Why Use HILIC and HIC Chromatography?

Theory and method development strategies

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Agenda

1. What is HILIC and when should you consider it?
2. How to approach HILIC method development
   - Agilent HILIC column options
   - Mobile phase considerations
3. Common HILIC application areas
4. Tips and tricks for successful HILIC column use and care
   - Column equilibration
   - Sample solvent compatibility
   - Inert HILIC solution for metal-sensitive compounds

Agenda

Agilent InfinityLab

6 What is HIC and its separation mechanism?

7 Requirements for HIC

- Factors affecting selectivity

8 Common HIC application areas

- Post-translational modification (PTM) analysis
- Separation of NIST mab (RM 8671)
- Monitoring oxidation in mAbs

What Is HILIC and When Should You Consider It?

HILIC compared to RPLC

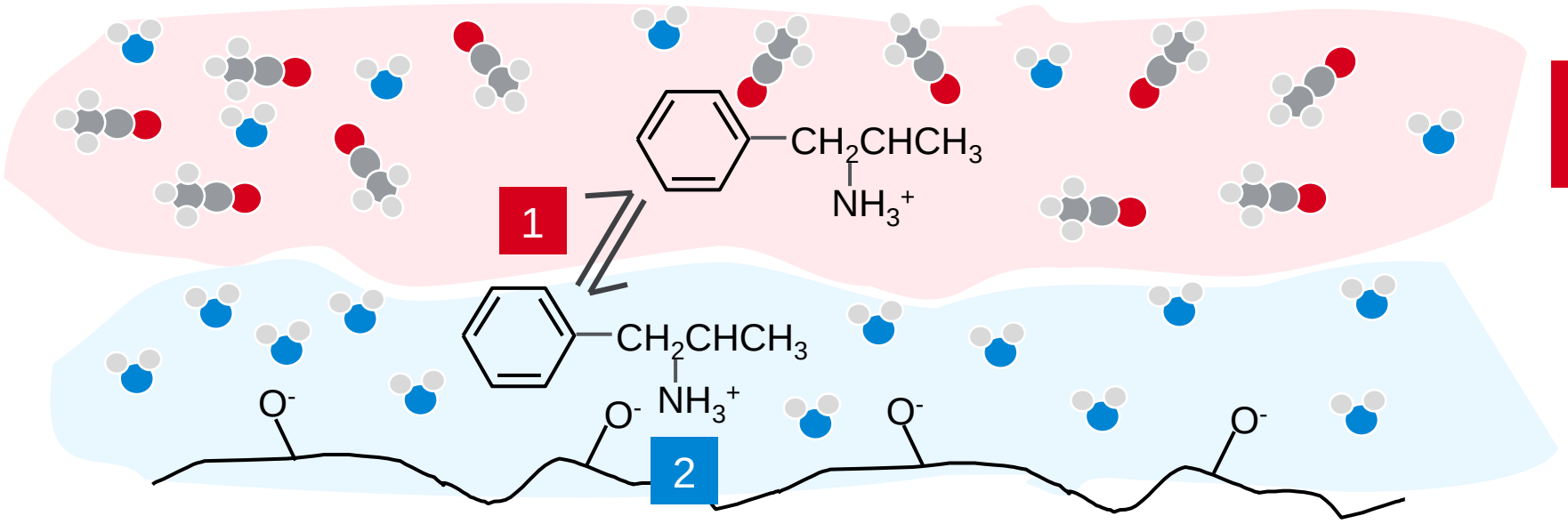
What Is HILIC and When Should I Consider It?

HILIC retention mechanism

A water layer is adsorbed onto the polar silica surface, creating a liquid/liquid extraction system.

1 Polar analytes can partition into and out of the water layer, with more polar analytes having a stronger interaction.

2 Charged polar analytes can also undergo ion exchange with the silica surface.



Elution is typically from least to most polar, the opposite of RPLC.

Solvent strengths in HILIC mode are: THF < acetone < acetonitrile < isopropanol < ethanol < methanol < water.

What Is HILIC and When Should I Consider It?

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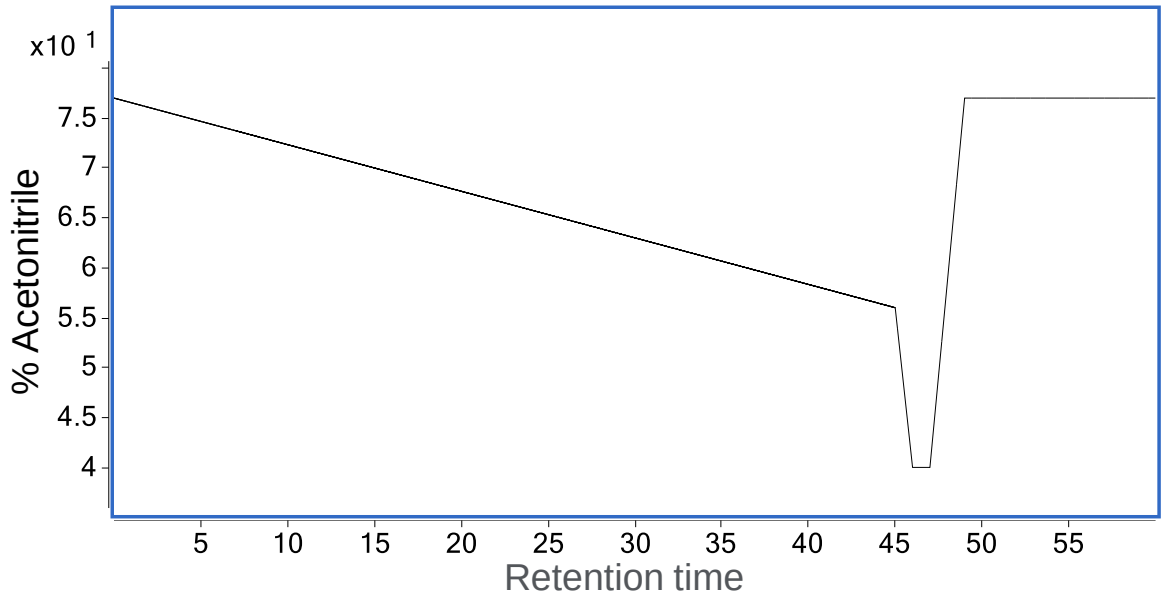
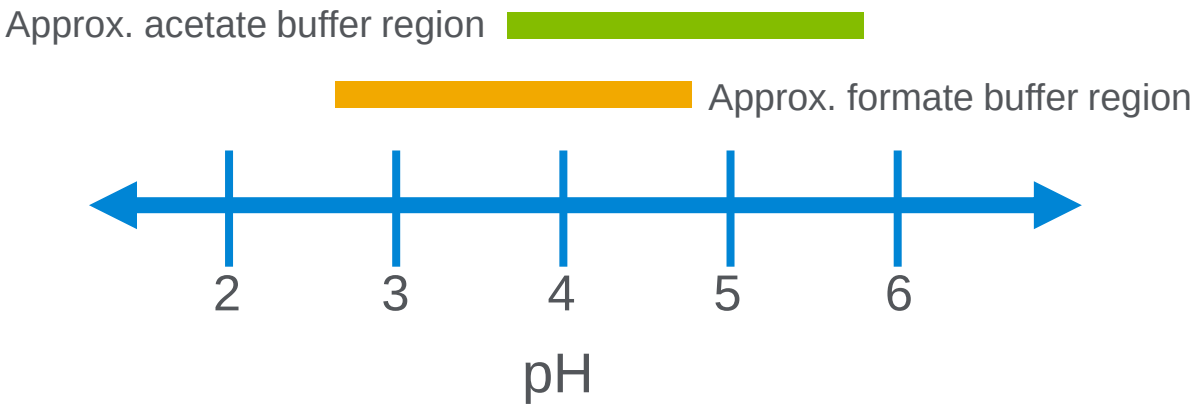
HILIC complements RPLC

Reversed-phase LC		Hydrophilic interaction LC (HILIC)
Nonpolar stationary phase (for example, C18)	Polarity	Polar stationary phase (for example, silica)
Polar mobile phase H_2O/CH_3OH, H_2O/CH_3CN	Mobile phase	Polar mobile phase H_2O/CH_3CN
Decrease retention by decreasing the polarity of the mobile phase ddH_2O ↓ = retention ↑ CH_3CN ↑ = retention ↓	Gradient	Retains hydrophilic (polar and ionized) compounds well and often reverses elution order vs RPLC ddH2O ↑ = retention ↓ CH_3CN ↓ = retention ↑
Polar to nonpolar	Elution order	Nonpolar to polar

HILIC Mobile Phases for Glycans

- Typical gradients use an acetonitrile/aqueous buffer mixture
- Volatile buffers made from ammonium formate or ammonium acetate are most common to allow for downstream MS analysis
- Most common setup:
  - Bottle A: 50 mM ammonium formate, pH 4.4
  - Bottle B: Acetonitrile





What Is HILIC and When Should I Consider It?

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InfinityLab Poroshell 120 offers a broad portfolio to suit your needs

Best All Around	Best for Low pH Mobile Phases	Best for High pH Mobile Phases	Best for Alternative Selectivity	Best for More Polar Analytes	Chiral
EC-C18 1.9 µm, 2.7 µm, 4 µm	SI 1.9 µm,	**RP chemistries for polar analytes**	**Bonus-RP** 2.7 µm	**SB-Aq** 1.9 µm, 2.7 µm, 4 µm	Chiral-V 2.7 µm
EC-C8 1.9 µm, 2.7 µm, 4 µm	S 2		**PFP** 1.9 µm, 2.7 µm, 4 µm	**EC-CN** 2.7 µm	Chiral-T 2.7 µm
Phenyl-Hexyl 1.9 µm, 2.7 µm, 4 µm				**HILIC** 1,9 µm, 2.7 µm, 4 µm	Chiral- CD 2.7 µm
	AdvanceBio Amide HILIC - N-Glycan separations	**HILIC chemistries**		**HILIC-Z** 1.9 µm, 2.7 µm, 4 µm	Chiral-CF 2.7 µm
				HILIC- OH5 2.7 µm	

What Is HILIC and When Should I Consider It?

Agilent InfinityLab

When to chose which separation mode for your sample

01 Find the best column to retain and separate all analytes

02 Consider the sample: analyte solubility and sample solvent

03 Ensure reliable detection of your sample

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Agilent InfinityLab

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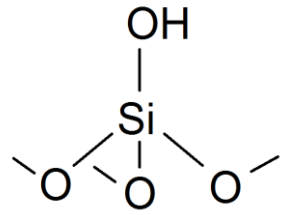
HILIC Method Development – Column Selection

Agilent InfinityLab

InfinityLab Poroshell 120 HILIC column options

Best for polar analytes

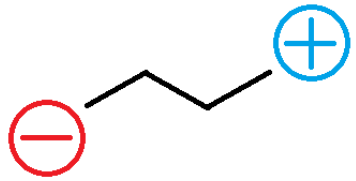
InfinityLab Poroshell
HILIC
1.9 µm, 2.7 µm, 4 µm



HILIC

- Bare silica chemistry
- For very simple mixtures, low column bleed

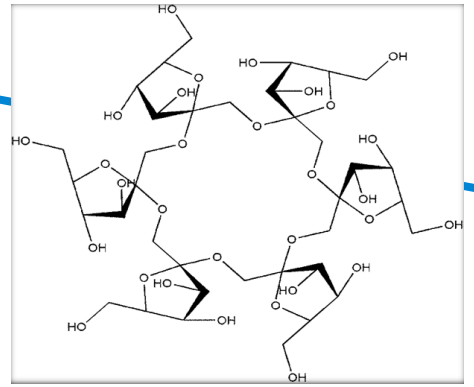
InfinityLab Poroshell
HILIC-Z
1.9 µm, 2.7 µm, 4 µm



HILIC-Z

- Proprietary zwitterionic chemistry, high pH stable
- **Most modern and robust column – start method development here**
- PEEK-lined version available

InfinityLab Poroshell
HILIC-OH5
2.7 µm



HILIC-OH5

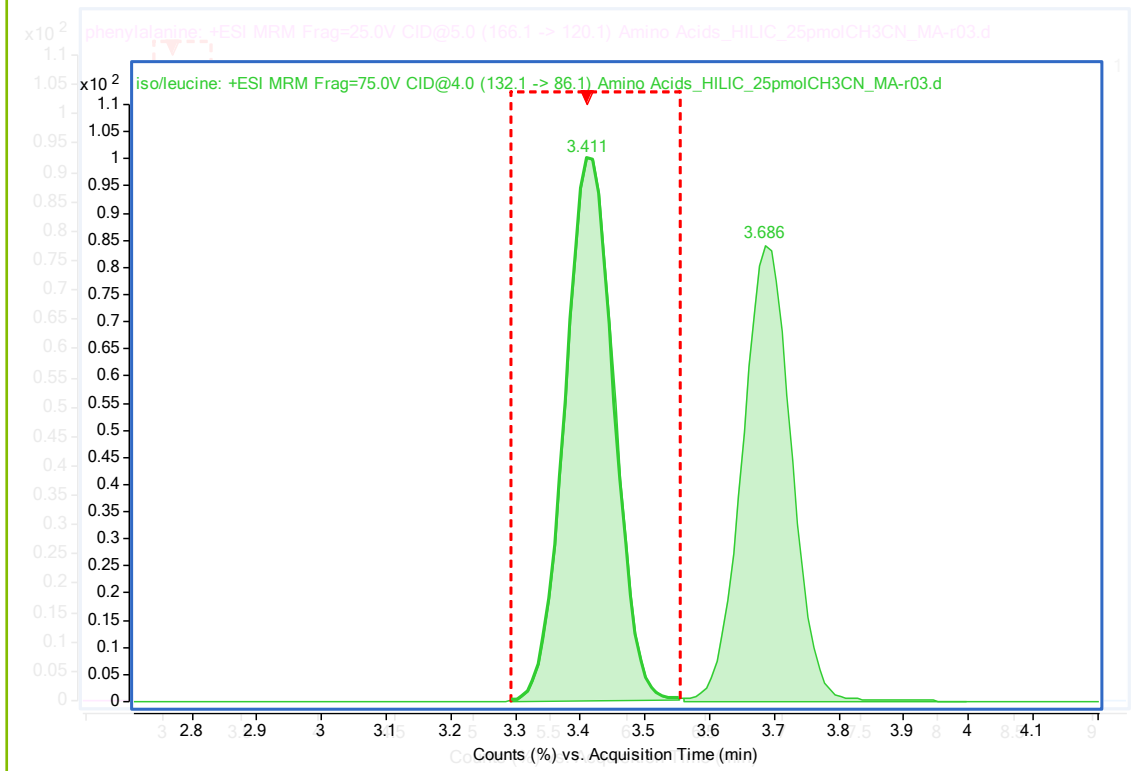
- Brushed fructan chemistry
- Alternative selectivity

Find the Best Column to Retain and Separate All Analytes

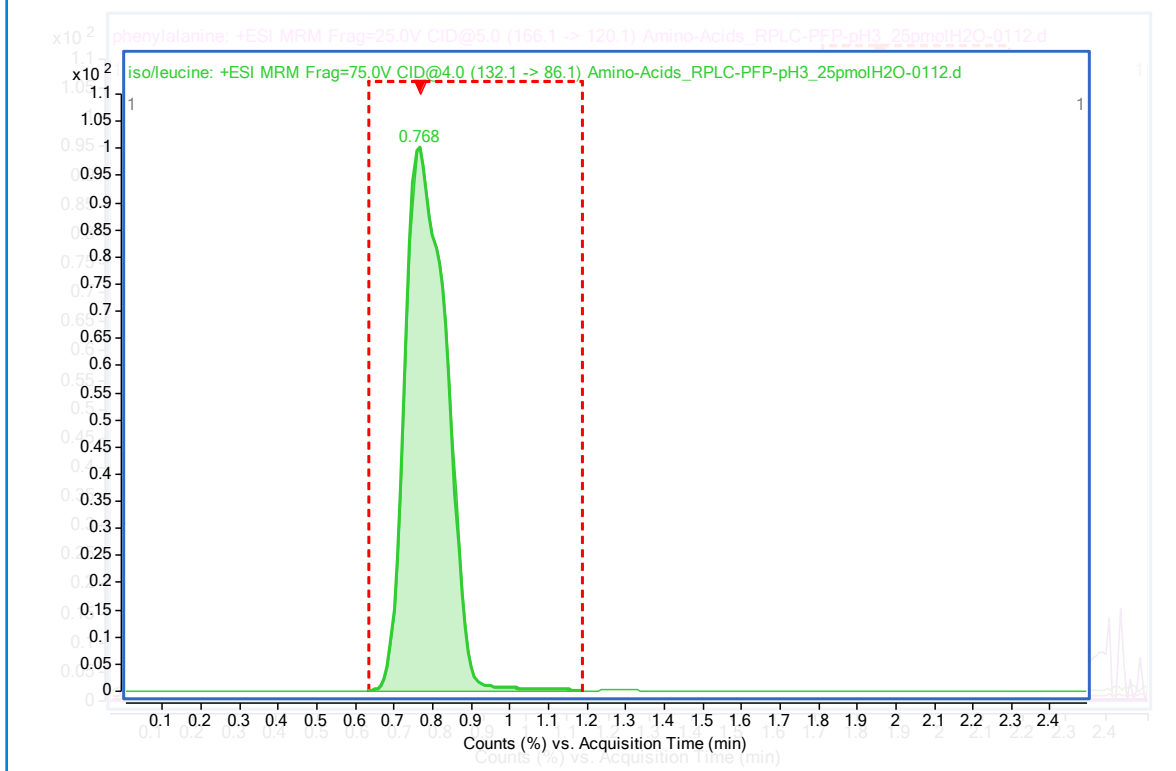
Agilent InfinityLab

HILIC retains amino acids and separates isobars, while RPLC struggles

HILIC: Poroshell 120 HILIC-Z



RPLC: Poroshell 120 PFP



Separation of isobars leucine/isoleucine

Find the Best Column to Retain and Separate All Analytes

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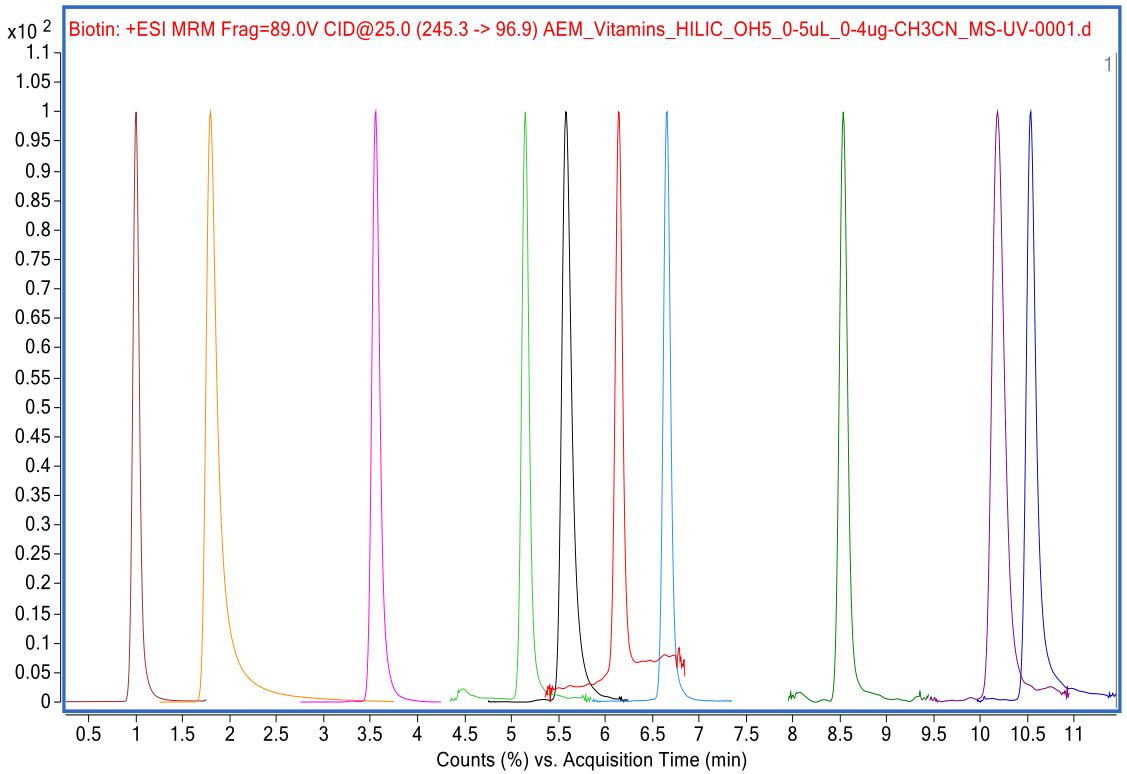
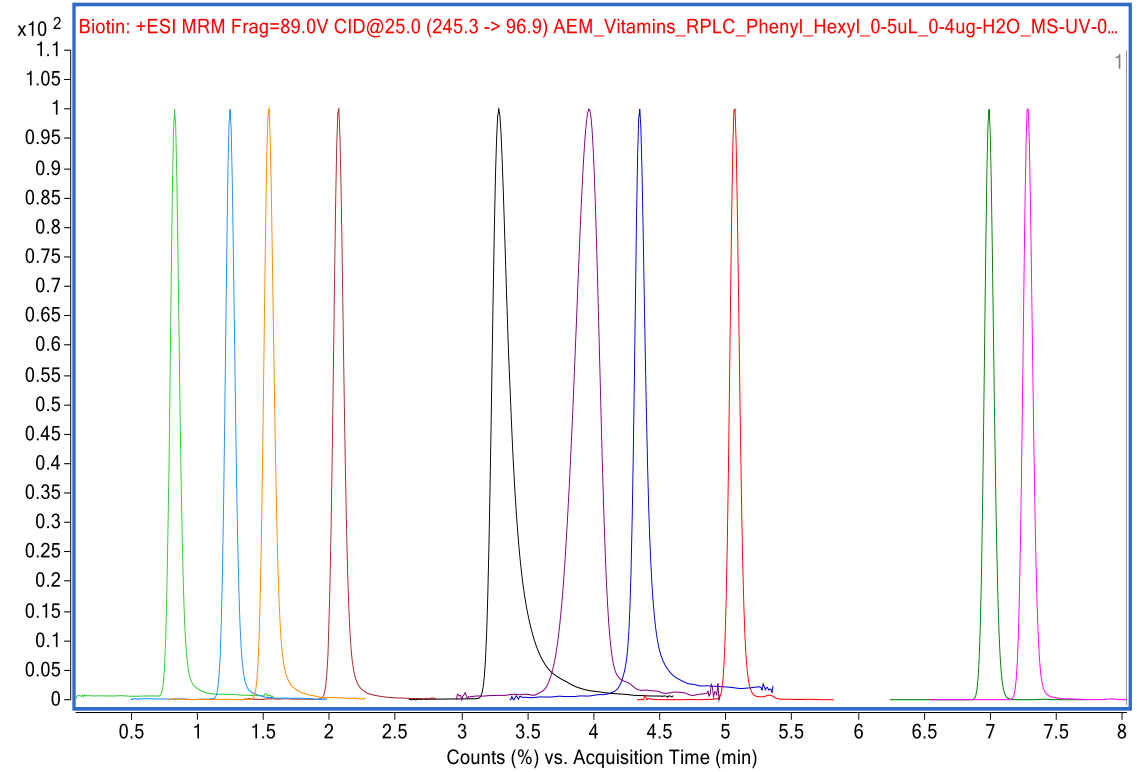
Water-soluble vitamins

HILIC: Poroshell 120 HILIC-OH5



Mobile phase A: H_2O; mobile phase B: CH_3CN; mobile phase D: 200 mM ammonium acetate (no pH adjustment), pH ~6.7; flow rate: 0.5 mL/min; gradient: 95-65% B in 10 min, hold 5% D constant throughout analysis, 5 min postrun; injection: 0.5 µL injection of 0.4 µg/mL vitamin standard in CH_3CN; column: 25 °C, 2.1 x 100 mm, 2.7 µm Agilent InfinityLab Poroshell 120 HILIC-OH5; detection: Ultivo LC/TQ ESI+ dMRM (parameters above), DAD sig = 260 nm, 80 Hz.

