Mixed-Mode Chromatography in the Purification of Recombinant Proteins

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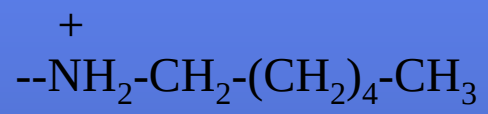
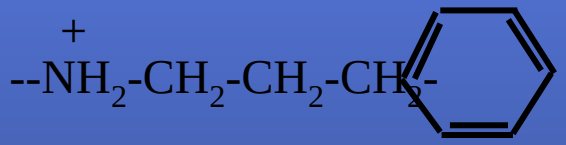
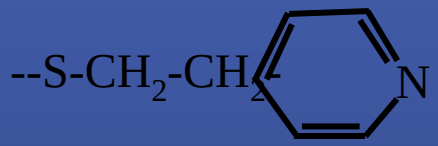
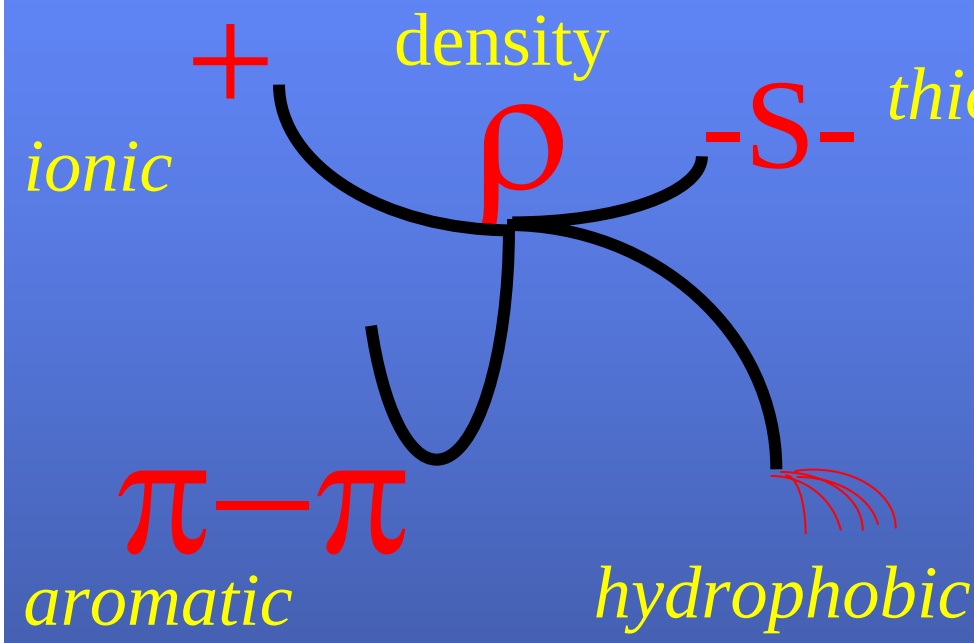
Thank-you to clients who were kind enough to allow me to share their work

Mixed Mode Ligands

(Pall Life Sciences/BioSepra)

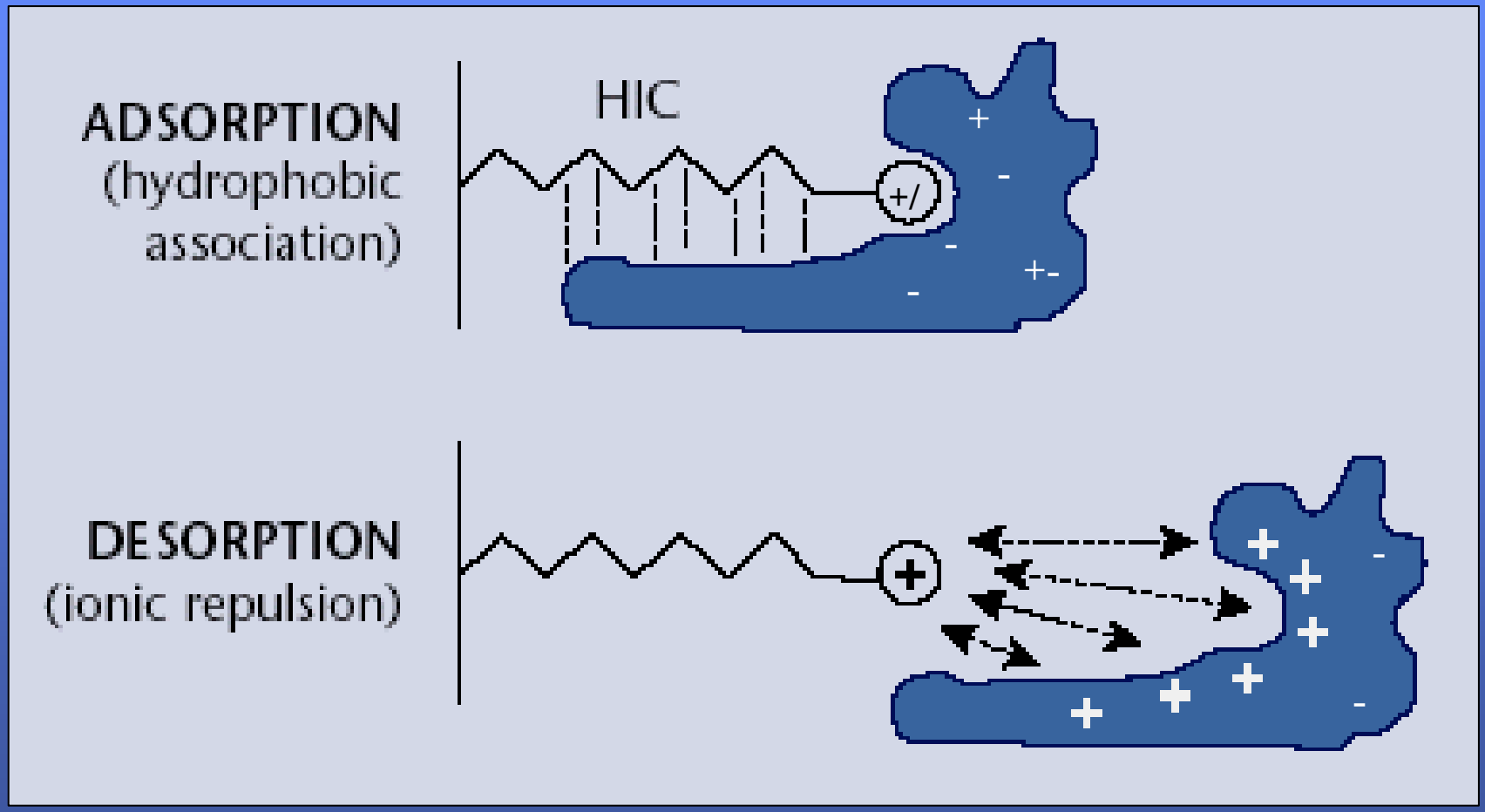
		pK_a amine
	HEA (amine + n-hexyl)	>9
	PPA (amine + phenylpropyl)	>9
	MEP (4-mercaptoethylpyridine)	4.8

Mixed mode ligand interactions



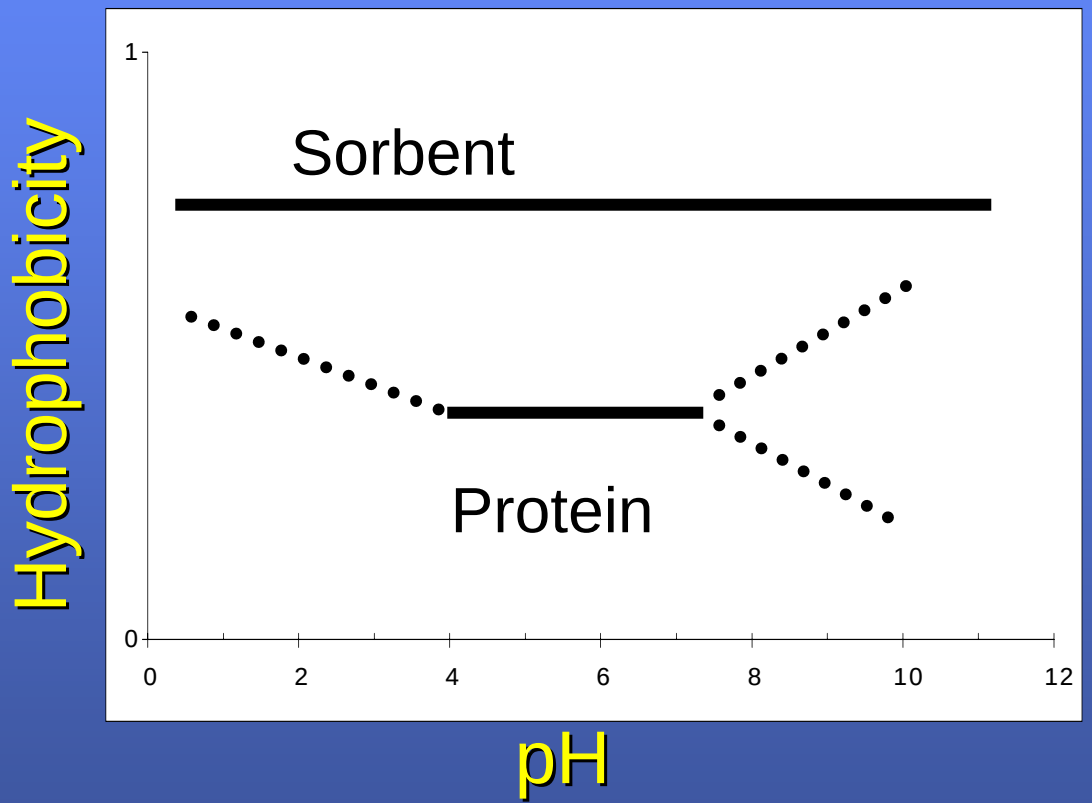
- Site interactions
  - "charge assisted HIC binding"
- Spatial effects- "pseudo-affinity"
  - Relative position of charges & hydrophobic patches
- Kinetics & thermodynamics
  - Timing & order effects
  - "getting comfortable"

