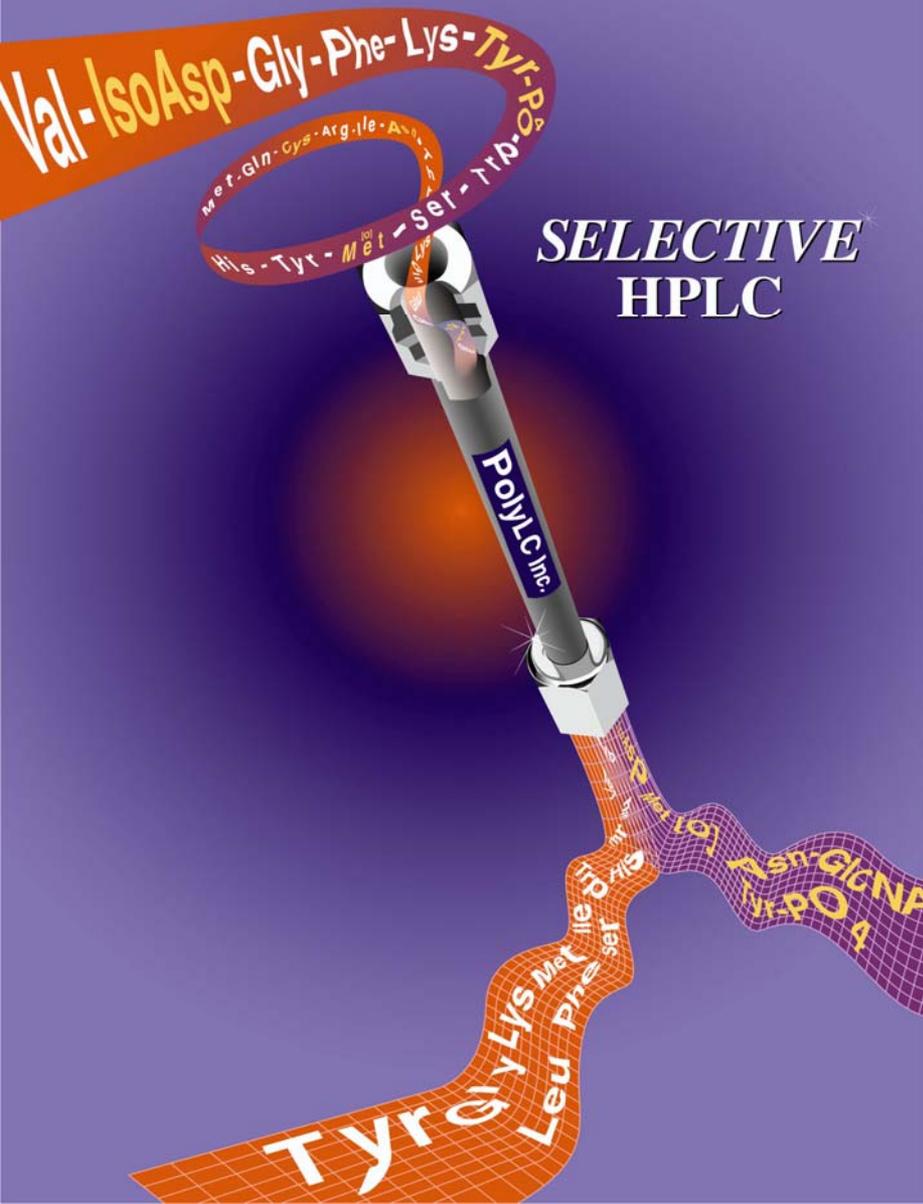


HPLC OF *REALLY* DIFFICULT PROTEIN MIXTURES: Prions to Proteomics

Andrew J. Alpert, Ph.D.
PolyLC Inc.
Columbia, MD U.S.A.



TRADITIONAL BIOCHEMISTRY METHODS:

Isolation: Keep enzymes active; resolve proteins.

Detergents; nonvolatile salts; urea or Gd.HCl (for solubility)

Analysis: Gel electrophoresis (1- or 2-D); enzyme assays.

CURRENT REQUIREMENTS:

Isolation: Not necessary in many cases; MS can handle mixtures. Objective: Reduce large mixture to small mixtures. Identification not dependent on enzymes being active.

Analysis: MS: Volatile solvents required.

Chromatography: Organic solvents, yes; detergents, no.

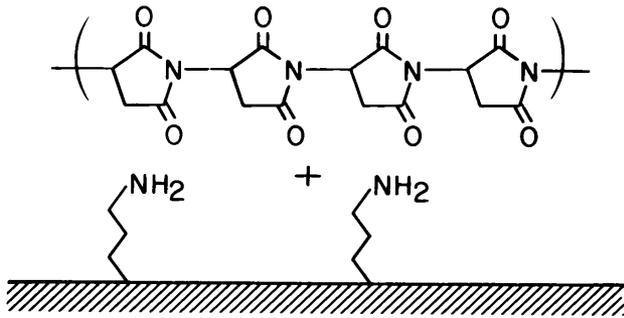
ALTERNATIVES TO REVERSED-PHASE

1) Separation by charge

- a. Anion-exchange (WAX; SAX)
- b. Cation-exchange (WCX; SCX)

2) Separation by polarity

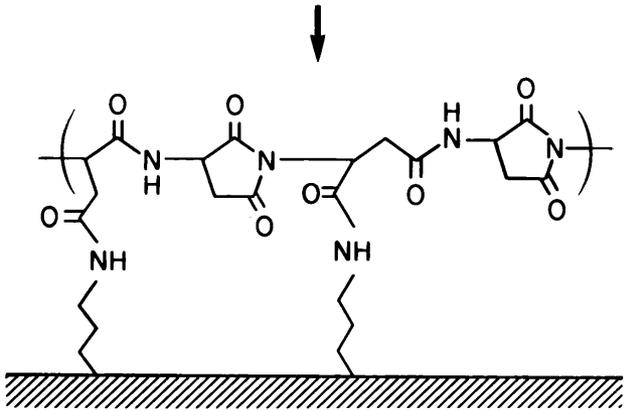
- a. Hydrophobic Interaction (HIC)
- b. Hydrophilic Interaction (HILIC)



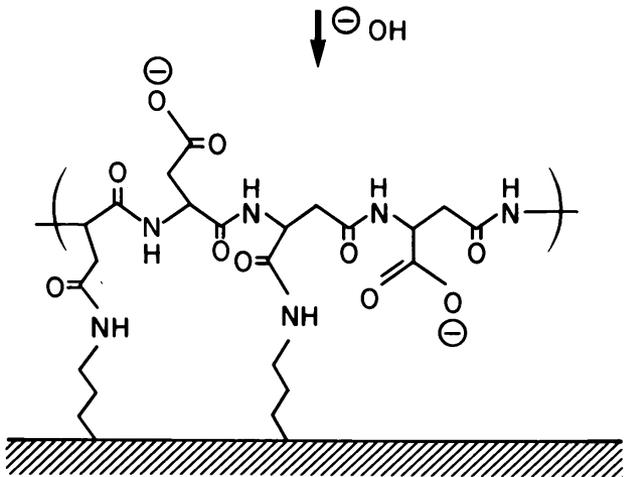
Polysuccinimide

Aminopropyl-silica

**Preparation of
a Weak
Cation-Exchange
(WCX) material**



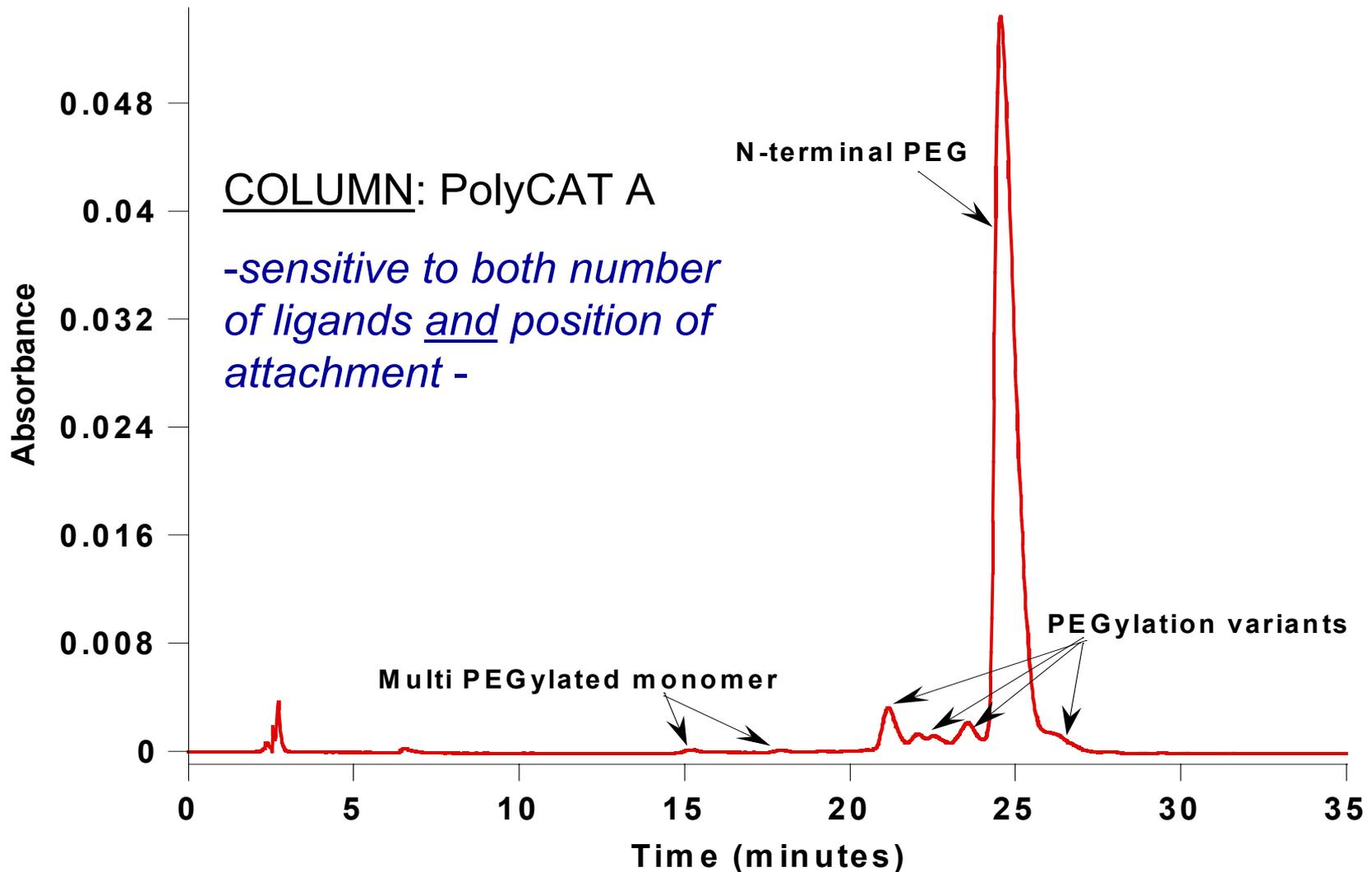
Poly(succinimide)-silica



Poly(aspartic acid)-silica

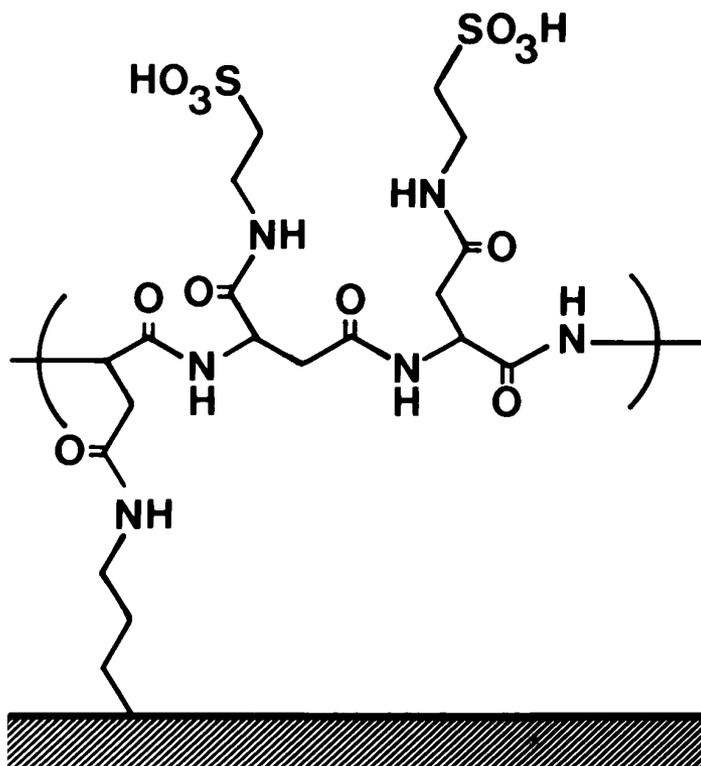
(PolyCAT A)

NEUROTROPHIC FACTOR: Polyethylene Glycol (PEG) Attached



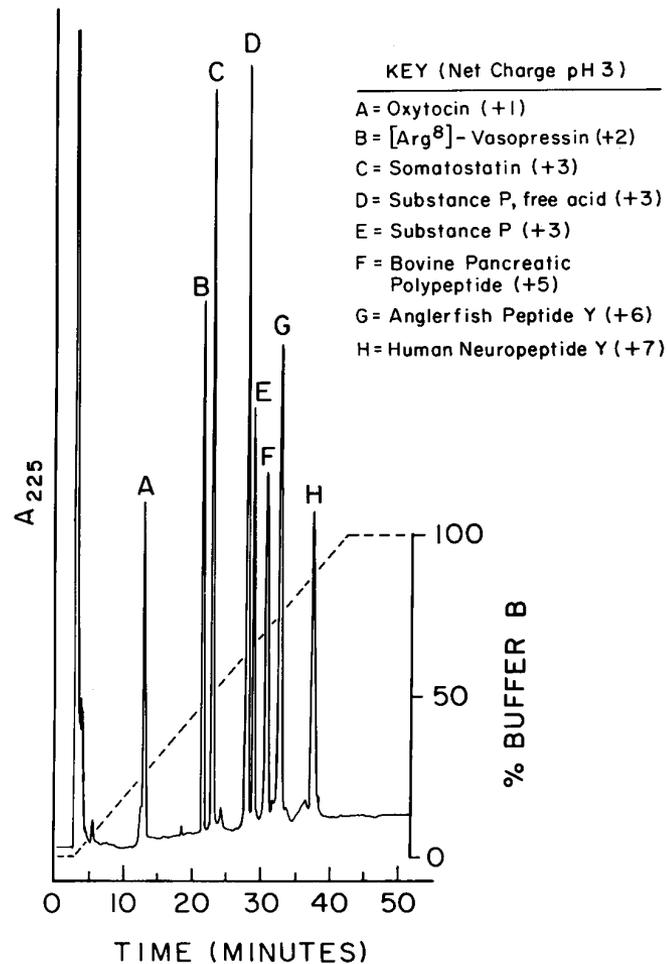
CATION-EXCHANGE OF PEPTIDES (SCX)

PolySULFOETHYL
Aspartamide

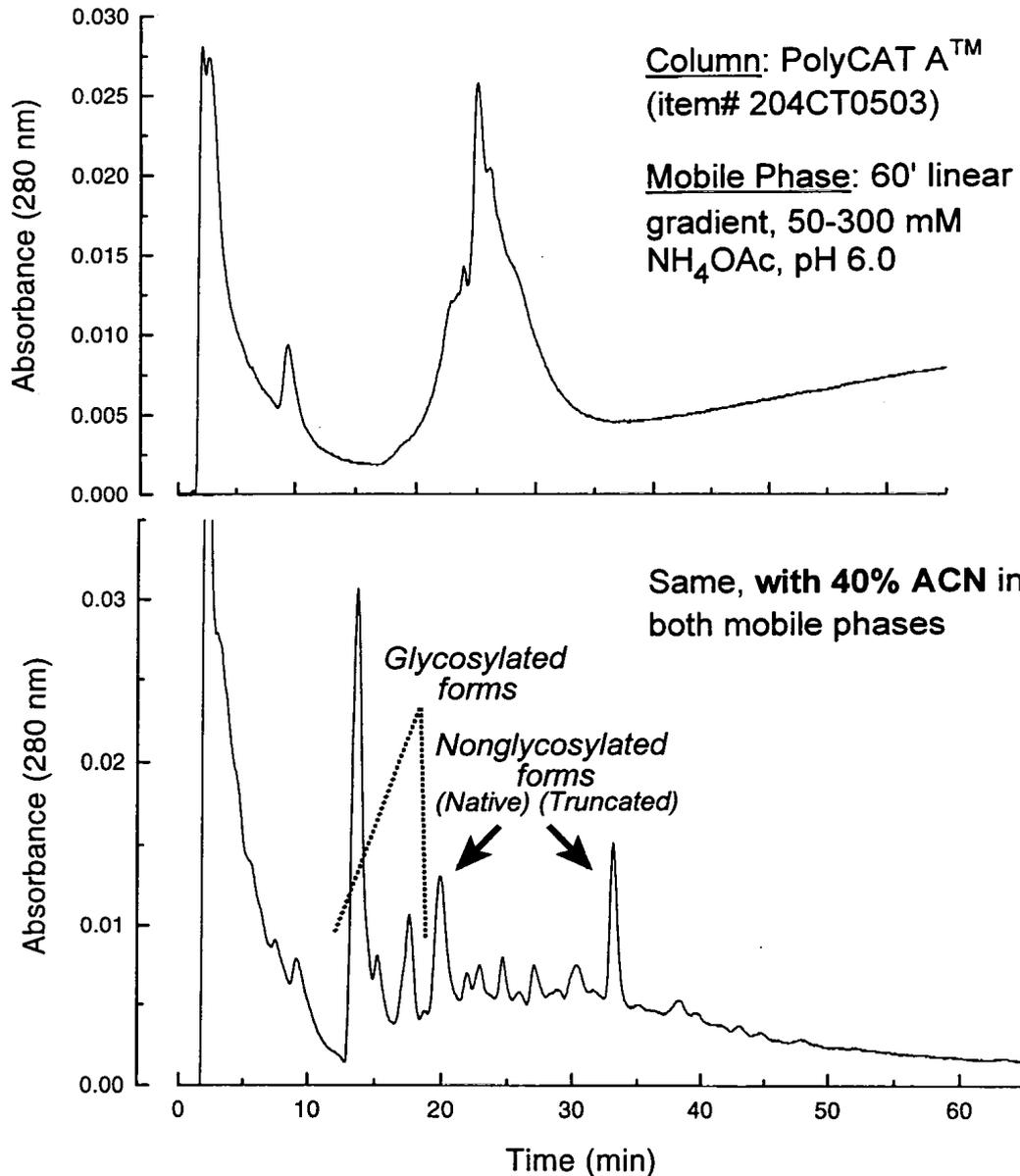


PEPTIDE STANDARDS

Column: PolySULFOETHYL A, 5 micron, 4.6 x 200 mm
 Buffer A: 5 mM K_2PO_4 , pH 3.0, with 25% acetonitrile
 Buffer B: Same + 0.25 M KCl



