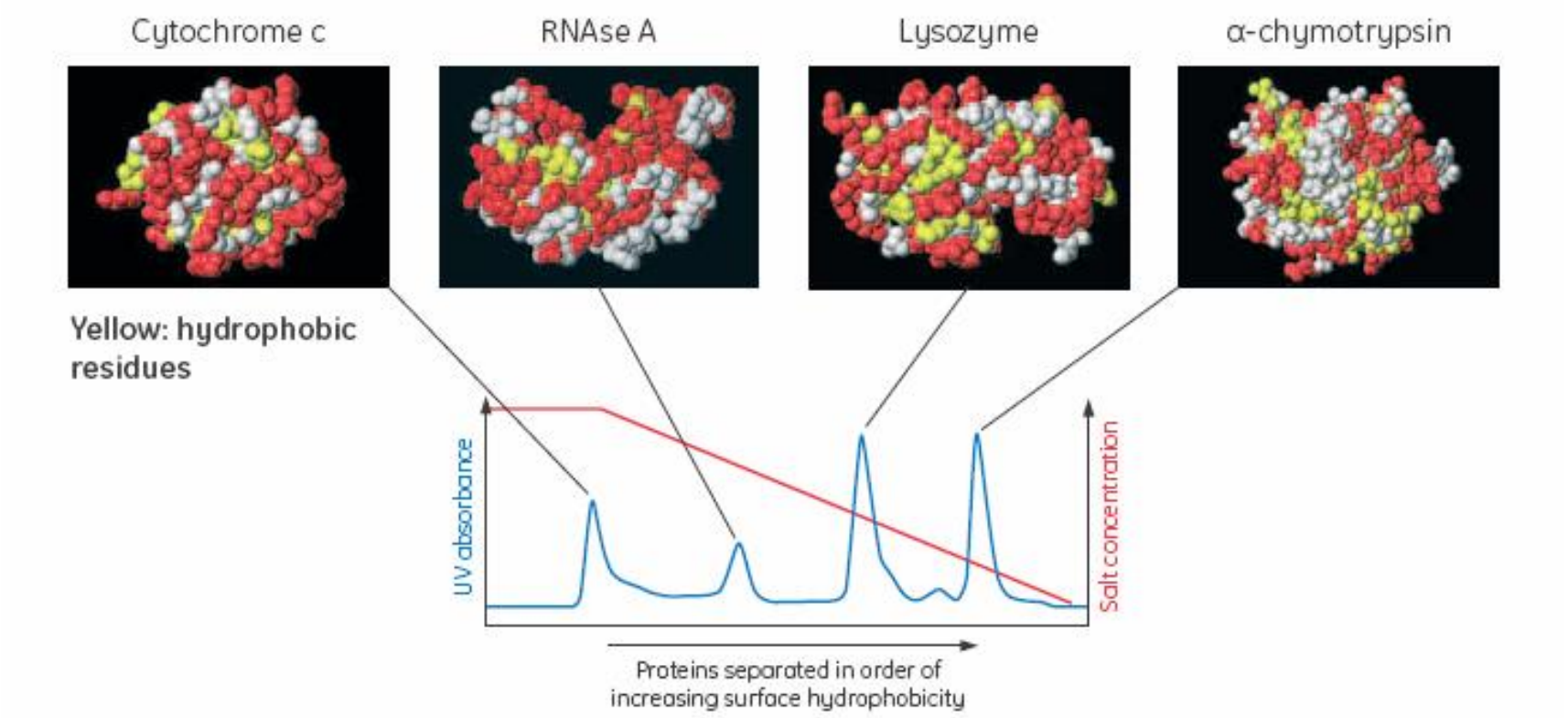
Hydrophobic Interaction Chromatography (HIC)

Hydrophobic interaction chromatography (HIC)

- Principles of HIC
- Main stages in HIC
- HIC Parameters for development optimization : ligand, salt concentration, etc
- Troubleshooting
- Example

What is HIC?

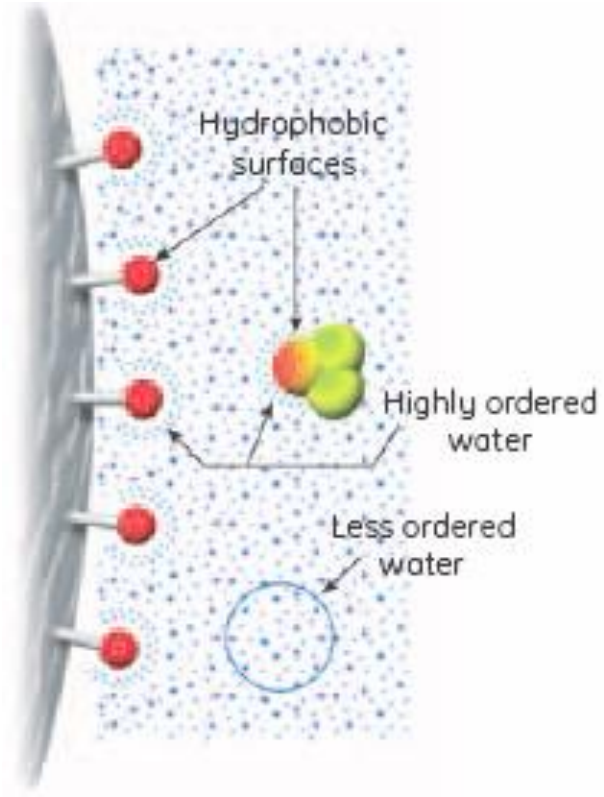
Hydrophobic interaction chromatography (HIC) is a liquid chromatography technique that separates biomolecules according to their hydrophobicity



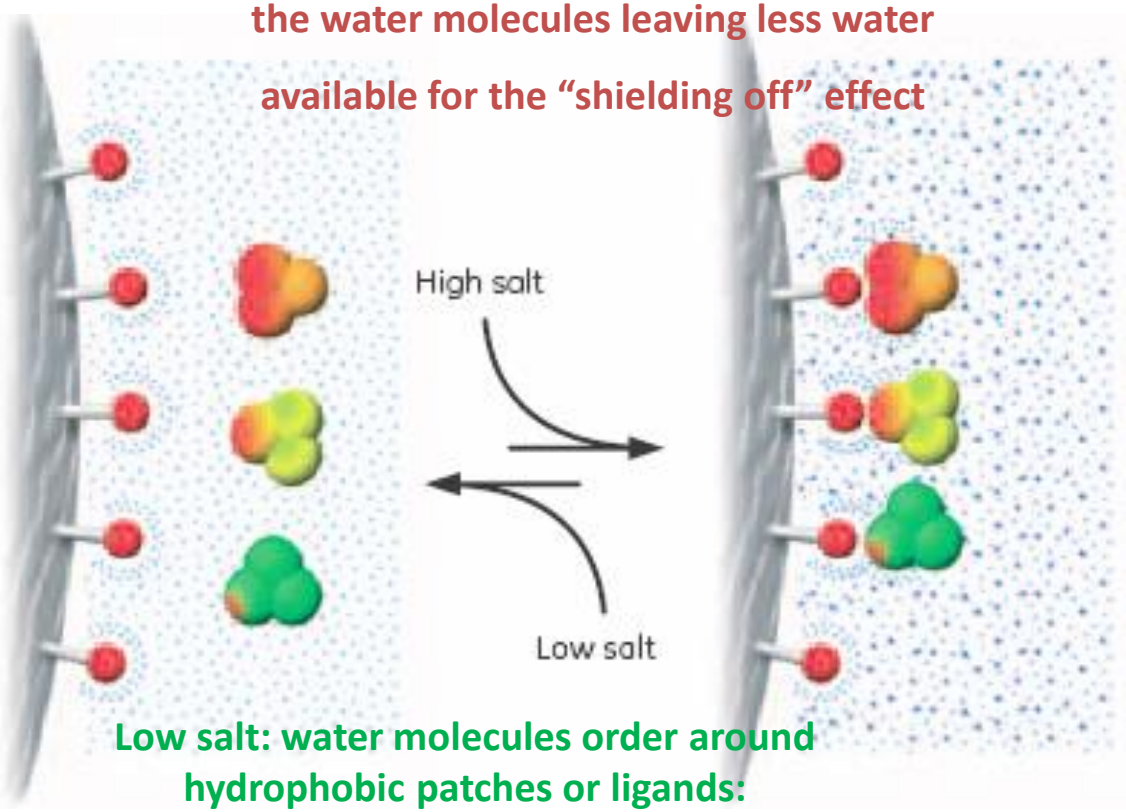
Interaction principle

According to Hydrophobic Interaction and Reversed Phase Chromatography Principles and Methods - GE Healthcare

Close to the surface of the hydrophobic ligand and solute, the water molecules are more highly ordered than in the bulk water and appear to “shield off” the hydrophobic ligand and solute molecules.



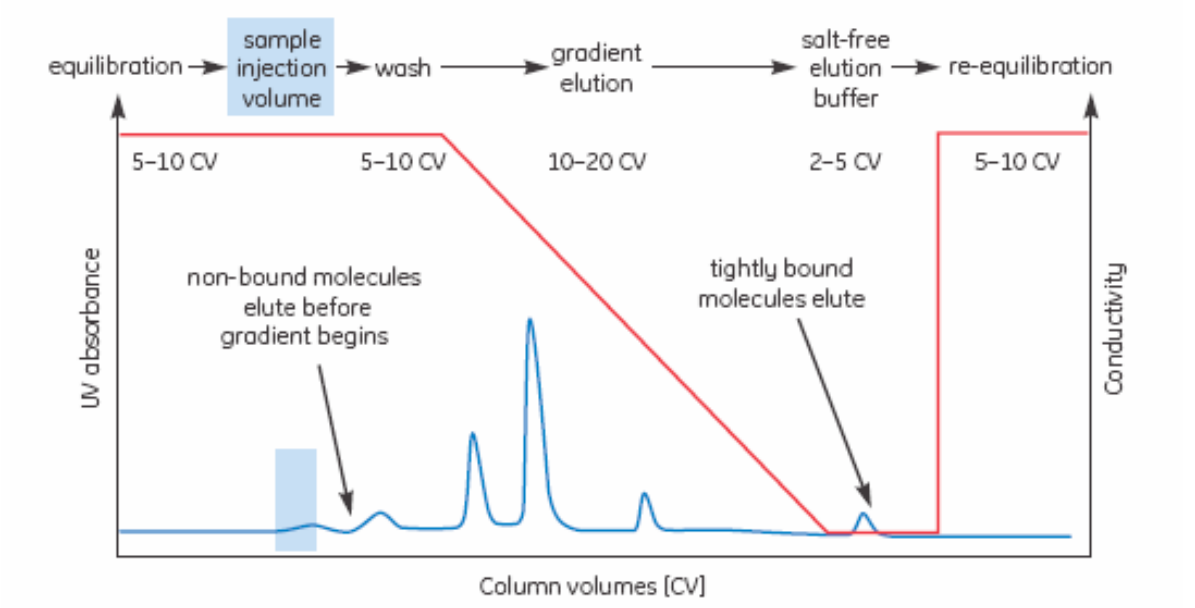
High salt concentration interact strongly with the water molecules leaving less water available for the “shielding off” effect



Low salt: water molecules order around hydrophobic patches or ligands: “shield off” effect

Main stages in HIC

- Equilibrate the gel and the sample to binding conditions
- Apply the sample
- Wash out contaminants
- Elute
- Wash and Regenerate column
- https://www.youtube.com/watch?v=v6SPK6ZovgA



Before starting any HIC separation

- Establish the “salt stability window” for the sample: add increasing amounts of salt to the crude sample in order to establish the concentration at which precipitation occurs.

