Multiplelabs
Turnkey Laboratories Solutions

Analytical®
Technologies Limited

Portable-HPLC™

Complete, Portable High Pressure Gradient HPLC System

- Compact, Low Cost and Mobile for the Lab or Field
- Fully Featured HPLC – High End Components
- 255 nm Fixed Wavelength Detector or any other application
- Manual Injector with Replaceable Injection Loop
- Binary High Pressure Gradient Pumps
- On Board Reagent and Waste Bottles
- Tablet PC or Laptop with Installed Control Software
- Easy End User Service or Send the Entire HPLC to Factory for Repair – Just Close the Case and Ship!

EPCC / PRODUCTS / APPLICATION / SOFTWARE / ACCESSORIES / CONSUMABLES / SERVICES

Analytical Technologies Limited

An ISO 9001 Certified Company

www.analyticalgroup.net

The Portable-HPLC instrument offers a complete HPLC system, with all the functionality required by any laboratory, packaged in a portable, ergonomic hard shell case, specifically designed to run most chromatography applications. The Portable-HPLC system is rugged and compact, and includes in a single enclosure: Pumps, Fluid Path, Manual Injector, Column Holder, High Performance Detector, and Tablet PC with full instrument control and data acquisition software (pre-installed and tested). Additional features include: On-Board Reagent, Wash and Waste Containers, External USB Ports, 100 – 240 VAC Power Source, Automotive Power Jack, and Storage Compartment for power sources, injection syringe and sample prep items.

The Portable-HPLC is easy to service with all major components (Pumps, Detector, Injector and other key parts) easily accessible for service or swap-out replacement. A hinged door exposes the interior, and the chassis is removable from the case with two fasteners and quick disconnect electrical/fluid fittings. Alternatively, the entire system can be readily sent to the factory for repair. Just close the case and ship!

The Portable-HPLC is designed and built for consistent performance and reliability. Automatic Piston Wash with on-board Wash Bottle, provides extended pump seal life. Reagents are filtered to prevent fluid clogging. Pump seal change, filter replacements and other maintenance items are trouble-free with easy access to Pumps and other components. The Injection Valve has a replaceable sample port and an external injection loop. The system has rugged fittings on all bottles and fluid path connections to prevent leakage during system transport.

Easy Component Serviceability through Access Door

Specifications & Capabilities:

Reagent Pumps and Flow Path	
Flow Rate Range:	0.001 – 5.000 mL/min
Flow Rate Accuracy:	< +/- 2% of Set Point
Pressure of Operation:	3,000psi
Binary High Performance Pumps	
Automatic Piston Wash	
Prime Purge Valve	
Analog Pressure Gauge	
High Pressure Injector with External Loop	

Detector	
Flow Cell:	10 mm (tapered); PEEK; 15 µl Volume
Noise:	< 3 x 10-5 AU RMS
Drift:	< 1.5 x 10-4 AU RMS
Data Rate:	Up to 40 Samples/sec
255 nm Fixed Wavelength Standard	
Easily Interchangeable with Other Wavelength Models	
LED Light Source – USB Powered	
50,000+ hours of Operation	
Virtually No Requirement for Light Source Replacement	

Interactive User Interface
Dell Venue 8 Tablet PC
Windows 10 Operating System
Clarity Instrument Control & Data
Binary High Pressure Gradients
Simple New Method Development
Automatic Peak Detection and Area Integration
Detector Zero and Calibration
Comprehensive Report Generation

Injector

Detector

Pump

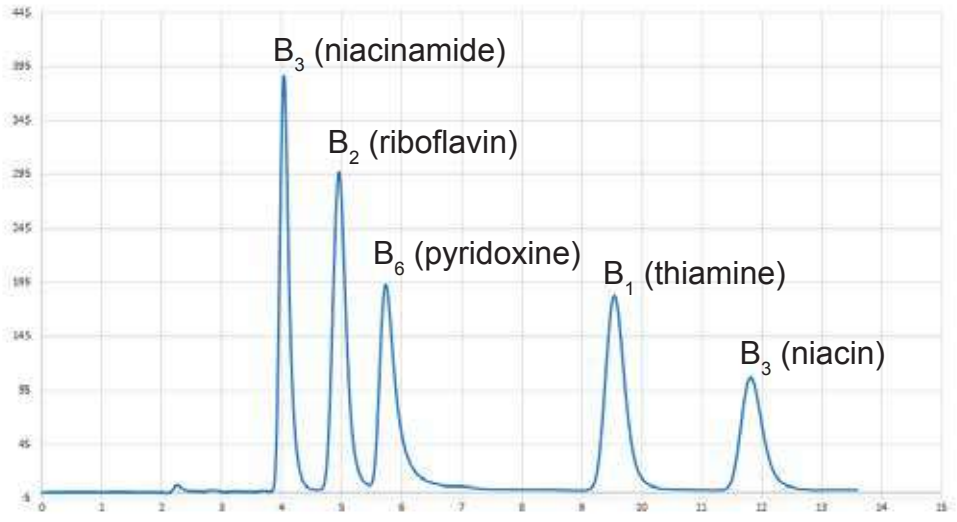
All Portable-HPLC critical components are individually available and easily replaceable by the user

Physical Specifications	
Dimensions:	16.20" x 12.70" x 6.60" (L x W x H) completely enclosed in Pelican Case
Weight:	25lbs (11.3Kg)
Electrical:	100-240 VAC, 50/60 Hz; or 12 VDC Power Source (3.0A) – Optional Mobile Power Pack

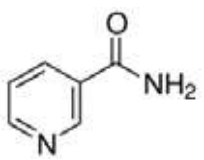
Optional Items:

- 280 and 415 nm Wavelength Detectors - easily interchangeable
- Custom Software and Applications
- Higher Flow Pumps and Isocratic Models
- Battery Pack for 9 hours of continuous operation in the field without access to other power source
- 12-Volt Automobile Adaptor for remote locations

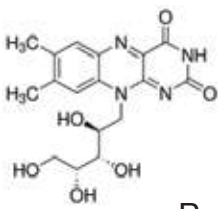
ANALYSIS OF B-VITAMINS VIA HILIC
Column: HYDROXYETHYL A (204HY05006)



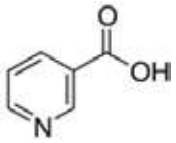
Niacinamide (B_3)



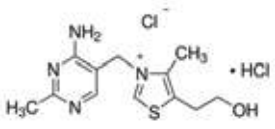
Riboflavin (B_2)



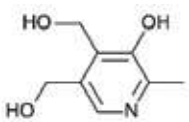
Niacin (B_3)



Thiamine (B_1)



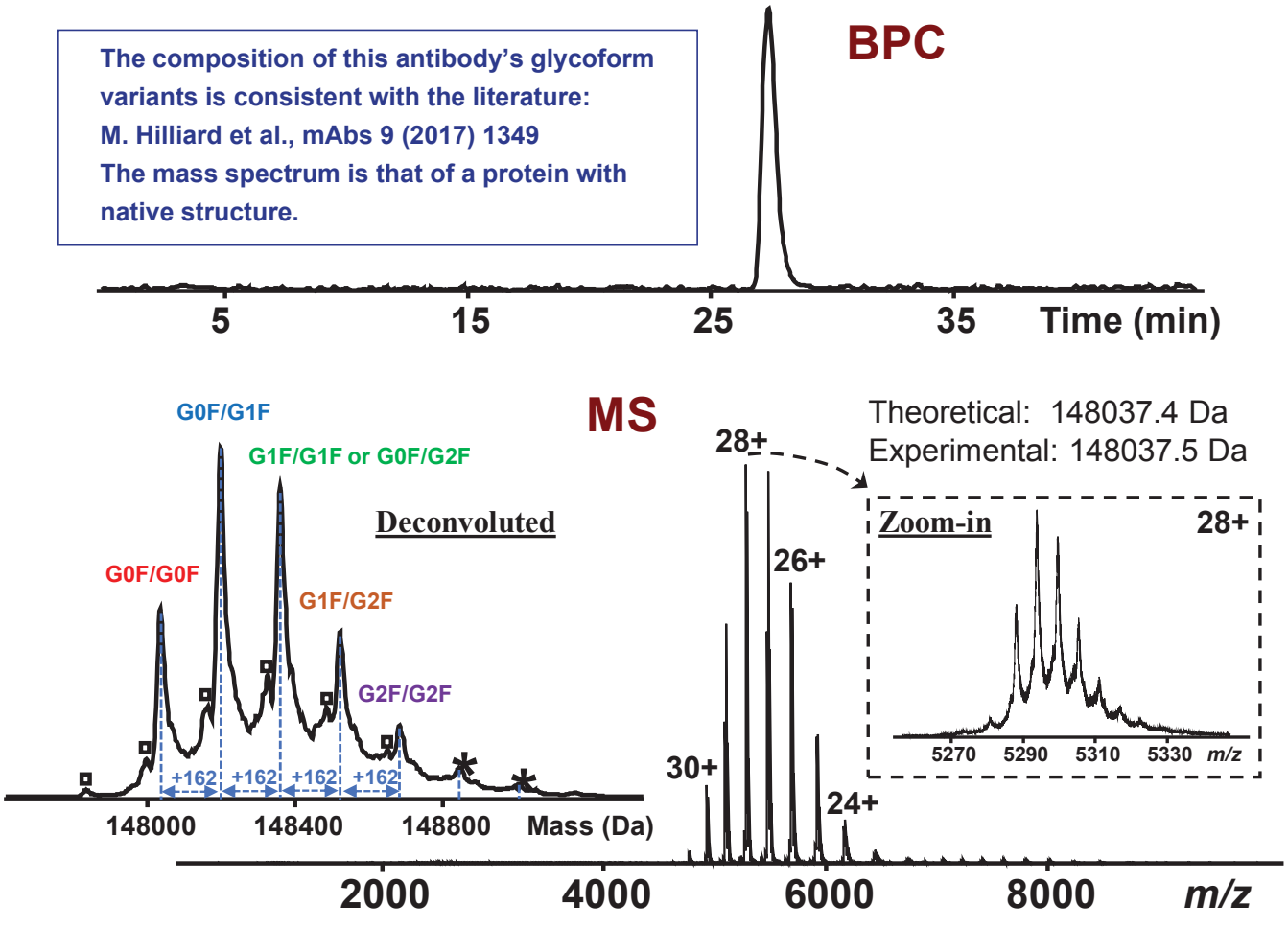
Pyridoxine (B_6)



HIC-MS of Antibodies!

- Sample: NIST mAb standard 8671(IgG1k)
- Column: PROPYL A capillary, 100x0.2-mm, 2-µm, 1000-Å
- Flow rate: 2.6 µl/min
- Mobile Phase: Decreasing gradient of NH4-OAc
- MS: maXis II QTOF (Bruker)

The composition of this antibody's glycoform variants is consistent with the literature:
M. Hilliard et al., mAbs 9 (2017) 1349
The mass spectrum is that of a protein with native structure.

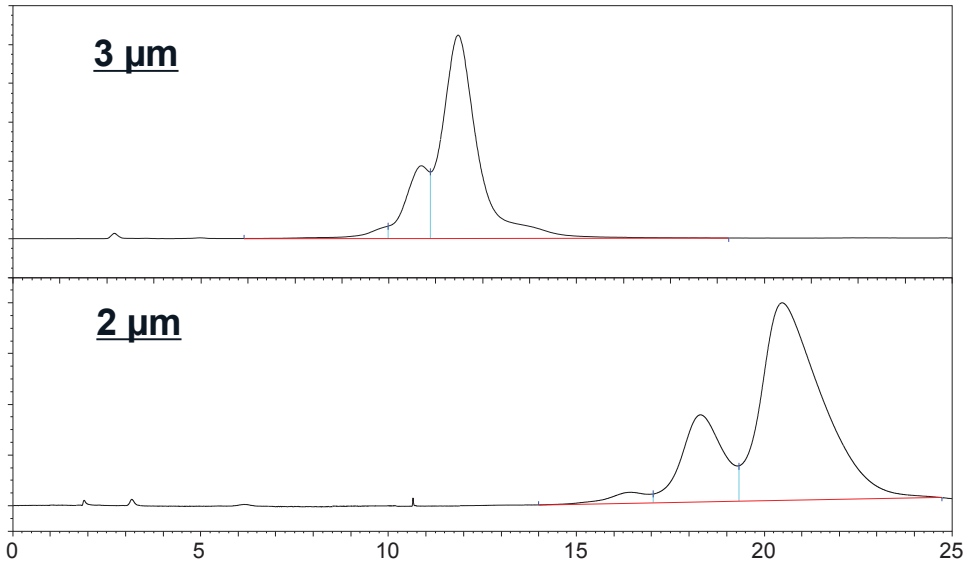


□ -203 Da (-GlcNAc)

* +162 Da

HIC of mAb: 3- vs. 2-µm columns

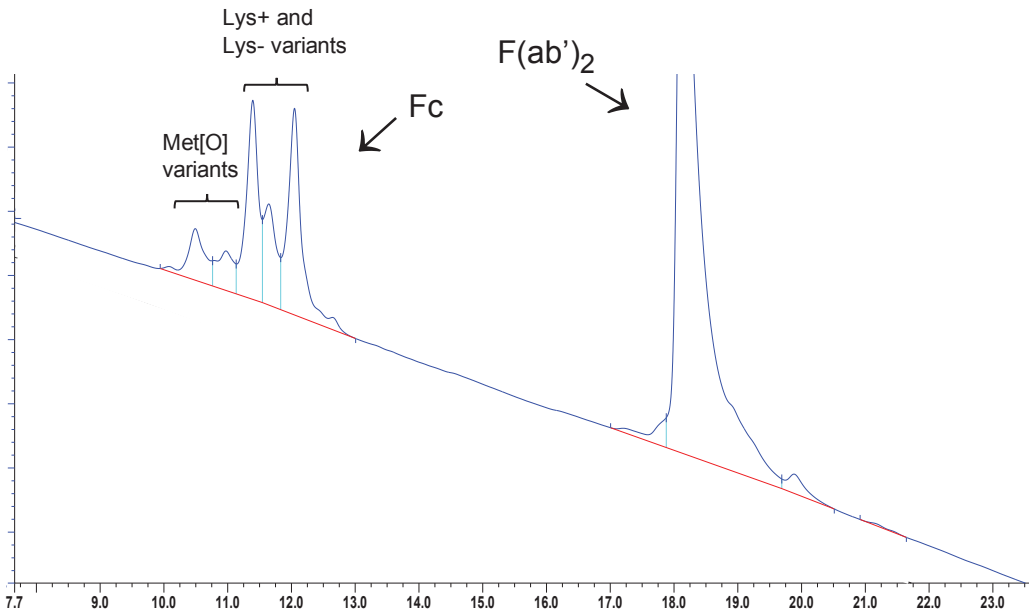
• Column: PROPYL A, 100x4.6-mm, 1000-Å pore diameter;
decreasing NH_4-SO_4 gradient (regular HIC mode)



Oxidized IdeS digest of mAb:

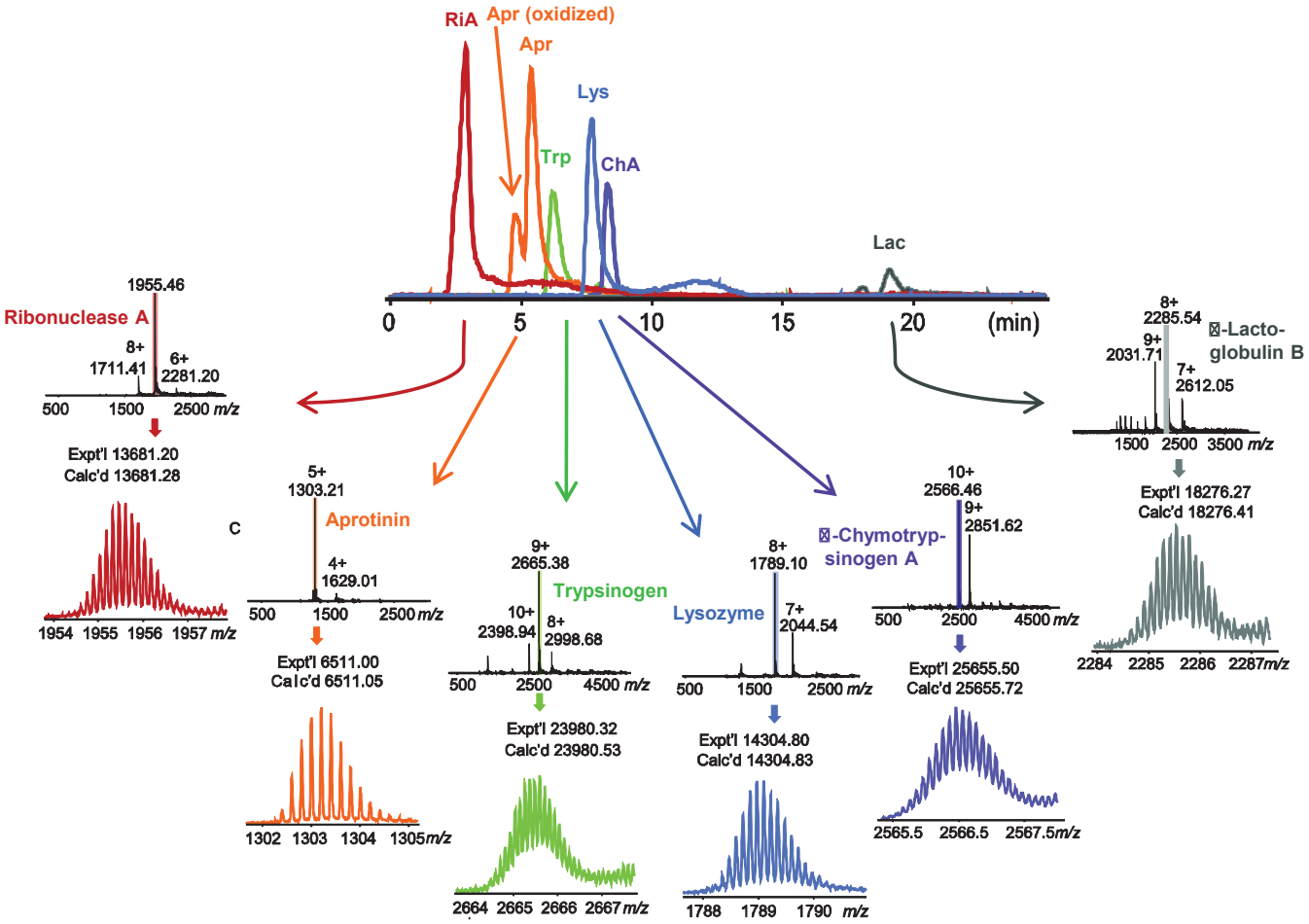
RPLC cannot separate the unoxidized Fc fragment with one C-terminal Lys residue from the form with one oxidized Met residue, but our 2-µm HIC columns can!

IdeS digest of oxidized mAb on a PROPYL A column; item# 104PR0210



NEW! HIC-MS for Top-Down Proteomics

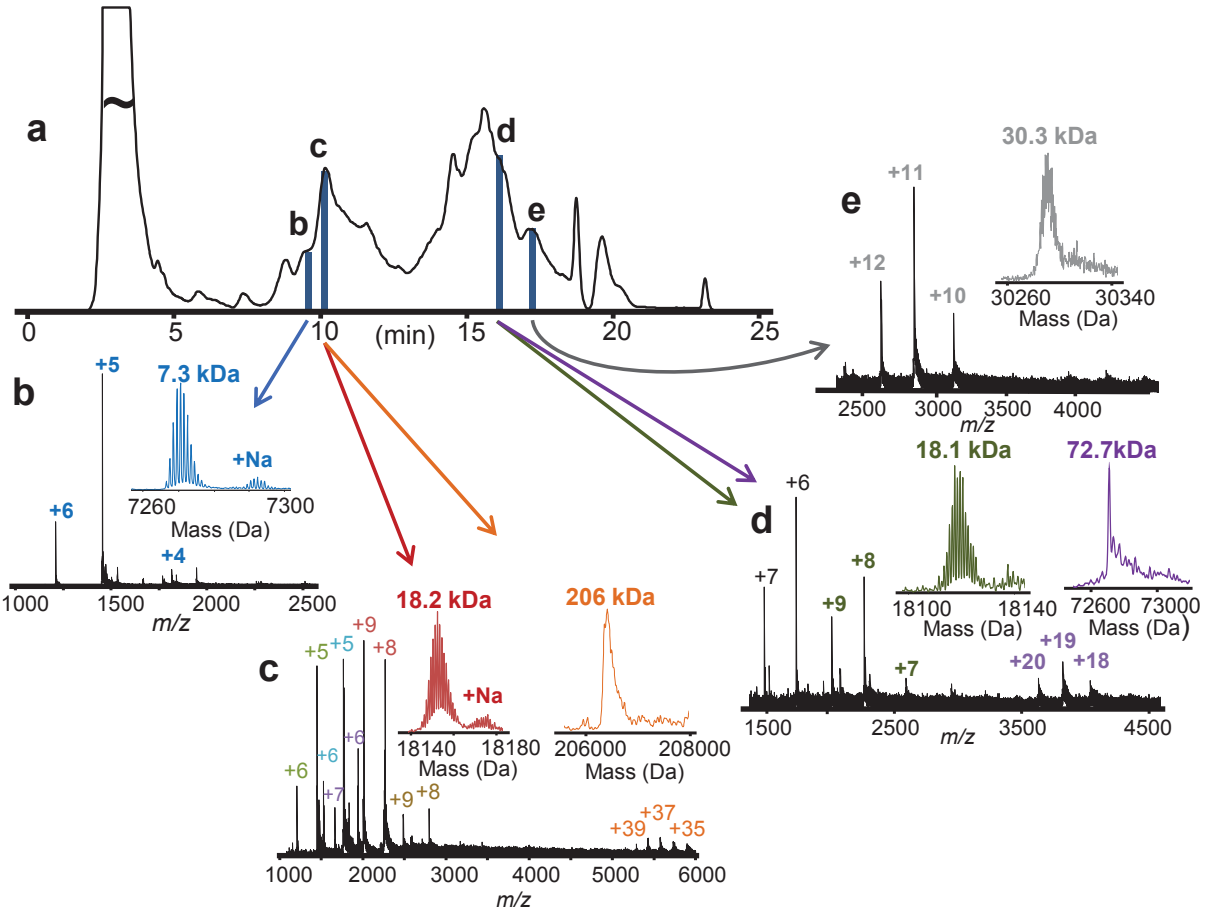
Our new series of materials for HIC (Hydrophobic Interaction Chromatography) permit the retention of proteins using concentrations of ammonium acetate that are compatible with direct analysis via mass spectrometry. Proteins can be separated and eluted with their native structures intact. This is useful for top-down proteomics and permits the analysis of some proteins that are not compatible with the conditions of reversed-phase chromatography. The example below shows base peak chromatograms of some protein standards, their mass spectral charge state patterns, and isotopic resolution in the deconvoluted mass spectra. Note the separation of aprotinin from an oxidized variant.



- Column: HEPTYL A capillary, 100x0.2 mm; 3-µm, 1500-Å
- Mobile Phase: A) 1 M ammonium acetate; B) 20 mM ammonium acetate with 50% ACN
- Gradient: 15' linear, 0-100% B, then 5' at 100% B
- Flow: 2.4 µl/min
- Detection: maXis™ Plus Q-TOF mass spectrometer (Bruker Daltonics)

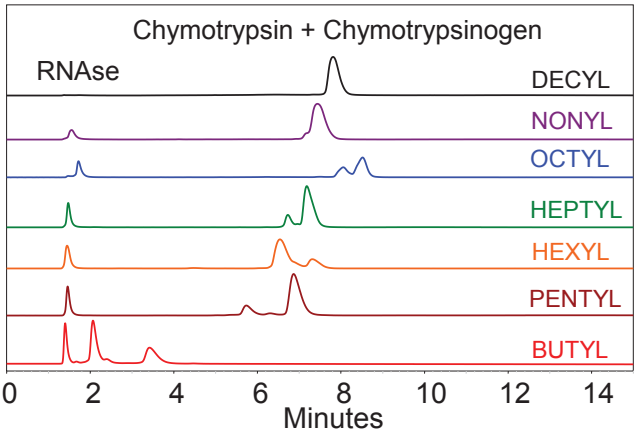
HIC-MS of E. coli lysate

• Column: HEXYL A capillary, 100x0.2-mm; 3-µm, 1500-Å



Comparison of different HIC materials

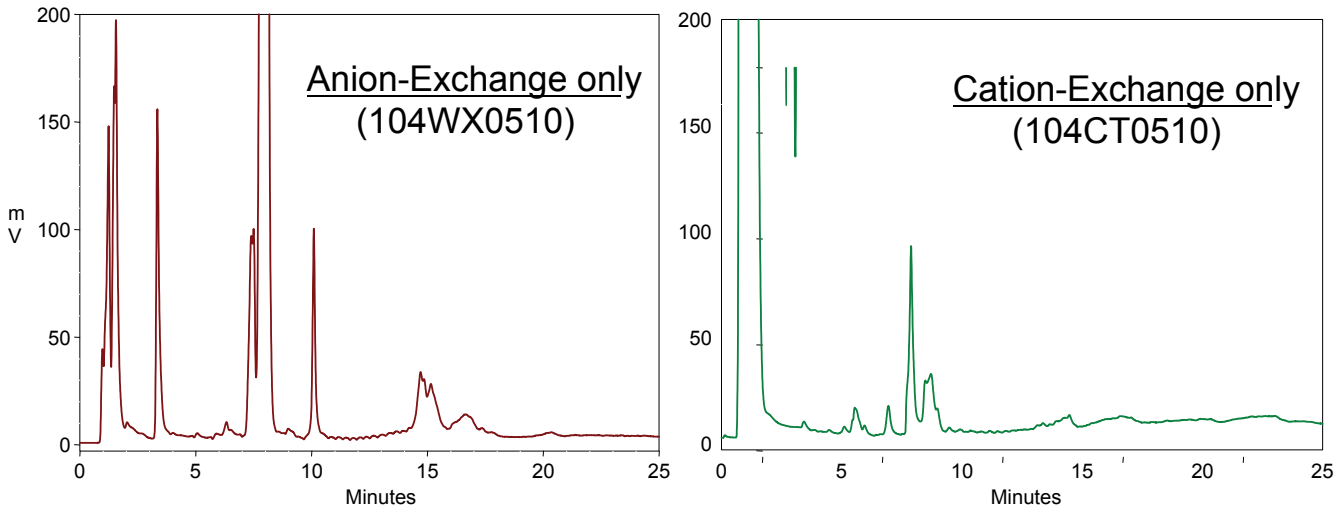
Conventional HIC materials like BUTYL A are not hydrophobic enough to retain many proteins well with Am-OAc buffers. Our PENTYLA and more hydrophobic new materials do retain them. However, sensitive proteins (here, RNAse) may denature on OCTYLA and more hydrophobic materials before they can elute. The new method is probably going to be implemented using PENTYLA, HEXYLA, and HEPTYLA. A particularly hydrophobic protein, such as an antibody-drug conjugate (ADC), might best be run using PENTYLA or even BUTYL A.

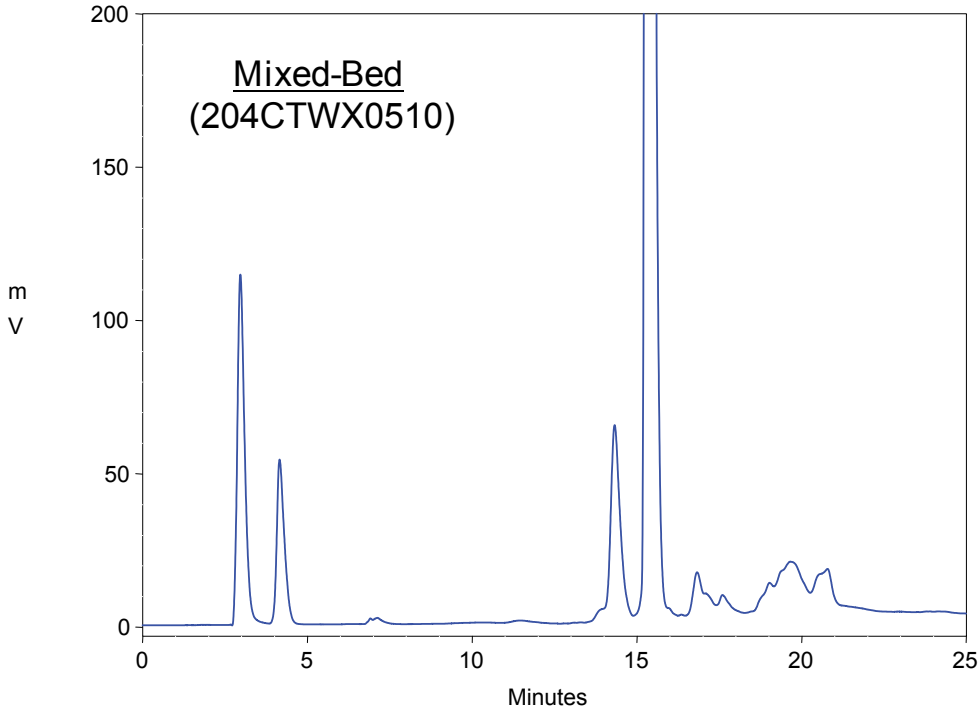


BUTYL A, PENTYL A, HEXYL A, HEPTYL A, OCTYL A, NONYL A, and DECYL

Mixed-Bed Ion-Exchange of Proteins

With complex protein mixtures like lysates or serum, some protein will elute in the void volume from any single ion-exchange column. With a mixed-bed column, though, almost all proteins are retained, per the following example (a yeast lysate with a NaCl gradient in MES buffer, pH 6):

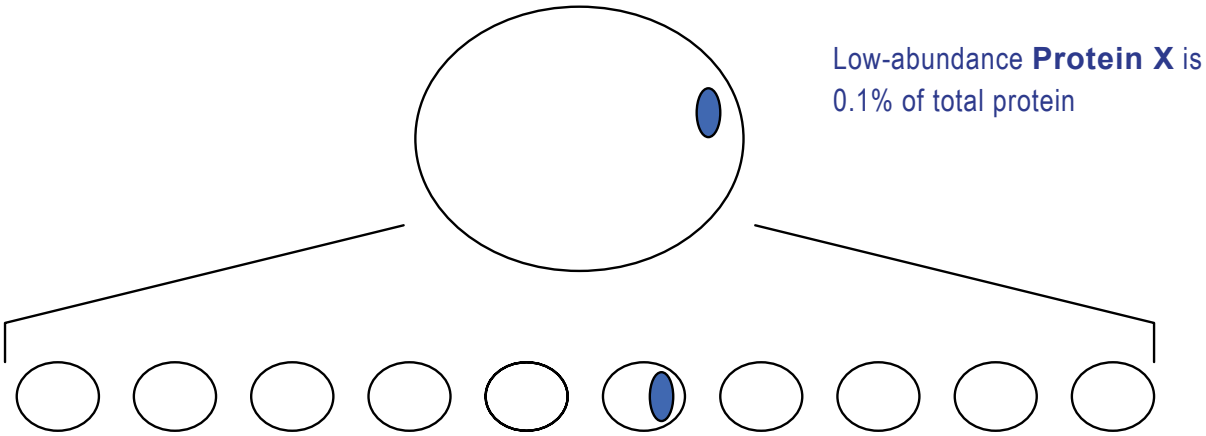




The 200x4.6-mm mixed-bed contains the cation-exchange (CAT A) and anion-exchange (WAX LP) materials in equal amounts. Particle diameter: 5 µm. Pore diameter: 1000 Å.