ION-EXCHANGE OF PROTEINS WITH ORGANIC SOLVENTS



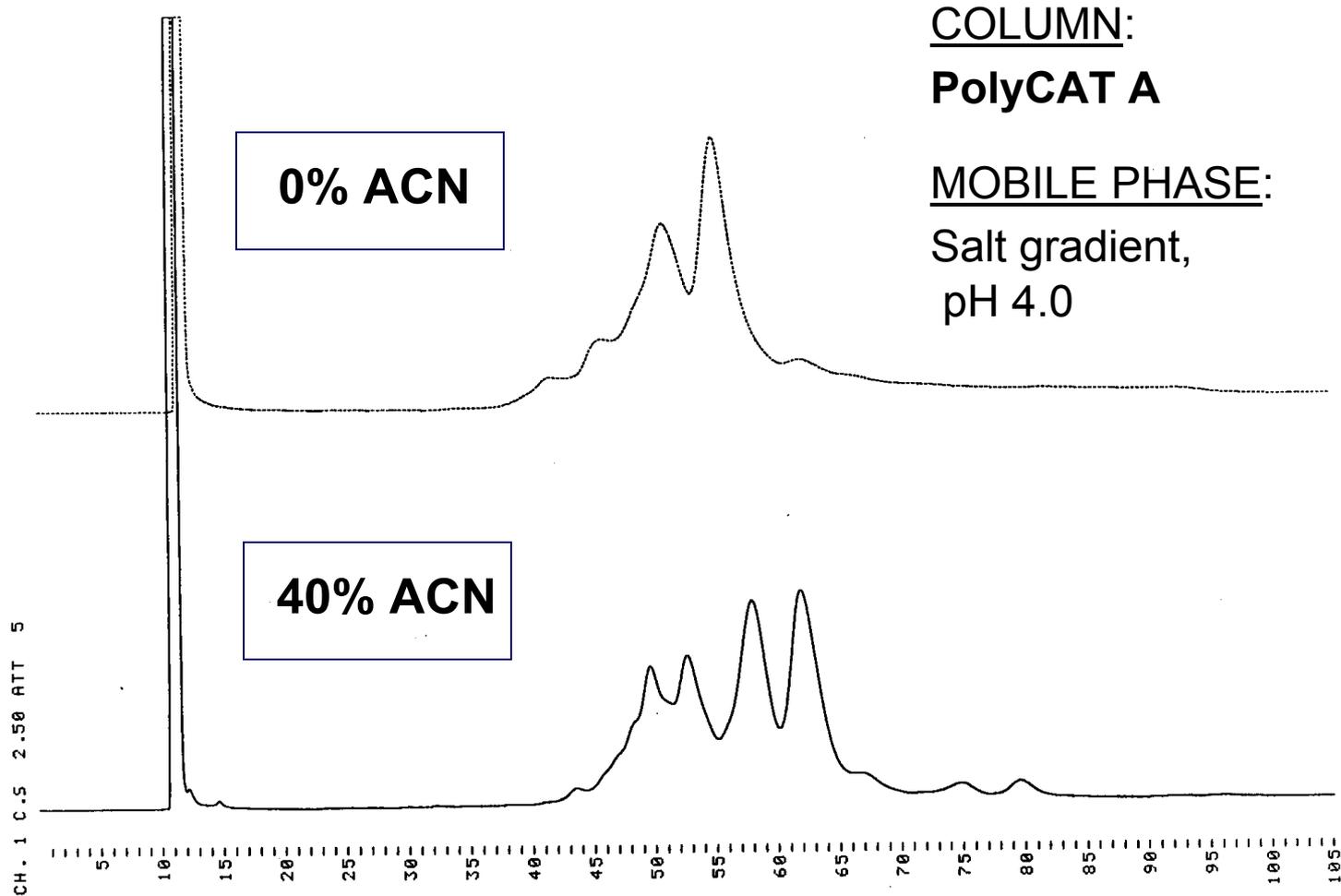
Sample: *rec*
 α -Bungarotoxin
from *P. Pastoris*

- **Selectivity**
Increases
dramatically -

- data courtesy of Robert Rogowski and Edward Hawrot (Brown Univ.) -

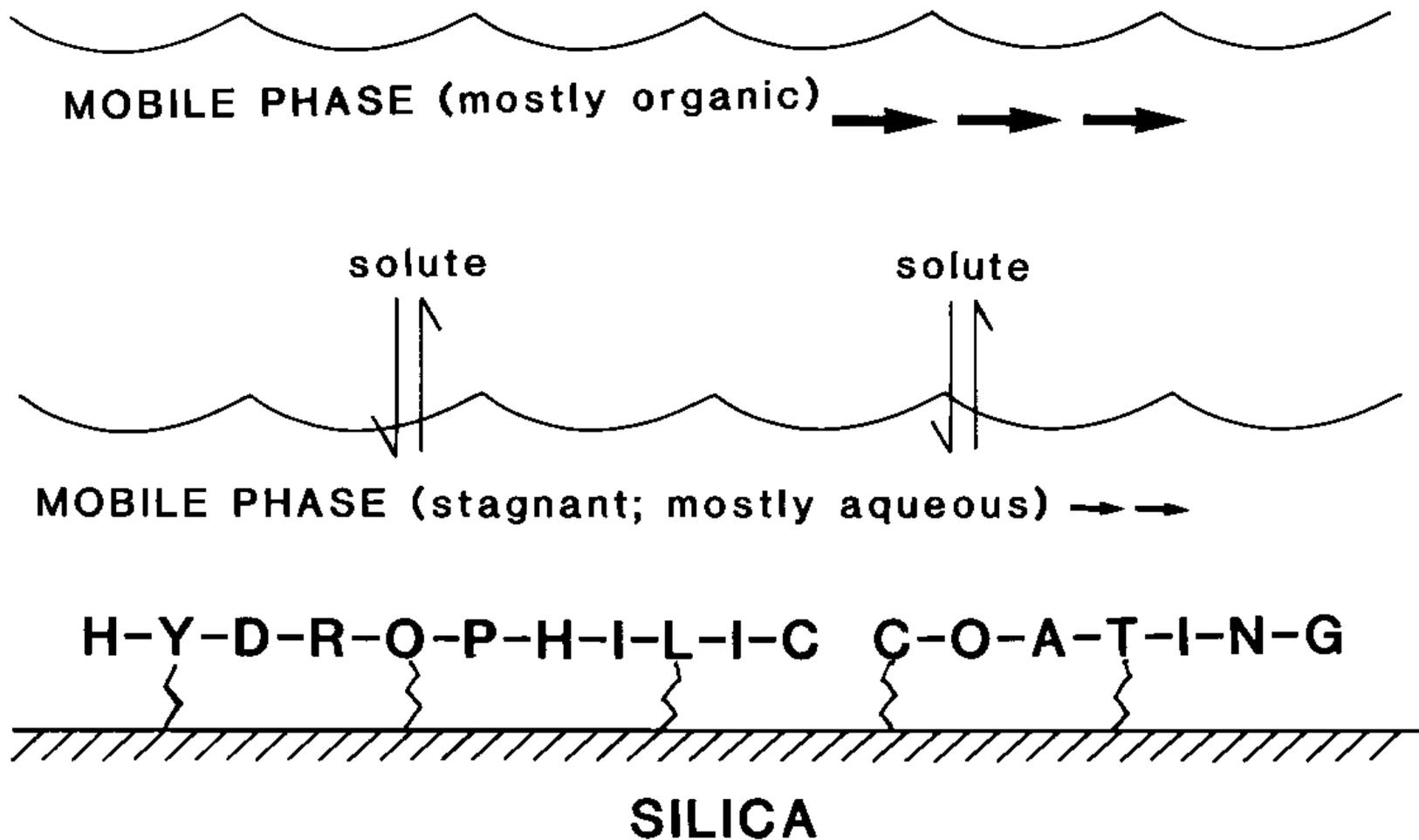
ION-EXCHANGE WITH ORGANIC SOLVENTS

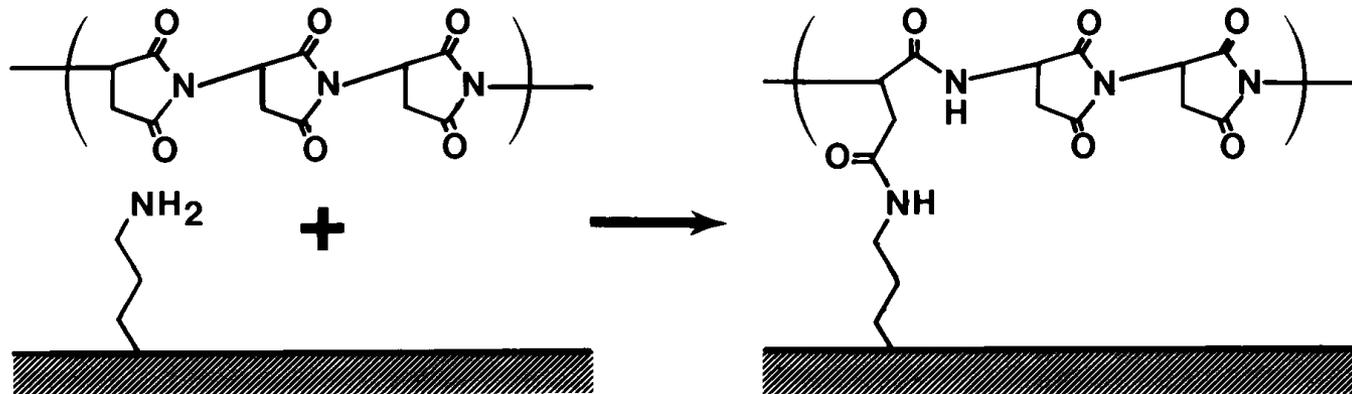
- *rec* Human Growth Hormone -



- *data courtesy of Benny Welinder, Novo Nordisk* -

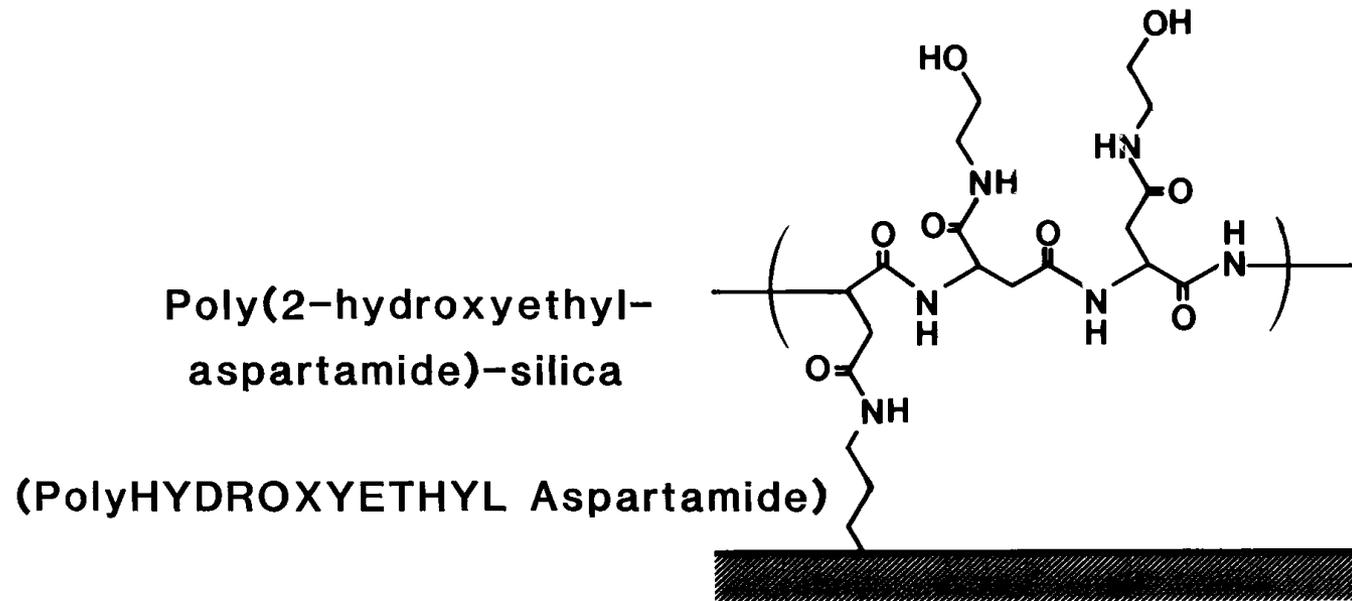
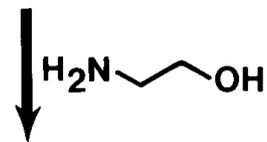
HYPOTHETICAL PARTITION MECHANISM OF HYDROPHILIC-INTERACTION CHROMATOGRAPHY (HILIC)





Poly(succinimide)
+
Aminopropyl-silica

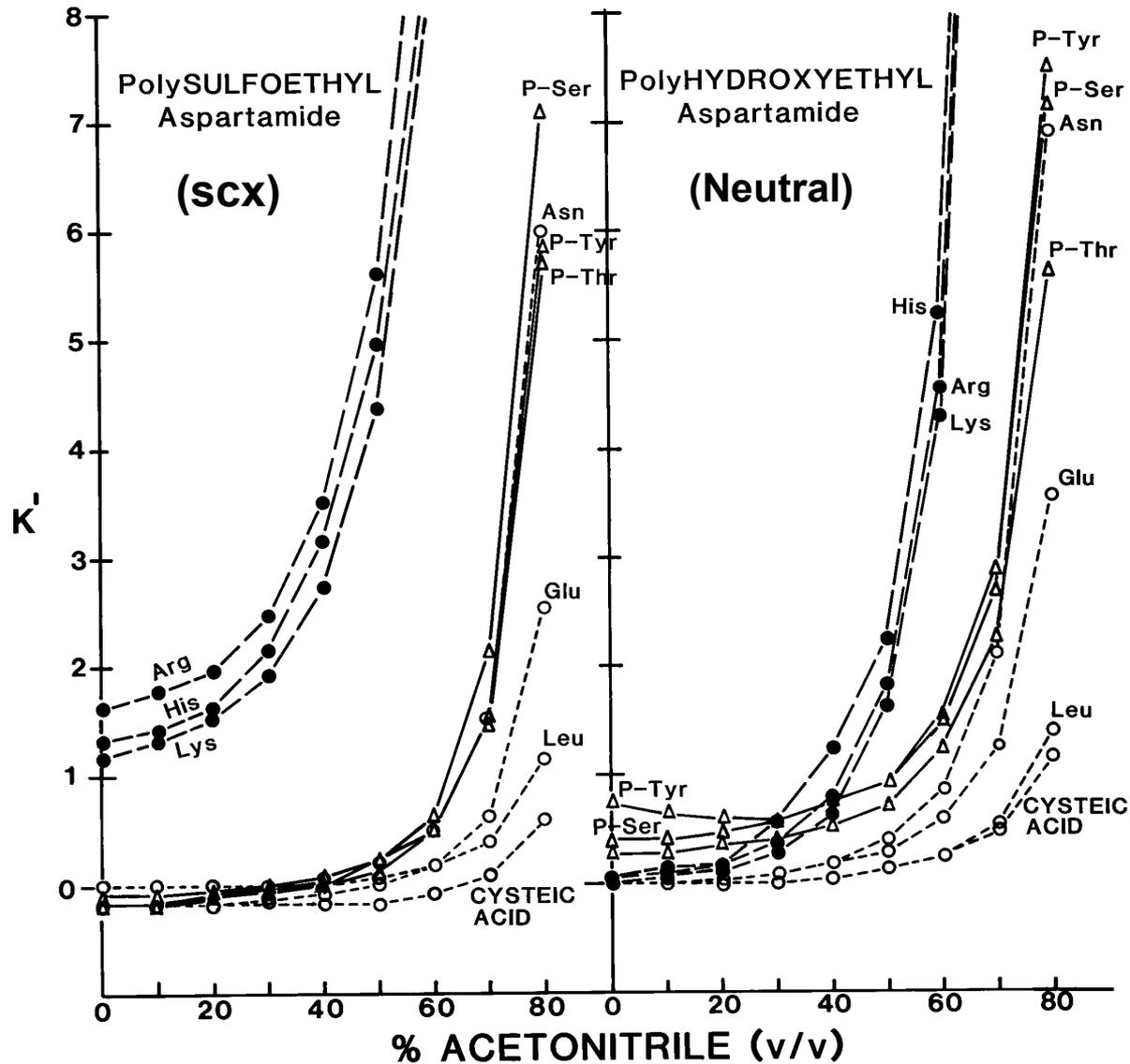
Poly(succinimide)-silica



Poly(2-hydroxyethyl-
aspartamide)-silica

(PolyHYDROXYETHYL Aspartamide)

