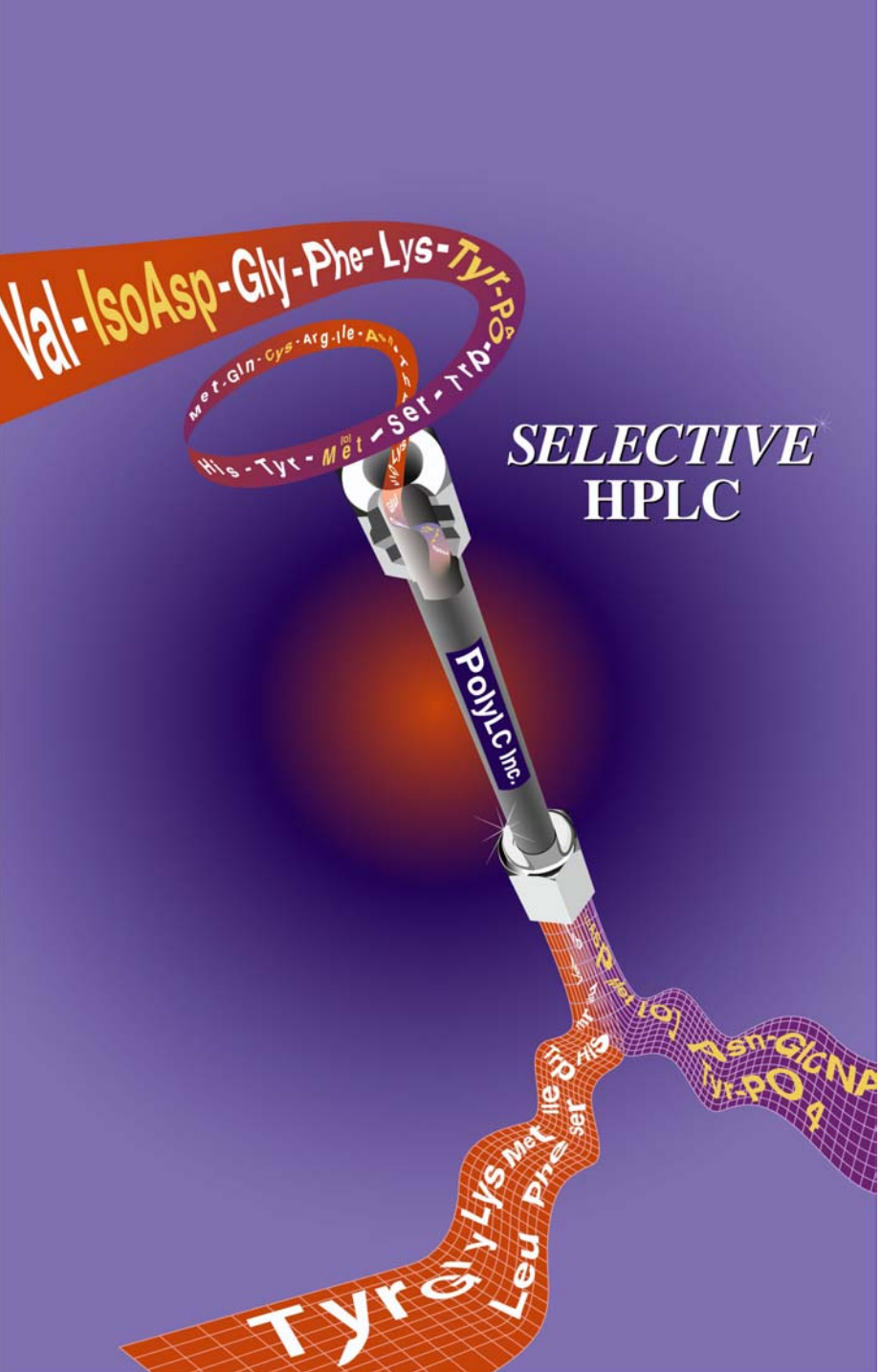



# HPLC OF *REALLY* DIFFICULT PROTEIN MIXTURES:

## 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:

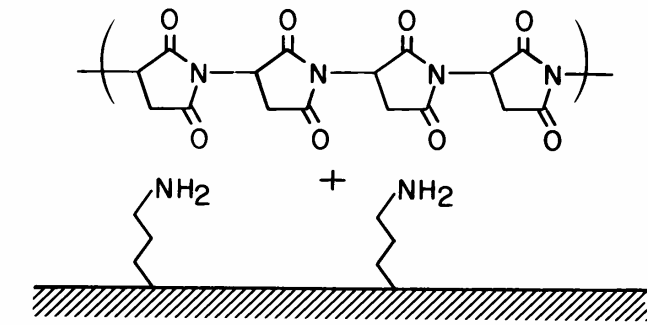
Isolation: Not necessary in many cases; MS can handle mixtures. Objective: Reduce large mixture to small mixtures. Identification not dependent on enzymes being active.

Analysis: MS: Volatile solvents required.
Chromatography: Organic solvents, yes; detergents, no.

# ALTERNATIVES TO REVERSED-PHASE

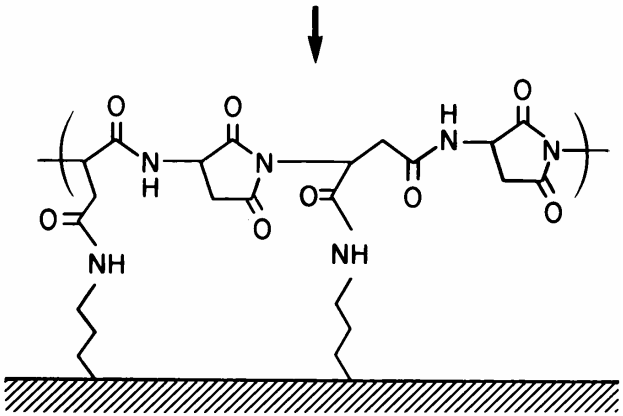
1) Separation by charge
   a. Anion-exchange (WAX; SAX)
   b. Cation-exchange (WCX; SCX)

2) Separation by polarity
   a. Hydrophobic Interaction (HIC)
   b. Hydrophilic Interaction (HILIC)

