method Conditions

Column: InfinityLab Poroshell 120 Chiral-V (10 cm x 4.6 mm, 2. 7µm)
Mobile phase: 90/10: Methanol/15 mM Ammonium Formate (pH 3.6)
Flow Rate: 1.0 mL/min
Temperature: Ambient (23 °C)
Injection Volume: 1.0 µL
Detection: UV 220 nm



Hydrophobic Interaction Chromatography

HIC

Hydrophobic interaction chromatography separates protein molecules based on differences in their hydrophobicity. There is an interaction between the protein sample and the hydrophobic surface of the HIC packing

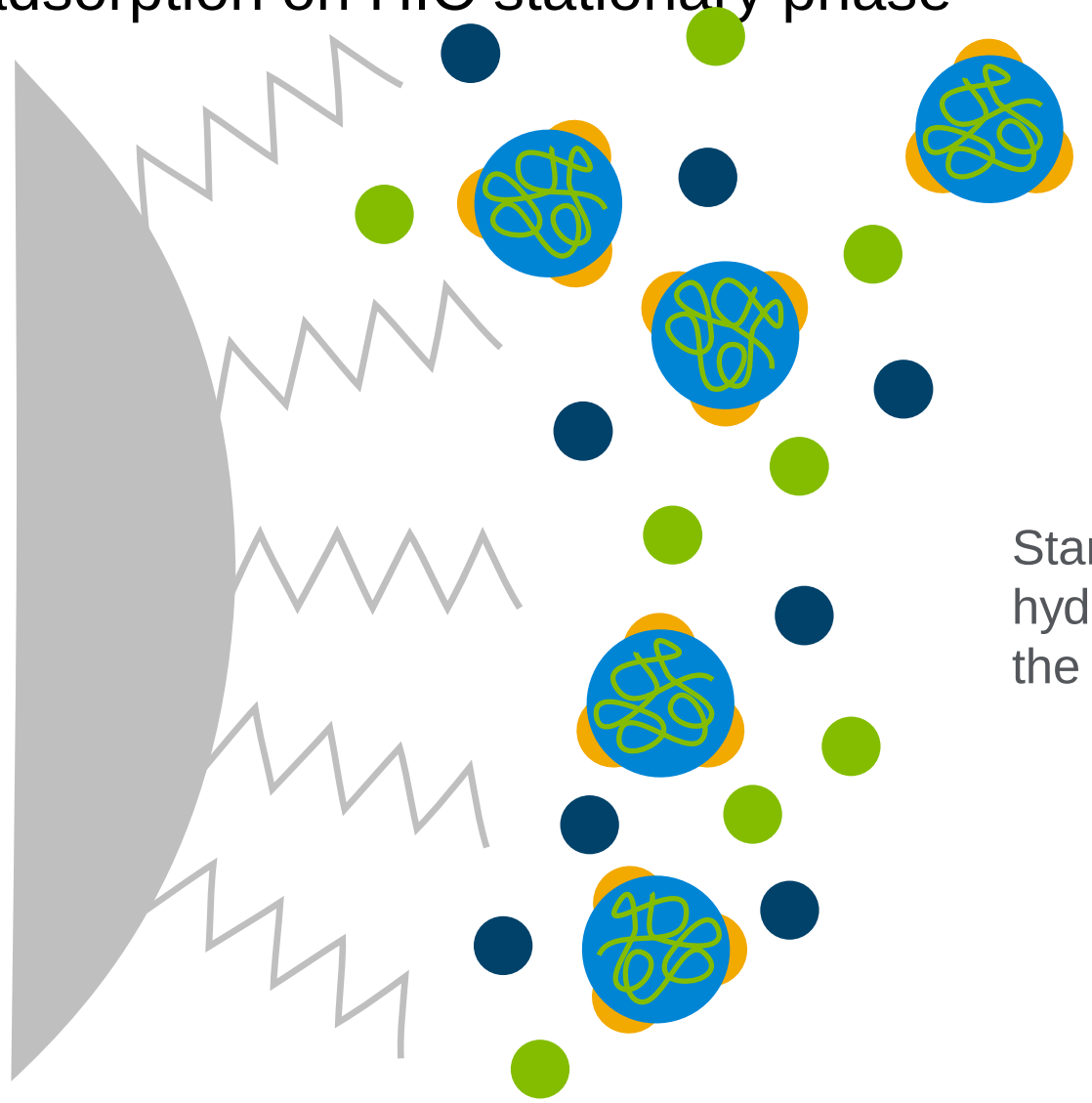
HIC is most commonly used for separating proteins because, unlike reversed-phase chromatography which denatures proteins, HIC conditions maintain proteins in their intact, native (and therefore active) state.

HIC is used for:

- Separating proteins
- Separating variants (impurities) from individual proteins
- Separating antibody drug conjugate species

How Does HIC Work?

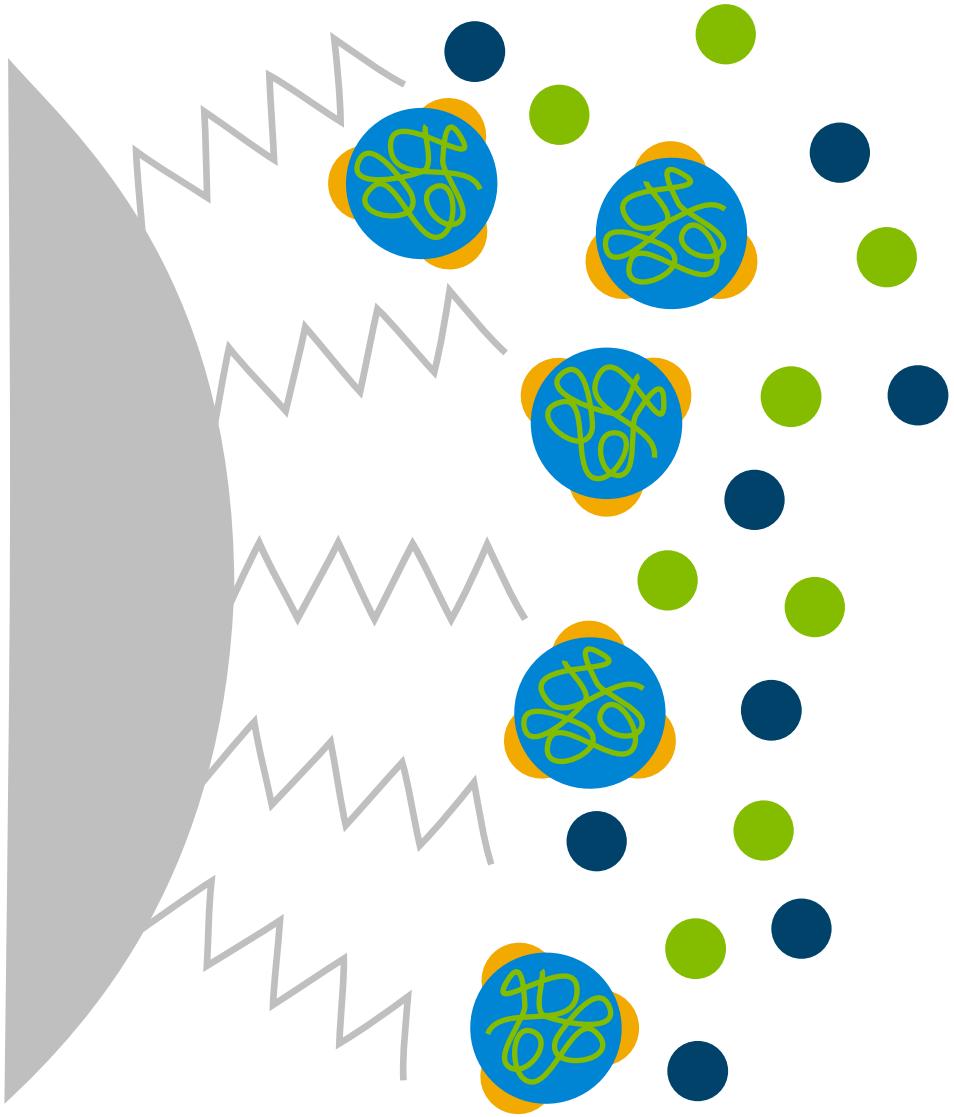
Protein adsorption on HIC stationary phase



Salt

Start at higher levels of salt to promote hydrophobic interaction with ligands in the stationary phase.

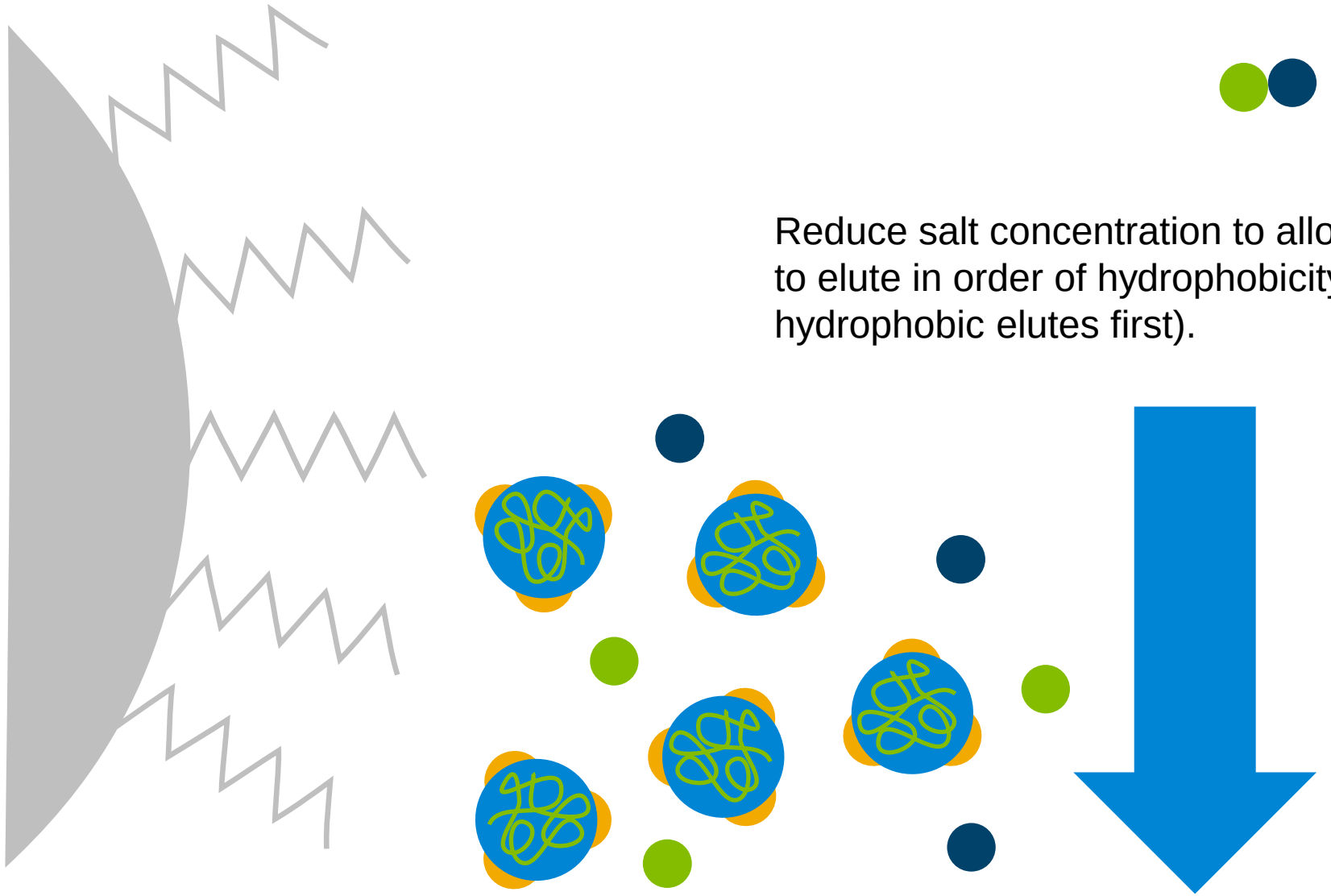
Protein Adsorption on HIC Stationary Phase



Salt

At the start of the gradient with a HIGH salt concentration, the protein is absorbed onto the column. Its concentration is almost certainly increased.

