Hydrophobic Interaction Chromatography (HIC)
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- 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, which is the driving force for the interaction of the solute with the ligand.
Main stages in HIC

- Equilibrate the gel and the sample to binding conditions
- Apply the sample
- Wash out contaminants
- Elute
- Wash and Regenerate column
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

- **Binding buffer:** 50 mM phosphate buffer pH 7.0 with 1-2 M ammonium sulphate or 3M NaCl
- **Elution buffer:** 50 mM phosphate buffer pH 7.0
- **Gradient:** 10-15 column volumes
- **Flow rate:** according to manufacturer's instructions
- **Gel:** Phenyl Sepharose 6 Fast Flow (high sub)
HIC pro´s and con´s

- Predictability
  - High salt/viscosity
  - Precipitation/denaturation

+ Mild/stabilizing
  - Concentrating
  - High selectivity/ recovery.
  - Complements IEX, GF and affinity
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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.
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

Butyl

Octyl

Ether

Isopropyl
CRITICAL FACTORS - SELECTING MEDIA

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

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 (NH₄)₂SO₄

1 M Na₂SO₄

3 M NaCl

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: $\text{PO}_4^{3-}, \text{SO}_4^{2-}, \text{CH}_3\cdot\text{COO}^-, \text{Cl}^-, \text{Br}^-, \text{NO}_3^-, \text{ClO}_4^-, \text{I}^-, \text{SCN}^-$

Cations: $\text{NH}_4^+, \text{K}^+, \text{Na}^+, \text{Cs}^+, \text{Li}^+, \text{Mg}^{2+}, \text{Ca}^{2+}$

The most commonly used salts are $(\text{NH}_4)_2\text{SO}_4$, $\text{Na}_2\text{SO}_4$, $\text{NaCl}$, $\text{KCl}$ and $\text{CH}_3\text{COONH}_4$. 
CRITICAL FACTORS - SELECTING ADSORPTION CONDITIONS

Effect of pH

✓ Protein binding to HIC-media is generally not changed much between pH 5 and 8.5
CRITICAL FACTORS - SELECTING ADSORPTION CONDITIONS

Effect of temperature 1

Temperature Impact on Adsorption Efficiency

**23°C Sample & System**

**4°C Sample, 23°C System**

Hydrophobic interaction generally decreases with decreasing temperature.

**SOURCE™ 15ISO**
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. Decrease 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
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 additives
- Degree of ligand substitution
- Particle and pore size
- Salt type
- Buffer concentration
- pH
- Temperature
- Type of Matrix
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.
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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**: Q-Sepharose XL
- **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
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