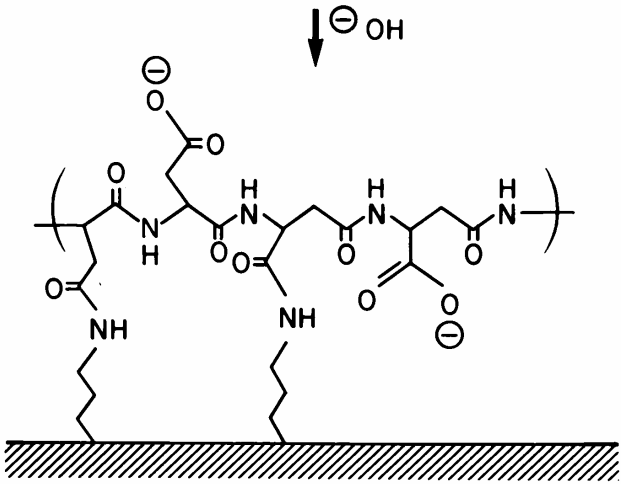



Polysuccinimide

Aminopropyl-silica




Poly(succinimide)-silica

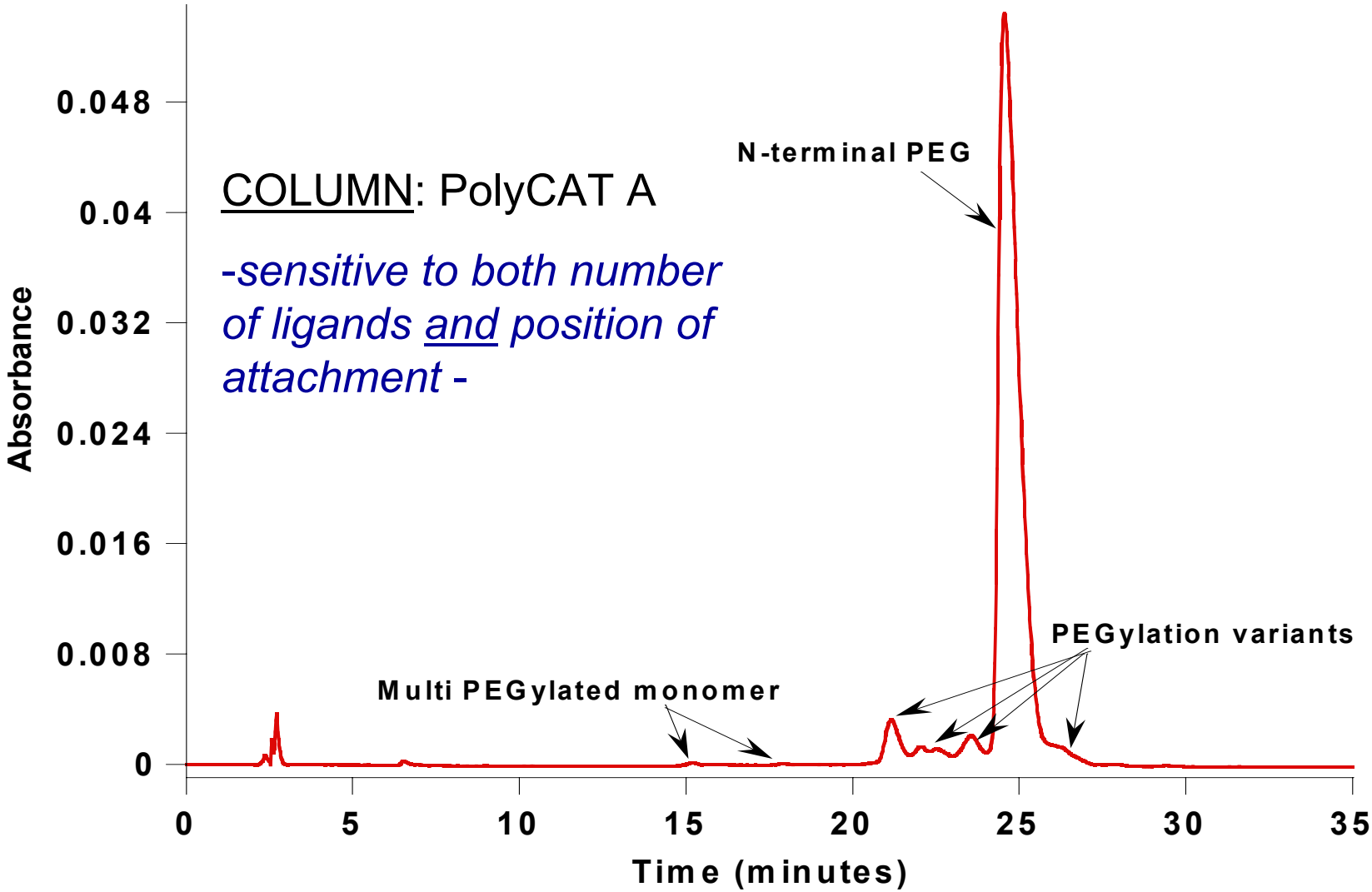



Poly(aspartic acid)- silica

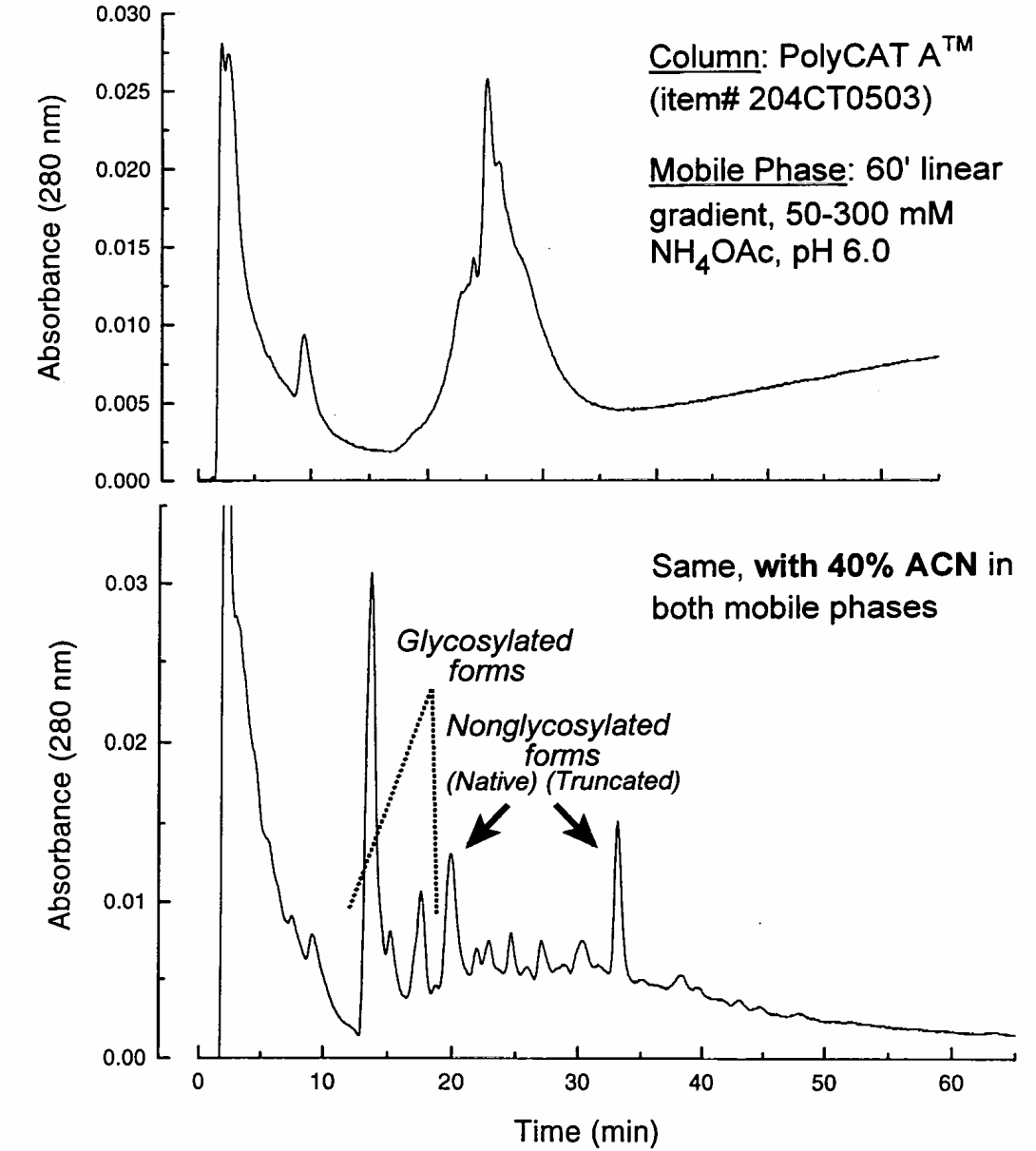
**(PolyCAT A)**

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

# NEUROTROPHIC FACTOR: Polyethylene Glycol (PEG) Attached



# ION-EXCHANGE OF PROTEINS WITH ORGANIC SOLVENTS



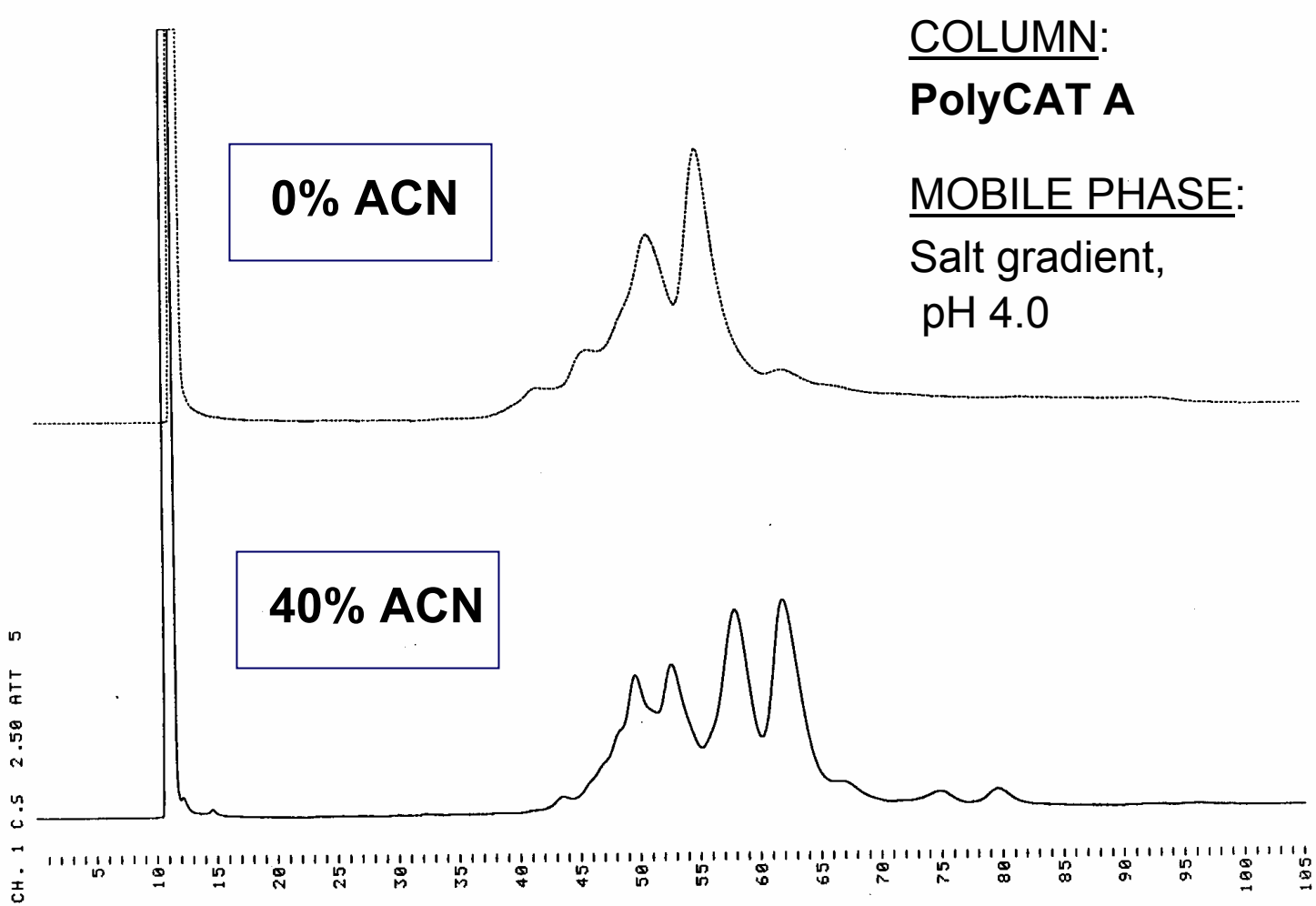

Sample: *rec* α-Bungarotoxin from *P. Pastoris*

**- Selectivity Increases dramatically –**

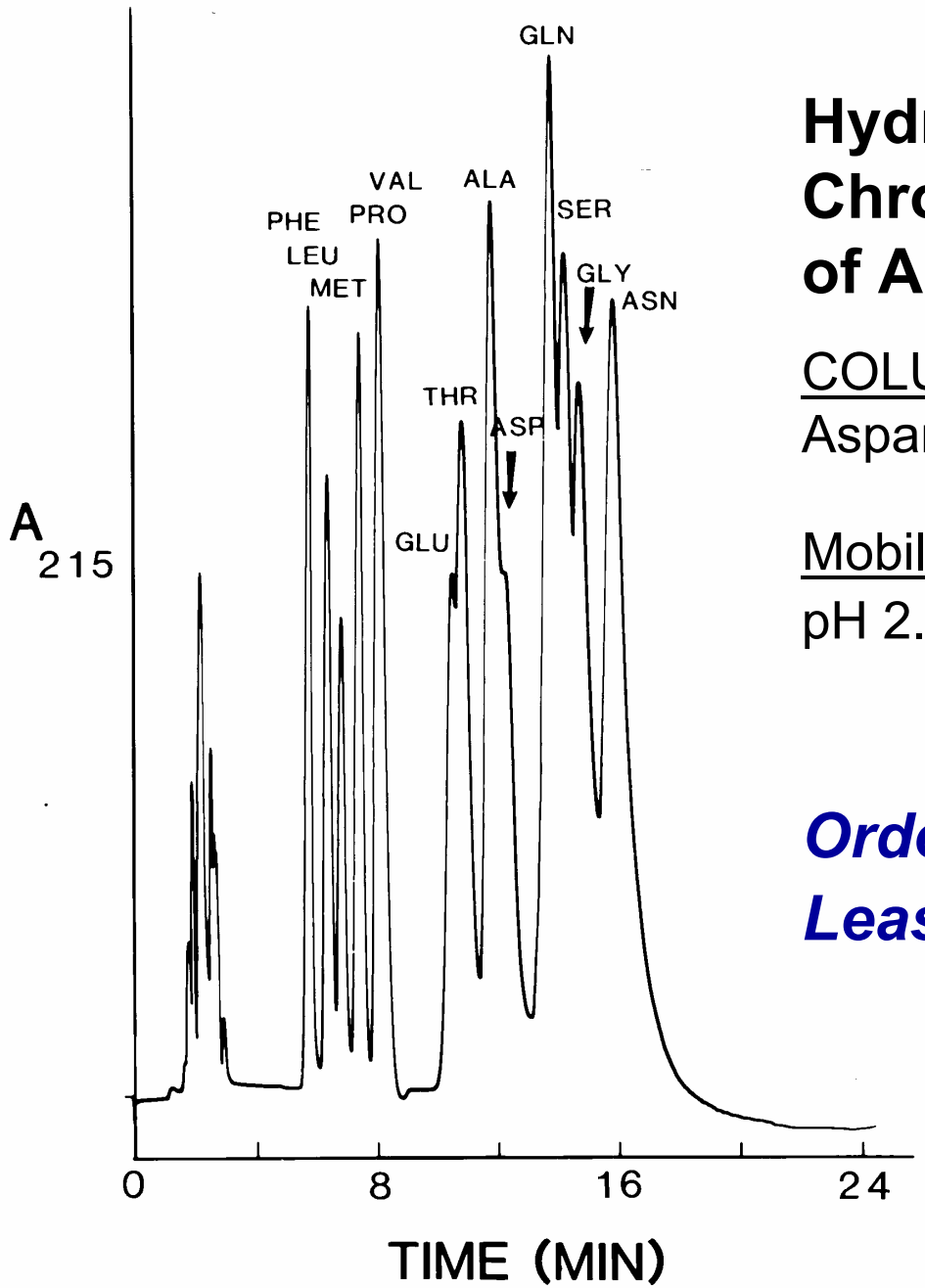
*- data courtesy of Robert Rogowski and Edward Hawrot (Brown Univ.) -*

# ION-EXCHANGE WITH ORGANIC SOLVENTS

- *rec* Human Growth Hormone -




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



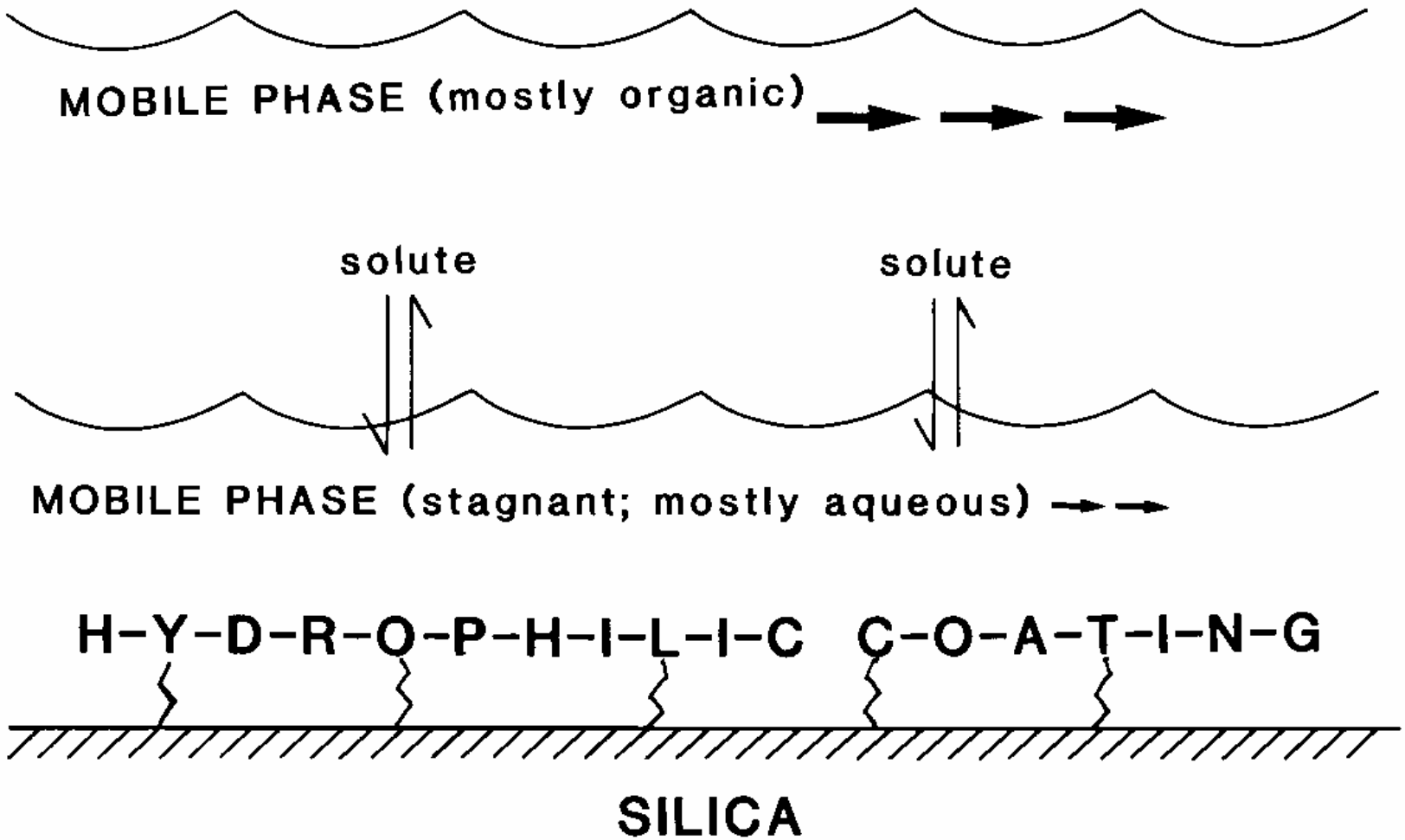

# Hydrophilic Interaction Chromatography (HILIC) of Amino Acids

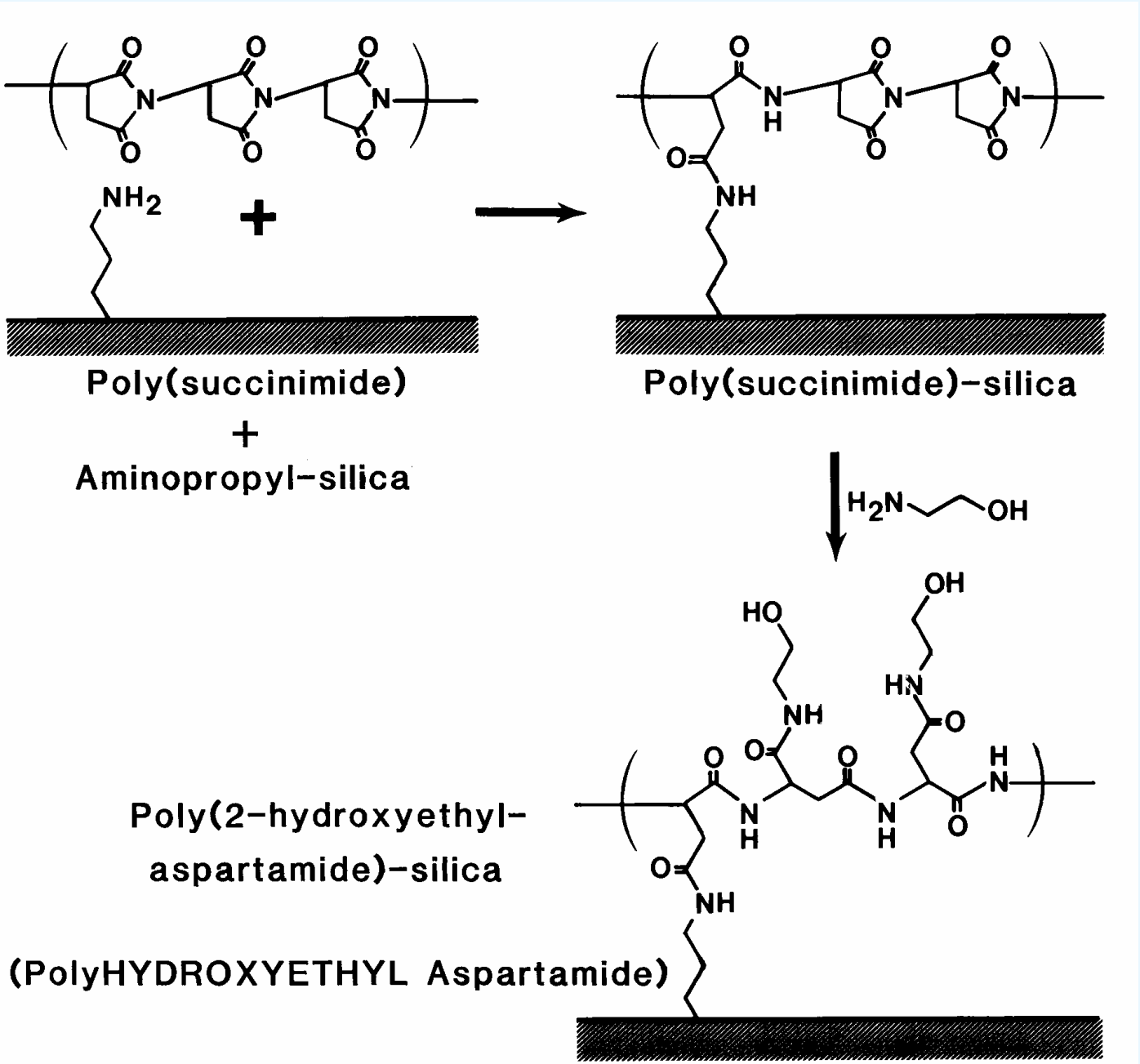
COLUMN: PolySULFOETHYL Aspartamide, 200x4.6-mm

Mobile Phase: 5 mM TEAP, pH 2.8, in 80% ACN

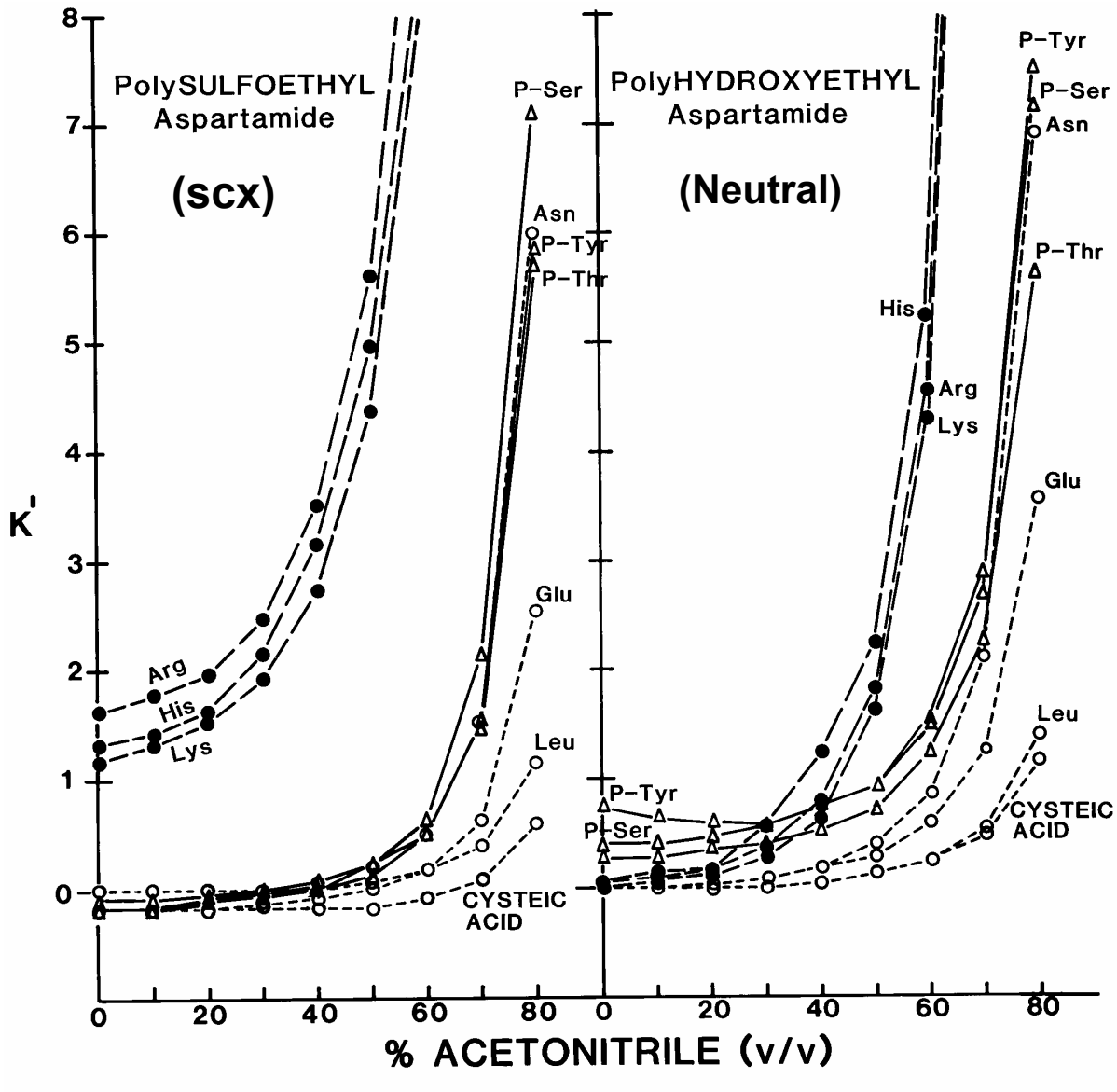
***Order of elution:***
***Least to most polar***

# HYPOTHETICAL PARTITION MECHANISM OF HYDROPHILIC-INTERACTION CHROMATOGRAPHY (HILIC)




Poly(succinimide)
+
Aminopropyl-silica
Poly(succinimide)-silica
Poly(2-hydroxyethyl-
aspartamide)-silica
(PolyHYDROXYETHYL Aspartamide)

