Quality Control:
Alternatives to Reversed-Phase Chromatography for Protein and Peptide Analysis

Andrew Alpert
PolyLC Inc.
Columbia, MD U.S.A.
TOPICS

1) Effect of pore and particle diameter

2) Effect of organic solvents

3) Alternatives to reversed-phase HPLC for protein variants
   a) Size-Exclusion
   b) HIC
   c) Ion-exchange
   d) HILIC

4) Alternatives for peptide QC
Growth Factor on PolyCAT A: Comparison of 3- vs. 5-µm

COLUMNS: 35x4.6-mm; 1000-Å

MINOR VARIANTS RESOLVED WITH THE 3-µm COLUMN
Effect of Pore Diameter

- Resolution is better with wider pores –

Sample: rec Human Growth Hormone (21 kDa)

COLUMN: PolyPROPYL A
(Hydrophobic Interaction Chromatography)

- Data courtesy of Benny Welinder, Novo Nordisk -
SCX Analysis of multiPEGylated Protein

- selectivity increases with % solvent -

**Column:** 102SE0510 (PolySULFOETHYL A)

**Mobile Phase:**
- A: 20mM KH2PO4, pH3.0
- B: A + 500mM NaCl

**Gradient:**
- 0% B in 5min, 0% to 20% B in 10 min, 20% B to 40% B in 30min, 40% B to 0% B in 5min and 0% B in 5min

**Flow:**
- 0.3 ml/min
### Effect of Solvents on Protein HPLC

<table>
<thead>
<tr>
<th>PROTEIN</th>
<th>OPTIMUM % SOLVENT</th>
</tr>
</thead>
<tbody>
<tr>
<td>bFGF</td>
<td>0</td>
</tr>
<tr>
<td>Bovine Somatotropin</td>
<td>20</td>
</tr>
<tr>
<td>Many growth factors</td>
<td>30-40</td>
</tr>
<tr>
<td>Histones, Membrane proteins</td>
<td>60-70</td>
</tr>
</tbody>
</table>

Optimal concentration must be determined on a case-by-case basis.
SEC

（separation by size）
SEC-TOF-MS of intact proteins: Reduced Antibody

Mobile phase: 0.1% formic acid, 0.1 ml/min

Column: PolyHYDROXYETHYL A, 250x2.1 mm, 300-Å;

An SEC column was chosen with pores narrow enough for intact proteins to elute in the V₀ peak, which is run into the MS. Total analysis time: 8’.
SEC of Iron-Containing Proteins in Meat

COLUMNS: PolyHYDROXYETHYL A™, 200x9.4-mm; 200- and 500-Å (in series)

- COURTESY OF JAMES MURPHY AND JAMES HARNLY, USDA (Beltsville, MD) -
Screening Combinatorial Libraries for Potential Drug Candidates via **ALIS**: Automated Ligand Identification System

Combinatorial Drug Screening via ALIS:

**Theory**

- Target protein + bound high affinity ligands (sent to mass spectrometer)
- Nonbound low affinity ligands (sent to waste)

**Practice**

- Target protein + bound high affinity ligands (sent to mass spectrometer)
- Nonbound low affinity ligands (sent to waste)

**COLUMN:**
PolyHYDROXYETHYL A; 60-Å

**IMPORTANT:** The Vo peak must elute in < 30 seconds or even high-affinity ligands will start to diffuse off the target protein.
POLARITY MODES OF CHROMATOGRAPHY

Water + Salt  Water  Water + Organic  Organic Solvent

HYDROPHOBIC INTERACTION  LESS POLAR

MORE POLAR  HYDROPHILIC INTERACTION

REVERSED PHASE  NORMAL PHASE
HIC

( Hydrophobic Interaction Chromatography )

Gradient: high to low [salt]; non-denaturing

Elution order: Least to most hydrophobic on the surface of the tertiary (3-D) structure
HIC of Fab and Fc Oxidation Products (Met[O])

COLUMN: PolyPROPYLA, 100x4.6-mm; 3-µm, 1500-Å

GRADIENT: Decreasing [(NH₄)₂SO₄]

∴ HIC is a good way to separate polarity variants of proteins
Ion–Exchange
CATION-EXCHANGE OF PEPTIDES (SCX)

PolySULFOETHYL Aspartamide

PEPTIDE STANDARDS

Column: PolySULFOETHYL A, 5 micron, 4.6 x 200 mm
Buffer A: 5 mM K₂PO₄, pH 3.0, with 25% acetonitrile
Buffer B: Same + 0.25 M KCl