- Ensure that the sample is below this salt concentration when applied to a column in order to avoid precipitation.

- When possible, test for biological activity of the target protein to establish the concentration range over which activity can be maintained

First trial, general conditions

- Gel: Phenyl Sepharose 6 Fast Flow
- Binding buffer: 50 mM phosphate buffer pH 7.0 with 1-2M SO_4 $[NH_4]_2$ or 3M NaCl
- Elution buffer: 50 mM phosphate buffer pH 7.0
- Gradient: 10-15 column volumes
- Flow rate: according to manufacturer's instructions

HIC pro´s and con´s

Advantages

Mild / stabilizing

Non-denaturative as RPC

Concentrating for capture

High range of selectivity

High diversity of columns

Good recovery

Complements IEX, GF and affinity

Disadvantages

Predictability

High salt – viscosity *(SDS PAGE sample problems)*

Precipitation

Possible denaturation

No use of detergents

Not for membrane proteins

Hydrophobic interaction chromatography (HIC)

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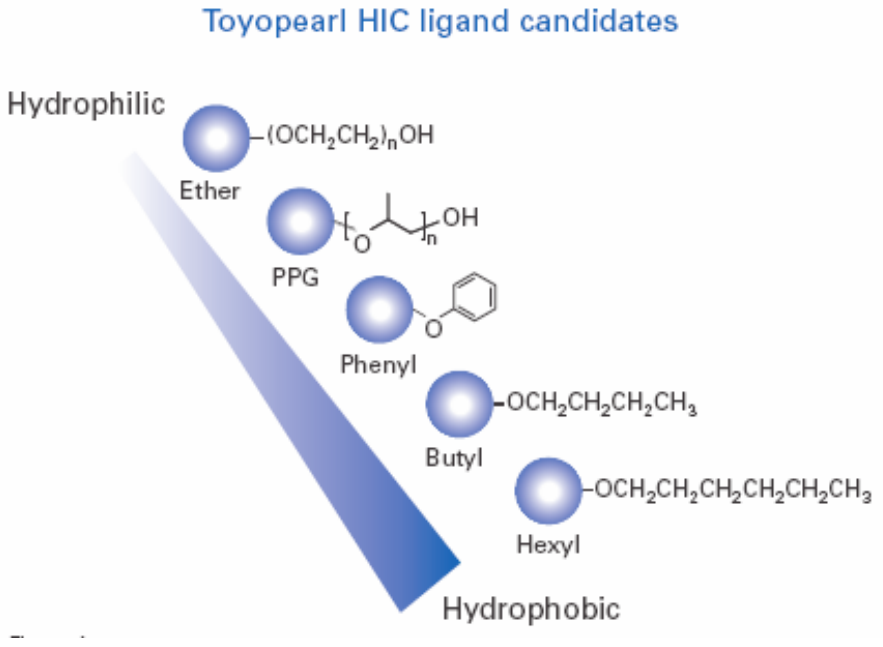
Selectivities of HIC columns



✓The hydrophobicity of the resin determines the salt concentration necessary to adsorb the target

✓With low-hydrophobic ligands, you need more [salt] to bind your protein

✓Very high-hydrophobic ligand might cause irreversible binding of hydrophobic proteins

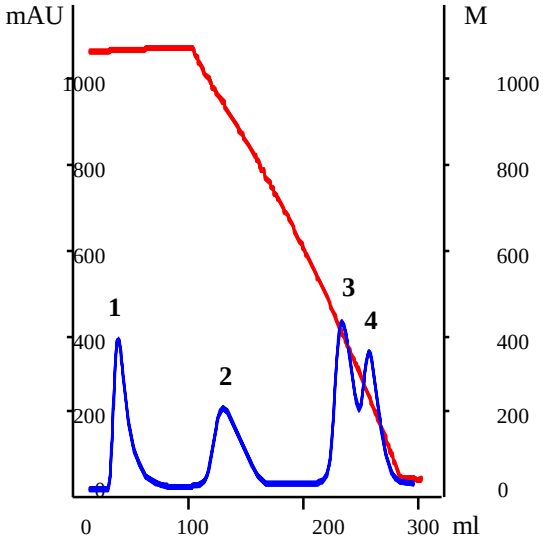
CRITICAL FACTORS - SELECTING MEDIA

Different selectivity of HIC media

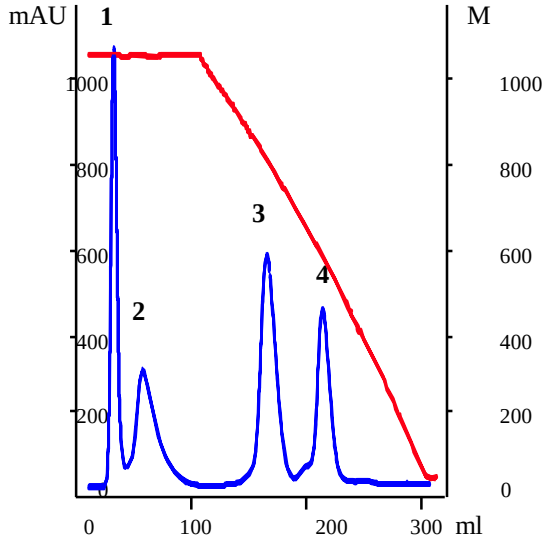
The interaction strength of adsorbents generally varies in the order phenyl>octyl>butyl.

A recommendation is to use a strong adsorbent to allow for lower salt concentration of adsorption buffer and reduce the risk of protein precipitation

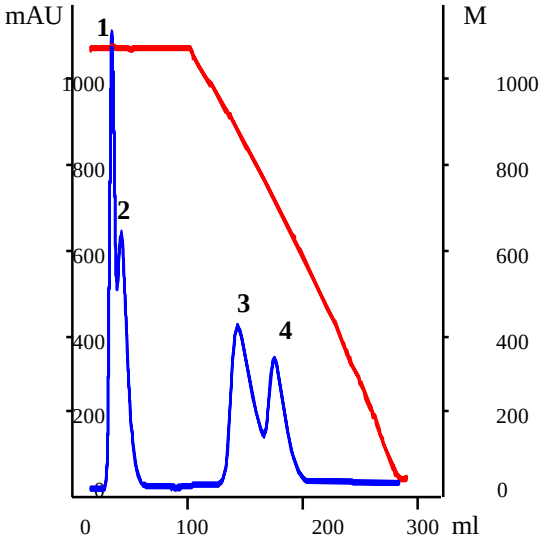
Phenyl (high sub)



Butyl



Octyl



*Columns:*HiPrep 16/10

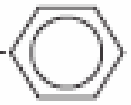
Sample: Cytochrome C (1), lysozyme (2), ribonuclease A (3) and a-chymotrypsinogen (4)

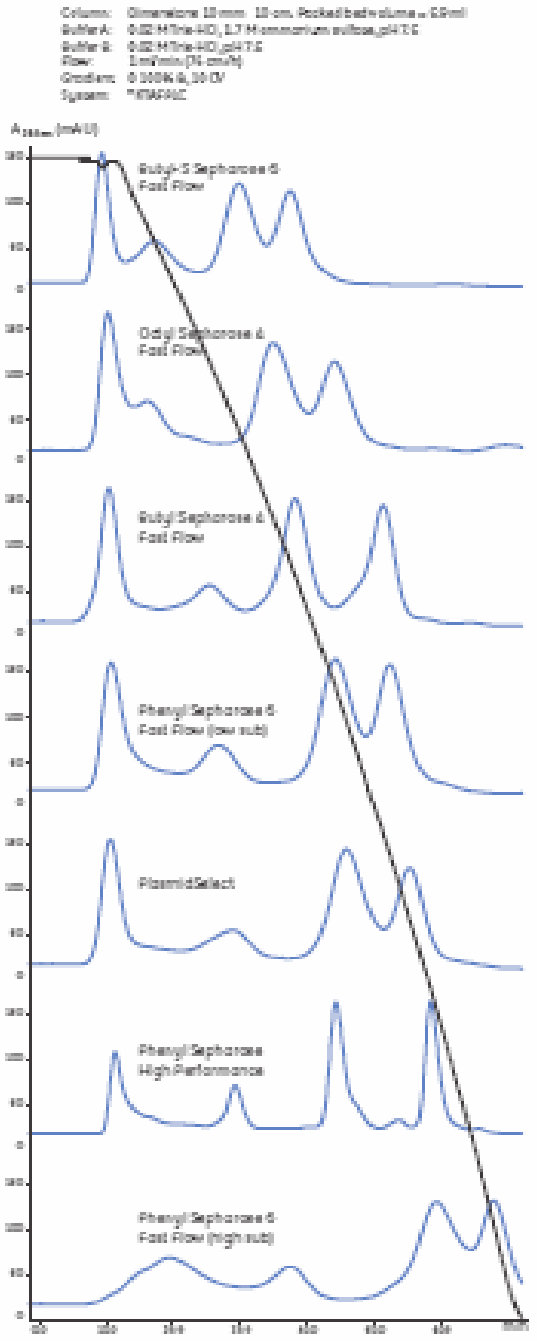
Selectivities of HIC columns

According to Downstream 39 from GE Healthcare

10cv gradient 1.7 to 0M Ammonium Sulfate

Sample: Cytochrome C, RNaseA, Lysozyme and α Chymotrypsinogen

Phenyl	
Butyl-S	$-S-(CH_2)_3-CH_3$
Butyl	$-O-(CH_2)_3-CH_3$
Octyl	$-O-(CH_2)_7-CH_3$
Ether	$-O-CH_2-CHOH-CH_2-OH$
Isopropyl	$-O-CH-(CH_3)_2$



Butyl S-Sepharose 6 FF

Octyl Sepharose 4 FF

Butyl Sepharose 4 FF

Phenil-Sepharose 6 FF (low sub)

Plasmid Select

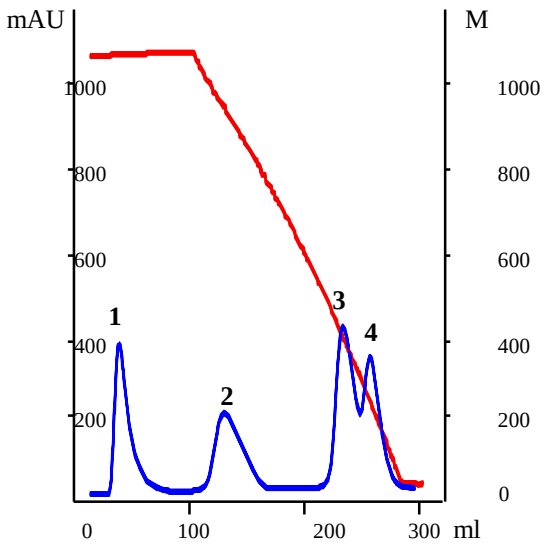
Phenil-Sepharose High Performance

Phenil-Sepharose 6 FF (high sub)

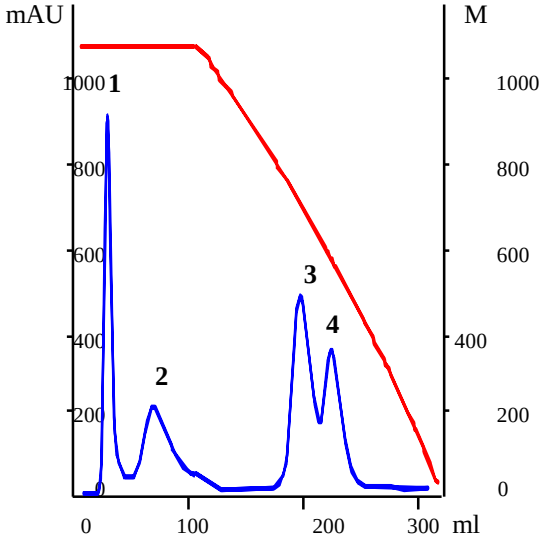
Degree of substitution

Generally, there is an increase in protein binding capacity concomitant with an increase in substitution level.