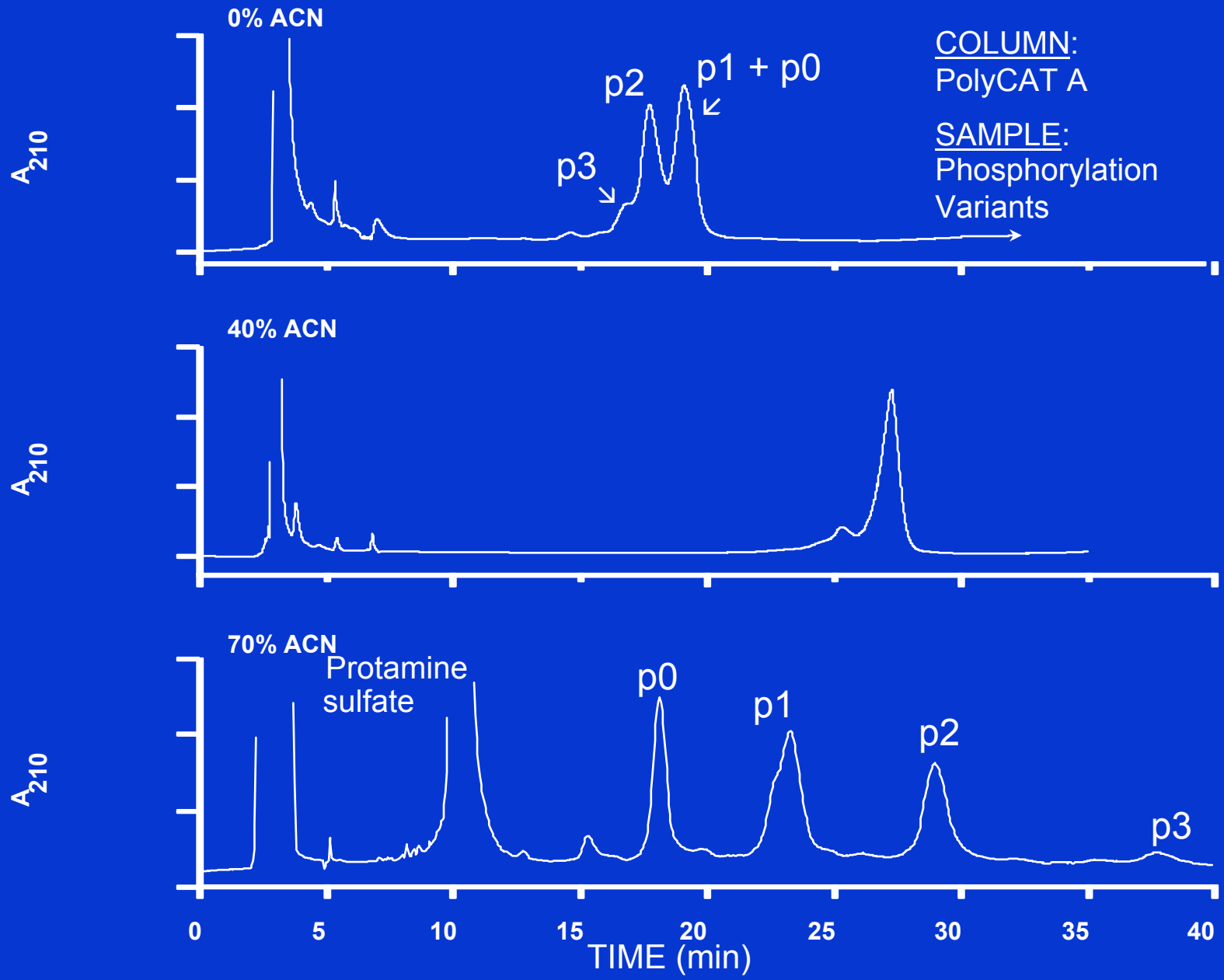
# HYDROPHILIC INTERACTION CHROMATOGRAPHY OF AMINO ACIDS



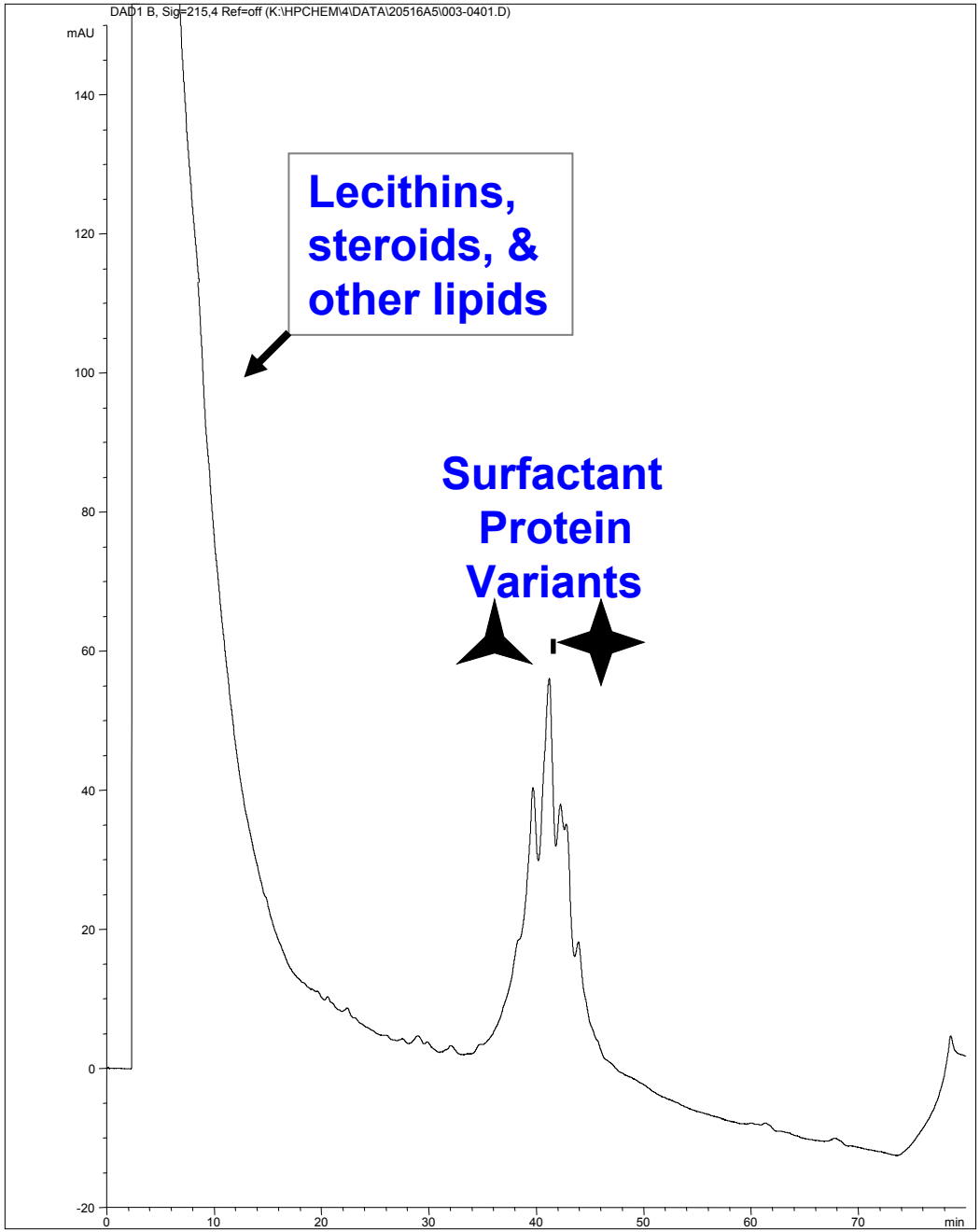

COLUMNS:
200 x 4.6 mm;
5-µm, 300-Å

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-µm; 1000-Å

**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

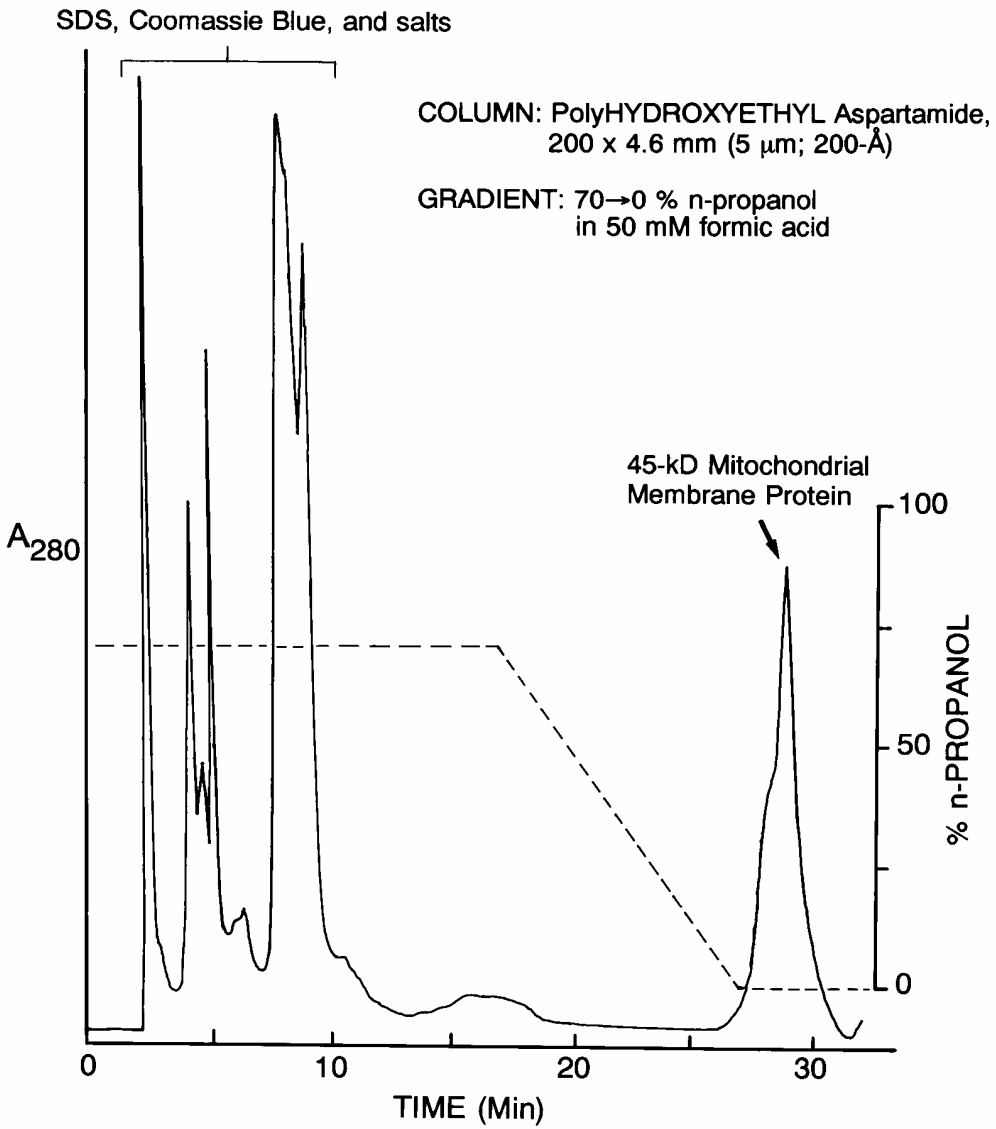
B) Same but 100 mM $NaClO_4$

**DETECTION**: A215

**SAMPLE**: 24 µg protein/80 µl

# Hydrophilic Interaction Chromatography (HILIC)

*- works well for membrane proteins -*



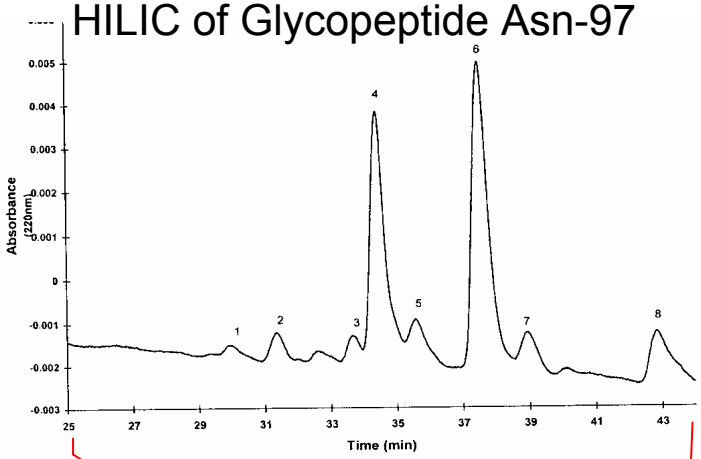

**Removal of SDS, Coomassie Blue, and Salts from an Electroeluted Membrane Protein**

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

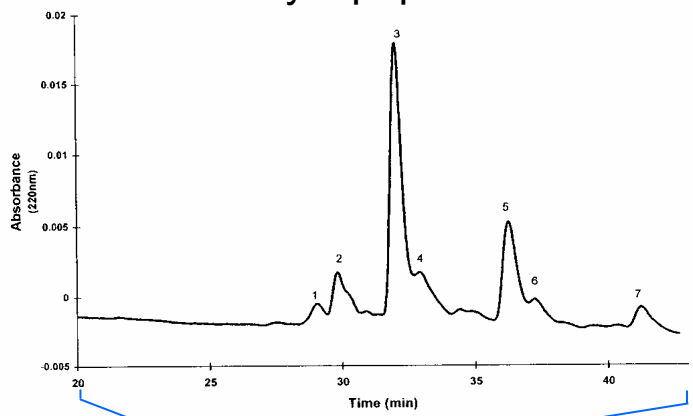
# RPC-HILIC Purification of Variant Glycopeptides from a Tryptic Digest of γ-Interferon

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

HILIC of Glycopeptide Asn-97

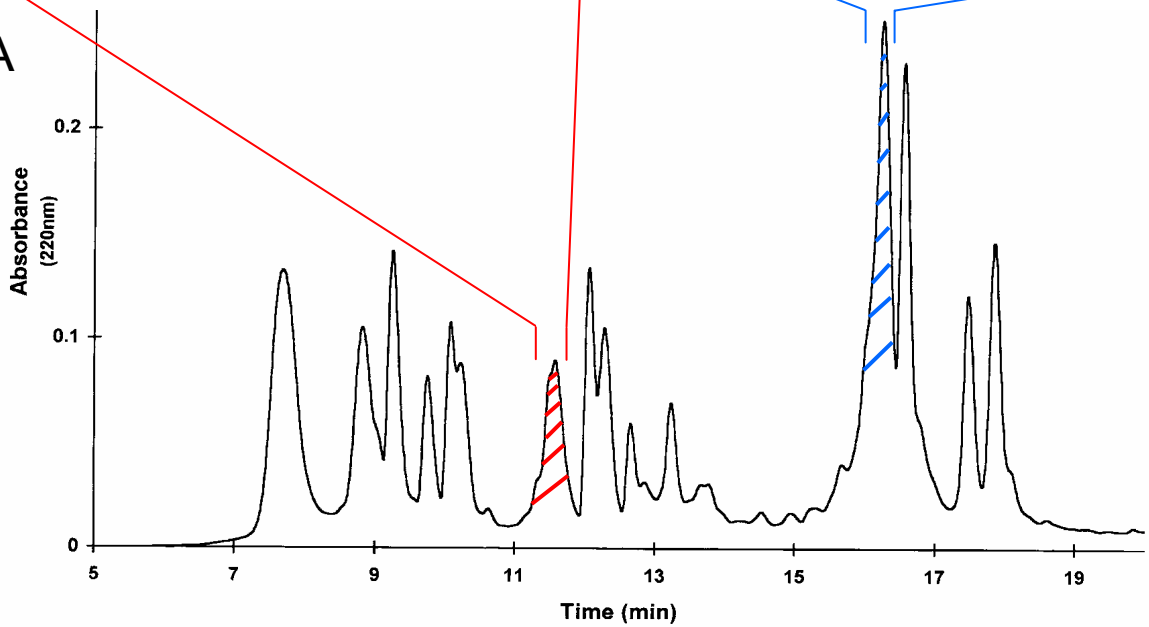



HILIC of Glycopeptide Asn-25



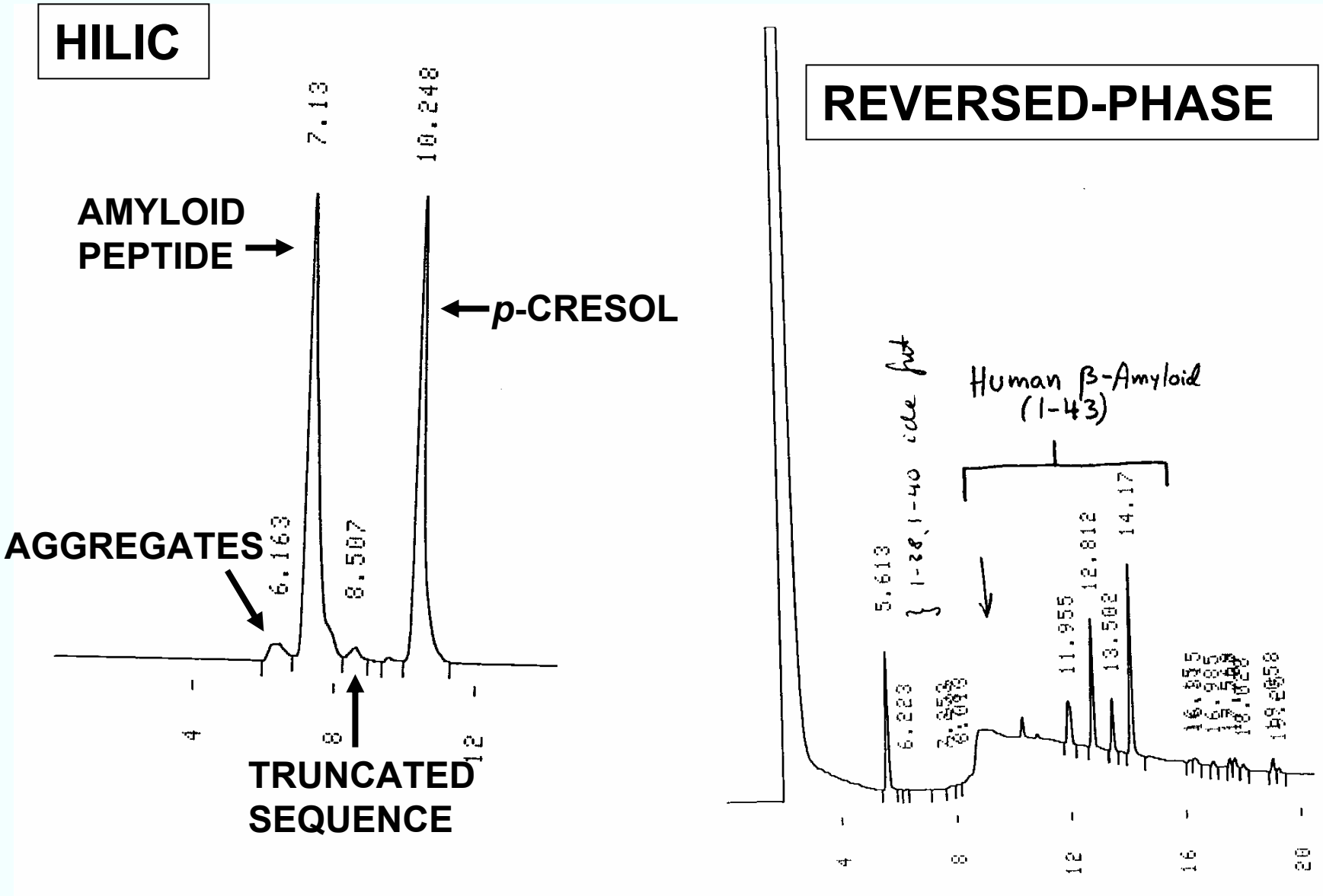

PolyHYDROXYETHYL A (150x1.0-mm)