RPLC: Poroshell 120 Phenyl-Hexyl



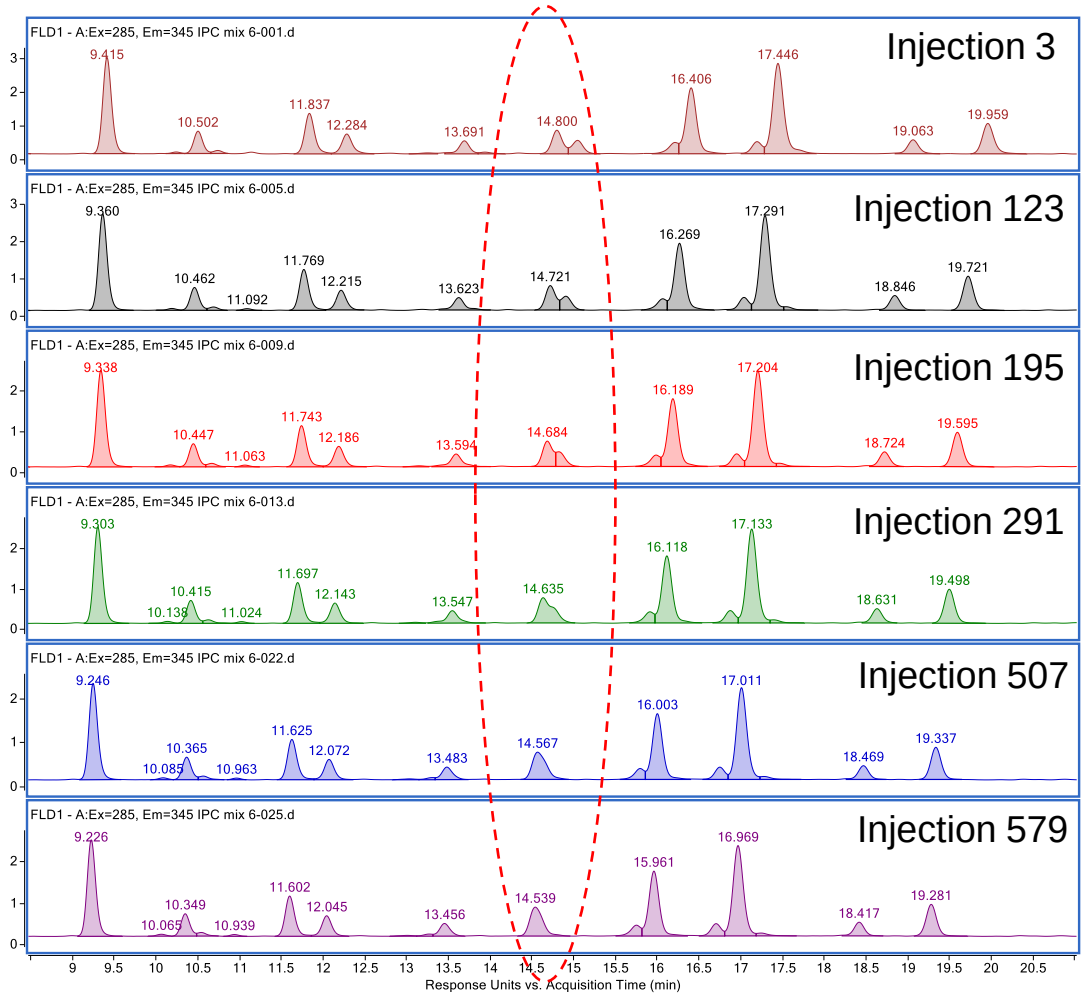
Mobile phase A: H_2O; mobile phase B: CH_3CN; mobile phase D: 200 mM ammonium acetate + 0.2% acetic acid, pH ~5.3; flow rate: 0.5 mL/min; gradient: 0% B for 1 min, 0-25% B in 8 min, hold 5% D constant throughout analysis, 3 min postrun; injection: 0.5 µL injection of 0.4 µg/mL vitamin standard in H_2O; column: 25 °C, 2.1 x 100 mm, 2.7 µm Agilent InfinityLab Poroshell 120 Phenyl-Hexyl; Detection: Ultivo LC/TQ ESI+ dMRM, DAD sig = 260 nm, 80 Hz.

Released Glycan Analysis

Important factors for column selection

- Ability to sufficiently and consistently resolve key glycan pairs
- Column dimensions
- pH and temperature limits
- Longevity and performance throughout lifetime
- Time required for desired separation
- Compatibility with existing HPLC equipment

Competitor 2.1 x 150 mm, 1.7 µm column
Selectivity changes over column lifetime



Find the Best Column to Retain and Separate All Analytes

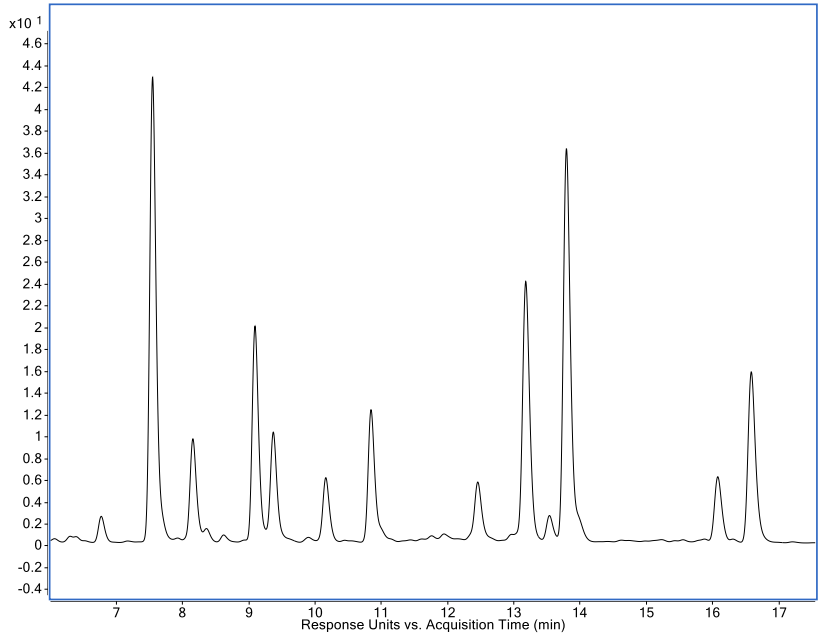
Agilent InfinityLab

N-Glycan separation

The new Agilent AdvanceBio Amide HILIC column is designed with several key improvements to facilitate glycan analysis.

- It is the newest Agilent HILIC LC column for released N-glycan analysis
- Available in:
  - 2.1 x 150 mm (859750-913),1.8 µm, 300 Å
  - 2.1 x 100 mm (858750-913), 1.8 µm, 300 Å
- The AdvanceBio Amide HILIC column demonstrates advantages in critical pair separations, temperature stability, and column lifetime
- Increased charge group separation
- Ability to modulate charge group selectivity with mobile phase buffer strength
- Improved peak capacity
- Allows sub-2 µm column runs on LC instruments with 600 bar pumps (for example, Agilent 1260)

AdvanceBio Amide HILIC



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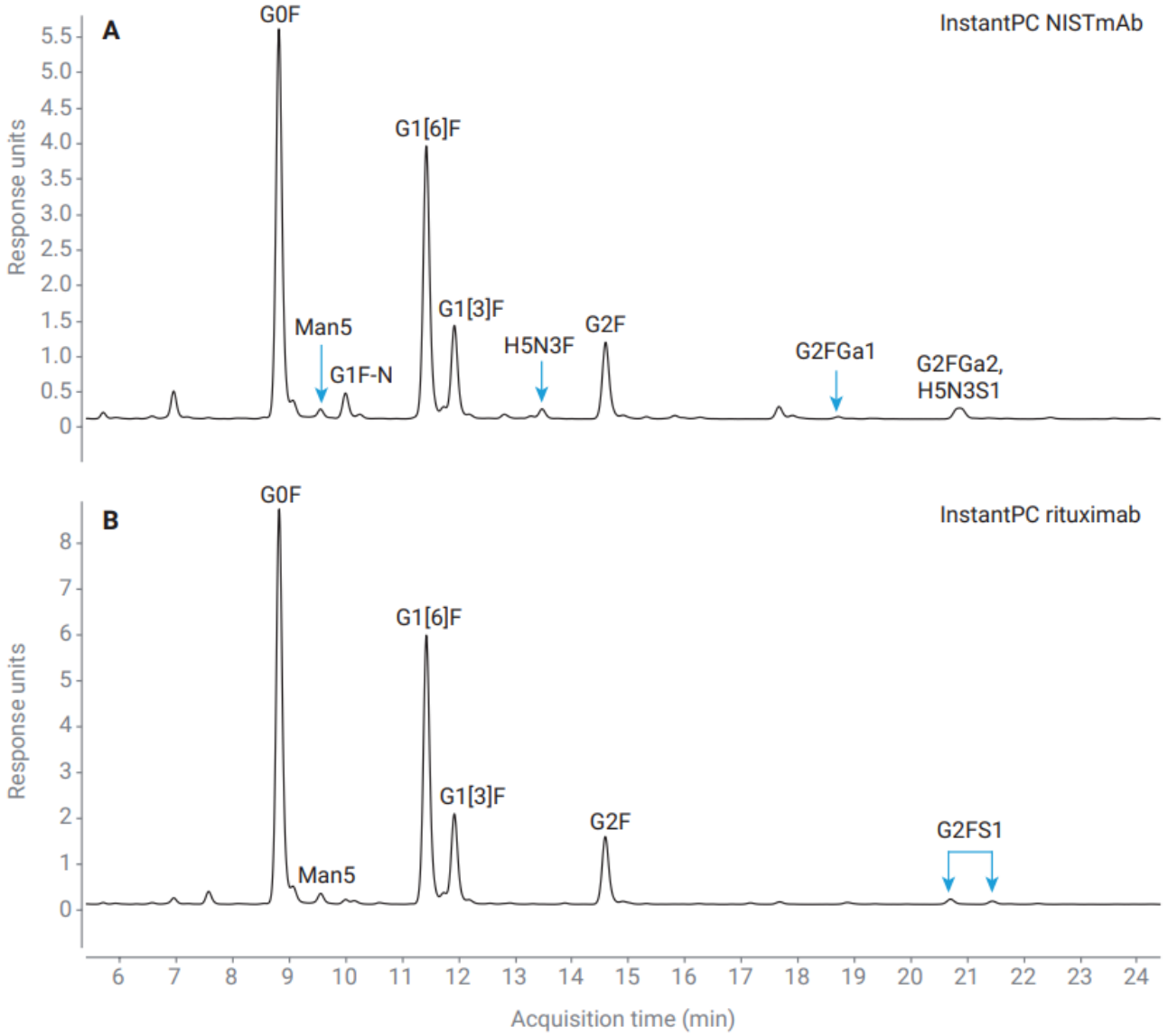
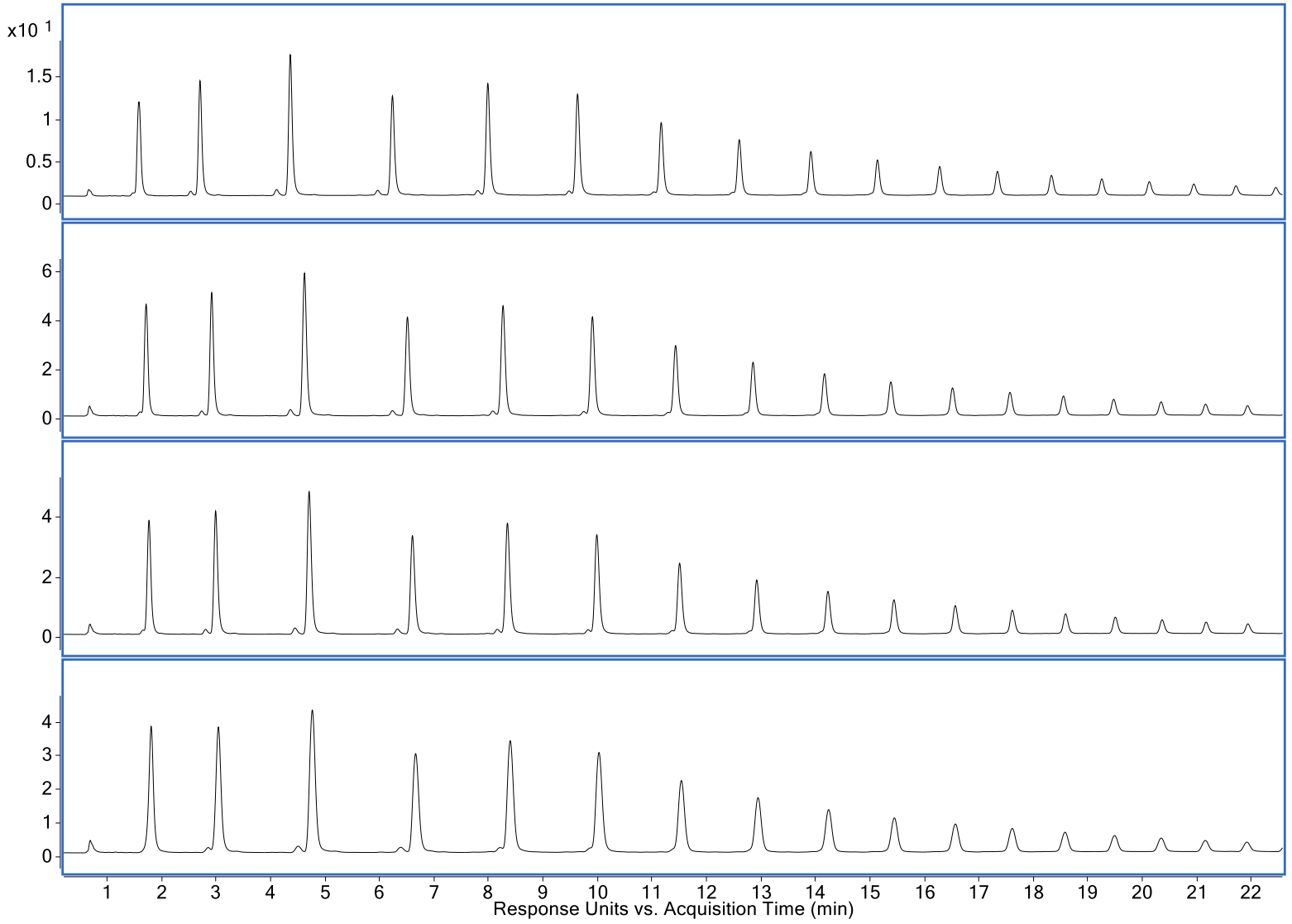
AdvanceBio Amide HILIC Column – Separation of IgG N-Glycans



LC/FLD chromatograms of InstantPC labeled N-glycans from (A) NISTmAb and (B) rituximab.

Agilent application note
5994-6916

Amide HILIC 80 °C Lifetime Study – InstantPC Maltodextrin (GKPC-503)



Injection 2

Injection 194

Injection 410

Injection 578

What Is HILIC and When Should I Consider It?