KEY (Net Charge pH 3)
- A = Oxytocin (+1)
- B = [Arg^8]-Vasopressin (+2)
- C = Somatostatin (+3)
- D = Substance P, free acid (+3)
- E = Substance P (+3)
- F = Bovine Pancreatic Polypeptide (+5)
- G = Anglerfish Peptide Y (+6)
- H = Human Neuropeptide Y (+7)

A265

% BUFFER B

TIME (MINUTES)
SCX of Degraded Peptide: Pramlintide (Amylin)

- Good selectivity for charge variants -

SEQUENCE (37 residues):
KCNTATCATQRLANFLVHSS-
NNFGPILPPTNVGSNTY-NH₂

COLUMN: PolySULFOETHYL A

Anion-Exchange of Ovalbumin Phosphorylation Variants

COLUMN: PolyWAX LP (#104WX0510)    SAMPLE: Sigma Grade VI (99%)
GRADIENT: 10 mM K-PO4, pH 7.0; 60-300 mM NaCl in 20’
Preparation of a Weak Cation-Exchange (WCX) material

Polysuccinimide

Aminopropyl-silica

Poly(succinimide)-silica

Poly(aspartic acid)-silica

(PolyCAT A)

- from Alpert, J. Chromatogr. 266 (1983) 23-37 -
PEGylation Positional Variants of TNF Soluble Receptor Type I

COLUMN: PolyCAT A (204CT0510)  
(data courtesy Scott Buckel - Amgen, Inc.)

GRADIENT: 30’, 1-160 mM KCl in 20 mM Na-OAc, pH 5.0, with 20% ACN

Absorbance (280 nm)

Time (minutes)
CEX of Monoclonal Antibody
COLUMN: PolyCAT A (204CT0510)

The 3-peak pattern is characteristic. Minor peaks usually reflect additional deamidation, oxidation or sialylation.

AFTER CARBOXY-PEPTIDASE B TREATMENT

# Lys or Arg lost from C-termini of heavy chains
2
1
0 (native sequence)
CEX of Monoclonal Antibody

COLUMNS: PolySULFOETHYL A (104SE0315)
PEGylated Growth Factor: Comparison of three CEX columns

TSK SP-NPR; selectivity OK

PolyCAT A (#104CT0315); Good selectivity and capacity

TSK SP-5PW; capacity OK

PEGylated

multi-PEGylated

unPEGylated
Degraded PEGylation Reaction Mixture:
Analysis with a PolyCAT A column (item# 104CT0315)

"GIGO":
"Garbage In, Garbage Out"
DEGRADATION OF PROTEINS
- DEAMIDATION AND DEHYDRATION -

1) Favored if the residue on the C-terminal side is unhindered (Gly; Ala)
2) Consequences for biological activity: Negligible to serious!

Aspartic acid

Asparagine

H$_2$O

NH$_3$

Aspartyl linkage

Succinimide

Isoaspartyl linkage

(pKa $\sim$ 4.1)

(pKa $\sim$ 3.1)
CASE STUDY: Degradation of Human Growth Hormone
(from Benny Welinder [Novo Nordisk], WCBP ’98)

191 residues total; pI = 5.0

Asn 149 and Asn 152 (*): prone to deamidation

Asp 130 (**): prone to dehydration
Degradation of rec Human Growth Hormone: Effect of pH

High pH:  
↑ Deamidation of Asn-

Neutral/Acid pH:  
↑ Dehydration of Asp-

COLUMN: PolyCAT A  
GRADIENT: 130-145 mM NH₄–acetate, pH 4.0, with 40% ACN; 30°
DEAMIDATION AND DEHYDRATION OF rHGH

- Effect of pH on Kinetics of Variant Formation -

1) Stability of rec HGH is maximal for formulation ~ pH 6.1.

2) IMPORTANT TO OBTAIN KINETICS DATA LIKE THIS FOR EVERY BIOPHARMACEUTICAL IF DEAMIDATION IS A PROBLEM!
Method Development:
Analysis of a Growth Factor via Cation-Exchange
Growth Factor on PolyCAT A

- Effects of pore & particle size -

3 µm, 300 Å

Best selectivity with small particles & wide pores

3 µm, 1000 Å

Deamidation variants

Met[O] or incorrect -S-S- (biologically inactive)

5 µm, 1000 Å
Growth Factor Variants on PolyCAT A (104CT0315)

- batch selectivity differences -

Lot-to-lot differences $\propto$ difficulty of separation

.: Test different lots to find the selectivity your protein requires
Analysis of Growth Factor on PolyCAT A

- PEG (Polyethylene Glycol) sometimes improves selectivity when added to the mobile phase -
Growth Factor Variants on EDTA-treated PolyCAT A column (104CT0315)

EDTA treatment passivates metal surfaces → ↓ nonspecific protein interactions (but may let 3-µm silica escape the frits!). Test on a case-by-case basis.