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

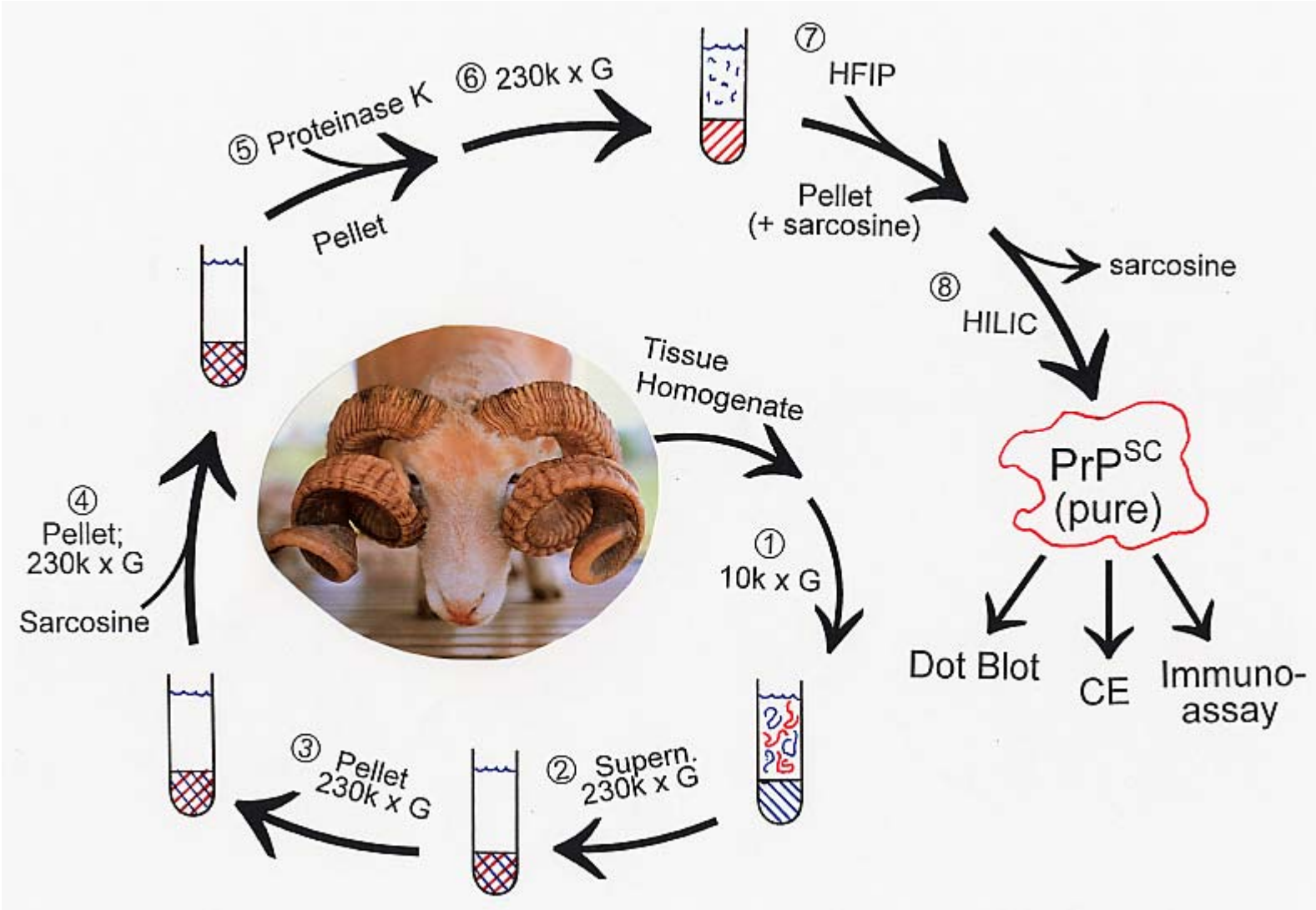



*Data courtesy of J. Zhang and D. Wang (MIT)*

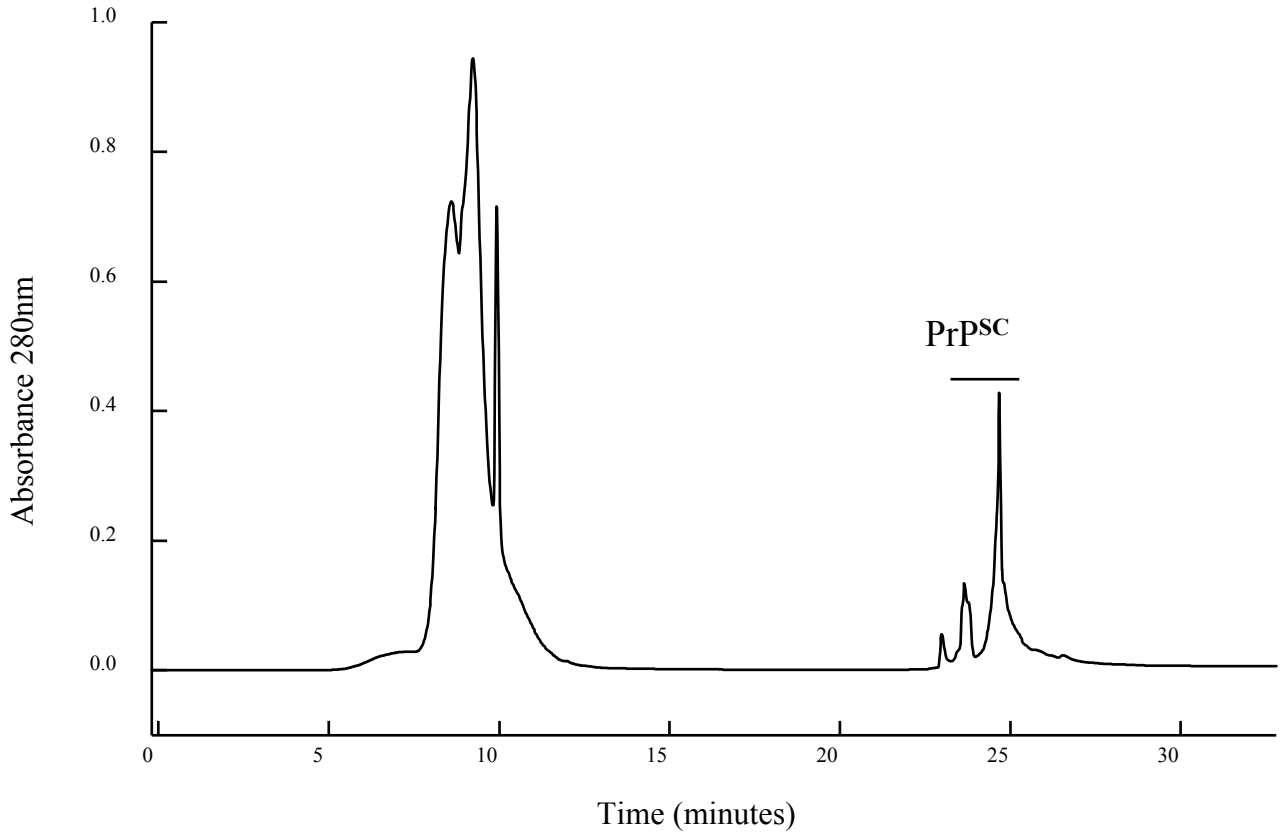
# HPLC of β-Amyloid Peptide (1-43)



# PREPARATION OF SCRAPIE PRION PROTEIN



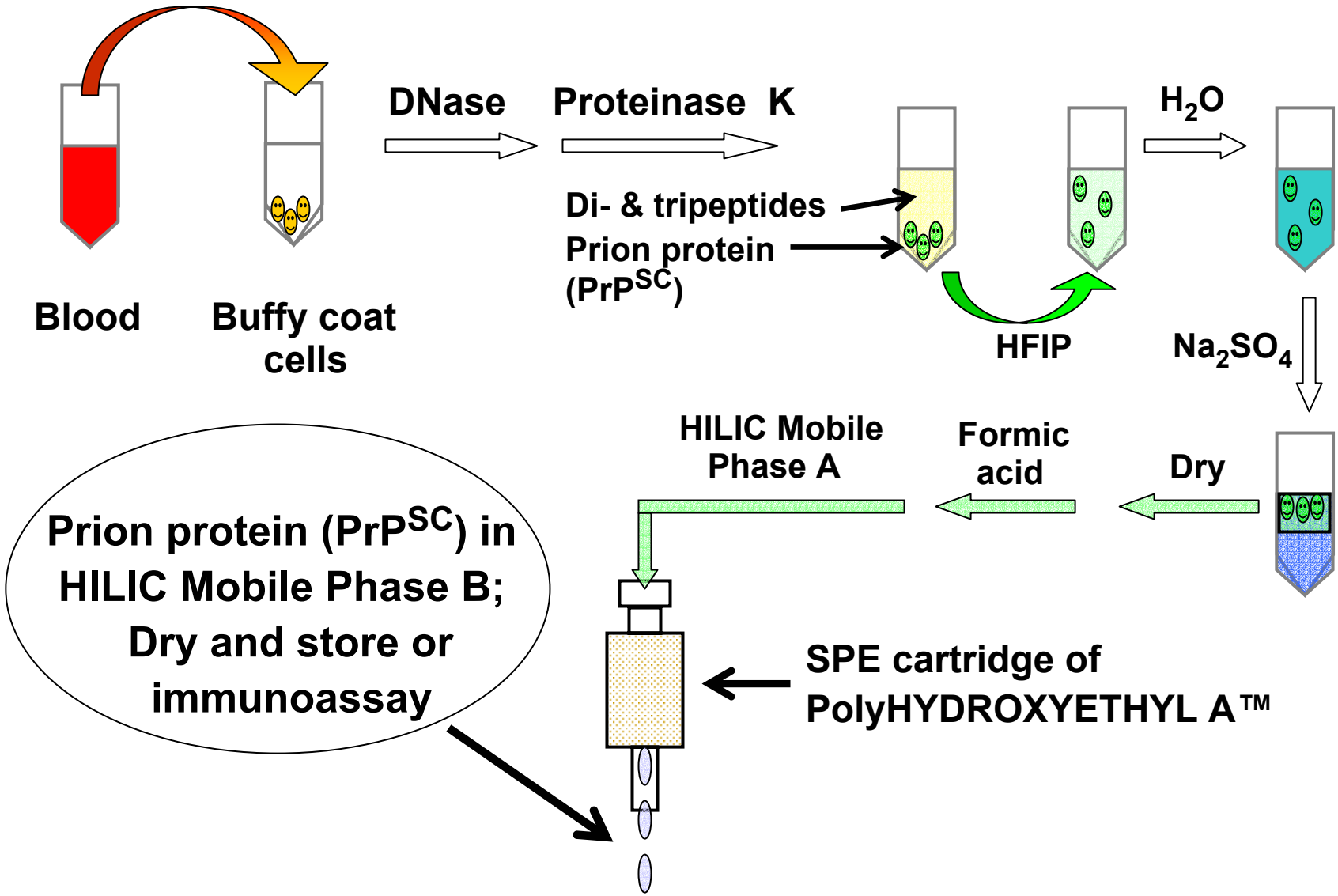
# HILIC of Scrapie Prion Protein

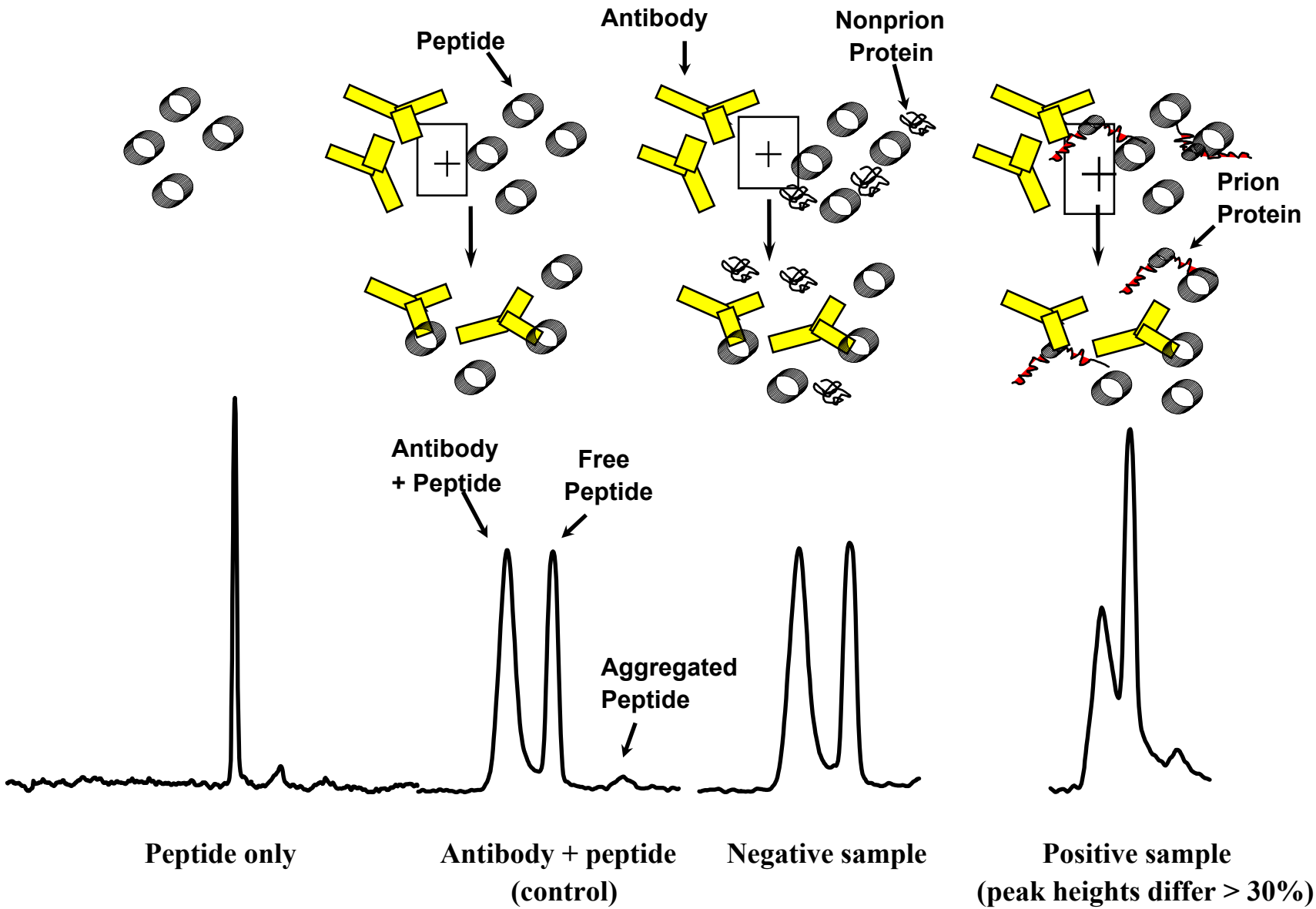



Column: PolyHYDROXYETHYL A

Gradient: Decreasing [ACN]