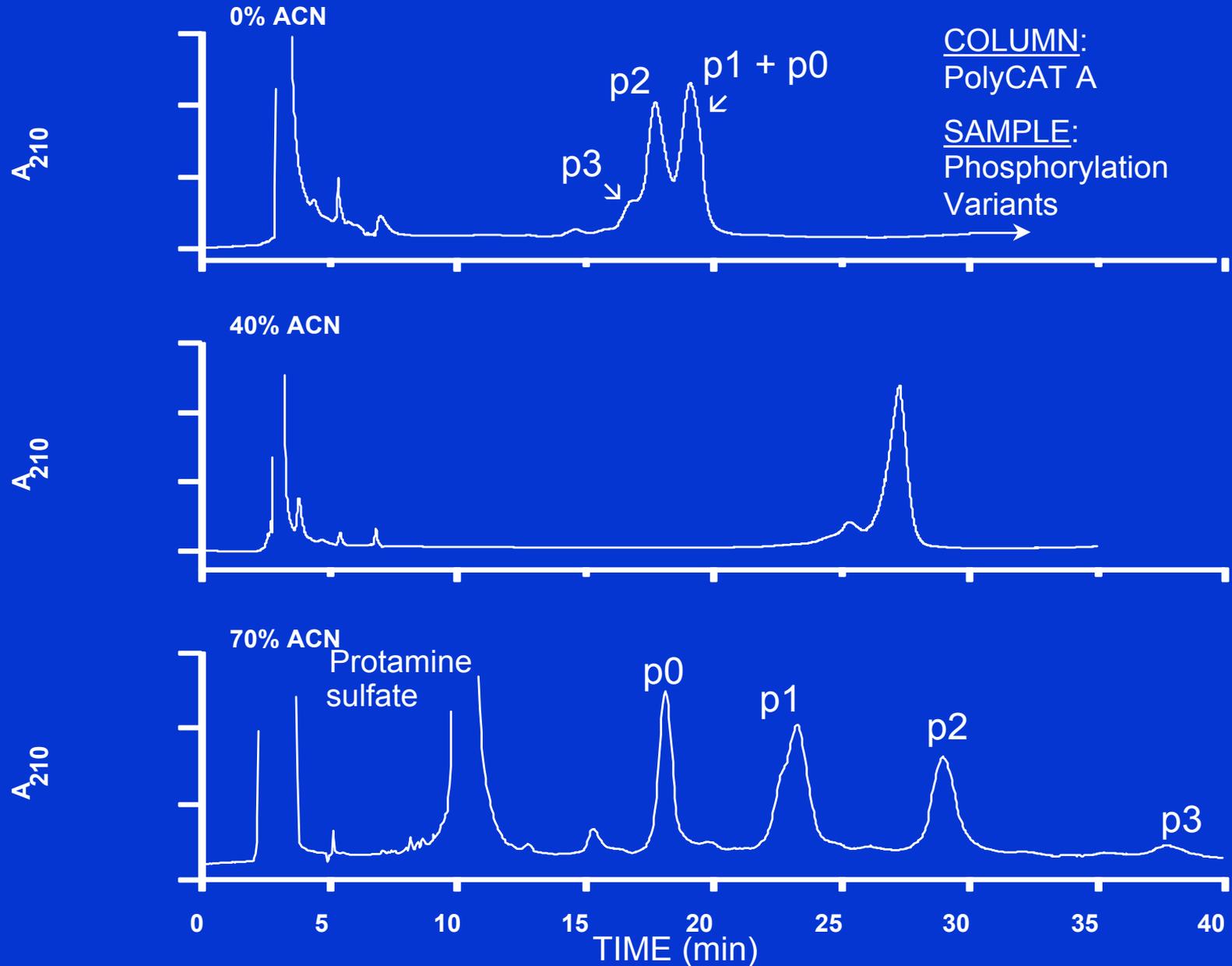
HYDROPHILIC INTERACTION CHROMATOGRAPHY OF AMINO ACIDS



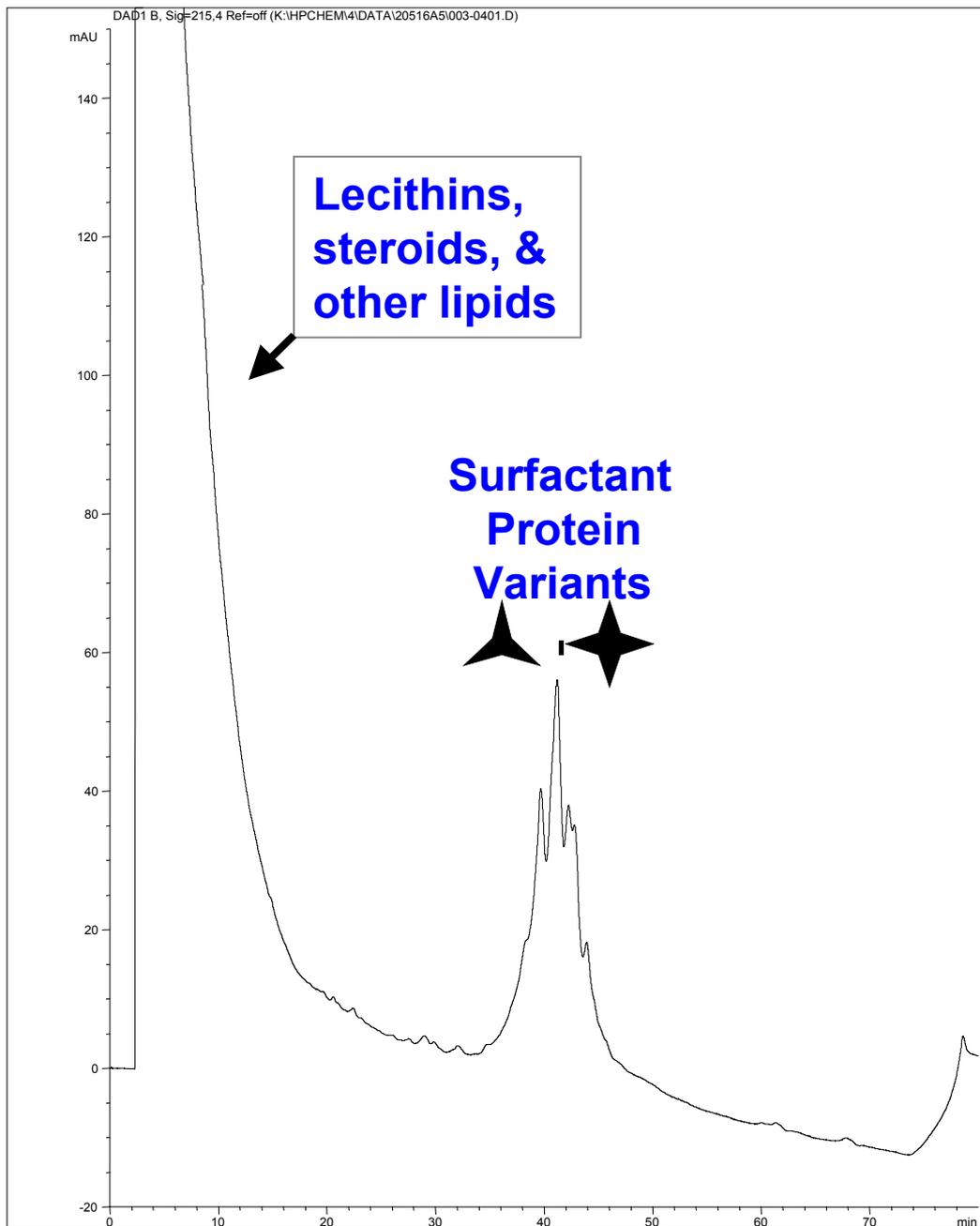
COLUMNS:
 200 x 4.6 mm;
 5- μ m, 300- \AA

MOBILE PHASE:
 25 mM TEAP,
 pH 5.0, with
 ACN as noted

CEX-HILIC OF HISTONE H1.1



COURTESY OF HERBERT LINDNER, UNIV. INNSBRUCK



SCX-HILIC of Lung Surfactant Protein

(lipid:protein = 500:1)

COLUMN: PolySULFOETHYL A,
200x4.6-mm; 5- μ m; 1000- \AA

MOBILE PHASE (1 ml/min):
5' hold, then 0-100% B in 60'

A) 0.1% methylphosphonic acid +
5 mM NaClO_4 , pH 3, with 70% ACN

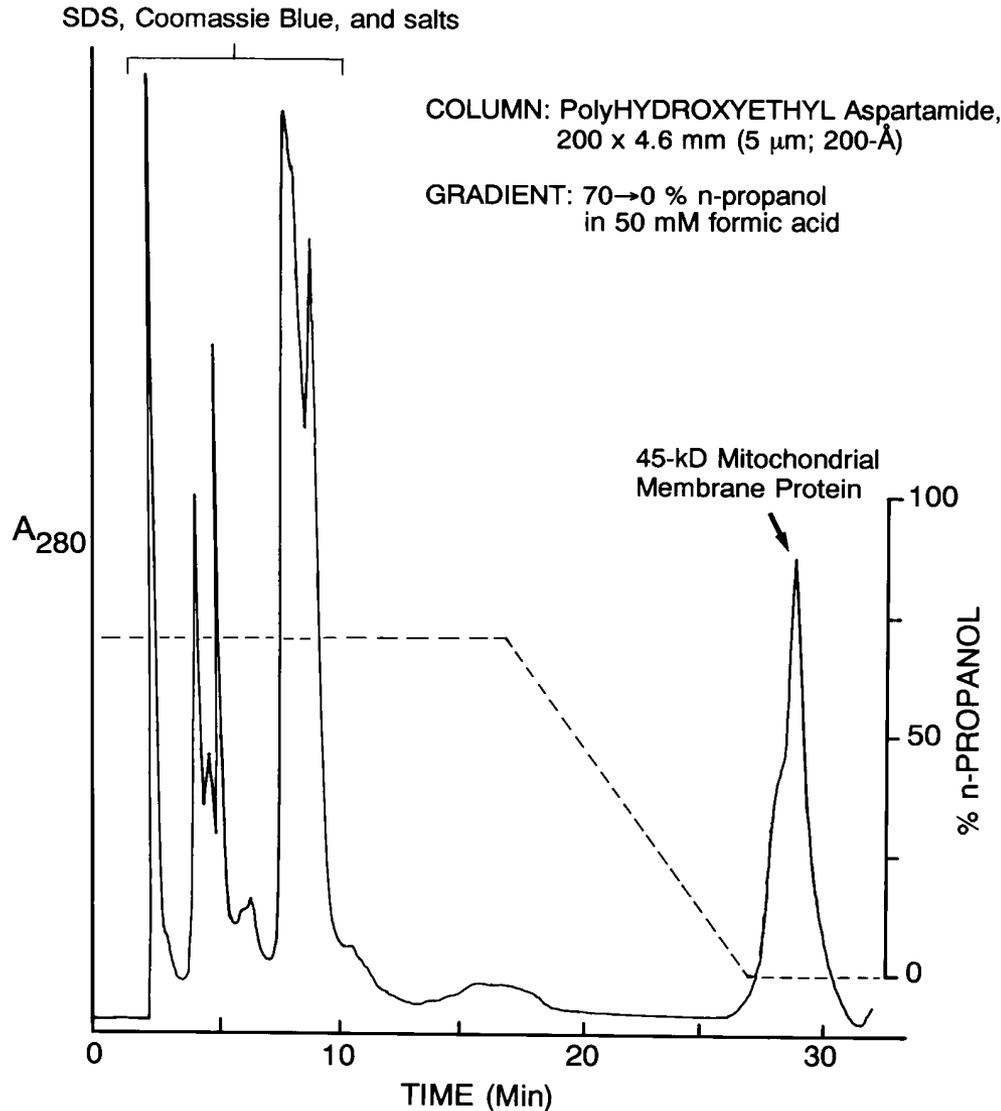
B) Same but 100 mM NaClO_4

DETECTION: A215

SAMPLE: 24 μ g protein/80 μ l

Hydrophilic Interaction Chromatography (HILIC)

- *works well for membrane proteins* -

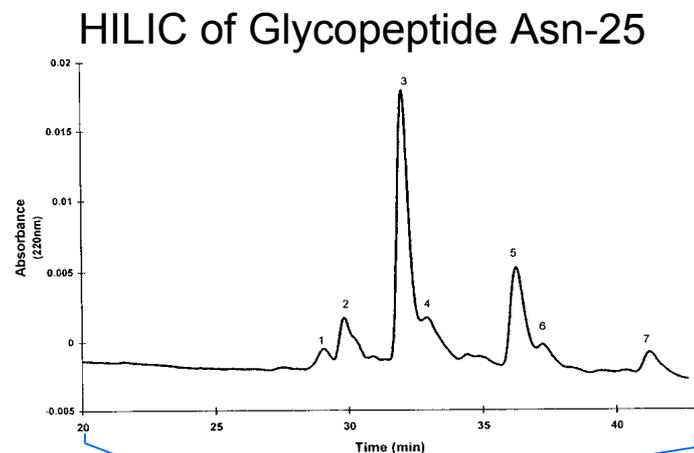
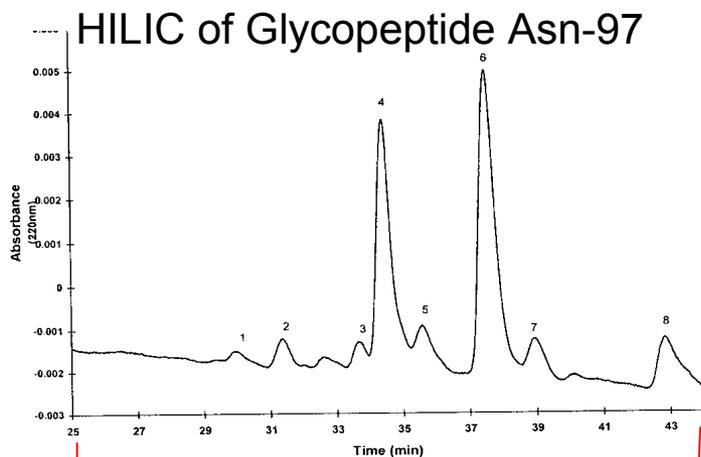


**Removal of SDS,
Coomassie Blue, and Salts
from an Electroeluted
Membrane Protein**

- data courtesy of Paul Jenö,
Univ. of Basel -

RPC-HILIC Purification of Variant Glycopeptides from a Tryptic Digest of γ -Interferon

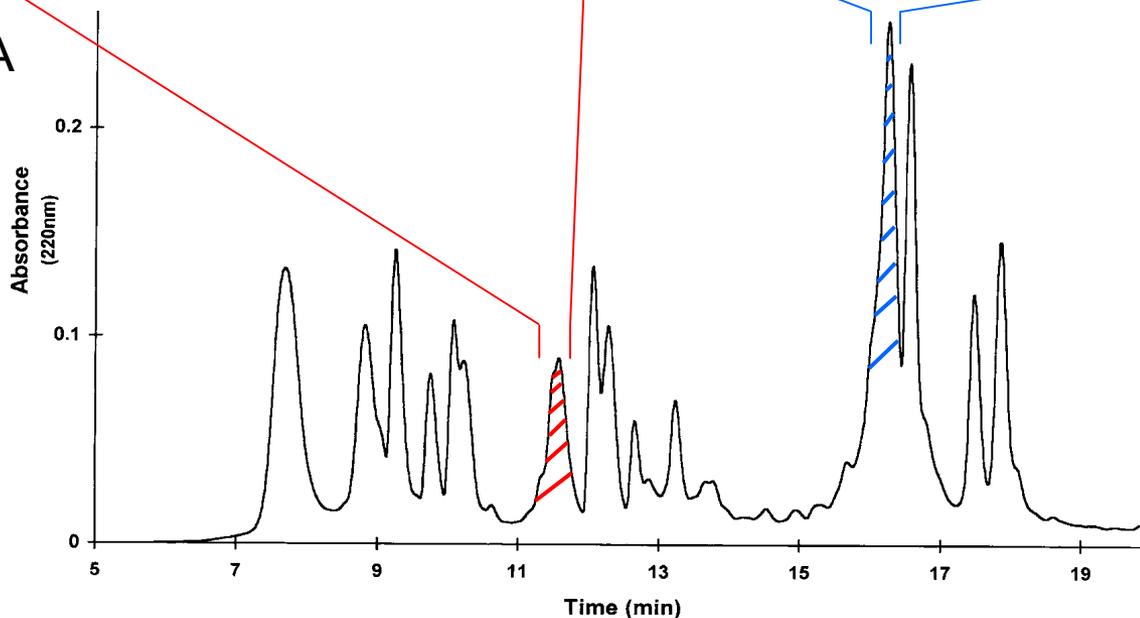
**ADJACENT PEAKS
DIFFER BY ONE
CARBOHYDRATE
RESIDUE OR
POSITION**



PolyHYDROXYETHYL A
(150x1.0-mm)

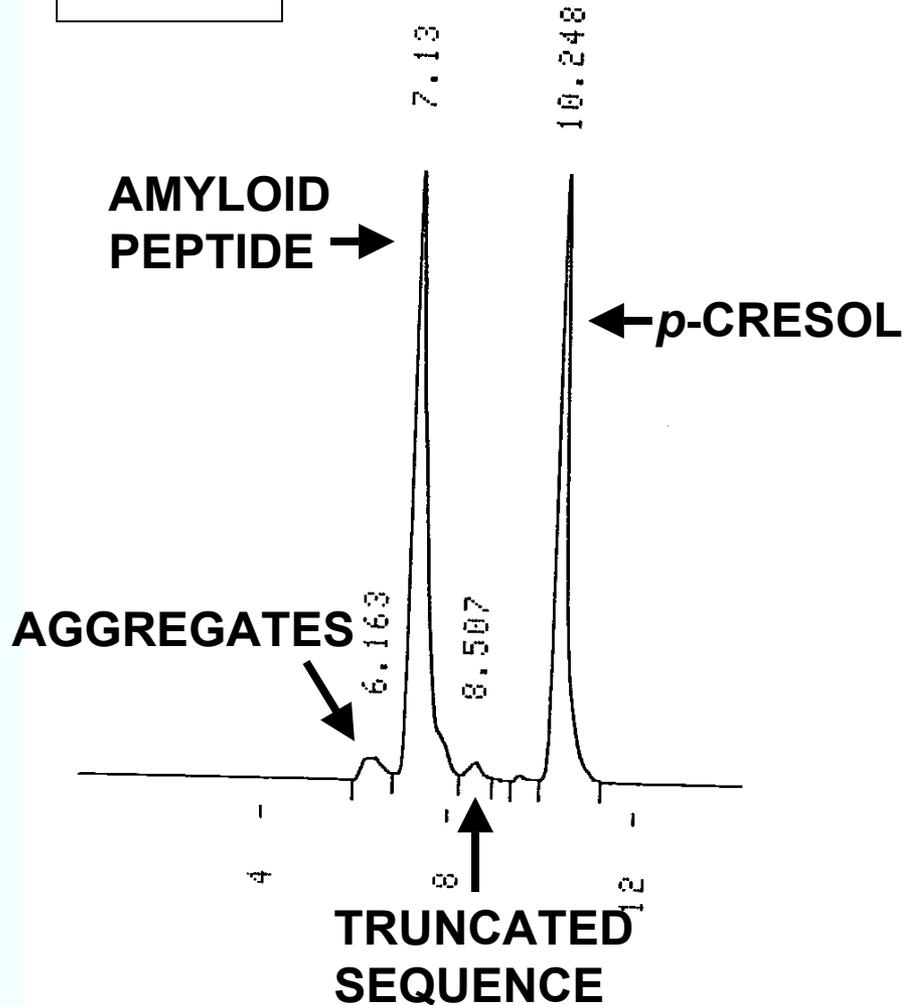
RPC (Vydac C-18) of
total digest from CHO
batch culture

*Data courtesy of J. Zhang
and D. Wang (MIT)*

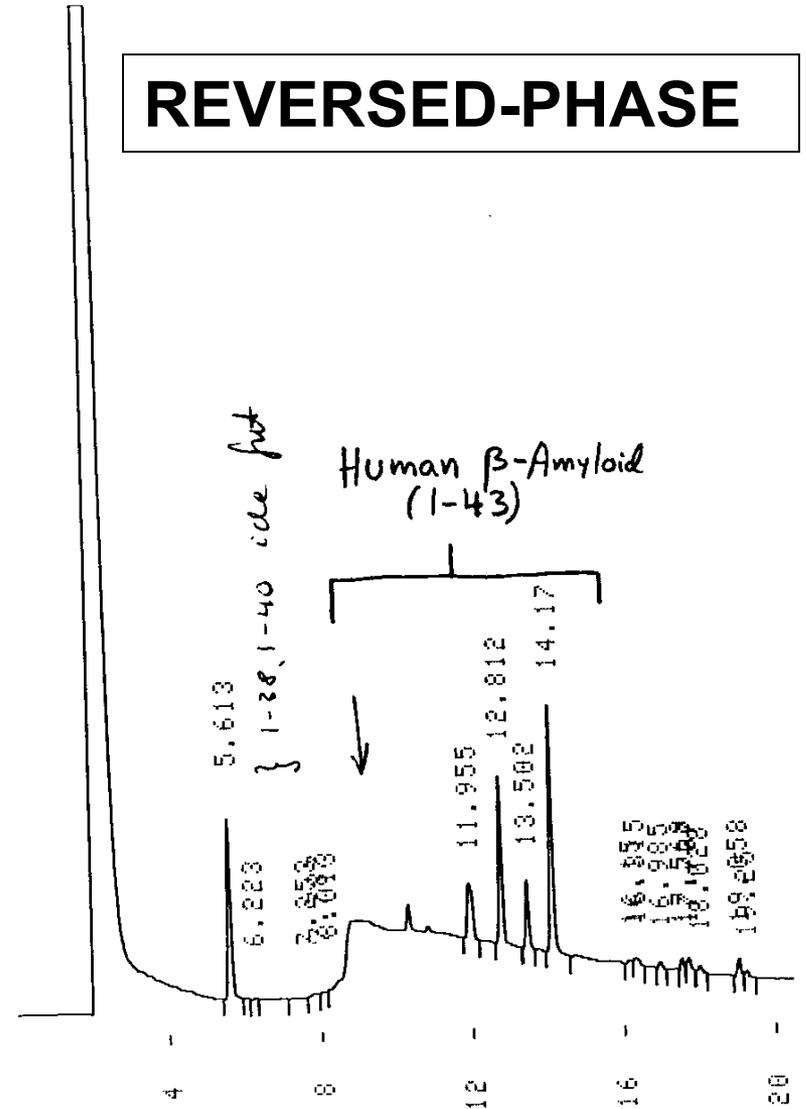


HPLC of β -Amyloid Peptide (1-43)