Why fractionate intact proteins for proteomics? The schematic below rationalizes the increase in protein identifications that result:

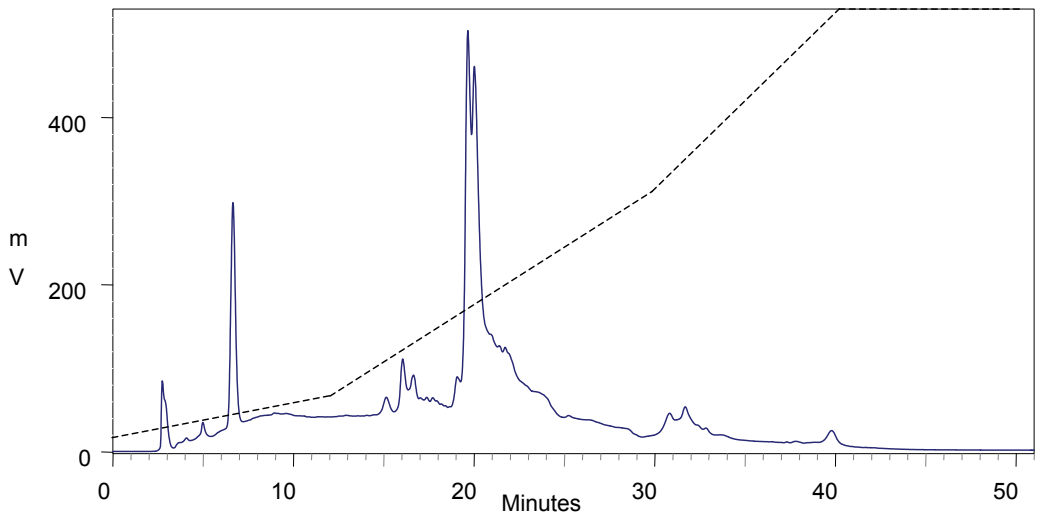
Fractionating Intact Proteins Increases Detection of Peptides from Proteins of Low Abundance



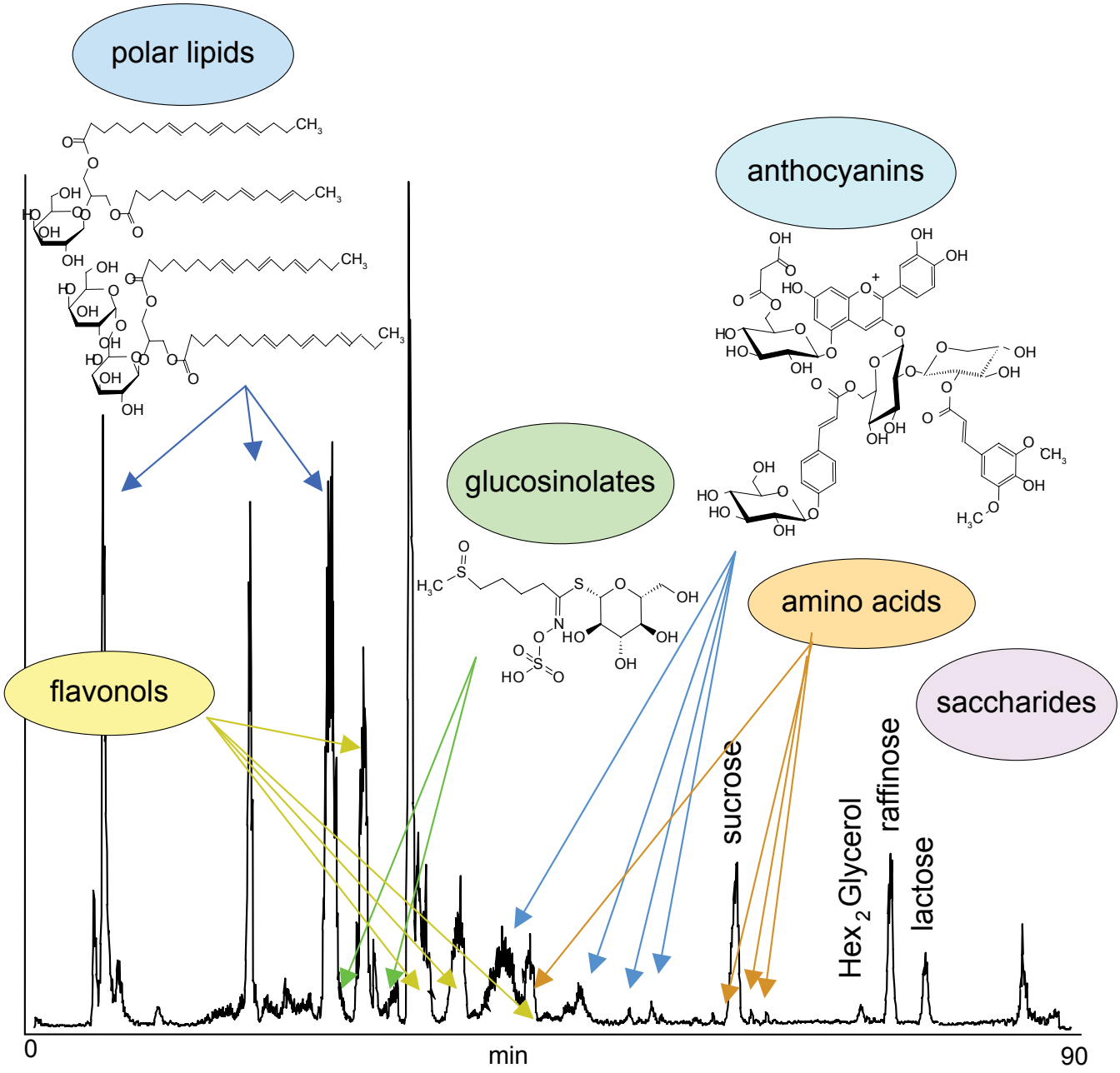
Now Protein X is 1.0 % of total protein in Fraction #6. After digestion, its peptides will be 10x higher a percentage of the total in that fraction than would have been true in a digest of the unfractionated mixture. That greatly increases the chances of identifying Protein X through 2-3 of its fragments rather than just one.

It is also possible to use volatile mobile phases for protein ion-exchange:

Serum Proteins on a Mixed-Bed Ion-Exchange Column - gradient from 20-800 mM NH_4OAc, pH 7 -



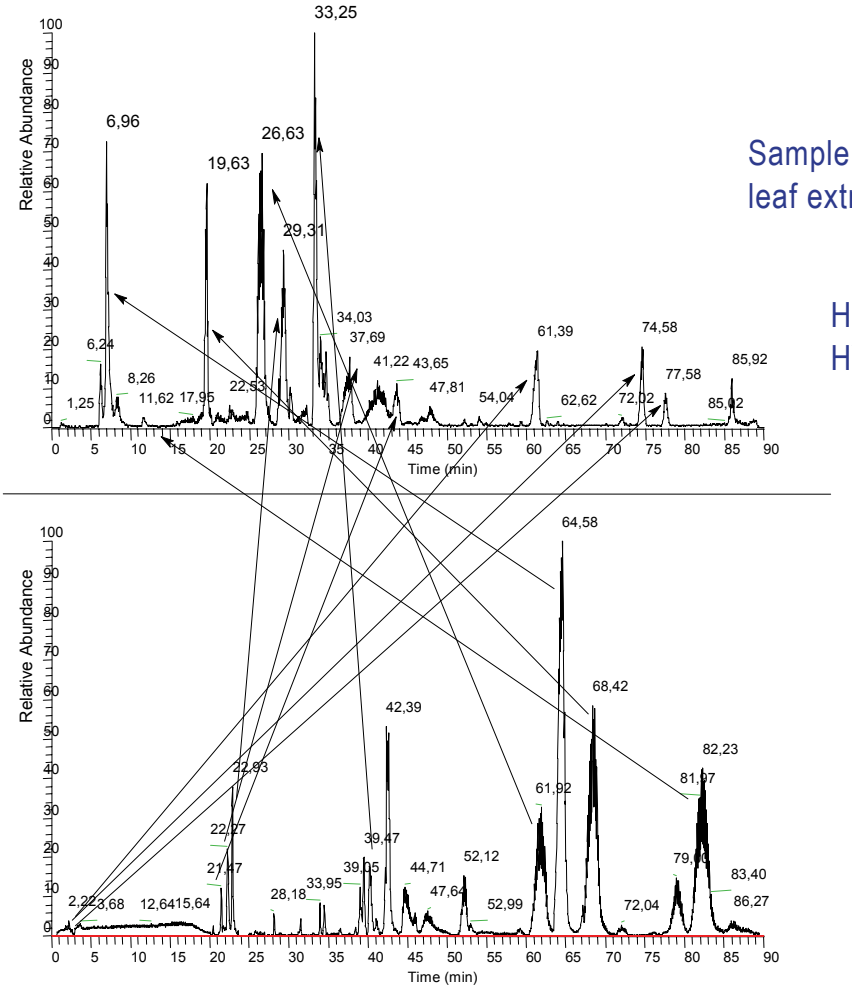
METABOLOMICS 2004



Arabidopsis thaliana leaf extract

HILIC-ESI-MS with a HYDROXYETHYL A capillary 3 µm, 100 Å, 150x0.32 mm
Gradient: Decreasing ACN (Courtesy of V.V. Tolstikov, Max Planck Institute-Potsdam)

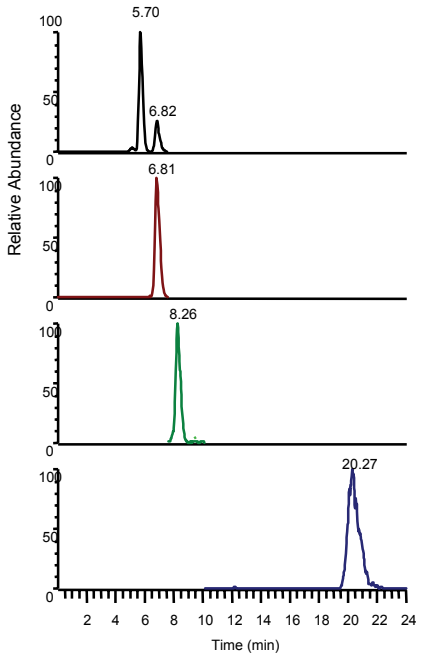
THE WORST-RETAINED SOLUTES IN REVERSEDPHASE ARE THE BEST-RETAINED IN HILIC!



Sample: Arabidopsis thaliana leaf extract

HILIC mode: Capillary of HYDROXYETHYL A

RPC mode: Capillary of C-18 Hypersil

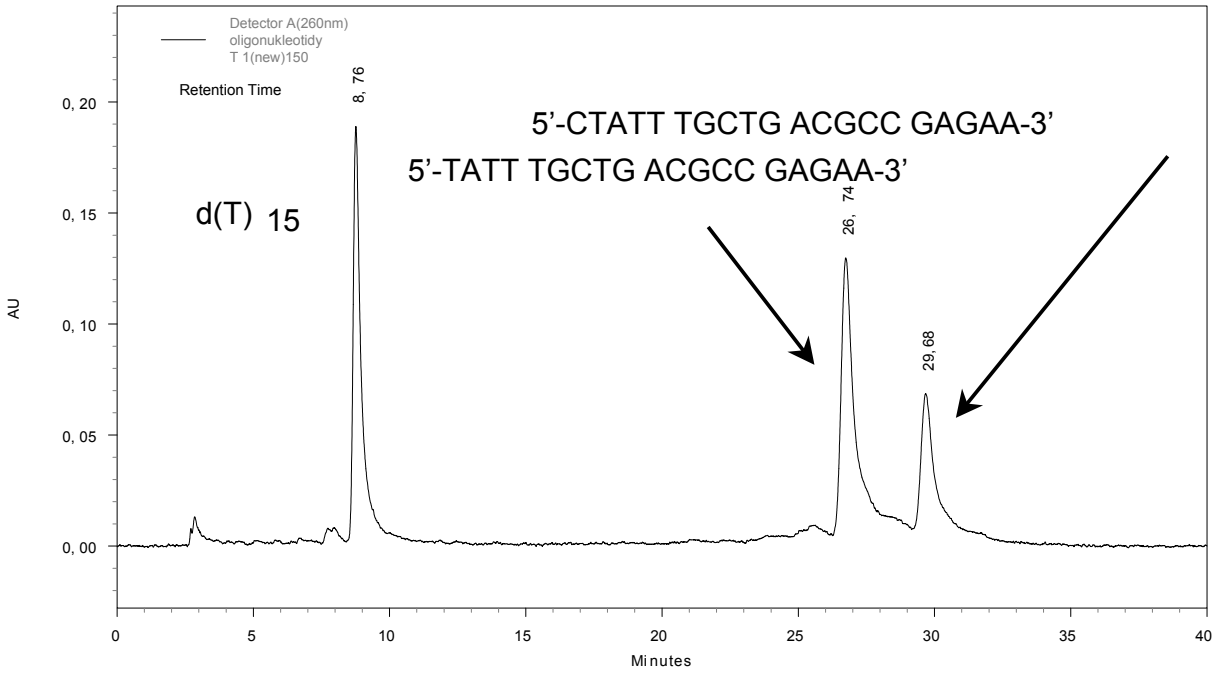


HILIC-MS/MS -
Isocratic analysis of individual compounds from serum or crude extracts in minutes!

Sample: Crude soybean extract on HYDROXYETHYL A column

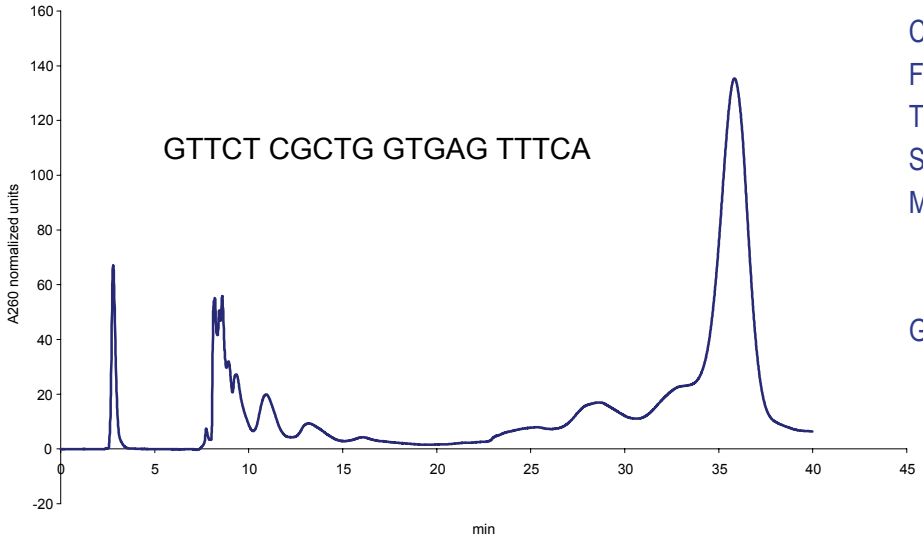
Nucleic Acids

Anion-exchange: Excellent separation of oligonucleotides by size!