Protein Elution on HIC Stationary Phase



Reduce salt concentration to allow proteins to elute in order of hydrophobicity (least hydrophobic elutes first).

Agilent InfinityLab Chromatography Columns

Agilent InfinityLab Chromatography Column Offerings

Affinity	Ion Exchange	HIC	Size Exclusion	Chiral
Selective affinity between phase and molecule	*Complementary attraction between opposite charges*	*Hydrophobic interactions between phase and protein*	*Noninteractive with stationary phase – size in solution*	*Multiple types of interactions between phase and molecule*
Bio-Monolith Protein A	**Bio IEX** (SCX, WCX, SAX, WAX)	**AdvanceBio HIC**	**AdvanceBio SEC**	**Poroshell Chiral CF** (Derivatised cyclofructan)
Bio-Monolith Protein G	**Bio MAb** (WCX)		**Bio SEC-3**	**Poroshell Chiral CD** (Hydroxypropylated b cyclodextrin)
	PL-SAX, PL-SCX		**Bio SEC-5**	**Poroshell Chiral V** (Vancomycin)
	Zorbax IEX (SCX, SAX)		ProSEC 300S	**Poroshell Chiral T (Teicoplanin)**
	Bio-Monolith (QA, DEAE, SO_3^-)		Zorbax GF250 and GF450	

AdvanceBio HIC

Key features

New wider pore 3.5 µm ZORBAX particle

- Rapid mass transfer
- Reduced operating pressures
- Increased speed of analysis of large biomolecules

Unique bonded phase

- Optimized hydrophobicity for HIC bioseparation at lower salt concentrations
- Versatile for a wide range of modalities, from hydrophilic proteins to hydrophobic ADCs

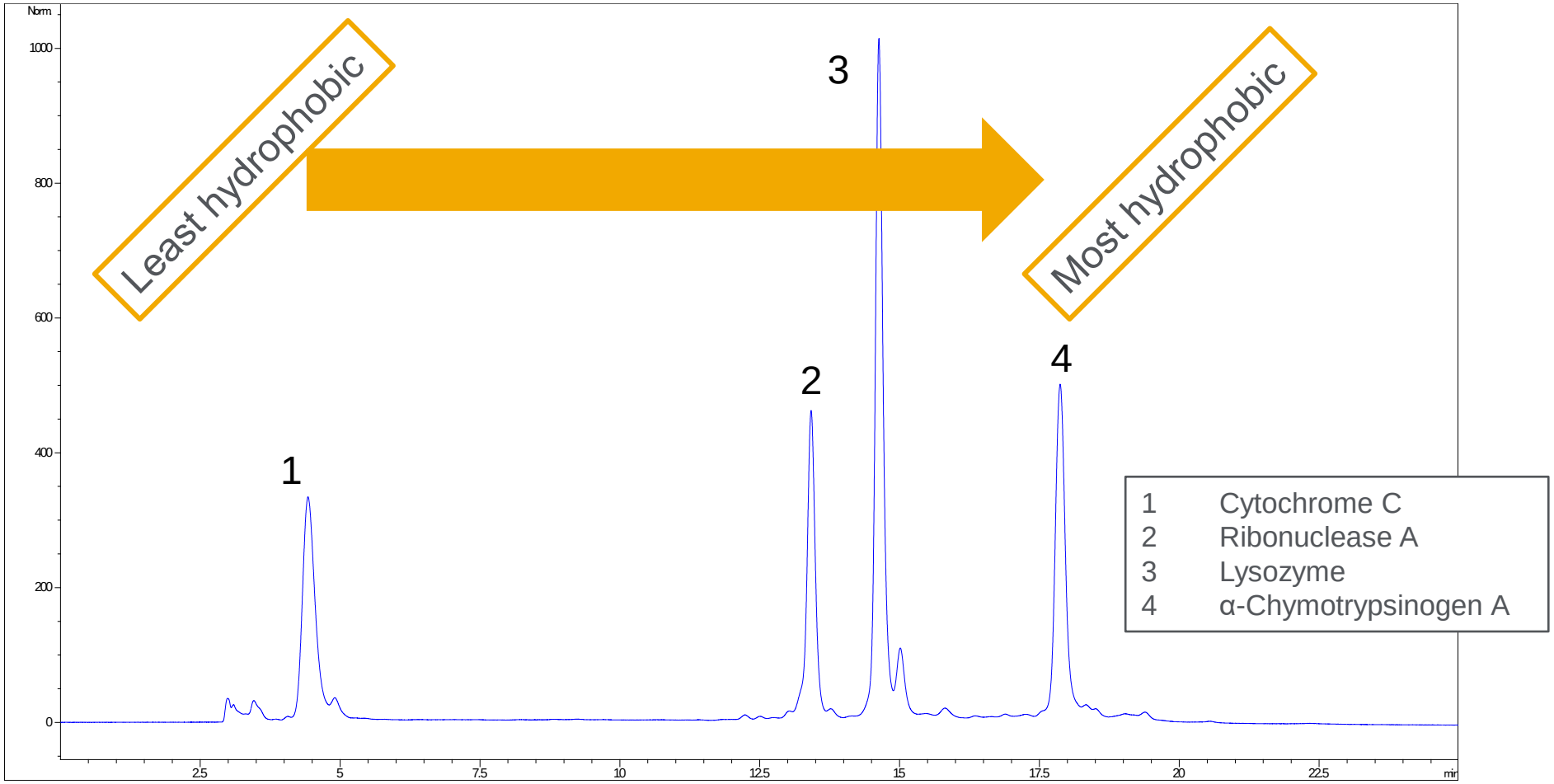
AdvanceBio HIC columns for mAb CQA and ADC DAR analysis

Column dimensions

- **4.6 x 100 mm (p/n 685975-908)** for higher resolution separations
- **4.6 x 30 mm (p/n 681975-908)** for faster separations

HIC Separation of Standard Proteins

Example on a 4.6 x 100 mm column



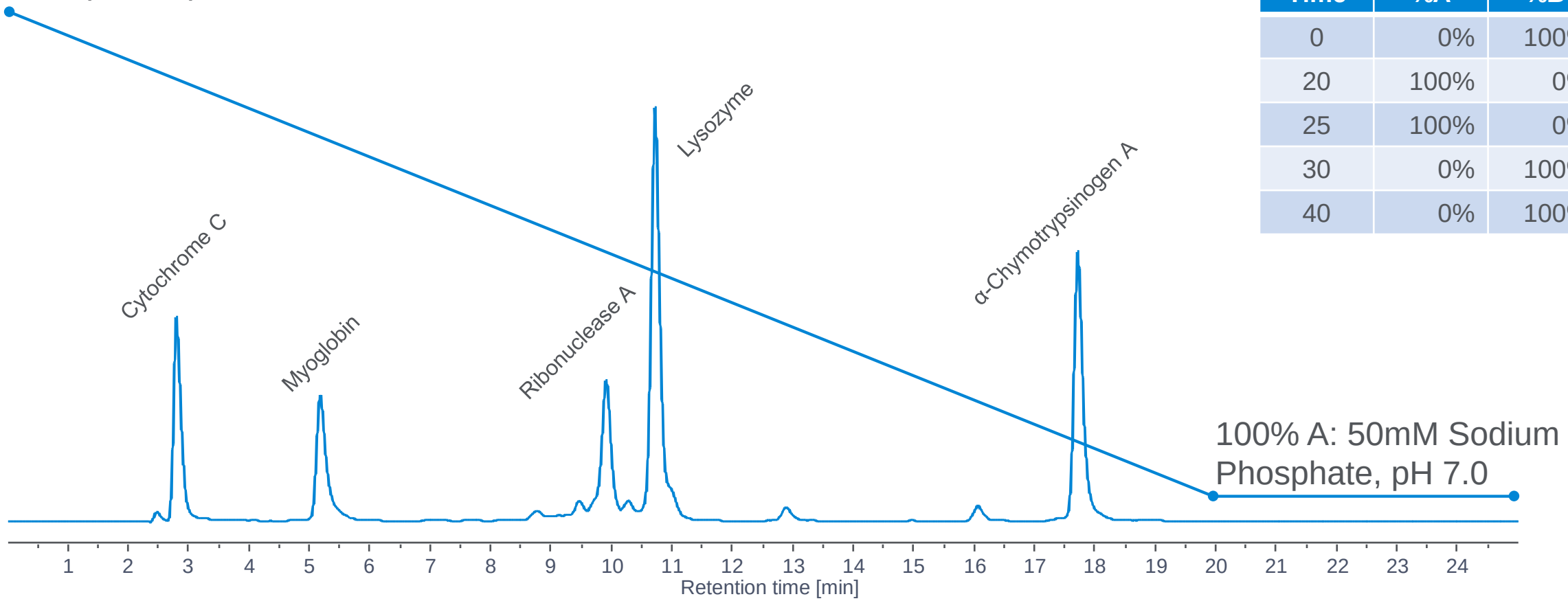
HIC Separation of Standard Proteins

Example on a 4.6 x 100 mm column

Agilent InfinityLab

100% B: 2M $(NH_4)_2SO_4$ + 50mM Sodium Phosphate, pH 7.0

Time	%A	%B
0	0%	100%
20	100%	0%
25	100%	0%
30	0%	100%
40	0%	100%



Agilent

HIC Separation of NIST mAb (RM 8671)