Binding & Desorption

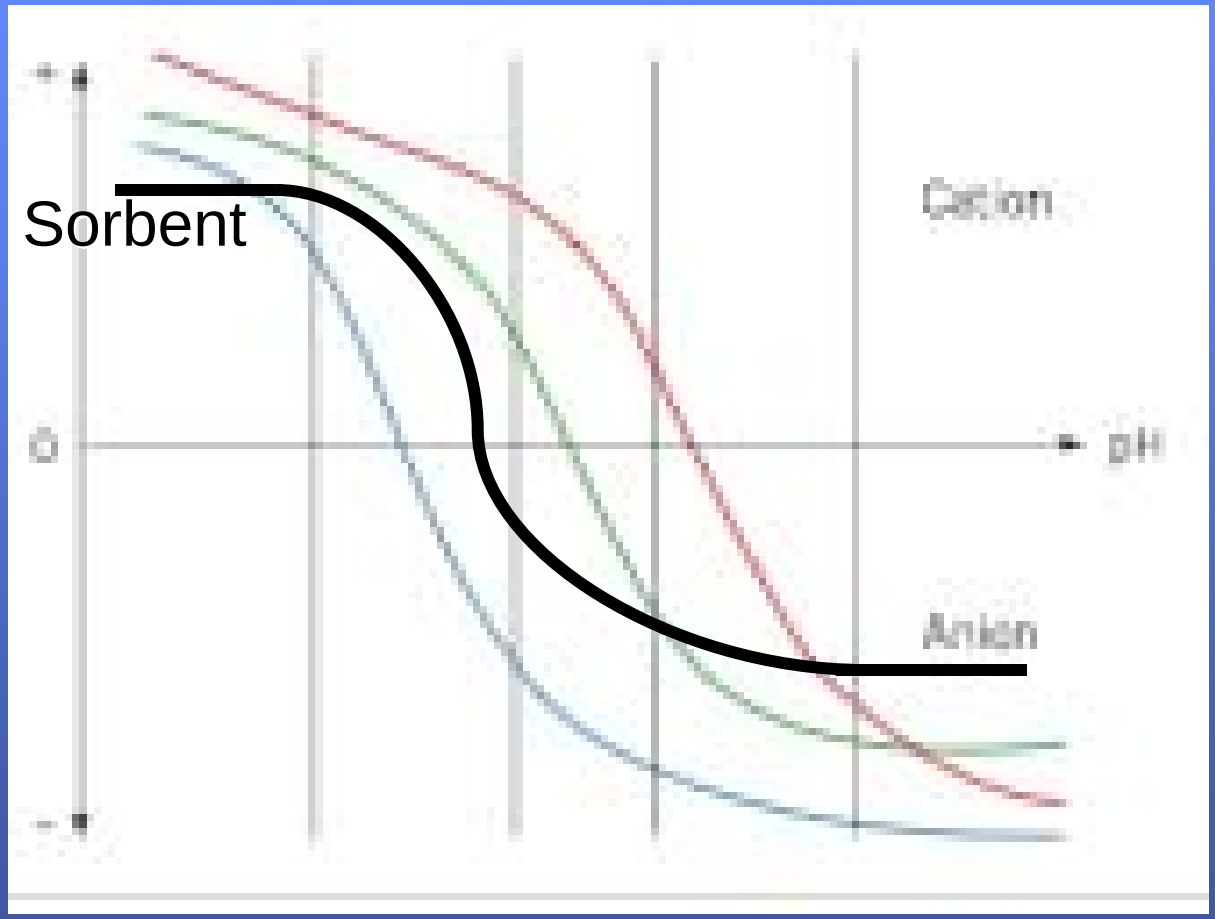


“Between pH 5–8.5, pH values have very little significance on the final selectivity and resolution of a HIC separation. An increase in pH weakens hydrophobic interactions and retention of proteins changes more drastically at pH values above 8.5 or below 5.0.”

GE Healthcare HIC book

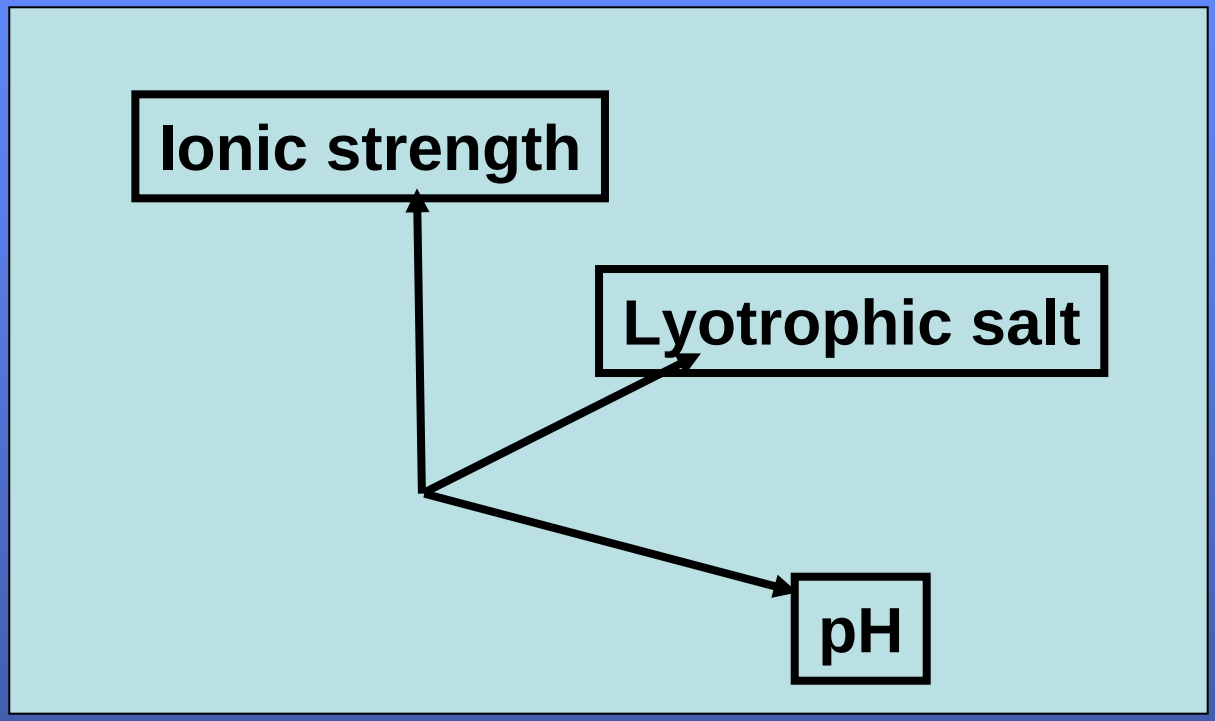


Proteins are polyions



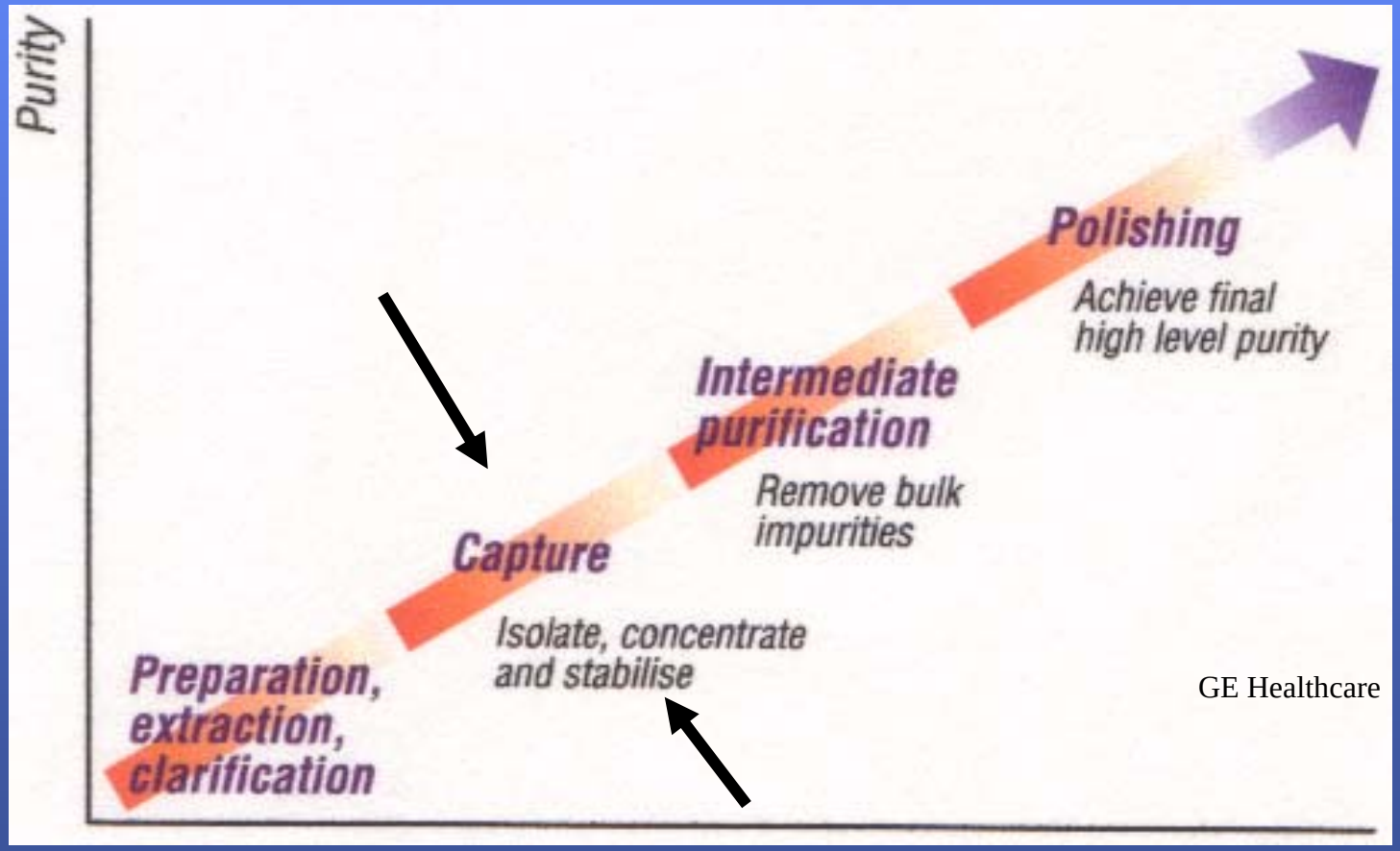
Ligand titration curve is relatively sharp while protein titration curve is flatter