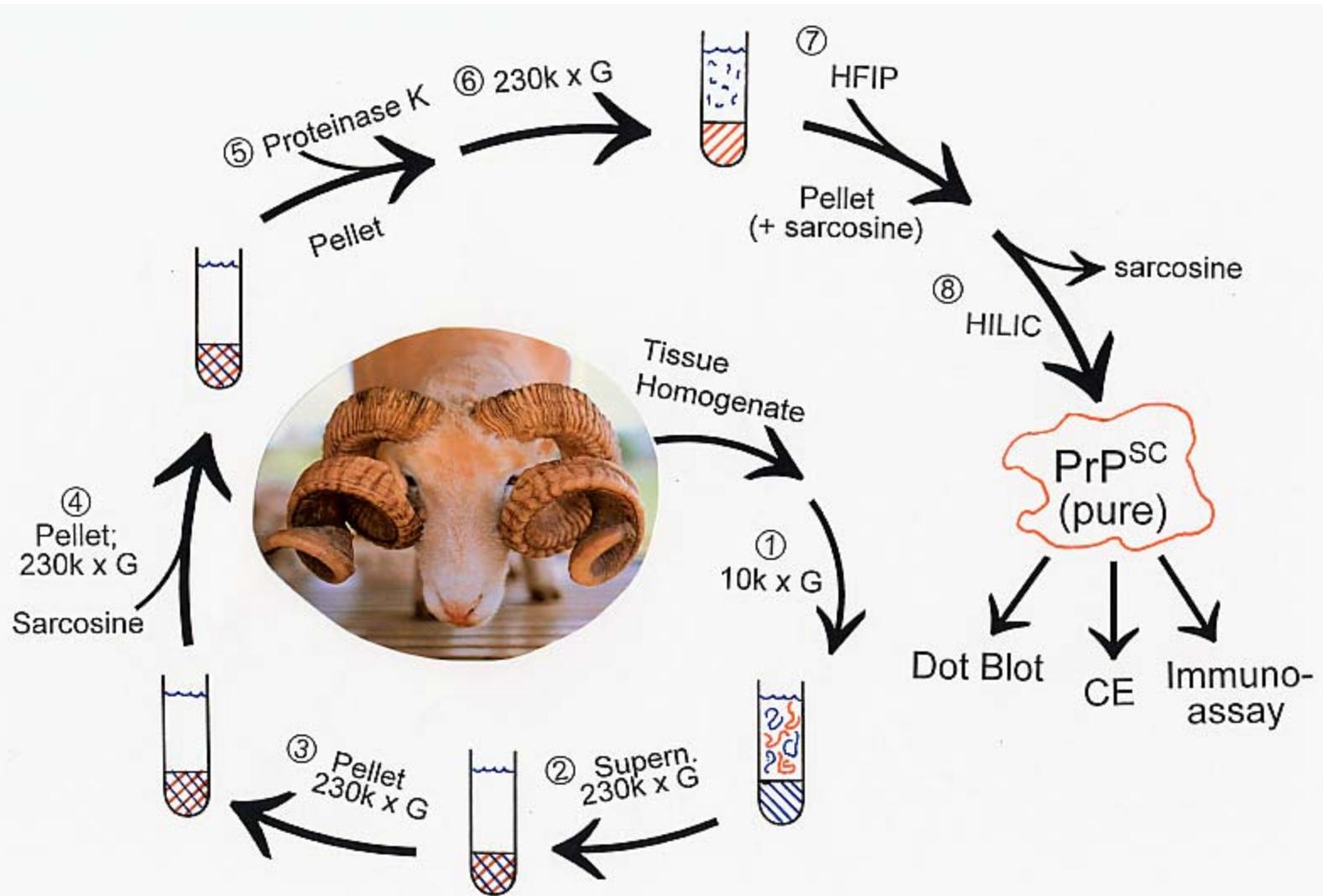
HILIC



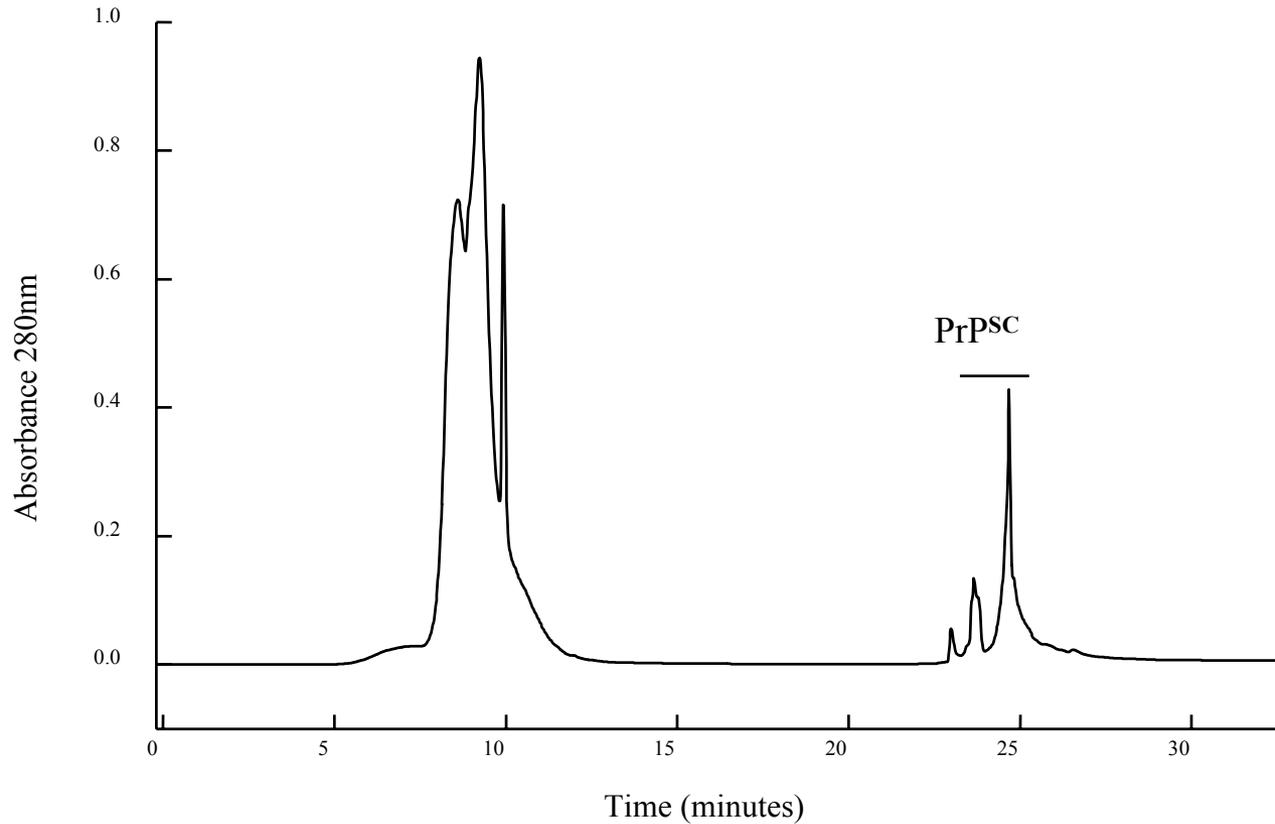
REVERSED-PHASE



PREPARATION OF SCRAPIE PRION PROTEIN



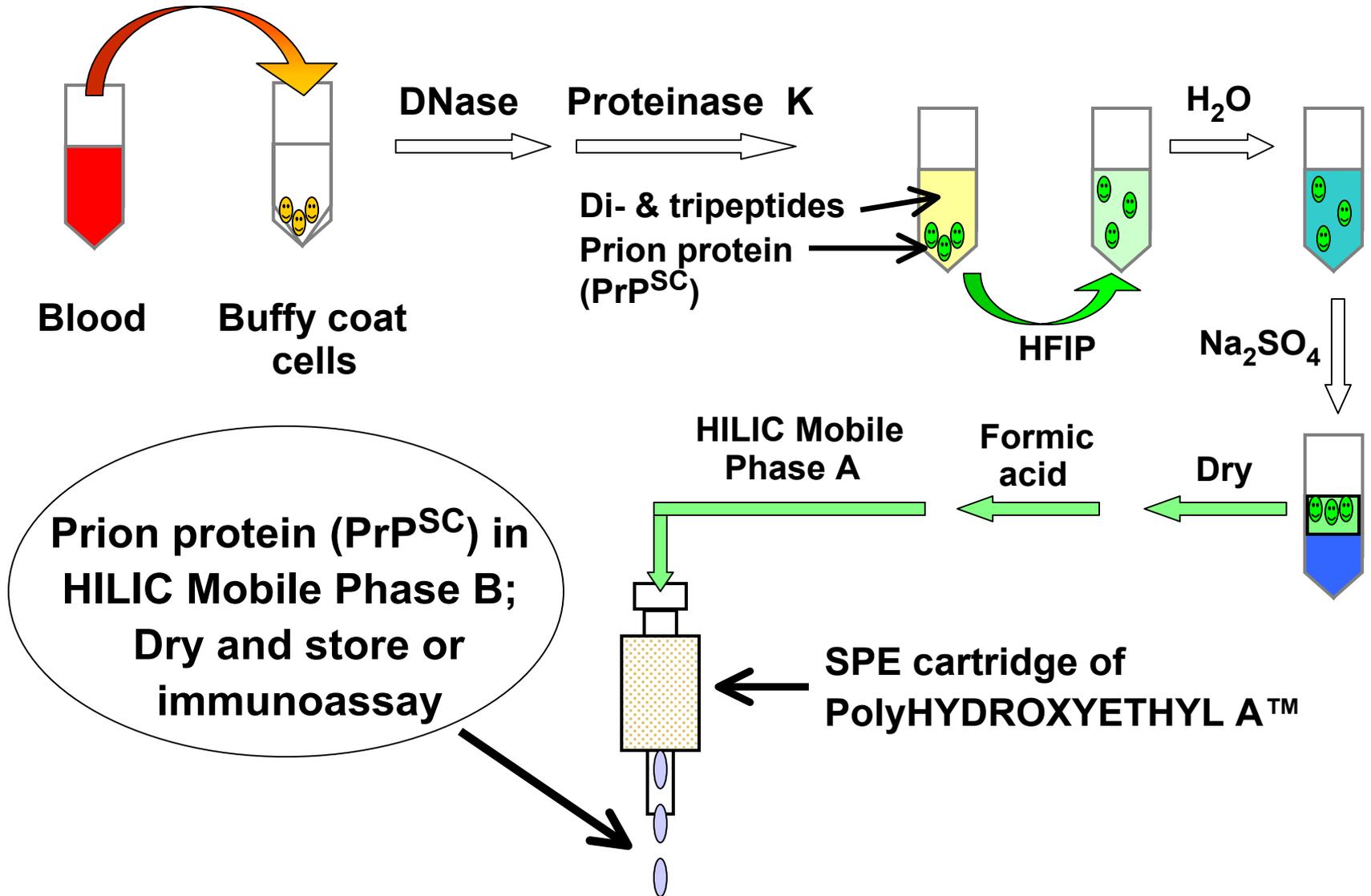
HILIC of Scrapie Prion Protein

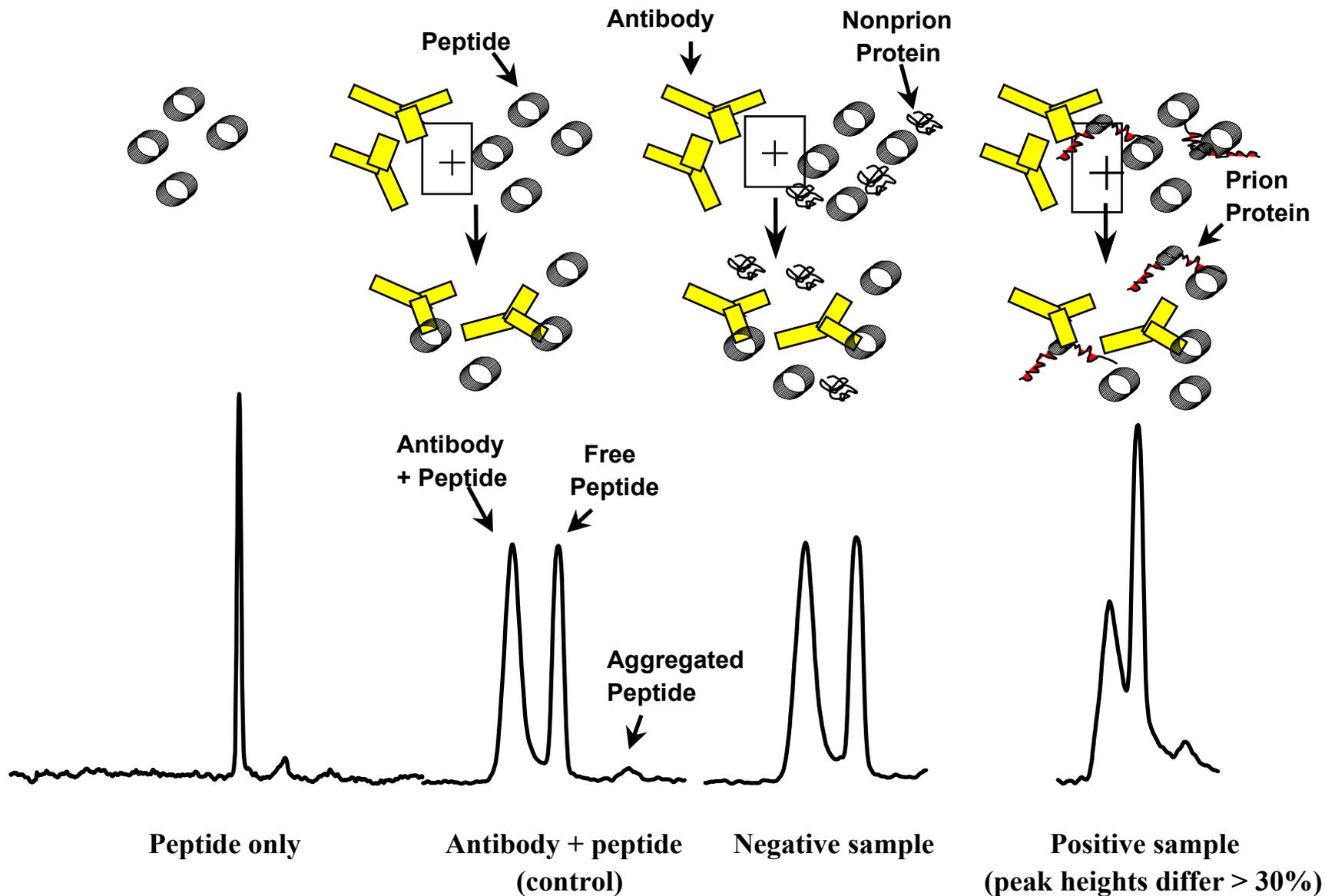


Column: PolyHYDROXYETHYL A

Gradient: Decreasing [ACN]

EXTRACTION OF PRION PROTEIN BY HILIC





COMPETITIVE IMMUNOASSAY OF SCRAPIE PRION BY CE.

Elapsed time of each electropherogram: 4'.

SHEEP: Assays of Normal Controls

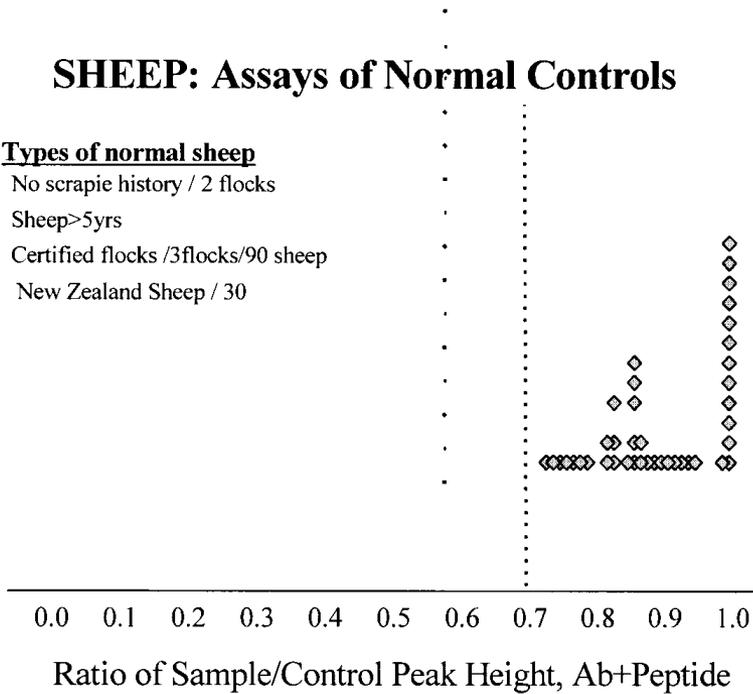
Types of normal sheep

No scrapie history / 2 flocks

Sheep > 5 yrs

Certified flocks / 3 flocks / 90 sheep

New Zealand Sheep / 30



SHEEP: Assays of Scrapie Victims

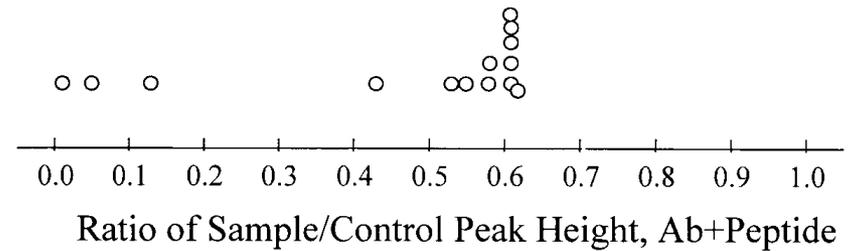
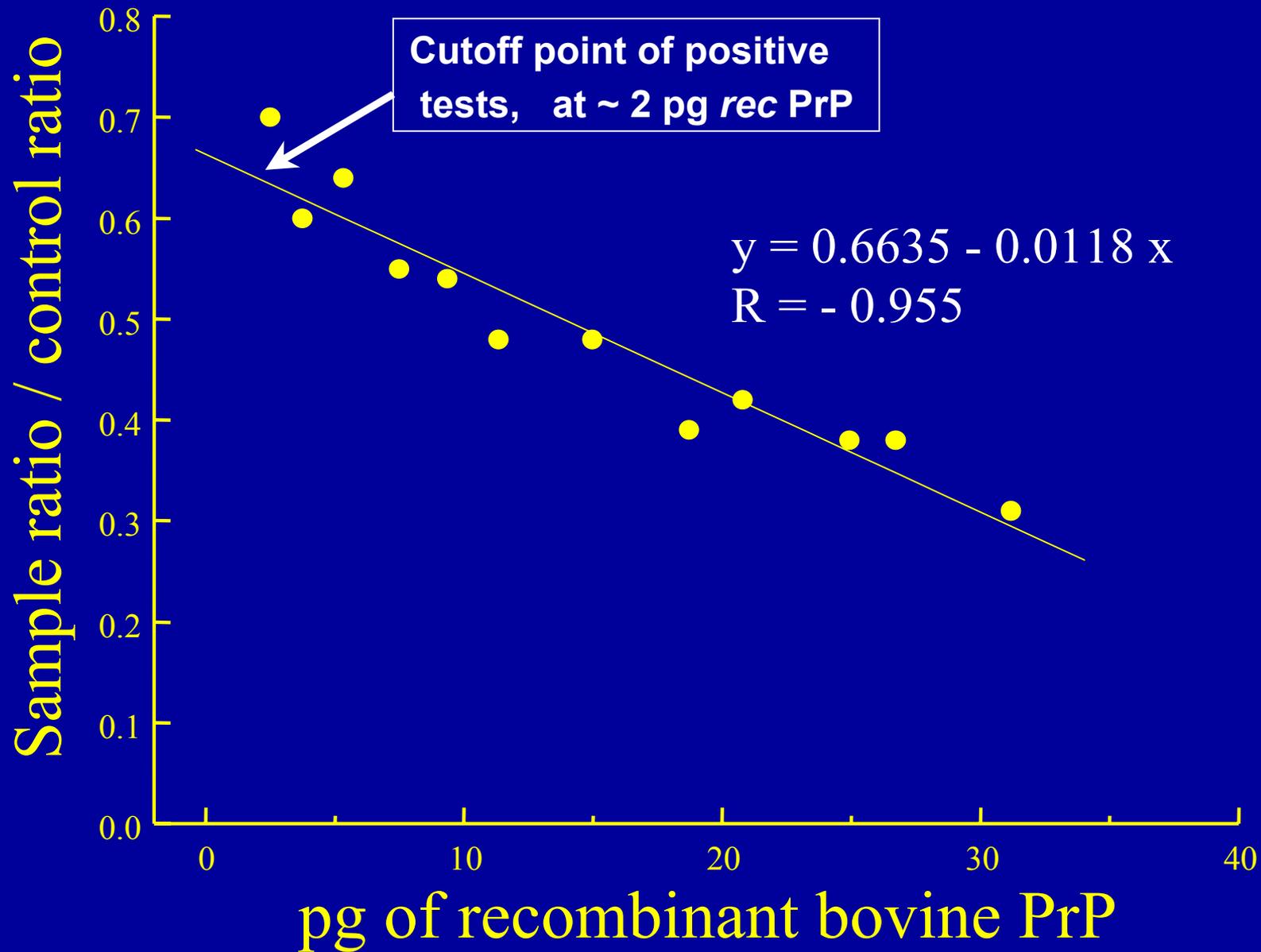


Fig. 4b. Response of CE immunoassay to extracts from blood of normal sheep and sheep with scrapie.