Method conditions

Column:	AdvanceBio HIC 4.6 x 100 mm
Eluent A:	50 mM NaPO, pH 7.0
Eluent B:	2M $(NH_4)_2SO_4$, 50 mM NaPO, pH 7.0
Flow rate:	0.3 mL/min
Temperature:	25 °C
Injection:	5 µL (1 mg/mL)
Sample:	NIST mAb (RM 8671)

4.6 x 100 mm = 1.7 mL column volume

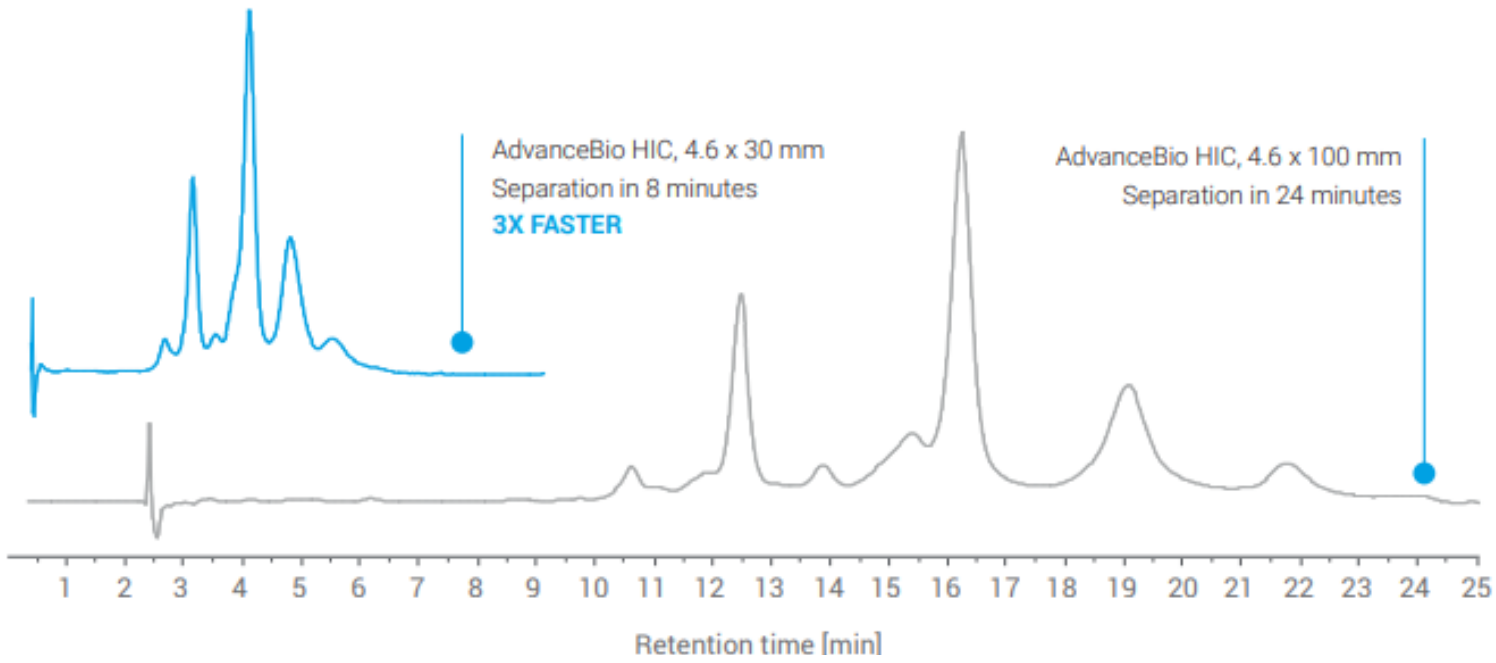
- For higher resolution separations

Gradient profile

Time	%A	%B
0	50%	50%
40	100%	0%
45	100%	0%
50	50%	50%
60	50%	50%

Column Options to Meet Your Needs

Increase throughput with faster separations of ADCs



Instrument:	Agilent 1260 Infinity II Bio-inert LC System
Software:	Agilent OpenLab CDS
Flow rate:	1.0 mL/min, 0.5 mL/min
Eluent A:	2 M ammonium sulfate, 50 mM sodium phosphate, pH 7.0
Eluent B:	50 mM sodium phosphate, pH 7.0
Eluent C:	propan-2-ol
Temperature:	30 °C
Injection volume:	5 µL
Detection:	UV, 220 nm

Column: AdvanceBio HIC, 4.6 x 30 mm

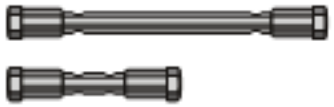
Gradient:

Time (min)	%A	%B	%C
0	50	45	5
5	0	75	25
10	0	75	25
15	50	45	5
20	50	45	5

Column: AdvanceBio HIC, 4.6 x 100 mm

Gradient:

Time (min)	%A	%B	%C
0	50	45	5
20	0	75	25
25	0	75	25
30	50	45	5
40	50	45	5



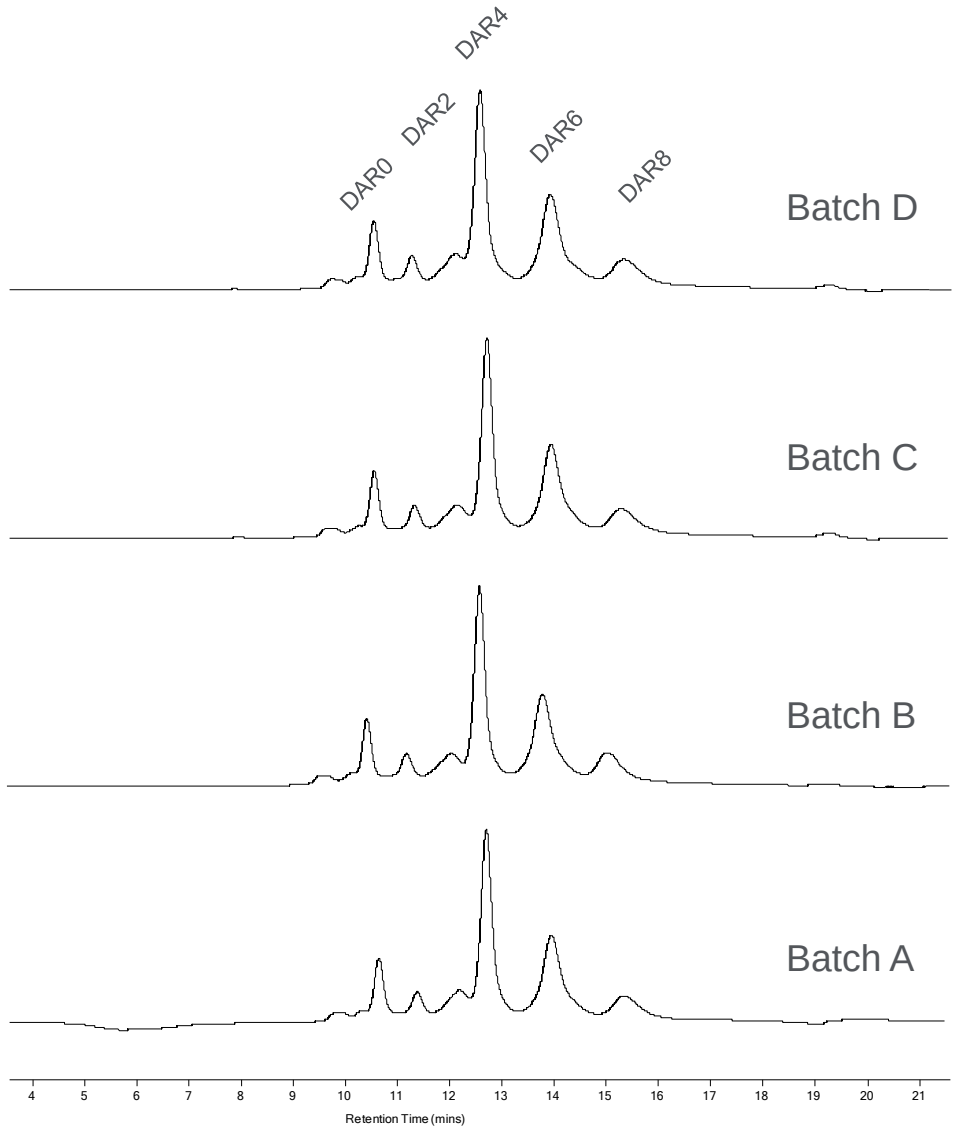
4.6 x 100 mm = 1.7 mL column volume
✓ for higher resolution separations

4.6 x 30 mm = 0.5 mL column volume
✓ for faster separations

Batch to Batch Reproducibility

Example of ADC Separation

- Consistent retention times
- Consistent peak shapes
- Consistent results



What is Needed for HIC ?

A mobile phase containing a salt that encourages the protein to absorb onto the stationary phase, but does not cause the protein to denature.

- Ammonium sulfate, typically 1 – 2 M concentration

A mobile phase that contains a buffer salt to ensure consistent pH and to keep the protein dissolved.

- Sodium phosphate, pH 7, typically 50 – 100 mM concentration

Gradient elution from high to low salt concentration.

- Gradient times from 10 – 20 column volumes are ideal

A stationary phase that is hydrophobic, but will work in the aqueous environment needed for HIC and does not always require organic solvents.

Ion Exchange Chromatography
Common terminology and acronyms

SAX/WAX – Strong anion exchange and weak anion exchange

SCX/WCX – Strong cation exchange and weak cation exchange

Resin Type	Cation Exchange	Anion Exchange
Net charge of molecule of interest	+	–
Charge of resin	–	+

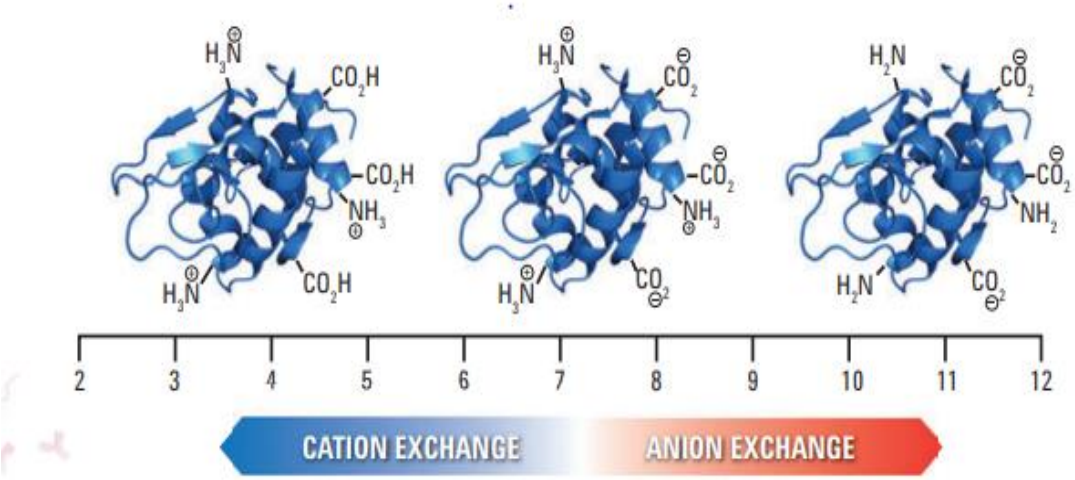


Figure 1 – Effect of pH on net protein charge

Ion Exchange (IEX) Technique

Proteins/samples interact with the stationary phase due to the charge present.