"Tuning" of parameters can yield high purity in one step



Each step, binding, washing, and elution, can be optimized by adjusting the pH, ionic strength and the lyotropic salt.

Purification with mixed mode sorbents



Capture step

Target protein 1 is an E. coli recombinant protein:

MW 26 kDa
pI ~10
Intracellular
Zinc metalloenzyme
Not stable at pH>7.5

Problem: Protein 1 was not soluble at the low ionic strength needed for capture by cation exchange.

Initial protocol

Water extraction → conditioning to 150 mM NaCl

Did not work for E. coli

Needed for solubility

SP capture → Q column → HIC

Only ~50% capture
Needed repeated runs

Load @ 0.8 M AmSulfate

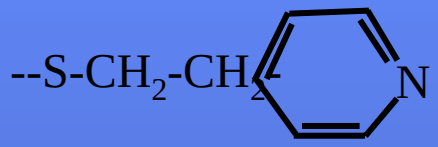
IMAC capture

Efficient capture on a metal affinity column (IMAC with copper).

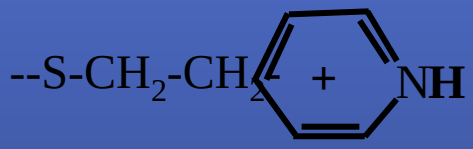
However:

- *Enzymatic activity was ~ 50%*
- *Precipitate formed after IMAC, requiring additives*
- *Protein was blue*
- *Metals promote oxidation*

MEP HyperCel



- High ligand density
  - Very hydrophobic
  - Binding at lower salt conc.



- Hydrophobic Charge Induction
  - Charge-charge repulsion
  - High recovery

Optimization of elution pH on MEP HyperCel

pH 7.5

pH 7.0

pH 6.5

pH 6.0

pH 5.5

pH 5.2

pH 4.9

pK_a 4.8

pH 4.6

pH 4.3

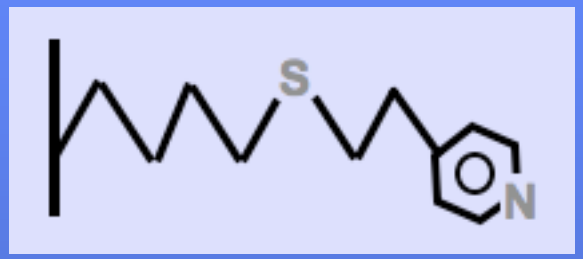
pH 4.0

Elution of relatively basic proteins.

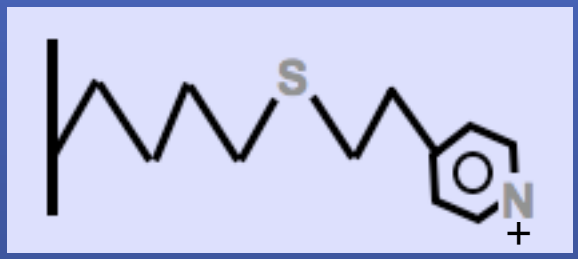
Elution of relatively acidic proteins.

Elution of relatively hydrophilic proteins.

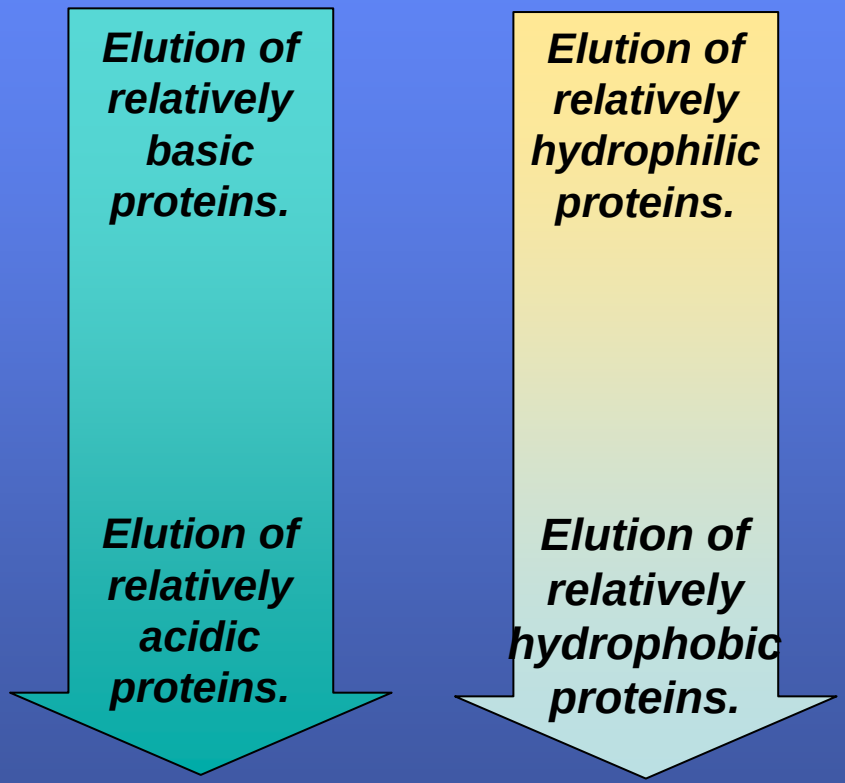
Elution of relatively hydrophobic proteins.



As the pH becomes more acidic, the MEP ligand becomes progressively more positively charged.



Optimization of elution pH on MEP HyperCel



Determine:

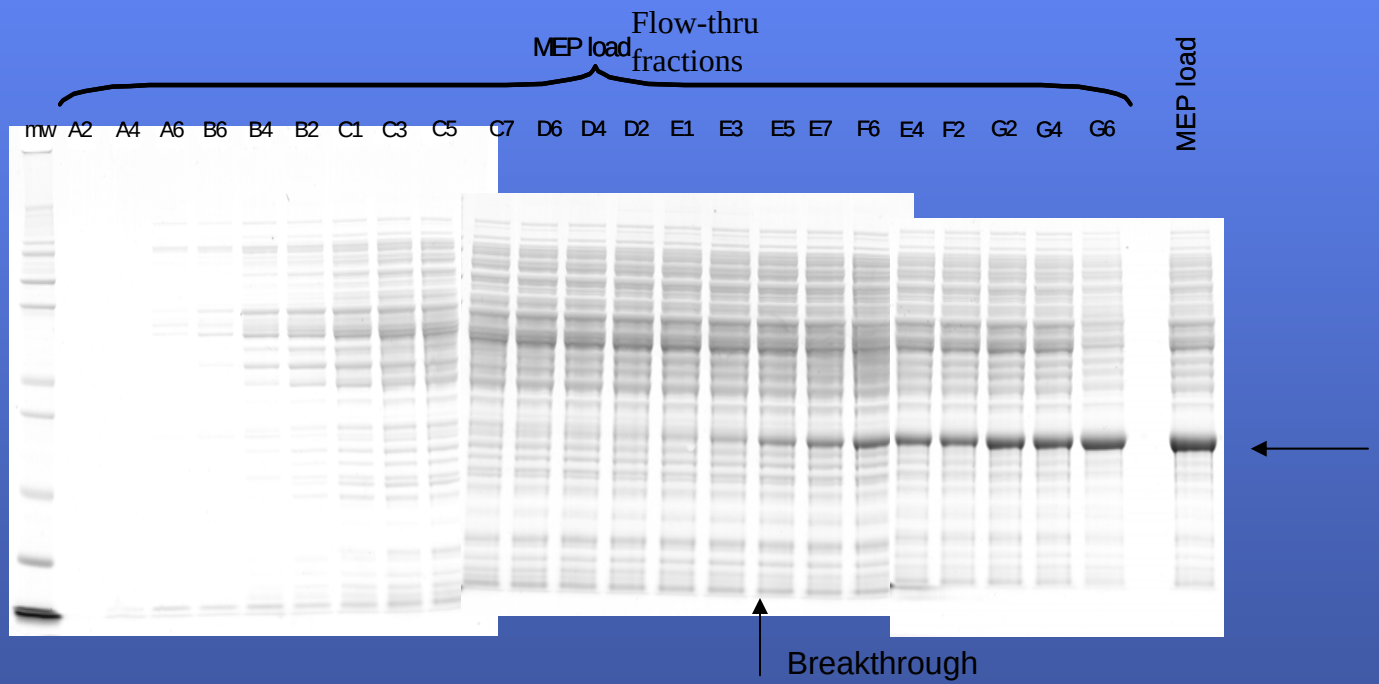
the lowest salt conditions for adsorption.

the lowest pH & salt at which the target remains bound.

the highest pH & salt at which the target elutes.