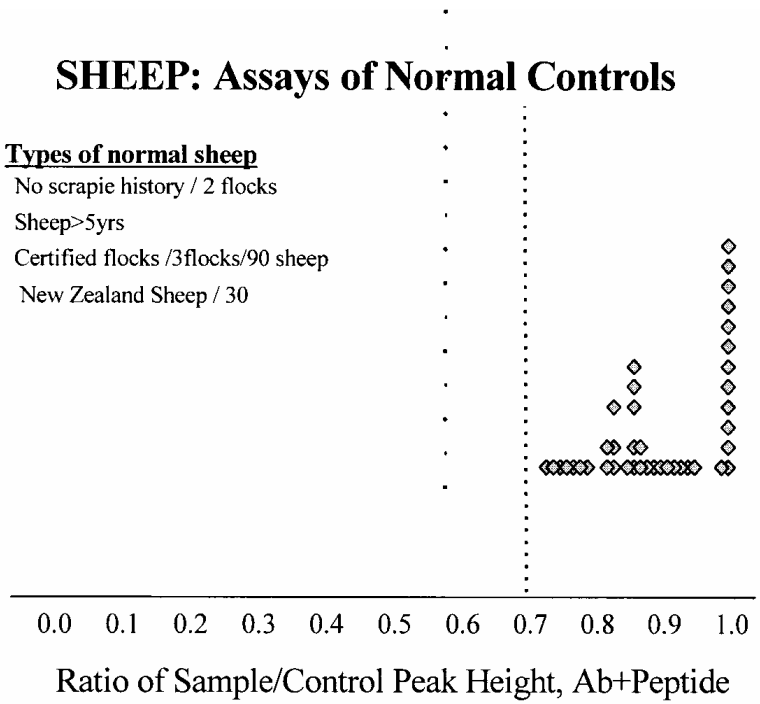
# EXTRACTION OF PRION PROTEIN BY HILIC






**COMPETITIVE IMMUNOASSAY OF SCRAPIE PRION BY CE.**

**Elapsed time of each electropherogram: 4'.**



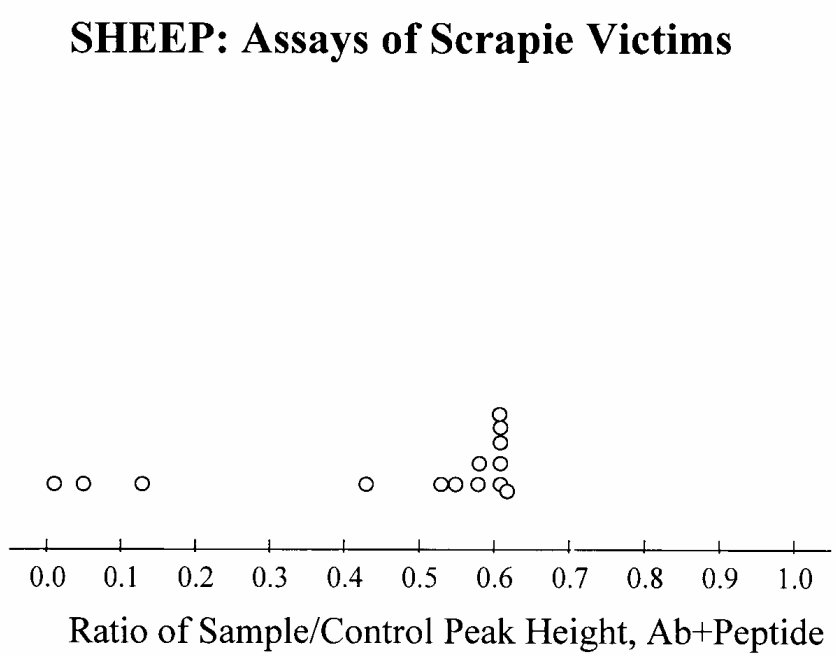
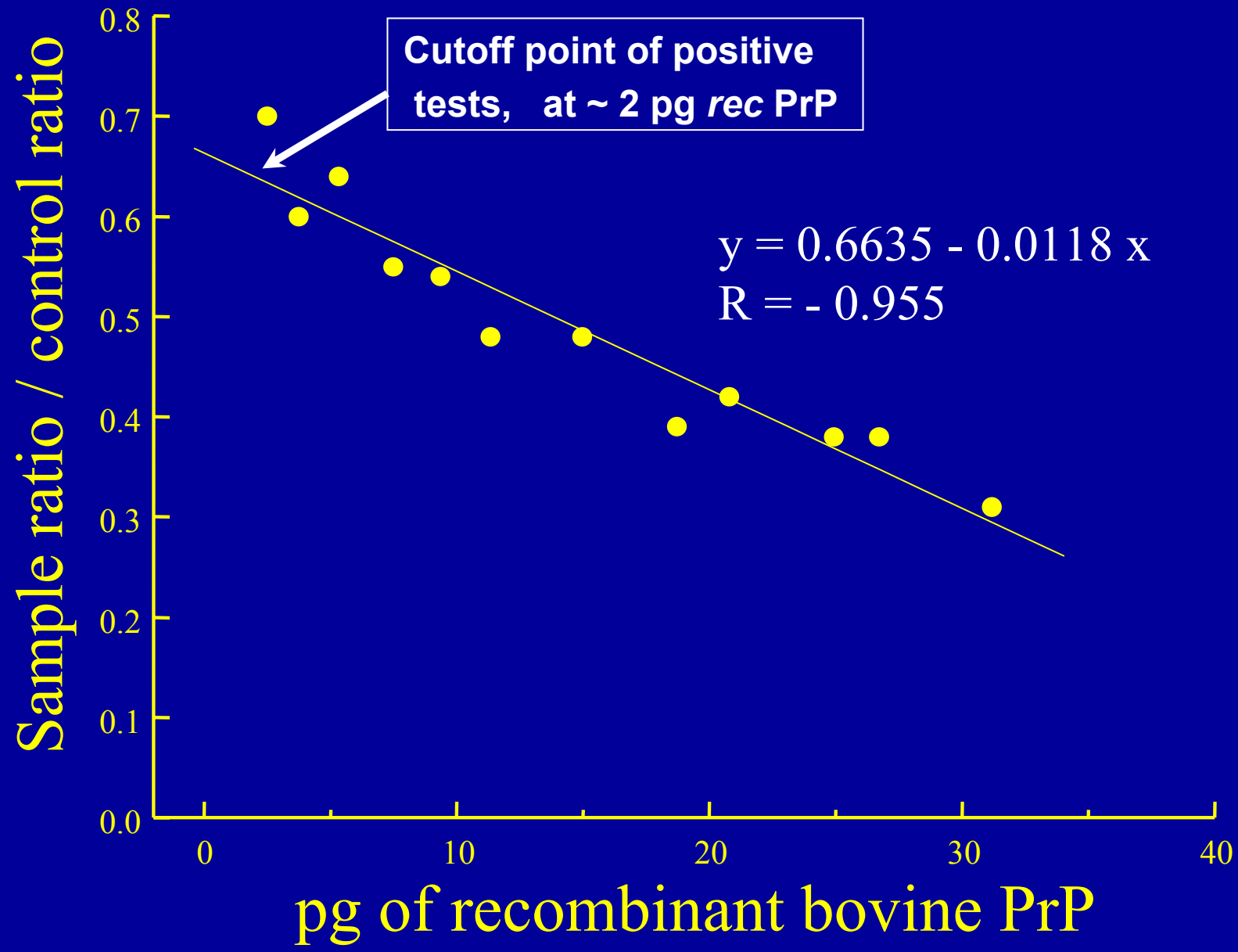
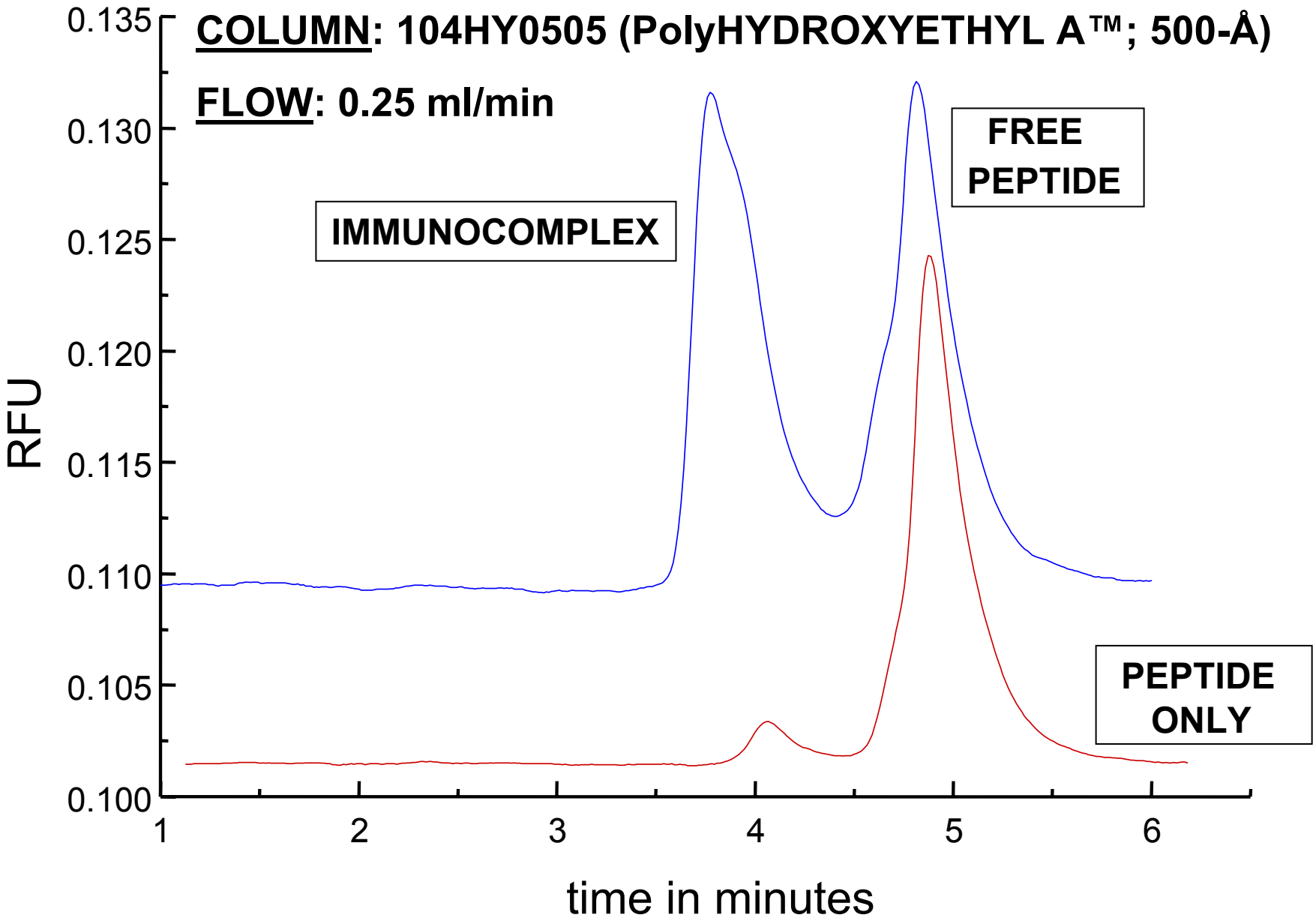




**Fig. 4b. Response of CE immunoassay to extracts from blood of normal sheep and sheep with scrapie.**

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


Cutoff point of positive tests, at ~ 2 pg *rec* PrP
y = 0.6635 - 0.0118 x
R = - 0.955
Sample ratio / control ratio
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0
10
20
30
40
pg of recombinant bovine PrP

# SEC OF FLUORESCENT PRION PEPTIDE + ANTIBODY



# PROTEOMICS ANALYSES

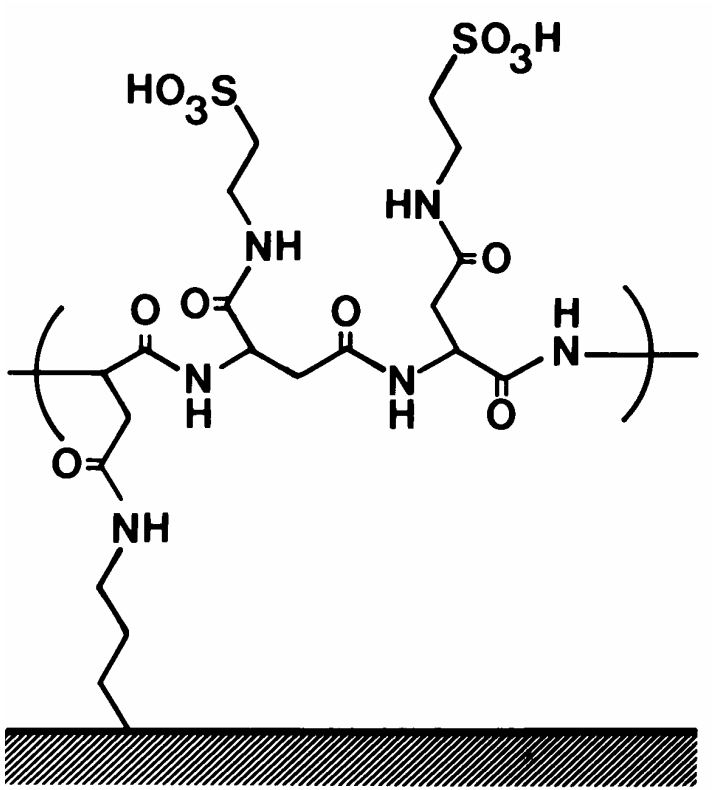
"***A butterfly and a caterpillar have the same genome but different proteomes.***" - Ron Orlando

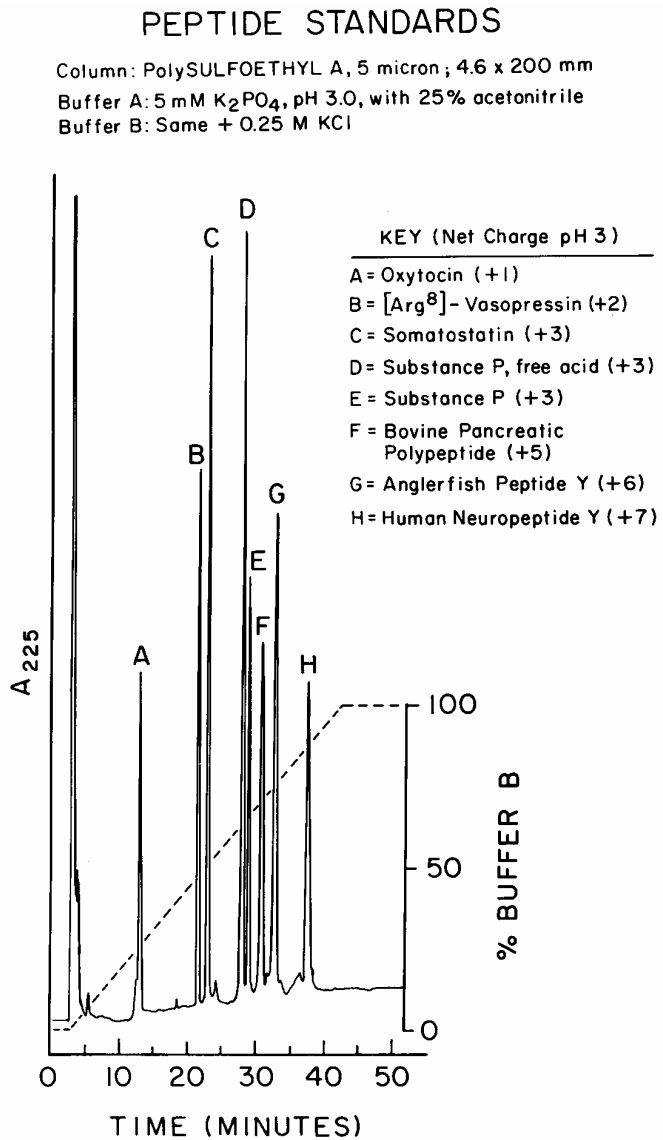
## Techniques for resolving complex protein mixtures:

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.

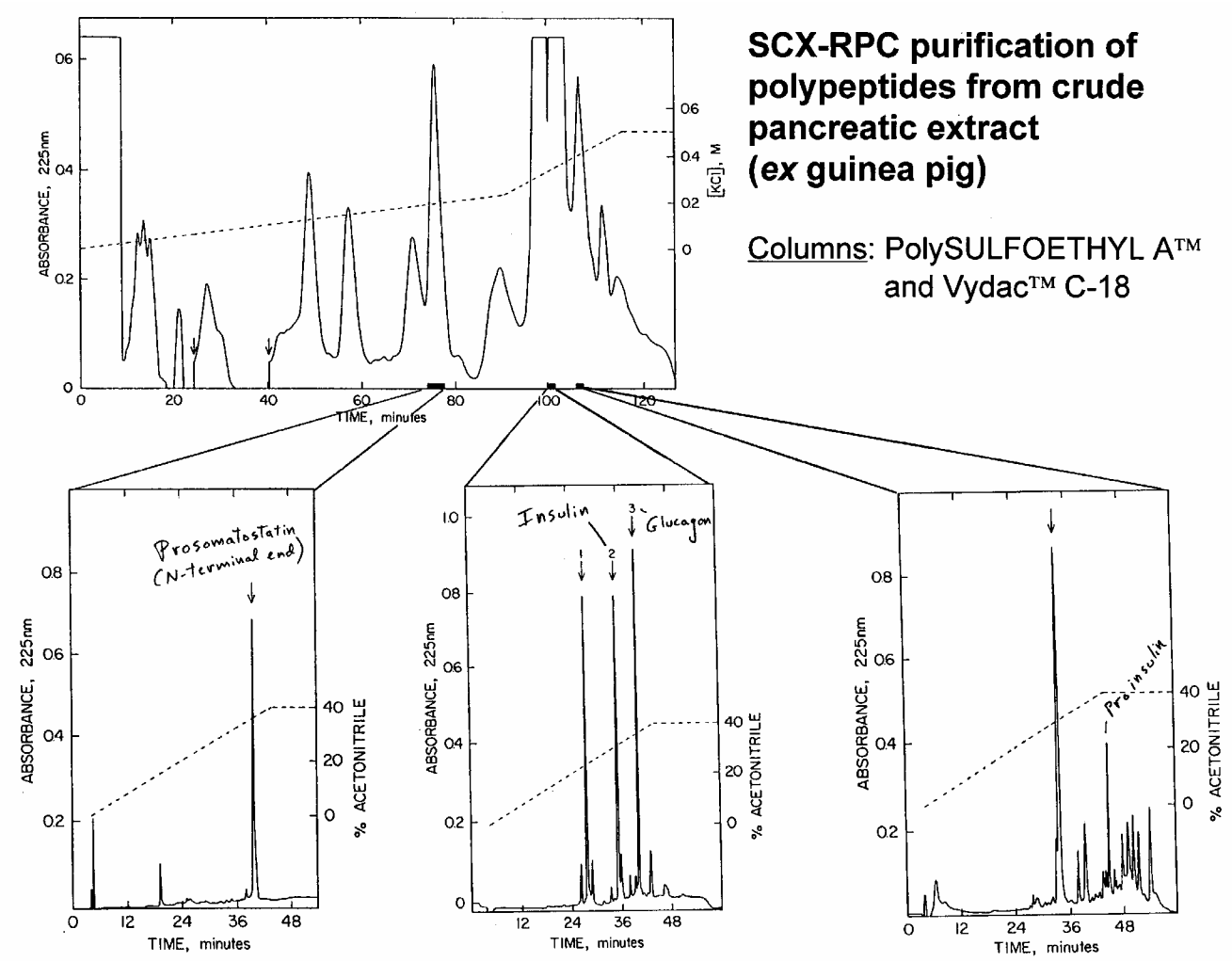
# CATION-EXCHANGE OF PEPTIDES (SCX)

PolySULFOETHYL
Aspartamide



# PEPTIDE PURIFICATION BY A CHARGE-POLARITY SEQUENCE




**SCX-RPC purification of polypeptides from crude pancreatic extract (*ex* guinea pig)**

Columns: PolySULFOETHYL A™ and Vydac™ C-18

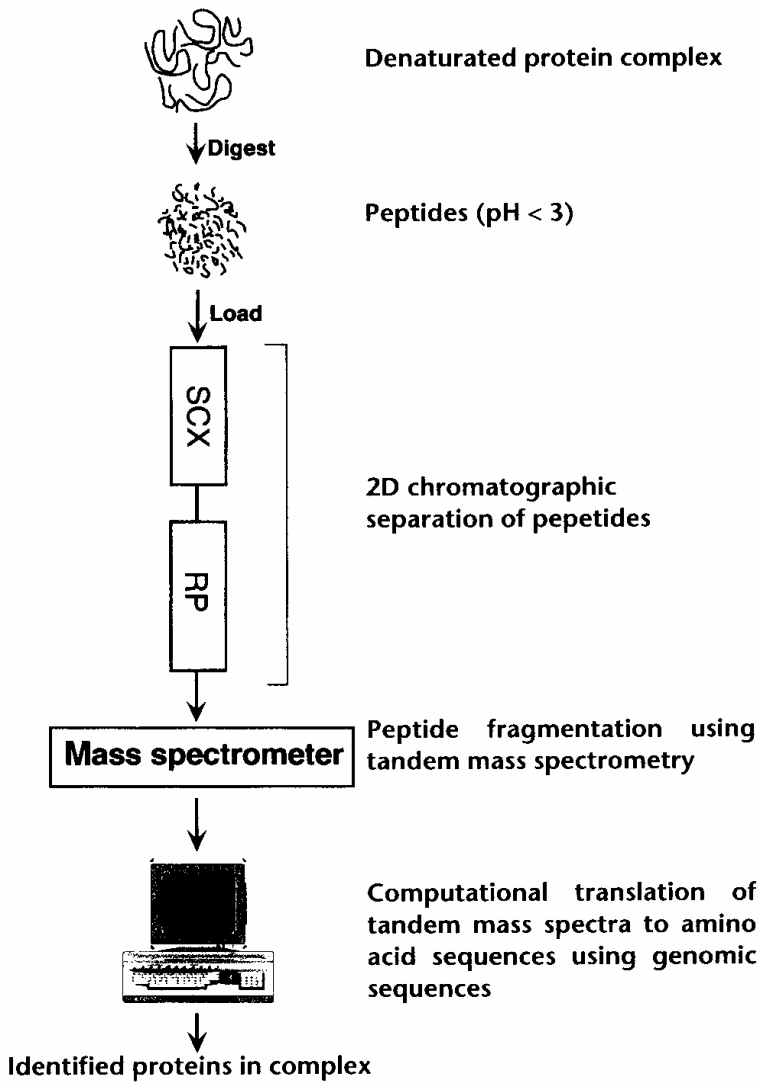
ADVANTAGES OF A SCX-RPC SEQUENCE:

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 ELECTROPHOPHORESIS

1) Greater dynamic range:

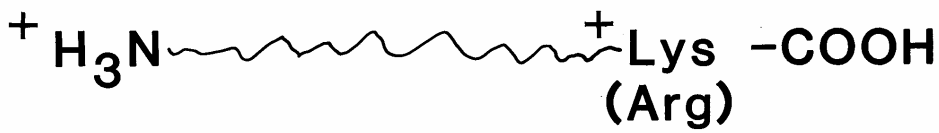
Most abundant protein detected

Least abundant protein detected

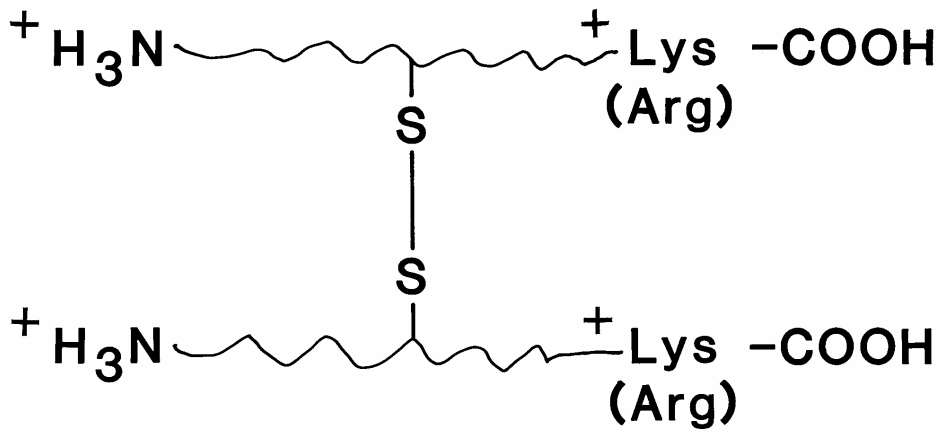
2) Works better for hydrophobic and very basic proteins