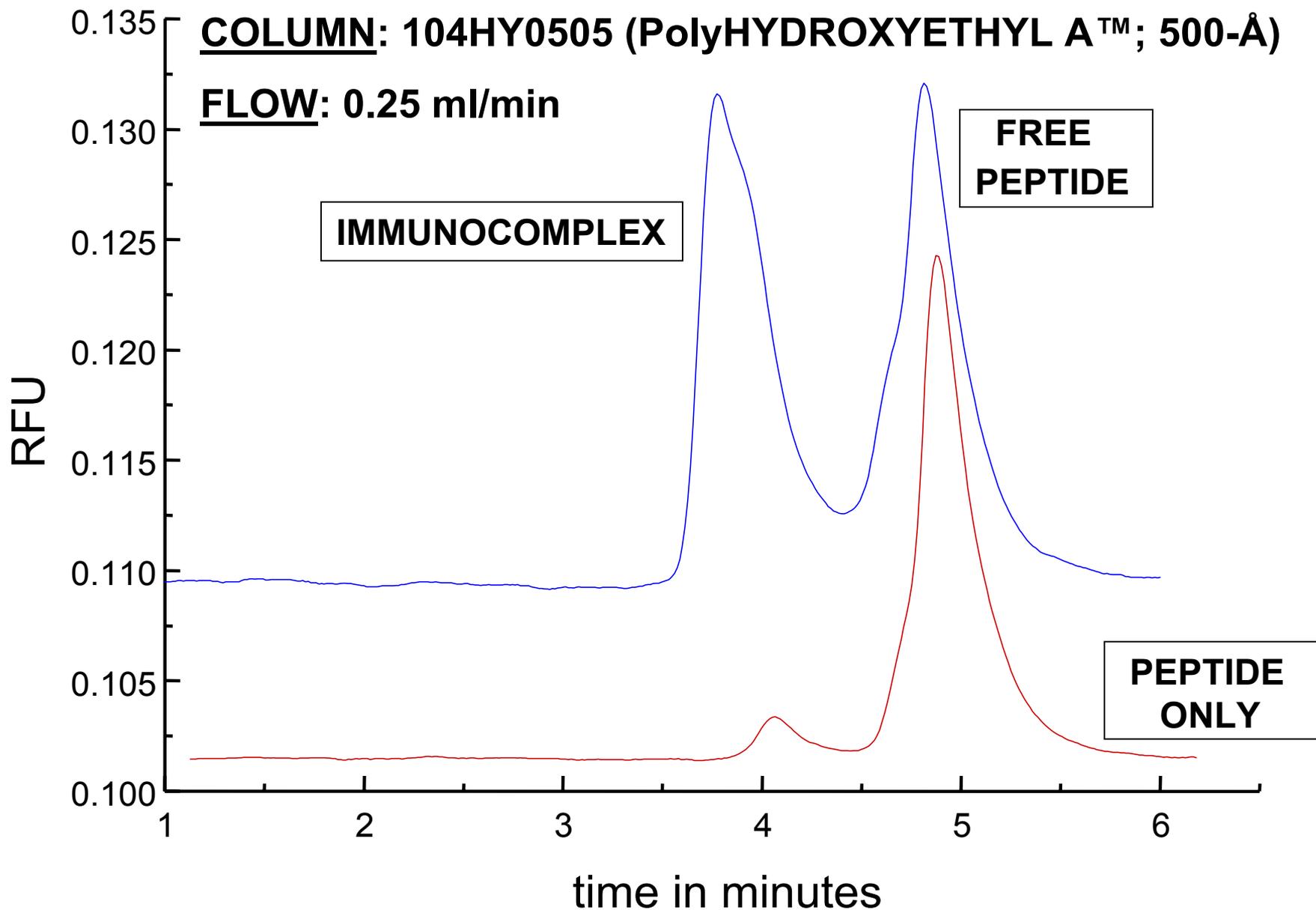
A test with a ratio < 70% is considered positive. This occurs with > 2 picograms/ml of prion protein in the blood.



SEC OF FLUORESCENT PRION PEPTIDE + ANTIBODY

COLUMN: 104HY0505 (PolyHYDROXYETHYL A™; 500-Å)

FLOW: 0.25 ml/min



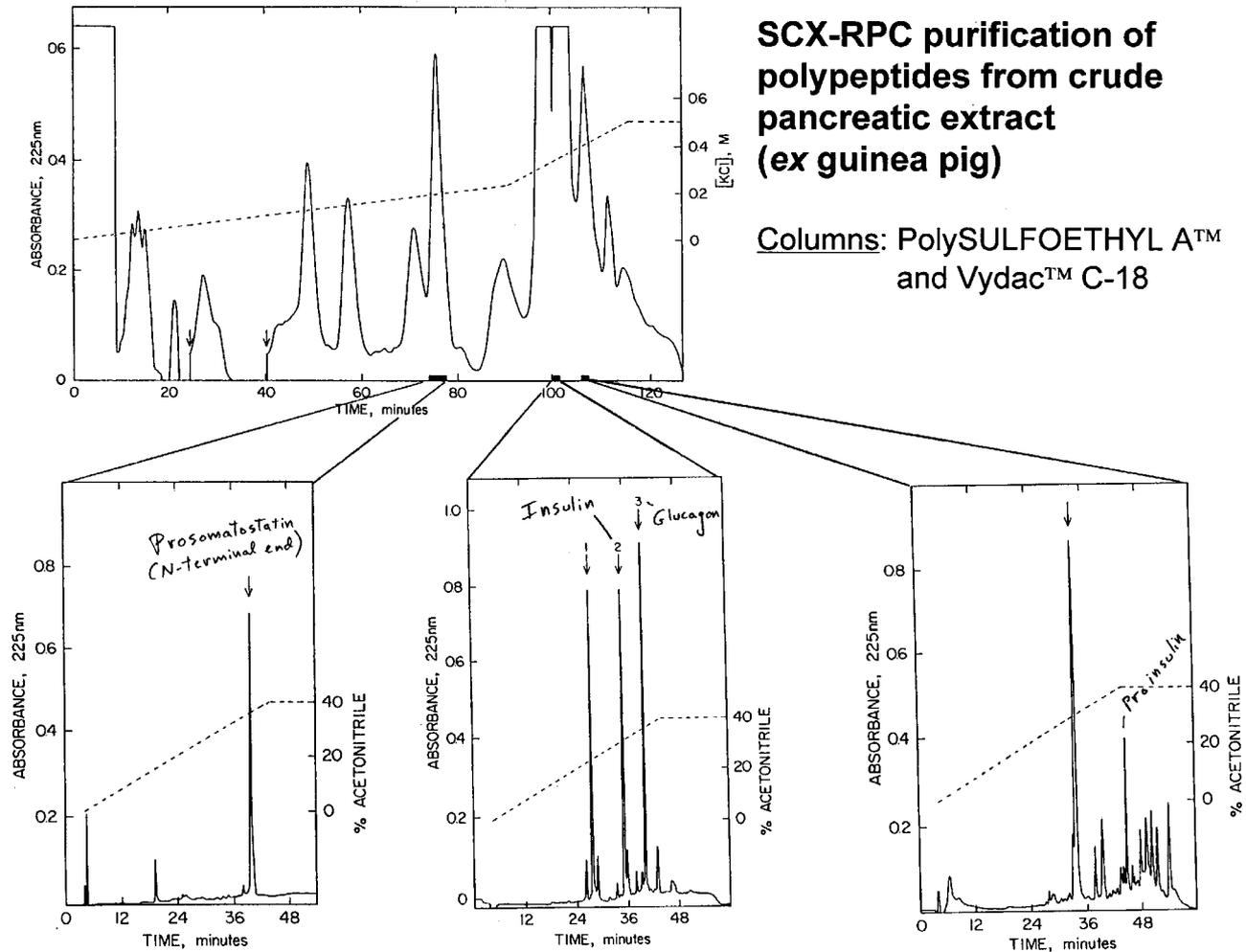
PROTEOMICS ANALYSES

“A butterfly and a caterpillar have the same genome but different proteomes.” - Ron Orlando

Techniques for resolving complex protein mixtures:

- 1) (TRADITIONAL) 2-Dimensional gel electrophoresis.
- 2) (NEW) Digest the proteins with trypsin, identify the peptides via tandem mass spectroscopy (MS/MS), and search a genome database to identify the original proteins.
 - a) Need >15 fmol of each peptide, and < 15 peptides per scan window (~ 10”) or they’ll suppress each other’s ionization.
 - b) **16-300 Peptides**: Use an RPC capillary to break up the peptides into sets of < 15 peptides per scan window.
 - c) **300-12,000 Peptides**: **2-D LC**: Use SCX to break up the peptides into sets [same charge-different polarity] small enough for RPC to handle.
 - d) **>12,000 Peptides** : **3-D LC** needed.
- 3) OBJECTIVE: Elution of components uniformly throughout the run, not to get individual sharp peaks. Ugly chromatograms may actually contain thousands of sharp peaks.

PEPTIDE PURIFICATION BY A CHARGE-POLARITY SEQUENCE



ADVANTAGES OF A SCX-RPC SEQUENCE:

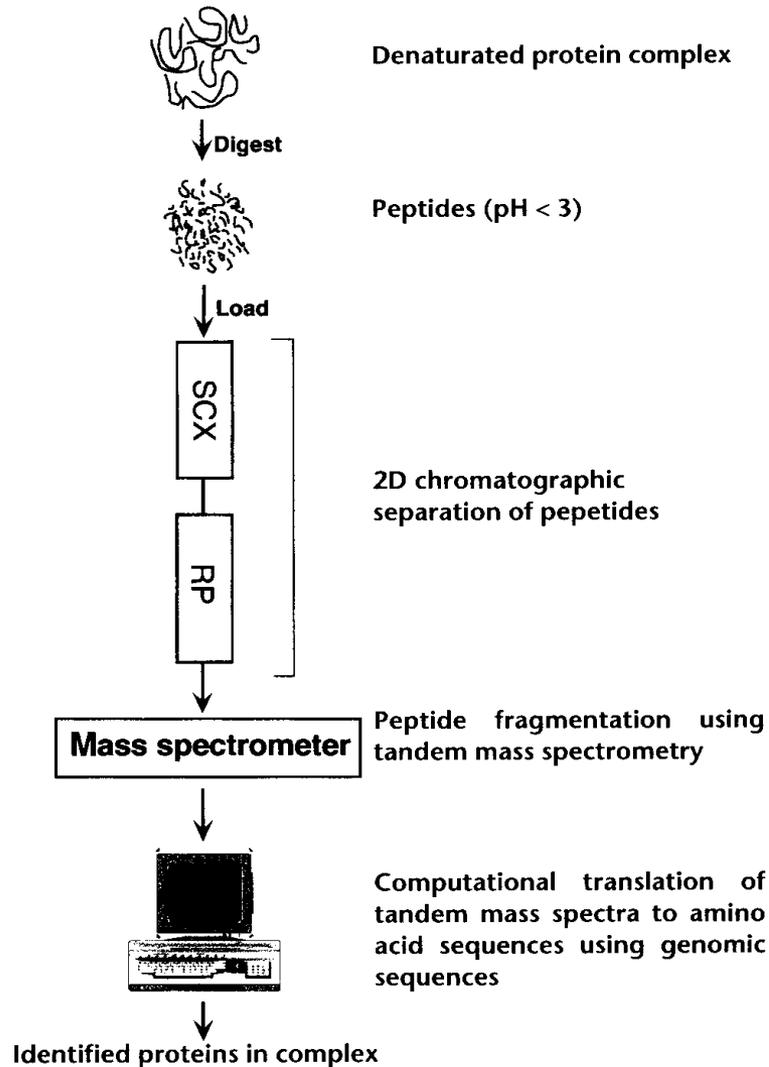
- 1) Capacity of SCX is 4x higher than RPC; better for first step
- 2) RPC step desalts the SCX fractions

**Always be alert
and then wait.
Perhaps what
you're looking
for, will find
you...**



PROTEOMICS BY 2-DIMENSIONAL LC/ MS/MS

MudPIT: Multidimensional Protein Identification Technology



Typically identifies 2.5-3 peptides per protein; rugged method.
Good where sample size is limited

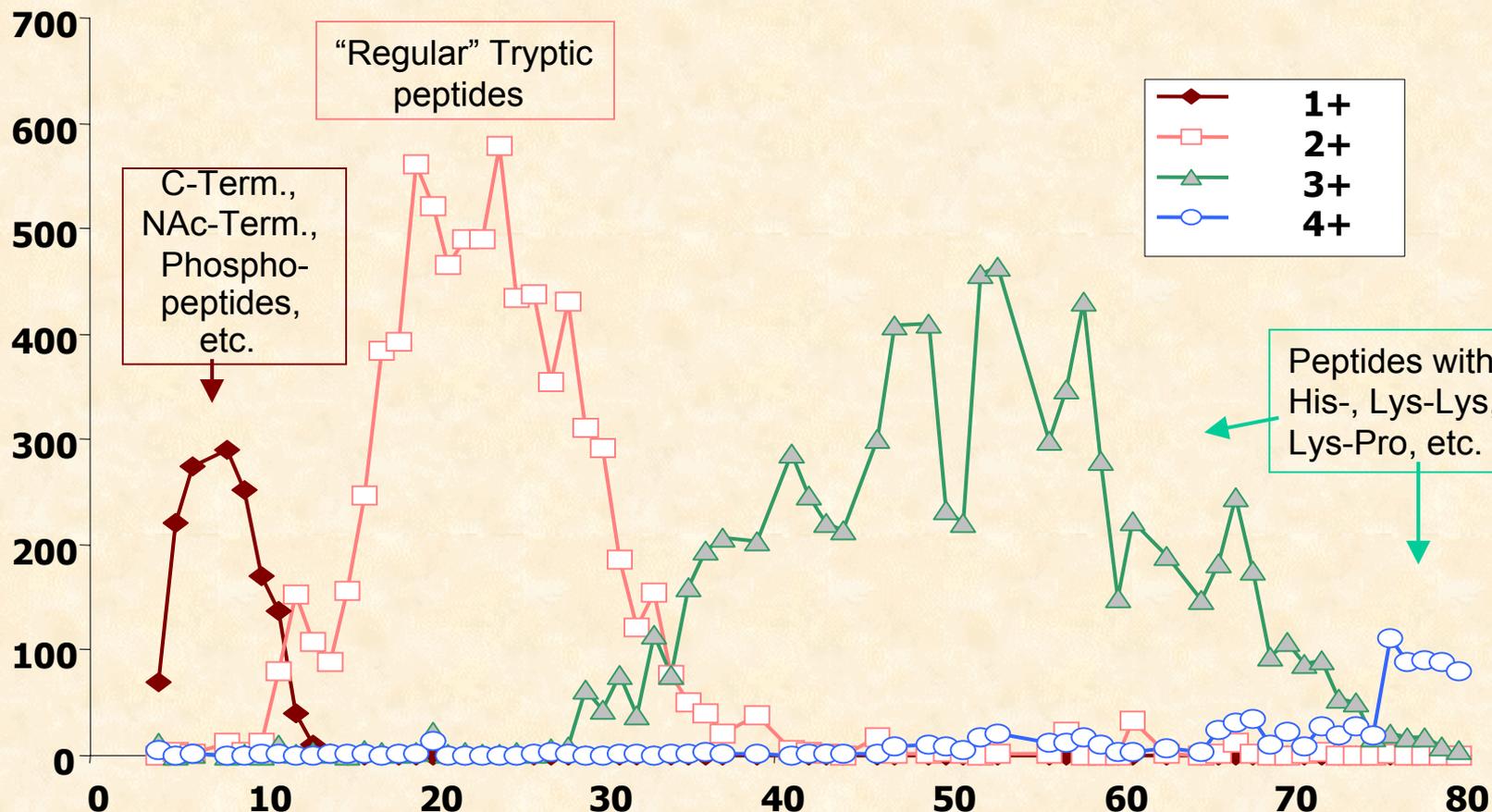
ADVANTAGES OVER 2-D ELECTROPHORESIS

- 1) Greater dynamic range:
Most abundant protein detected
Least abundant protein detected
- 2) Works better for hydrophobic and very basic proteins
- 3) Handling and automation easier

- from Link *et al.*, *Nat. Biotechnol.*
17 (1999) 676-682 -

SCX of Tryptic Digest of HeLa Cells: Retention Time vs. Charge

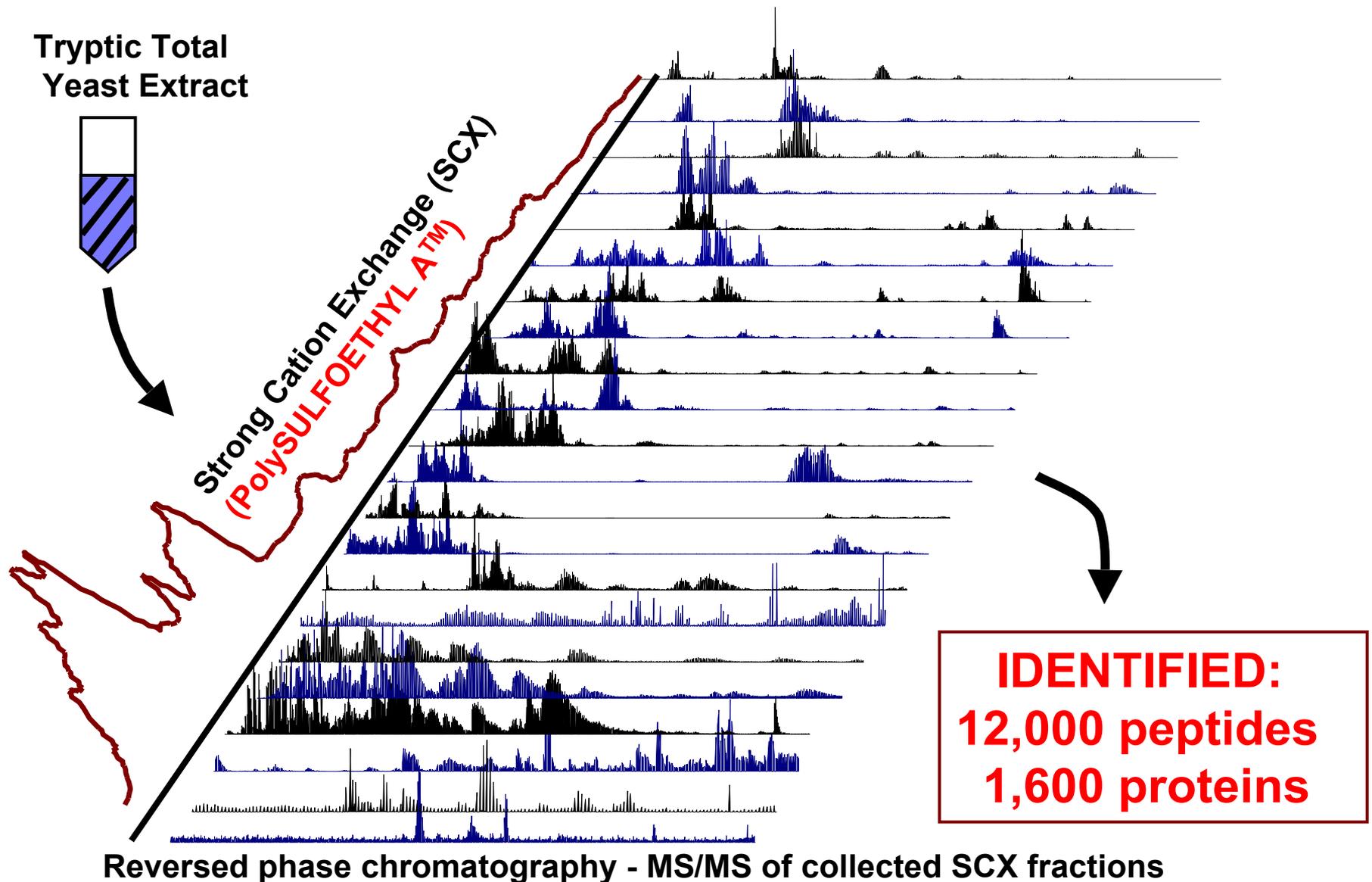
COLUMN: PolySULFOETHYL A (item# 202SE0502)



NOTE: Y-Axis = number of peptides **identified** in each fraction, not the total number in the fraction. There are many more peptides in the +2 group than in the others.