Phenyl (high sub)



Phenyl (low sub)



Columns: HiPrep 16/10

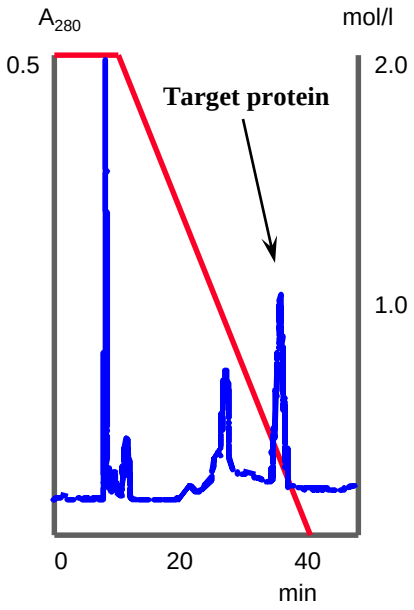
Sample: Cytochrome C (1), lysozyme (2), ribonuclease A (3) and a-chymotrypsinogen (4)

CRITICAL FACTORS - SELECTING ADSORPTION CONDITIONS

Effect of salt concentration on adsorption

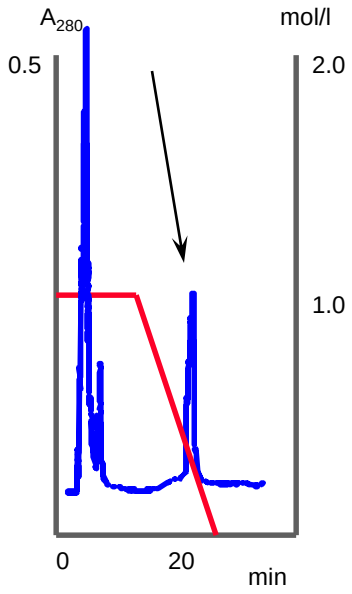
Too high

2 M $(NH_4)_2SO_4$



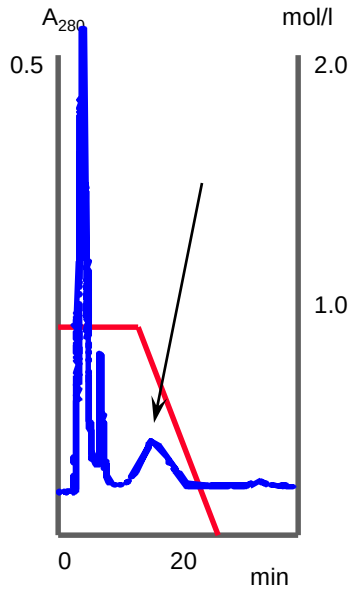
Optimal

1 M $(NH_4)_2SO_4$



Too low

0.8 M $(NH_4)_2SO_4$



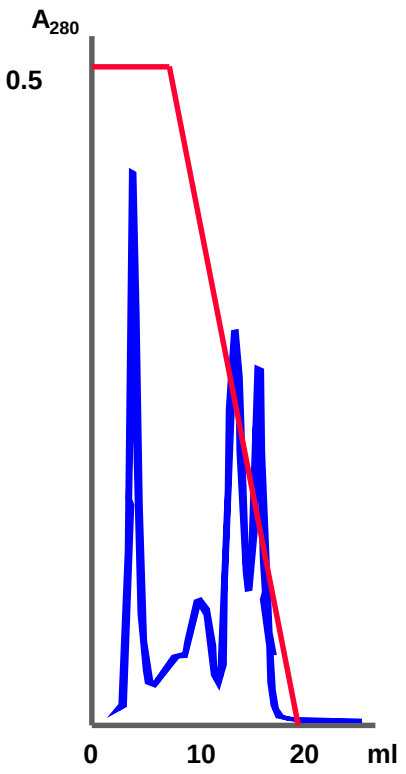
In general the adsorption process is often more selective than the desorption process and it is therefore important to optimize the starting buffer conditions

Ideal situation: salt concentration with selective binding of the target protein while the majority of the impurities pass through the column

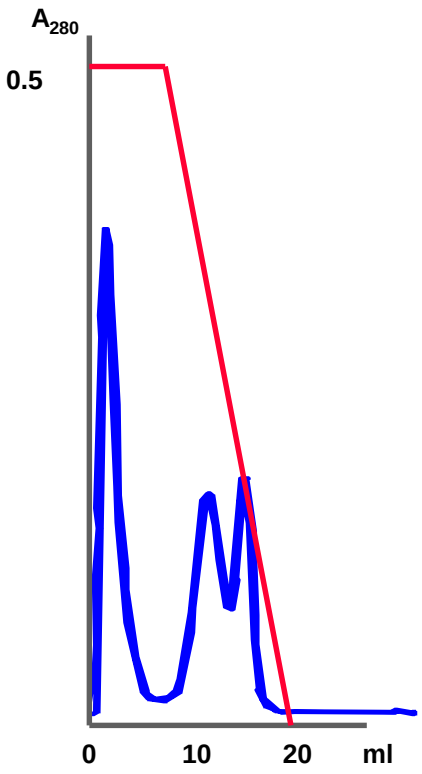
CRITICAL FACTORS - SELECTING ADSORPTION CONDITIONS

Effect of different salts

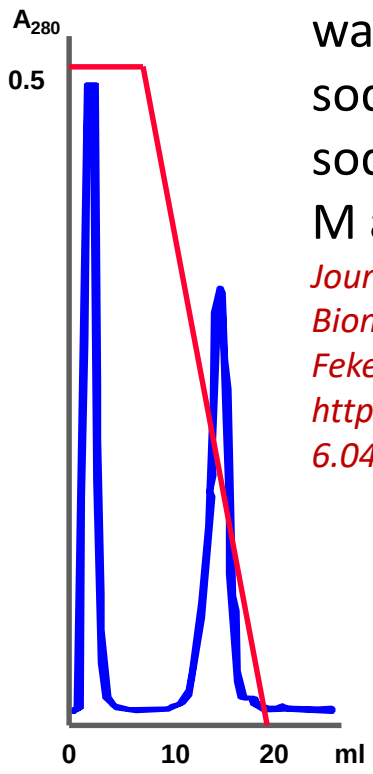
1.7 M $(NH_4)_2SO_4$



1 M Na_2SO_4



3 M NaCl



Thermo HIC 10 column

1M ammonium sulfate was equivalent to 2.2 M sodium acetate, 2.6 M sodium chloride and 3.3 M ammonium acetate

Journal of Pharmaceutical and Biomedical Analysis
Fekete S et al. 2016
http://dx.doi.org/10.1016/j.jpba.2016.04.004

Different salts, different binding and elution effects

CRITICAL FACTORS - SELECTING ADSORPTION CONDITIONS

The Hofmeister series

⟵ Increasing precipitation ("salting-out") effect

Increasing chaotropic ("salting-in") effect ⟶

Anions: PO_4^{3-}, SO_4^{2-}, $CH_3 \cdot COO^-$, Cl^-, Br^-, NO_3^-, ClO_4^-, I^-, SCN^-

Cations: NH_4^+, K^+, Na^+ Cs^+, Li^+, Mg^{2+}, Ca^{2+}

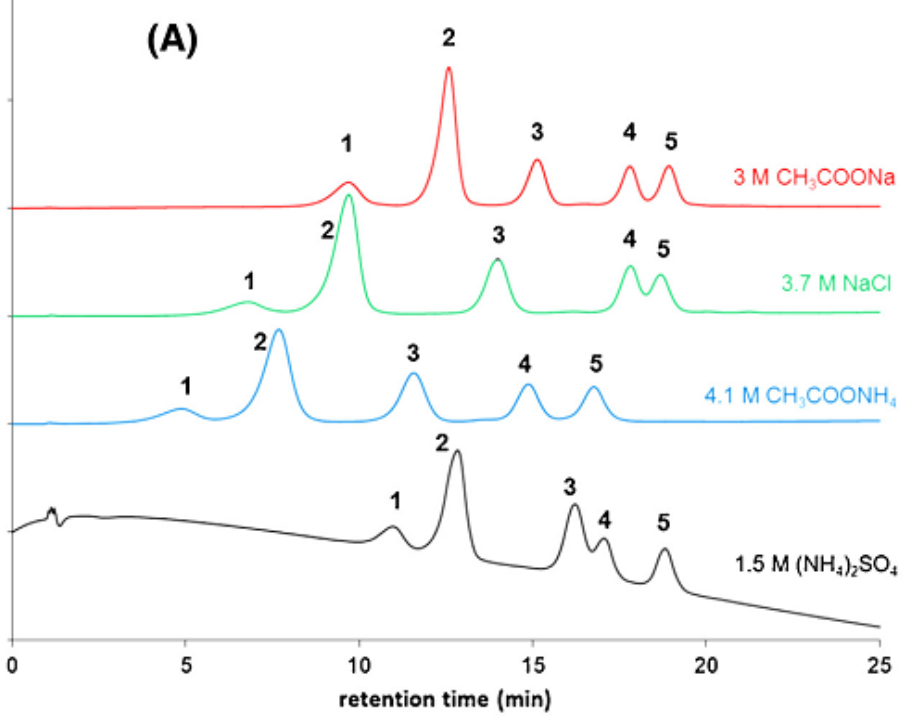
The most commonly used salts are $(NH_4)_2SO_4$, Na_2SO_4, NaCl, KCl and CH_3COONH_4.

1M ammonium sulfate is equivalent to 2.2 M sodium acetate, 2.6 M sodium chloride and 3.3 M ammonium acetate

CRITICAL FACTORS

Different salt systems

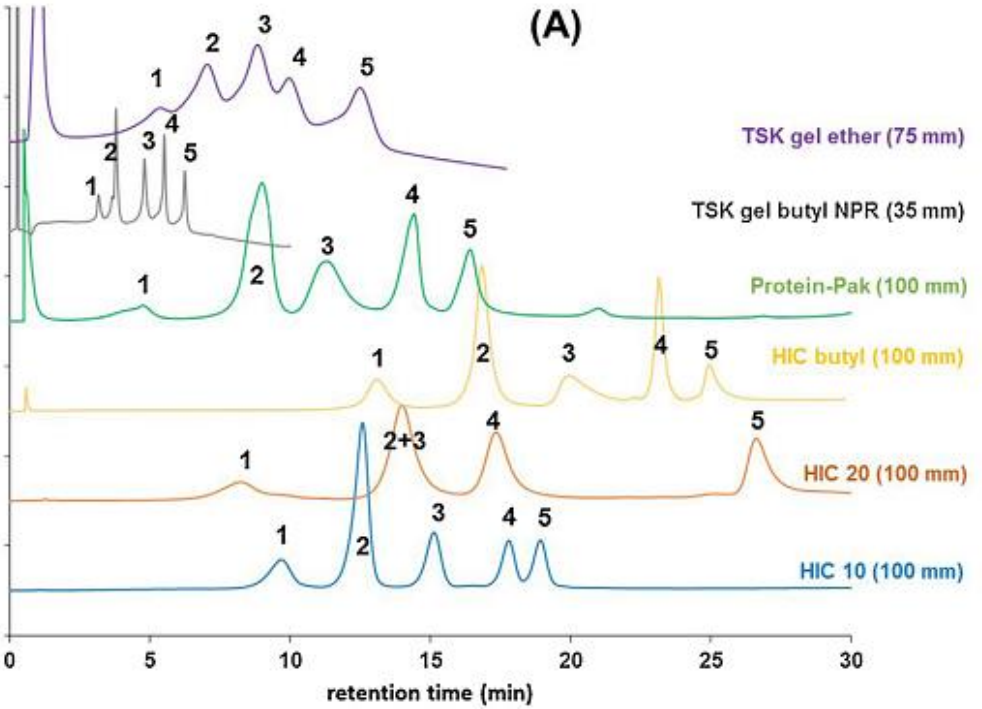
MabPac HIC 10 (100 × 4.6 mm, 5µ)
column Flow rate: 1 mL/min,
gradient: 0–100% B in 30 min, 20◦C