3) Handling and automation easier

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




Typical Tryptic Peptide

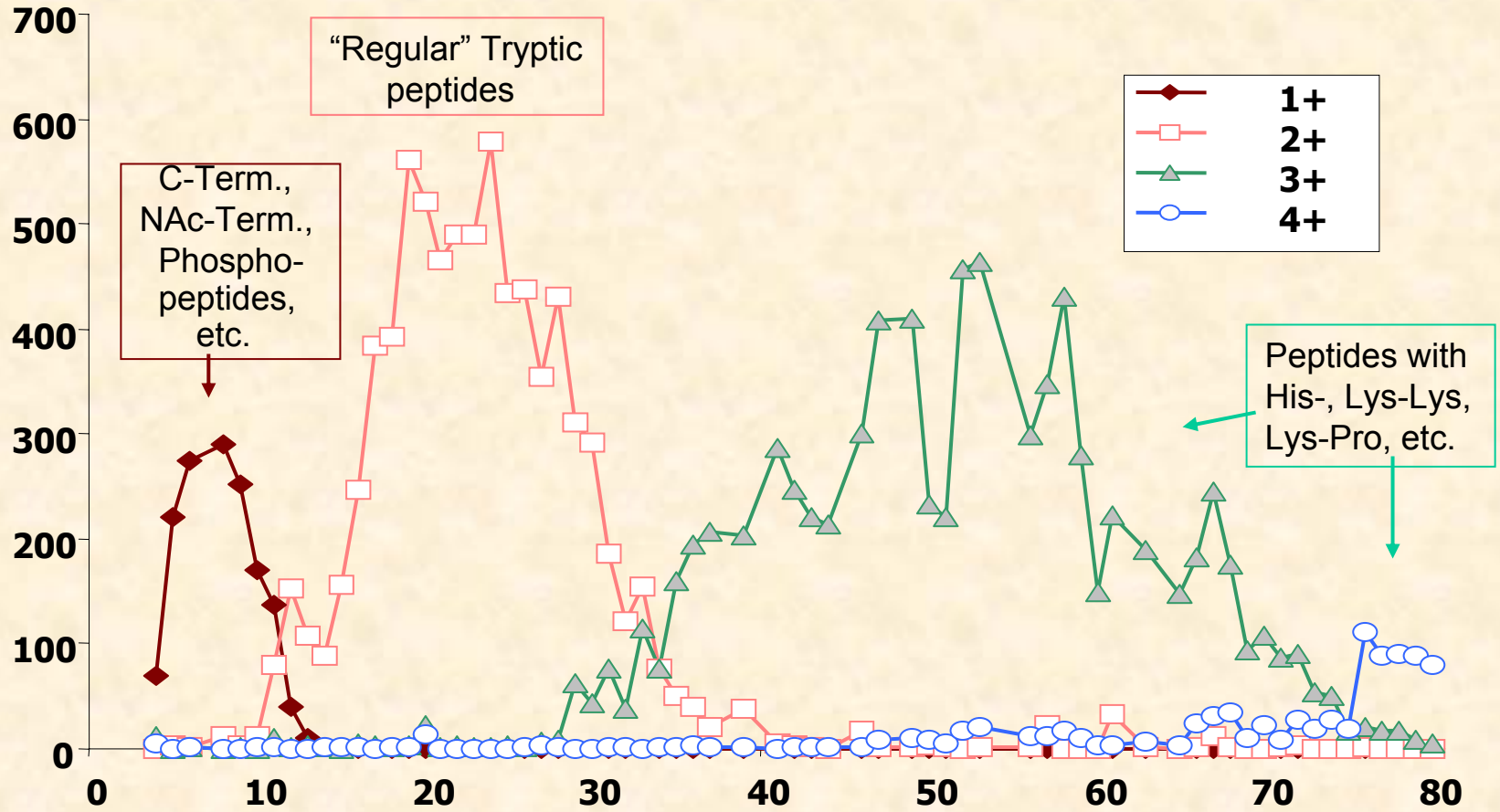



***WHY USE TRYPTIC PEPTIDES FOR PROTEOMICS ANALYSES?***

At pH 3, Asp- and Glu- are uncharged and all peptides have the same charge: +2 (Exceptions: Peptides with His-, -S-S- bridges, the C-terminal peptide, etc.); should elute within a narrow window in SCX. Also, good size for MS/MS (< 20 residues, typically).

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

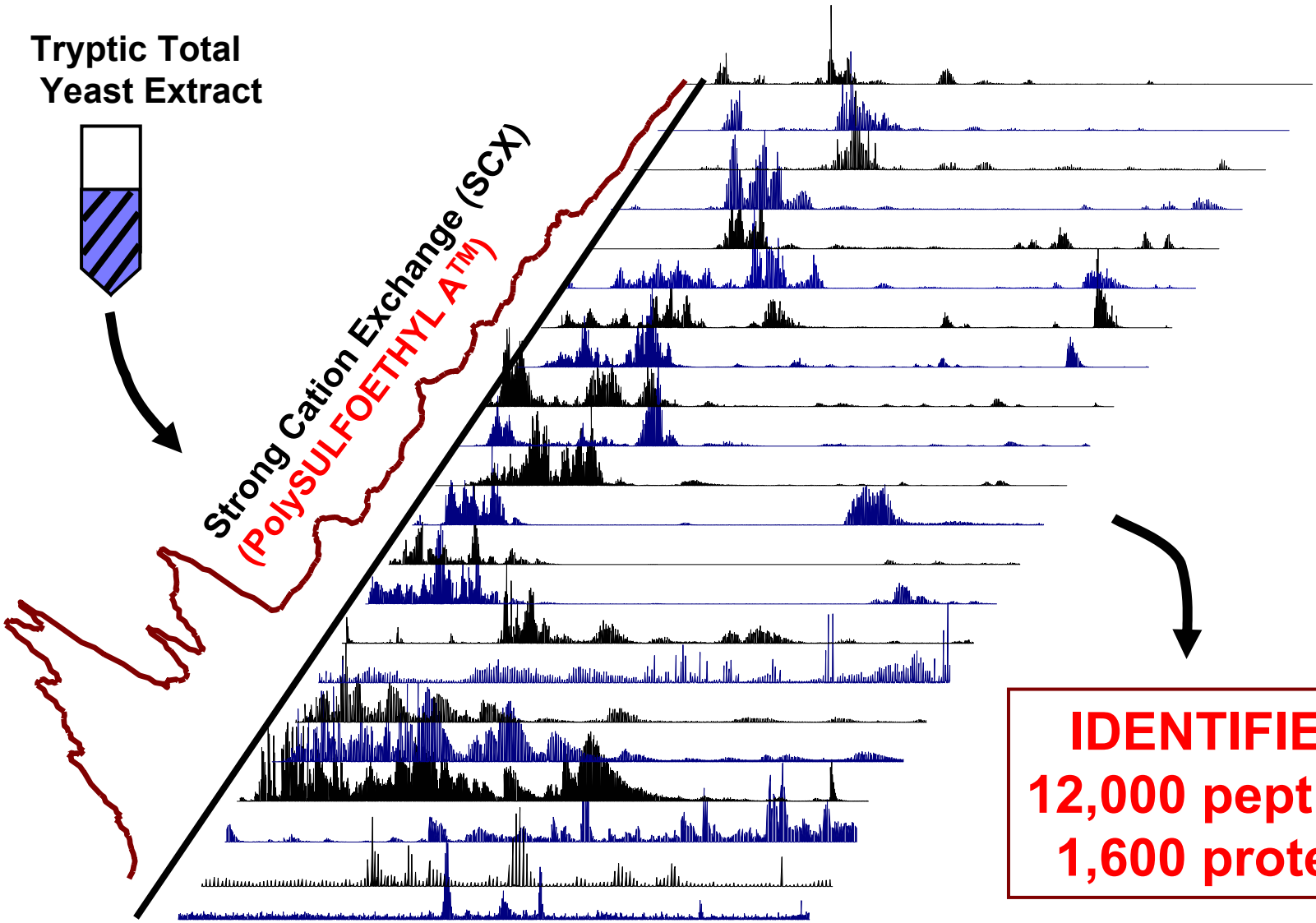
**COLUMN: PolySULFOETHYL A (item# 202SE0502)**




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




**IDENTIFIED:**
**12,000 peptides**
**1,600 proteins**

**Reversed phase chromatography - MS/MS of collected SCX fractions**

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

# STRATEGY FOR PROTEOMICS ANALYSES

**1) KEEP FRACTIONS SMALL**

**a) Peptides suppress each others' ionization ∴ 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 ( ↑ loading) and faster flow rates; facilitates collection of smaller, more numerous fractions.**

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

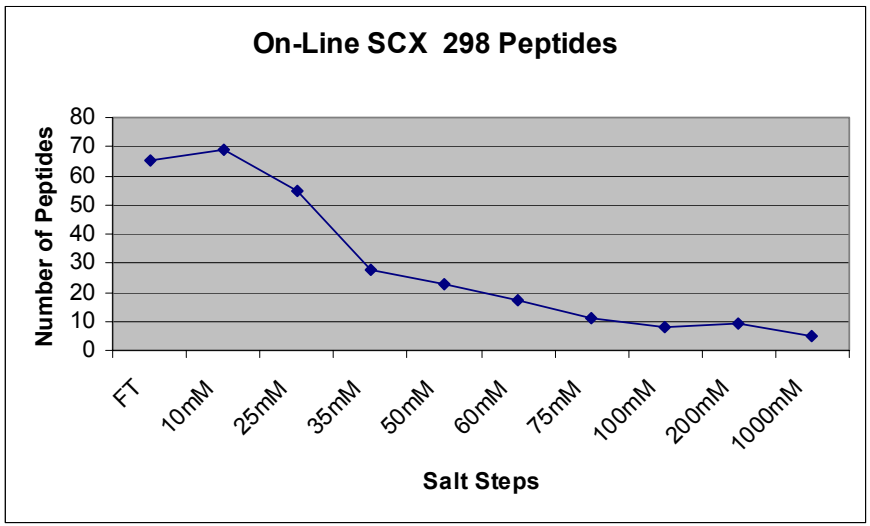
# Comparison of On-Line vs. Off-Line SCX of Peptides in 2-D LC/ MS/MS

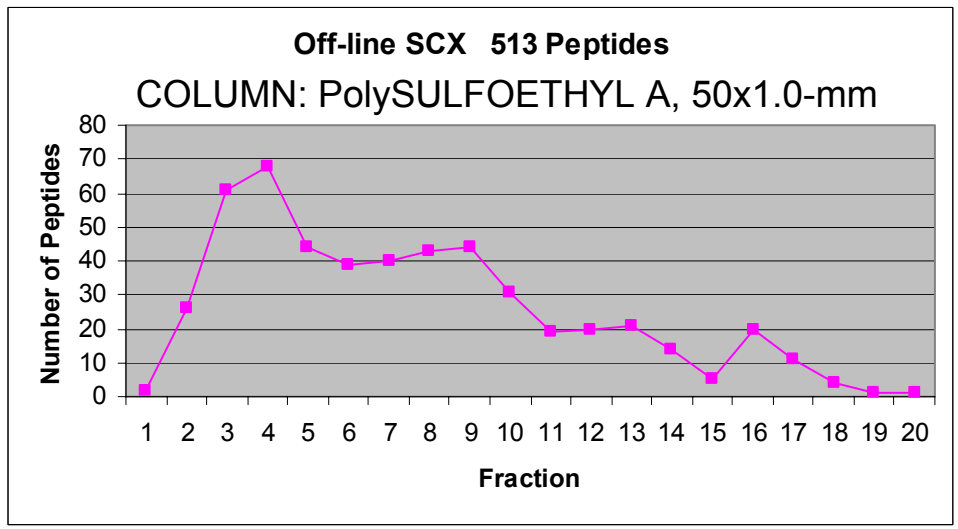
Sample: LysC/Trypsin digest of rat liver microsomes
250 µg digested protein loaded

**∴ 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




*- data courtesy Kevin Blackburn, Glaxo SmithKline -*

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

# 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

$\therefore$ 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:

$2.25\ \mu g \times 10^5 =$ **225 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

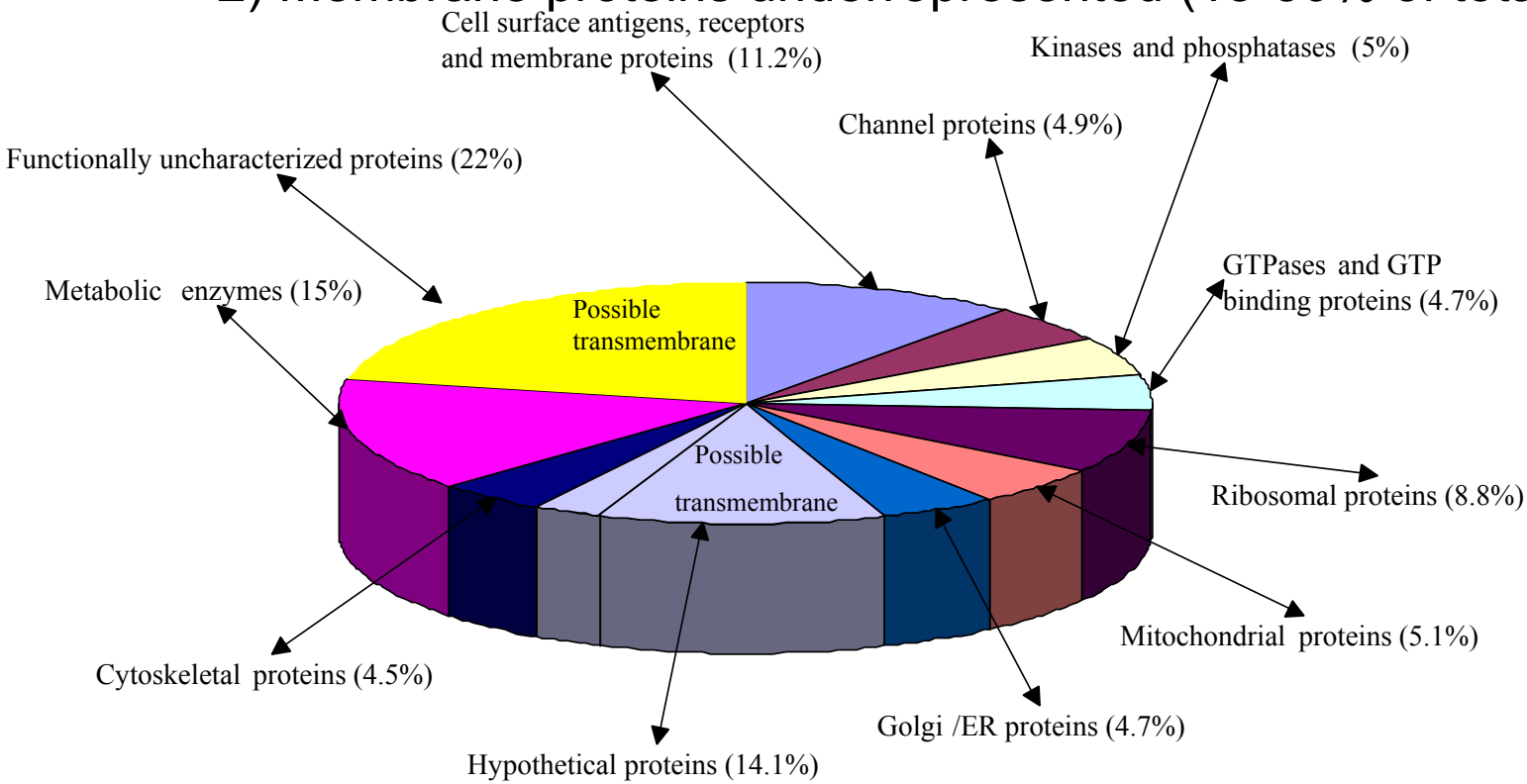
$\therefore$ **1) BIG MISMATCH BETWEEN SIZE OF SCX AND RPC COLUMNS**

**2) NEED TO COLLECT AND PROCESS 500+ FRACTIONS AT SCX STEP?**

# Be determined in achieving your goals…

# 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**

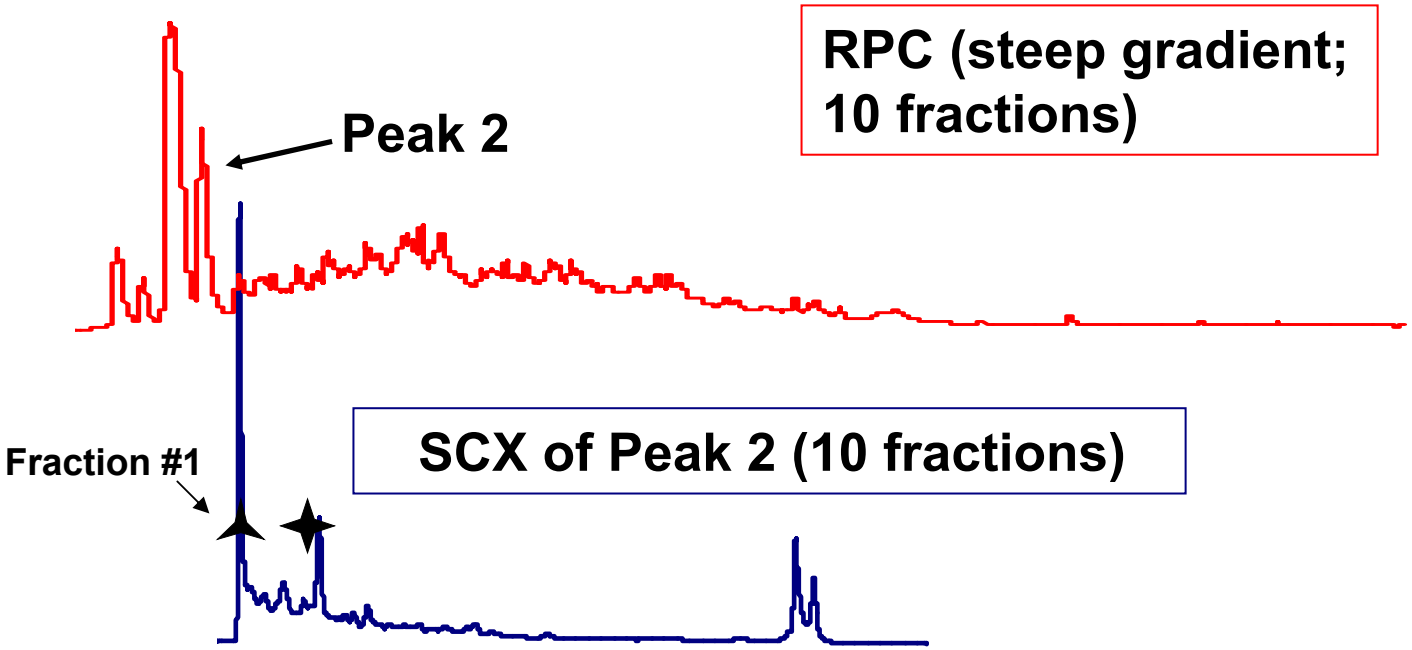
**- from Han *et al.*, *Nat. Biotechnol. 19* (2001) 946-951**