The technique requires gradients for elution.

Separation is based on differences in degree of charge.

- The sample is injected in a mobile phase buffer with a low salt concentration – this binds the proteins to the column.
- Proteins are typically eluted at a constant pH with increasing salt gradients (mobile-phase ionic strength) to displace the proteins from the stationary phase.
- Higher charge proteins bind more strongly and an increased salt gradient is needed to elute them.
- A typical mobile phase will contain salt, NaCl, and KCl.

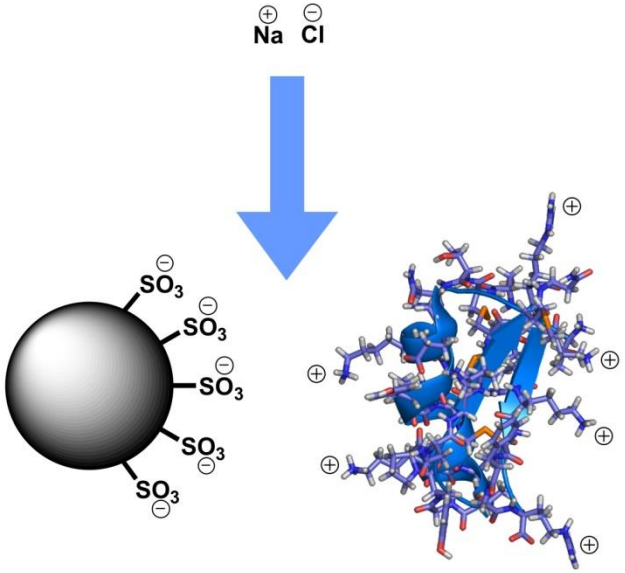
This technique does not denature.

IEX Mechanism Example

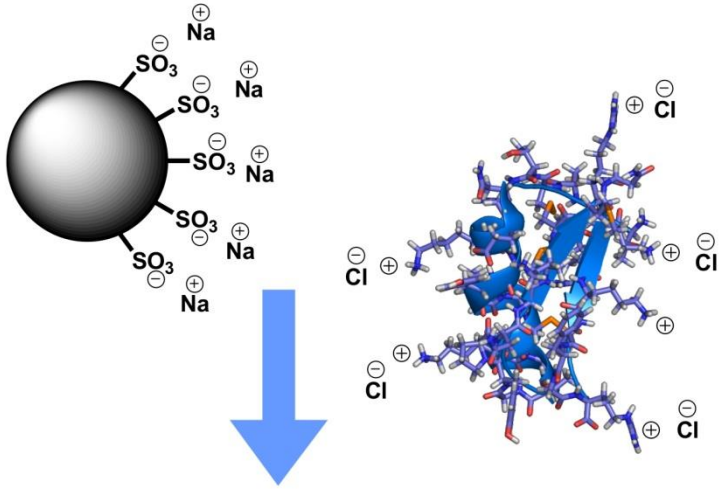
Basic protein on strong cation exchange packing

Agilent InfinityLab

Low salt to bind



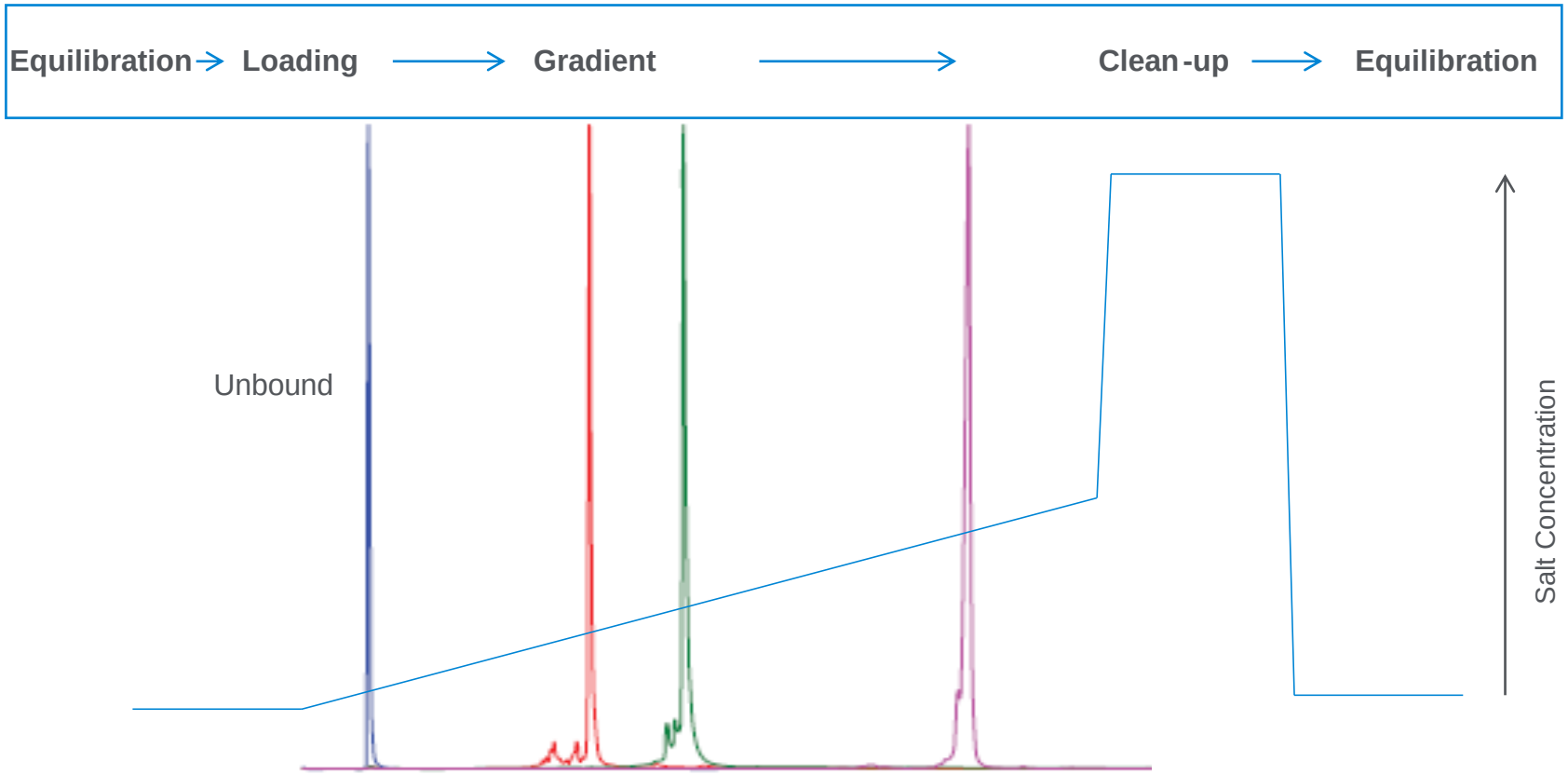
High salt to elute



Elution order will correlate with number of positive charges

Agilent

Ion Exchange Separation Sequence



Equilibration/clean-up is typically 5 to 10 column volumes – essential for reproducibility

Agilent InfinityLab Chromatography Columns

Agilent InfinityLab

Agilent InfinityLab Chromatography Column Offerings

Affinity	Ion Exchange	HIC	Size Exclusion	Chiral
Selective affinity between phase and molecule	*Complementary attraction between opposite charges*	*Hydrophobic interactions between phase and protein*	*Noninteractive with stationary phase – size in solution*	*Multiple types of interactions between phase and molecule*
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	Zorbax IEX (SCX, SAX)		ProSEC 300S	**Poroshell Chiral T (Teicoplanin)**
	Bio-Monolith (QA, DEAE, SO_3^-)		Zorbax GF250 and GF450	

Agilent

Agilent Columns for Ion Exchange

Column specifics

	Particle	Porosity	Functionalities	Particle Sizes	Pore Size	Application
Agilent Bio-IEX	Polymer	Nonporous	SAX, WAX, SCX, WCX	1.7 µm, 3 µm, 5 µm 10um	N/A	Peptides proteins
Agilent Bio MAb	Polymer	Nonporous	WCX	1.7 µm, 3 µm, 5 µm 10 µm	N/A	IgG
PL-SAX	PS/DVB	Fully porous	SAX	5 µm, 8 µm, 10 µm 30 µm	1000 Å, 4000 Å	Peptides, oligos, proteins
PL-SCX	PS/DVB	Fully porous	WCX	5 µm, 8 µm, 10 µm 30 µm	1000 Å, 4000 Å	Peptides, proteins
Bio-Monolith IEX	Polymer	Monolith	QA, DEAE, SO_3	N/A	N/A	Biomacromoleucles

1. Nonporous particles for high-efficiency analytical separations
2. Porous particles for scale up to purification
3. Monoliths for high-speed separations

Agilent BioHPLC Columns
Publication number: 5994-0974EN

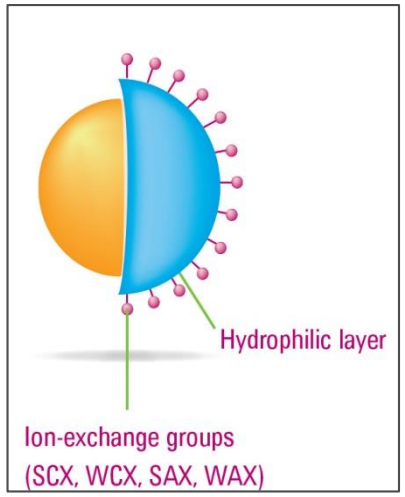
BioIEX – Nonporous Particle Technology

Agilent InfinityLab

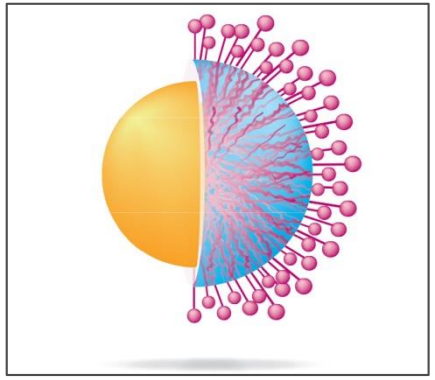
Bio MAb column is a ***WCX*** specifically designed for mAb separations.