A. Cusumano et al. / Journal of Pharmaceutical and Biomedical Analysis 121 (2016) 161–173

Different column Suppliers

Sodium acetate salt system.
Flow rate: 1 mL/min for 4.6 mm columns and 0.21 mL/min on 2 mm columns,
Gradient: 0–100% B



CRITICAL FACTORS - SELECTING ADSORPTION CONDITIONS

Effect of pH

- ✓ Binding at a pH value close to the protein's pI as well as conditions near the solubility limit resulted in increased protein binding

- ✓ These findings indicated a structural change upon binding in HIC, which are favored under destabilizing conditions

Effects of Mixed Electrolytes on Protein Separations: DBC (dynamic binding capacity)

(TOSOH BIOSCIENCE)

- ✓ While the capacities and recoveries of HIC applications cannot compete with those of modern IEX resins, its performance can be improved by adjusting the operating parameters
- ✓ The salt most recognized for use in HIC separations is ammonium sulfate. Its salting-out (kosmotropic) potential is well-known and it is also used in non-chromatography based purification methods such as protein precipitation. Others: sodium citrate and sodium sulfate

Table 1a. Lysozyme dynamic binding capacity

Salt	TOYOPEARL PPG-600M Capacity (g/L)	TOYOPEARL Phenyl-600M Capacity (g/L)	TOYOPEARL Butyl-600M Capacity (g/L)
2.0 mol/L $(NH_4)_2SO_4$	30	46	15
1.0 mol/L sodium sulfate + 1.0 mol/L sodium acetate	39	63	18
1.0 mol/L $(NH_4)_2SO_4$ + 1.0 mol/L NaCl	31	54	10
0.9 mol/L trisodium citrate + 0.9 mol/L NaCl	38	43	20

Lysozyme dynamic binding capacities measured at 10% breakthrough. Capacities were evaluated in various mobile phases with TOYOPEARL PPG-600M, TOYOPEARL Phenyl-600M, and TOYOPEARL Butyl-600M resins. The capacity measured in 2.0 mol/L ammonium sulfate serves as a point of reference for the other samples.

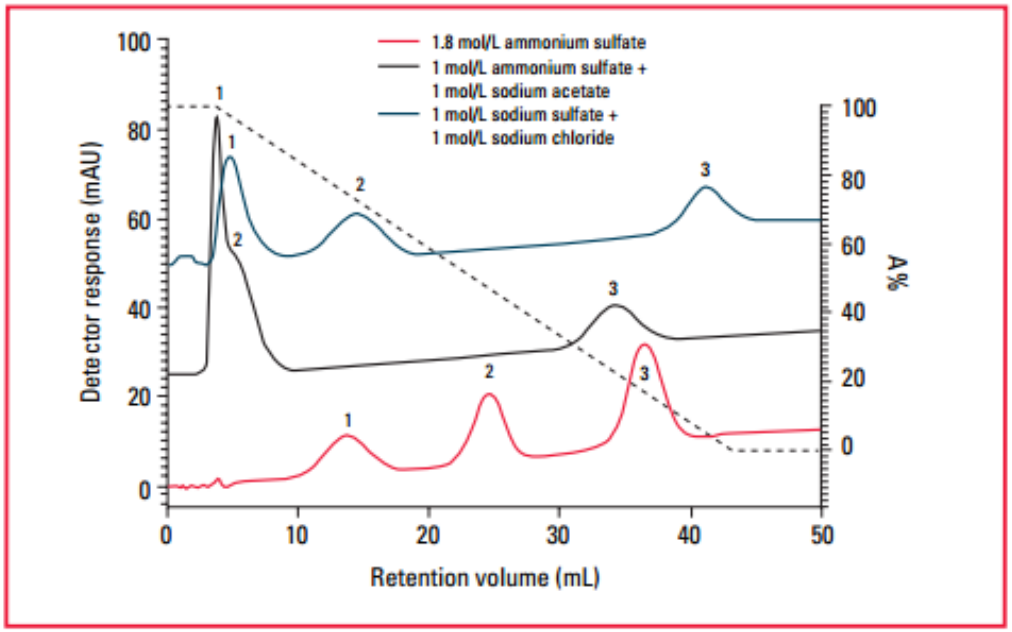
- ✓ Sodium acetate and sodium chloride are common mobile phase salts used in manufacturing scale purifications. Are not typically used with HIC as their kosmotropic properties are relatively weak.
- ✓ **By mixing chaotropic with kosmotropic salts, the DBC can strongly be influenced**
- ✓ **Salt mixtures with a higher amount of chaotropic compared to kosmotropic salt mainly increased the DBCs** whereas higher ratios of kosmotropic salt decreased the binding capacities even compared to single salt experiments.

Effects of Mixed Electrolytes on Protein Selectivity

(TOSOH BIOSCIENCE)

- ✓ Using mixed electrolytes in HIC introduces an additional opportunity to improve HIC separations as an alternative to traditional HIC applications using ammonium sulfate
- ✓ The kinetics followed an inverse trend compared to the DBCs. The highest DBCs were obtained for the setups of slowest kinetics

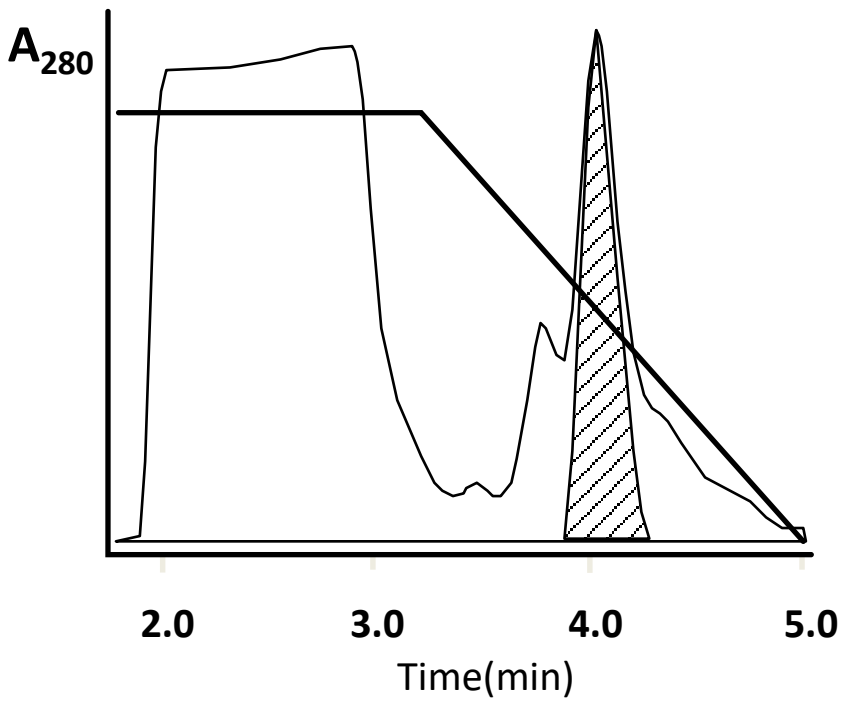
Figure 3. Separation of cytochrome C, ribonuclease A, and lysozyme on TOYOPEARL Butyl-600M



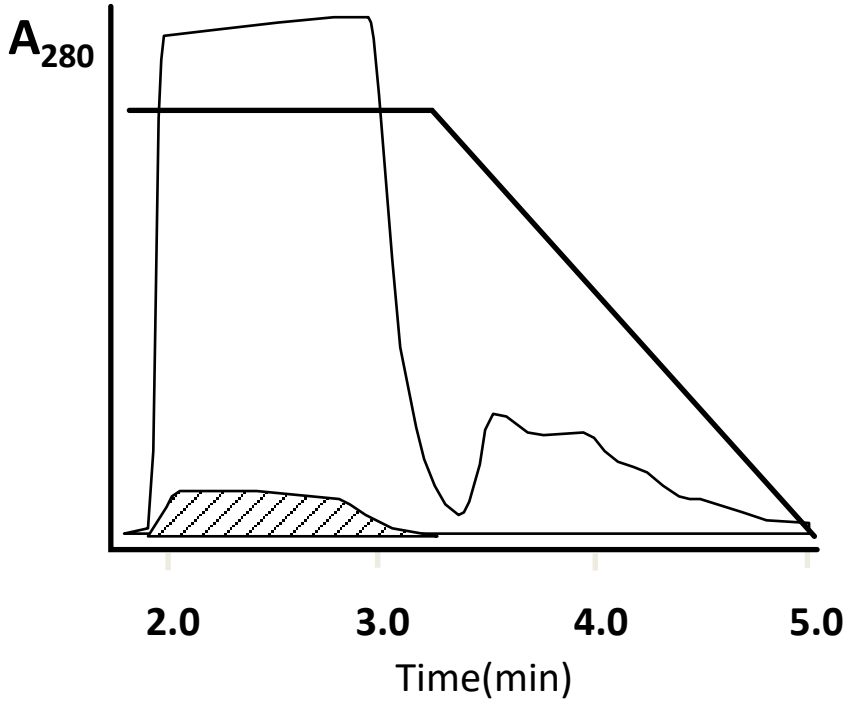
Influence of Mixed electrolytes in HIC selectivity

Effect of temperature 1

23°C Sample & System



4°C Sample, 23°C System



SOURCE™15ISO

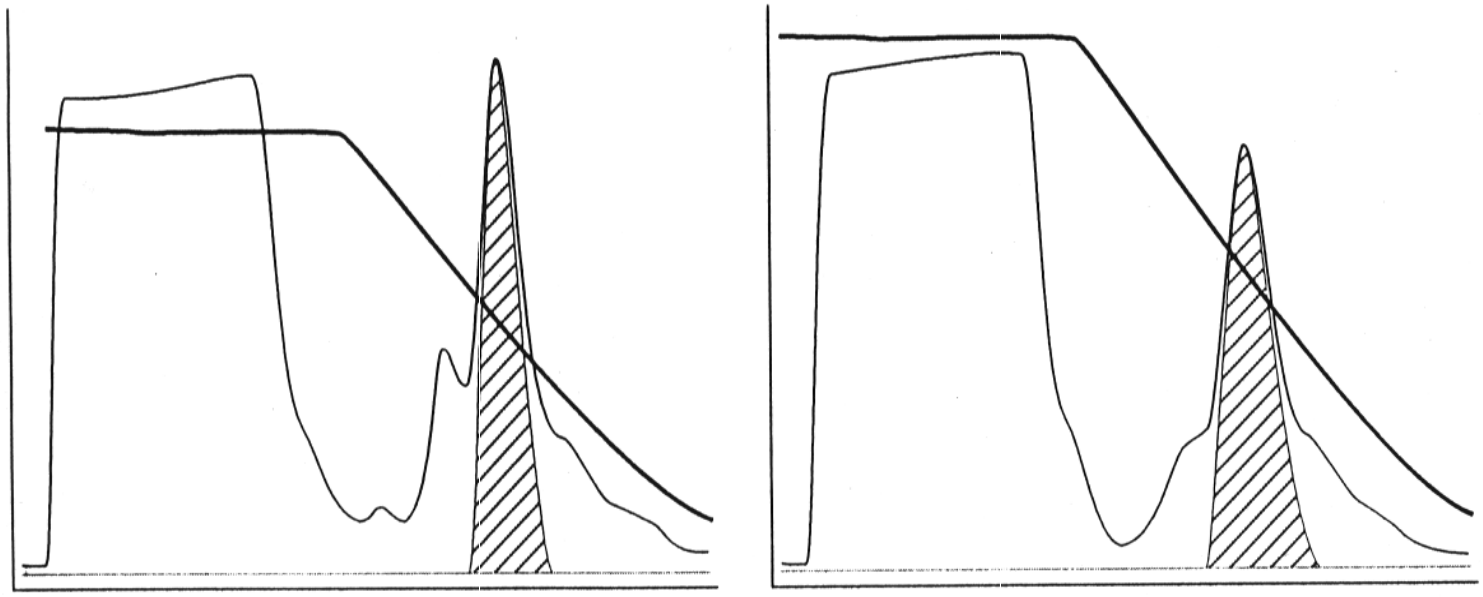
Hydrophobic interaction generally decreases with decreasing temperature