- Data courtesy of Steven Gygi (Harvard Medical School) -

Analysis of Yeast Proteome by SCX-RPC-MS/MS



(COURTESY OF STEVEN GYGI & JUNMIN PENG, HARVARD MEDICAL SCHOOL)

STRATEGY FOR PROTEOMICS ANALYSES

1) KEEP FRACTIONS SMALL

a) Peptides suppress each others' ionization \therefore Better MS spectra obtained with less peptides.

b) Decreases chances of a high-abundance peptide sharing a fraction - and MS scan window - with a low-abundance peptide.

2) OFFLINE FRACTION COLLECTION SUPERIOR TO ONLINE

a) Permits larger SCX columns (\uparrow loading) and faster flow rates; facilitates collection of smaller, more numerous fractions.

b) Permits inclusion of 25% ACN in SCX buffers \rightarrow sharper peptide peaks \rightarrow \uparrow % peptides eluting in a single collected fraction \rightarrow \uparrow sensitivity to low-abundance peptides (NOTE: Not compatible with C-18 trap cartridges)

Comparison of On-Line vs. Off-Line SCX of Peptides in 2-D LC/ MS/MS

Sample: LysC/Trypsin digest of rat liver microsomes
250 μ g digested protein loaded

\therefore 2x more peptides identified with off-line SCX!

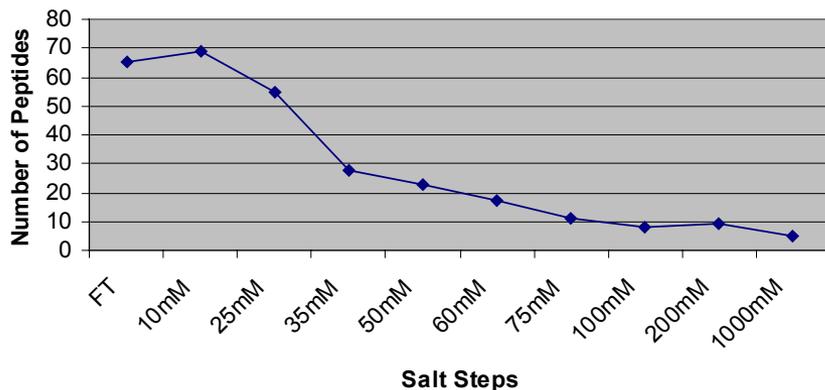
SCX Buffer: 5mM KH_2PO_4 , pH 3, 5% MeCN, X mM KCl

SCX Buffers:

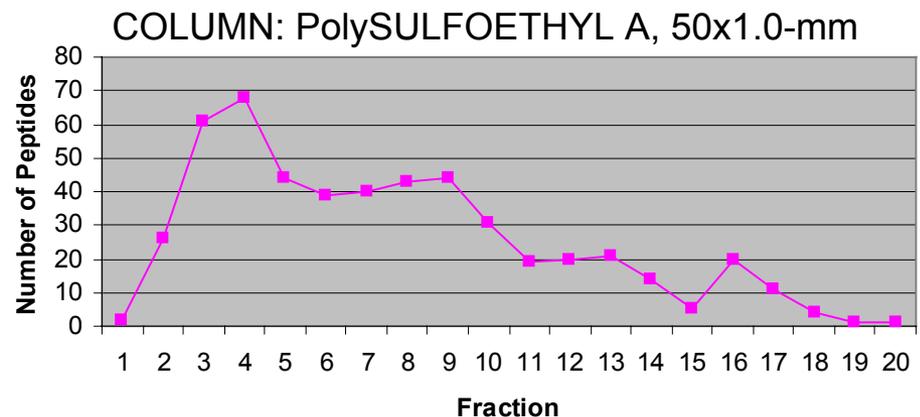
Buffer A - 5mM KH_2PO_4 , 5% MeCN, pH 3

Buffer B - 5mM KH_2PO_4 , 5% MeCN, pH 3, 500mM KCl

On-Line SCX 298 Peptides



Off-line SCX 513 Peptides



Gradient : 1-25% B in 25 minutes, 25-50% B in 10 minutes

- data courtesy Kevin Blackburn, Glaxo SmithKline -

PROTEOMICS OF *REALLY* COMPLEX MIXTURES

ASSUMPTIONS:

- 1) 5000 PROTEINS IN THE MIXTURE (YIELDING 40,000 TRYPTIC FRAGMENTS)
- 2) AVERAGE MOL. WT. OF PROTEINS = 30 KDa
- 3) YOU NEED AT LEAST 15 FMOL TO GET A PEPTIDE SEQUENCE VIA MS/MS

∴ YOU NEED (450 pg OF EACH PROTEIN) x (5000 PROTEINS) = 2.25 μg TOTAL PROTEIN

HOWEVER, PROTEINS DIFFER BY 10^6 IN REL. ABUNDANCE. NOW ASSUME RELATIVELY FEW PROTEINS MAKE UP MOST OF THE SAMPLE. TO GET 15 FMOL OF THE LEAST ABUNDANT PROTEIN, YOU WILL NEED AT LEAST 10^5 MORE SAMPLE:

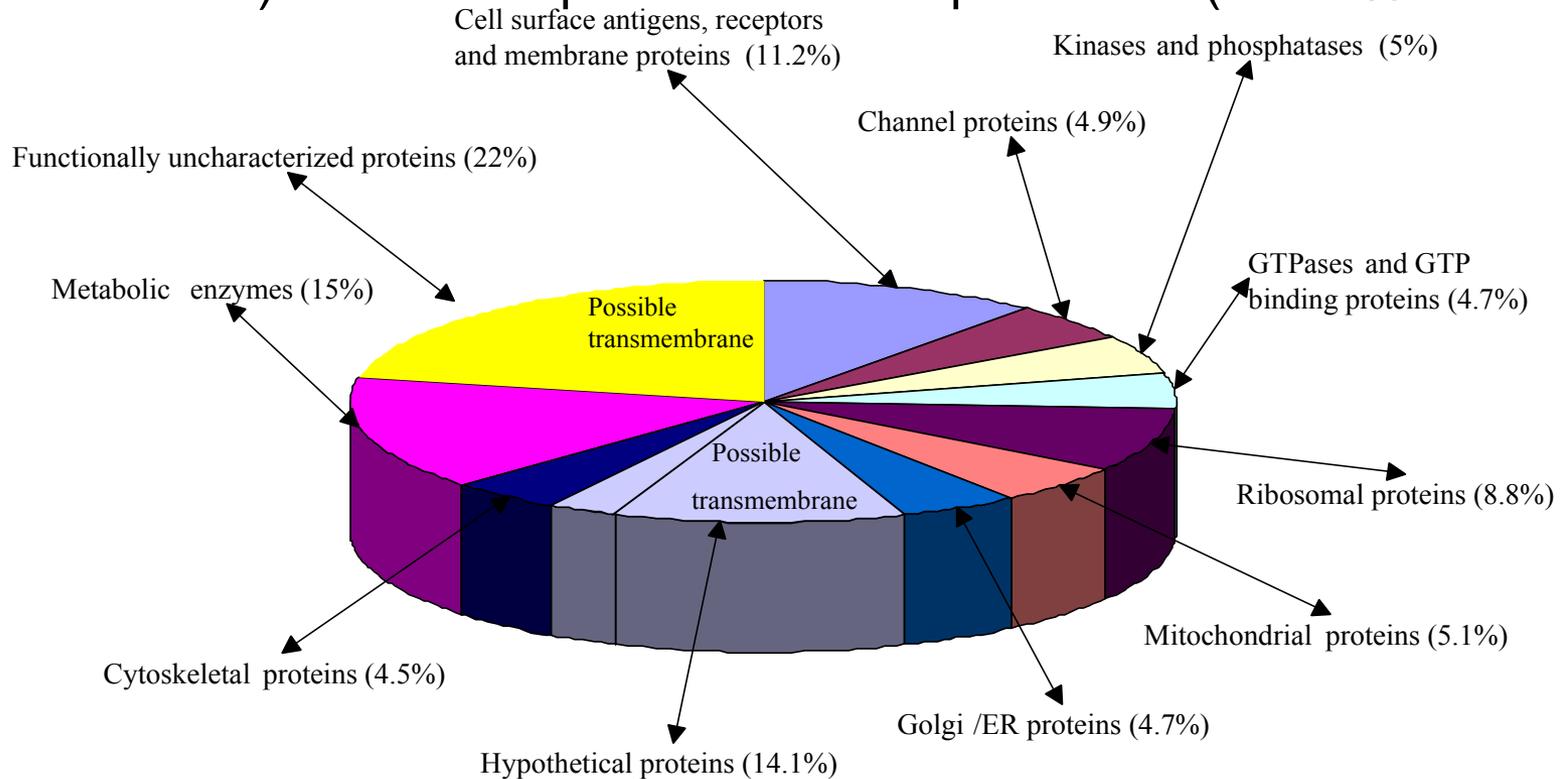
$$2.25 \mu\text{g} \times 10^5 = \mathbf{225 \text{ mg TOTAL PROTEIN NEEDED}}$$

THIS REQUIRES A **LARGE** SCX COLUMN TO PROCESS. EVEN IF YOU DECREASE #PEPTIDES 90% VIA ICAT® [usually done *after* SCX], THAT STILL LEAVES 22.5 mg TO PROCESS

- ∴ 1) **BIG MISMATCH BETWEEN SIZE OF SCX AND RPC COLUMNS**
- 2) **NEED TO COLLECT AND PROCESS 500+ FRACTIONS AT SCX STEP?**

Proteomics of *Really* Complex Mixtures: Simplification Through Organelle Isolation

- PROBLEMS: 1) Contamination by other organelles
2) Membrane proteins underrepresented (15-30% of total)



Distribution of Proteins in Microsomal Extract, Identified via SCX-RPC-MS/MS

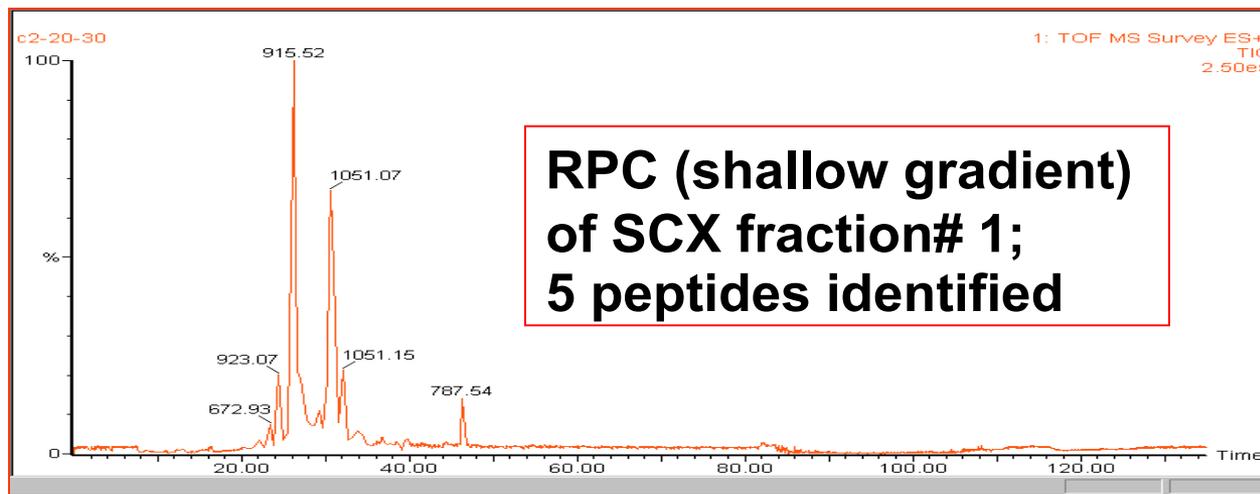
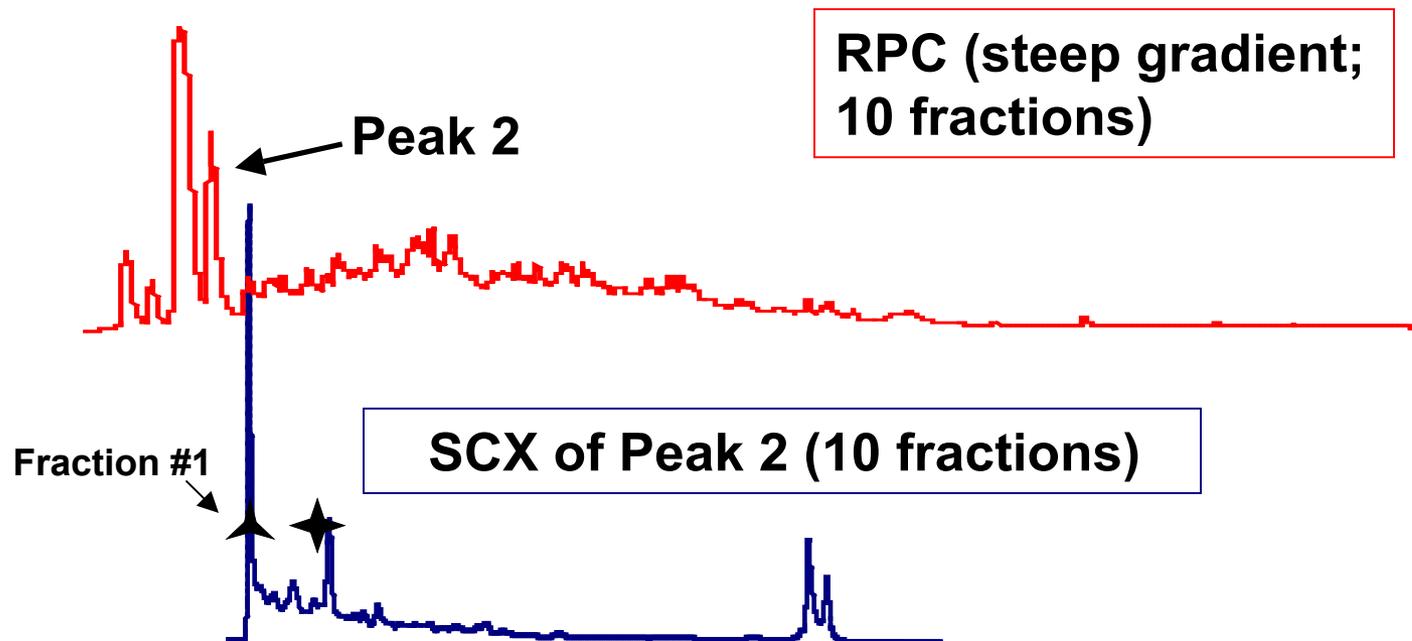
- from Han *et al.*, *Nat. Biotechnol.* 19 (2001) 946-951

Alternative strategies to 2DE are MS-based and involve multiple separation steps

	1 st dimension	2 nd dimension	3 rd dimension	Acronym
	IEF	SDS-PAGE →		2DGE
Chait and colleagues	RP-LC	SDS-PAGE →	RP- μLC	
Gygi, Lee, Mann, etc.	SDS-PAGE →	RP-μLC		geLC-MS
Yates and colleagues →	SCX-LC	RP- μLC		MuDPIT
Isobe and colleagues →	SAX-LC	RP- μLC		