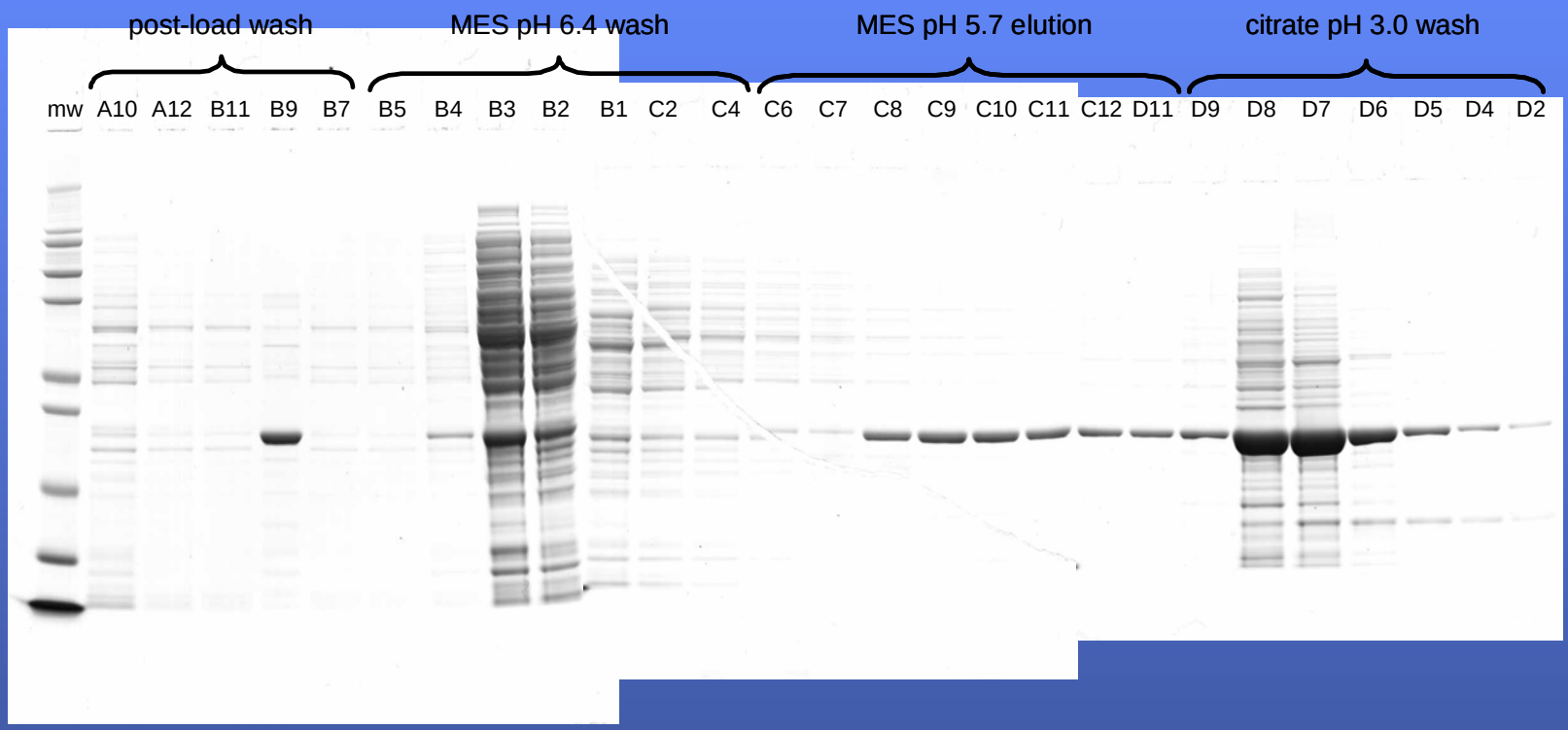
Binding to MEP at lower salt

Complete adsorption in 0.5 M ammonium sulfate, 25 mM $NaPO_4$, pH 7.2



22.5 x 1.6 MEP column (45 ml)
1.5 liters @2.9 g/liter loaded before breakthrough
Capacity: ~98 mg target protein/ml MEP

Scale-up



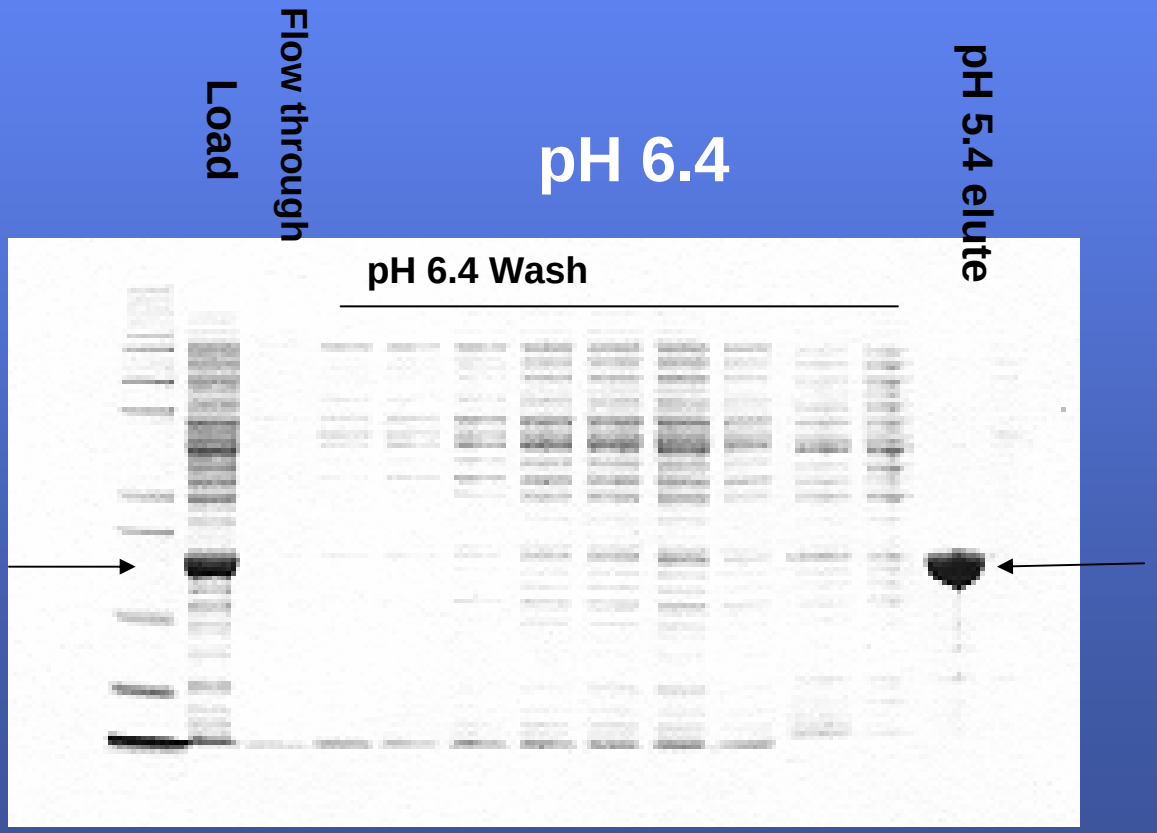
Lab scale conditions were not exactly replicated on scale-up.

Modify buffer composition

Widen pH range for robustness

100 liter fermentation: Capture

A clarified extract from a 100 liter fermentation (~4 g/L) on a 10 liter MEP column