CRITICAL FACTORS - SELECTING ADSORPTION CONDITIONS

Effect of temperature 2

System at 23°C, 1.25 M salt

System at 4°C, **1.55** M salt



Higher temperature, higher binding effect and lower salt needed for binding or elution

CRITICAL FACTORS - SELECTING ELUENTS CONDITIONS

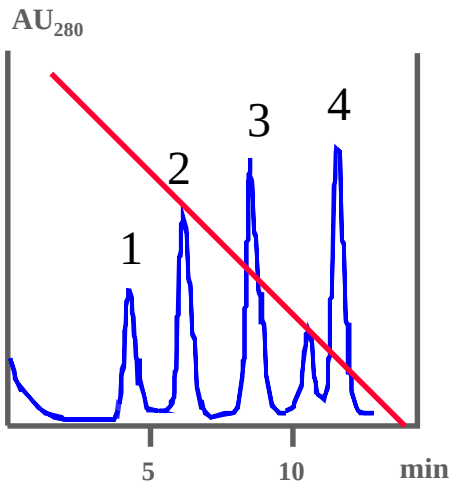
Type of Eluent - Use of Additives

- **Low salt concentration** - *Best solution*
- **Organic solvents** (Up to 10% ethanol or 30% isopropanol or 10% glycerol or 20–80 v/v % ethylene glycol) - *Change Buffer Polarity. Weak hydrophobic interactions by decreasing surface tension. May affect the conformation of the protein.*
- **Detergents** (From 0.1% up to 1% v/v Triton X-100) – *Non-polar regions compete with the proteins for the hydrophobic ligands, causing dissociation. Change Buffer Polarity. Decrease Surface tension. May affect the conformation of the protein. Detergent can strongly bind to the resin ligand*
- **Chaotropic salts** ($MgCl_2$, $CaCl_2$, NaCNS, up to 8M Urea) - *Decrease the hydrophobic effect in solution. May affect the conformation of the protein. Ca^{2+} increases stability during purification of calcium-binding proteins; Mg^{2+} decreases stability.*
- **Additives** like Arginine, other amino acids, osmolytes, etc - *Can influence a separation by improving protein solubility, modifying protein conformation and helping elution of bound proteins*.

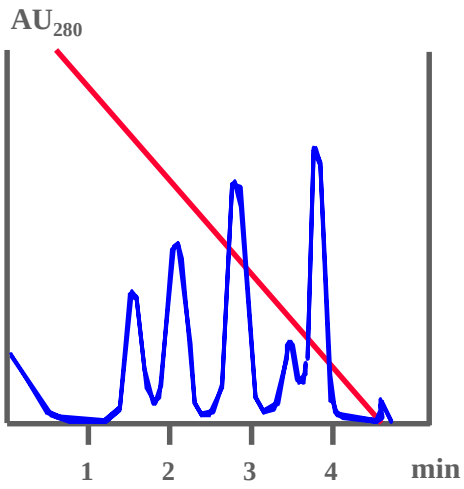
CRITICAL FACTORS - SELECTING ELUENTS CONDITIONS

Effect of flow rate on resolution

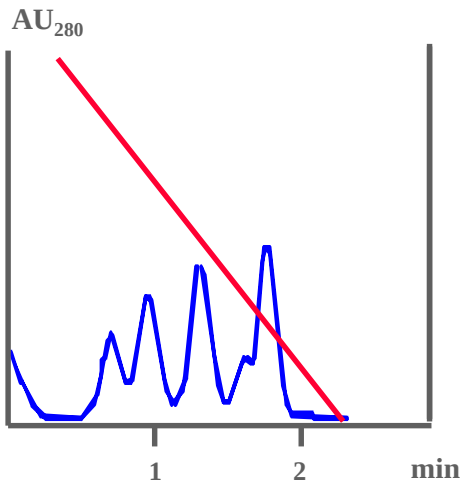
300 cm/h

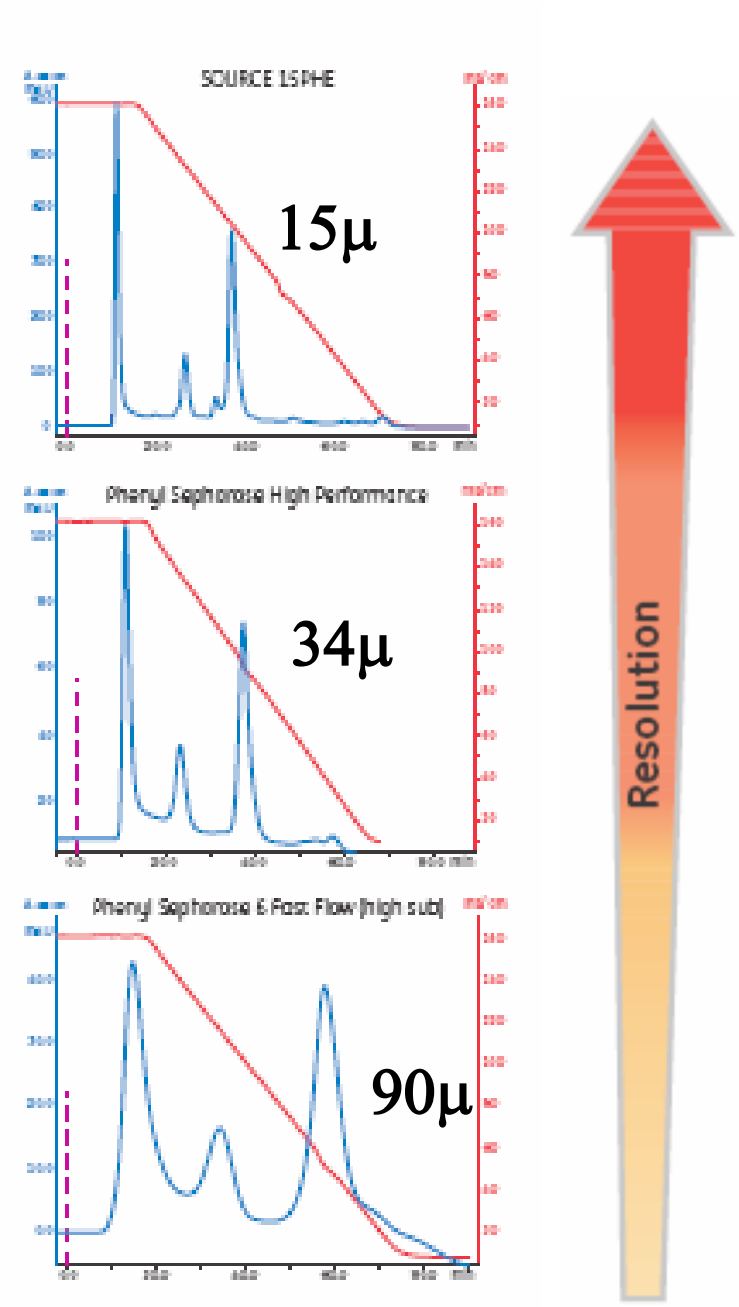


900 cm/h



1800 cm/h





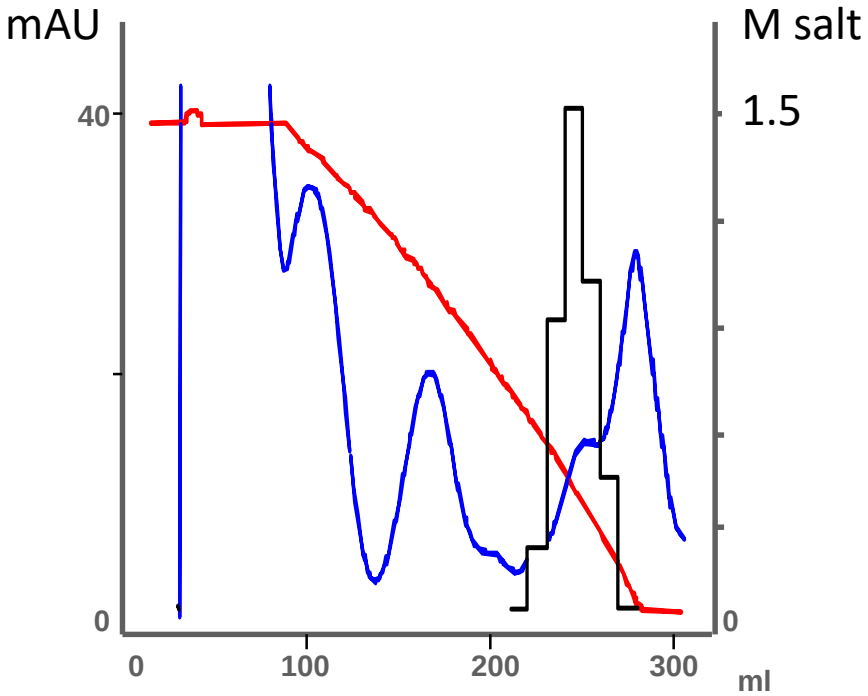
CRITICAL FACTORS - SELECTING ELUENTS CONDITIONS

Effect of particle size on resolution

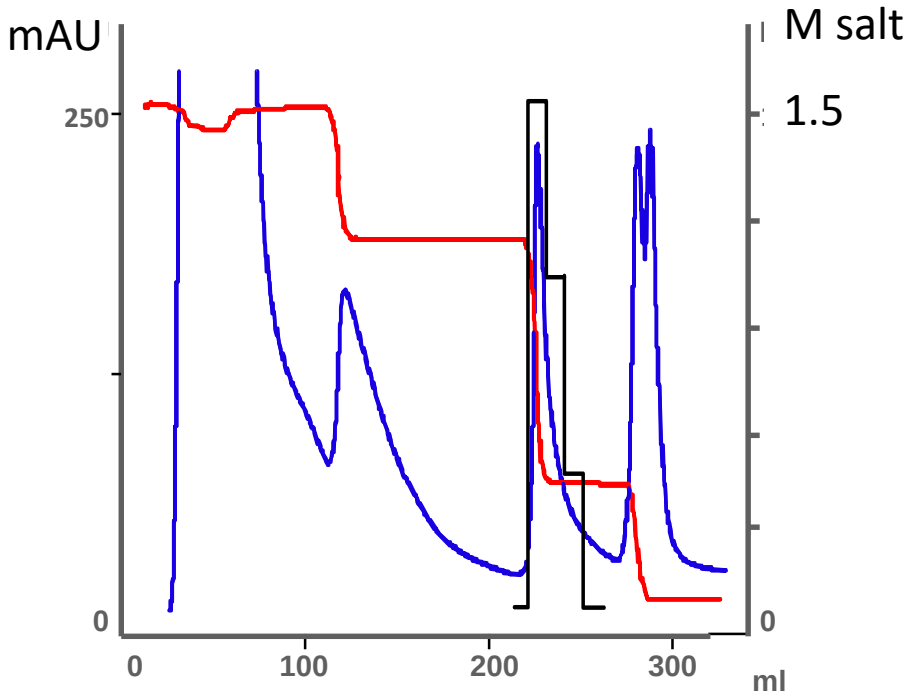
CRITICAL FACTORS - SELECTING ELUENTS CONDITIONS

Effect of gradient shape on separation

Linear gradient



Step gradient



Aim of a step elution: reduced separation time & reduced buffer consumption

Apply up to 20% of the total binding capacity of the column for optimal resolution with gradient elution.