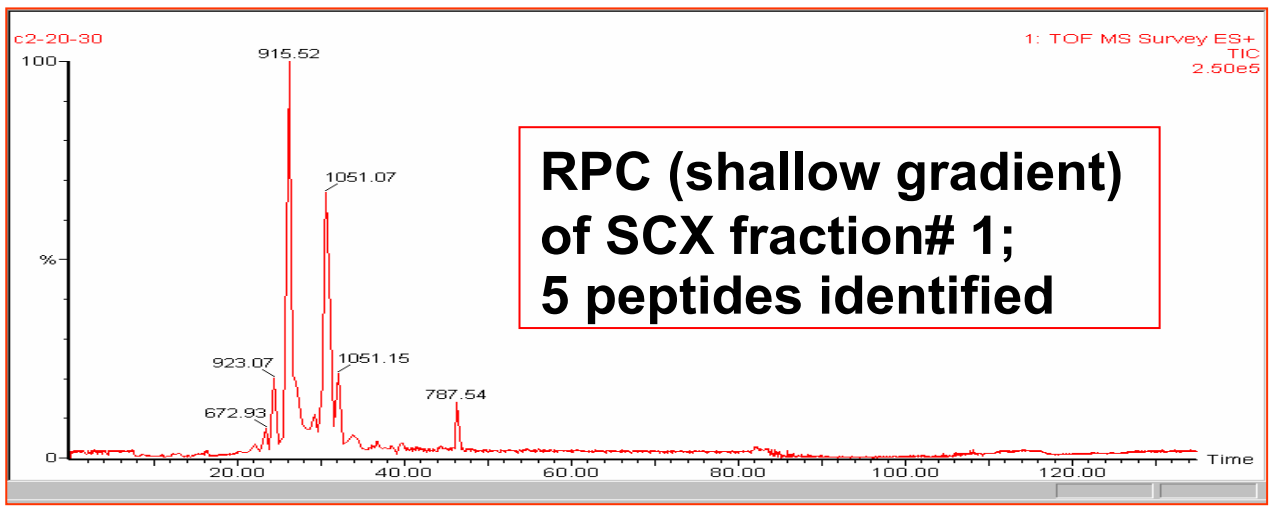
# Alternative strategies to 2DE are MS-based and involve multiple separation steps

| | 1st dimension | 2nd dimension | 3rd 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

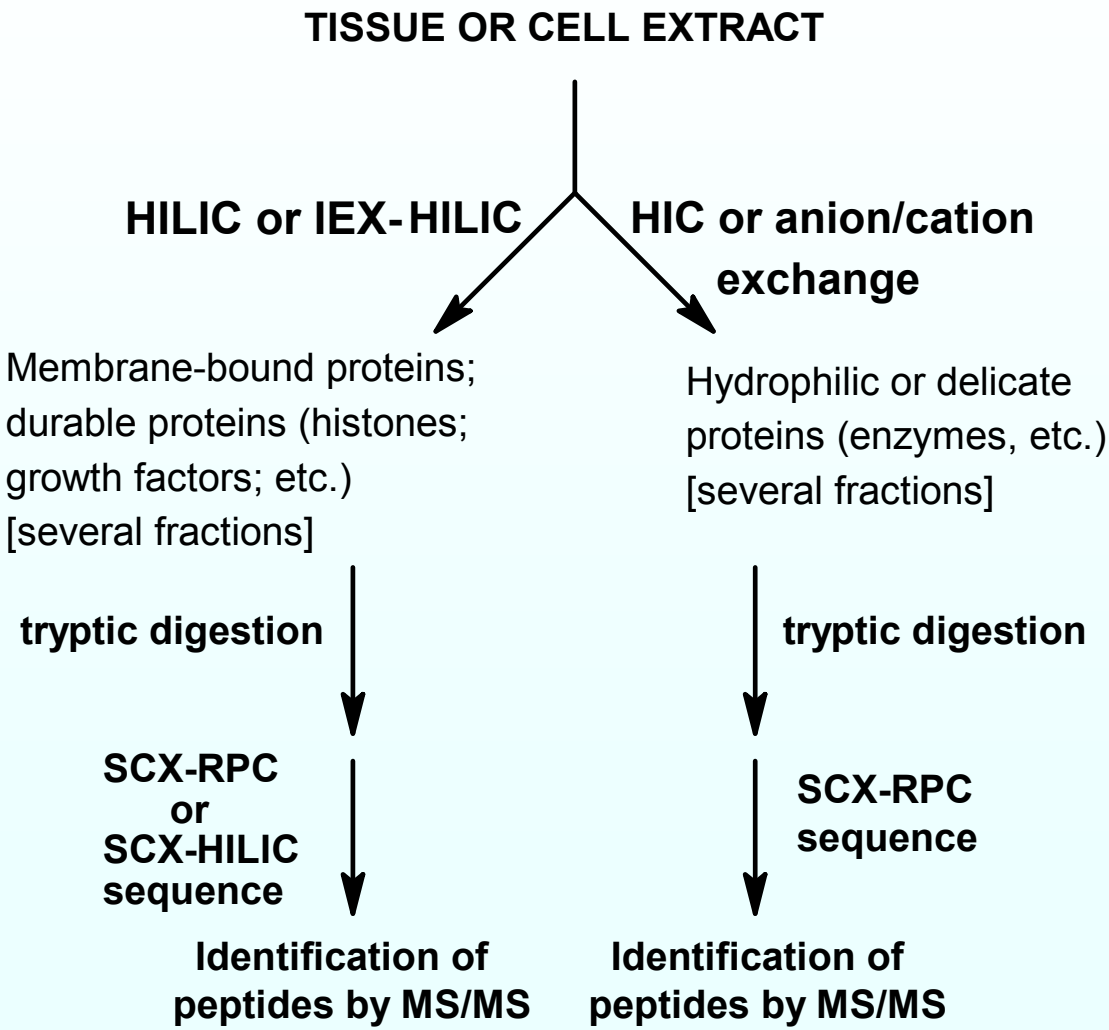


# PROTEOMICS OF *REALLY* COMPLEX MIXTURES

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

**Predigest fractionation of proteins by HPLC**



# Mixed-Bed IEX of Cell Extract Proteins

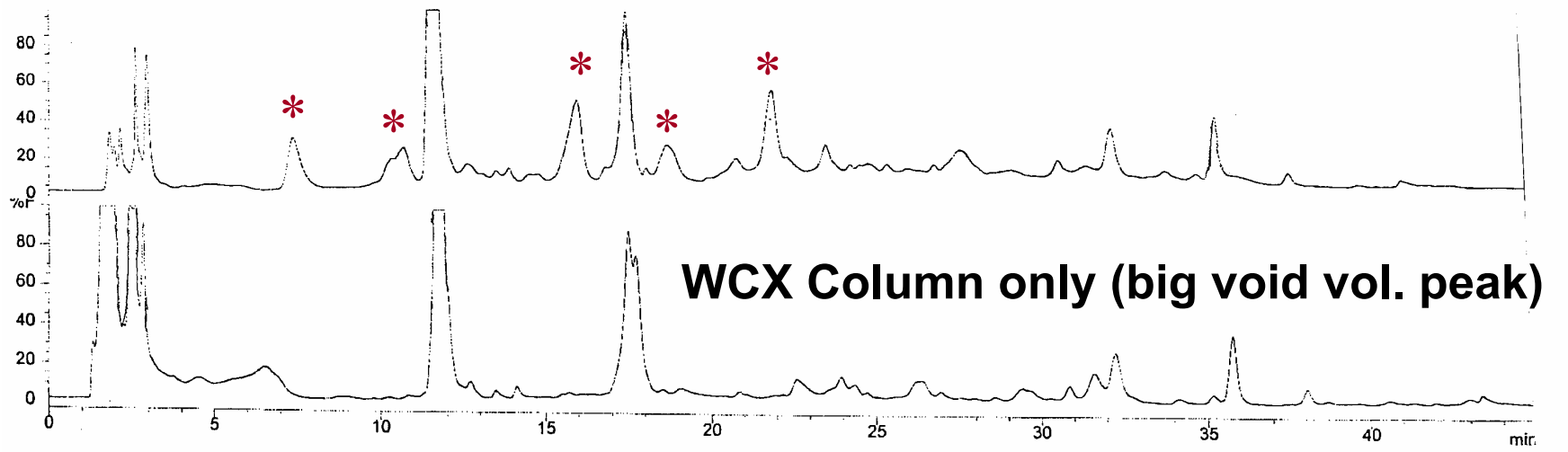
**COLUMN (WCX)**: PolyCAT A, 200x4.6-mm; 5-µm, 1000-Å

**GUARD CARTRIDGE (WAX)**: PolyWAX LP, 10x4-mm; 5-µm, 1000-Å

**GRADIENT (40' linear)**: 0-0.6 M NaCl in 10 mM $KH_2PO_4$, pH 6.2, with 5% ACN

**DETECTION**: Fluorescence (λex = 280 nm; λem = 350 nm)

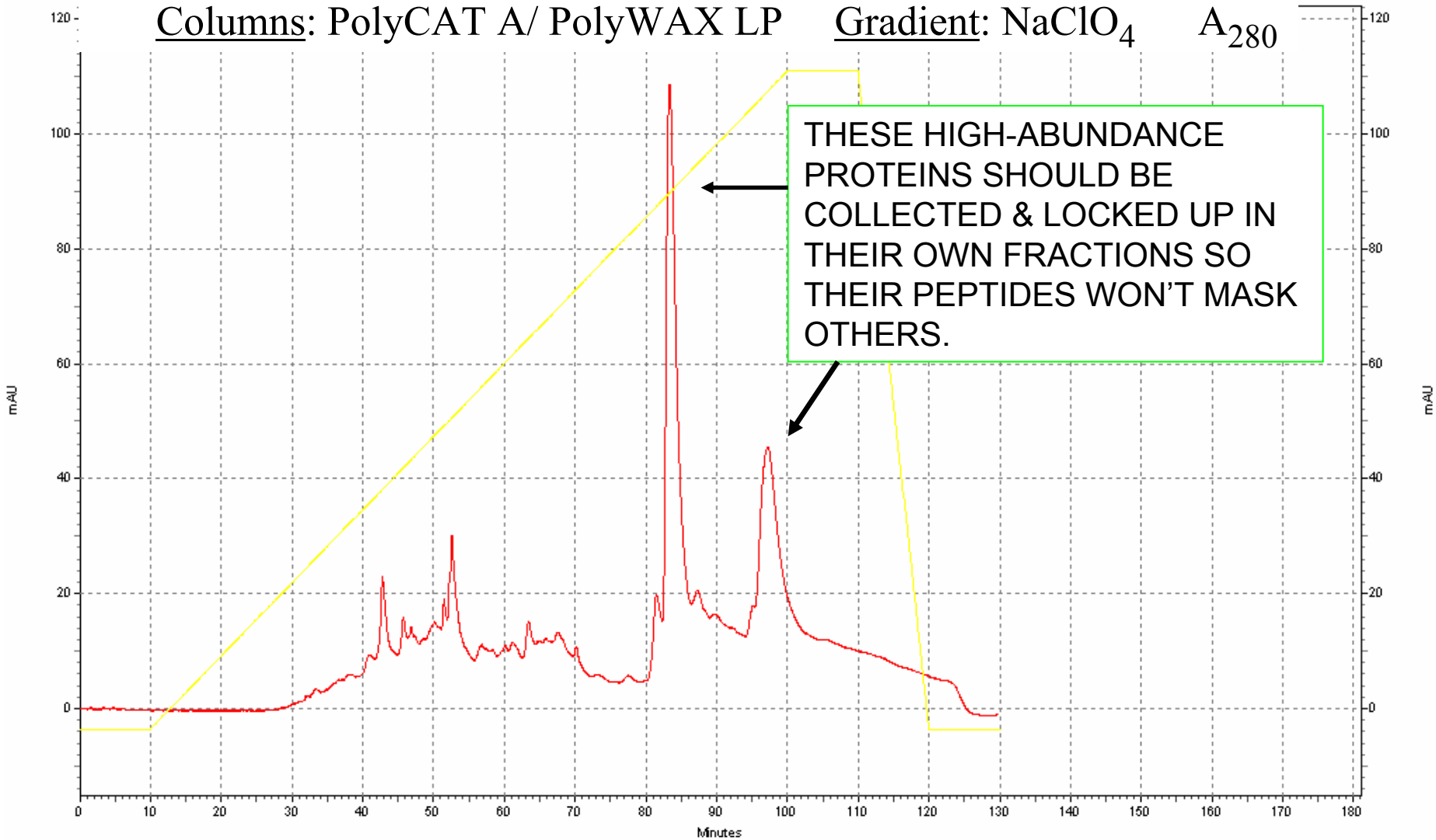
**WCX Column + WAX Cartridge (* = acidic proteins?)**



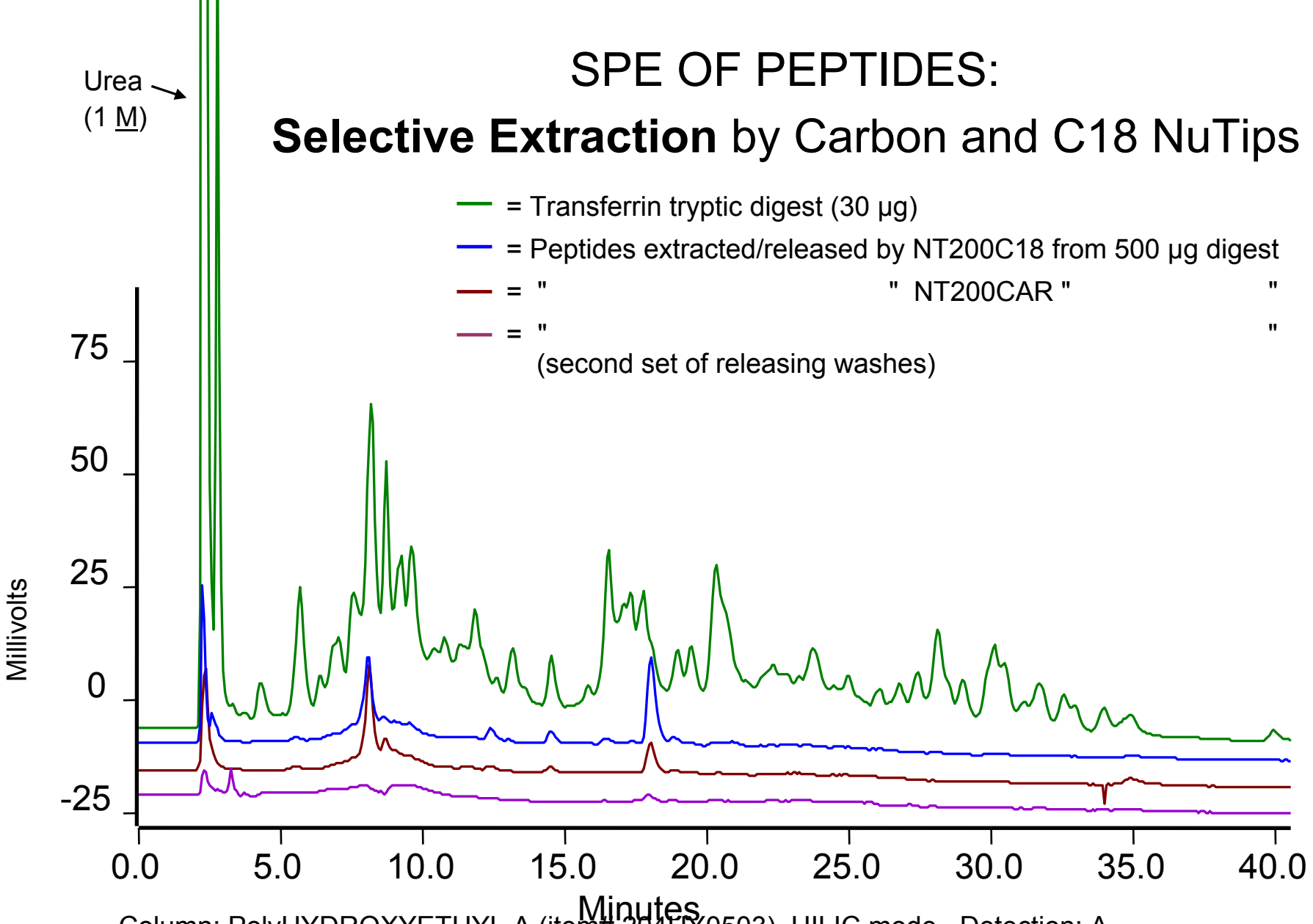

**WCX Column only (big void vol. peak)**

# Mixed-Bed IEC of Intact Proteins from THP-1 Monocyte Cell Lysate

(~ $6x10^6$ cells; water-soluble fraction)