Step yields at 100 liter scale

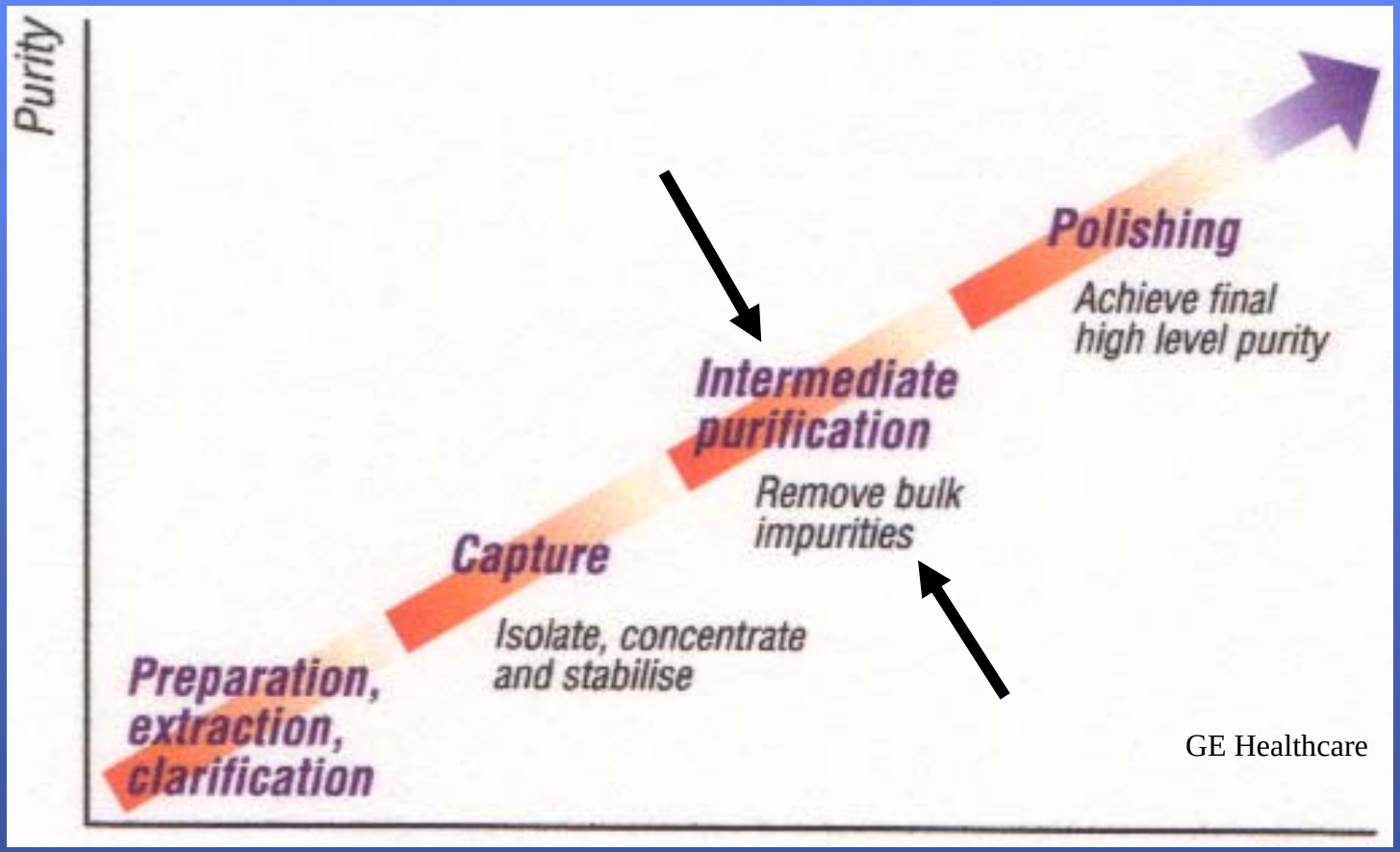
		Step yield
nonGMP	Run #1	74%
	Run #2	94%
GMP	Run #1	73%
	Run #2	109%
	Run #3	116%

MEP capture & purification

High recovery and purity by SDS PAGE & RP HPLC

- Late eluting fractions had less enzymatic activity than the main peak
  - Mass spec revealed that the target protein in the trailing edge of the peak was deaminated.
- Deaminated products are more acidic.
  - retarded by the anion exchange properties of the sorbent.
- By rejecting the late eluting materials:
  - significantly improve specific enzymatic activity
  - improved resolution on the next chromatographic step due to lower contamininant load.

Purification strategy



INTERMEDIATE STEP

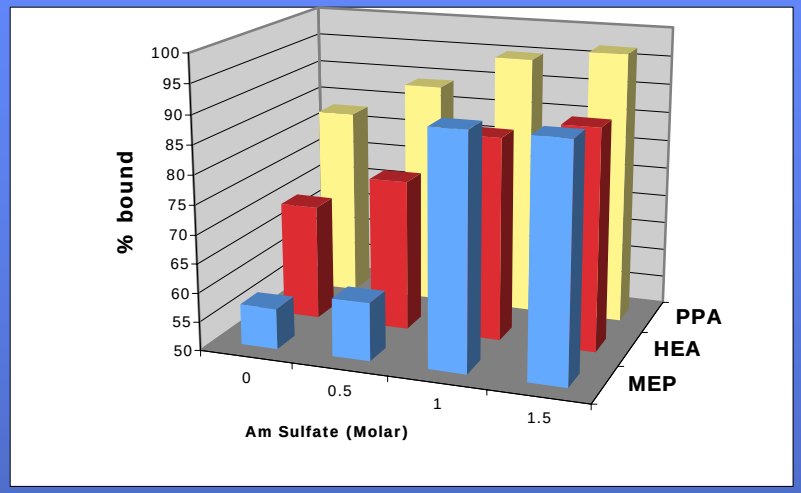
Target protein 2 is an E. coli recombinant protein:

MW 18 kDa
pI ~9
Intracellular
Excellent capture on a cation exchange column (SP Fast Flow)
SP eluant was ~80% pure by reverse phase HPLC

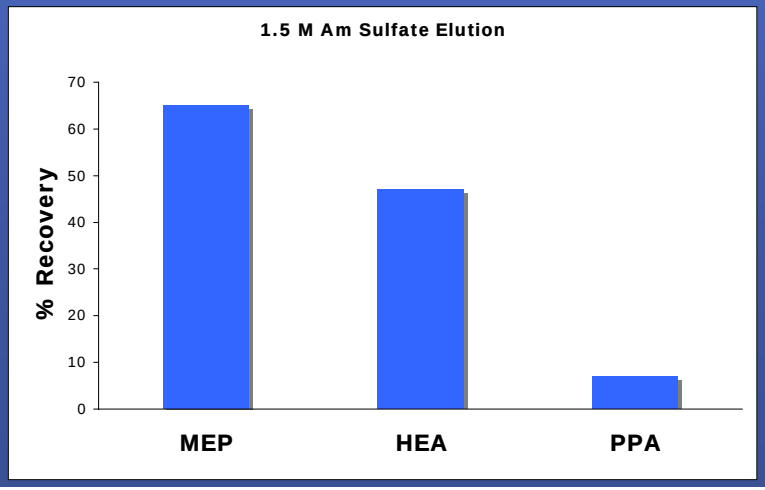
Problem: Needed >99% purity product to begin formulation studies, using a protocol suitable for scale-up.

Screening of Mixed-Mode Sorbents

Binding



Elution



Based on these results, we developed a purification process using MEP HyperCel, with loading at pH 8 and 1 M ammonium sulfate.

Binding: MEP vs HEA: dynamic binding

	100mM NaCl	500 mM NaCl	1 M AmSulfate
MEP	+/-	++	++++
HEA	++++	n.d	++++

Intermediate purification protocol

CAPTURE

↓

MEP HyperCel

1 M Am Sulfate pH 8

Wash 50 mM Tris pH 8

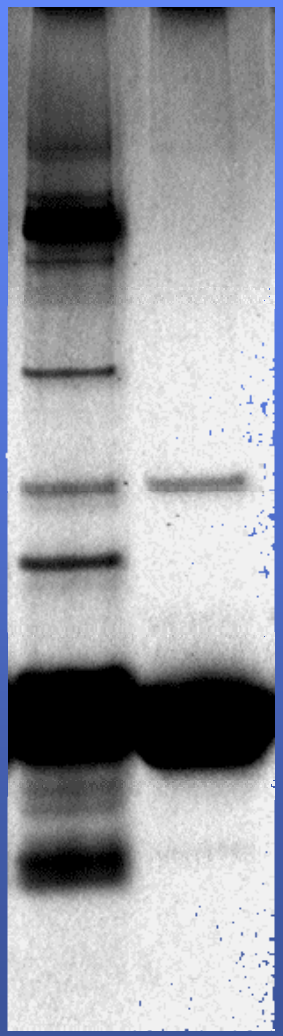
Elute 100 mM $NaPO_4$ pH 6.7

- 1 M ammonium sulfate needed for binding
  - Protein is very hydrophilic
  - Unlike HIC, once bound, salt could be removed
- No target protein found in flow thru or wash.
- Target protein eluted at pH 6.7.
  - MEP ligand is essentially uncharged.