Sample load can be increased if resolution is satisfactory or when using a step elution.

Variables for HIC method development

- Hydrophobicity of the ligand
- Gradient type and slope
- Column dimensions
- Flow rate
- Use of aditives
- Degree of ligand substitution
- Particle and pore size
- Salt type and mixture of salts
- Buffer concentration
- pH
- Temperature
- Type of Matrix / Supplier

Short practical optimization strategy

- Screen media for proper selectivity
- Optimize type and concentration of salt during adsorption
- Optimize gradient shape for maximum resolution and/or throughput
- If resolution or throughput are not ideal, continue optimizing with flow rate, temperature, additives and/or pH adjustments.

Hydrophobic interaction chromatography (HIC)

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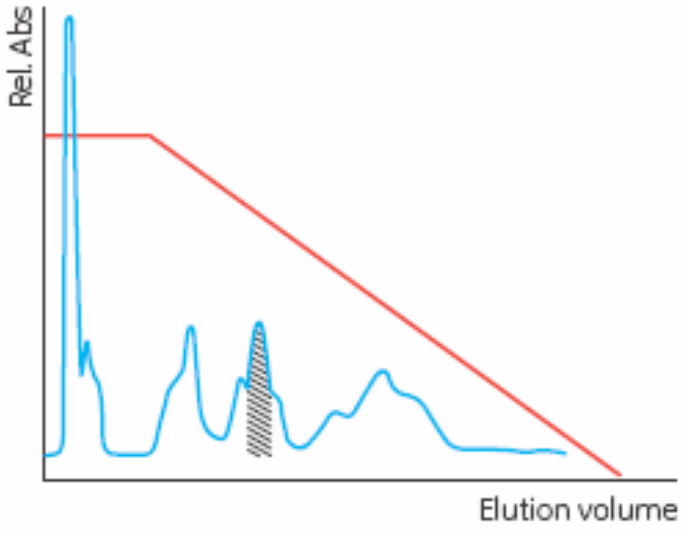
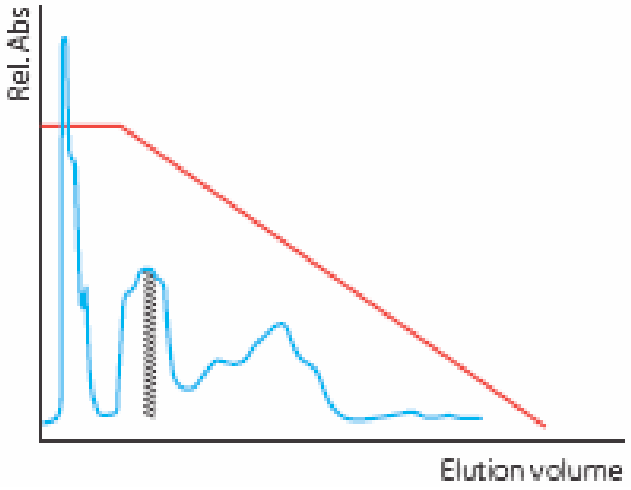
Unexpected results



The ideal HIC separation: target protein is well resolved by gradient elution

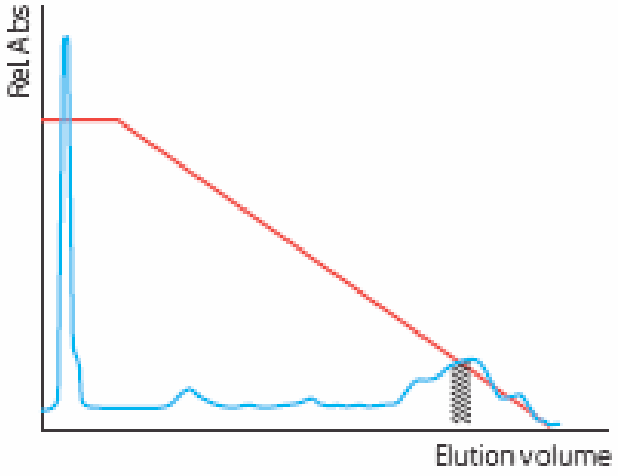


Target protein is eluted early in the gradient. Poor resolution.

Repeat the separation at a higher salt concentration in the start buffer or with a salt of higher 'salting-out' strength.

If no improvement in selectivity is obtained, try a medium with a more hydrophobic ligand or with a higher degree of ligand substitution, if available.

Unexpected results

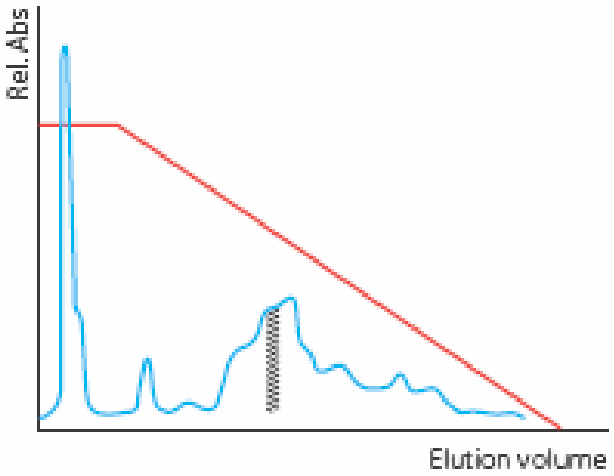


Target protein is eluted near the end of the gradient. Poor resolution.

Repeat the separation with a lower salt concentration in the start buffer or using a salt with a lower salting-out strength

If no improvement in selectivity is obtained, try a medium with a less hydrophobic ligand or with a lower degree of ligand substitution.

A decrease in the initial salt concentration will weaken the binding, resulting in earlier elution of the protein. It may,however, not have a positive effect on selectivity, since the contaminants are eluted very close to, both before and after, the protein of interest.



Target protein is eluted in the middle of the gradient. Poor resolution.

Optimize the gradient around the target protein; for example, use a segmented gradient with a shallower region around the target protein.

Also consider the use of additives to improve resolution.

Check column with different ligand.

If resolution cannot be improved, use an alternative chromatography technique such as ion exchange for further purification.

Purification examples

Optimization steps for intermediate purification of a recombinant protein.

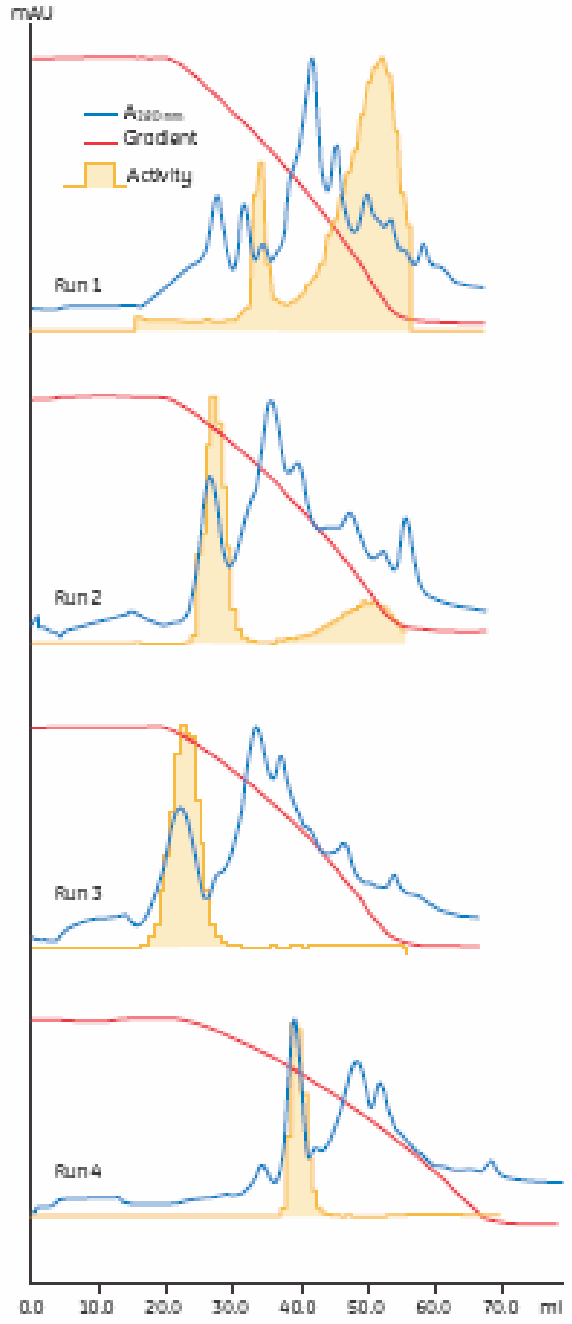
- Starting material: Diluted *E. coli* supernatant
- Capture: QSepharoseXL
- Intermediate purification: SOURCE 15PHE
- Polishing: Superdex 75

Start buffer (run 1): 1.5 M (NH4)2SO4, 25 mM Tris, 1 mM EDTA, 2 mM DTT, pH 7.5
Elution buffer (run 1): 25 mM Tris, 1 mM EDTA, 2 mM DTT, pH 7.5

Start buffer (run 2): 1.5 M (NH4)2SO4, 50 mM Tris, 1 mM EDTA,
2 mM DTT, pH 7.5
Elution buffer (run 2): 50 mM Tris, 1 mM EDTA, 2 mM DTT, **10% glycerol**, pH 7.5

Start buffer (run 3): 1.5 M (NH4)2SO4, 20 mM MES, 1 mM EDTA,
2 mM DTT, pH 6.5
Elution buffer (run 3): 20 mM **MES pH 6.5**, 1 mM EDTA, 2 mM DTT, **10% glycerol**

Start buffer (run 4): **2 M (NH4)2SO4**, 20 mM MES, 1 mM EDTA,
2 mM DTT, pH 6.5
Elution buffer (run 4): 20 mM MES, 1 mM EDTA, 2 mM DTT, **10% glycerol, pH 6.5**



HIC & AEIX

Sup Insect cellexpression of rec Protein

Industrial case

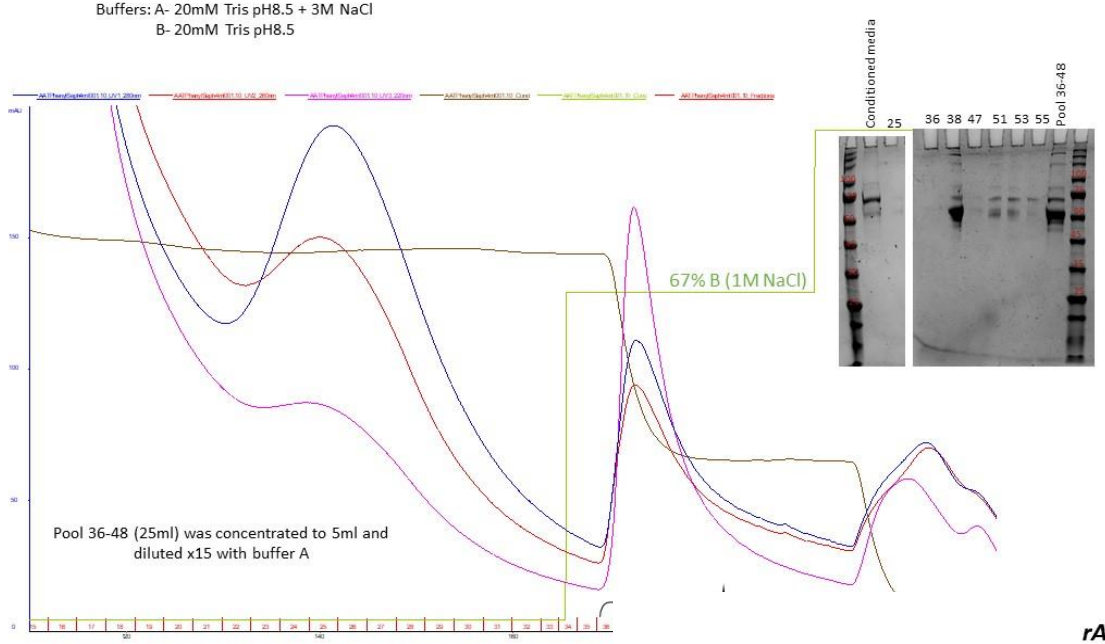
Rec Protein Insect cells extracellular expression