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When to chose which separation mode for your sample

01 Find the best column to retain and separate all analytes

02 Consider the sample: analyte solubility and sample solvent

03 Ensure reliable detection of your sample

Analyte solubility and sample solvent

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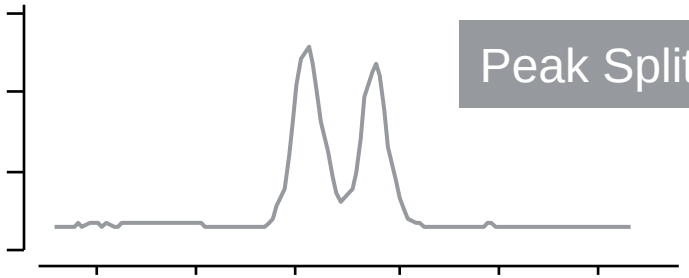
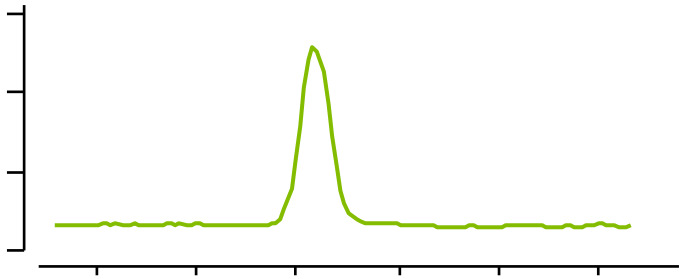
Strong Injection Solvents Distort Peak Shapes for HILIC and RPLC

HILIC: Glufosinate

in **100% H_2O**



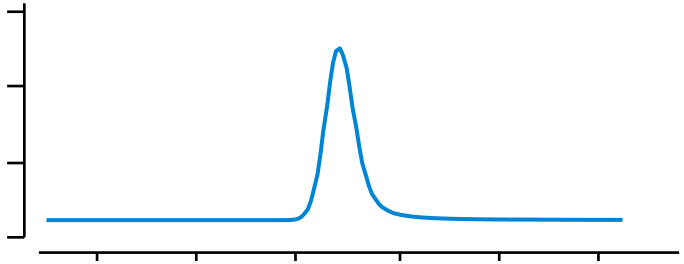
in **1:1 CH_3CN/H_2O**



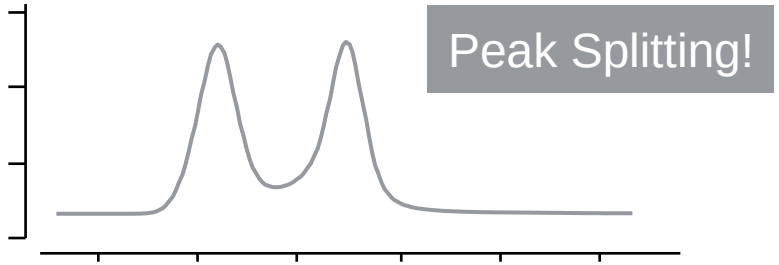
4 uL injection of Glufosinate, 100 ppb, InfinityLab Poroshell HILIC-Z, 2.1x100mm, Temperature: 30 ºC, Flowrate: 0.6 mL/min, Mobile Phase A: 10 mM ammonium acetate, pH 9, Mobile Phase B: 100 mM ammonium acetate, pH 9 in 90% ACN (final concentration: 10 mM), Gradient: 90% B => 60% B in 10 minutes, System: Agilent 6490 LC/QQQ

RPLC: Pyridoxine (Vitamin B6)

in **100% H_2O**



in **9:1 CH_3CN/H_2O**



0.5 uL injection of 13 ug/mL pyridoxine, A: H_2O; B: CH_3CN; D: 200 mM Ammonium Acetate + 0.2% Acetic Acid, pH ~5.3; 0.5 mL/min; Gradient: 0% B for 1 min, 0-25% B in 8 min, hold 5% D constant throughout analysis; Column: 25 ºC, 2.1 x 100 mm, 2.7 µm Agilent InfinityLab Poroshell 120 Phenyl-Hexyl; Detection: Ultivo TQ/MS ESI+ dMRM

Analyte solubility and sample solvent

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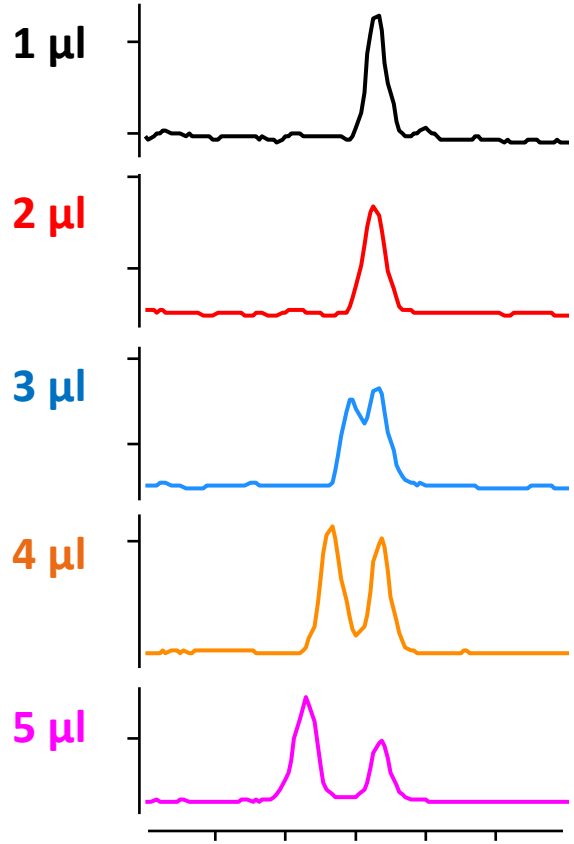
Strong Solvent Effects are Greater with Larger Injection Volumes

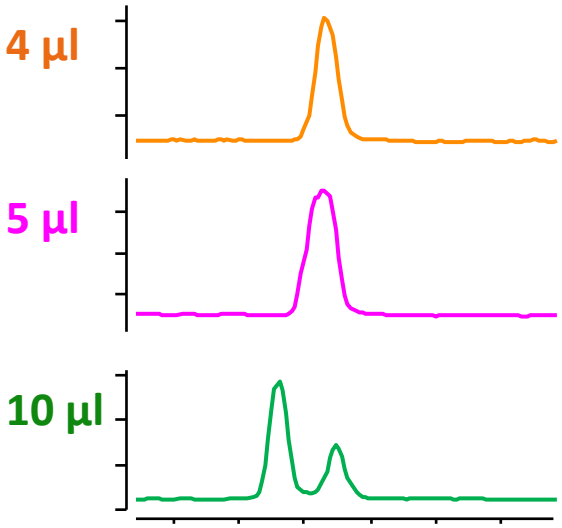
Sample Solvent: **100% Water**



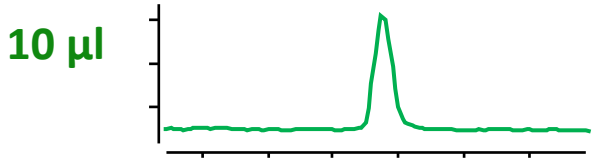
Sample: Glufosinate, 100 ppb
Column: InfinityLab Poroshell HILIC-Z, 2.1x100mm
Temperature: 30 ºC
Flowrate: 0.6 mL/min
Mobile Phase A: 10 mM ammonium acetate, pH 9
Mobile Phase B: 100 mM ammonium acetate, pH 9 in 90% ACN (final concentration: 10 mM)
Gradient: 90% B => 60% B in 10 minutes
System: Agilent 6490 LC/QQQ

Note: System required phosphoric acid wash

Sample Solvent: **50:50 CH_3CN/H_2O**



Sample Solvent: **80:20 CH_3CN/H_2O**



Ensure Samples are Completely Soluble

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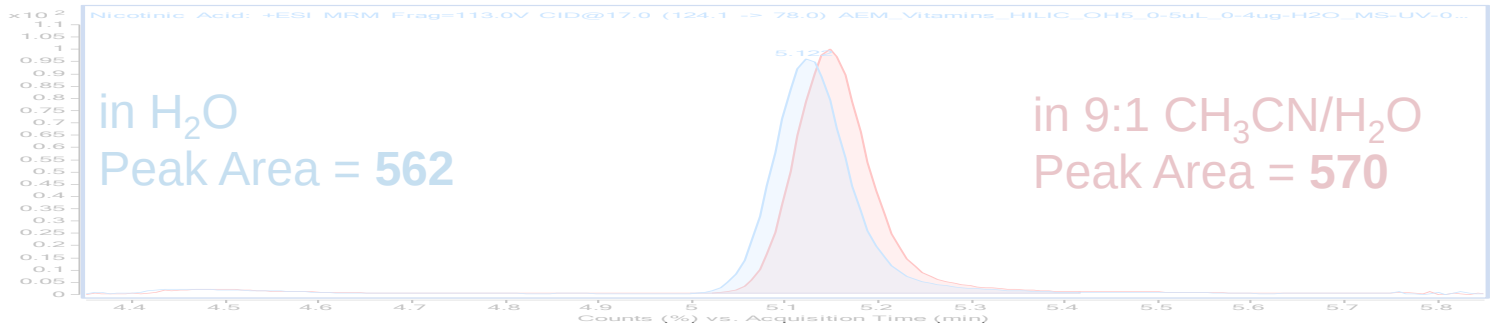
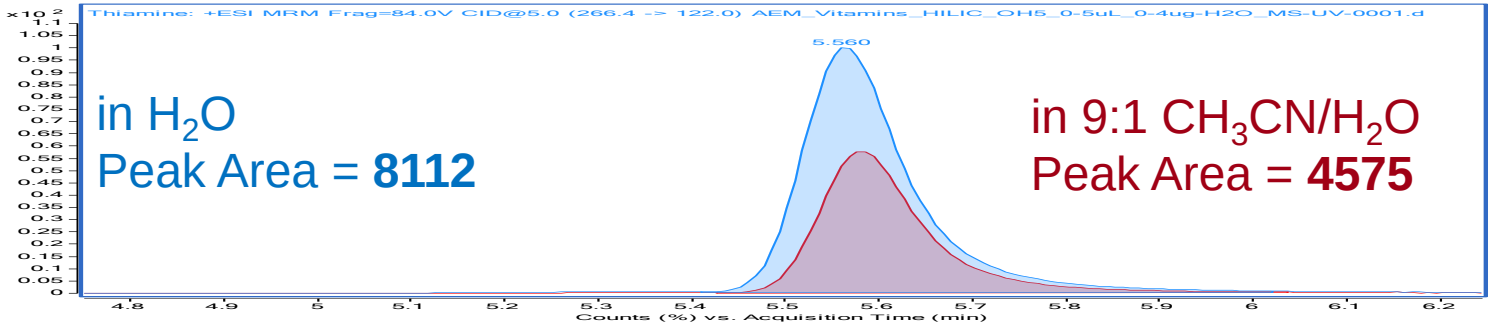
Ensure Samples are Completely Soluble

HILIC

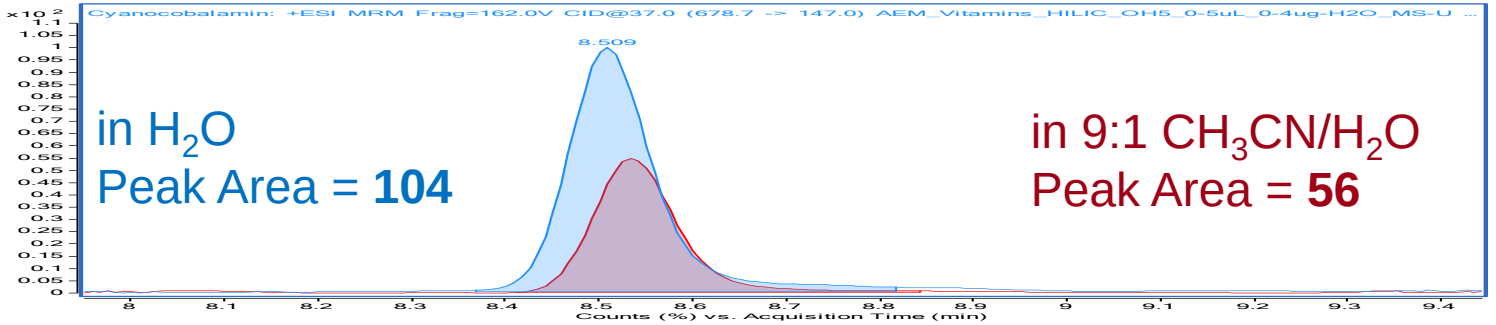
Nicotinic Acid
0.4 µg/mL



Thiamine
0.4 µg/mL



Cyanocobalamin
0.4 µg/mL



~50% sample lost due to poor solubility in CH_3CN

What is HILIC and when should I consider it?

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When to chose which separation mode for your sample

01 Find the best column to retain and separate all analytes

02 Consider the sample: analyte solubility and sample solvent

03 Ensure reliable detection of your sample

Ensure Reliable Detection of Your Sample

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Choose a detector that can analyze compounds of interest

UV, VIS absorbance

- For light-absorbing compounds

Refractive index

- Universal detection, but poor sensitivity; can only run isocratic

Evaporative light scattering

- For nonvolatile analytes

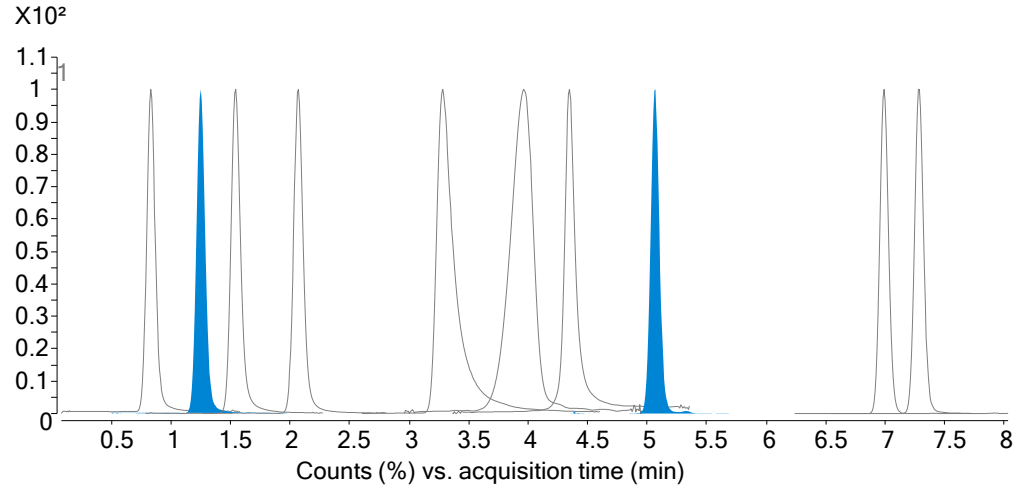
Mass spectrometer

- Low limits of detection based on molecular weight

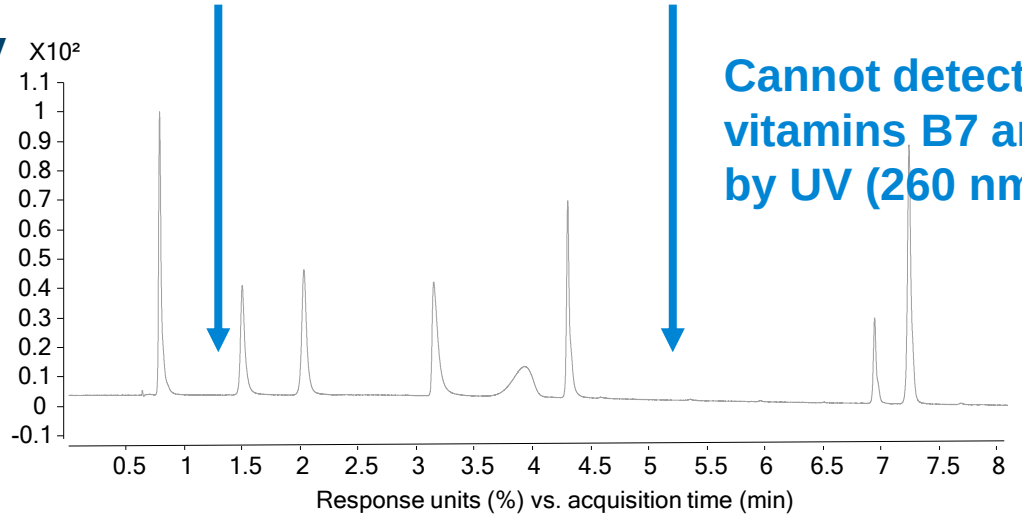
Fluorescence

- For compounds that fluoresce or can be derivatized to do so

MS



UV



Cannot detect vitamins B7 and B5 by UV (260 nm)

Ensure Reliable Detection of Your Sample

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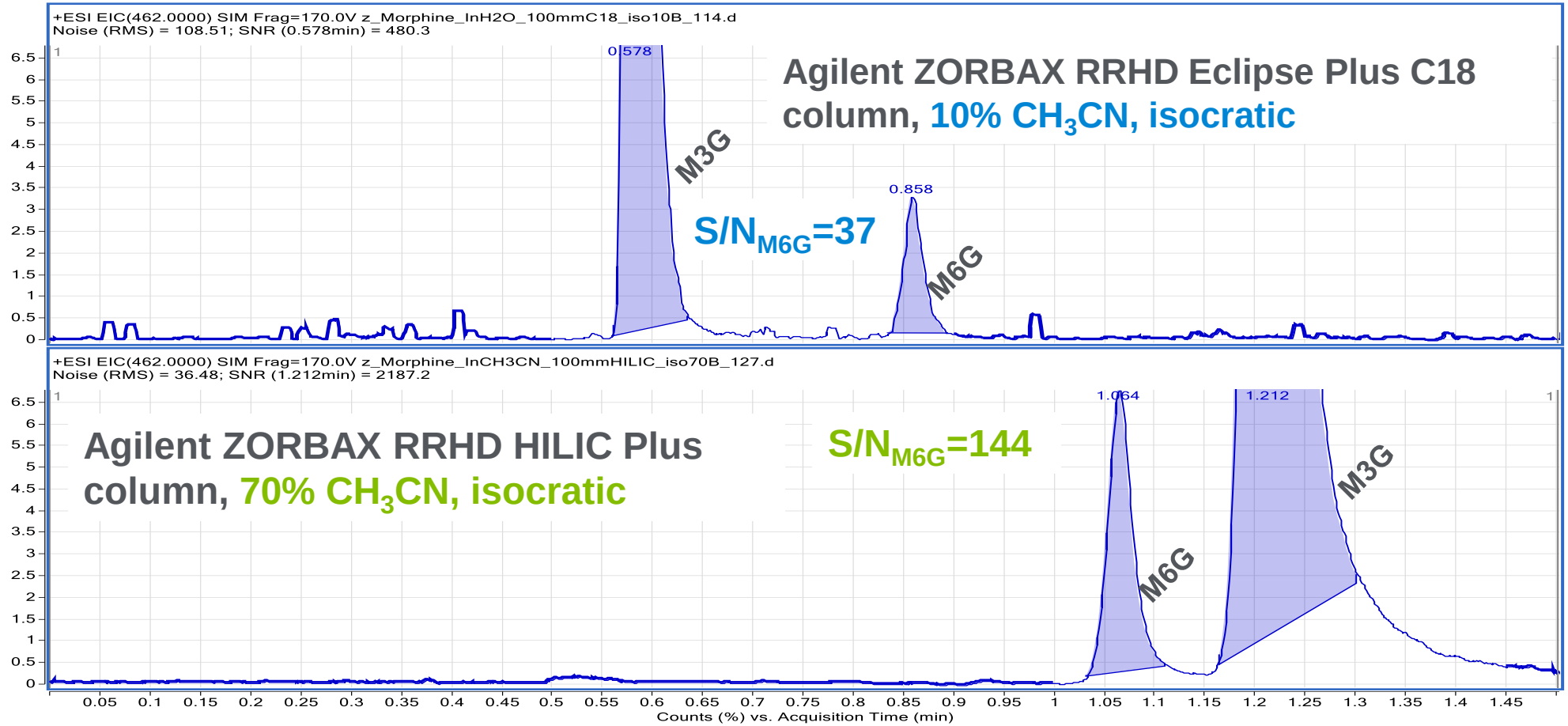
HILIC pairs well with LC/MS and can improve sensitivity compared to RPLC for opioid metabolites



Columns used were 2.1 x 100 mm, 1.8 µm. (A) 10 mM Ammonium formate pH 3.2 in water. (B) Acetonitrile/100 mM ammonium formate pH 3.2 in water (9:1), isocratic elution, 0.4 mL/min, 2 µL injection of 1 µg/mL each of morphine-3-β-D-glucuronide, and morphine-6-β-Dglucuronide; 25 °C, MS source: ESI+, 200 V, 250 °C, 11 L/min, 30 psi, 4000 V; SIM: 462, frag 170 V, Agilent publication: 5991-0245.

Reversed-Phase LC and UV Detection are Compatible with a Wider Range of Mobile Phases, Especially at Low pH

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Mobile Phase	Useable pH/Range	Recommended for HILIC?	Recommended for MS?	Recommended for RPLC and UV?
TFA	<1.5	No	No	Yes
Phosphate	1.1 to 3.1	No	No	Yes
Formic acid	<2.8	No	Yes	Yes
Acetic acid	<3.8	No	Yes	Yes
Formate	2.8 to 4.8	Yes	Yes	Yes
Acetate	3.8 to 5.8	Yes	Yes	Yes
Carbonate	5.4 to 7.4	Yes	Yes	Yes
Phosphate	6.2 to 8.2	No	No	Yes
Bicarbonate	6.6 to 8.6	Yes	Yes	Yes
Ammonia	8.2 to 10.2	Yes	Yes	Yes
Phosphate	11.3 to 13.3	No	No	Yes

What Is HILIC and When Should I Consider It?

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Summary of when to use which separation mode

01

Find the best column to retain and separate all analytes.

- RPLC cannot retain all polar/ionized analytes, HILIC may work for these
- Some analytes can be retained and separated equally well in both modes of LC

02

Consider the sample: analyte solubility and sample solvent

- Injecting strong solvent in both RPLC and HILIC will negatively affect chromatographic quality
- Strong solvent effects get worse with larger injection volumes
- Polar compounds are generally more soluble in water than acetonitrile, which is good for RPLC

It's a balancing act

03

Ensure reliable detection of your sample

- Ensure analytes are compatible with detector choice
- HILIC can improve LCMS analyses due to more volatile mobile phases
- UV and RPLC are compatible with a wider variety of mobile phases, which may improve analyte retention and separation

Other Techniques for Polar Compounds

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HILIC pairs well with LC/MS and can improve sensitivity compared to RPLC for opioid metabolites

	Advantages	Disadvantages
Ion Pairing	Fast. Uses standard system and reverse phase columns	Often contaminates the system, reagents can cause ion suppression, restricted to only positive or only negative mode MS
Ion Chromatography	Well understood mechanism, established for over 40 years	Slower than modern HPLC, expensive systems and consumables, cannot resolve cations and anions simultaneously, difficult to make MS-compatible
Ion Exchange	Strong retention and separation	Slower than HPLC, cannot resolve cations and anions simultaneously, difficult to make MS-compatible
Normal Phase	Fast. Uses standard HPLC and common columns	Safety and compatibility of organic solvents, smaller selection of stationary phases, sample solubility issues
Derivatization	Tailored selectivity, adds chromophore or fluorophore	Lengthy sample preparation, repeatability issues, complex MS spectra

What Is HILIC and When Should I Consider It?