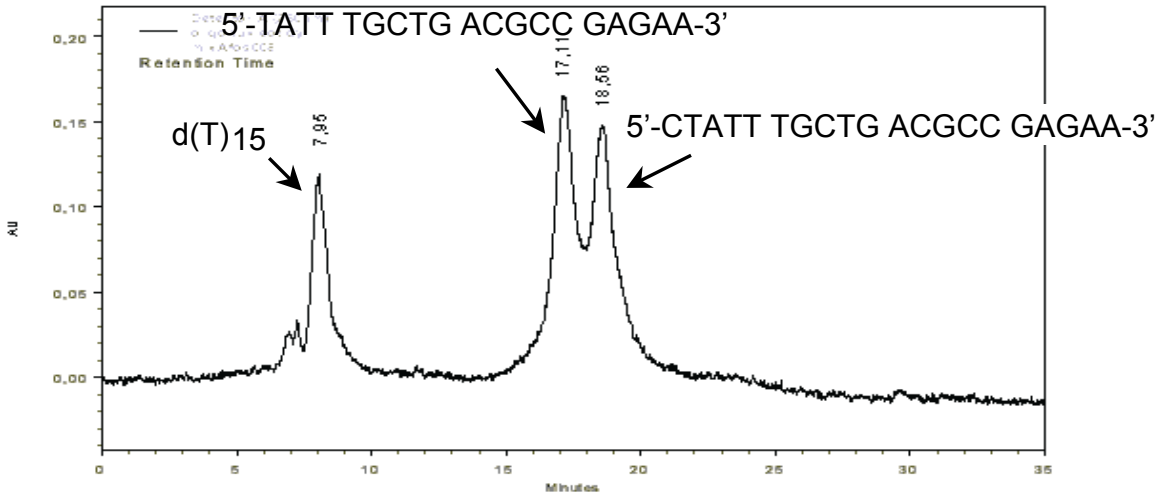
COLUMN: WAX LP, 100x4.6-mm; 3 µm, 1500-Å (item# 104WX0315)
Mobile phase: A) 25 mM Tris-HCl, pH 8.0, with 30% ACN; B) Same + 1 M NaCl
Gradient: 60-100% B in 50'. Flow rate: 0.5 ml/min. Temp: 60°C. Detection: A_{260}

Crude phosphorothioate: Good selectivity for failure sequences



COLUMN: 104WX0315
Flow rate: 0.5 ml/min
Temp: Ambient
Sample: 1.7 µg/25 µl
Mobile Phase: A) 25 mM Tris, pH 8, with 30% ACN
B) 1 M $NaClO_4$ in A
Gradient: 0-3': 0% B
3-4': 0-60% B
4-34': 60-100% B
34-38': 100% B

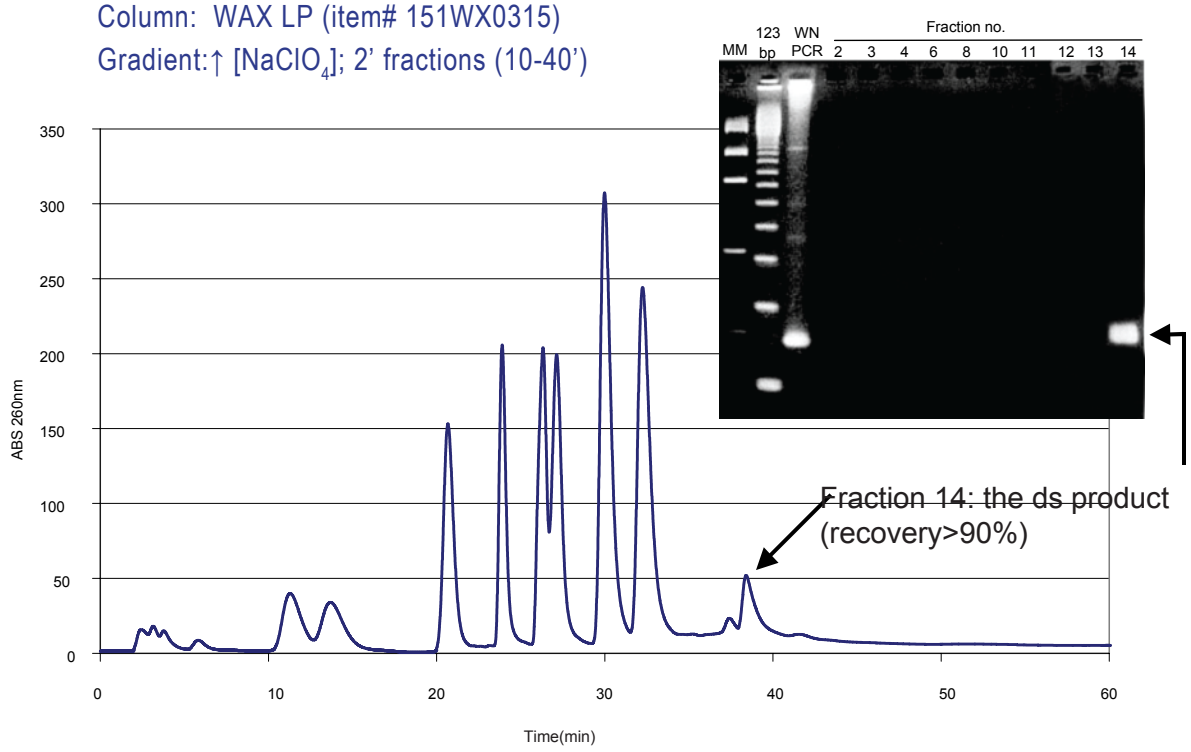
Phosphorothioates by **Hydrophilic Interaction Chromatography**



COLUMN: HYDROXYETHYL A, 200x4.6-mm; 5 µm, 300 Å (#204HY0503)
Gradient: 75-60% ACN in 100 mM TEAA

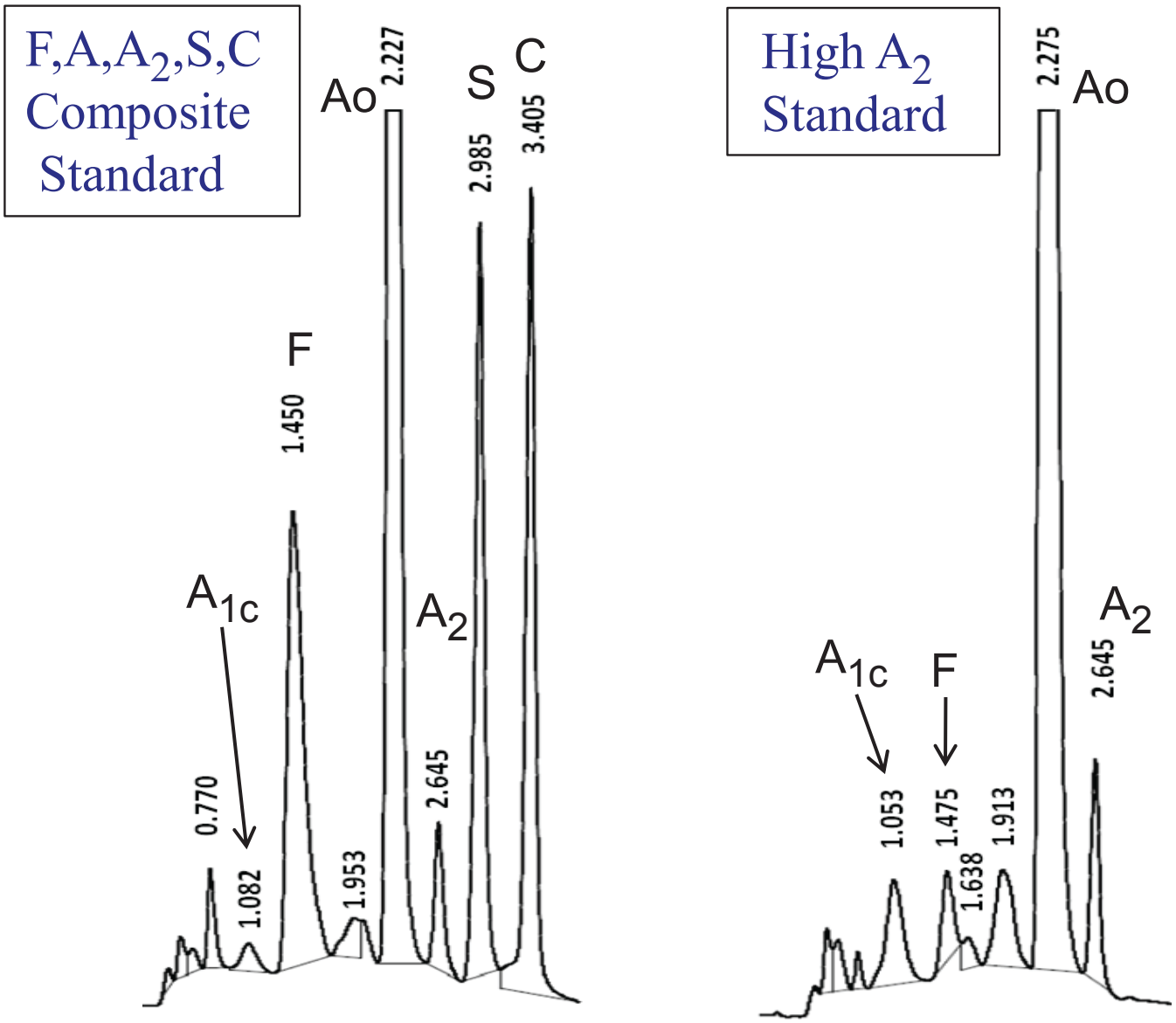
Double-Stranded DNA; **PCR Reaction Mix**
(West Nile Virus template; 202-bp GC-rich product)

Column: WAX LP (item# 151WX0315)
Gradient:↑ [$NaClO_4$]; 2' fractions (10-40')



HEMOGLOBINS

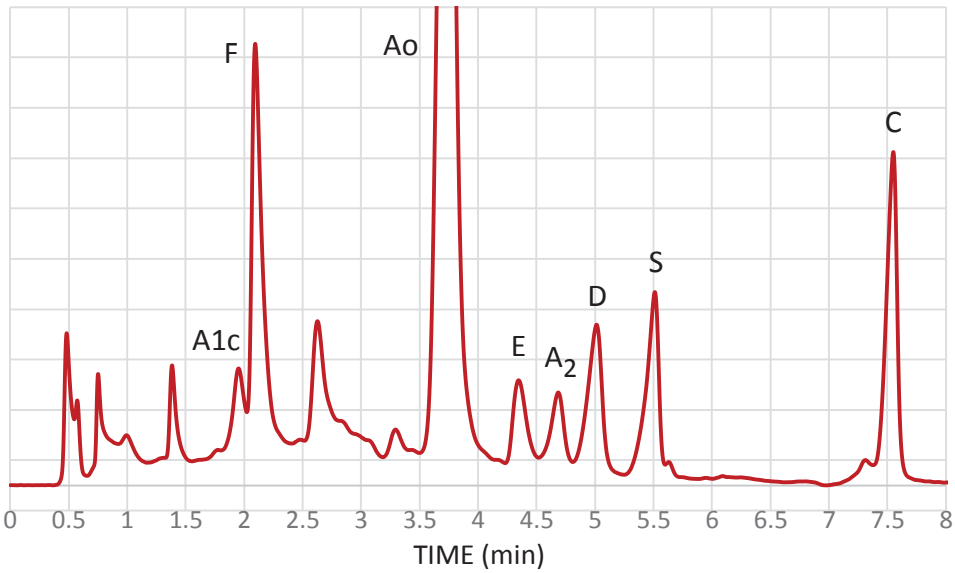
CAT A is the gold standard worldwide for analysis of hemoglobin variants by cation-exchange HPLC. The most common variants can be separated in 3.5 minutes with good quantitation and with a turnaround time between samples of 4 - 4.5 minutes. This includes quantitation of Hb A_{1c} for diagnosis of diabetes. The examples below were obtained with the widely-used 35x4.6-mm column packed with the 3-µm, 1500-Å pore version of CAT A (item 3.54CT0315).



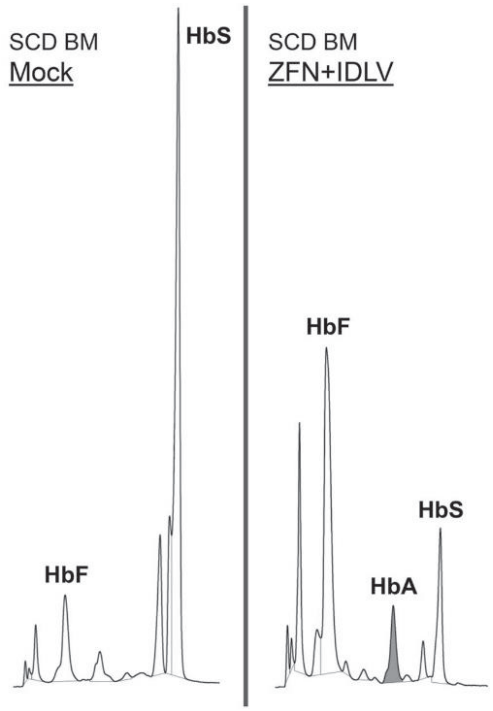
the most popular sizes of CAT A columns are as follows (all 3-µm, 1500-Å)

If the objective is to optimize resolution of variants and not A1c or the speed of analysis, then the gradient can be modified accordingly, as per this example.

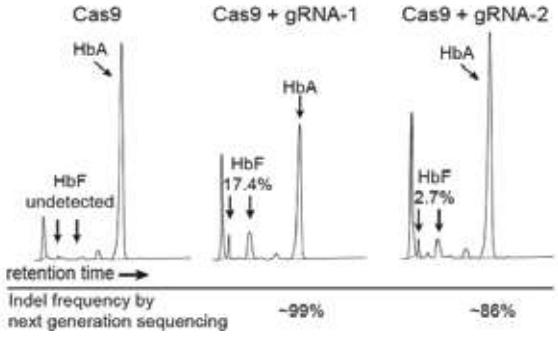
Column: CAT A, 50x4.6-mm; 3-µm, 1500-Å



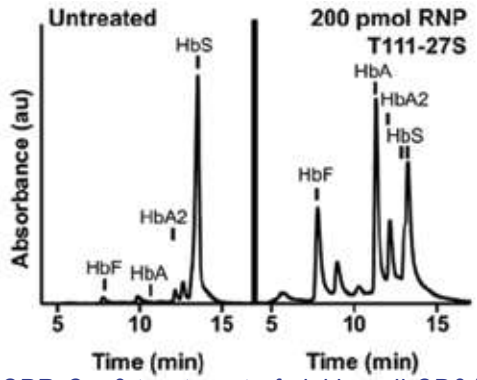
Genetic engineering to cure hemoglobinopathies - CAT A is the column of choice -



Functional correction of sickle bone marrow CD34⁺ cells (zinc finger nucleases)



CRISPR-Cas9 treatment of CD34⁺ stem cells



CRISPR-Cas9 treatment of sickle cell CD34⁺cells

Introducing ERLIC

Electrostatic Repulsion-Hydrophilic Interaction Chromatography

ERLIC is a new, general-purpose mode of chromatography. With ERLIC,

1) Many gradient separations become isocratic separations;
2) Phosphopeptides can be isolated selectively from tryptic digests and separated with high resolution.