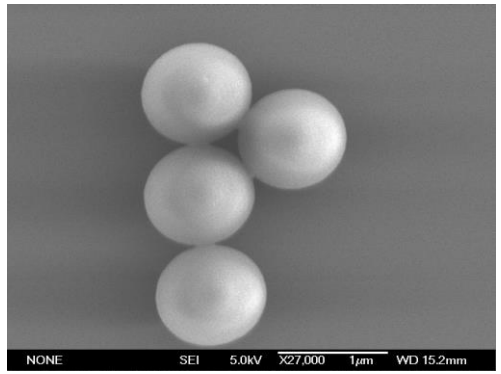
Multiple functionalities:
- Anion exchange
- Cation exchange
- Strong
- Weak

Agilent Bio IEX
High Resolution Ion Exchange Columns



Agilent Bio MAb
High Resolution Separations of Monoclonal Antibodies



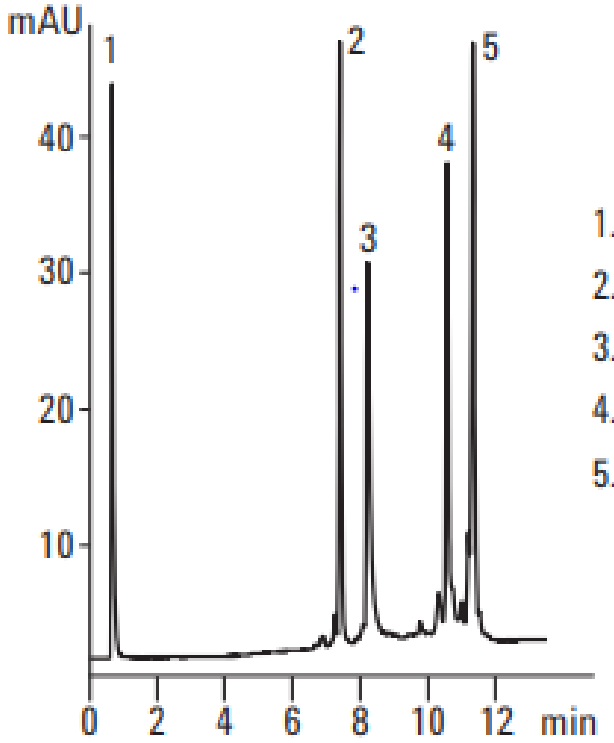


Bio IEX columns are general purpose for high resolution IEX (SCX, WCX, SAX and WAX) separations of proteins.

Nonporous particles do not suffer from slow diffusion into and out of pores and so protein peaks are narrower, allowing higher resolution separations.

Example IEX Applications

Column:	**Bio SCX, stainless steel** **5190-2423** **4.6 x 50 mm, 3 µm**
Buffer:	10 mM phosphate, pH 6.0
Flow Rate:	0.5 mL/min
Gradient:	0-1.0 M NaCl, 15 min
Detector:	280 nm

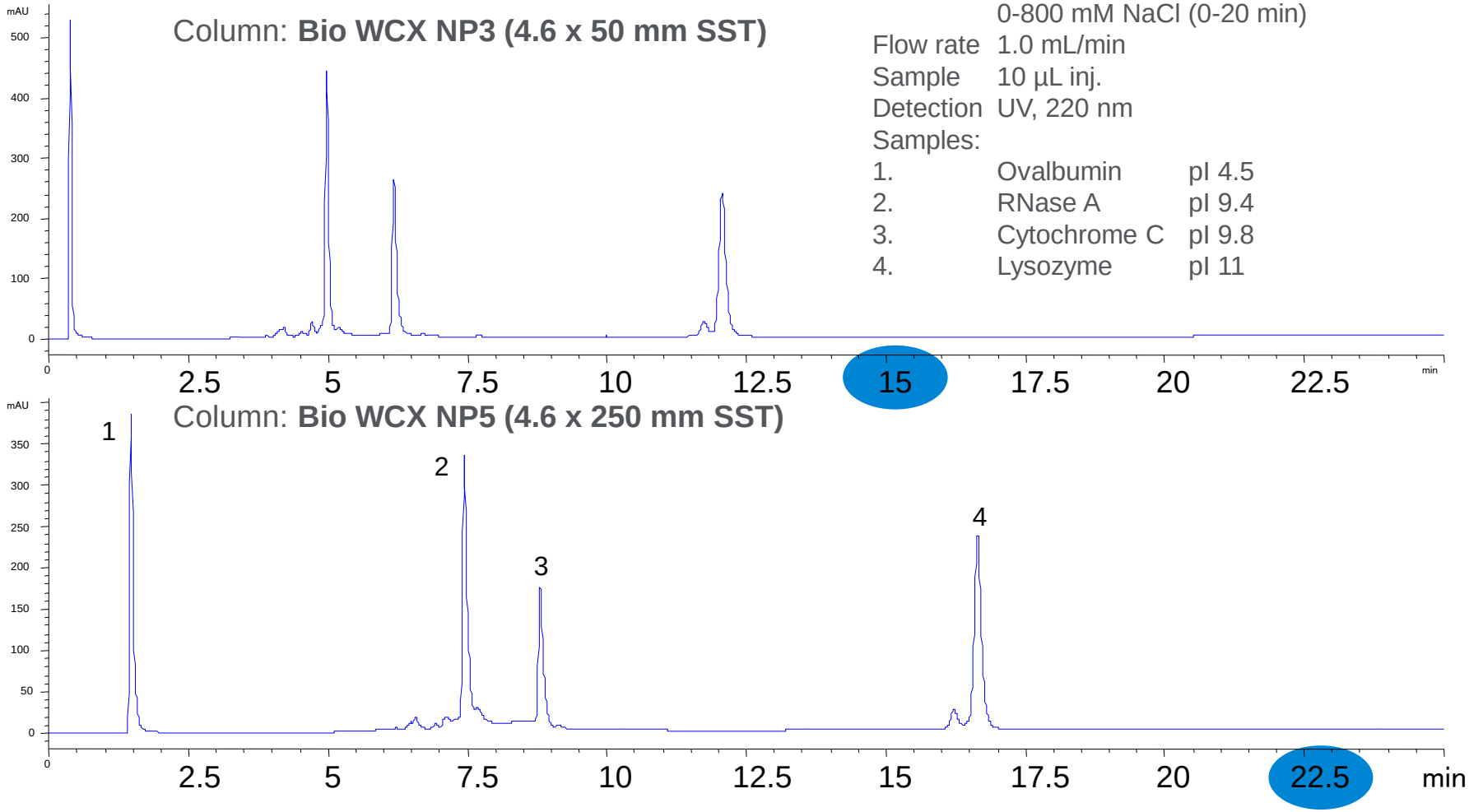


1. Ovalbumin, pI = 4.6
2. Ribonuclease A, pI = 8.7
3. Cytochrome c, pI = 9.6
4. Aprotinin, pI = 10.0
5. Lysozyme, pI = 11.0

N > 100,000/50 mm for lysozyme

Increase Speed of Analysis While Maintaining Resolution

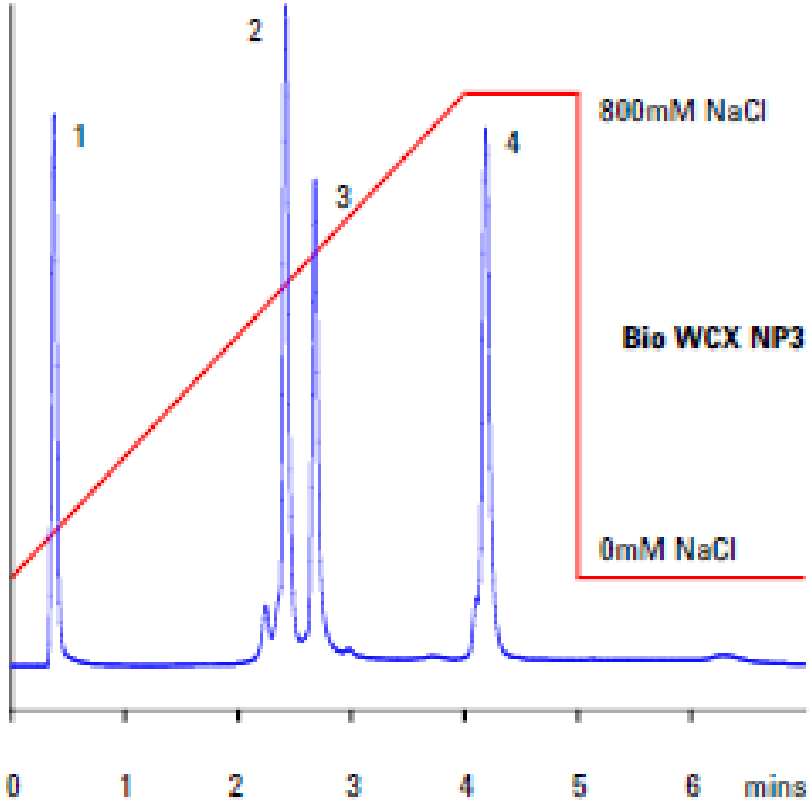
Use smaller IEX particle size



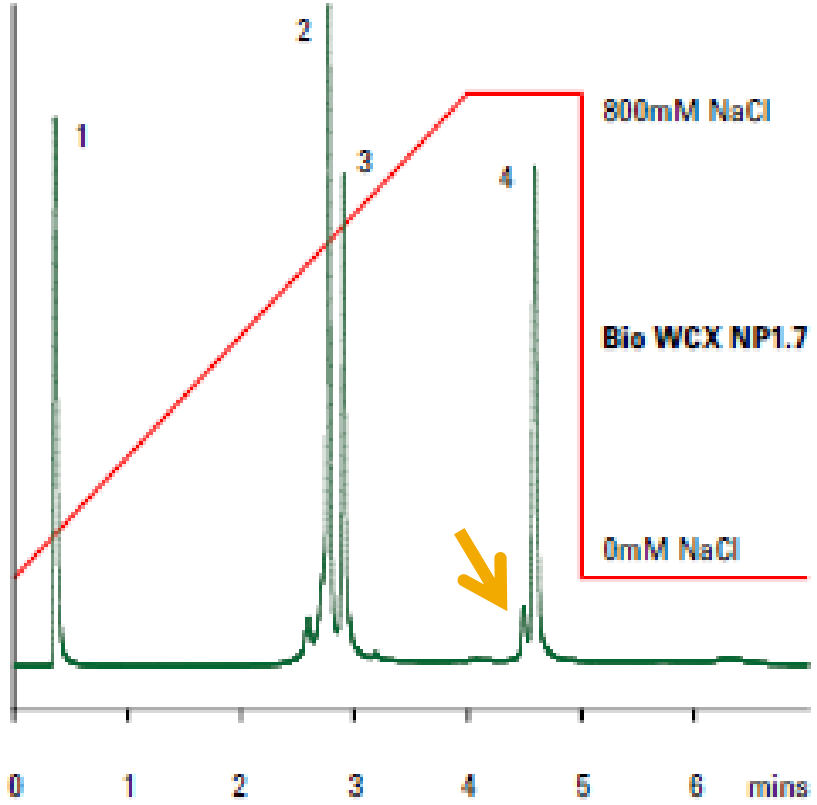
Gradient	20 mM sodium phosphate buffer, pH 6.5 0-800 mM NaCl (0-20 min)	
Flow rate	1.0 mL/min	
Sample	10 µL inj.	
Detection	UV, 220 nm	
Samples:		
1.	Ovalbumin	pI 4.5
2.	RNase A	pI 9.4
3.	Cytochrome C	pI 9.8
4.	Lysozyme	pI 11