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Why we choose HILIC

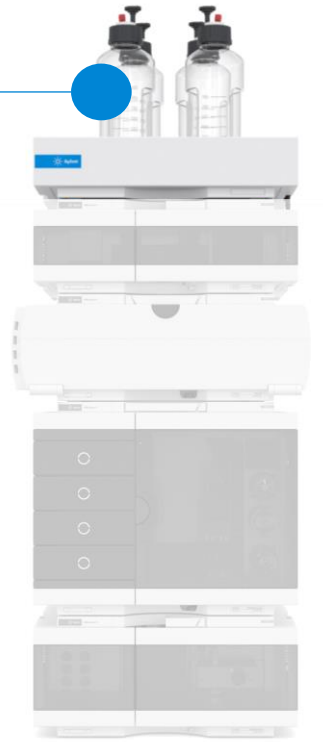
Advantages

- Uses a standard system and solvent, just swap columns
  - Easily adopted by labs currently performing reversed-phase analysis
- Retains with cations, anions, and polar neutrals
  - Widely applicable across all major polar samples
- Fully MS-compatible
  - Operate in positive or negative mode with high sensitivity

HILIC Method Development

Mobile phase considerations

	Recommendations
Organic solvent concentration	• Solvent strength in HILIC mode: *THF < acetone < CH_3CN < IPA < EtOH < MeOH < H_2O* • H_2O must be present — need >3% H_2O for hydration of silica • Mobile phase will typically be >50% acetonitrile
Ionic strength of buffer	• Concentration of (salt) buffer increases strength • Different anions and cations can also affect analyte retention
Type of buffer	• Acetates, Formates, soluble in CH_3CN—also MS-friendly • Phosphate salts are bad due to low CH_3CN solubility



Useful application notes

Hydrophilic Interaction Chromatography Method Development and Troubleshooting - **5991-9271EN**
Mastering HILIC-Z Separation for Polar Analytes- **5994-5949EN**

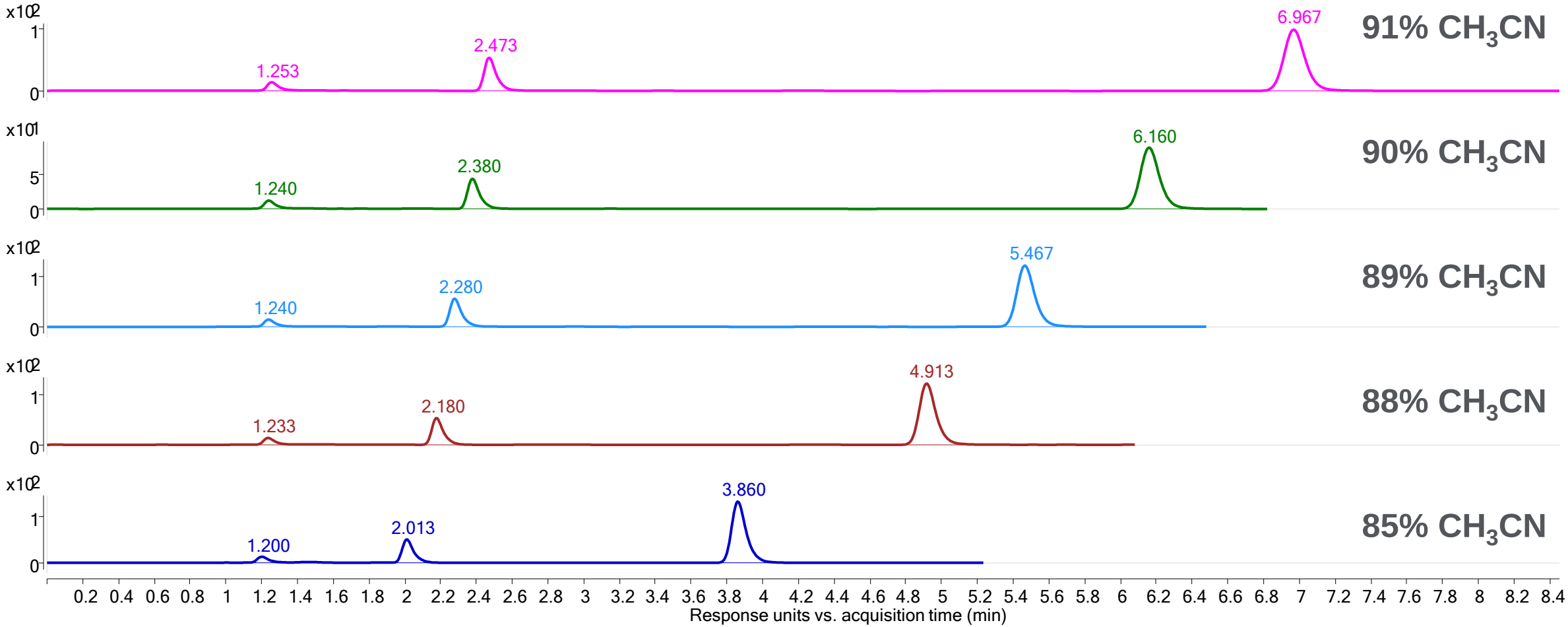
HILIC Method Development

Common starting conditions for HILIC method development

	Method Parameter
Column	Agilent InfinityLab Poroshell 120 HILIC-Z
Buffer	• **Acidic analytes**: mid to high pH (HILIC-Z only) • **Basic analytes**: low to mid pH • **Mixed analytes**: mid pH
Isocratic	Column equilibration is faster as you move from high to low aqueous • **50% ACN** – Column wash (typically no retention) • **70% ACN** – Very polar analytes • **80% ACN** – Polar analytes, mixtures • **90% ACN** – Less polar analyte, retention and separation
Gradient	• **90% → 50% ACN** – Scouting gradient • Isocratic holds or shallow gradients (1 to 3% per minute) recommended for critical pair separation

HILIC Method Development

Less CH_3CN makes a HILIC mobile phase stronger, causing less retention



Column used was 2.1 x 150 mm, 2.7 µm Agilent InfinityLab Poroshell 120 HILIC-Z (PEEK-lined). (A) 100 mM pH 3 Ammonium formate in water. (B) Acetonitrile, % B, isocratic elution, 0.25 mL/min, 30 °C, 1 µL injection of toluene, cytosine, uracil QC mixture, 254 nm.

Effect of pH on Retention of Acidic Compounds with HILIC

Starting mobile phases

In HILIC mode, ionizable compounds are better retained when they are ionized.

- Acids at high pH
- Bases at low pH

Once the analyte is fully ionized, retention should stabilize.

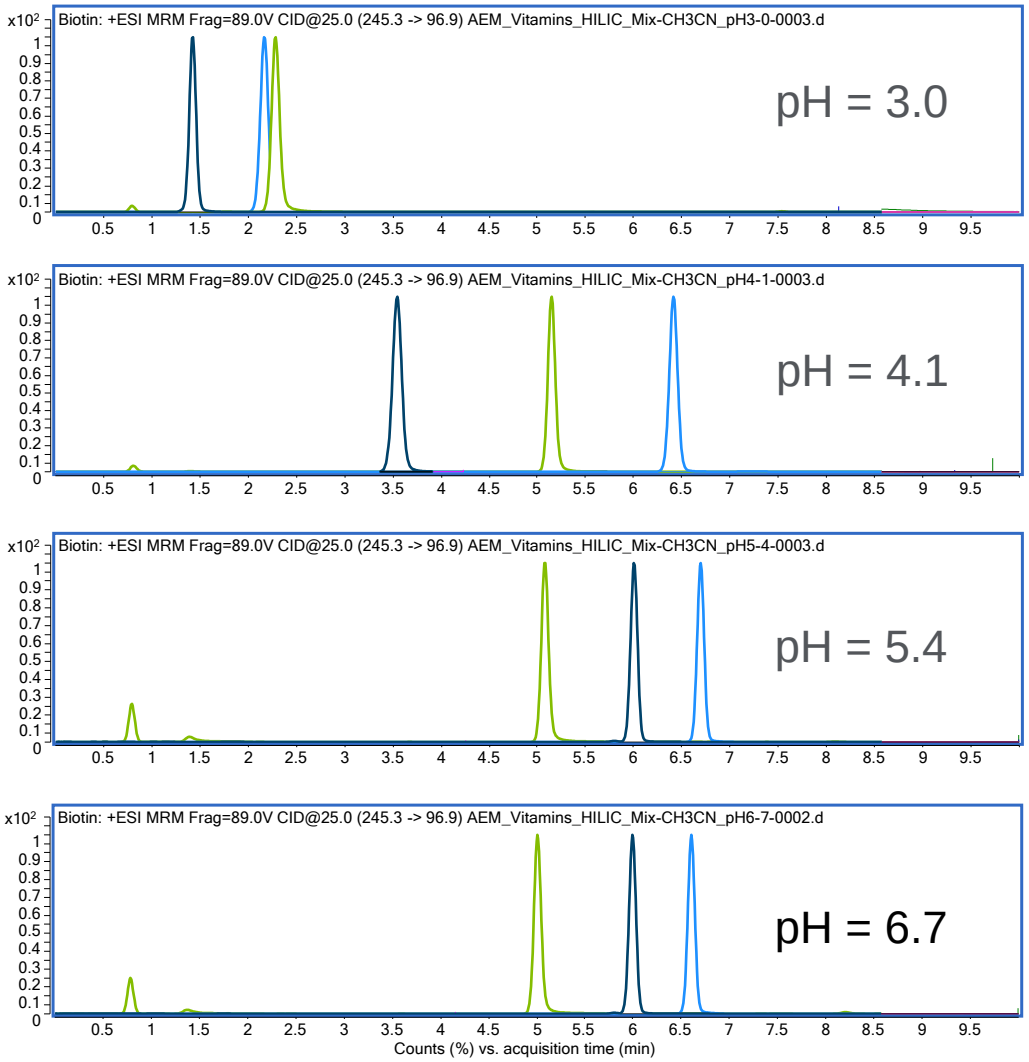
- Note: if other retention mechanisms are occurring, this may not be true

Biotin pKa = 4.5

Nicotinic acid pKa = 4.8

Pantothenic acid pKa = 4.3

Mobile phase A: H_2O, B: CH_3CN, D: varies, 200 mM ammonium formate or acetate; flow rate: 0.5 mL/min; gradient: 95% B for 1 min, 95-65% B in 9 min, hold 5% D constant throughout analysis, 5 min postrun; injection: 0.5 µL of 13.3 µg/mL each in CH_3CN/H_2O 19:1; column: 25 °C, 2.1 x 100 mm, 2.7 µm Agilent InfinityLab Poroshell 120 HILIC-Z; detection: Ultivo LC/TQ ESI+ dMRM.



Tips and Tricks for Your HILIC Separations

Considerations on solvent and sample handling

	Impact
Add 10% aqueous to your organic	• Buffer solubility increases drastically with addition of 10-20% water • HILIC columns equilibrate faster with more aqueous
Have the same ionic strength in both mobile phases	• Ionic strength gradients have more variability than constant ionic strength • Near 90-100% ACN, many buffers crash out, causing serious clogs
Increasing buffer concentration can improve peak shape and sample loadability	• High buffer concentrations can cause ion suppression when using MS detection
Follow good measurement practices when mixing buffers	• Retention can vary from bottle-to-bottle if the eluent is not mixed accurately and consistently
Prepare samples in as much acetonitrile as possible and keep injection volumes small	• Avoid peak shape and retention issues from strong solvent effects
Use inert solution, if needed	• Reduce unwanted interactions of analytes with metal in the flow path

InfinityLab solvent bottles

InfinityLab Stay Safe caps

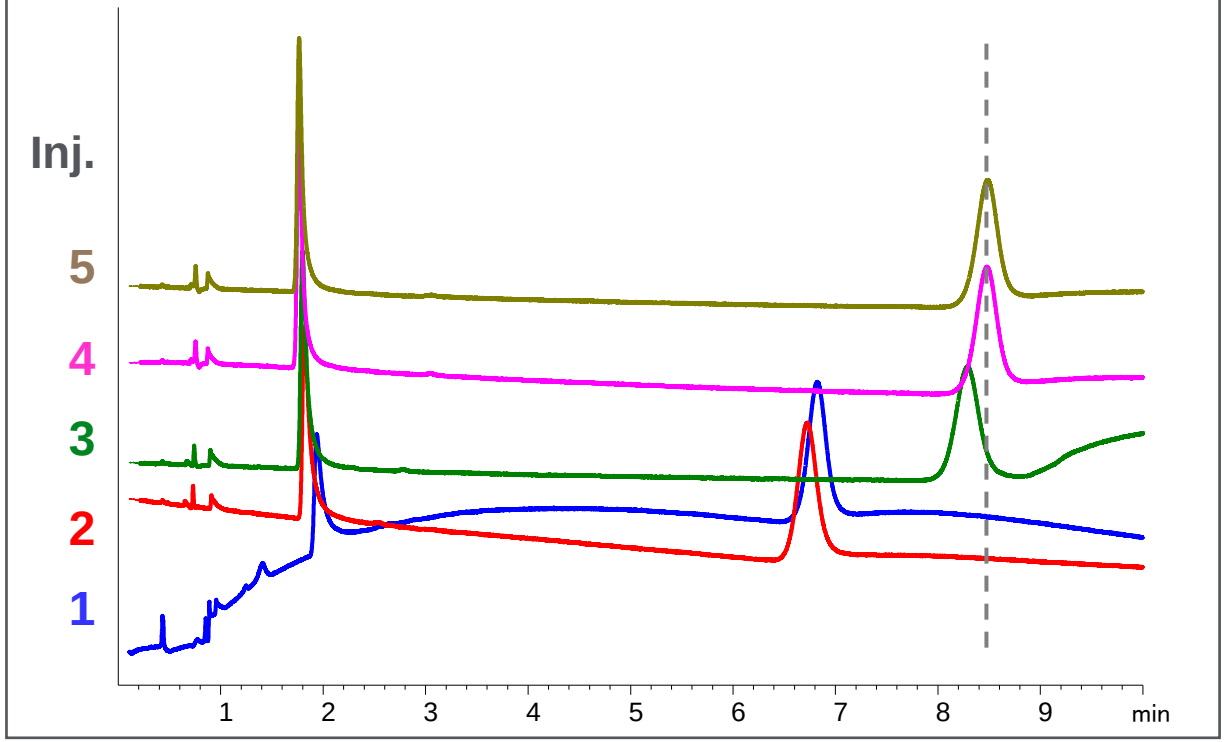
HILIC Column Equilibration is Faster with Higher Amounts of Aqueous

Agilent InfinityLab

B vitamins on Agilent InfinityLab Poroshell 120 HILIC-OH5 (2.1 x 100 mm, 2.7 µm)

4% aqueous, isocratic

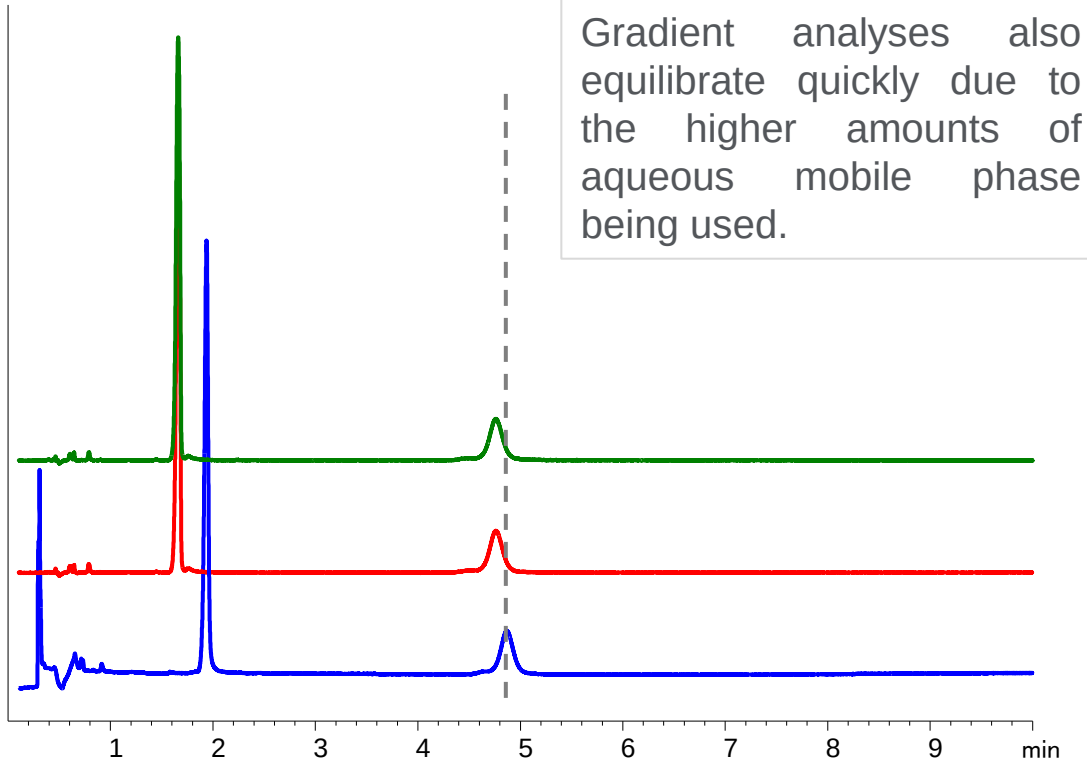
Equilibrated in 30 minutes (75 column volumes)



Column stored in 100% CH_3CN before analysis. (A) 100 mM Ammonium formate pH 3.0. (B) CH_3CN, 96% B isocratic, 0.5 mL/min, 1 µL injection of B2+B6, 25 °C, 260 nm, 80 Hz.

20% aqueous, isocratic

Equilibrated in <10 minutes (<25 column volumes)



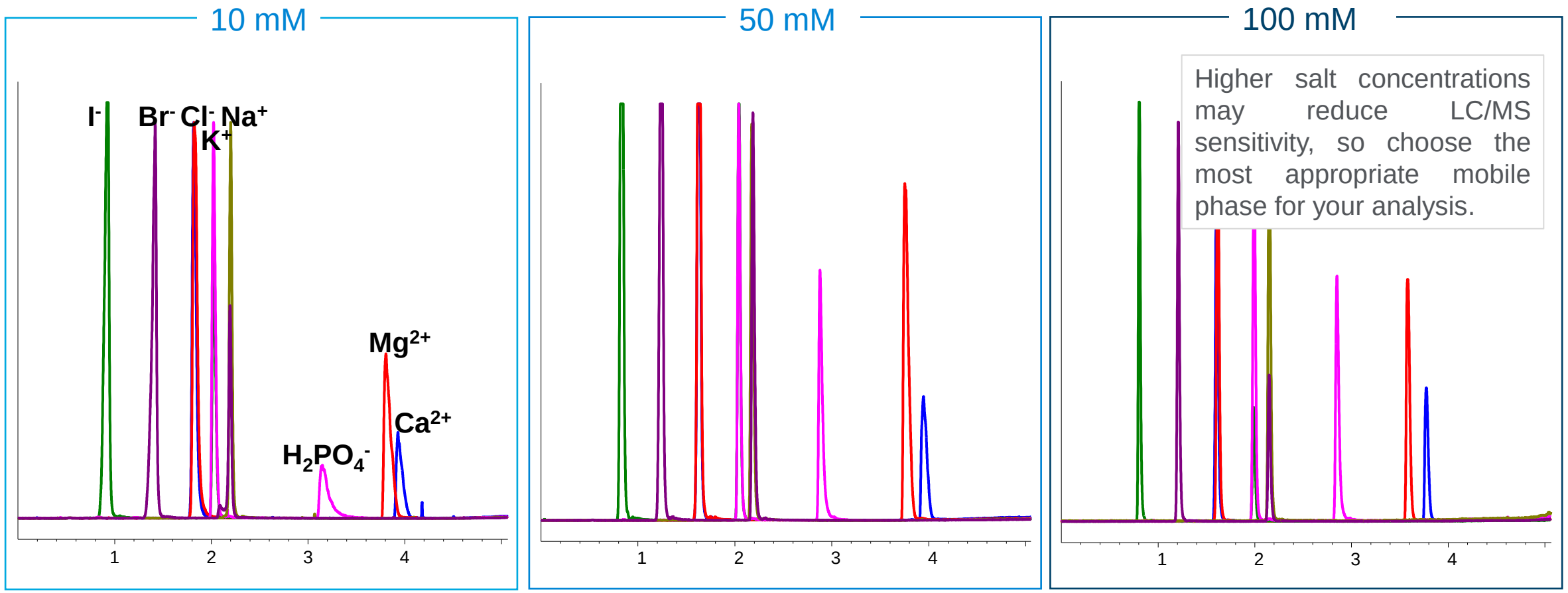
Gradient analyses also equilibrate quickly due to the higher amounts of aqueous mobile phase being used.

Column stored in 100% CH_3CN before analysis. (A) 100 mM Ammonium formate pH 3.0. (B) CH_3CN, 80% B isocratic, 0.5 mL/min, 1 µL injection of B9+B12, 25 °C, 260 nm, 80 Hz.

Higher Salt Concentrations Can Improve Peak Shapes and Resolution

Agilent InfinityLab

Inorganic ions on Agilent InfinityLab Poroshell 120 HILIC-Z



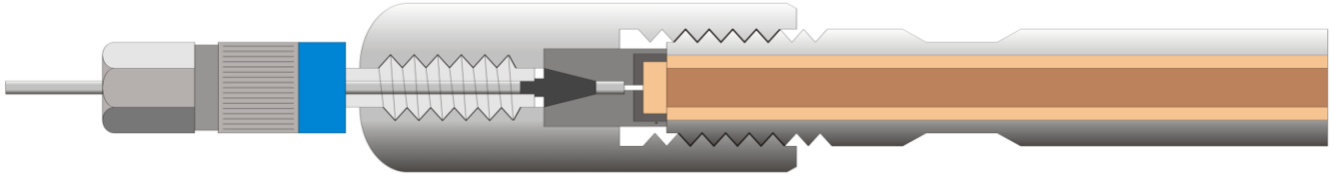
Agilent InfinityLab Poroshell 120 HILIC-Z 2.1 x 100 mm, 2.7 µm. (A) 10, 50, or 100 mM pH 3 ammonium formate. (B) Acetonitrile, 80-20% B in 5 min, 3 min re-equilibration, 0.4 mL/min, 30 °C, 2 µL injection of individual standards (0.3 to 0.5 mg/mL), ELSD 40 °C/3.5 psi/30 Hz.