- better separation of variants -
A different growth factor yields a similar profile in cation-exchange HPLC
Structure of a Protein in the TGF-β Family (many growth factors):
Two ~ 120-aa Polypeptide Chains Linked in a Cysteine Knot

- Three disulfide (-S-S-) links within each chain
- One disulfide (-S-S-) link between chains
Cation-Exchange HPLC of **BMP-14** (Bone Morphogenetic Protein 14)

COLUMN: PolyCAT A, 100x4.6-mm; 3-µm, 1000-Å (item# 104CT0310; ser# A2562C)

MOBILE PHASE: A) 20 mM K-PO₄, pH 6.0, with 20% ACN;  B) Same + 0.6 M NaCl

GRADIENT: 20’ linear, 0-100% B, then 5’ at 100% B  1.0 ml/min   A220

SAMPLE: 25 µg BMP-14 in 20 µl of (Mobile Phase A:10 mM HCl = 2:1)
BMP-14: Blowup to show deamidation variants
**BMP-14**: More gradual gradient to improve separation of variants; resembles preceding growth factor’s profile

**QUESTION**: Do all recombinant growth factors contain ~ 8% Met[O] or mismatched -S-S- variants (@ cysteine knot motif)?

[Graph showing a new shoulder and another new shoulder]
Method Development:
Analysis of a Sialylated, MultiPEGylated Protein (GCSF) via Cation-Exchange
SCX Analysis of multiPEGylated Protein

- Effect of sialidase treatment -

COLUMN: 202SE0510 0.1 ml/min
MOBILE PHASE: 5 mM K-P04, pH 3.0, with 30% 1-PrOH + 10% 1-BuOH; 0-50 mM NaCl

PER MOLECULE:
4-5 PEGs (at Lys-);
1-2 sialic acid res.

before sialidase

after sialidase
SCX Analysis of multiPEGylated Protein

- Preliminary characterization before sialidase treatment -

**Column:** PolySULFOETHYL A, 200x2.1mm I.D., 5µm, 1000Å

**Mobile Phase:**
A: 5mM KH2PO4, pH 3.0; 30% 1-PrOH, 10% 1-BuOH
B: A + 50mM NaCl

**Flow:** 0.1 ml/min

**Gradient:** 10 – 25 mM NaCl over 80 min
SCX Analysis of multiPEGylated Protein

- Characterization after sialidase treatment; the PAGE analysis permitted post-sialidase peaks to be paired with their pre-sialidase versions -
Mixed-Bed Ion-Exchange HPLC of Intact Proteins
Mixed-Bed IEX of *E. coli* Lysate 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$_2$PO$_4$, pH 6.2, with 5% ACN

**DETECTION:** Fluorescence (λ$_{ex}$ = 280 nm; λ$_{em}$ = 350 nm)

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

**WCX Column + WAX Cartridge ( * = acidic proteins?)**
Acidic proteins; Basic proteins;
Use anion-exchange Use cation-exchange

pI Distribution of the Predicted Mouse Proteome

From: H. Wang et al.,
J. Proteome Res. 5
(2006) 361
Fig. 1. Comparison of regular IEX columns to the mixed-bed column. Sample: Yeast lysate. All materials were 5-µm, 1000-Å pore diameter. The mixed-bed column was 200x4.6-mm while the others were 100x4.6-mm. Flow rate: 1 ml/min. Detection: A280. Gradient: 0-300 mM NaCl in 20 mM MES, pH 6.0.

A complex mixture of proteins always contains some that elute in the void volume on a single IEX column. With a mixed-bed column the number that do this is minimal\textsuperscript{1,2}. This helps to insure more uniform distribution of the proteins for proteomics fractionations.
Fig. 2. Mixed-bed IEX of yeast lysate with a volatile mobile phase.

COLUMN: Same mixed-bed as in Fig. 1. DETECTION: 280 nm
MOBILE PHASE: 20-800 mM ammonium acetate, pH 6.0  FLOW: 1 ml/min
GRADIENT: 0-12’: 0-10%B; 12-30’: 10-60%B; 30-40’: 60-100%B 40-50’: 100%B
Example of Sample Simplification via General-Purpose Chromatography: Mixed-bed IEX-SPE of serum

HPLC: Mixed-bed column (204CTWX0510)   1 ml/min.
Gradient: 3-segment, 20-800 mM NH$_4$-OAc, pH 7.0   A280
Sample: 25µl serum + 75 µl 10 mM NH$_4$-OAc
Fractionation of serum via mixed-bed IEX-SPE

Analysis: Mixed-bed HPLC column
Gradient: 20-800 mM NH$_4$-OAc, pH 7.0

Fractionation seems to be lossless
Fractions from mixed-bed IEX-SPE of serum - blowup