Faster, Higher Resolution Separation

Separation with 1.7 µm IEX columns



Bio WCX NP3, 4.6 x 50 mm
p/n 5190-2443
0.5 mL/min



Bio WCX NP1.7, 4.6 x 50 mm
p/n 5190-2441
0.5 mL/min

PL-SAX PL-SCX

Strong anion and strong cation exchange

Agilent InfinityLab

Wide pore polymeric based packings

1000 Å and 4000 Å pore size offerings

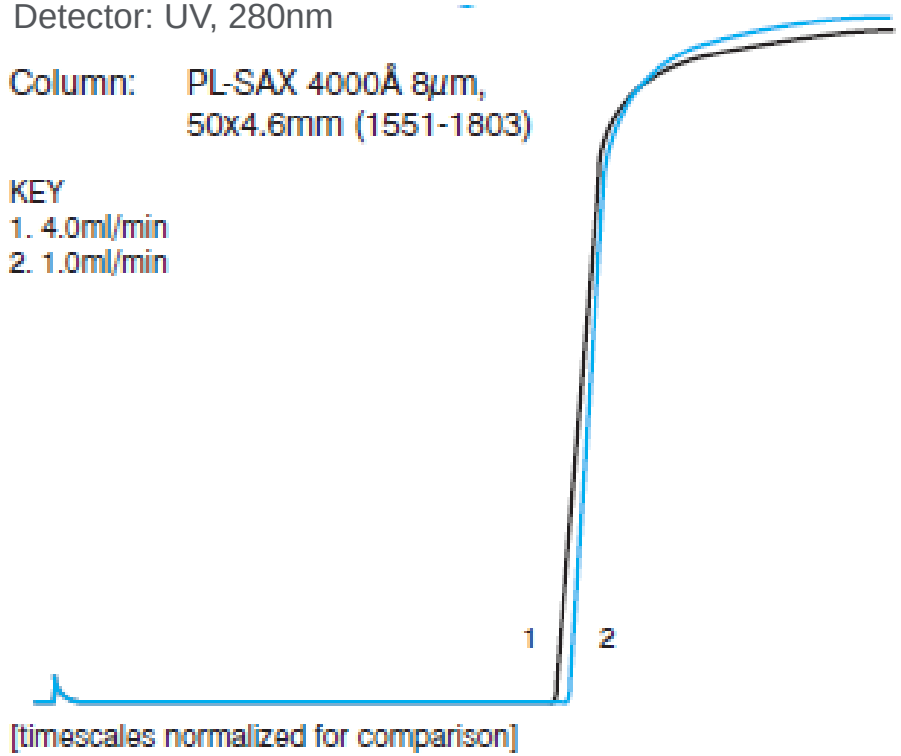
Technical specifications:

PL-SAX 4000 Å	BSA dynamic loading 35 mg/mL
PL-SAX 1000 Å	BSA dynamic loading 80 mg/mL

PL-SCX 4000 Å	Lysozyme dynamic loading 30 mg/mL
PL-SCX 1000 Å	Lysozyme dynamic loading 60 mg/mL

BSA Frontal Loading Curves

Eluent A: 0.01M Tris HCl, pH 8
Eluent B: A + 0.5M NaCl, pH 8
Gradient: Linear 0-100% B in 2 min
Detector: UV, 280nm



Column Selection – Ion Exchange

Application	Agilent Columns	Notes
Monoclonal antibodies	Bio MAb	Thorough characterization of monoclonal antibodies includes the identification and monitoring of acidic and basic isoforms. Agilent Bio MAb HPLC columns feature a unique resin specifically designed for high-resolution charge-based separations of monoclonal antibodies.
Peptides and proteins	Bio IEX	Agilent Bio IEX columns are packed with polymeric, nonporous, ion-exchange particles. The Bio IEX columns are designed for high resolution, high recovery and highly efficient separations.
Proteins, peptides and deprotected synthetic oligonucleotides Globular proteins and peptides Very large biomolecules/ high speed	PL-SAX 1000 Å PL-SAX 4000 Å PL-SAX 1000 Å PL-SAX 4000 Å	The strong anion exchange functionality, covalently linked to a fully porous chemically stable polymer, extends the operating pH range. In addition, the anion-exchange capacity is independent of pH. For synthetic oligonucleotides, separations using denaturing conditions of temperature, organic solvent, and high pH are all possible. The 5 µm media delivers separations at high resolution with the 30 µm media used for medium pressure liquid chromatography.
Small peptides to large proteins Globular proteins Very large biomolecules/ high speed	PL-SCX 1000 Å PL-SCX 4000 Å PL-SCX 1000 Å PL-SCX 4000 Å	PL-SCX is a macroporous PS/DVB matrix with a very hydrophilic coating and strong cation-exchange functionality. This process is controlled to provide the optimum density of strong cation-exchange moieties for the analysis, separation and purification of a wide range of biomolecules. The 5 µm media delivers separations at higher resolution with the 30 µm media used for medium pressure liquid chromatography.
Antibodies (IgG, IgM), plasmid DNA, viruses, phages and other macro biomolecules Viruses, DNA, large proteins Plasmid DNS, bacteriophages Proteins, antibodies	Bio-Monolith QA Bio-Monolith DEAE Bio-Monolith SO_3 Bio-Monolith QA Bio-Monolith DEAE Bio-Monolith SO_3	Strong cation-exchange, strong and weak anion-exchange phases. Bio-Monolith HPLC columns are compatible with preparative LC systems, including Agilent 1100 and 1200 Infinity Series.

Table from: Agilent BioColumns: Charge Variant Analysis
Publication number: 5994-0034EN

Affinity

Affinity chromatography is a method of separating biochemical mixture based on a highly specific interaction between antigen and antibody, enzyme and substrate, receptor and ligand, or protein and nucleic acid.

Affinity chromatography is a powerful technique which takes advantage of highly specific molecular interactions.

The Protein A affinity column is designed for the analytical separation of all IgG (human and mouse), except for IgG class 3.

Protein G offers alternate selectivity for those IgG molecules that do not bind to Protein A.

Agilent InfinityLab Chromatography Columns

Agilent InfinityLab Chromatography Column Offerings

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Bio-Monolith Protein G	**Bio MAb** (WCX)		**Bio SEC-3**	**Poroshell Chiral CD** (Hydroxypropylated b cyclodextrin)
	PL-SAX, PL-SCX		**Bio SEC-5**	**Poroshell Chiral V** (Vancomycin)
	Zorbax IEX (SCX, SAX)		ProSEC 300S	**Poroshell Chiral T (Teicoplanin)**
	Bio-Monolith QA, DEAE, SO_3^-)		Zorbax GF250 and GF450	

Analytical Bio-Monolith Protein A and G Columns

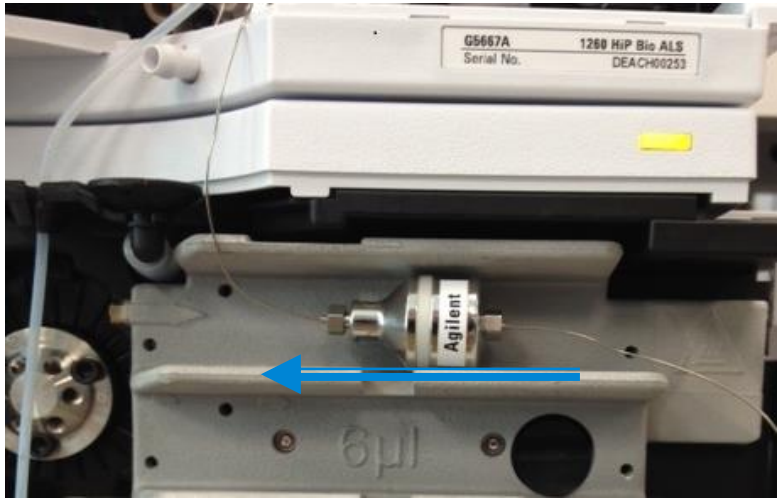
Agilent InfinityLab

Used for

- Fast screening of harvest cell culture samples for IgG – process optimization
- Accurate analysis of mAb quantities to determine protein harvest
- Capture and purification of protein for further characterization

Features

- Bio-Monolith Protein A and G (immunoaffinity)
- Monolith type material for fast, flow rate-independent separations
- Monolith material does not clog easily with cell debris
- Attaches easily to all LCs with standard fittings



Bio-Monolith Protein A Column p/n 5069-3639

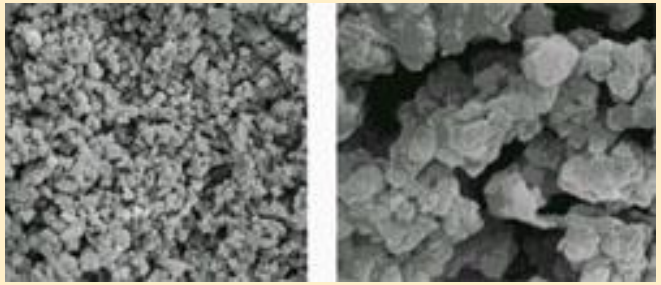
What is a Monolith – Agilent BioMonolith Specifics

Agilent InfinityLab

Agilent BioMonoliths

- Highly cross-linked polymer material – poly(glycidyl methacrylate –co-ethylene dimethacrylate)
- Well-defined channels of 1200 – 1500 nm for large molecules
- Disc with short bed format for fast analysis (desirable for Protein A & G)

Protein A Column



Monolith packing

SEC
Terminology and why do SEC?

SEC refers to the chromatographic technique that separates compounds by their size.