→ Proteins trypsinized to peptides

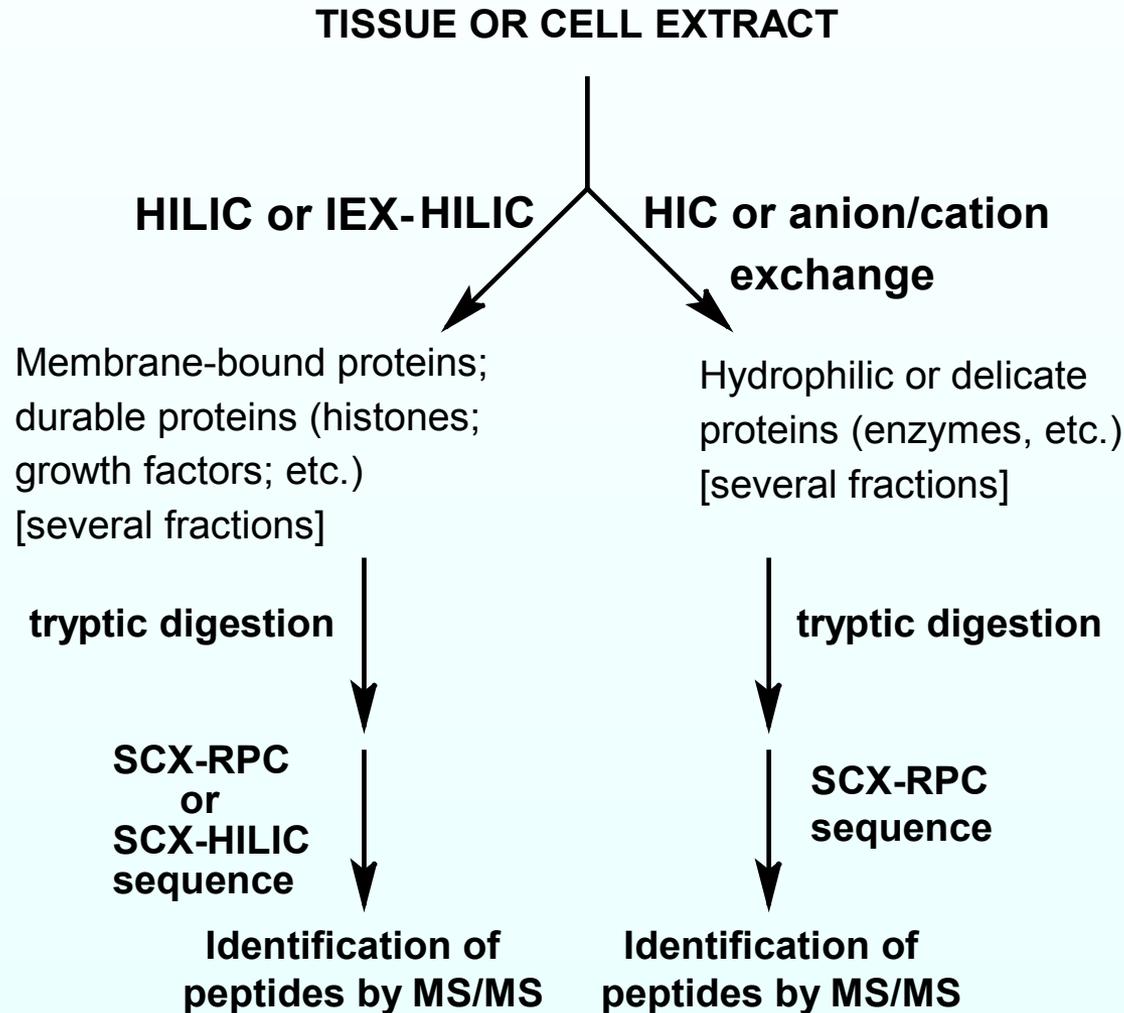
Orlando Model: 3-D LC-MS/MS



PROTEOMICS OF *REALLY* COMPLEX MIXTURES

Alternative to organelle fractionation or *n*-D LC of peptides:

Predigest fractionation of proteins by HPLC

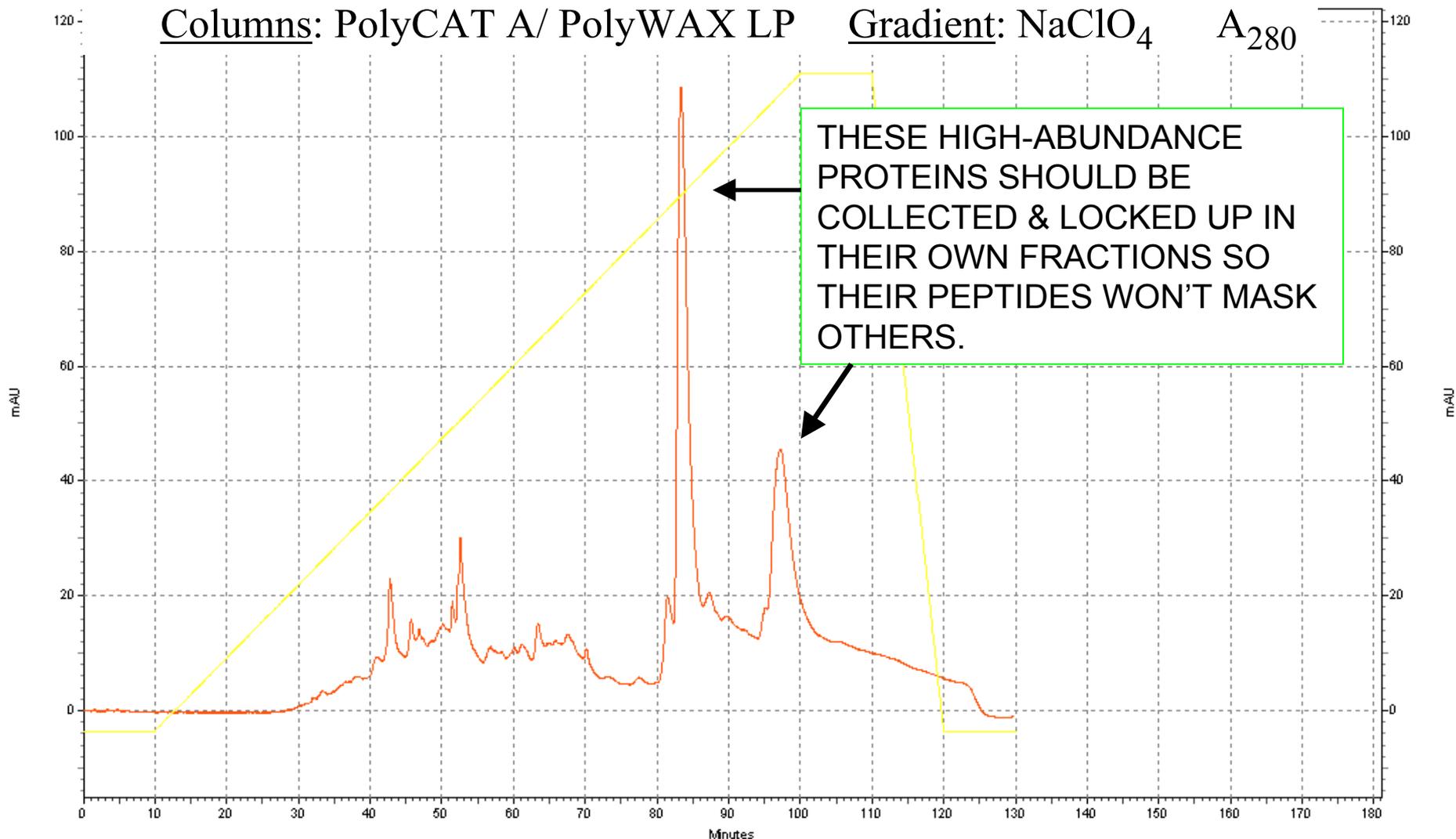


Suggestions for Analysis of Whole Cells and Membrane Proteins

- 1) DISSOLVE PELLETS IN HFIP:FORMIC ACID = 50:50.
- 2) FRACTIONATE/DELIPIDATE VIA HILIC OR MIXED-BED IEX-HILIC.
- 3) LYOPHILIZE FRACTIONS; REDISSOLVE PROTEINS IN (MINIMAL) HFIP AND SLOWLY ADD WATER/PrOH.
- 4) DIGEST FRACTIONS WITH TRYPSIN USING 30% PrOH/NH₄HCO₃
 - a) Digestion more thorough than with urea, esp. for membrane proteins. Ref.: Russell *et al.*, *Anal. Chem.* 73 (2001) 2682-2685, and ASMS 2002 poster# T005 [limit digests obtained in 6'].
 - b) Effect of ~ 5% HFIP: Slows trypsin's action; ~ 16-20 hr required to get a limit digest, not 6-60'.
- 4) *REALLY* HYDROPHOBIC TRANSMEMBRANE PEPTIDES:
Try SCX-HILIC instead of SCX-RPC.

Mixed-Bed IEC of Intact Proteins from THP-1 Monocyte Cell Lysate ($\sim 6 \times 10^6$ cells; water-soluble fraction)

Columns: PolyCAT A/ PolyWAX LP Gradient: NaClO₄ A₂₈₀



- Data courtesy of Leticia Cano (City of Hope) -

Delipidation of Whole Cell Lysates via HILIC¹

NEEDED: PolyHYDROXYETHYL A™ SPE cartridge [PolyLC item# SPEHY1203]. Lysates (approx. 2×10^7 cells) or CNBr-digested lysates (approx. 4×10^7 cells) were loaded in 90% organic and eluted in steps of 70%, 40%, 20%, 0% and again 0% organic solvent.

Loading/flushing solution: 20mM ammonium formate, 90% ACN, 0.1% HFIP

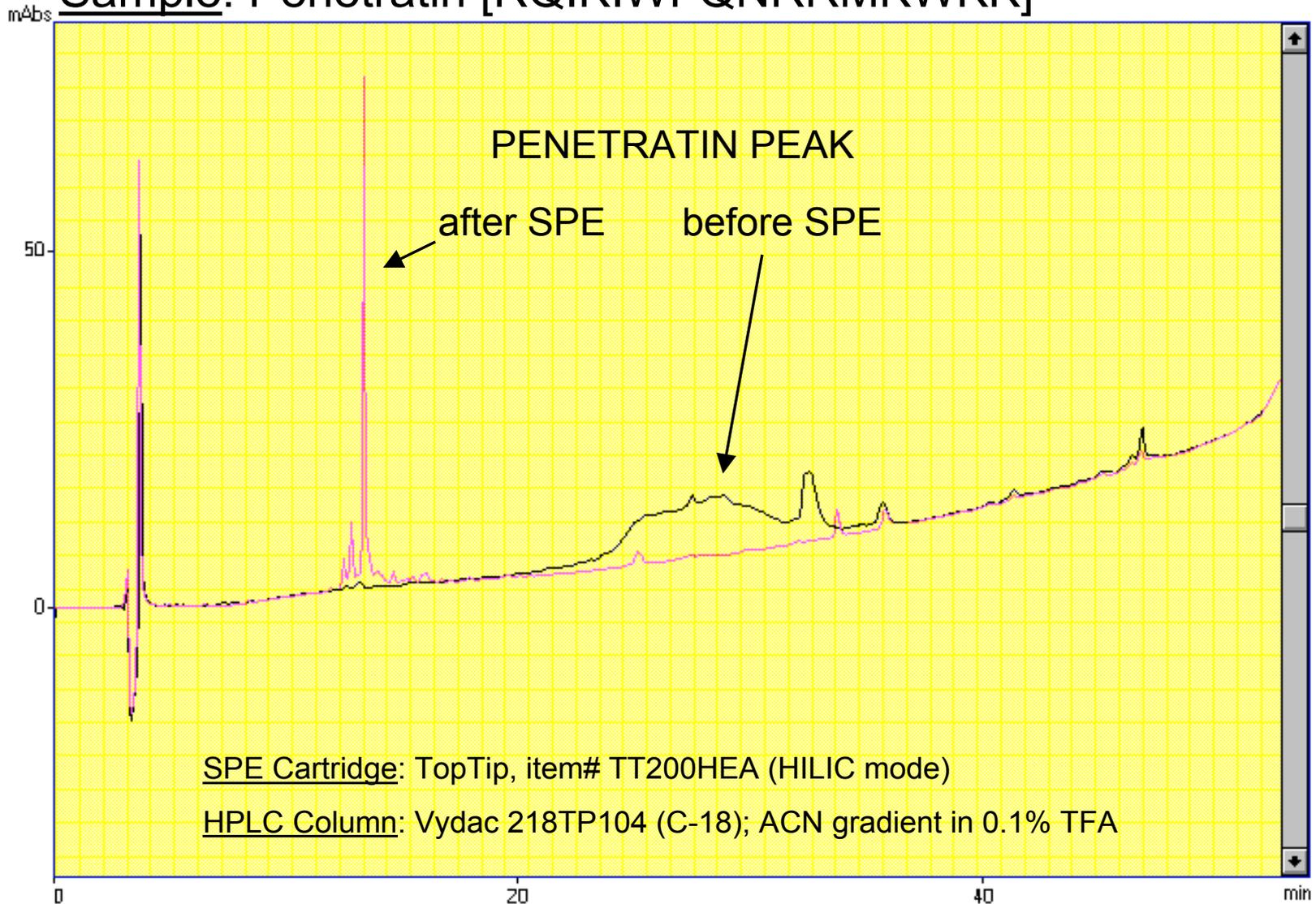
Eluting solution: 0.2% formic acid, 0.1% HFIP.

¹ A.J. Alpert, *J. Chromatogr.* 499 (1990) 177-196

**THIS METHOD ALSO ELIMINATES DETERGENTS, SALTS,
AND VARIOUS REAGENTS. GOOD CLEANUP STEP.**

Removal of 8.6% SDS from Peptide by SPE-HILIC

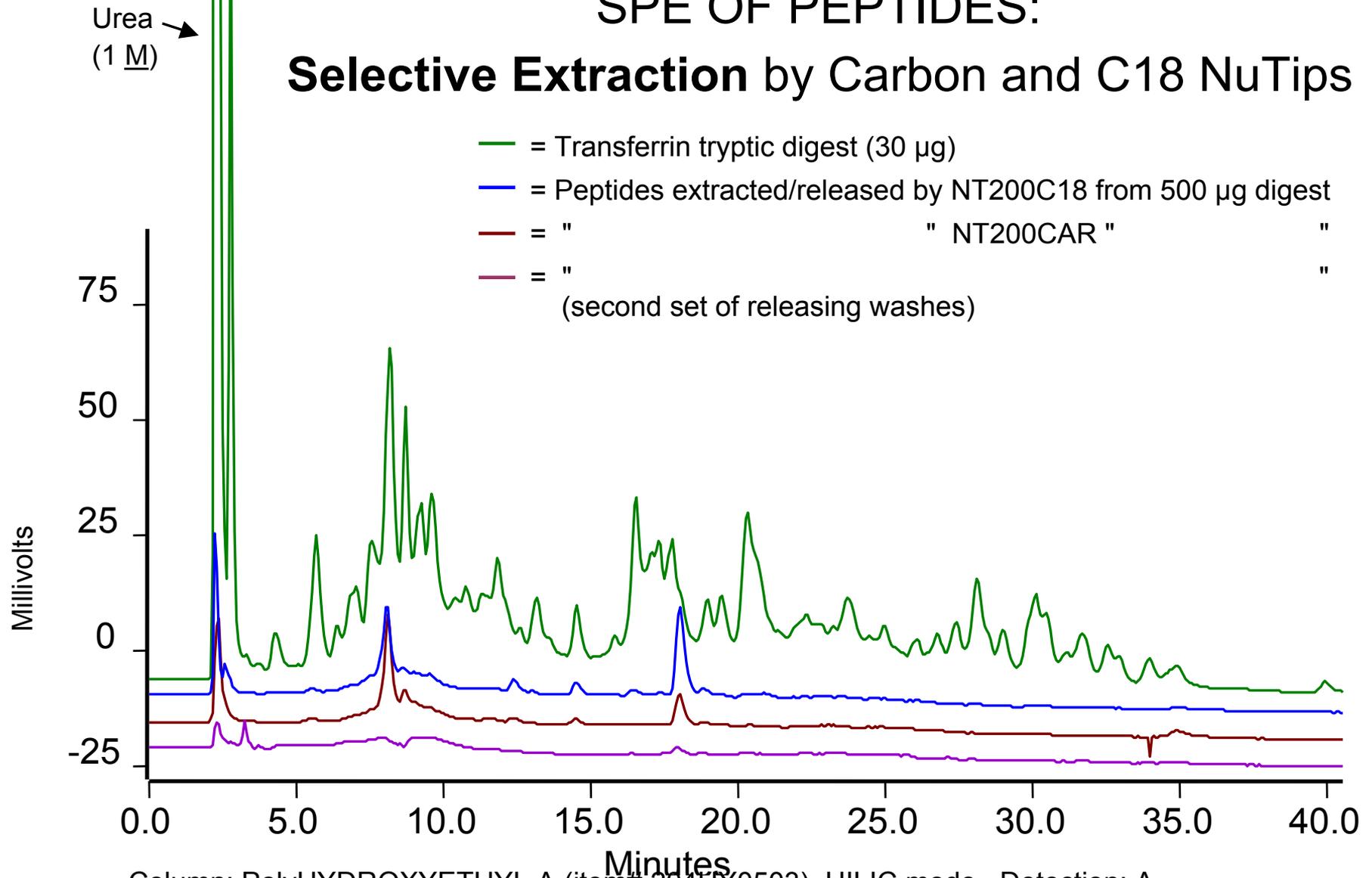
Sample: Penetratin [RQIKIWFQNRRMKWKK]



- Data courtesy of Britt-Marie Olsson (Stockholm Univ.) -

SPE OF PEPTIDES:

Selective Extraction by Carbon and C18 NuTips



Column: PolyHYDROXYETHYL A (item# 204HY0503), HILIC mode. Detection: A₂₂₀

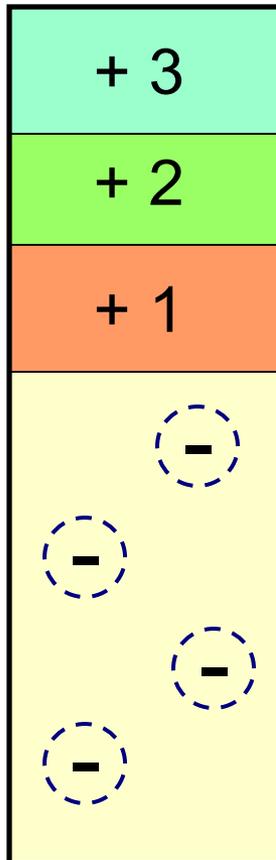
Gradient: 40' linear, 0-70% B. A) 15 mM TEAP, pH 2.7, with 85% ACN

B) " " " 10 "

Selectivity in Elution vs. Displacement Chromatography

COLUMN

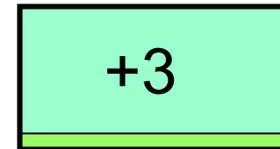
Excess capacity; everything retained
Separation through **selective elution**



OVERLOADED SPE CARTRIDGE

Capacity limited; the most strongly-retained solutes displace those less strongly-retained