Intermediate purification

SP eluant/ MEP load

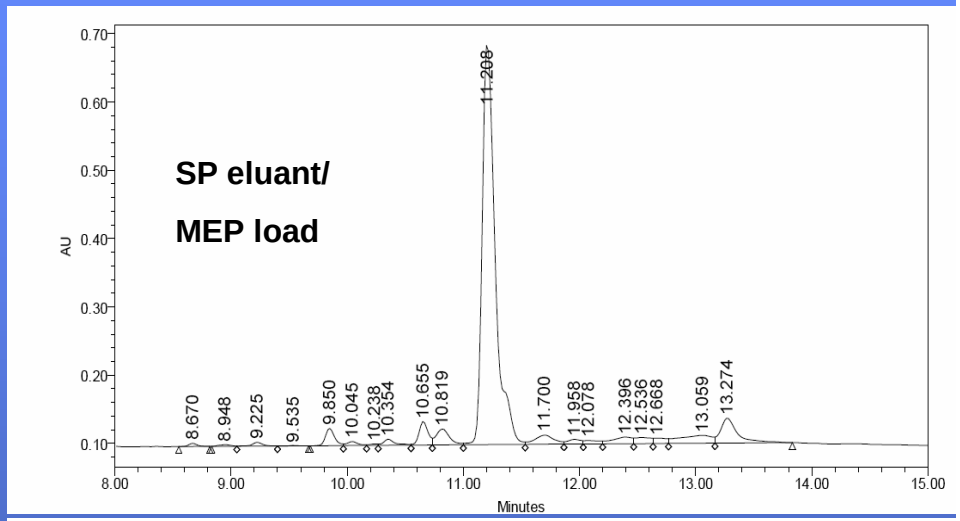
MEP eluant pH 6.7

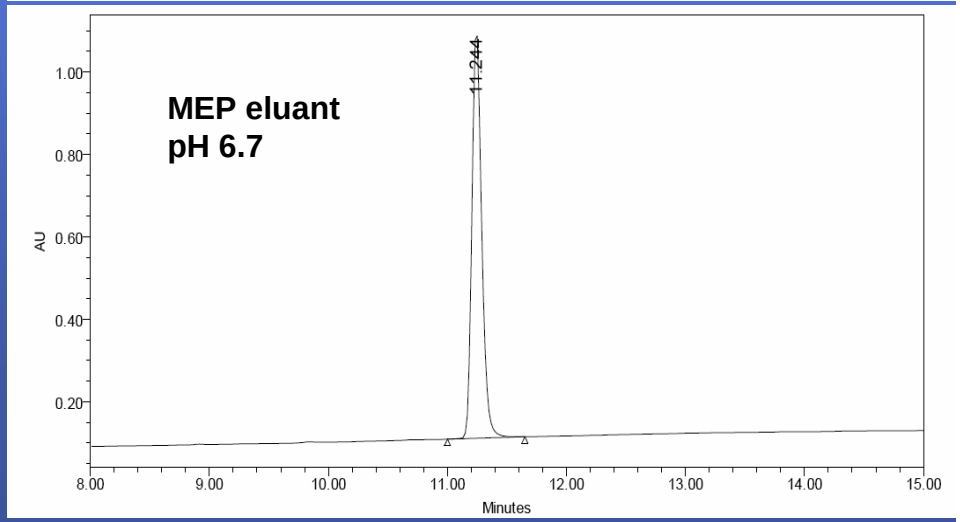


Silver Stained SDS PAGE

Step recovery was ~65%

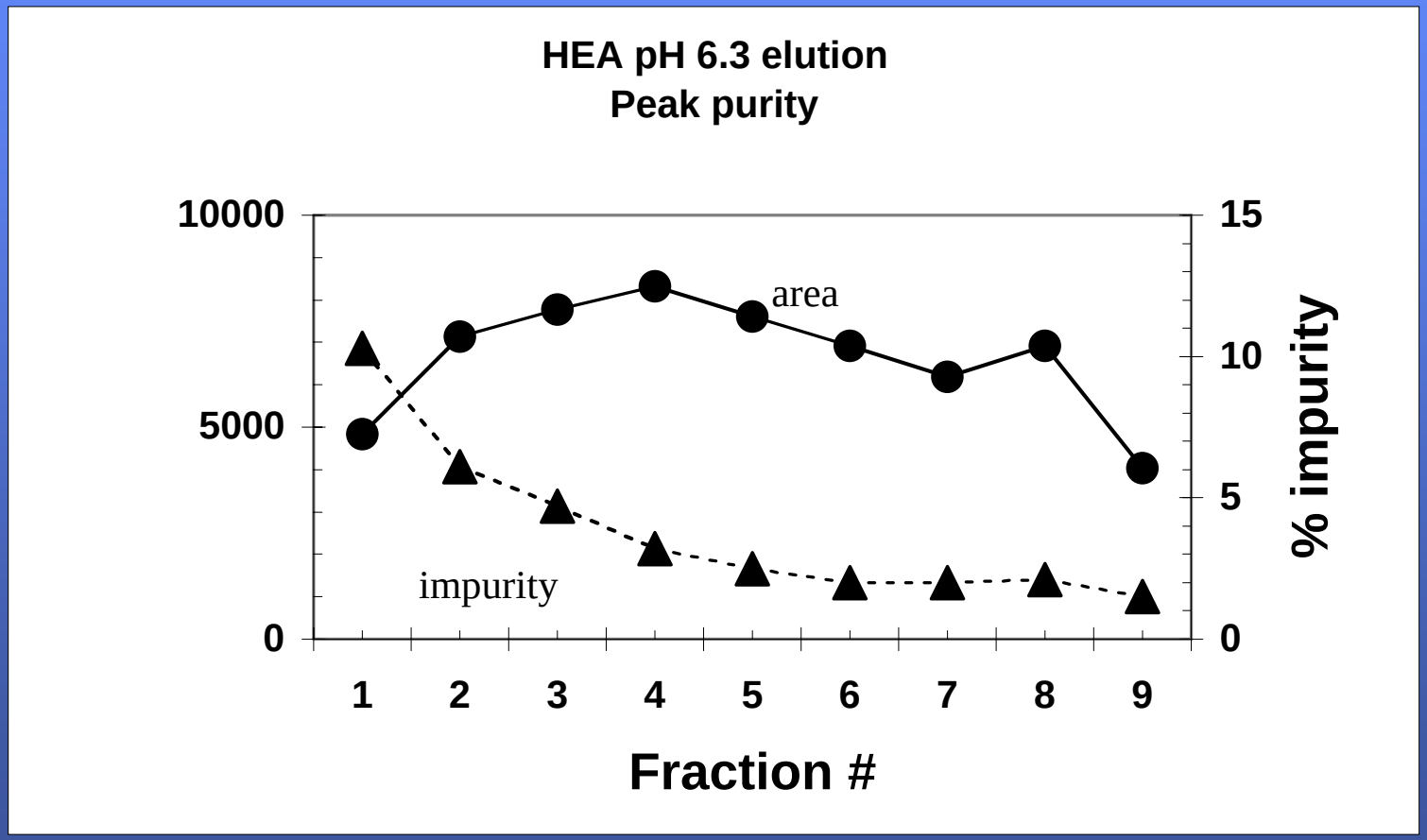
Purity was >99%, as judged by RP HPLC.





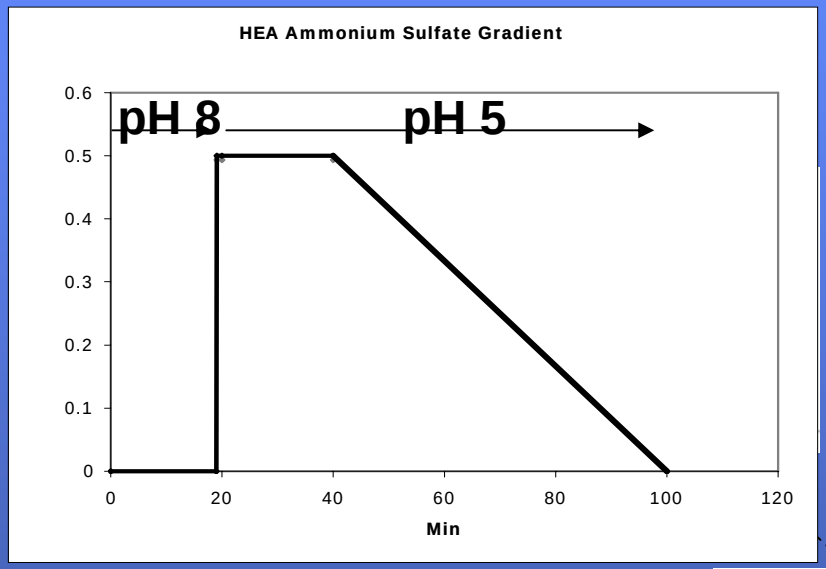
RP HPLC analysis

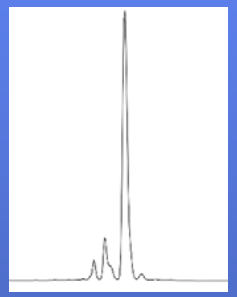
Improving recovery

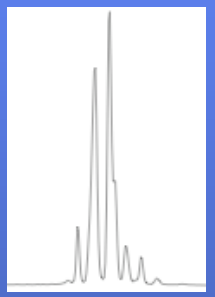


A less clean SP eluant was adsorbed onto HEA in 1 M ammonium sulfate, pH 8, washed with the same buffer without ammonium sulfate & eluted at pH 6.3. Individual fractions were assayed by RP HPLC

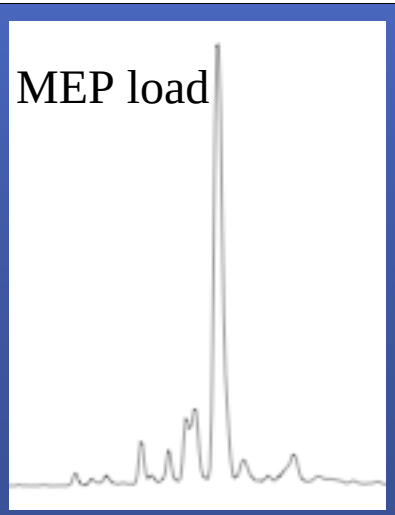
"Semi-classical HIC"