SPE: 100 µg TopTip™ of PolyCAT A & PolyWAX LP; TT1000CATWAX-2015
HPLC: Mixed-bed column (204CTWX0510) 1 ml/min. A280 Gradient: 20-800 mM NH₄-OAc, pH 7.0
Samples: 100µl of 200µl collected fraction (Filtrate: 100µl of 400µl)

Result: reproducible fractions with minimal overlap that elute in predictable ranges
SPE-IEX Fraction 4 on Mixed-Bed IEX Column (fast flow): Control, Gout and Rheumatoid Arthritis Serum Samples

Sample: 6 mg. of each serum → 300 µl each Fraction 4; 100 µl analyzed per run

Gradient: 300-800 mM NH₄-OAc, pH 7.0 (load at 20 mM)  
Column: 102CTWX0510
Flow: 0.8 ml/min  
Detection: 280 nm  
UHPLC System: SSI Corp.
HILIC

（Hydrophilic Interaction Chromatography）
HYPOTHETICAL PARTITION MECHANISM OF HYDROPHILIC-INTERACTION CHROMATOGRAPHY (HILIC)

MOBILE PHASE (mostly organic)

MOBILE PHASE (stagnant; mostly aqueous)

Solute


SILICA
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)
Sample: 2-PrOH/ACN (pH 3.7) extract of bovine heart mitochondria
Column: PolyHYDROXYETHYL A (100 x 2.1 mm)
Gradient: 20 mM ammonium formate, pH 3.7, + 0.5% HFIP; (63% 2-PrOH + 22.5% ACN) to 30% 2-PrOH


Courtesy of John Walker, Medical Research Council Dunn Human Nutrition Unit, Cambridge, U. K.
HILIC of Intact Apolipoproteins: Separation of Glycation Variants

Mobile Phase A: 50 mM NH4-formate, pH 3.7, in 50% 2-PrOH, 25% ACN, & 0.5% HFIP
Mobile Phase B: 50 mM formic acid + 10% 2-PrOH + 0.5% HFIP + ~ 5 mg/L Trp
Flow: 1 ml/min. Temp: 24°C Gradient: 0-5’: 0% B; 5-35’: 0-100% B

COLUMN: PolyHYDROXYETHYL A, 200x4.6-mm; 5µm, 300-Å
SAMPLE: 100 µg

COLUMN: TSK Amide-80, 250x4.6-mm; 5µm, 80-Å
SAMPLE: 1 mg apoM

---

apoA-I (human)
apoM (human)
Cyt C (equine)
HILIC on an Ion-Exchange Column

~ 60-70% organic solvent present during the salt gradient

RESULT

Hydrophilic interactions superimposed on electrostatic effects
ERLIC of synthetic peptide: LIFAGKQLEDGR

COLUMN: PolyWAX LP, 200x4.6-mm, 5µm, 300Å

MOBILE PHASE: 20 mM sodium methylphosphonate, pH 2.0, with %ACN as noted
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 NaClO₄, pH 3, with 70% ACN
B) Same but 100 mM NaClO₄

DETECTION: A215

SAMPLE: 24 µg protein/80 µl
Histone H4 Acetylation & Methylation Variants

COLUMN: PolyCAT A (104CT0315; HILIC mode)

The most minor variants can be the most critical

MITOSIS

INTERPHASE

(courtesy James Pesavento - U. of Ill.)
Histone H3(1-50) *ex* HeLa Cells; Acetylation & Methylation Variants

HILIC on PolyCAT A; $\uparrow$ [NaClO$_4$] and $\downarrow$ [ACN]

(courtesy Benjamin Garcia - U. of Ill.)
Chicken Erythrocyte Histone H1 Isoforms on PolyCAT A

CEX: 0% ACN

CEX: 40% ACN

CEX: 70% ACN - much better selectivity!

Retention time (min.)

Buffer acetonitrile content (% v/v)

Absorbance 214 nm

Time (min.)

10 mM Na-PO₄
0.6 M NaClO₄
70% CH₃CN
70% CH₃CN + 0.6 M NaClO₄

10⁻³ x [θ] (degrees cm² dmol⁻¹)

Wavelength (nm)
**Hemoglobin FASC Standard:** Fast Flow Analysis

COLUMN: PolyCAT A, 100x3.0-mm; 5-µm, 1000-Å  
FLOW: 3.5 ml/min  
$A_{415}$  
Pressure: 5379 psi

GRADIENT: 0-2’: 11-45% B; 2-2.5’: 45-100% B

A) 20 mM Bis-tris + 2 mM KCN, pH 6.96  
B) 20 mM Bis-tris + 2 mM KCN + 200 mM NaCl, pH 6.55