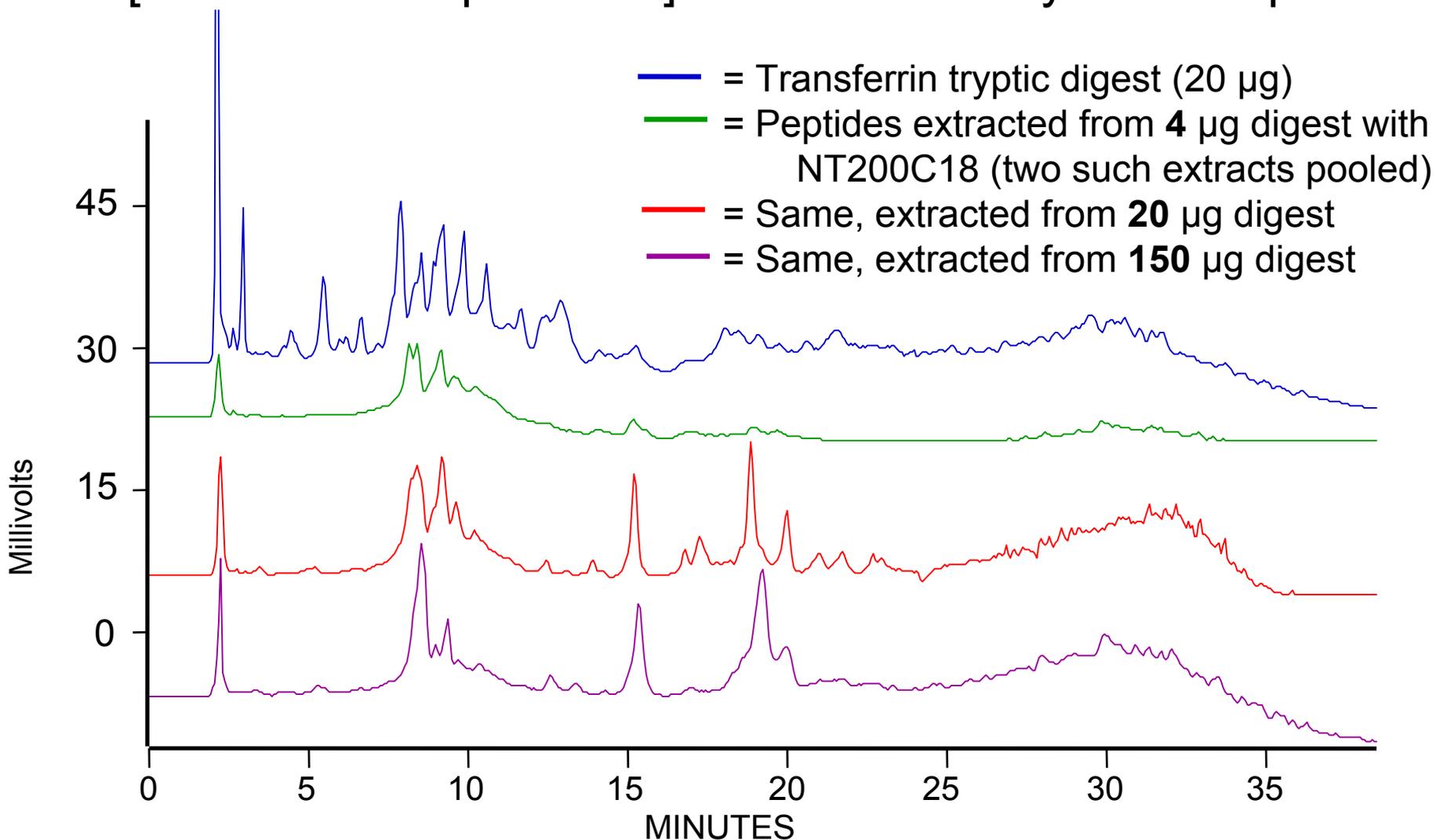
Result: Separation through **selective retention**



EXAMPLE: Cation-exchange chromatography

DISPLACEMENT EFFECTS IN SPE OF PEPTIDES:

↑ [Transferrin Peptide Mix] → ↑ Selectivity of Adsorption

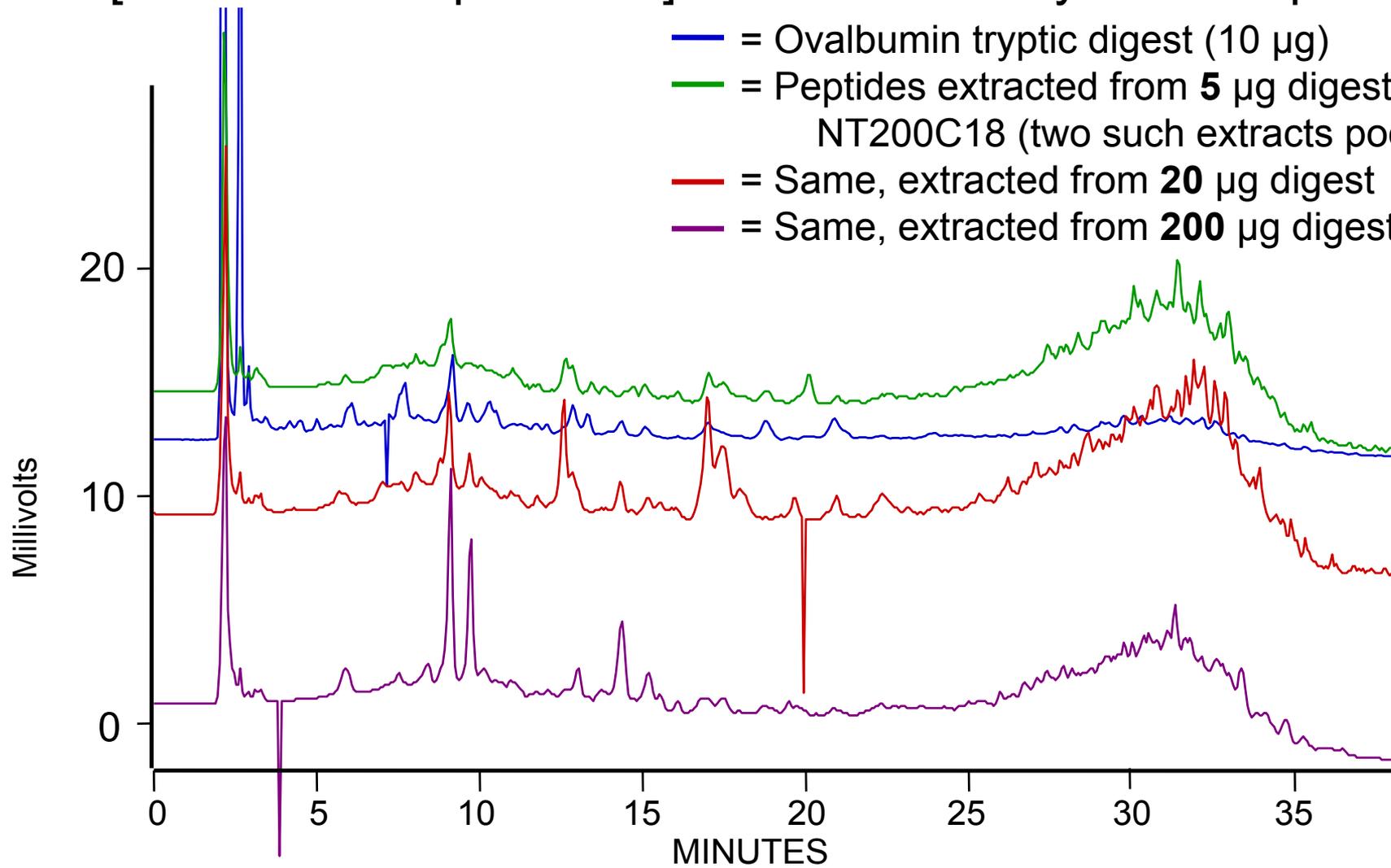


Column: PolyHYDROXYETHYL A (item# 204HY0503), HILIC mode. Detection: A₂₂₀
Gradient: 40' linear, 85-44% ACN in 15 mM TEAP, pH 2.7

DISPLACEMENT EFFECTS IN SPE OF PEPTIDES:

↑ [Ovalbumin Peptide Mix] → ↑ Selectivity of Adsorption

- = Ovalbumin tryptic digest (10 μg)
- = Peptides extracted from **5** μg digest with NT200C18 (two such extracts pooled)
- = Same, extracted from **20** μg digest
- = Same, extracted from **200** μg digest



Column: PolyHYDROXYETHYL A (item# 204HY0503), HILIC mode. Detection: A_{220}
Gradient: 40' linear, 85-44% ACN in 15 mM TEAP, pH 2.7

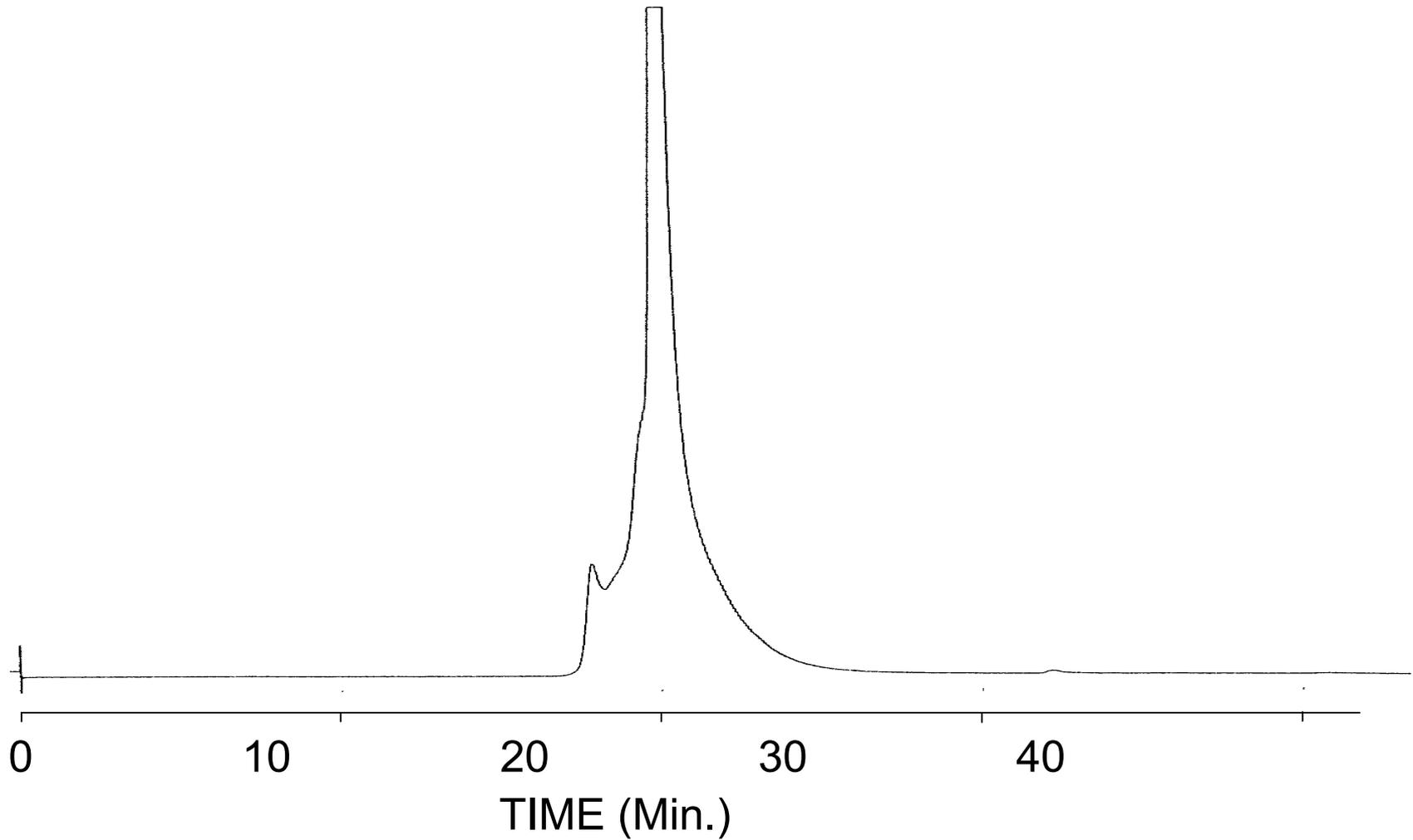
SELECTIVE PRECIPITATION TO REMOVE PROTEINS OF HIGH ABUNDANCE

PROTEIN CONTENT OF SERUM

Protein	Mol. Wt. (Da)	G. per 100 g. serum proteins
Albumin	66,000	52 - 68
IgG	150,000	13.5 - 23
Transferrin	80,000	3 - 7
α 1-Antitrypsin	52,000	2 - 4
IgA	180,000 - 500,000	0.8 - 2.9
IgM	950,000	0.7 - 1.8

These six proteins represent ~ 90% of the total protein in serum (disregarding lipoproteins). All have mol. wt. > 50,000 Da.

SEC of Crude Serum



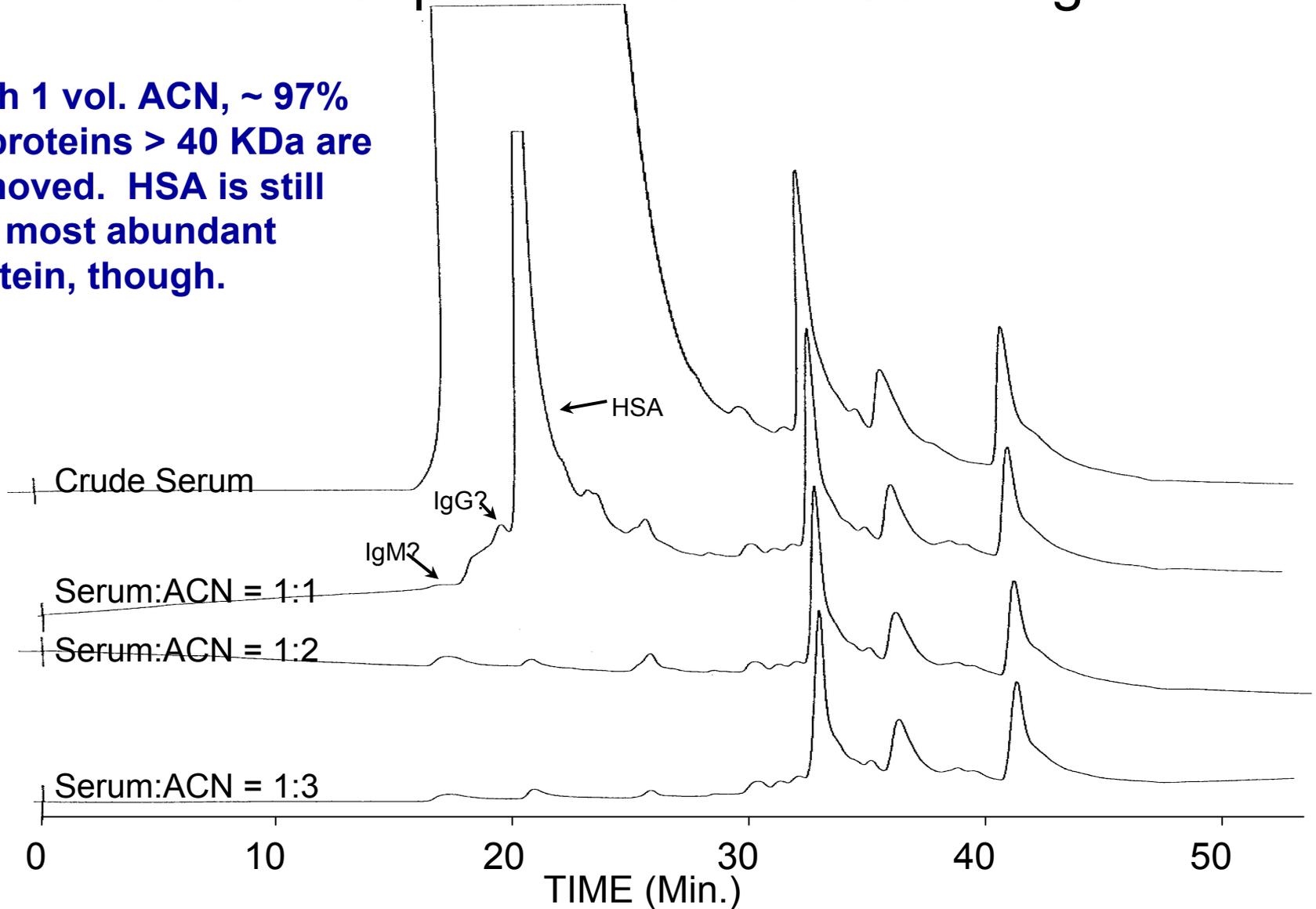
COLUMN: PolyHYDROXYETHYL A (item# 209HY0503, two in series)

MOBILE PHASE: 100 mM KH_2PO_4 , pH 6.6

Serum + Acetonitrile

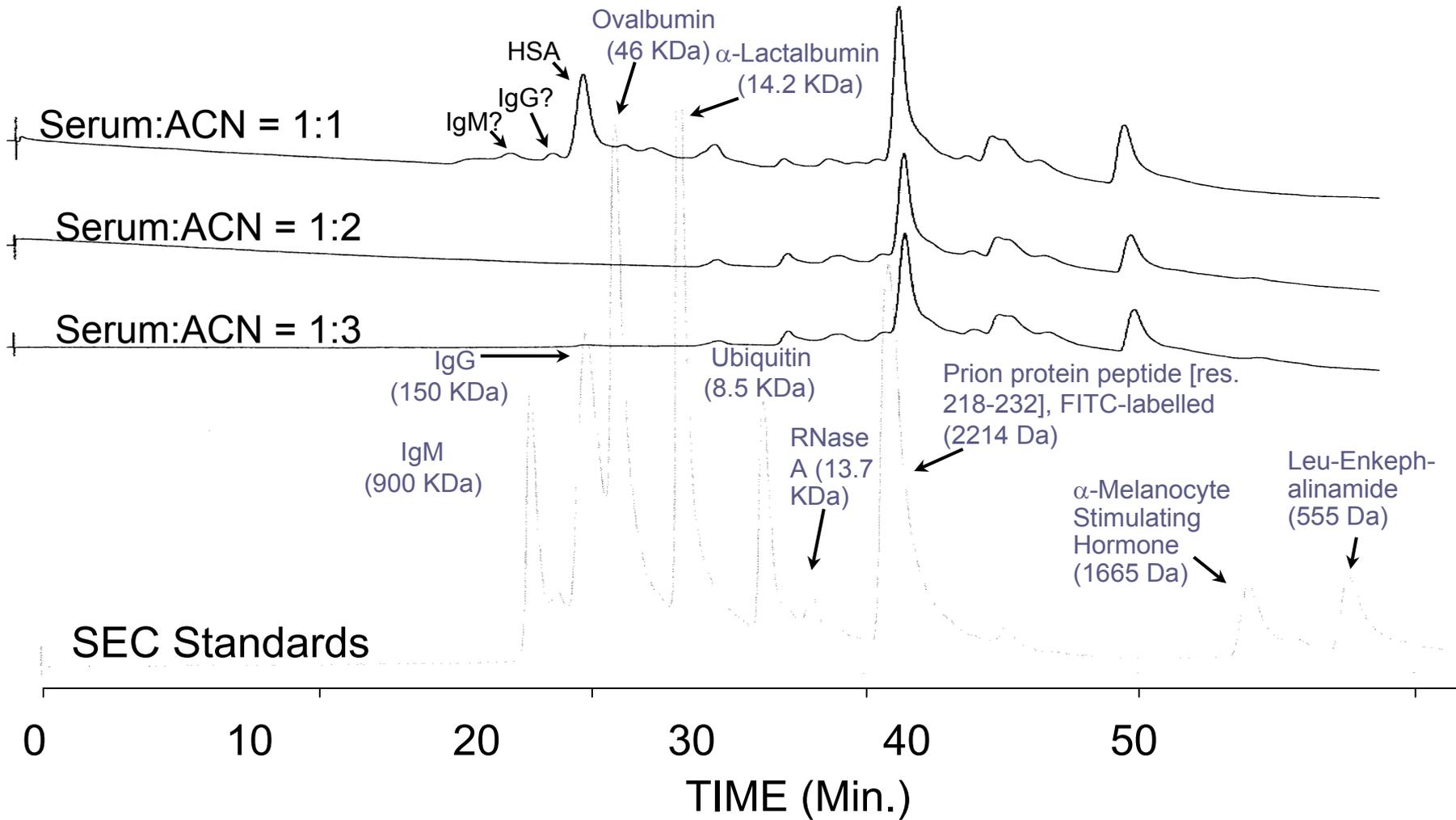
- SEC of Supernatants Post-Centrifugation -

With 1 vol. ACN, ~ 97% of proteins > 40 KDa are removed. HSA is still the most abundant protein, though.



Serum + Acetonitrile

- SEC of TopTip™ Filtrates -



Filtration is 10x more effective than centrifugation. The 1:1 supernatant is rich in proteins < 30 KDa.

MEANWHILE, JUST A FEW DOORS AWAY...

I WASN'T ALWAYS A HOMELESS VAGRANT. I'M A DOCTOR, A RESEARCH SCIENTIST.



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MY SPECIALTY, MAY HEAVEN HELP ME-- WAS chromatography!