Hadar Amartely

~80 ml media

rAAT purification – Phenyl sepharose 5ml column (MERCK)

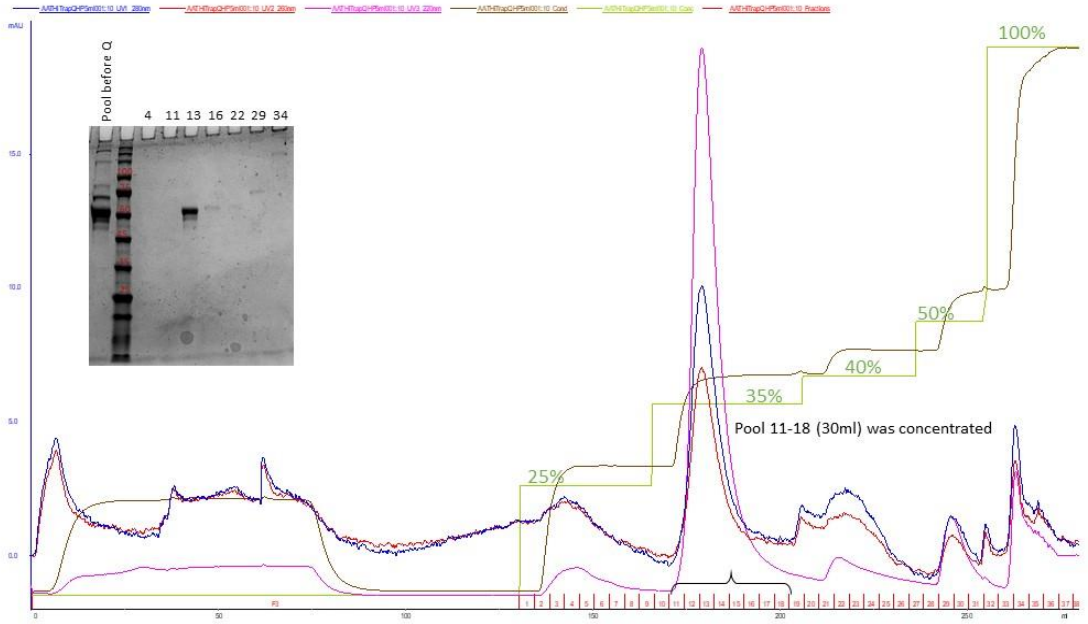
Buffers: A- 20mM Tris pH8.5 + 3M NaCl
B- 20mM Tris pH8.5



rAAT purification – Hi Trap Q HP 5ml column

Batch2

Buffers: A- 20mM Tris pH8
B- 20mM Tris pH8 + 0.5M NaCl



Case Study: HIC for the characterization of antibody-drug conjugates (ADCs)

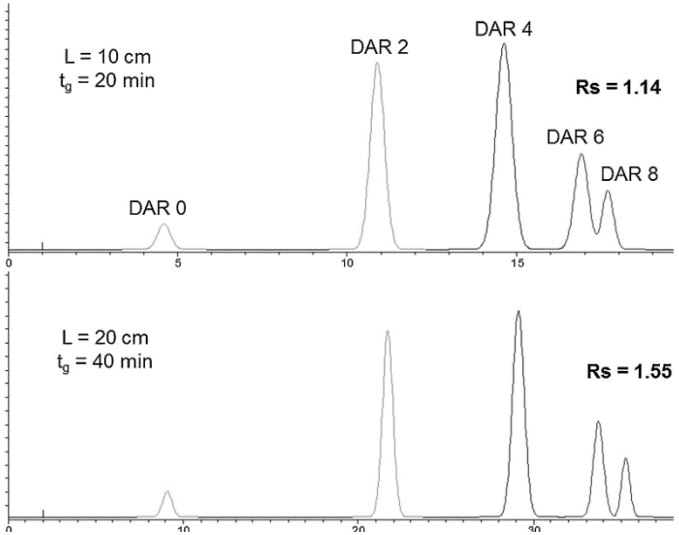
Journal of Pharmaceutical and Biomedical Analysis - Fekete S et al. 2016 - http://dx.doi.org/10.1016/j.jpba.2016.04.004

✓Is a valuable tool in downstream purification procedure

✓The main difference between HIC and RP is that proteins maintain their native structure with intra-molecular forces in HIC while they are denaturated in RP conditions

✓For the detailed characterization of Monoclonal antibodies (mAbs) and related products such as antibody-drug conjugates (ADCs) and bispecific antibodies (bsAbs)

✓To separate different populations of ADC molecules that differ in their number of drugs per antibody which are often known as DAR (drug-to-antibody ratio) in native conditions



Drug to antibody ratio (DAR)

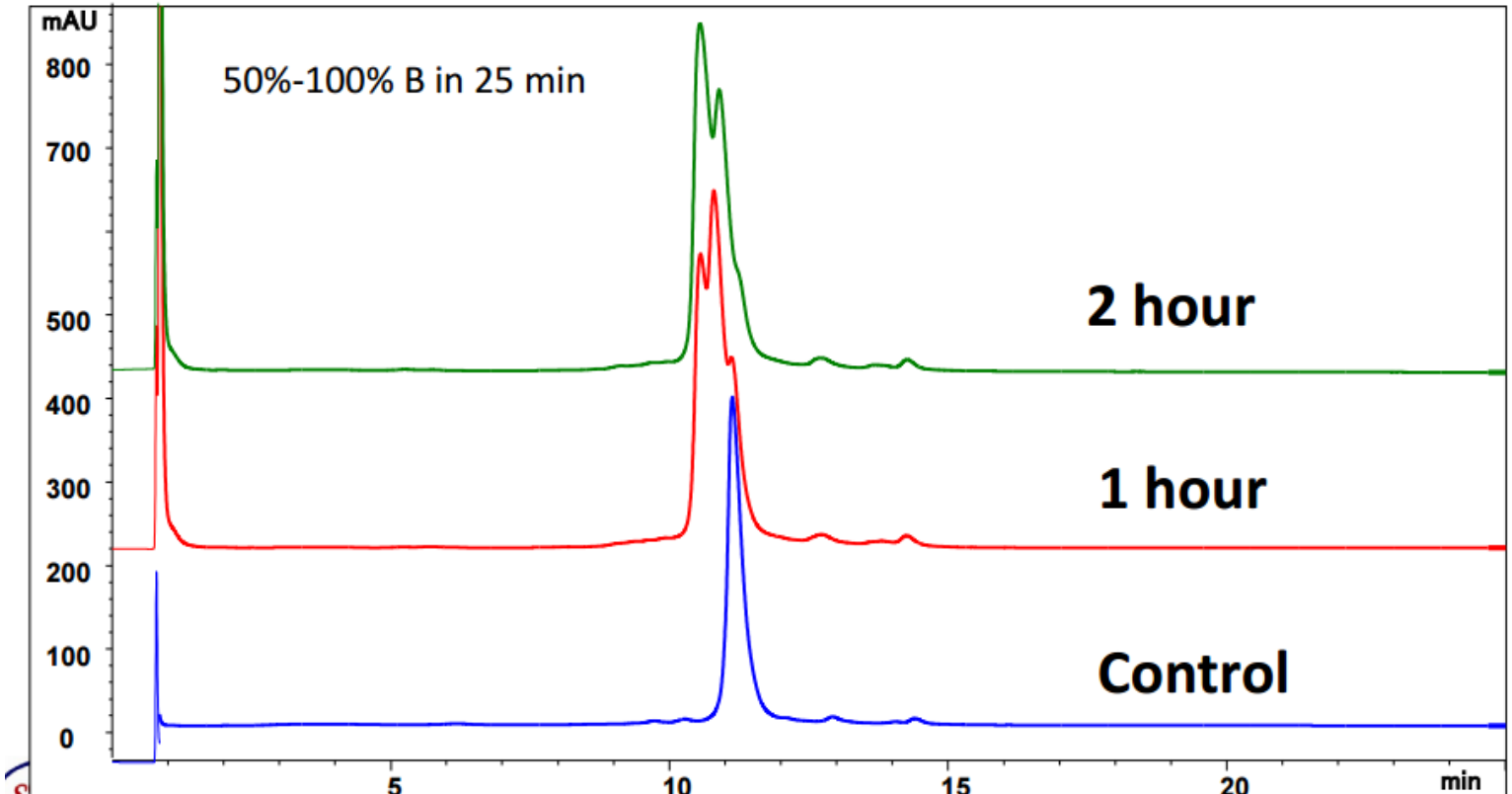
Case Study
Analytical HIC for the characterization of antibody oxidation