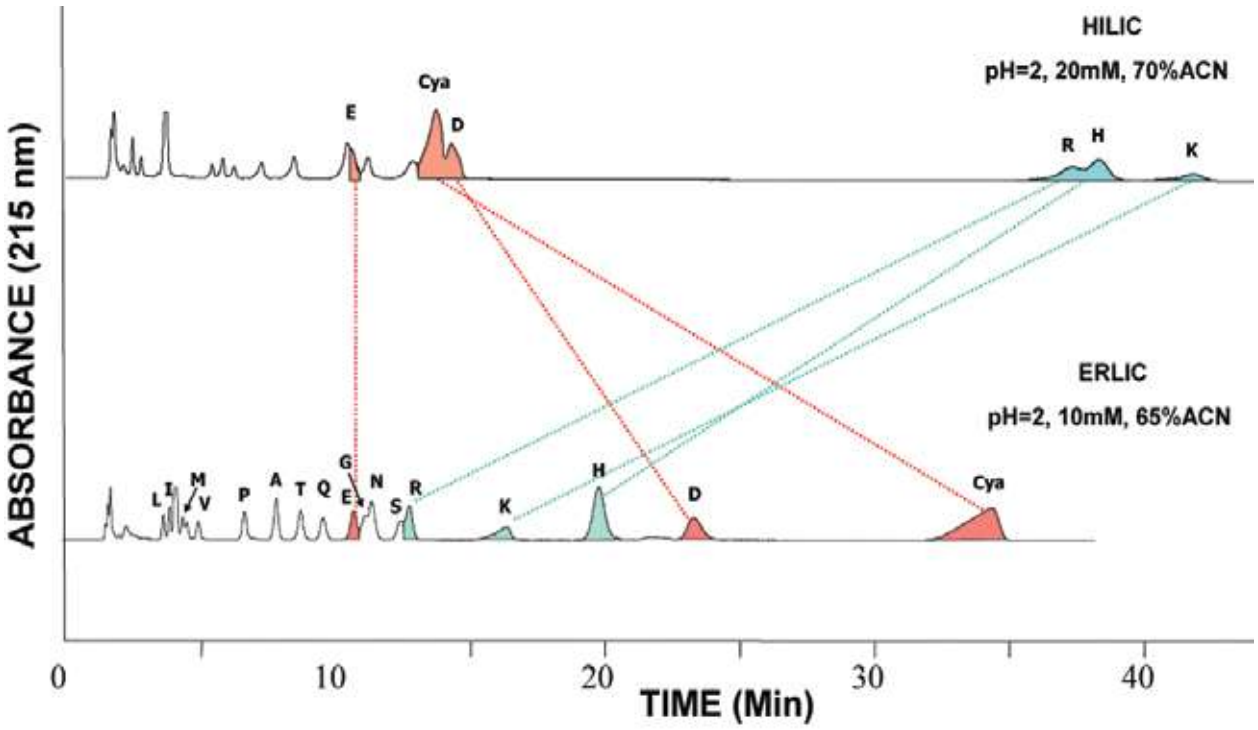
Biochemicals of high charge are frequently much better retained in chromatography than compounds of low charge. Example: ATP vs. AMP. This is true in both ion-exchange and hydrophilic interaction chromatography (HILIC). A gradient is necesssary to make both types of compounds elute in the same time frame. However, if HILIC is performed using an ion-exchange column of the same charge as the most highly-charged compounds, then their retention is selectively antagonized by electrostatic repulsion. This permits their elution in the same time frame as less highly-charged compounds using isocratic conditions.

A. Isocratic Separations

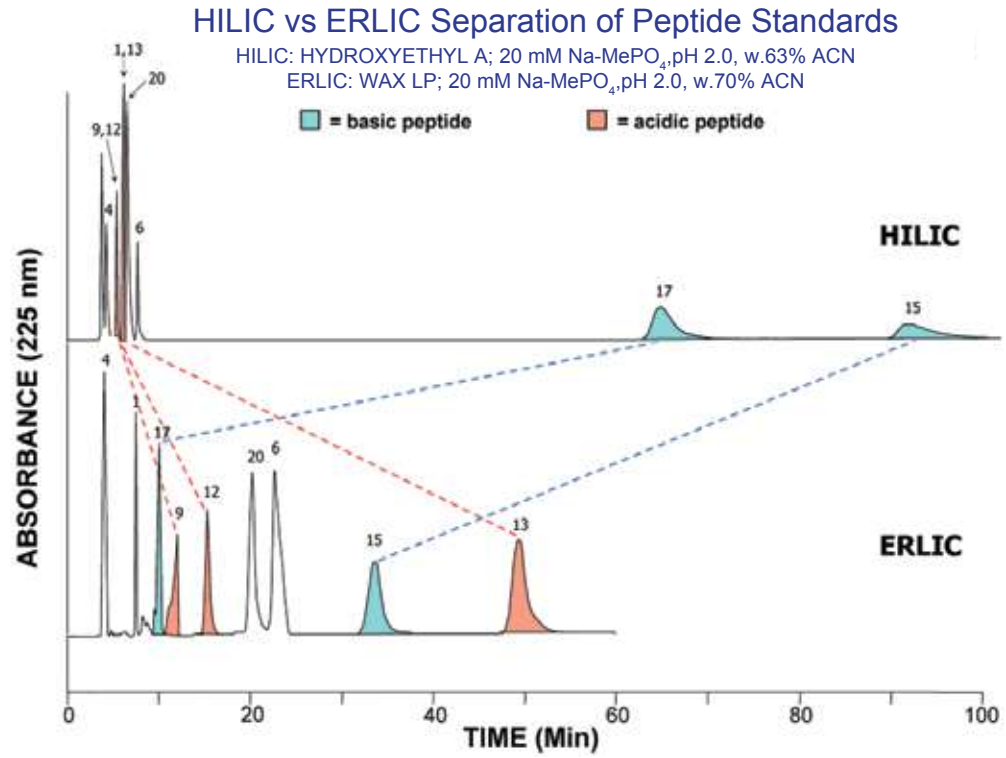
1) Amino acids, peptides and proteins: The most polar groups are basic residues. ERLIC of these compounds is performed with an anion-exchange column (e.g., our WAX LP™) at pH ~ 2.0, low enough for carboxyl- groups to lose their negative charge. Amino acids, peptides and proteins will have a net + charge and will all experience some degree of electrostatic repulsion. The most basic compounds, normally the best-retained, experience the most repulsion.

2) Nucleotides and nucleic acids: ERLIC is performed with a cation-exchange column (our SULFOETHYL A™) at a pH < 3.5, low enough for all phosphate groups to have a single negative charge (repulsion is too great at higher pH, where phosphate groups acquire a second negative charge).

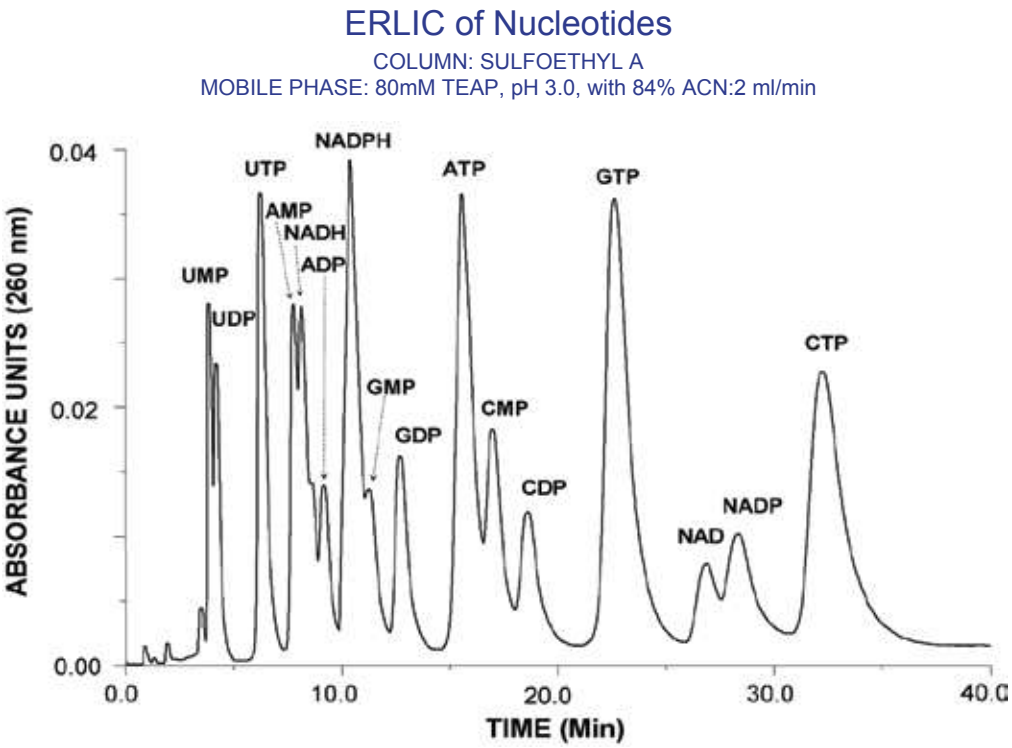
Example 1: ERLIC vs. HILIC of Amino Acids (100-Å pore columns)



Example 2: ERLIC vs. HILIC of Acidic, Basic, and Neutral Peptides (300-Å pore columns):



Example 3: ERLIC of Nucleotides:



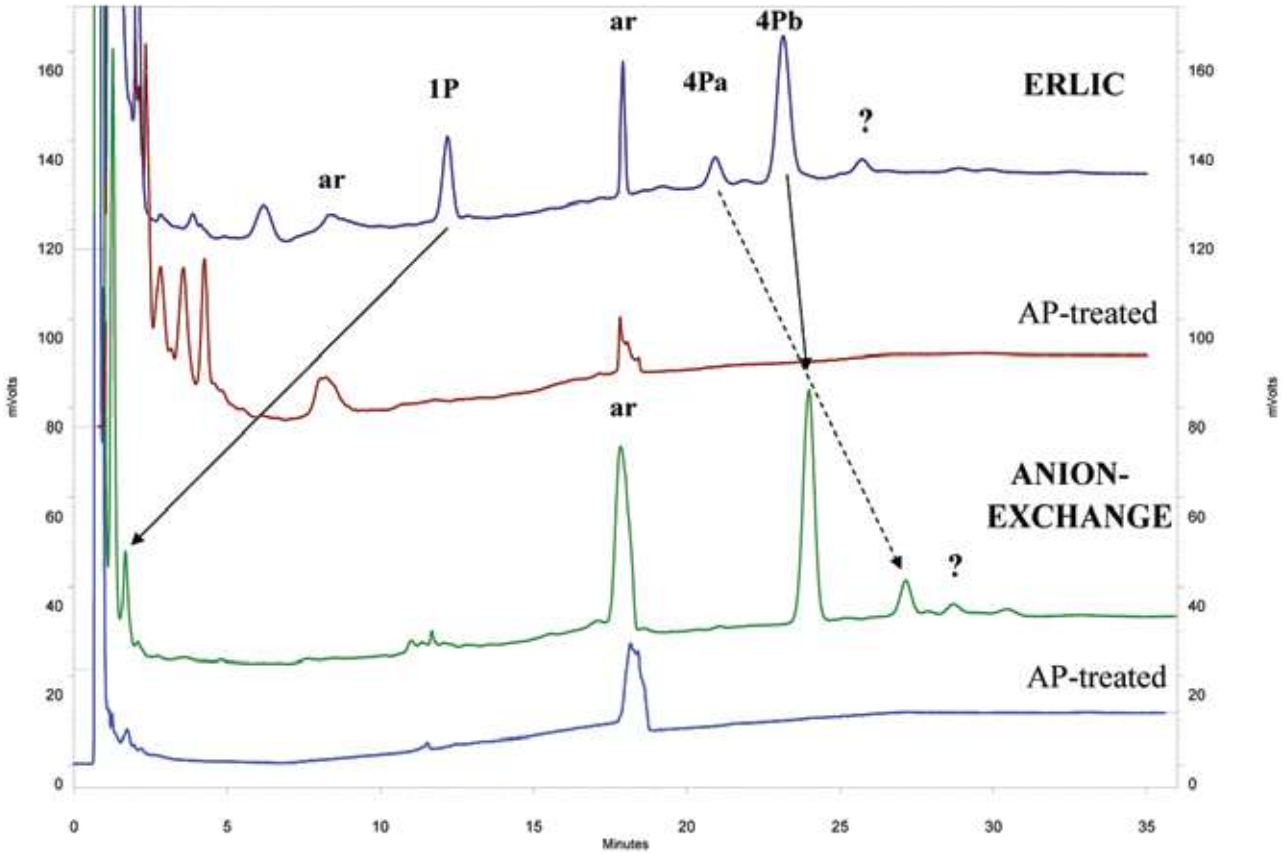
B. Isolation of Phosphopeptides

At pH 2.0, phosphate groups in peptides retain some of their negative charge. This does not permit the isolation by anion-exchange chromatography of singly phosphorylated peptides from tryptic digests, since the electrostatic attraction is not sufficient to overcome the electrostatic repulsion from the N-terminus and the C-terminal Lys- or Arg- residue. However, phosphate residues are quite hydrophilic. In the ERLIC mode, the combination of electrostatic attraction and hydrophilic interaction does suffice to pull singly phosphorylated peptides away from the nonphosphorylated peptides in tryptic digests. Also, unlike the situation with high-affinity media such as IMAC or titania, the phosphopeptides are well-resolved from each other. This permits their convenient separation into numerous fractions, an important tool in phosphoproteomics for identifying the sequences of thousands of phosphopeptides from a single sample. Peptides with multiple phosphate groups are retained so strongly that a salt gradient is necessary for elution.

Example 1: Tryptic Digest of Beta-Casein: Separation with the Same Column in the ERLIC and Anion-Exchange Modes:

Tryptic Digest of ß-Casein; ERLIC vs. Anion-Exchange

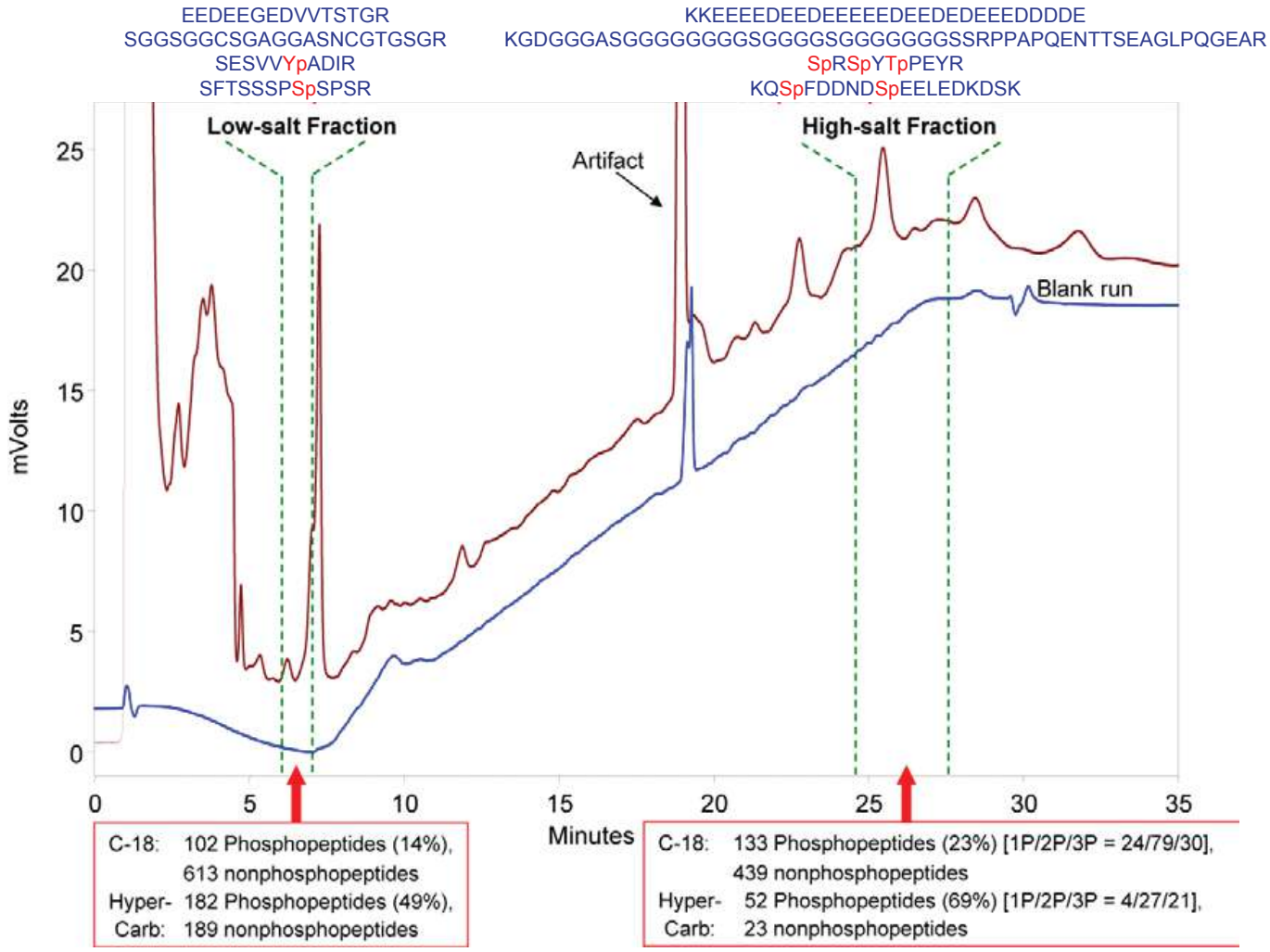
1P: FQSEEQQQTEDELQDK **ar:** artifact
4Pa: ELEELNVPGEIVESLSSSEESITER **AP:** Alkaline Phosphatase
4Pb: RELEELNVPGEIVESLSSSEESITER



A 100x4.6-mm column of WAX LP (5-µm, 300-Å) was used. Note the poor retention of the singly phosphorylated fragment in the anion-exchange mode.