HILIC Sensitivity Can Be Improved with a PEEK-lined Column

Agilent InfinityLab

PEEK-lined stainless steel and PEEK-coated titanium frits

- Metal-free flow path minimizes unwanted interactions
- Stainless steel provides strength for UHPLC use



For best results, use the full InfinityLab bio-inert solution:

- InfinityLab bio-inert LC system
- Bio-inert Quick Connect heat exchanger, p/n: G7116-60009
- All Agilent PEEK/SST bio-inert capillaries with Quick Turn fitting (5067-5966) or UHP-FF fitting bio-inert (5067-5695)

Tips and Tricks for Your HILIC separations

InfinityLab deactivator additive pairs well with PEEK-lined HILIC-Z

	Improvement
Reduce metal-analyte interaction	• Chelates free metals and covers exposed active sites in the sample flow path, reducing unwanted metal-analyte interactions and allowing lower detection limits through LC/MS
Amenable to LC/MS use	• Optimized for use at a 5 µM (1:1000 dilution) with minimal ion suppression effects • Does not persist in the LC/MS system after use (unlike traditional ion pairing reagents)
Operational time and cost savings	• Saves the time needed to passivate your system • Can avoid derivatization • Can avoid potential system contamination from ion pair agents • Limits of detection can be increased for challenging compounds, such as phosphorylated metabolites, phosphate pesticides, and organic acids

InfinityLab deactivator additive
50 mL: 5190-4506

Recommended read

More information can be found in the InfinityLab Deactivator Additive User Guide 5991-9516EN.

HILIC Column Care

How to clean and store a HILIC column

Cleaning a HILIC column:

- Use a strong HILIC solvent to clean HILIC columns
- Flush HILIC columns with 100% water
- If that is insufficient, add in 100 to 500 mM salt
  - You can use a strong salt like NaCl, or if you prefer to avoid that, you can use buffer salts, like ammonium acetate
- Increasing the temperature to between 35 and 55 ˚C can also help with the cleaning efficiency
- Flush the column with about 30 column volumes per step
- Be sure that, once you are finished flushing with high-concentration salt, you flush with pure water before reintroducing acetonitrile into the mobile phase.

Storing a HILIC column:

- Flush with acetonitrile/water (20/80) for 30 column volumes
- Flush with acetonitrile/water (80/20) for 30 column volumes
- Store at room temperature

Summary

When to consider a HILIC column:

- Are your analytes unretained with RPLC?
- Are your analytes at least somewhat soluble in acetonitrile?
- Are you using MS detection?
- Do your analytes interact with metals in the LC system?

Keep the sample solvent in mind for HILIC analyses: prepare the sample in as much acetonitrile as possible, and keep injection volumes as small as possible.

- Most common support issue with HILIC methods

Hydrophobic Interaction – Workflow Solution

What is HIC and its separation mechanism?

Hydrophobic Interaction Chromatography (HIC)

Hydrophobic interaction chromatography separates molecules based on differences in hydrophobicity. It 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

Proteins Are Made From Chains of Hydrophobic, Neutral/Polar, Acid, and Basic Amino Acids

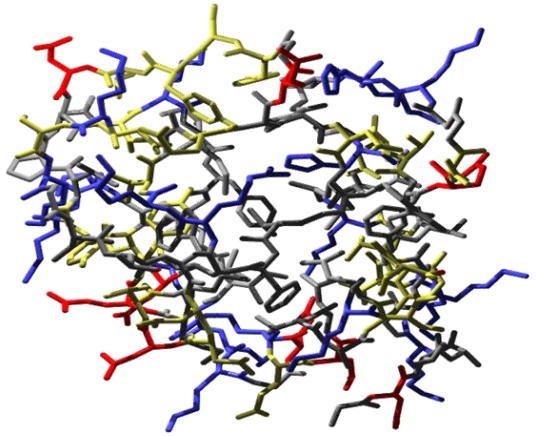
Agilent InfinityLab

Hydrophobic	Ala	Val	Leu	Ile	Pro
Hydrophobic	Met	Phe	Trp	Gly	Ser
Neutral/hydrophilic	Thr	Cys	Asn	Gln	Tyr
Acidic	Asp	Glu	Lys	Arg	His
Basic					

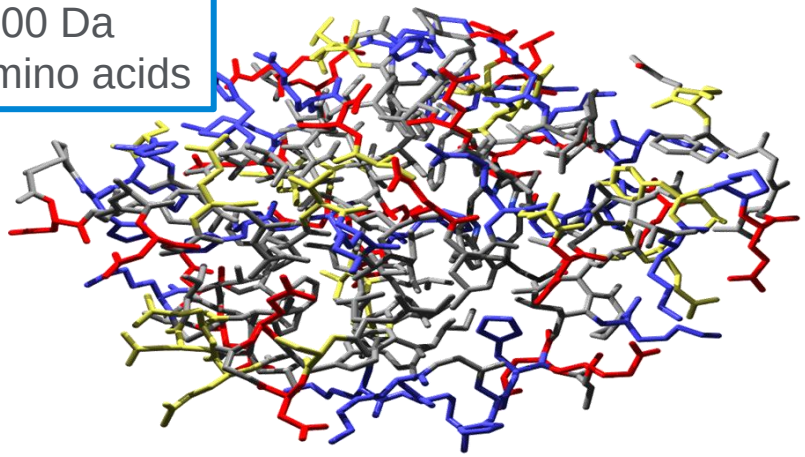
Protein Molecules Come in Different Shapes and Sizes…

Agilent InfinityLab

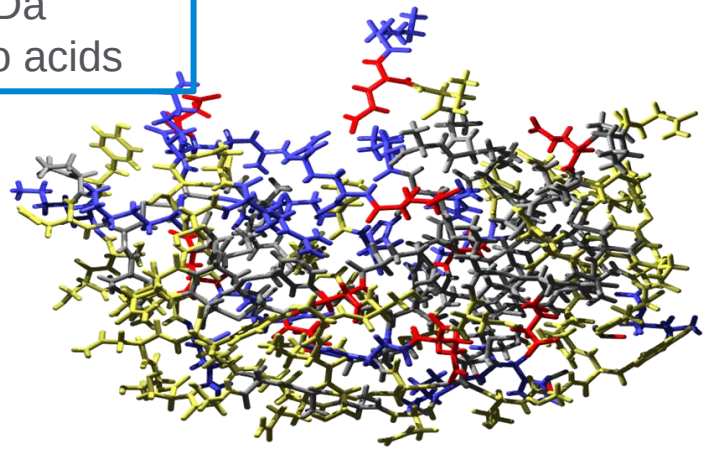
Cytochrome C
~ 12,000 Da
103 amino acids

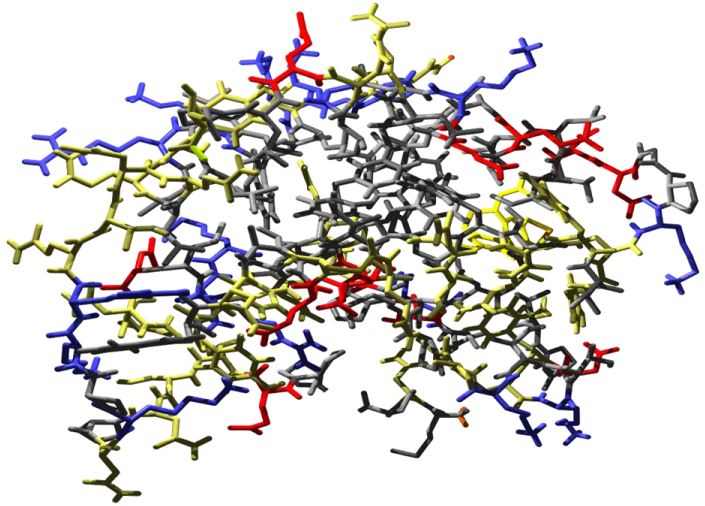


Myoglobin
~ 16,700 Da
153 amino acids



Ribonuclease A
~ 13,700 Da
124 amino acids

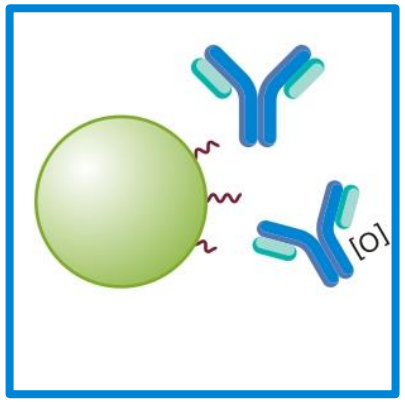




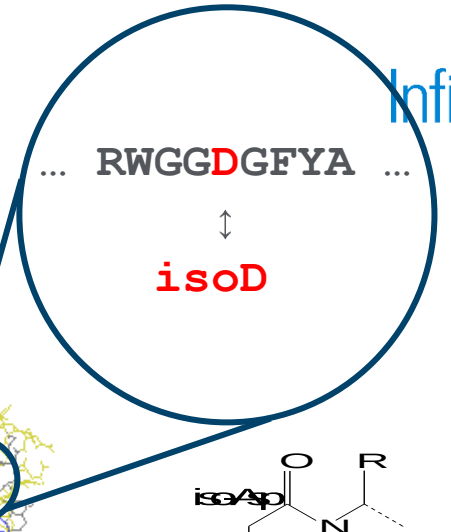
Lysozyme
~ 14,000 Da
129 amino acids

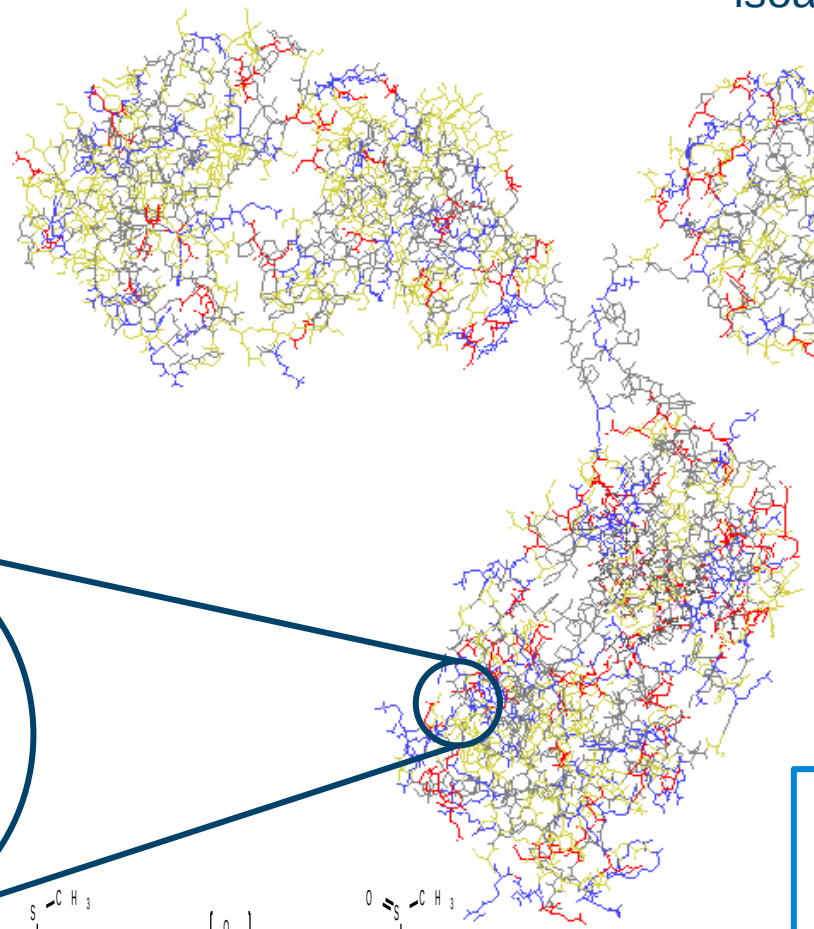
Monoclonal Antibody Variants (Impurities)

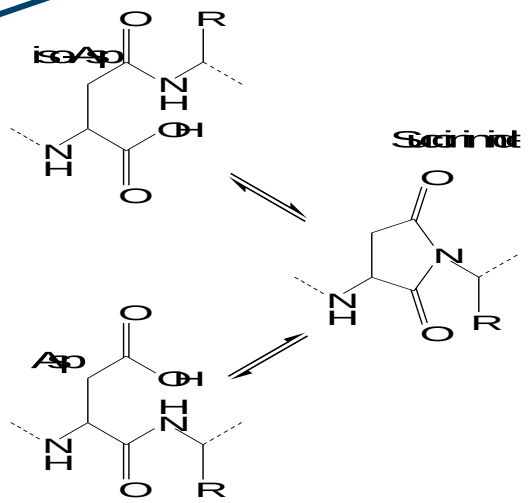
Agilent InfinityLab

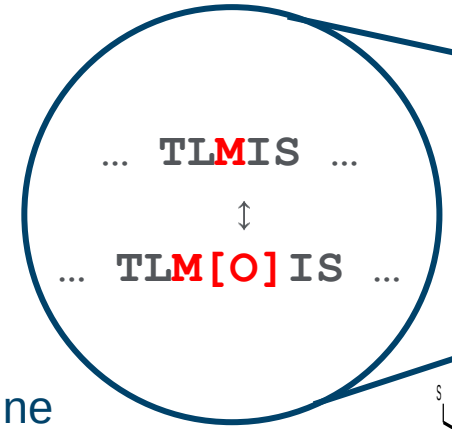


Isomerization (aspartic acid to isoaspartic acid)

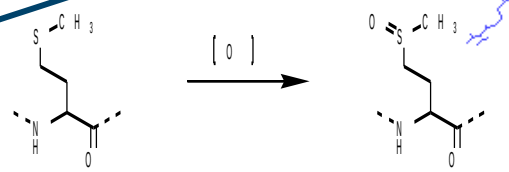






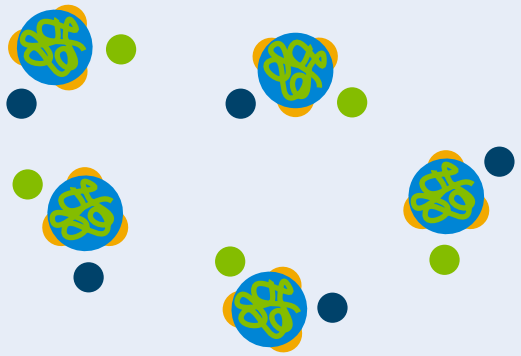
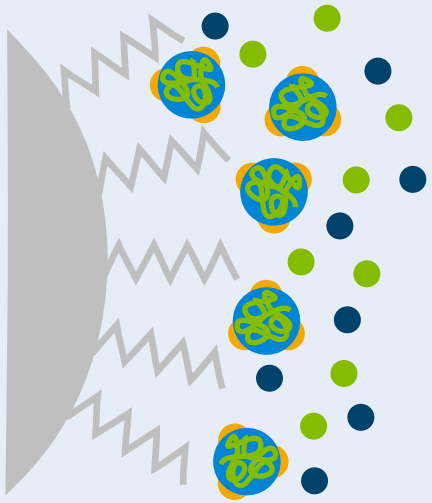
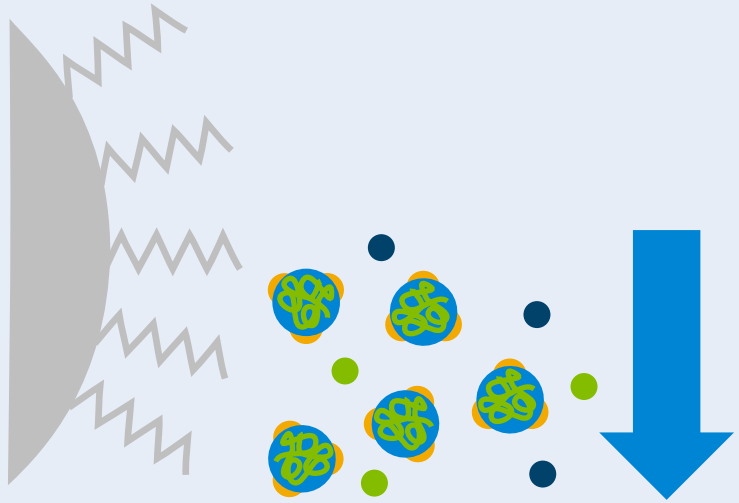


Methionine oxidation



Monoclonal antibody
~ 150,000 Da
> 1,300 amino acids

HIC – Mechanism

Step 1	Step 2	Step 3
In aqueous solution: • Water surrounds the molecule in an ordered manner • Favors ionic strength It is important that the sample does not precipitate at this stage.	Sample is applied to the column in high salt concentration: • Water molecules are displaced • Ionic charges are masked, exposing hydrophobic areas • Increased entropy Sample is absorbed onto the hydrophobic surface of the HIC stationary phase.	By reducing the amount of salt present in the mobile phase, the proteins will elute in order of increasing hydrophobicity: • The least hydrophobic proteins elute first • The most hydrophobic proteins elute last
		

LC Workflow for **Hydrophobic Interaction Analysis**

Agilent InfinityLab

Sample preparation – Manual | LC separation | Detection and data analysis

Manual

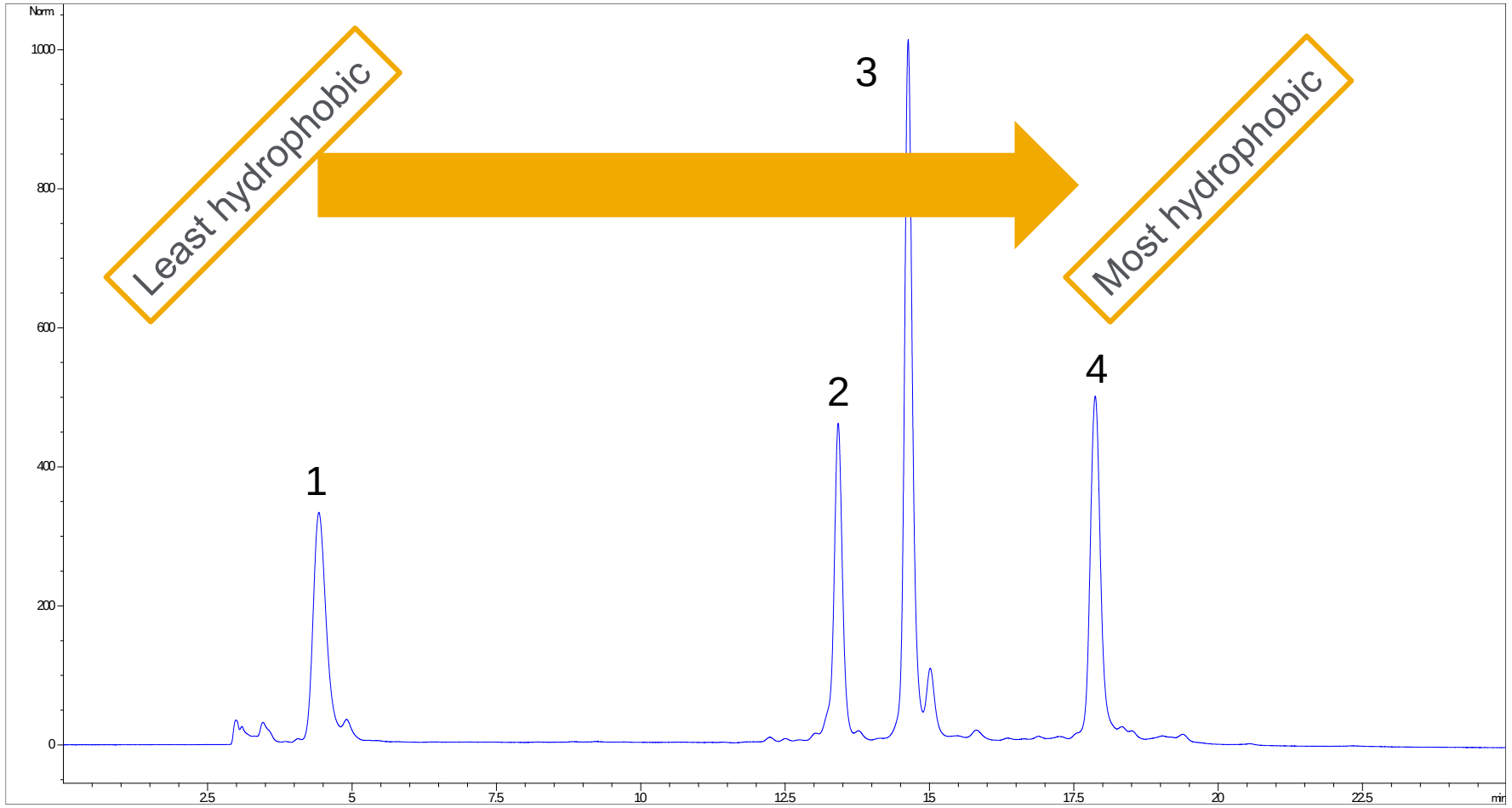
1260 Infinity II bio-inert LC

Manual methods of sample preparation are normally used. For high-efficiency separations, small injection volumes are recommended. Most proteins are basic so cation exchange will be used.