Sepax Technologies
Better Surface Chemistry for Better Separation

Rituximab Oxidation Analysis on HIC Butyl

HICM1006

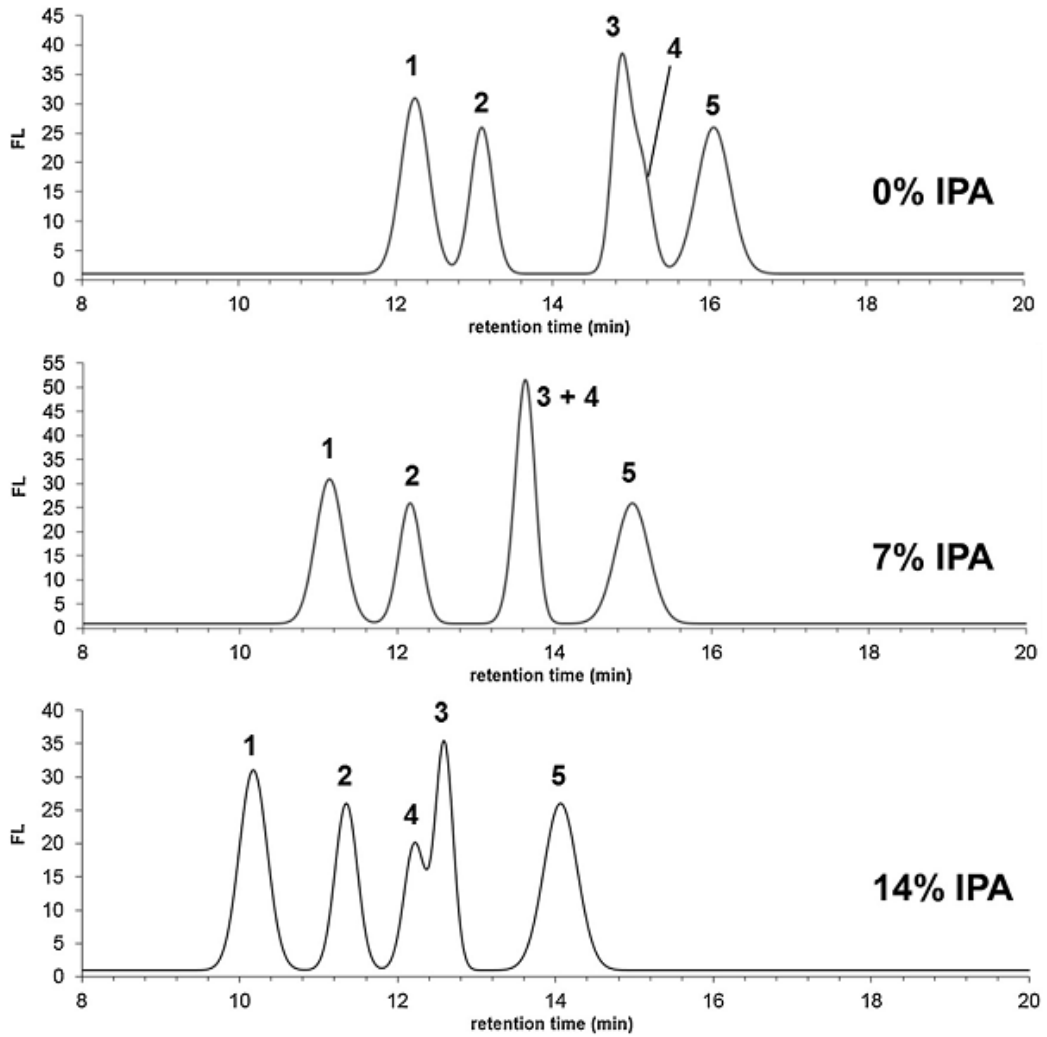
Column: Proteomix® HIC Butyl NP5, 5 µm, 4.6 x 100 mm (PN: 431NP5-4610)
Mobile phase: A: 100 mM sodium phosphate buffer, 2 M ammonium sulfate, pH 7.0, B: 100 mM sodium phosphate buffer, pH 7.0;
Flow rate: 0.8 mL/min, Pressure: 170 bar, System: UHPLC, Detector: UV 214 nm,, Column temperature: 25°C,
Injection: 10 µg mAb sample, 20 µg oxidized mAb,
MAb oxidation: 10 mg/mL was diluted to 5 mg/mL with water and 70% t-BHP was added to a final 5% concentration, incubate in dark and take time point, dilute to 2.5 mg/mL in 500 mM ammonium sulfate, 25 mM phosphate buffer



Impact of organic modifier on retention and selectivity

Separation of monoclonal antibody mixture [denosumab (1), palivizumab (2), pertuzumab (3), rituximab (4), bevacizumab (5)], using a generic gradient from 2 to 0 M ammonium sulfate on a 100 × 4.6 mm column. The mobile phase also contained 0.1 M phosphate buffer (pH = 7), and 0, 7 and 14% isopropanol (IPA) was added to mobile phase "B

Journal of Pharmaceutical and Biomedical Analysis - Fekete S. et al. 2016 -http://dx.doi.org/10.1016/j.jpba.2016.04.004



Conclusions

- Complementary to ion exchange (IEX) and gel filtration (GF)
- Separate proteins according to hydrophobic differences in protein surface. Mild, non-denaturing
- Useful mainly in capture and intermediate step purification. After Ammonium sulfate precipitation
- Many variables for optimization
- High recovery and reproducibility
- Non-predictable
- High salt, viscosity. PAGE: Samples with high salt

New resins: suitable for bind-elute and flow through applications at lower salt concentrations than classical HIC resins

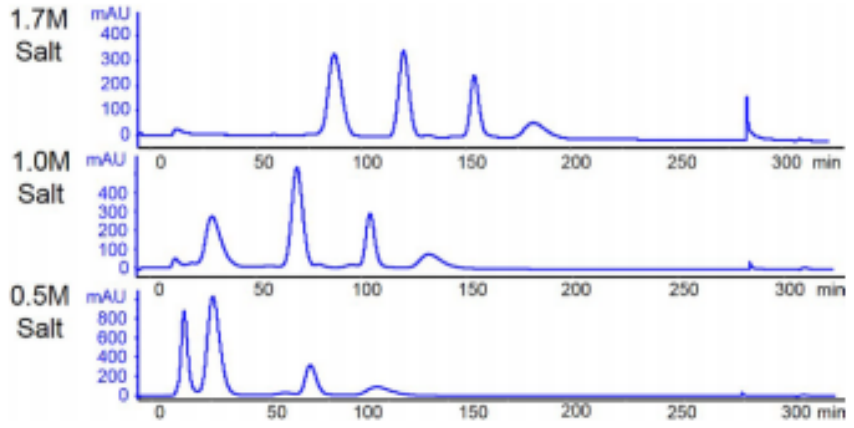
- 50 µm POROS polystyrene divinyl-benzene beads
- Very broad range of hydrophobicity
- Suitable for bind-elute and flow through applications at lower salt concentrations than classical HIC resins.
- higher capacity
- superior resolution
- a broad range of hydrophobicity

to enable differentiating selectivity for a variety of biomolecules.

Thermo Fisher Scientific

POROS Ethyl,
POROS Benzyl
POROS Benzyl Ultra

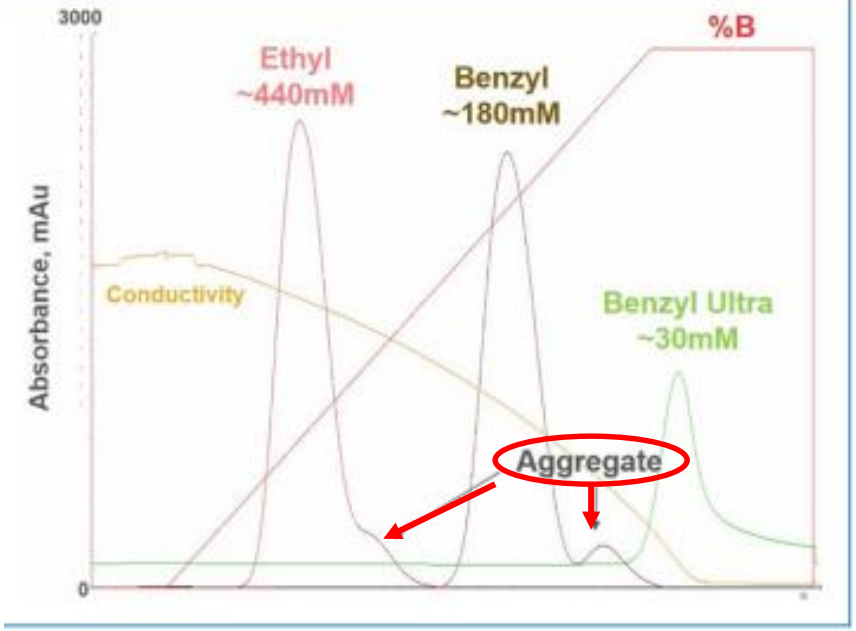
Figure 2 – Separation comparison in different salt concentrations
Experimental details: Protein mixture - ribonuclease A, lysozyme, chymotrypsin and chymotrypsinogen A; Eluent A: 1.7M, 1.0M or 0.5M ammonium sulfate/50 mM sodium phosphate pH 7.0; Eluent B: 50 mM sodium phosphate pH 7.0; Gradient: 0 to 100% B at 0.17M ammonium sulfate change per CV; Linear velocity: 100 cm/h; Detection: UV at 280 nm; Column format: 0.66 cmD x 20 cmL.



Thermo Fisher New HIC resins: case studies

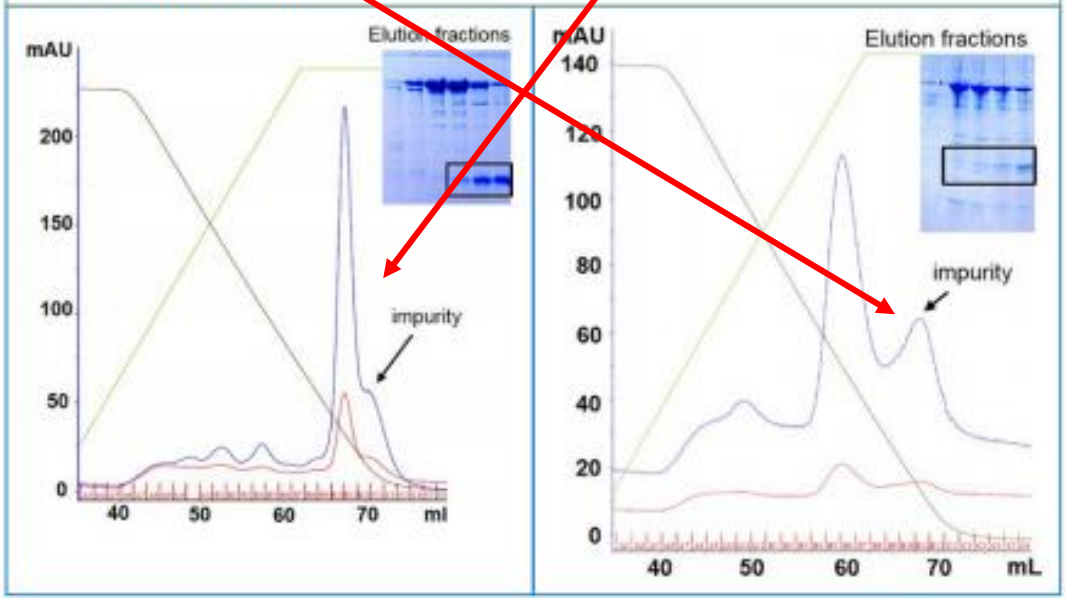
CLINICAL mAb-A REQUIRING AN IMPROVED STRATEGY FOR AGGREGATE CLEARANCE

Figure 3 – Retention analysis of mAb-A with sodium citrate gradient elution
Column: 0.5 cmD x 5 cmL, CV=1 mL. Residence time: 2 min. Buffer A: 600 mM sodium citrate, tris-acetate pH 7.0; Buffer B: tris-acetate pH 6.9. Linear gradient: 15 CV.



THERAPEUTIC ENZYME REQUIRING SEPARATION FROM A DOMINANT PROCESS-RELATED IMPURITY

Figures 6A and 6B – Impurity removal with the butyl 34 µm HIC resin (left) and POROS Ethyl (right), demonstrating increased impurity clearance due to the superior resolution of the POROS resin.
Process details: ~7 mg total protein separated with a 5 CV gradient from buffered 1.5 M ammonium sulfate containing 2 mM EDTA pH 7.4 to buffer containing 2 mM EDTA pH 7.4; Detection: A280 & A254nm; Column format: 1.0cmD x 7.5cmL; Flow rate: 100 cm/hr (6A) and 230 cm/hr (6B)



Thermo Fisher New HIC resins: case studies

CASE STUDY 3:
ADC PURIFICATION–DAR SEPARATION

ADC: Antibody-Drug Conjugates

DAR: Drug-Antibody Ratios

Figure 7B – DAR separation by POROS HIC resins, showing superior resolution over the butyl and phenyl HIC resins

Experimental details: Tricorn (5x50mm) column; HIC loading buffer: 15µL HEPES (0.1 M), NaCl (0.1 M), pH7.5 + 200 µL ammonium sulphate (3 M), HEPES (0.1 M), NaCl (0.1 M), pH = 7.5; HIC buffer A (ammonium sulphate (1 M), HEPES (0.1 M), NaCl (0.1 M), pH = 7.5); HIC buffer B (HEPES (0.1 M), NaCl (0.1 M), 20 % isopropyl alcohol v/v, pH = 7.5); 300 cm/hr (1 min residence time)