Same technique, but different acronyms:

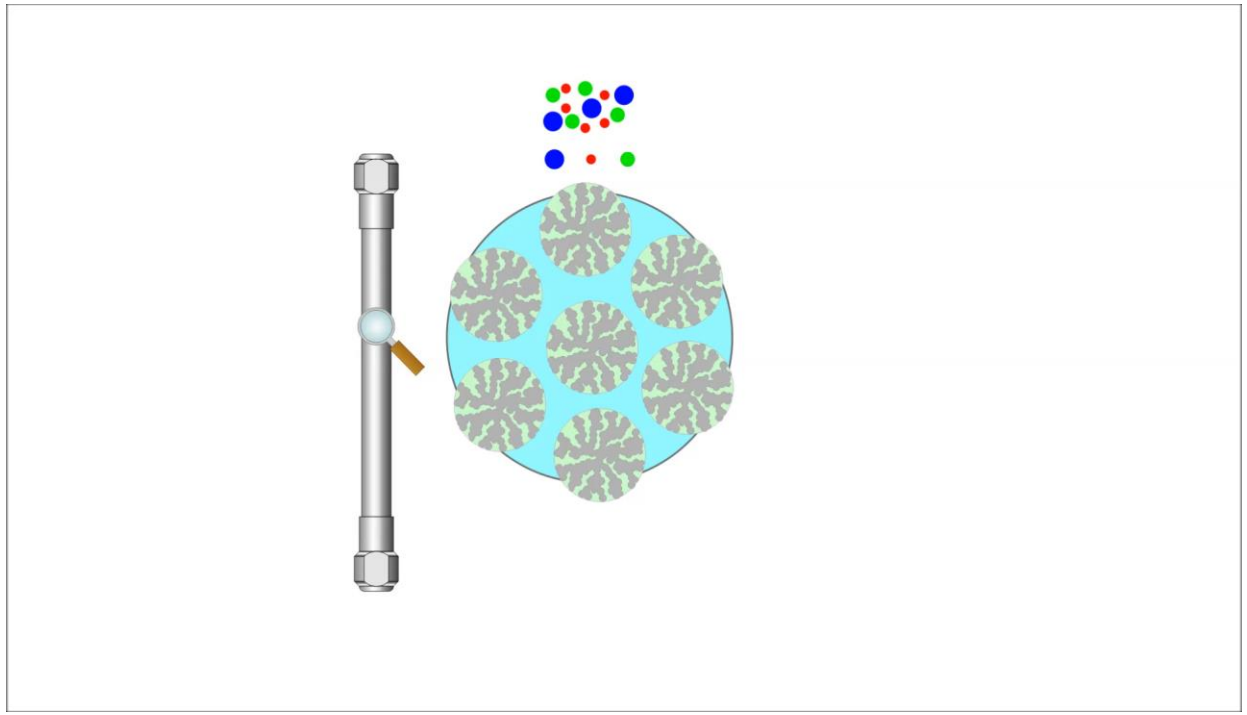
- **SEC** – Size exclusion chromatography
  - Primarily water and buffer
- **GFC** – Gel filtration chromatography
  - Water and buffer, common term for industrial purification step in the life sciences industry

SEC is a chromatography separations method typically used for the qualitative and quantitative analysis of protein aggregates, such as mAbs and antibody drug conjugates (ADCs).

It is important because aggregates are critical quality attributes (CQAs) since they may alter biotherapeutic efficacy or immunogenicity.

GPC/SEC Separation Mechanism

- An SEC column is packed with porous beads of controlled porosity and particle size
- Sample is prepared as a dilute solution in the eluent and injected into the system
- Large molecules are not able to permeate all of the pores and have a shorter residence time in the column
- Small molecules permeate deep into the porous matrix and have a long residence time in the column
- Sample molecules are separated according to molecular size, eluting largest first, smallest last



Agilent InfinityLab Chromatography Columns

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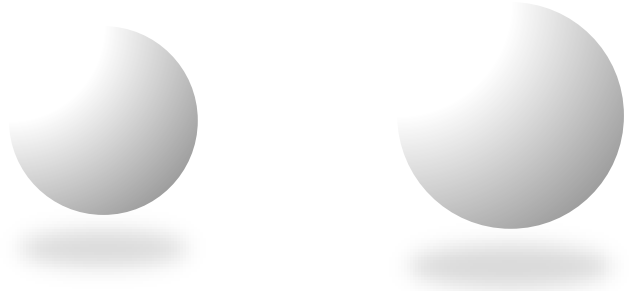
	Zorbax IEX (SCX, SAX)		ProSEC 300S	**Poroshell Chiral T (Teicoplanin)**
	Bio-Monolith (QA, DEAE, SO_3^-)		Zorbax GF250 and GF450	

Agilent Size Exclusion Columns

Agilent InfinityLab

AdvanceBio SEC	AdvanceBio SEC	Agilent Bio SEC-3	Agilent Bio SEC-5	ProSEC 300S	ZORBAX GF-250 and GF-450
1.9 µm	2.7 µm	3 µm	5 µm	5 µm	4 µm, 6 µm
200 Å, 120 Å	130 Å, 300 Å	100 Å, 150 Å, 300 Å	100 Å, 150 Å, 300 Å, 500 Å, 1000 Å, 2000 Å	Nominal 300 Å (linear resolving range)	150 Å, 300 Å
Coated silica (USP L59)	Coated silica (USP L59)	Coated silica (USP L59)	Coated silica (USP L59)	Silica Diol (USP L20)	Zirconium stabilized silica diol (USP L35)
mAb and ADC analysis • Dimer/monomer • LMW mAb fragments **Small proteins and peptides**	**mAb and ADC analysis** • Higher-order aggregates • Dimer/monomer **Small proteins and peptides**	• Polypeptide to small proteins • MS capable separations	• Broadest range of pore sizes for wide variety of biomolecules	• Unique linear resolving range • 30 cm and 60 cm column lengths	• Legacy product • Larger column dimensions • Ideal for GF-450 and GF-250 in series



Recommended Starting Conditions

Agilent InfinityLab

Choose initial columns and conditions for size-based separation of biomolecules, aggregation analysis—peptides, polypeptides, and proteins

Peptides, polypeptides, proteins
Mol Wt >0.1–1,250 kda

Peptides, polypeptides, proteins
Mol Wt >0.1–10,000 kda

Select column based on molecular weight range and pore size

AdvanceBio SEC (2.7 µm)

Pore size	Mol Wt range, kDa
130 Å	0.1–120
300 Å	5–1,250

Bio SEC-3 (3 µm)

Pore size	Mol Wt range, kDa
100 Å	0.1–100
150 Å	0.5–150
300 Å	5–1,250

Bio SEC-5 (5 µm)

Particle size, µm	Flow rate, mL/min
100 Å	0.1–100
150 Å	0.5–150
300 Å	5–1,250
500 Å	15–5,000
1000 Å	50–7,500
2000 Å	>10,000

Recommended Starting Conditions

Agilent InfinityLab

Select column based on molecular weight range and pore size

AdvanceBio SEC (2.7 µm)

Pore size	Mol Wt range, kDa
130 Å	0.1–120
300 Å	5–1,250

Bio SEC-3 (3 µm)

Pore size	Mol Wt range, kDa
100 Å	0.1–100
150 Å	0.5–150
300 Å	5–1,250

Bio SEC-5 (5 µm)

Particle size, µm	Mol Wt range, kDa
100 Å	0.1–100
150 Å	0.5–150
300 Å	5–1,250
500 Å	15–5,000
1000 Å	50–7,500
2000 Å	>10,000

Columns: AdvanceBio SEC
Bio SEC (3 µm and 5 µm)

Mobile phase: Phosphate buffer 150 mM, pH 7.0*

Gradient: Isocratic in 15 to 60 min range

Temperature: Recommended 10 to 30 °C, maximum 80 °C

Flow rate: 0.1 to 0.4 mL/min for 4.6 mm id columns
0.1 to 1.25 mL/min for 7.8 mm id columns
1.0 to 10.0 mL/min for 21.2 mm id columns

Sample size: ≤5% of total column volume

* Other aqueous buffers with high and low salt can be used

Buffer concentration and ionic strength can impact retention time, peak shape, and resolution
Adjustments can be made depending on your sample requirements.

Buffers and SEC: Criteria for Optimal Mobile Phase

The optimal eluent for the separation should be determined by the characteristics of the column stationary phase and the proteins/polymers to be analyzed, so that nonspecific interactions are minimized.

- Mobile phase should contain enough buffer salt (to overcome ionic interactions).
- Mobile phase should not contain too much buffer salt (to prevent hydrophobic interactions).
- Mobile phase should not alter the analyte (cause degradation/aggregation).
- Mobile phase should be made up fresh and used promptly (bacterial growth is rapid in dilute buffer stored at room temperature).
- Buffer shelf life <7 days unless refrigerated.
- Mobile phase should be filtered before use. Particulates may be present in water (less likely) or in buffer salts (more likely).

Resolution in SEC

Running two columns in series, same pore size

- Increase pore volume, increases resolution

Running two columns in series, different pore size

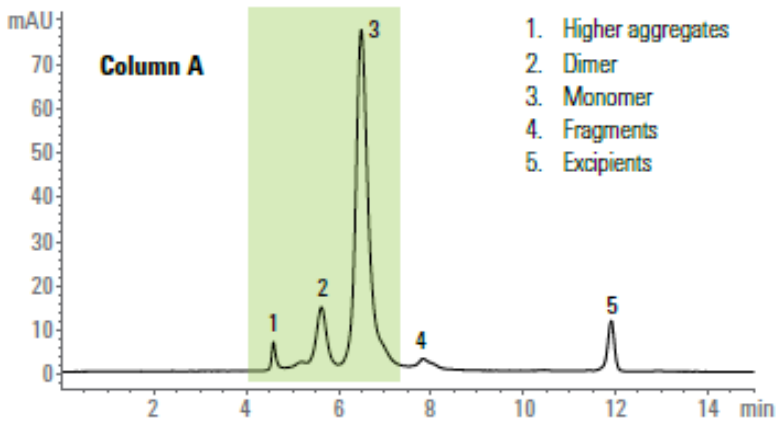
- Extends the resolving range and enables analysis of multiple attributes in one run

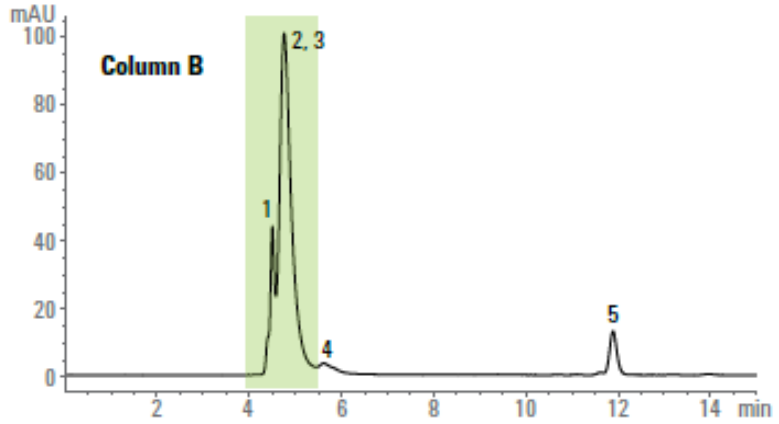
Use a packing with a smaller particle size