A chiller for the autosampler will help when analyzing protein samples. Buffer Advisor software can greatly enhance method optimization and separation robustness.

HIC eluents are rarely MS-friendly so HIC can only be combined with MS through 2D-LC or with desalting of samples taken offline. For protein HIC the best detector to use is the G1315D DAD (bio-inert).

HIC Separation of Standard Proteins
(4.6 x 100 mm column)



Method conditions

Column:	AdvanceBio HIC 4.6 x 100 mm
Eluent A:	50 mM NaPO, pH 7.0
Eluent B:	2 M $(NH_4)_2SO_4$, 50 mM NaPO, pH 7.0
Flow rate:	0.5 mL/min
Temperature:	25 °C
Injection:	5 µL (1 mg/mL)
Sample:	Protein mix
1	Cytochrome C
2	Ribonuclease A
3	Lysozyme
4	α-Chymotrypsinogen A

Gradient profile

Time	%A	%B
0	0%	100%
20	100%	0%
25	100%	0%
26	0%	100%
30	0%	100%

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 to 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 20 to 100 mM concentration**

Gradient elution from high to low salt concentration

- **Gradient times from 10 to 20 column volumes are ideal**

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

Factors affecting HIC selectivity

Resin hydrophobicity, salt (Hofmeister series - type, concentration)

pH, **temperature**, buffer type and organic additives

AdvanceBio HIC

Built using Agilent innovation

New wider pore 3.5 µm ZORBAX particle

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

+

Unique bonded phase

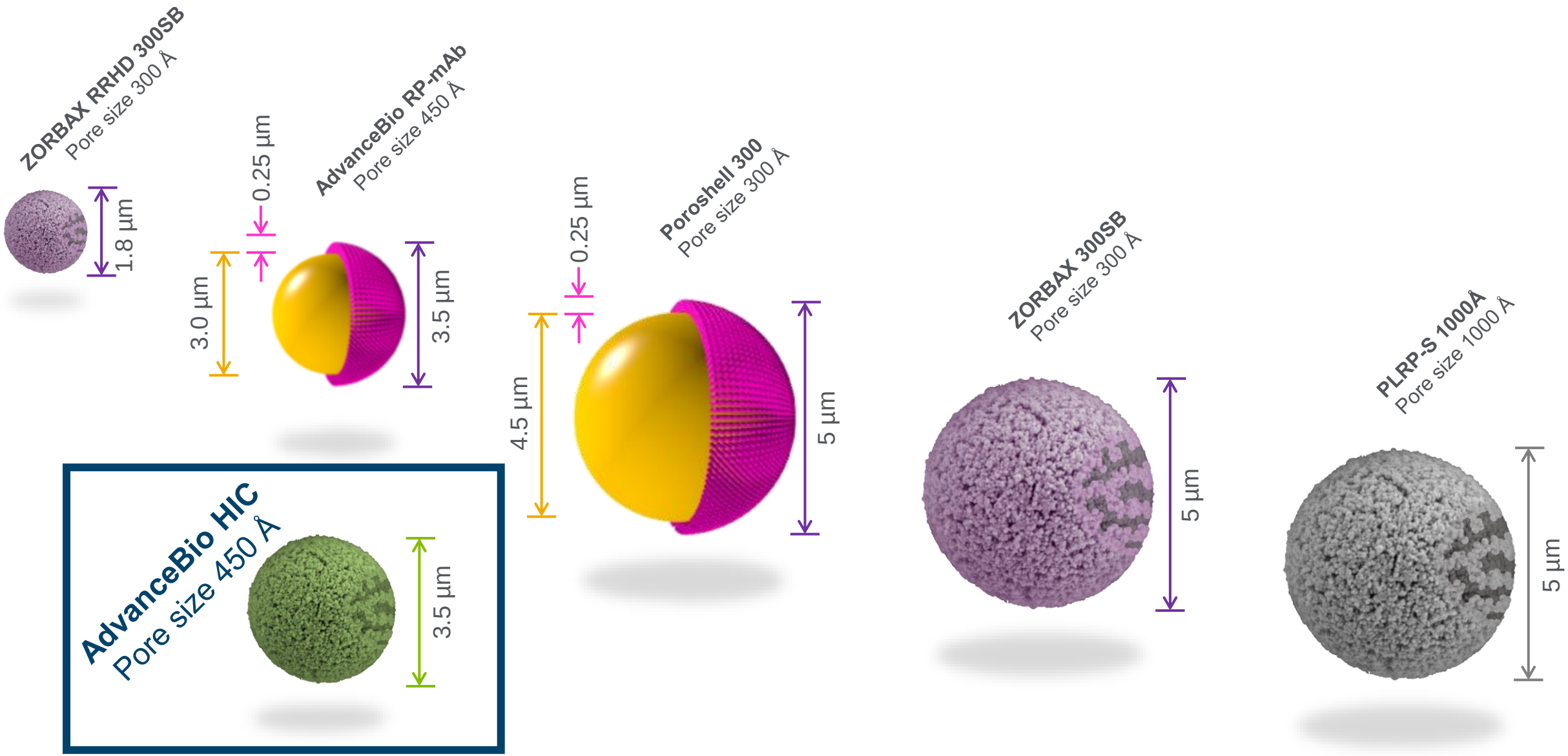
- Optimized hydrophobicity for HIC bioseparations at lower salt concentrations
- Versatile for a wide range of molecule types, from hydrophilic proteins to hydrophobic ADCs

=

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

AdvanceBio HIC: A New Addition to the Biocolumns Family



HIC for Post-translational Modification (PTM) analysis

Agilent InfinityLab

Post-translation modifications that impact efficacy or toxicity of a biotherapeutic must be characterized and quantified. As critical quality attributes (CQAs) can impact the structure of a protein, HIC is an ideal technique to use for characterization.

Critical quality attributes include:

Methionine oxidation (Met to Met[O])

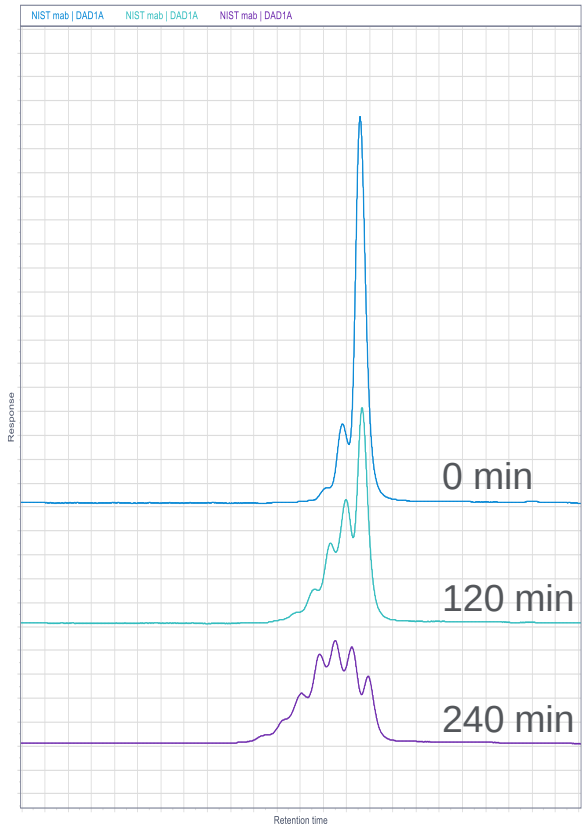
Tryptophan oxidation

Deamidation (Asn to Asp)

Free cysteine

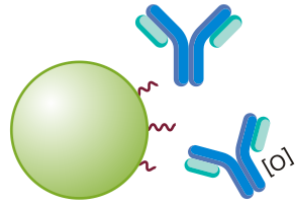
Aspartic acid isomerization (Iso-Asp in CDR region)

…any variant located in the complementary-determining region (CDR) may affect the ability to bind to the target antigen.

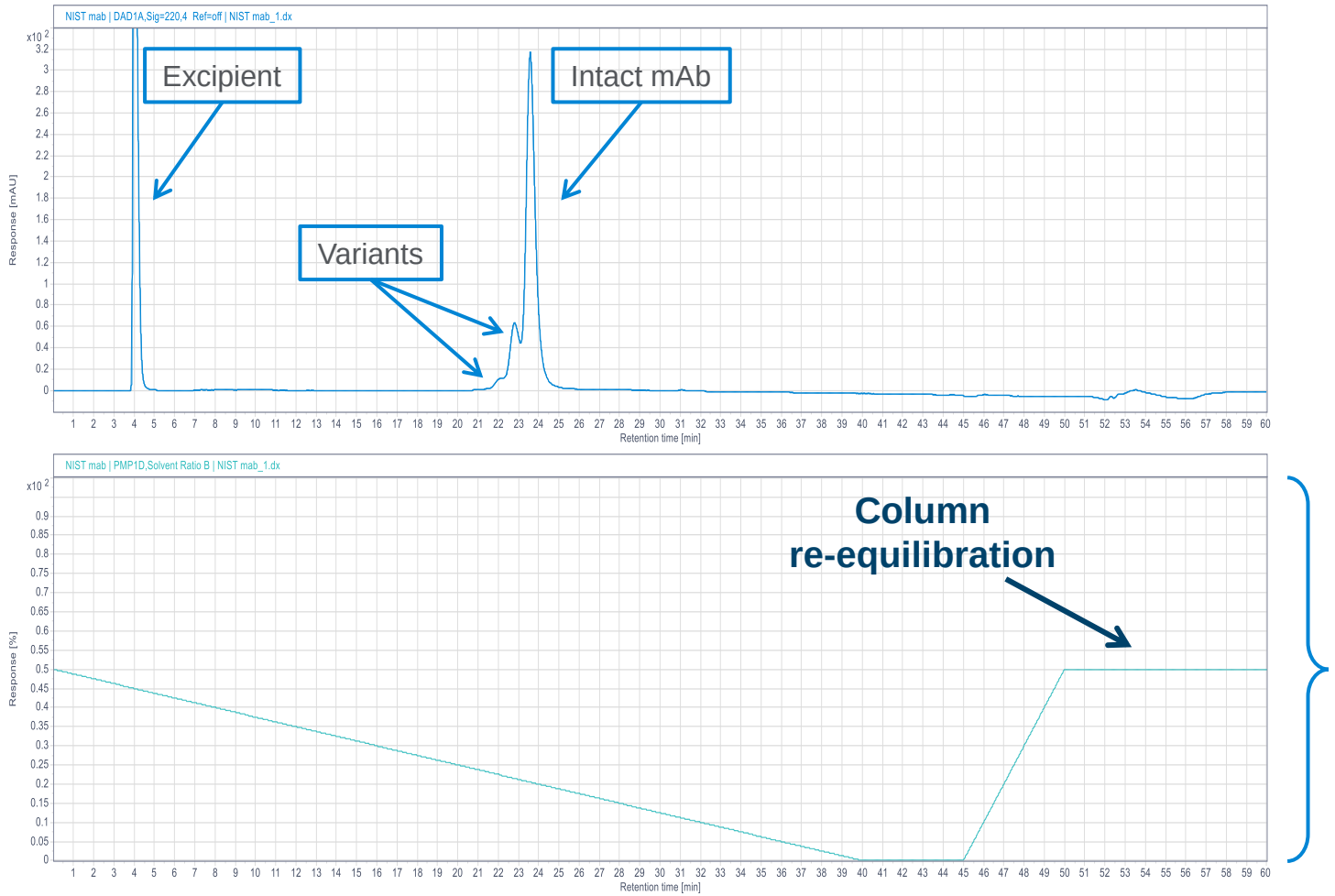


NIST mAb + tBHP Forced Oxidation

Column:	**AdvanceBio HIC, 4.6 x 100 mm**
Eluent A:	**50 mM sodium phosphate, pH 7.0**
Eluent B:	**2 M ammonium sulfate + 50 mM sodium phosphate, pH 7.0**
Gradient:	**60% B – 10% B, 0 - 40 mins**
Flow rate:	**0.3 mL/min**
Temperature:	**25 °C**
Detection:	**UV, 220 nm**



HIC Separation of NIST mAb (RM 8671)



Method conditions

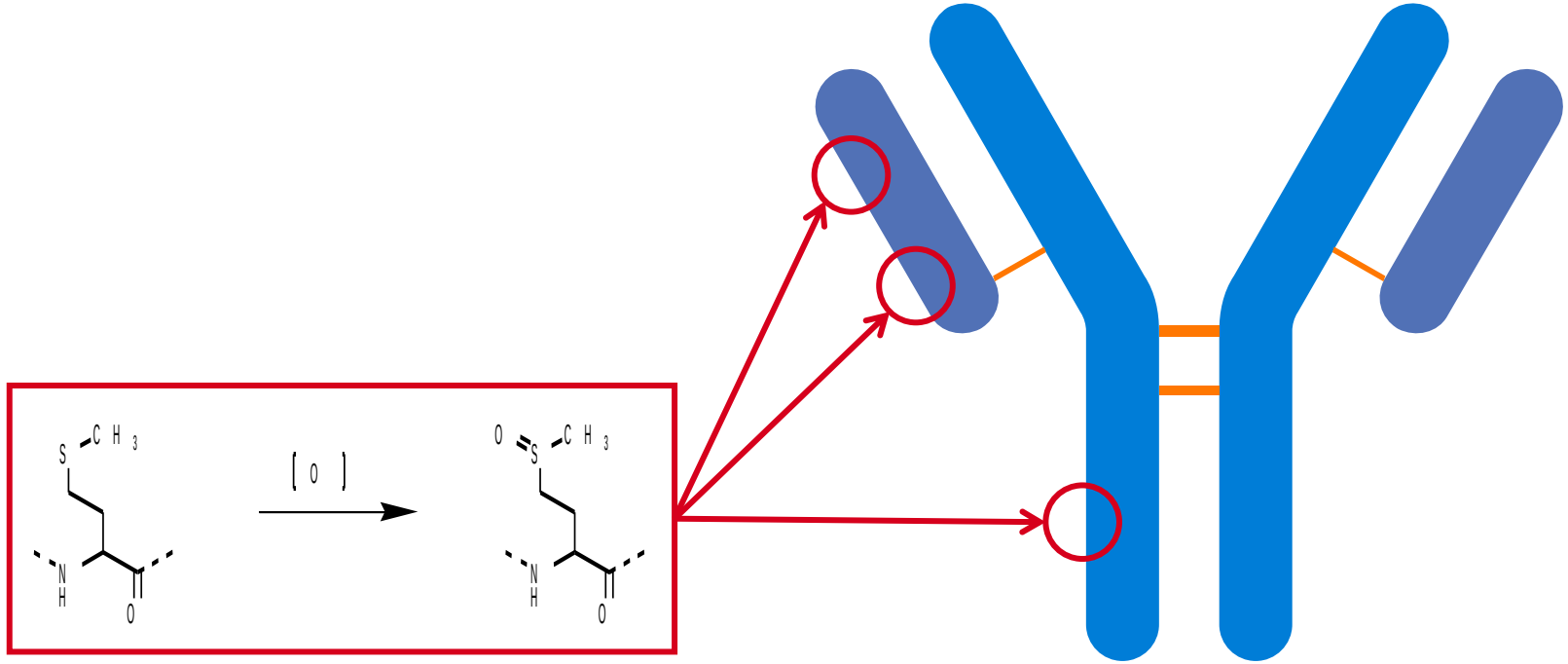
Column:	AdvanceBio HIC 4.6 x 100 mm
Eluent A:	50 mM sodium phosphate, pH 7.0
Eluent B:	2 M ammonium tartrate in 50 mM sodium phosphate, pH 7.0
Flow rate:	0.3 mL/min
Temperature:	25 °C
Injection:	5 µL (1 mg/mL)
Sample:	NIST mAb (RM 8671)

Gradient profile

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

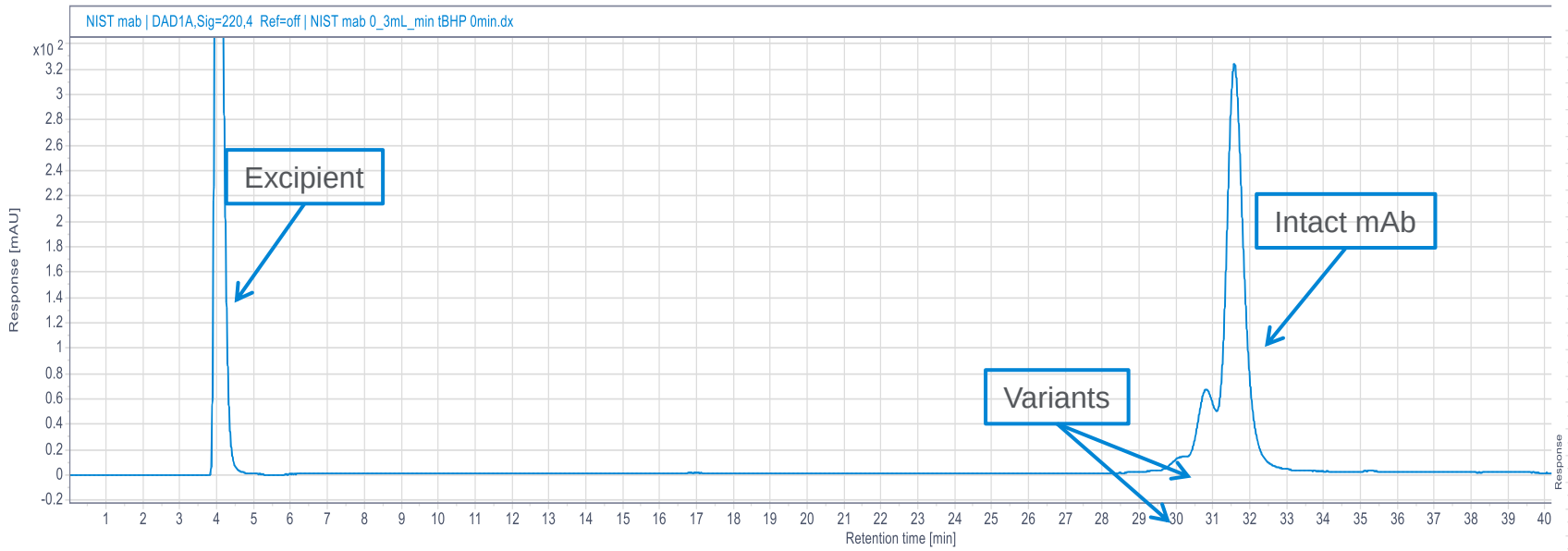
Monitoring Oxidation in mAbs

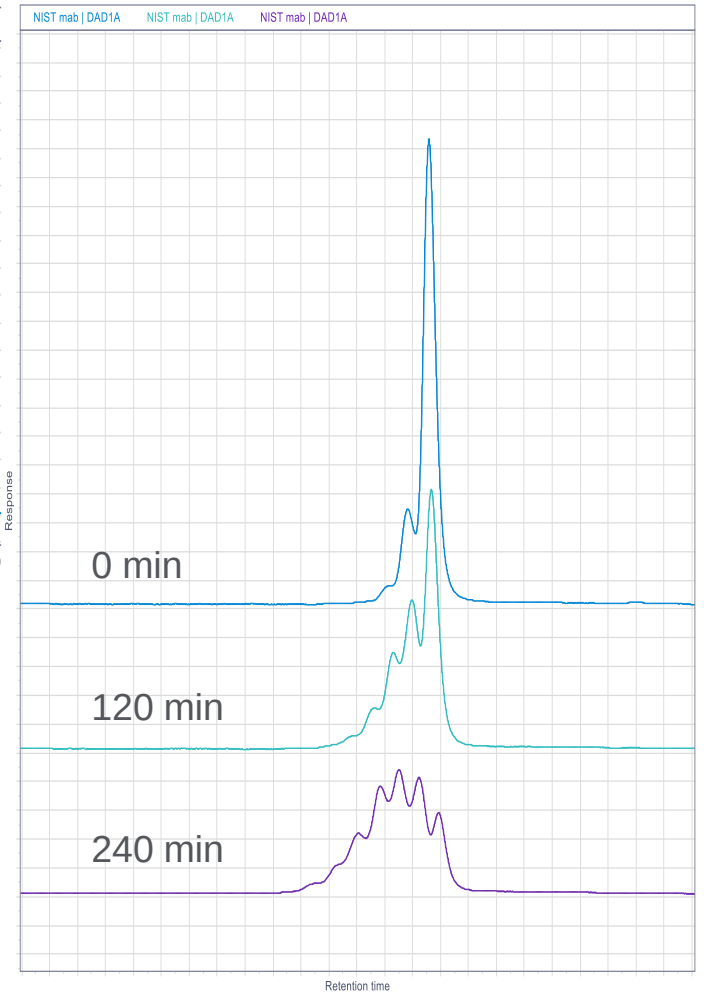
There may be 16 or more methionine residues distributed throughout a mAb. Not all of these are readily accessible, so oxidation is more likely to occur at other more accessible sites. Oxidation in the antigen binding region may be more problematical than oxidation in the Fc region of the heavy chain.