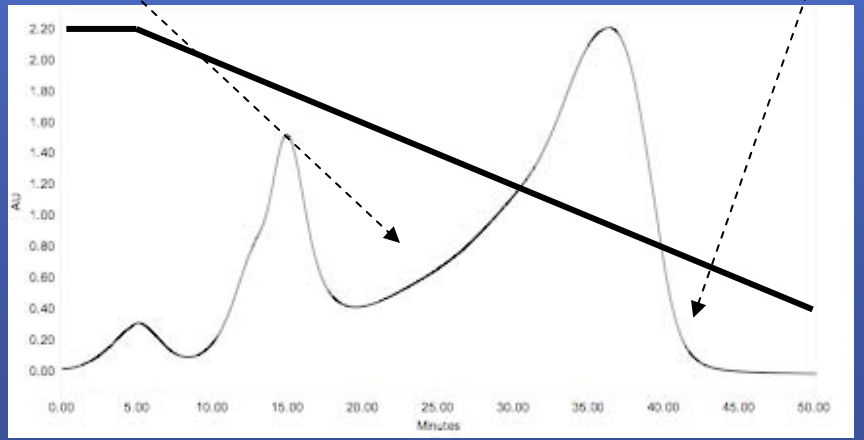




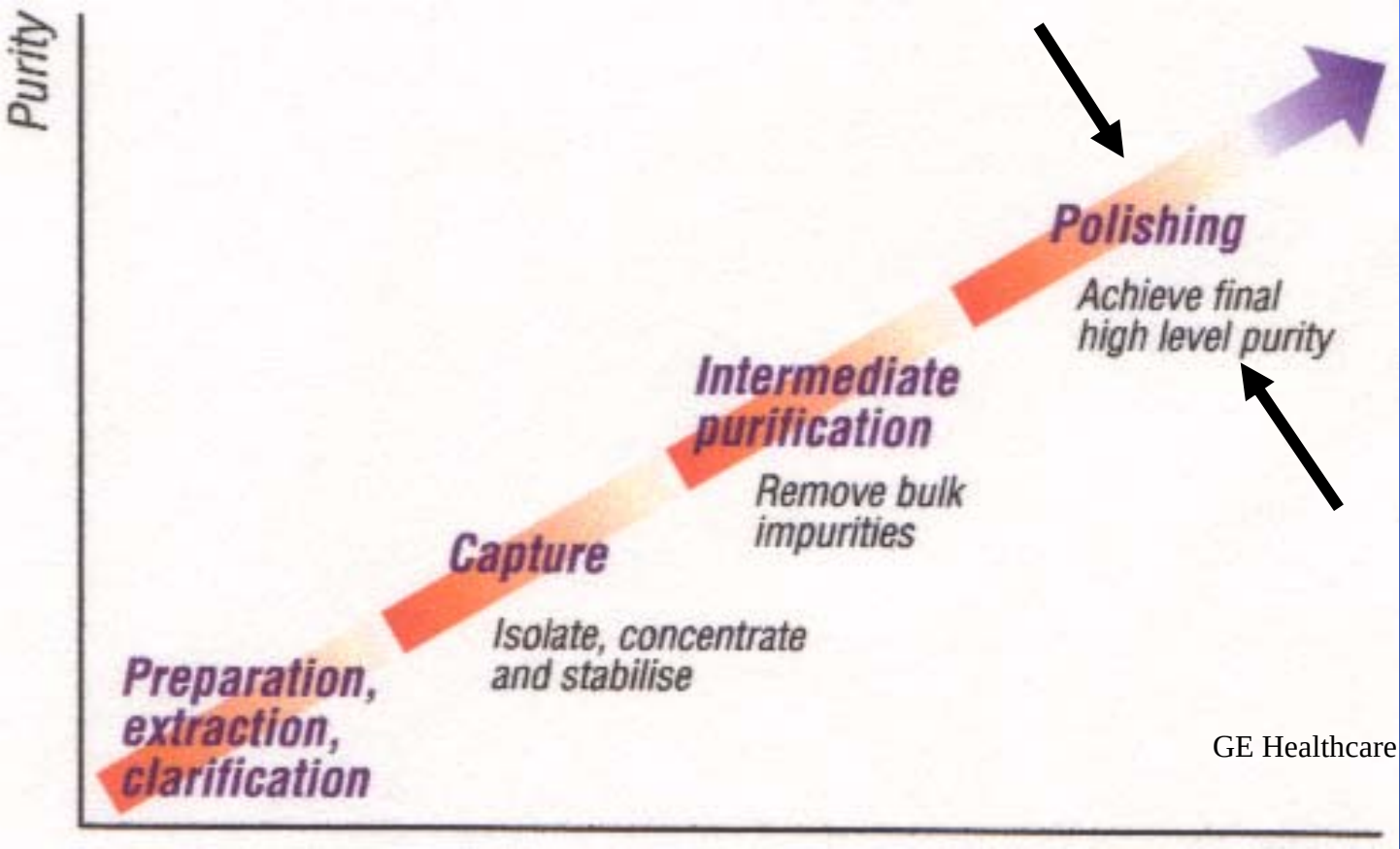


MEP load





Purification strategy



Polishing step

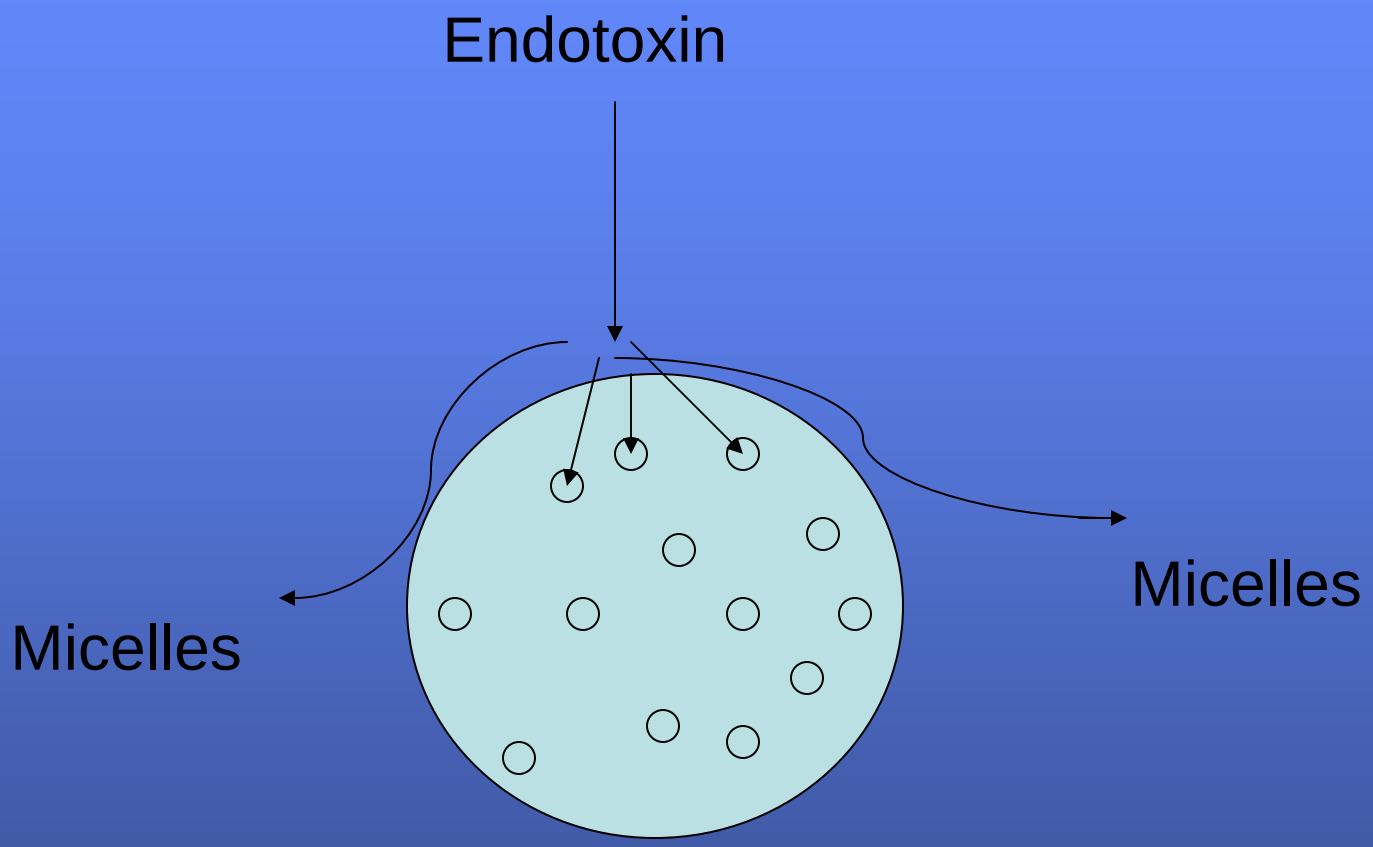
Problem: Trace contaminants that must be decreased to very low levels.