Example 2: Fractionation of the Tryptic Digest of HeLa Cell Lysate:

ERLIC of HeLa Cell Lysate Tryptic Digest: SPE Desalting of Phosphopeptides



Again, WAX LP column was used here. It should be noted that recovery of phosphopeptides is highest if low-salt fractions are desalted using HyperCarb; high-salt fractions, using C-18 silica.

Ordering information for the ERLIC columns used in these examples:

Amino acids: WAX LP column (200x4.6-mm; 5µm, 100-Å);
Peptides: WAX LP column (200x4.6-mm; 5µm, 300-Å);
Phosphopeptides: WAX LP column (100x4.6-mm; 5µm, 300-Å);
Nucleotides: SULFOETHYL A column (200x4.6-mm; 5µm, 300-Å):

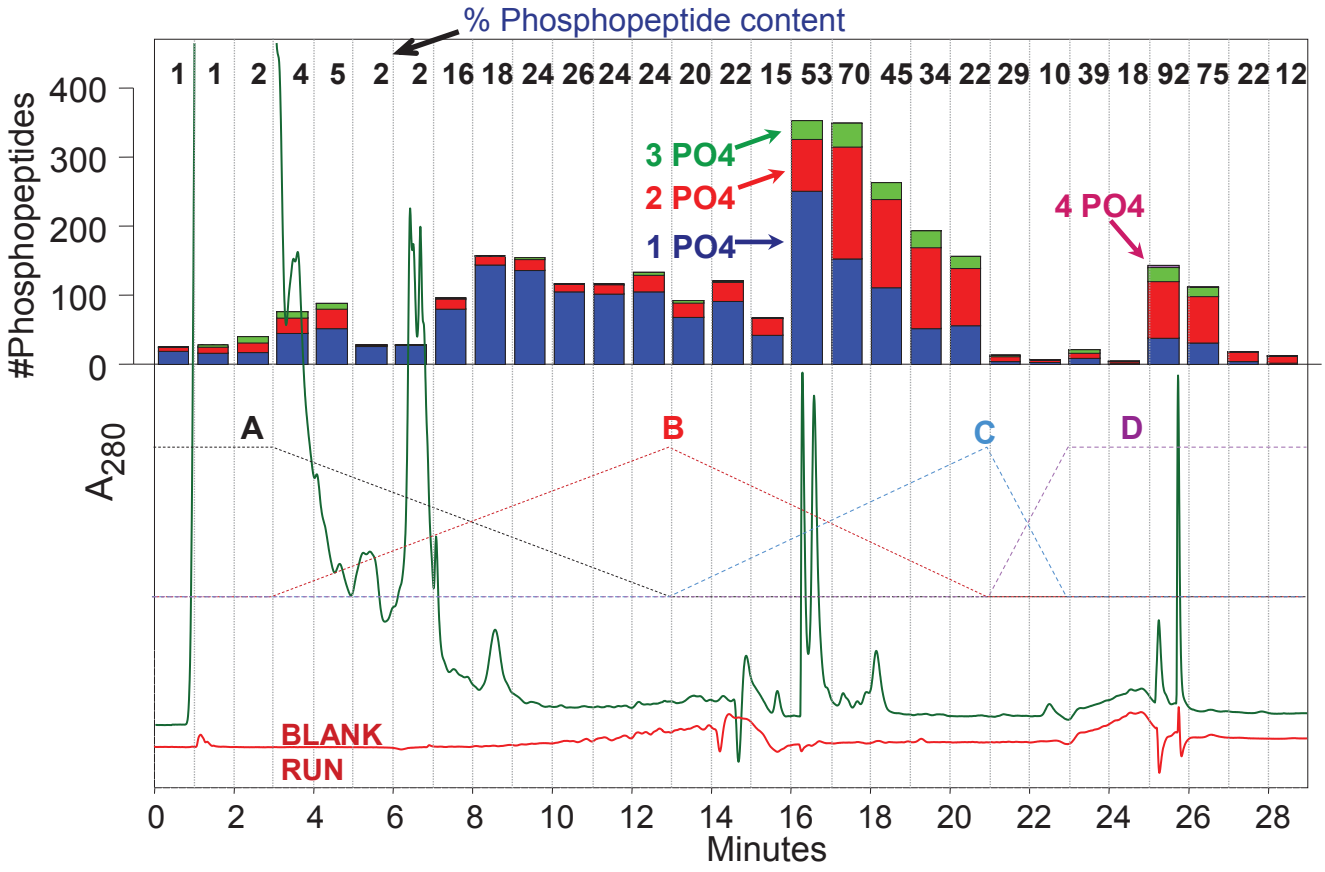
Isolation of Glyco- & Phosphopeptides via ERLIC

ERLIC is a new, general-purpose mode of chromatography; a column of the same electrostatic charge as the solutes is run in the HILIC mode. Both sialylated glycopeptides and phosphopeptides (as well as some nonsialylated glycopeptides) can be isolated selectively from tryptic digests via ERLIC.

At pH 2.0, peptides with phosphate and sialic acid residues retain some negative charge. This does not permit their isolation from tryptic digests by anion-exchange chromatography (AEX), since the electrostatic attraction is not sufficient to overcome the electrostatic repulsion from the N-terminus and the C-terminal Lys/Arg residue. When an AEX column is run in the ERLIC mode, though, then the combination of electrostatic attraction and hydrophilic interaction suffices to pull singly phosphorylated tryptic peptides and many glycopeptides away from the unmodified peptides. Unlike the situation with high-affinity media such as IMAC or titania, phospho- and glycopeptides can be well-resolved from each other in ERLIC. This permits their convenient separation into numerous fractions, an important tool in proteomics for identifying thousands of modified peptides from a single sample. Peptides with multiple phosphate or sialyl- groups are retained so strongly that gradient elution is necessary.

Example 1: ERLIC of HeLa Cell Lysate Tryptic Digest

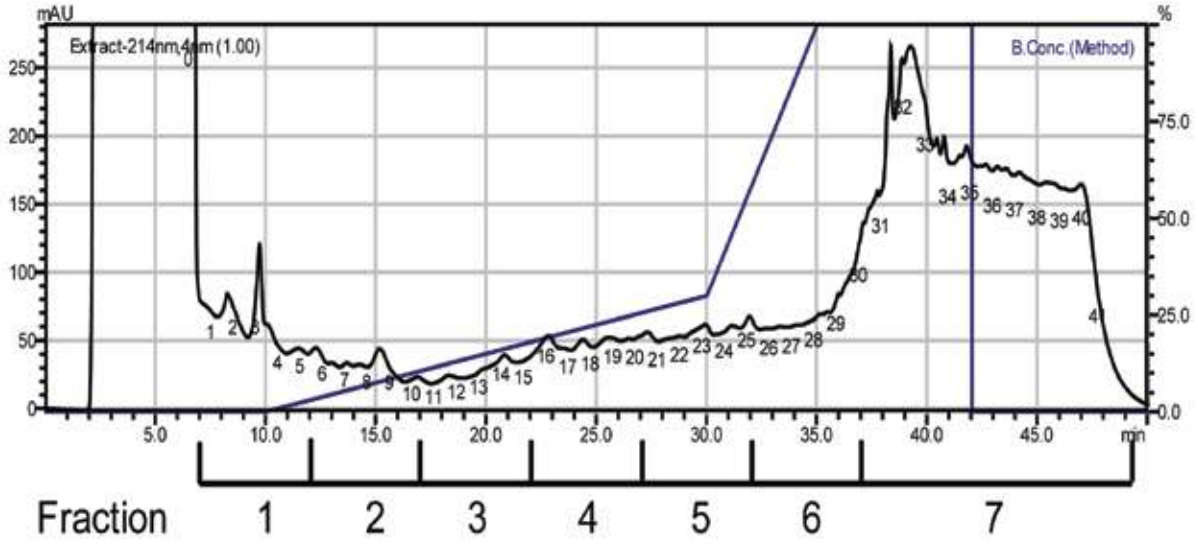
A 100x4.6-mm column of WAX LP (5-µm, 300-Å) was used. Over 3000 unique tryptic phosphopeptides were identified. Solvents through Fraction 20 were volatile.

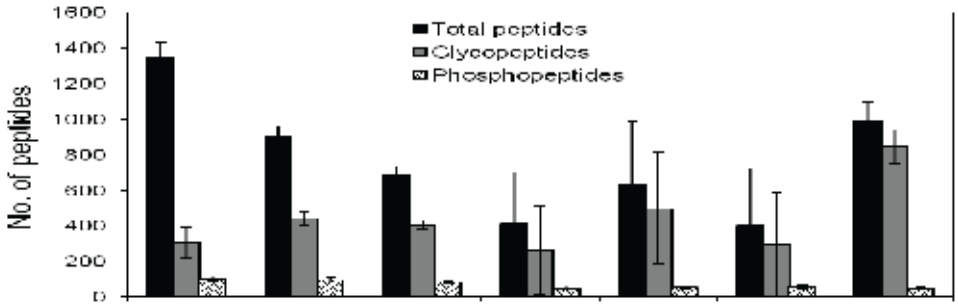


Example 2: Isolation of tryptic phospho- and glycopeptides

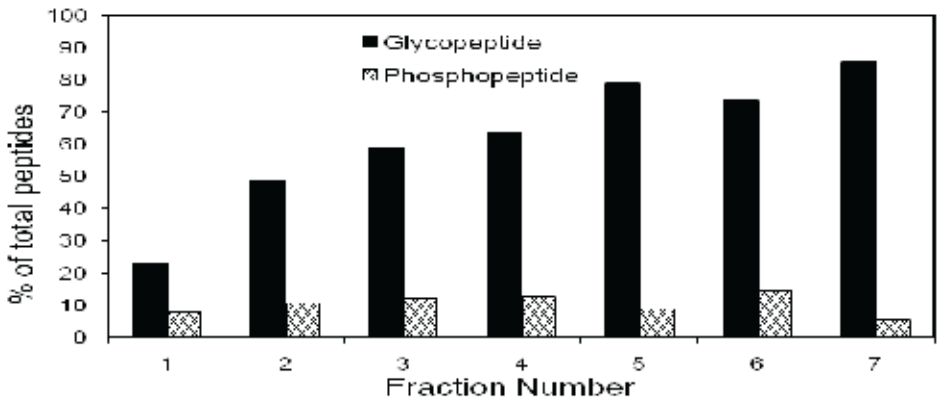
Column: 204WX0503 Flow rate: 1 ml/min. Detection: 214 nm
Gradient: 0-10': 0%B; 10-35': 0-30% B; 35-40': 30-100%B; 40-50': 100%B
MP A: 10 mM Na-methylphosphonate, pH 2.0, with 70% ACN
MP B: 200 mM TEA-phosphate, pH 2.0, with 25% ACN

Mouse Brain Extract (1 mg)





Phospho- and glycopeptide content is ~ 30-90% in fractions 1-7

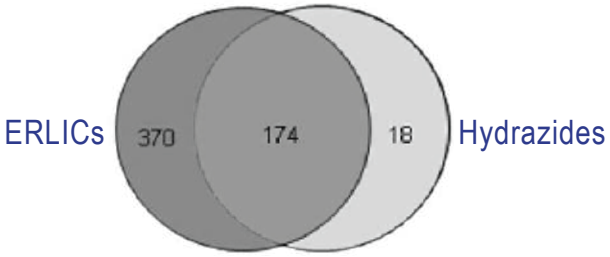


NOTE: Glycosylation sites were found in 41% of phospho-proteins id'd here

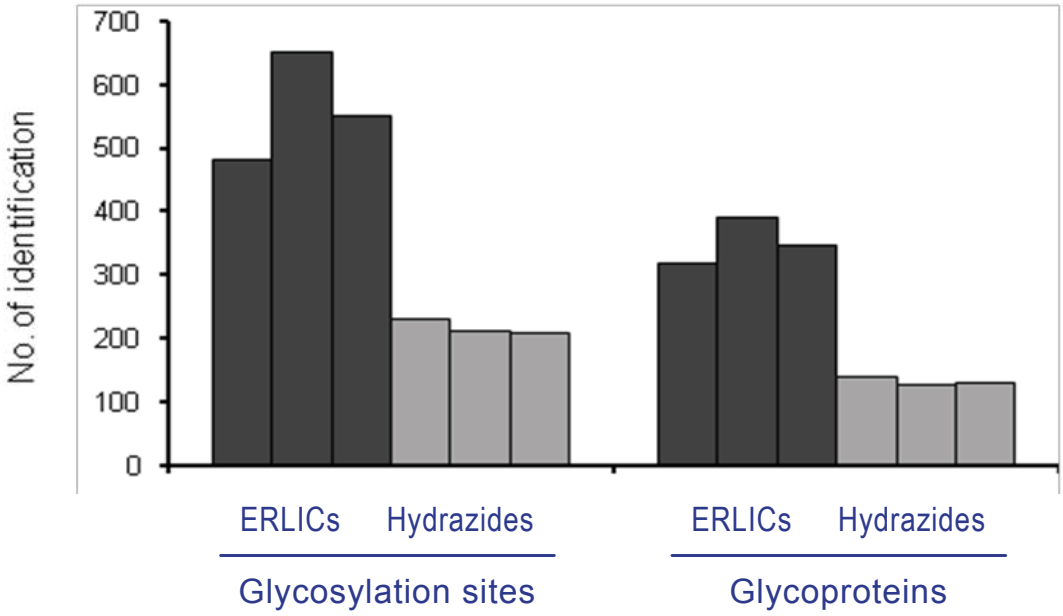
Isolation of N-linked Glycopeptides: Comparison of ERLIC with Hydrazide Covalent Chromatography

ERLIC: 50-minute run (same sample & conditions as in Example 2).
Hydrazide Method: 1) Oxidation with NaIO4; 2) Incubation overnight with Hydrazide gel; 3) Incubation overnight with PNGase F.
All fractions and isolates were analyzed via C18-MS (LTQ-FT).