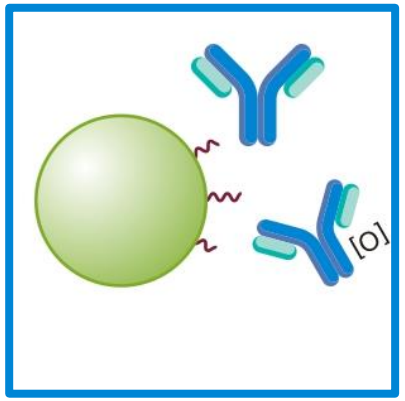
Further oxidation (to methionine sulfone) is less likely.

Forced oxidation of NIST mAb RM8671 with tBHP



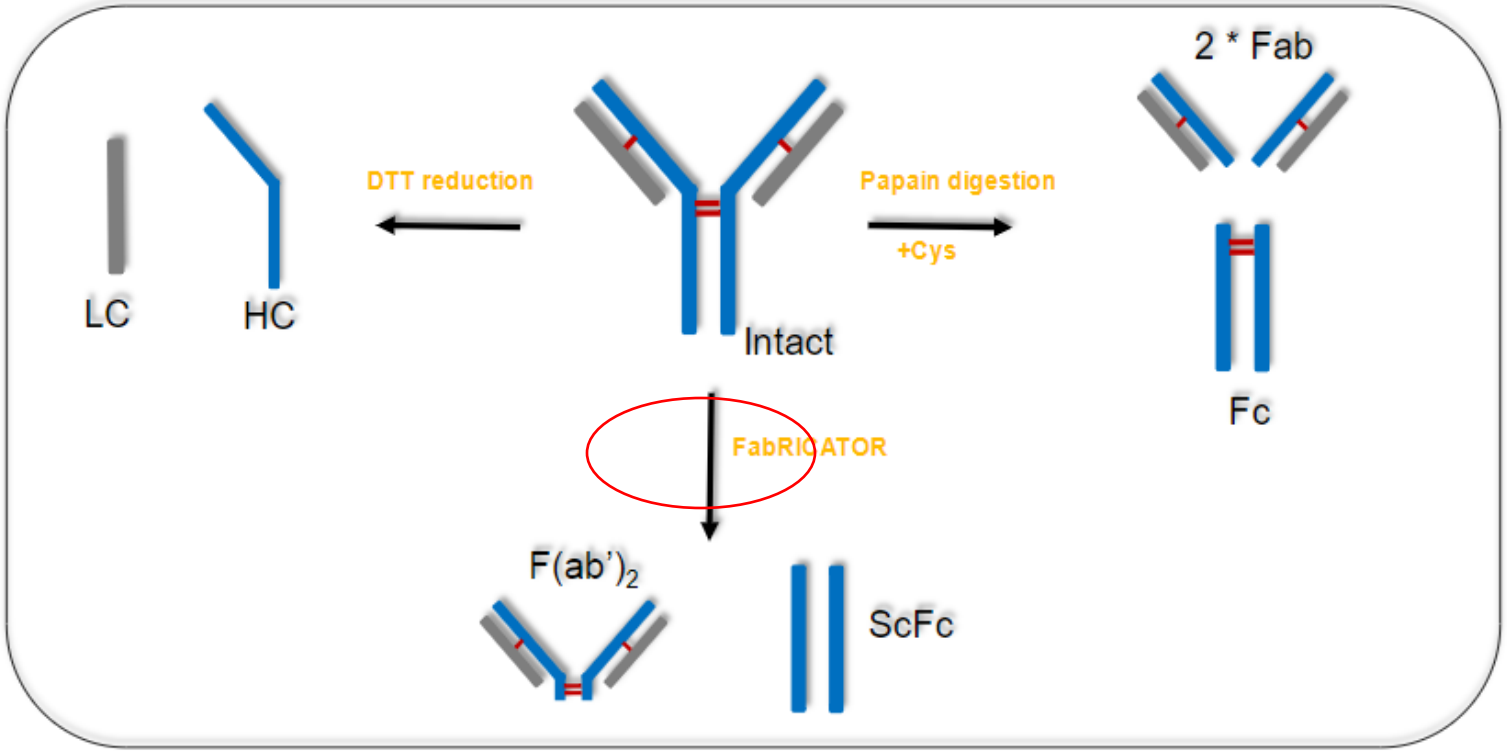


Eluent A	**50 mM sodium phosphate, pH 7.0**
Eluent B	**2 M $(NH_4)_2SO_4$ + 50 mM sodium phosphate, pH 7.0**
Flow rate	**0.3 mL/min**
Temperature	**25 °C**
Detector	**UV, 220 nm**
Gradient	**60% - 10% B, 0 - 40 mins**
Sample	**NIST mAb + tert-butylhydroperoxide (tBHP)**



More information in application note [5994-0199EN]

mAb Characterization: Fragments

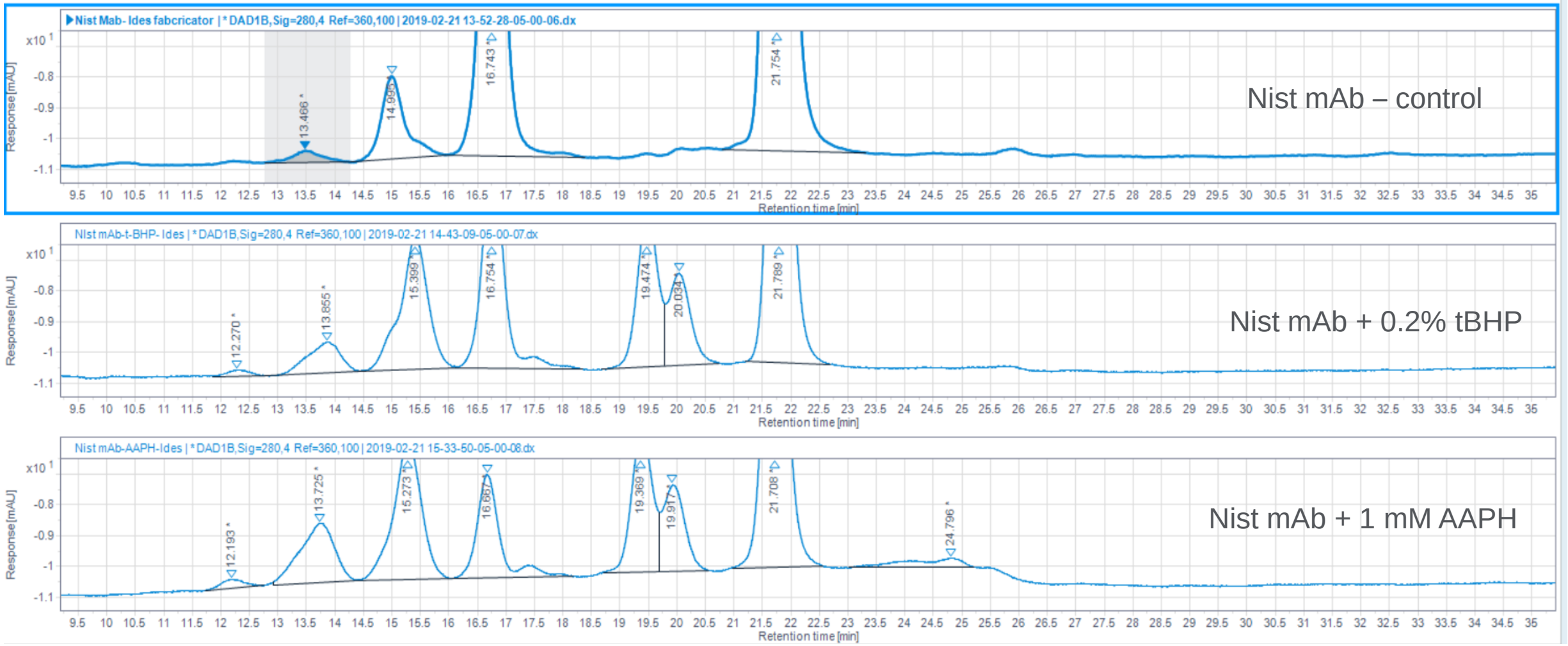


- **Fragment generation - Chemical and enzymatic reactions**
- **Molecules more amenable to chromatographic and mass spectrometric measurement**
- **Modifications can readily be traced back to specific domains.**

NIST mAb – Oxidation Samples Digested Using Fabricator (IDES) Enzyme Zoomed-in

Agilent InfinityLab

t-BHP: Tert-butyl hydroperoxide
AAPH: 2,2'-Azobis(2-amidinopropane) dihydrochloride



Analysis of Monoclonal Antibodies

Using Multiple Heart-cutting Hydrophobic Interaction/Reversed Phase 2D-LC/MS

Agilent InfinityLab

Analysis of Monoclonal Antibodies

Using Multiple Heart-cutting Hydrophobic Interaction/Reversed Phase 2D-LC/MS

Application Note

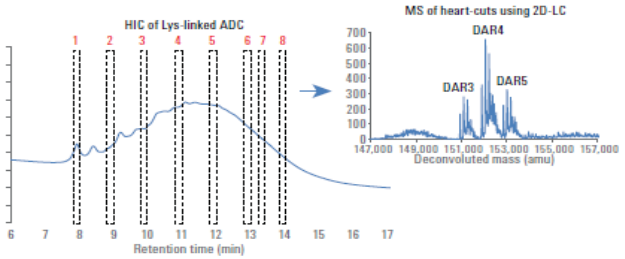
Biotherapeutics & Biologics

Author

Gregory Staples
Agilent Technologies, Inc.

Abstract

Hydrophobic interaction chromatography (HIC) is a popular approach for both downstream processing and analytical scale analysis of monoclonal antibodies (mAbs). In the latter case, HIC is a useful characterization tool because it can often separate mAb variants, for example certain isomers or oxidized species. HIC separations are also gaining popularity for the characterization of antibody-drug conjugates (ADCs). Such investigations often benefit from mass measurement using MS, but the high salt conditions used for HIC separations are completely incompatible with ESI. In this work, we overcome this obstacle using a 2D-LC system, which affords multiple heart-cutting and subsequent desalting/separation using reversed-phase chromatography on-line with TOF MS.



- The application note illustrates the ability of HIC by demonstrating separations of mAbs
- Use of the 2D-LC/MS platform, by combining HIC and RP, eliminates the effect of high salts for HIC, and uses the RP as a desalting step, providing information for unknown peaks

2D-LC Setup for Online Desalting

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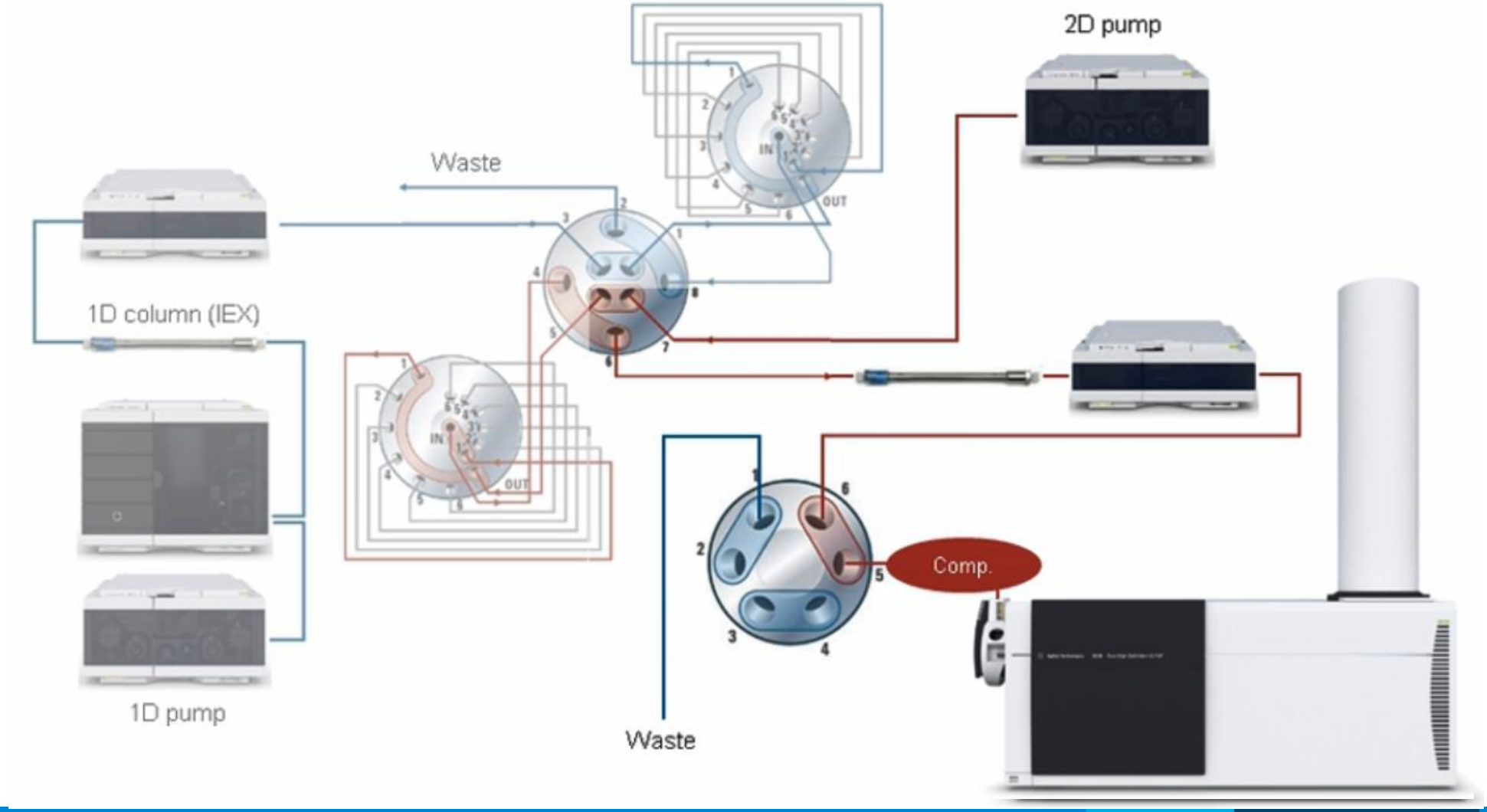
Compound send to MS after valve switch



HIC Separation of a mAB Followed by LC/MS Identification of Impurity Peaks

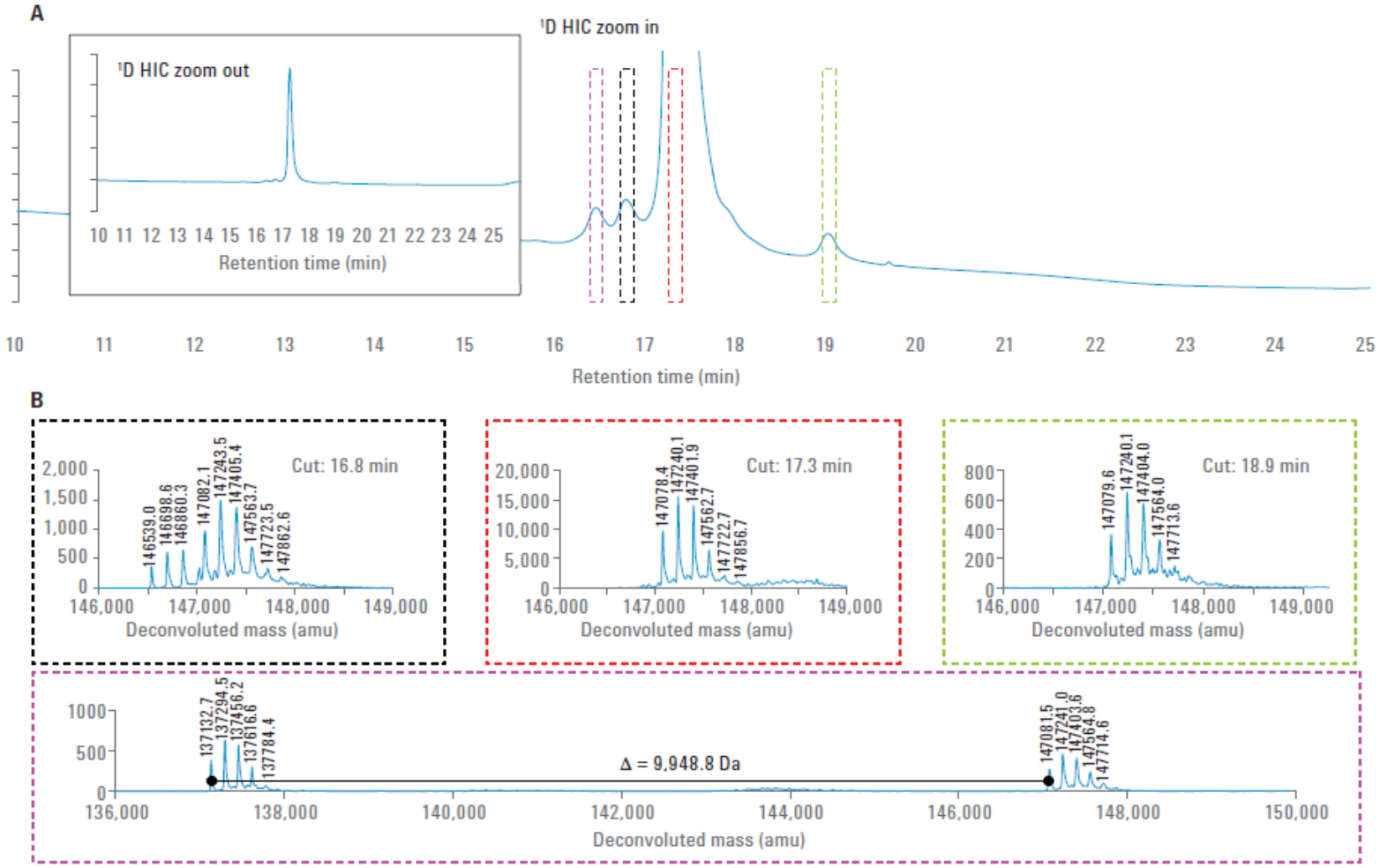
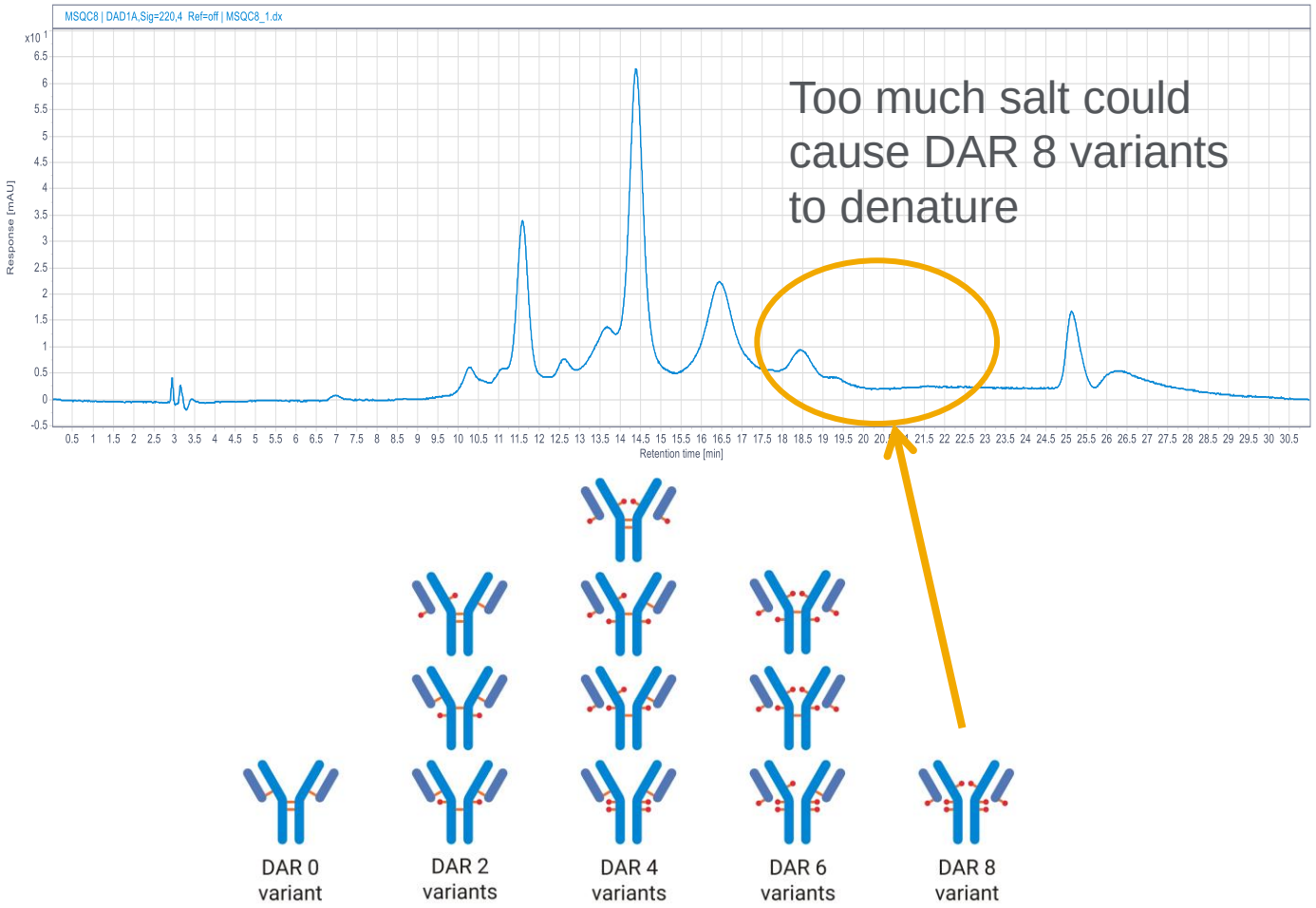


Figure 2. Analysis of mAb A using the HIC/RP 2D-LC/MS system. (A) HIC chromatogram of mAb A revealing multiple satellite peaks (see zoom in) as well as one major peak (see zoom out). (B) Heart-cuts from the first dimension at 16.4, 16.8, 17.3, and 18.9 minutes were stored, then separated on the second dimension followed by MS detection. Various impurities were detected that ranged in mass difference from the main peak.