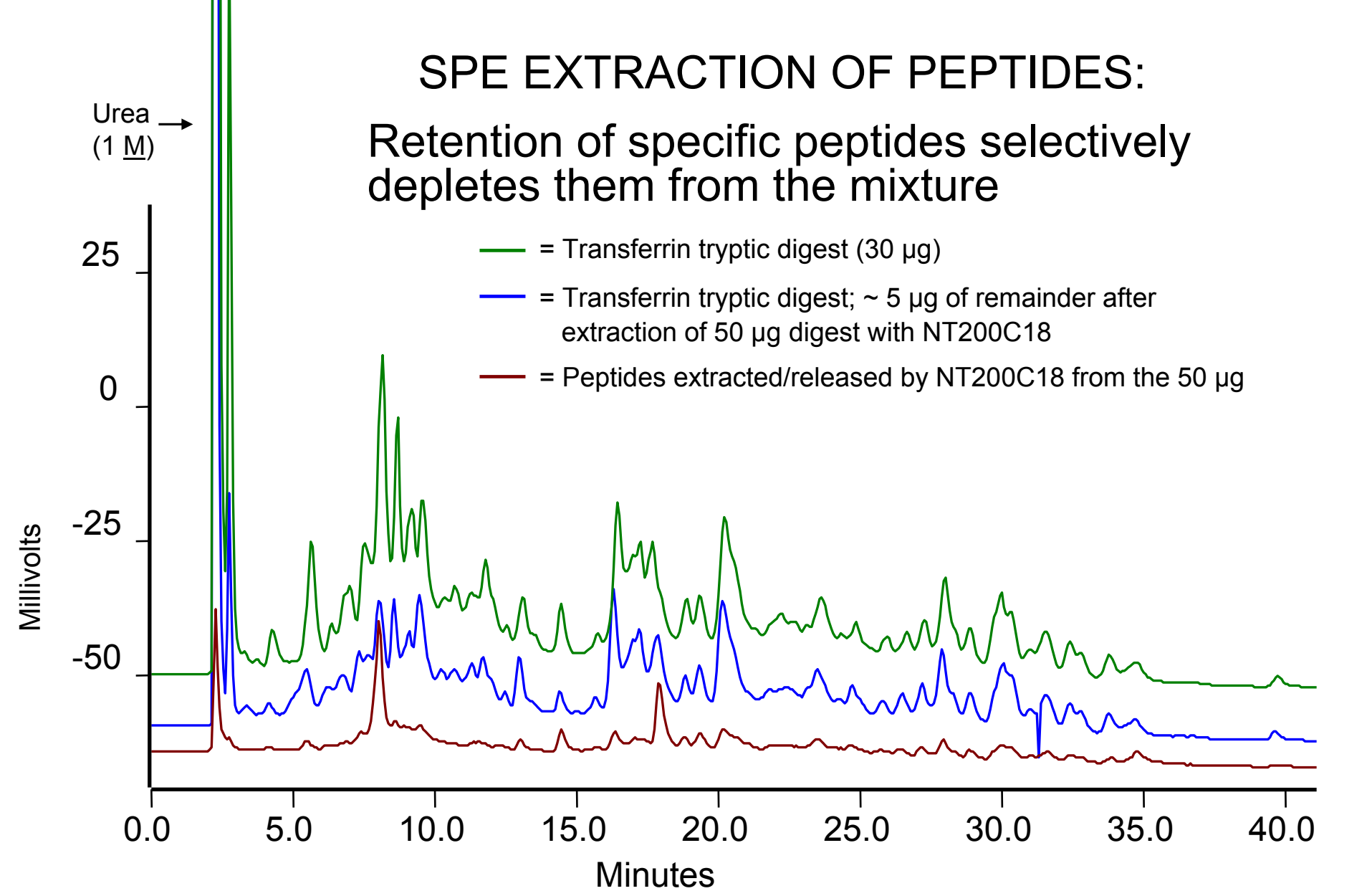



*- Data courtesy of Leticia Cano (City of Hope) -*




Column: PolyHYDROXYETHYL A (item# 204HY0503), HILIC mode. Detection: $A_{220}$

Gradient: 40' linear, 0-70% B. A) 15 mM TEAP, pH 2.7, with 85% ACN
B) " " 10 "




Column: PolyHYDROXYETHYL A (item# 204HY0503), HILIC mode. Detection: $A_{220}$

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

B) " " 10 "

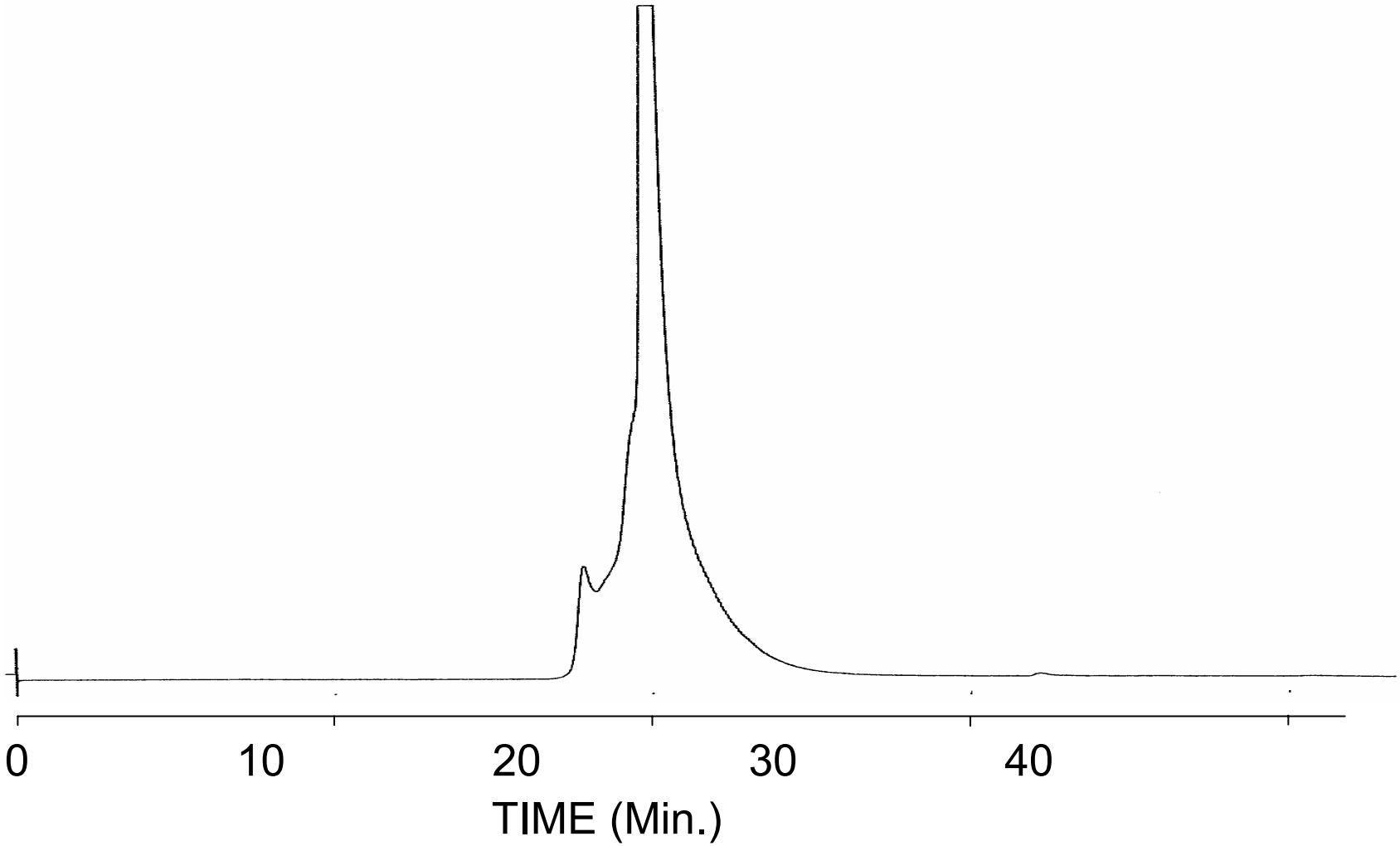
# SELECTIVE PRECIPITATION TO REMOVE PROTEINS OF HIGH ABUNDANCE

## 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 |
| α1-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



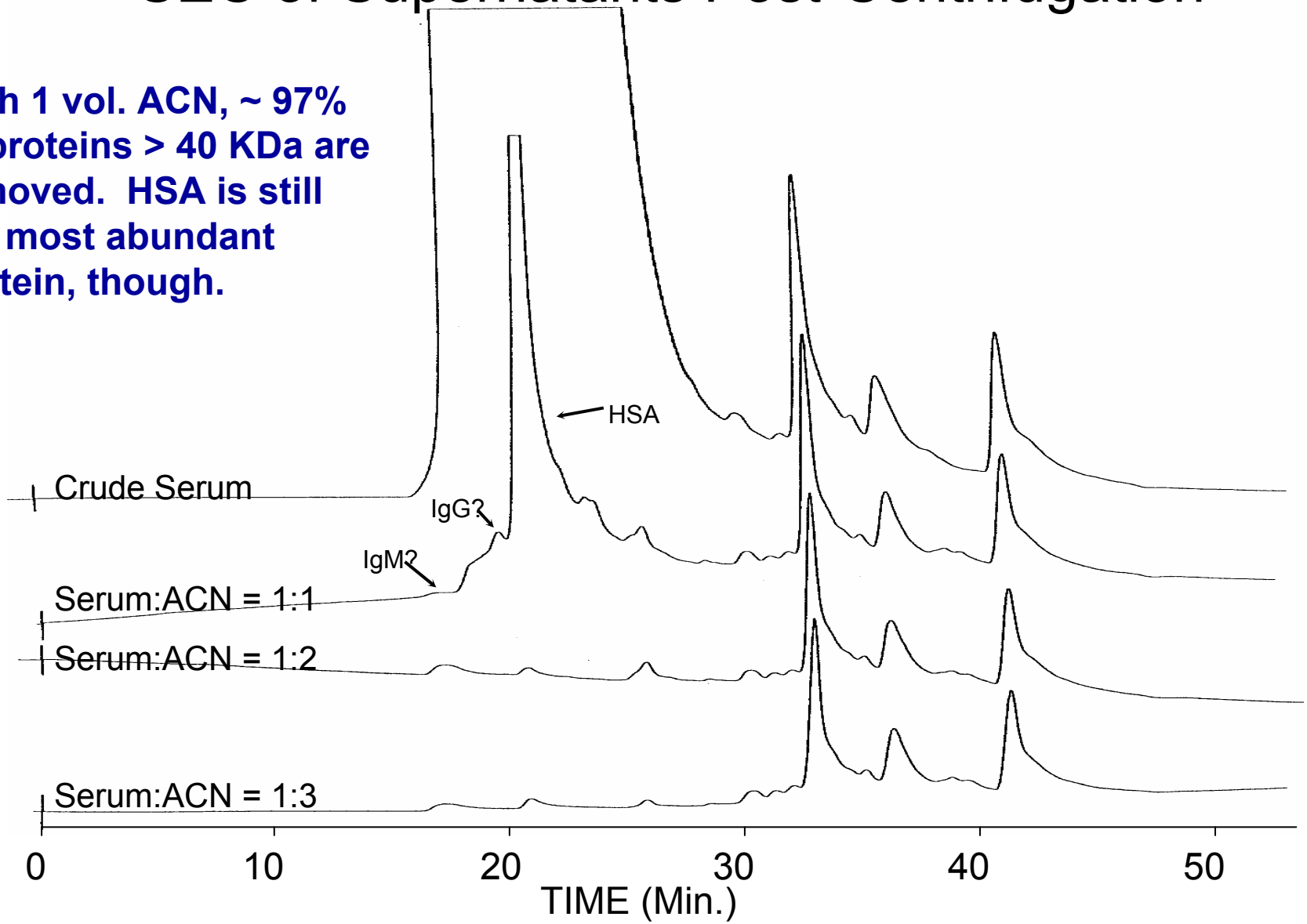

COLUMN: PolyHYDROXYETHYL A (item# 209HY0503, two in series)

MOBILE PHASE: 100 mM $KH_2PO_4$, pH 6.6

# Serum + Acetonitrile

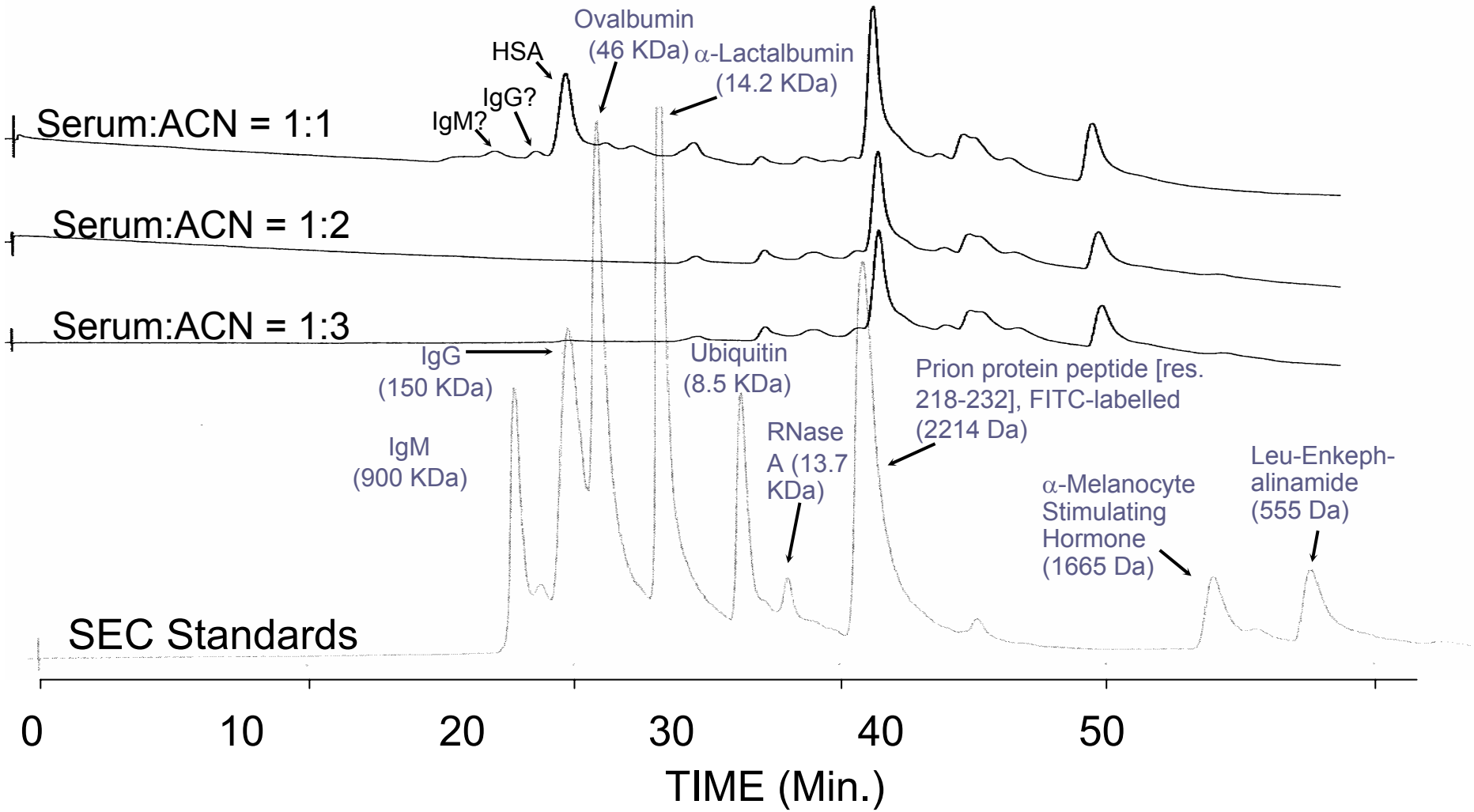
## - SEC of Supernatants Post-Centrifugation -

**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.**

# Suggestions for Analysis of Whole Cells and Membrane Proteins

**1) DISSOLVE PELLET IN HFIP:FORMIC ACID = 60:40.**

**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/$NH_4HCO_3$**

**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.**

# Delipidation of Whole Cell Lysates via HILIC[1]

NEEDED: PolyHYDROXYETHYL A™ SPE cartridge [PolyLC item# SPEHY1203]. Lysates (approx. 2 x $10^7$ cells) or CNBr-digested lysates (approx. 4 x $10^7$ 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

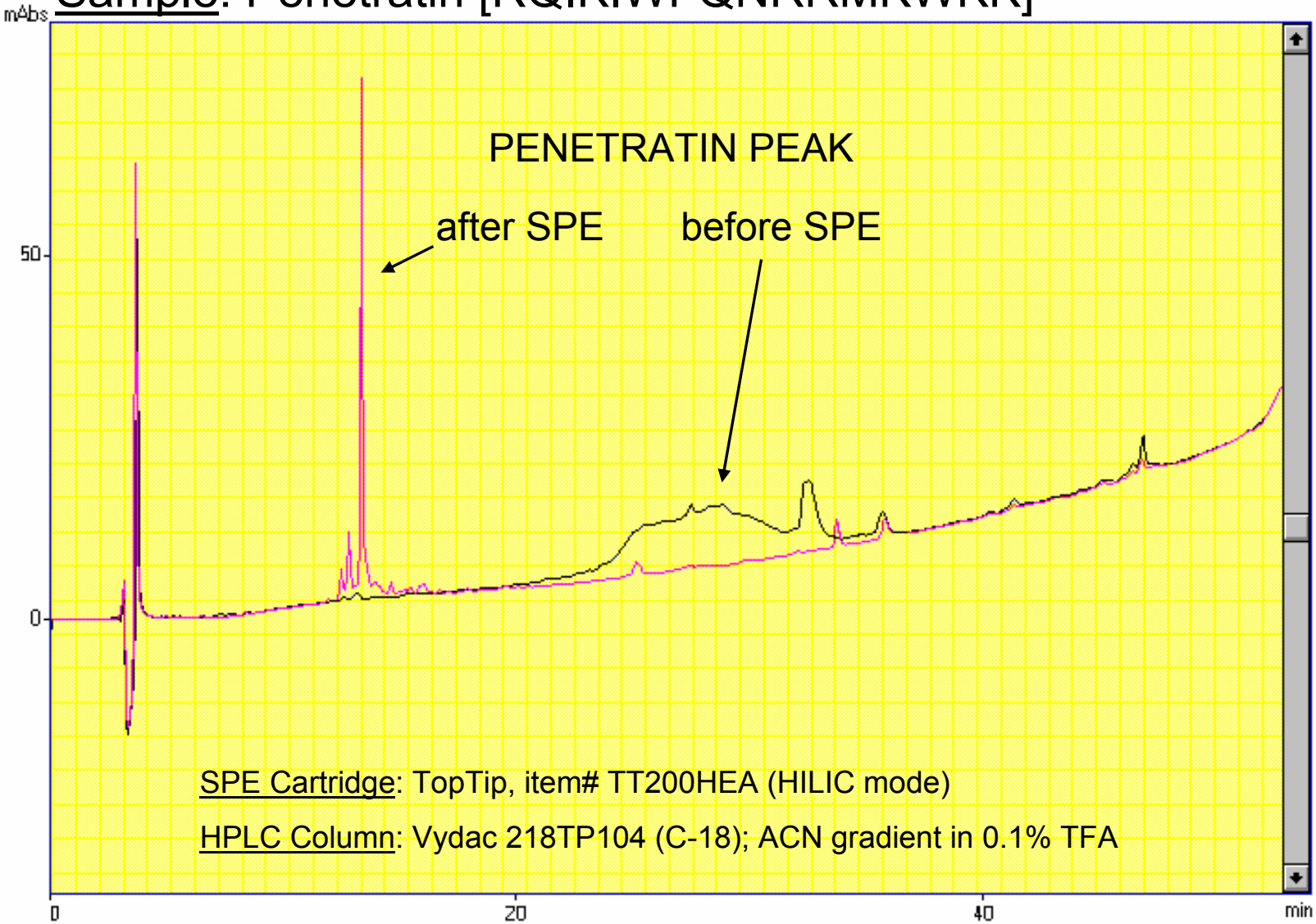
Eluting solution: 0.2% formic acid, 0.1% HFIP.

[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.) -*


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!