Glycosylation sites



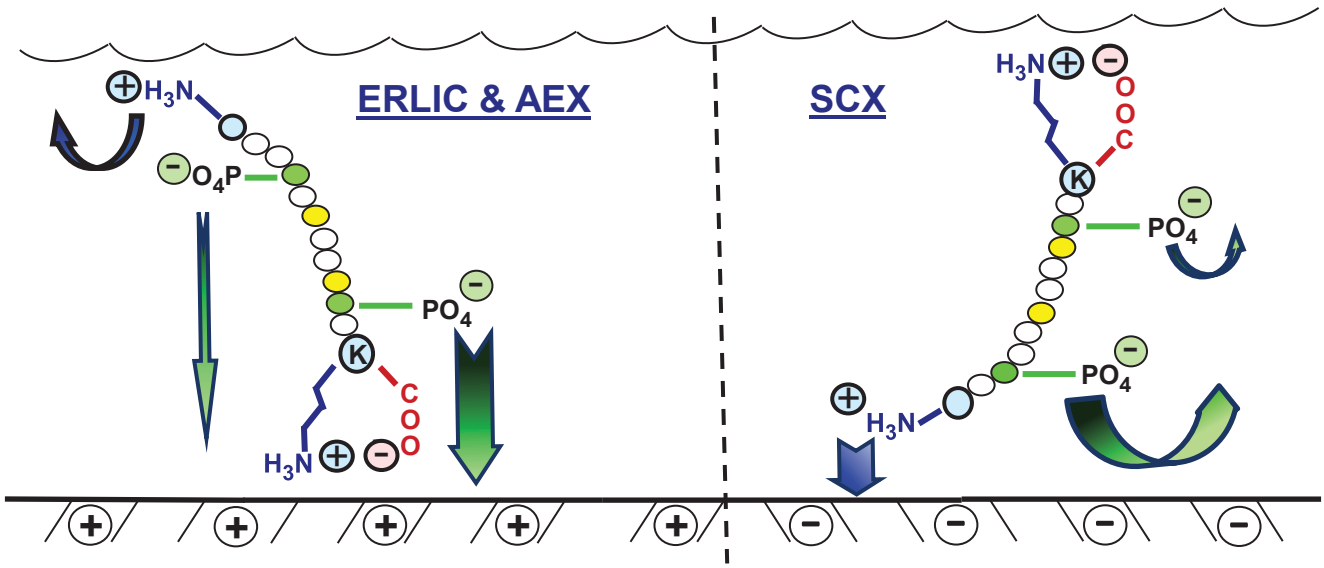
Glycoproteins



ERLIC is much more effective and convenient than the hydrazide method. In addition, glycopeptides elute in ERLIC with the glycan still attached, which is not true of the hydrazide method.

Applications of ERLIC in Proteomics:

1) ORIENTATION OF TRYPTIC PHOSPHOPEPTIDES



Peptides are frequently highly oriented in their migration through ion-exchange columns of all kinds. This affects the degree to which a charged group interacts with the surface and accounts for the separation of peptides of the same net charge. Ask for our paper on the subject.

2) FRACTIONATION OF TRYPTIC PEPTIDES IN GENERAL

ERLIC seems to be superior to SCX in some ways as a first dimension of chromatography for distribution of tryptic peptides into fractions. See our separate bulletin on the subject.

2) FRACTIONATION OF PHOSPHOPEPTIDES VIA SPE-ERLIC

Please ask for examples.

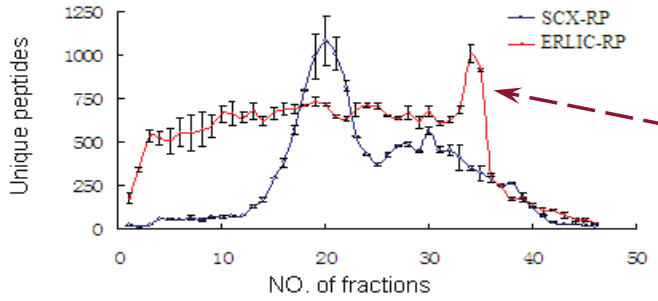
Buying the products used in these examples:

WAX LP column, 100x4.6-mm, 5-µm, 300-Å
WAX LP column, 200x4.6-mm
Other sizes of WAX LP columns are available. TopTips are available for SPE of samples 1-10 µl, 10-200 µl, and 200-1000 µl. Regular SPE cartridges (0.5-5 ml) of WAX LP are also available.

Superior Proteomics Fractionations with ERLIC-RPC

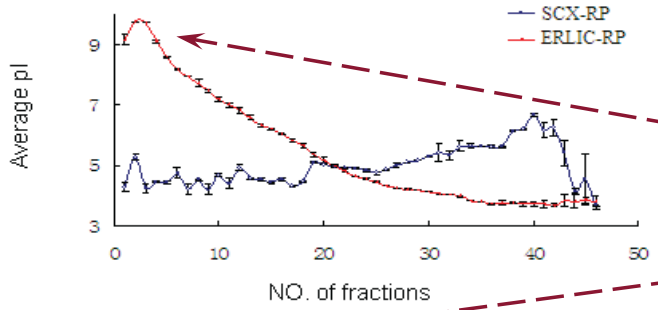
In bottom-up proteomics, more peptides of lower abundance are identified if complex digests are fractionated via two dimensions of chromatography. The most widely used combination at present is SCX-RPC. There are significant advantages to substitution of the new ERLIC mode for SCX. ERLIC of peptides is performed with an anion-exchange column operated in the HILIC mode at low pH. With a decreasing organic solvent gradient, hydrophilic interaction becomes so weak that electrostatic repulsion causes the peptides' elution. Volatile solvents can be used!

Example 2: mg. peptides from tryptic digest of rat kidney homogenate



Distribution of peptides:

Much more uniform in ERLIC than in SCX

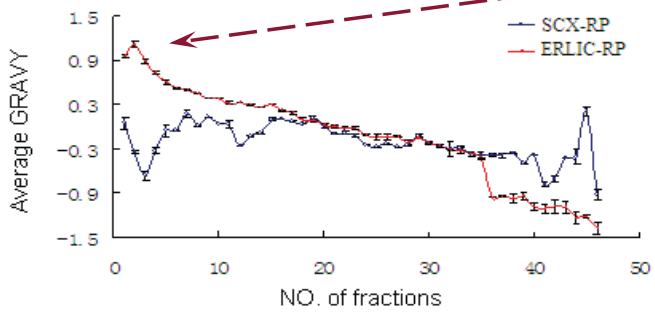


Basis of separation in ERLIC:

pI of peptide (high to low)

and

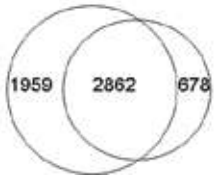
polarity (low to high)



- both variables are complementary to selectivity in RPC -

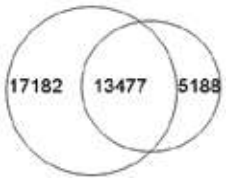
The number of identifications is significantly higher with ERLIC than with SCX

Protein id's

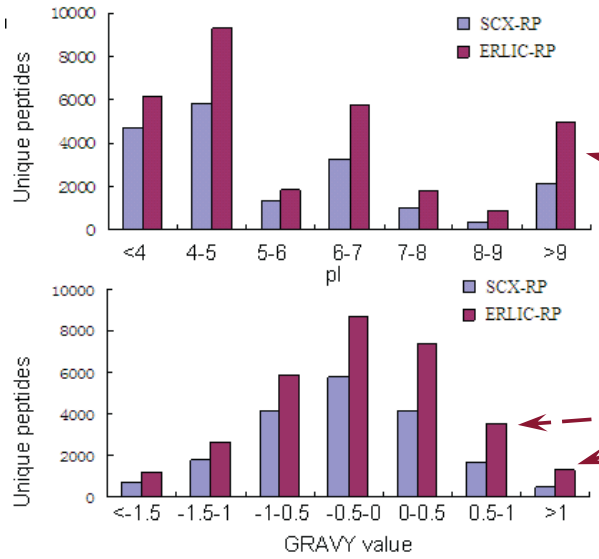


ERLIC -RPC SCX-RPC

Unique Peptide id's



ERLIC -RPC SCX-RPC

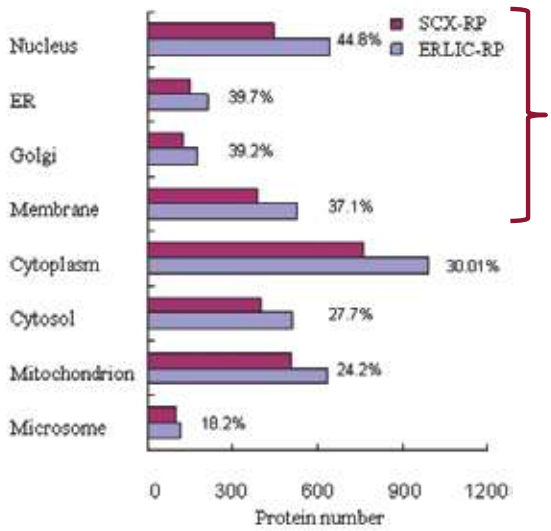


ERLIC affords > 120% more id's than does SCX with peptides that are:

very basic

or

very hydrophobic



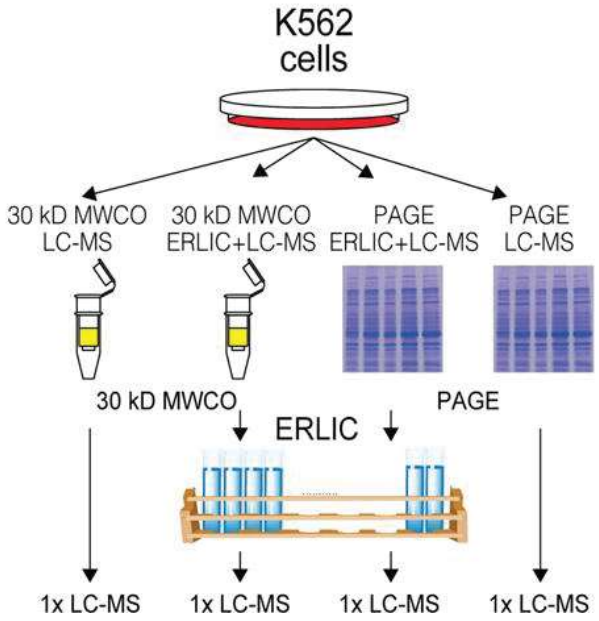
This makes it more effective at identifying peptides from membrane-rich organelles

Buying the products used in these examples:

ERLIC: WAX LP column, 200x4.6-mm, 5-µm, 300-Å
SCX: SULFOETHYL A column, 200x4.6-mm , 5-µm, 200-Å

FRACTIONATIONS FOR PROTEOMICS
- WHY USE ERLIC ? -

1) 5-7x more sORF-Encoded peptides (SEPs) detected



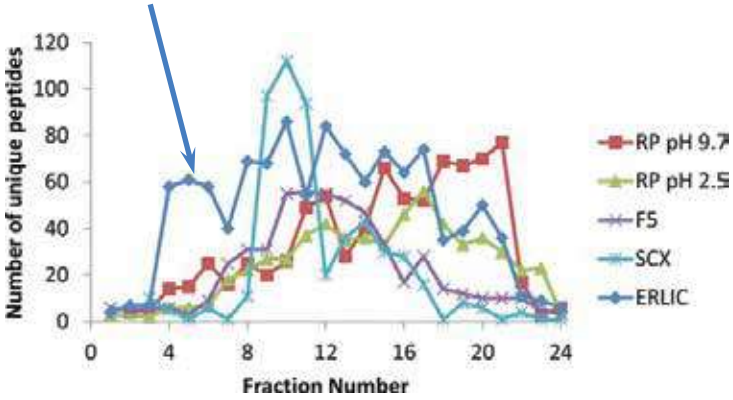
Workflow	Total SEPs Detected	Novel SEPs Detected
MWCO + LC-MS	13	6
MWCO + ERLIC + LC-MS	711	123
PAGE + ERLIC + LC-MS	433	98
PAGE + LC-MS	668	123

2) ERLIC-RPC id's more peptides than other 2-D methods

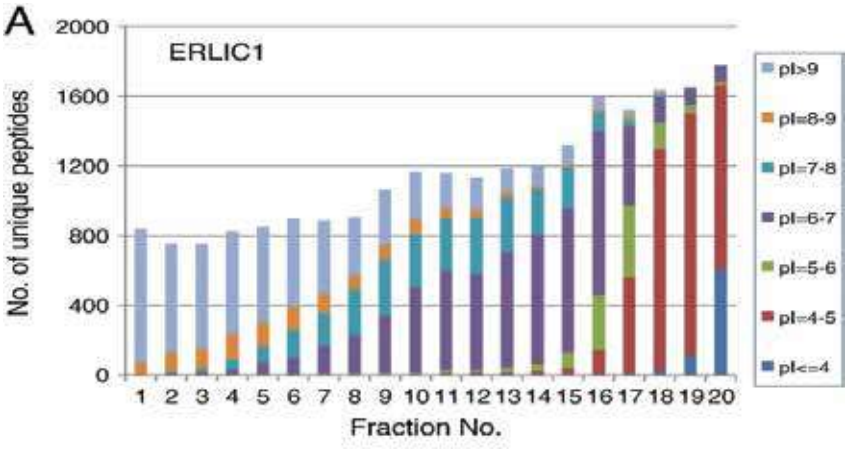
Table 1. Number of peptides and proteins identified in 2D LC-MS/MS experiments

First dimension separation mode	Second dimension separation mode	Number of peptides	Number of proteins
–	RP pH 2.6	185	45
SCX		452	95
ERLIC		711	123
F5		433	98
RP pH 9.7		668	123
RP pH 2.5		504	90

-with the most uniform distribution of peptides among the collected fractions -

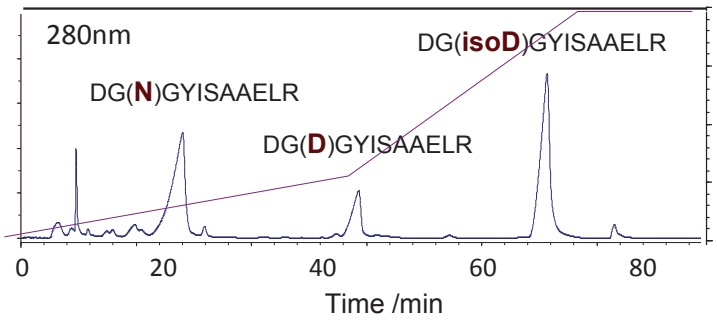


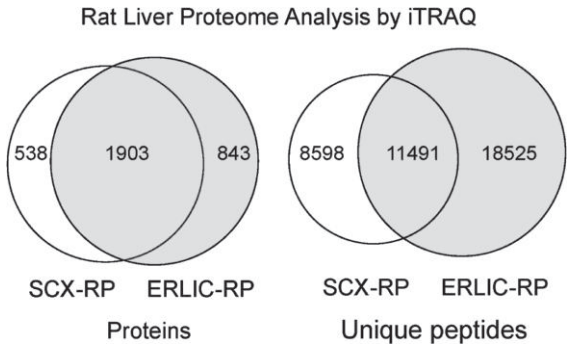
3) Peptides elute in order of decreasing pI…



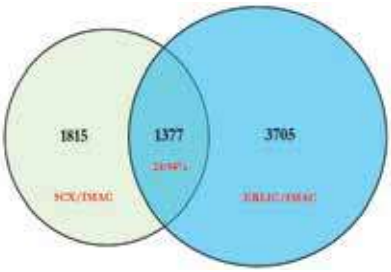
…which allows the separation of the products of deamidation

and...