- Lipopolysaccharide (LPS, endotoxin)
  - Must be reduced to very low levels for therapeutic use.
  - Basic proteins bind LPS tightly.
- Other contaminants include
  - Host cell proteins
  - Nucleic acids
  - Protein A leachate

Mixed mode sorbent with anion exchanger & HIC functionality

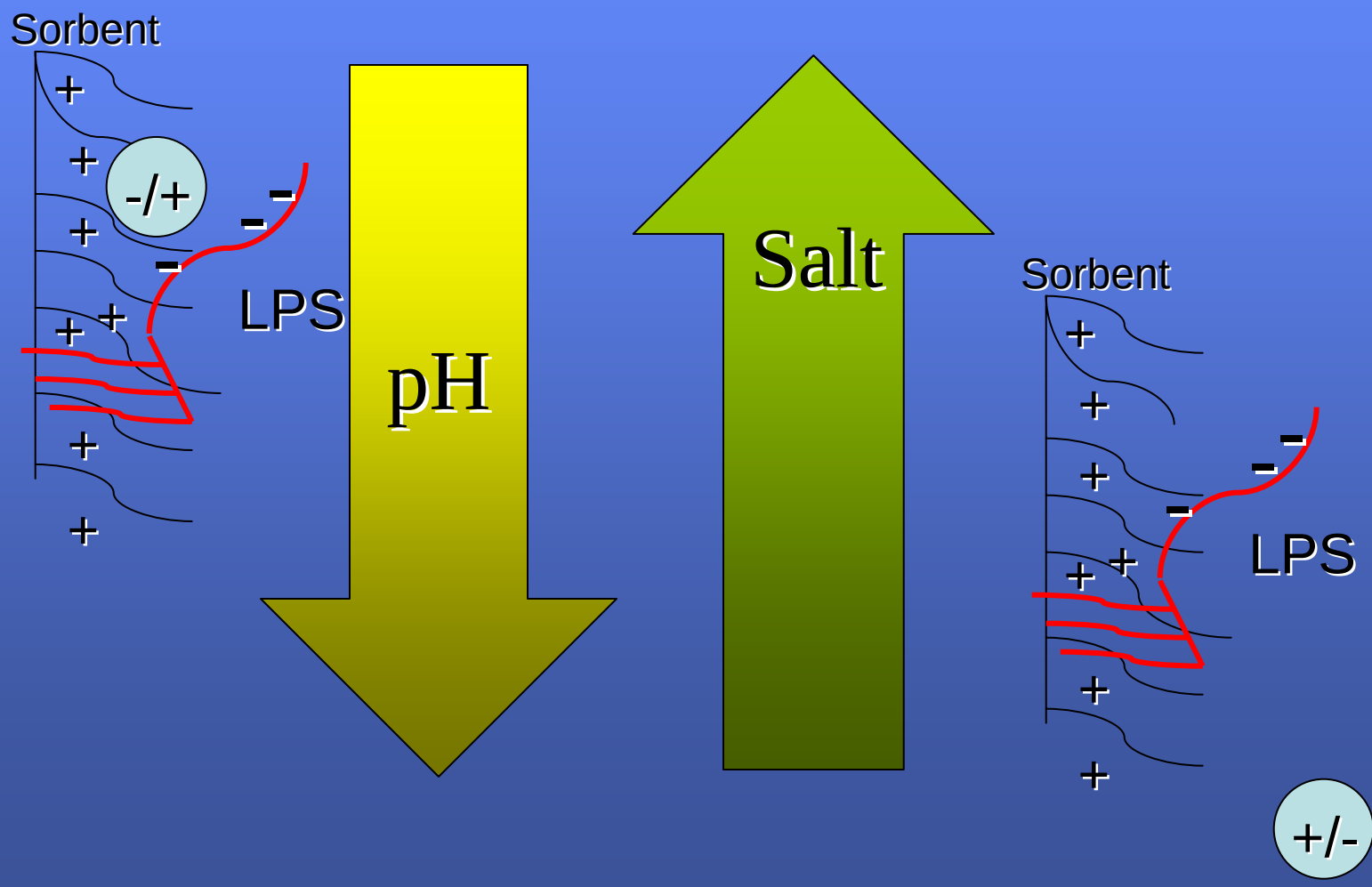
- Contaminants are acidic and/or hydrophobic
- They will all bind more tightly to AIX/HIC sorbents as pH is lowered

LPS binding to mixed mode sorbents



Micellular LPS is excluded from pores.

LPS binding to mixed mode sorbents

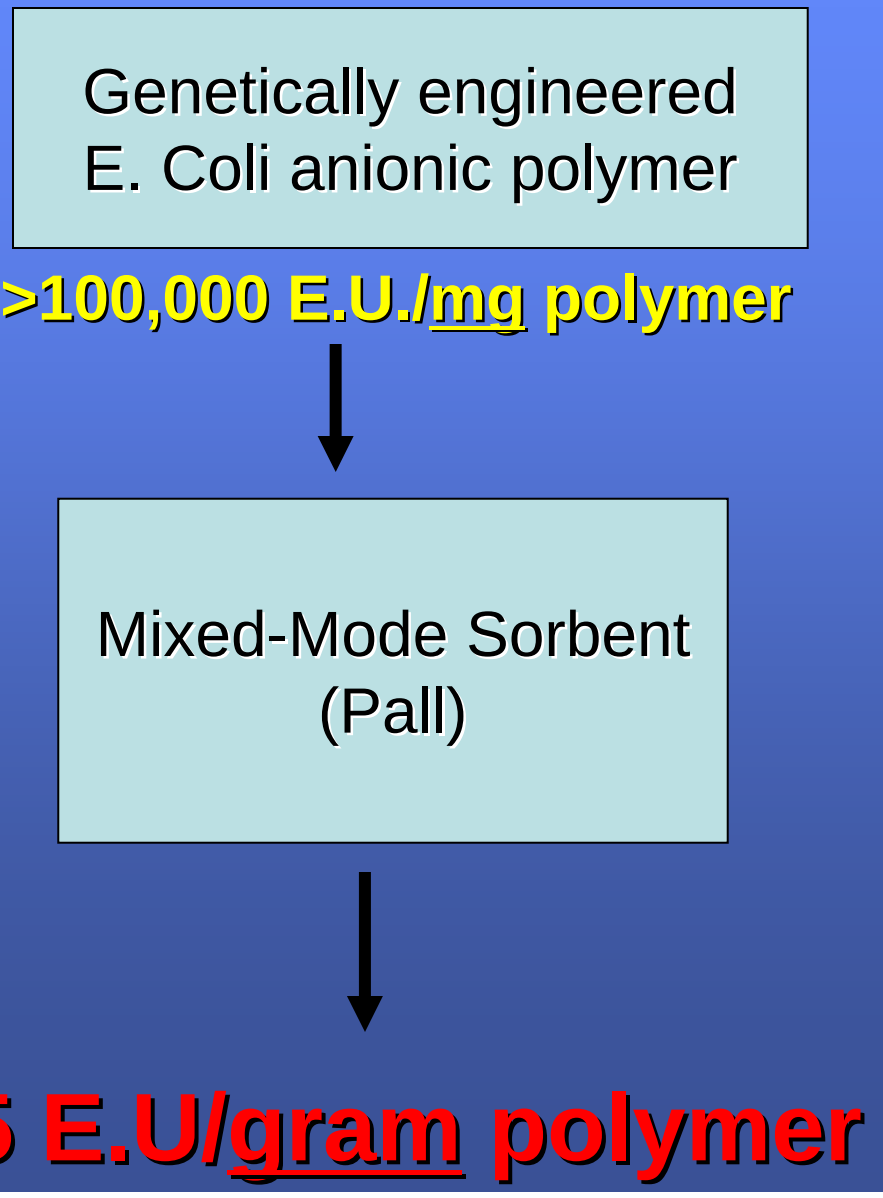


Polishing: Endotoxin reduction

	Sample stage	Conc. Target Protein	Endotoxin Units/ml	Endotoxin Units/mg
Target Protein 1 (capture step)	Fermenter culture	~4 g/L	>10,000 EU/ml	>2564 EU/mg
	MEP load	3.2 mg/ml	1190 EU/ml	377 EU/mg
	MEP eluant	5.65 mg/ml	1.8 EU/ml	0.3 EU/mg
Target Protein 2 (intermediate step)	MEP load	0.4 mg/ml	7500 E.U./ml	18,750 E.U./mg
	MEP eluant	2.2 mg/ml	1.1 E.U./ml	0.5 E.U./mg

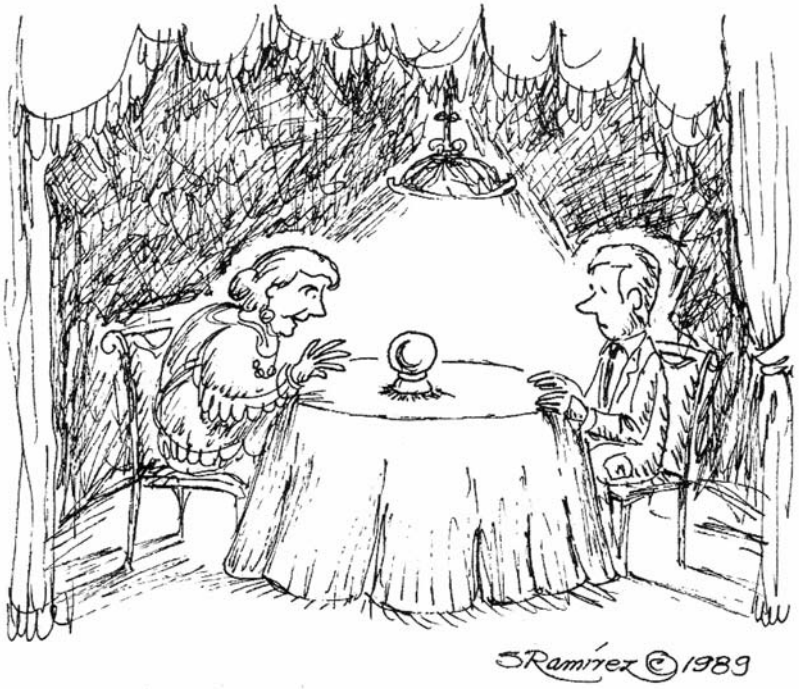
LPS levels were reduced to clinically acceptable levels in a single step

Polishing: Endotoxin reduction



Protein Purification

The art of protein purification involves both selecting the steps and arranging them in an optimal process



"I see you in the process of conducting a protein separation and –wait, you're not adding the proper amount of solvent and the pH of the solution is not right. Now I see your supervisor turning around and slowly begin to approach..."

Mixed-Mode Chromatography

Summary

- Applicable to all stages of purification
- Unique selectivities, including pseudo-affinity
- Mixed-mode sorbents reduce the number of columns needed by combining actions of multiple columns in one sorbent.
- HIC/AIX facilitates LPS removal.

Thank you!

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