- Decrease particle size, increase column efficiency

Importance of Pore Size Selection
Sample

Polyclonal IgG separation

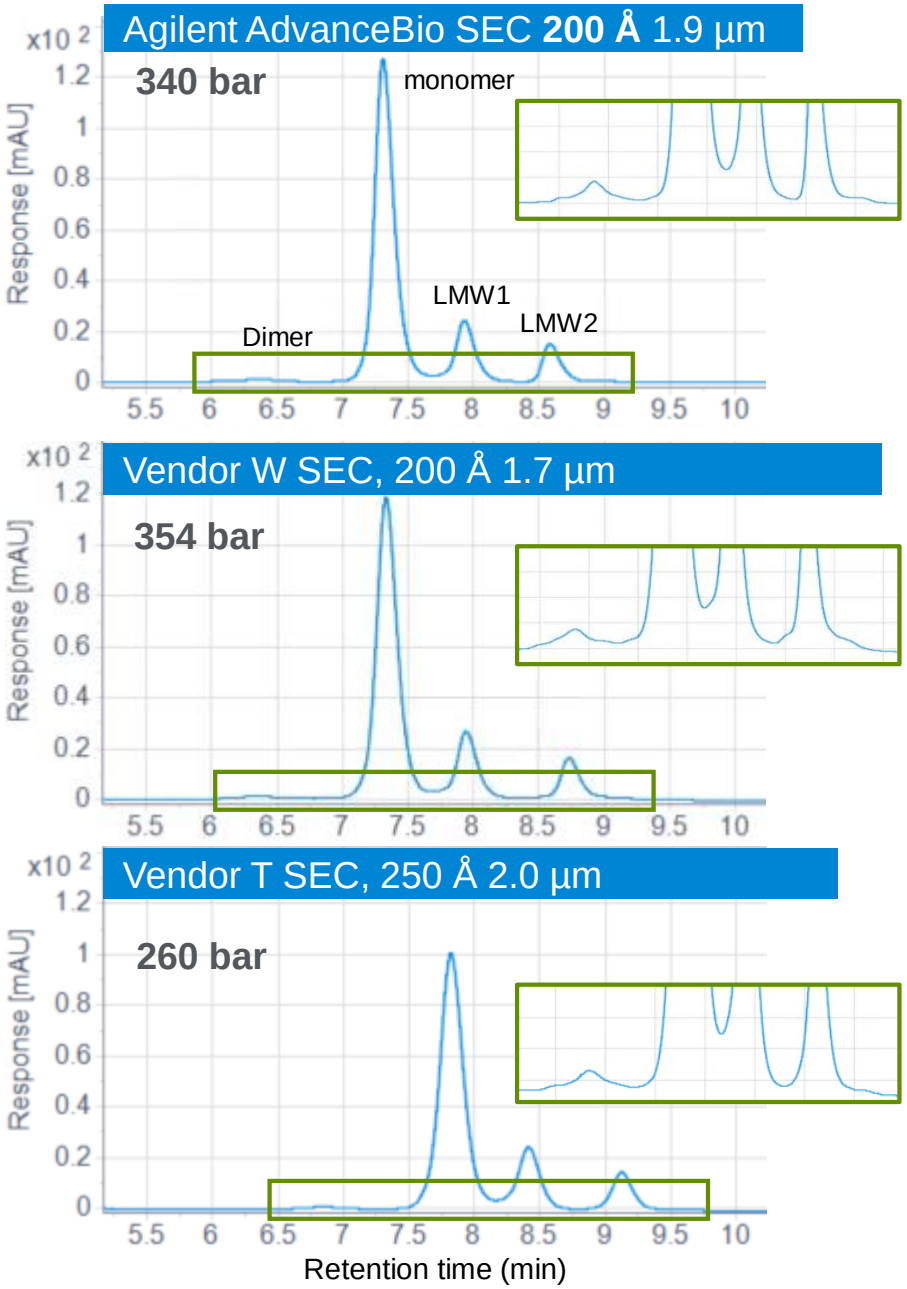




Column A: **AdvanceBio SEC 300Å**
4.6 x 300 mm, 2.7 µm (p/n PL1580-5301)

Column B: **AdvanceBio SEC 130Å**
4.6 x 300 mm, 2.7 µm (p/n PL1580-5350)

Instrument:	Agilent 1260 Infinity Bio-inert Quaternary LC System
Mobile phase:	150 mM phosphate buffer, pH 7.0
Flow rate:	0.35 mL/min
Detector:	UV, 220 nm
Sample:	Polyclonal IgG



LC Conditions	1260 Infinity II Bioinert LC System
Column dimension	4.6 x 300 mm
Mobile phase	50 mM sodium phosphate, 200 mM NaCl, pH 7.0
Temperature	25 °C
Sample	SigmaMAb (spiked with its $F(ab')_2$ and Fc fragments)
Flow rate	0.35 mL/min
UV detection	220 nm

	Peak Width at Half Height			Resolution (Rs)	
	Monomer	LMW1	LMW2	Dimer / monomer	Monomer / LWM1
AdvanceBio SEC 200 Å 1.9 µm	0.159	0.154	0.148	2.79	2.28
Vendor W SEC 200 Å 1.7 µm	0.172	0.166	0.160	2.46	2.09
Vendor T SEC 250 Å 2.0 µm	0.194	0.182	0.169	2.49	1.83

mAb aggregate and fragment separation

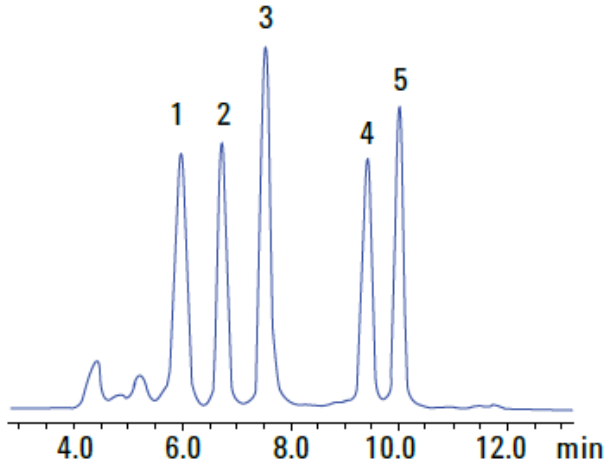
For AdvanceBio SEC, we see:

Better resolution

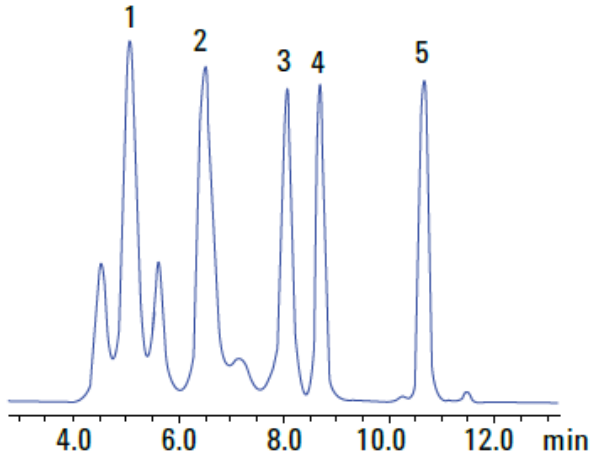
Sharper peaks

Lower back pressure than 1.7 µm

AdvanceBio SEC Protein Standards



AdvanceBio SEC 130Å Protein Standard separation on AdvanceBio SEC 130Å column



AdvanceBio SEC 300Å Protein Standard separation on AdvanceBio SEC 300Å column

AdvanceBio SEC 130Å Protein Standard p/n 5190-9416, 1.5 mL vial)	
Analyte	**MW**
1. Ovalbumin	45,000
2. Myoglobin	17,000
3. Aprotinin	6,700
4. Neurotensin	1,700
5. Angiotensin II	1,000

AdvanceBio SEC 300Å Protein Standard (p/n 5190-9417, 1.5 mL vial)	
Analyte	**MW**
1. Thyroglobulin	670,000
2. γ-globulin	150,000
3. Ovalbumin	45,000
4. Myoglobin	17,000
5. Angiotensin II	1,000

Agilent InfinityLab Chromatography Columns

Agilent InfinityLab Chromatography Column Offerings

Affinity	Ion Exchange	HIC	Size Exclusion	Chiral
Selective affinity between phase and molecule	*Complementary attraction between opposite charges*	*Hydrophobic interactions between phase and protein*	*Noninteractive with stationary phase – size in solution*	*Multiple types of interactions between phase and molecule*
Bio-Monolith Protein A	**Bio IEX** (SCX, WCX, SAX, WAX)	**AdvanceBio HIC**	**AdvanceBio SEC**	**Poroshell Chiral CF** (Derivatised cyclofructan)
Bio-Monolith Protein G	**Bio MAb** (WCX)		**Bio SEC-3**	**Poroshell Chiral CD** (Hydroxypropylated b cyclodextrin)
	PL-SAX, PL-SCX		**Bio SEC-5**	**Poroshell Chiral V** (Vancomycin)
	Zorbax IEX (SCX, SAX)		ProSEC 300S	**Poroshell Chiral T (Teicoplanin)**
	Bio-Monolith QA, DEAE, SO_3^-)		Zorbax GF250 and GF450	

Stationary Phase	Fully Porous (ZORBAX)	Superficially Porous (Poroshell)
Conventional RP	Eclipse Plus C8 Eclipse Plus C18	EC-C8 EC-C18 Bonus RP Phenyl-Hexyl
Pure Silica	Rx-SIL	--
HILIC	HILIC Plus	HILIC
Cyano	Eclipse XDB-CN	EC-CN

Columns for SFC

Thank you for attending

Any questions?

Contact Agilent Chemistries and Supplies Technical Support

- 1-800-227-9770 option 3, option 3:
- Option 1 for GC and GC/MS columns and supplies
- Option 2 for LC and LC/MS columns and supplies
- Option 3 for sample preparation, filtration, and QuEChERS
- Option 4 for spectroscopy supplies
- Option 5 for chemical standards

*Available 8am – 5pm EST – PST in U.S. and Canada

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- lc-column-support@agilent.com
- spp-support@agilent.com
- spectro-supplies-support@agilent.com
- Chem-standards-support@Agilent.com