HIC Column that Does not Require Much Salt

- Too much salt causes problems with subsequent analysis. We are interested in identifying those variants and often need to inject more sample.



Method conditions

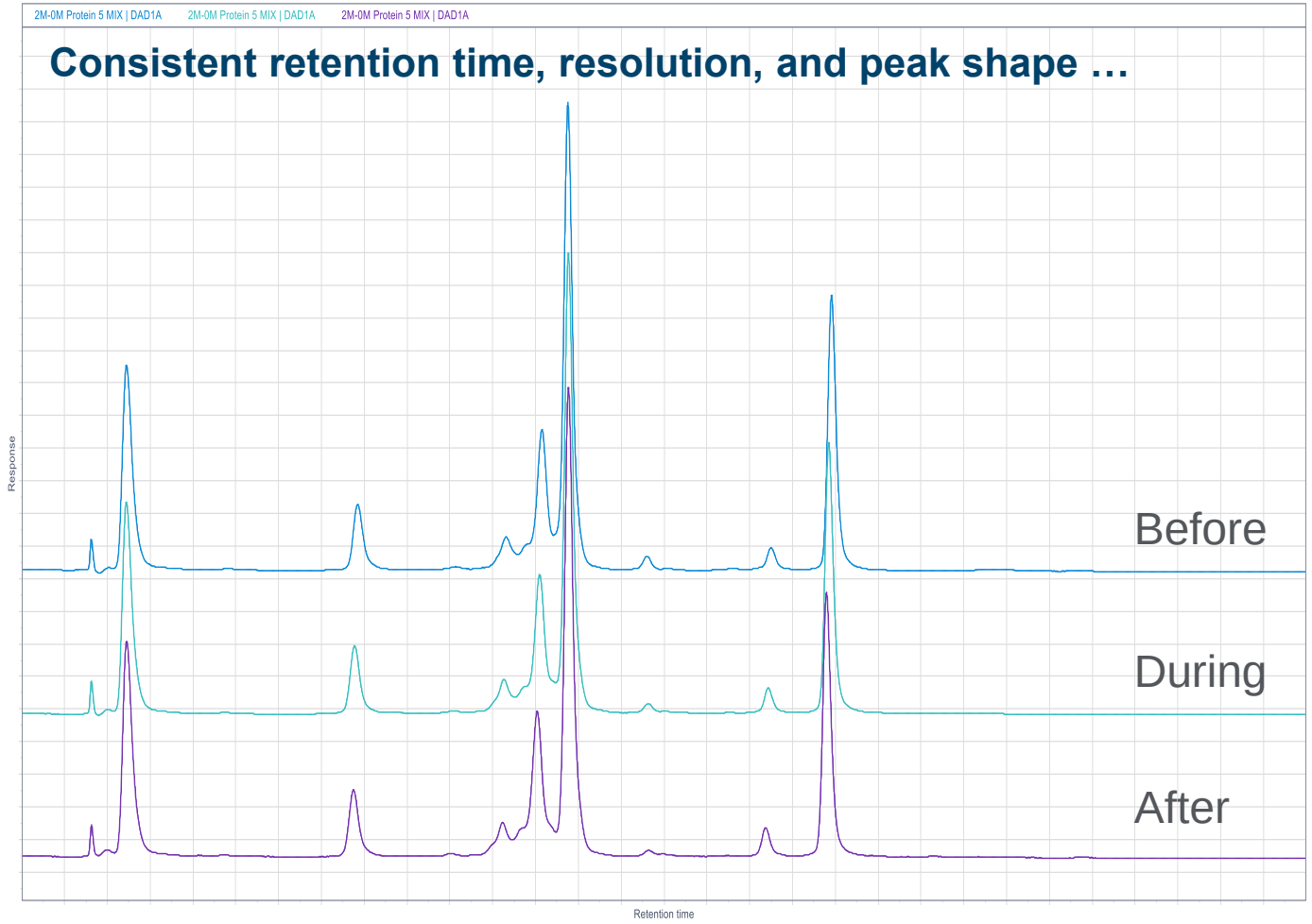
Column:	AdvanceBio HIC 4.6 x 100 mm
Eluent A:	50 mM NaPO, pH 7.0
Eluent B:	2 M $(NH_4)_2SO_4$, 50 mM NaPO, pH 7.0
Eluent C:	Propan-2-ol
Flow rate:	0.4 mL/min
Temperature:	25 °C
Injection:	5 µL (1 mg/mL)
Sample:	Sigma ADC Mimic (MSQC8)

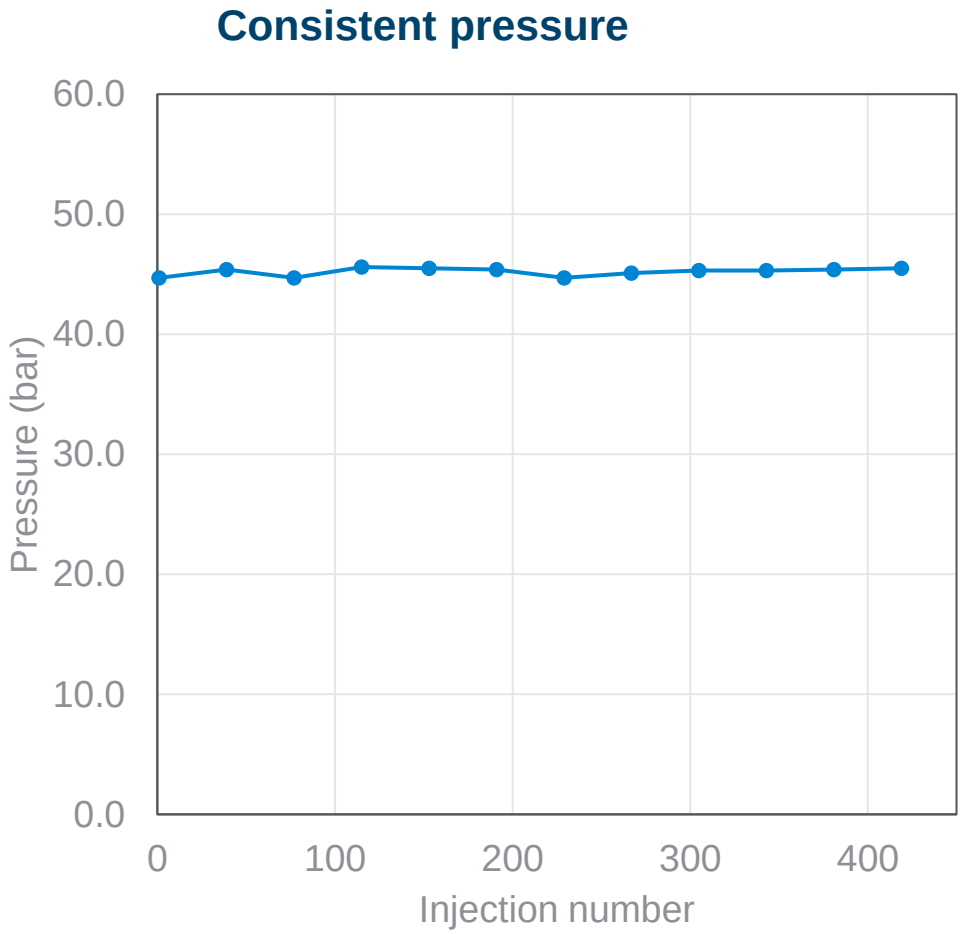
Gradient profile

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

HIC Column Offers Excellent Performance with Good Peak Shape and Resolution

- We see problems with pressure increasing during use, leading to column failure. We need to get good separation between the variant peaks.

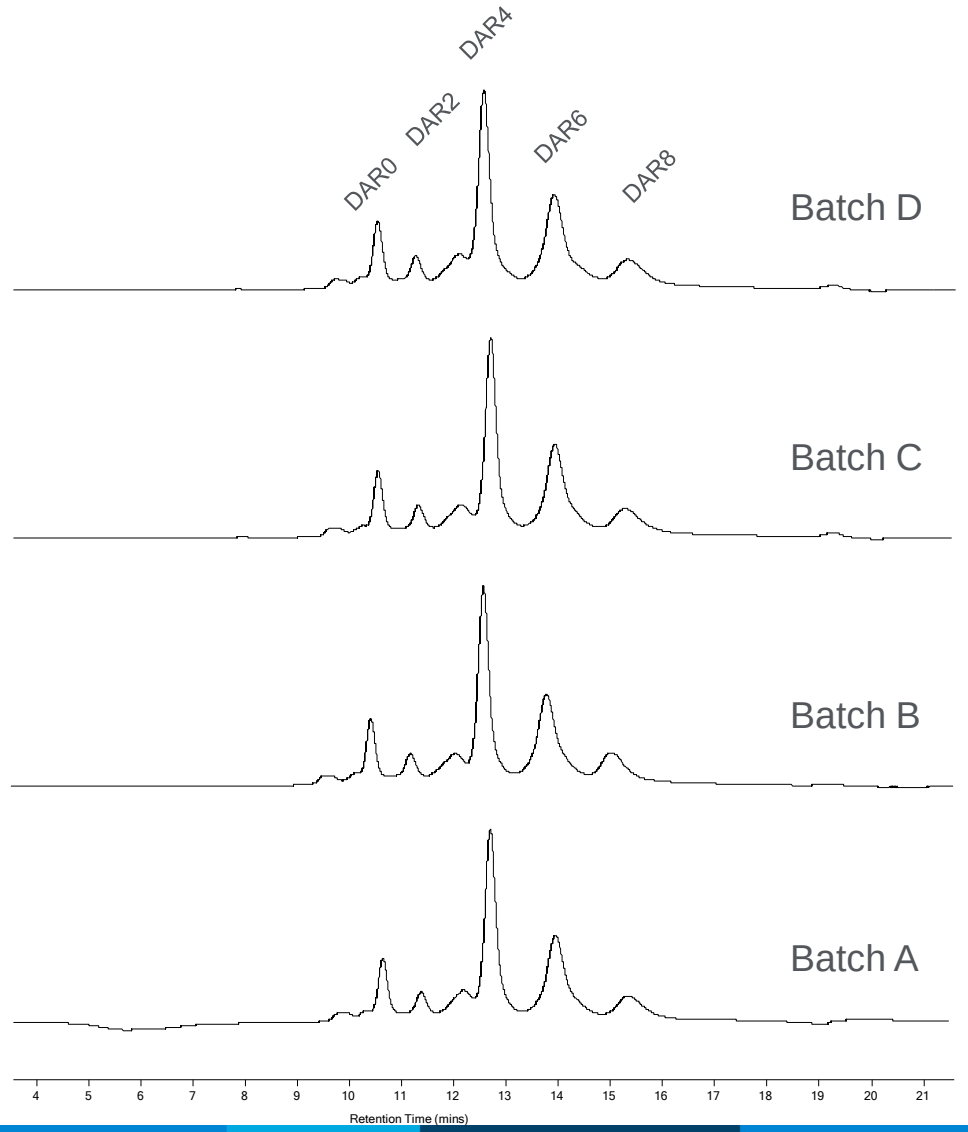




HIC Column Has Batch-to-Batch Reproducibility and Good Reliability

- We have not been able to transfer HIC methods to QC because of reproducibility issues.

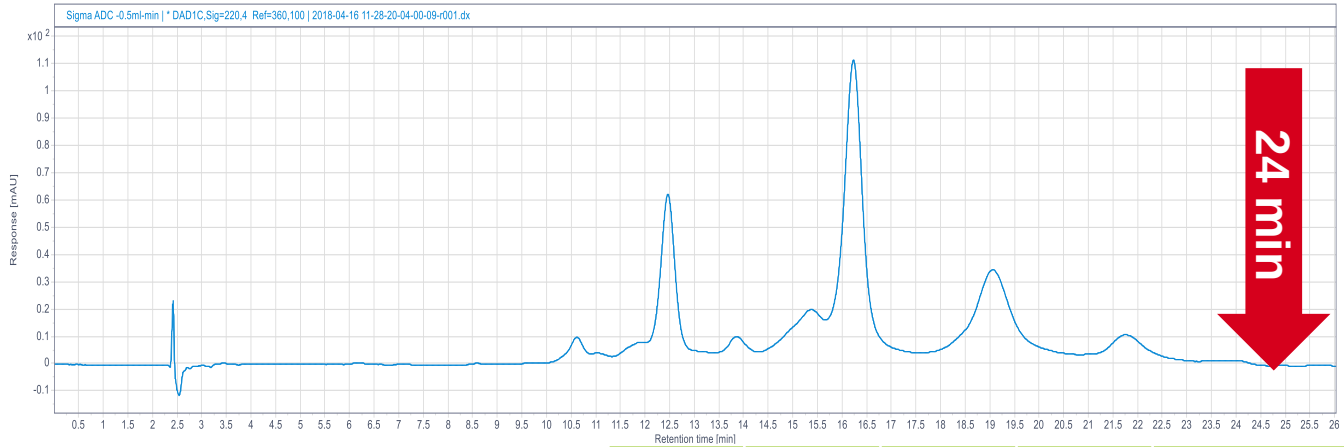
- Consistent retention times
- Consistent peak shapes
- Consistent results



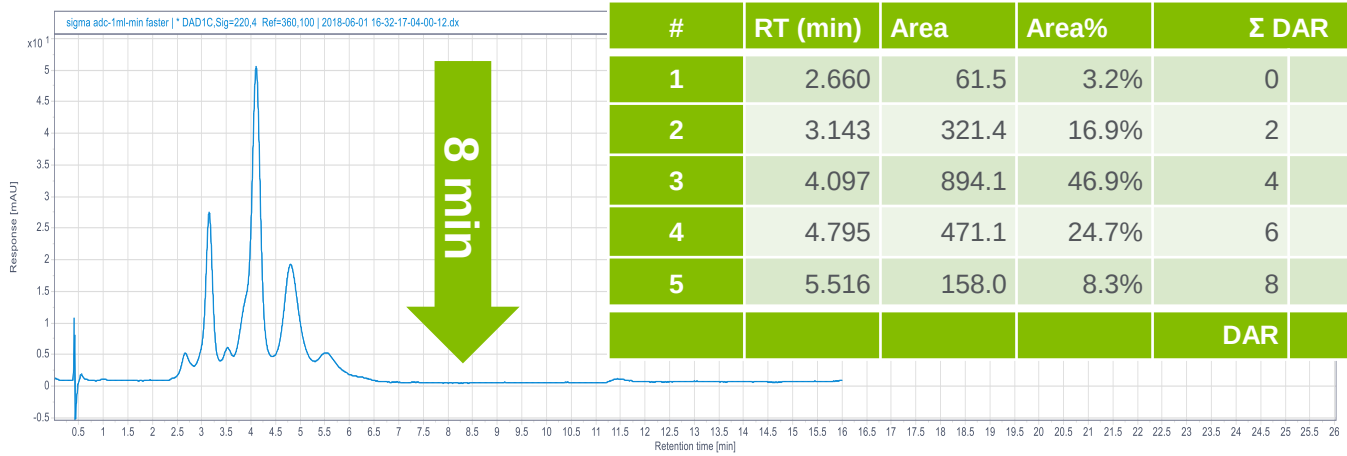
HIC Offers Faster Separations

DAR analysis of brentuximab vedotin

- Throughput is a bottleneck. Speed of analysis is a driver. I can only perform forty-eight 30 minute separations in one day.



#	RT (min)	Area	Area%	Σ DAR	
1	10.605	304.3	3.0%	0	0.00
2	12.457	1768.9	17.2%	2	0.34
3	16.223	4905.3	47.7%	4	1.91
4	19.042	2420.5	23.5%	6	1.41
5	21.760	879.1	8.6%	8	0.68
				DAR	4.35



#	RT (min)	Area	Area%	Σ DAR	
1	2.660	61.5	3.2%	0	0.00
2	3.143	321.4	16.9%	2	0.34
3	4.097	894.1	46.9%	4	1.88
4	4.795	471.1	24.7%	6	1.48
5	5.516	158.0	8.3%	8	0.66
				DAR	4.36

3x higher throughput by using 30 mm column at faster flow rate

Multiple Column Screening Is Not Needed

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- Different types of molecules may require screening of multiple column types from other vendors.

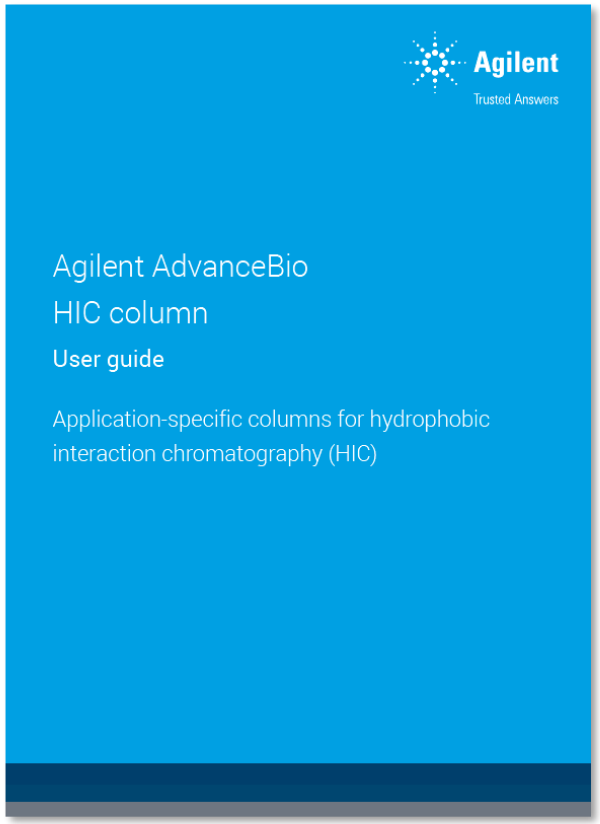
Column	Agilent AdvanceBio HIC	Vendor Column #1	Vendor Column #2	Vendor Column #3
Intact mAbs/Proteins	++++	++++	+++	++
mAb Fragments (F_{ab} and F_c)	++++	+++	++++	+++
Oxidized mAbs	++++	+++	++++	+++
ADCs	++++	+++	+++	++++
Fusion Proteins	++++	++++	+++	++
mAb Aggregates	++++	++++	+++	++

Characteristics and User Guide

AdvanceBio HIC	
Pore size	450 Å
Particle size	3.5 µm
Optimized bonding	For a wide range of molecules
Temperature limit	60 °C (at pH 7) *
Pressure limit	400 bar **
Flow rate	0.5 – 1.0 mL/min ***

Wider pores give better mass transfer for sharper peaks.
A fully porous particle has better loading capacity.

Particles of 3.5 µm size give good performance for proteins with less back pressure. Columns are less likely to clog.



* Protein retention is highly dependent on temperature. Lower operating temperatures can be beneficial for intact proteins.

** Typical operating pressures are <100 bar for high resolution applications

*** Performance is affected by flow rate. Lower flow rates (0.3 to 0.5 mL/min) may be beneficial for high resolution; higher flow rates are better for faster separations.

AdvanceBio HIC Advantages and Benefits

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Increased Performance

- Optimized particle chemistry provides better peak shape and resolution with lower back pressure
- Optimized hydrophobicity for lower salt separation

Better understanding of CQAs

Improved accuracy of quantitation

Confidence in your data

Single Versatile Chemistry

- Unique (proprietary) bonded phase with fine-tuned hydrophobicity offers universal chemistry for a wide range of modalities
- Eliminates the need for multiple columns

Faster method development

Platform methods

Time and cost savings

Increased Productivity

- Short columns for faster analysis without compromising performance

Higher throughput

Lower cost-per-sample

Faster data reporting

Enhanced Robustness

- Column efficiency testing
- Batch testing with NIST mAb
- Provides improved lifetime and reproducibility

Quality and dependability

Confidence in your data

Easier to transfer to QC

Be Agilent Sure of Your Biologic's Critical Quality Attributes

The importance of understanding the attributes of a biologic molecule, and the processes used to create it, cannot be understated.

www.agilent.com/chem/advancebio-columns