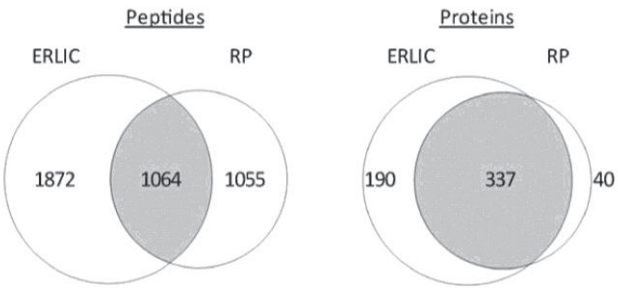




…ERLIC-RPC identifies more peptides and proteins than does SCX-RPC



…ERLIC/IMAC identifies more phosphopeptides than does SCX/IMAC

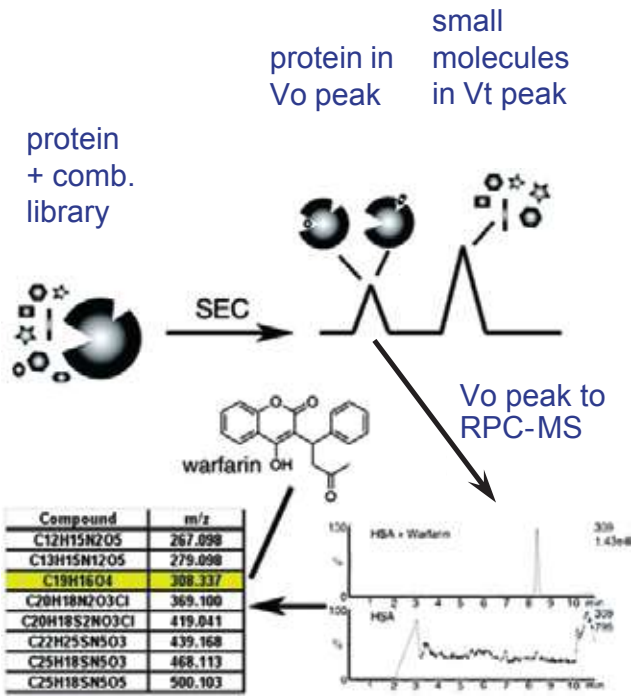


…online ERLIC-MS outperforms RPC-MS

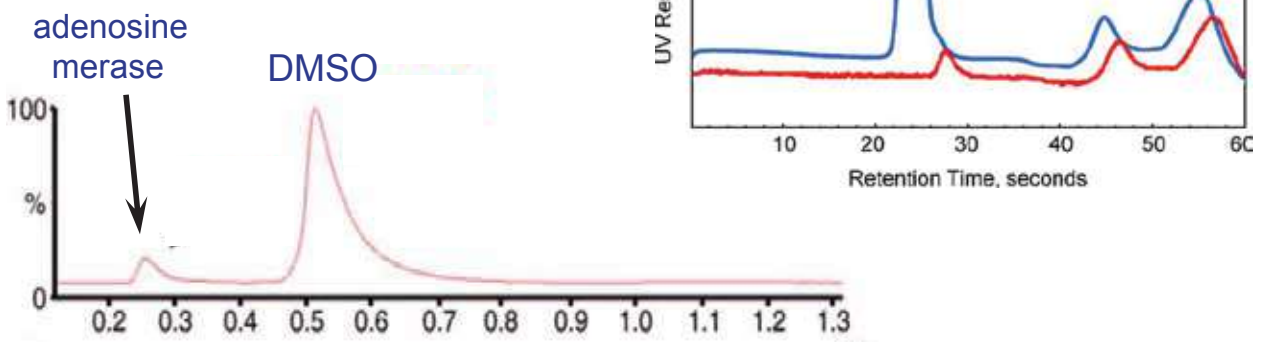
High-Throughput Screening (HTS)

For the past 13 years, pharmaceutical companies have successfully used our SEC columns to screen combinatorial libraries of up to 2500 components per run to identify small molecules that bind with high affinity to a target protein. Such molecules migrate through the SEC column with the protein and elute in the Vo peak instead of in the Vt peak with the rest of the small molecules. Small molecules in the Vo peak are then identified and a new library is synthesized with features in common with the high-affinity subset. Several such iterations may produce a drug candidate with very high affinity (Kd < 100 nM) and selectivity.

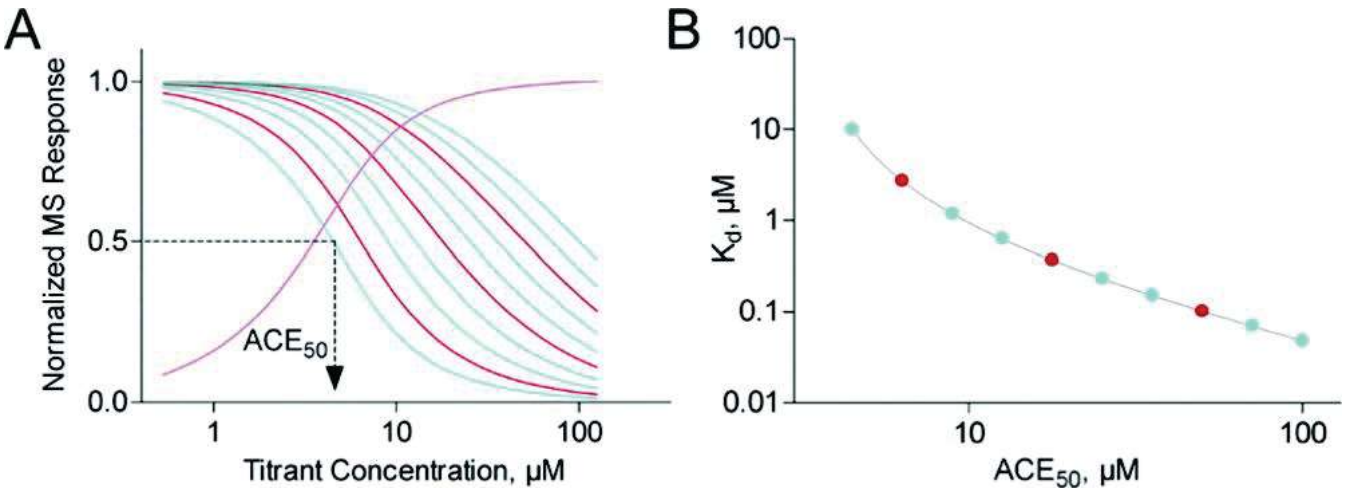
The SEC must be completed in less than one minute or even high-affinity molecules will start to diffuse off the target protein. ATL SEC columns can separate the Vo and Vt peaks to baseline under these conditions,which is essential for preventing false positives.



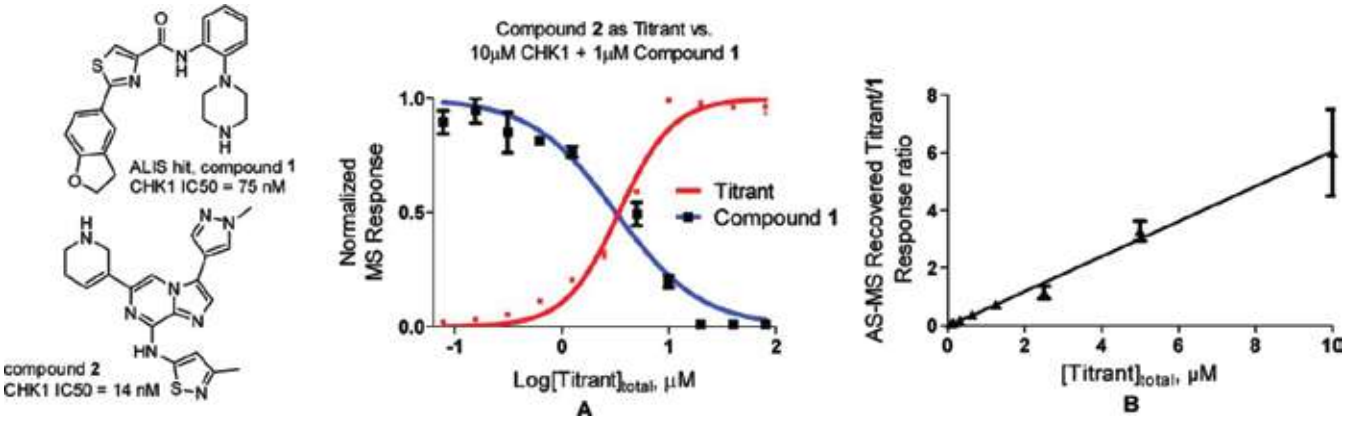
Example of SEC chromatograms from HTS



The SEC method can also be used to measure binding constants, effects of cofactors on binding, competitive and noncompetitive binding, and other ligand interactions with target proteins:

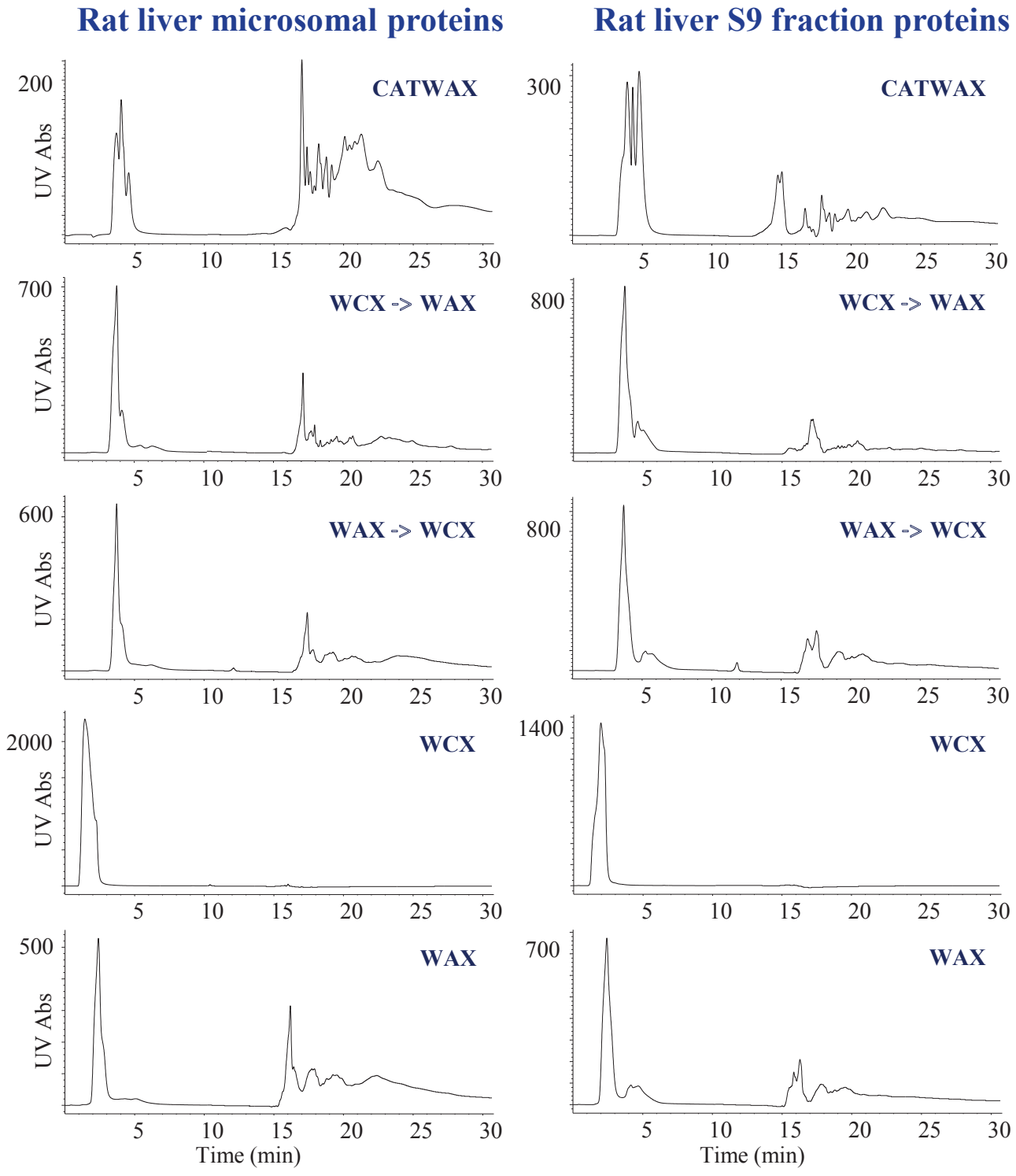


Affinity Competition Experiments enable protein–ligand binding affinity measurements in compound mixtures. As simulated in (A), a library of compounds of varying affinity (blue) is embedded with calibrant ligands of known Kd (red) and titrated with a compound of known Kd (purple) to yield MS-measured ACE_{50} curves. (B) A calibration curve generated from the calibrants' ACE_{50} and Kd values yields the other mixture components' Kds.



Mixed-Bed IEX column vs. CAT and WAX columns alone or in sequence

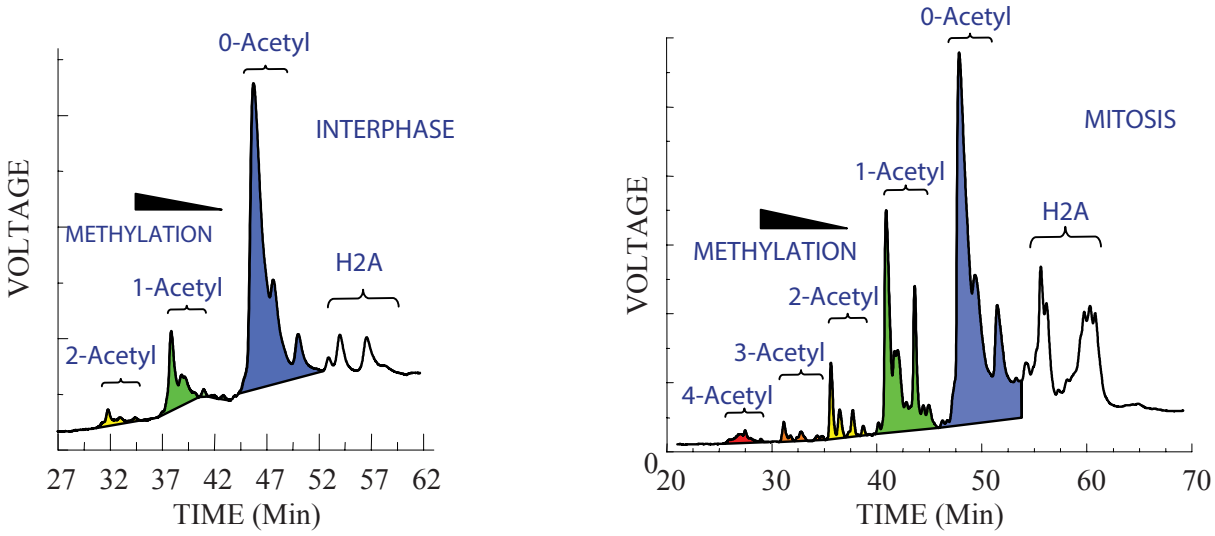
The mixed-bed column affords better fractionation
and better retention of weakly-retained proteins



Supplemental Figure S1. LC-UV traces at 220 nm from mixed-bed WCX/WAX (CATWAX), tandem dual-column WCX-WAX and WAX-WCX, stand-alone WCX and WAX separations. The y-axis (signal intensity) has been magnified 5x for the region eluting from 10-30 min for clarity

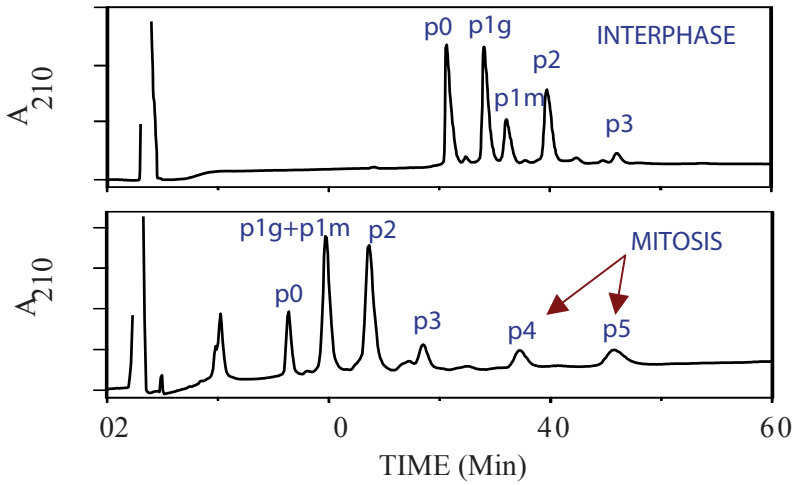
SEPARATE INTACT PROTEINS FOR PROTEOMICS

1) Histone H4 Acetylation & Methylation Variants



The most highly acetylated forms are present in the smallest amounts and for the shortest time, yet they are the most important in the cell cycle. It is only possible to detect and measure them by separating all forms by HPLC prior to digestion and MS analysis. COLUMN: CAT A in CEX-HILIC mode.

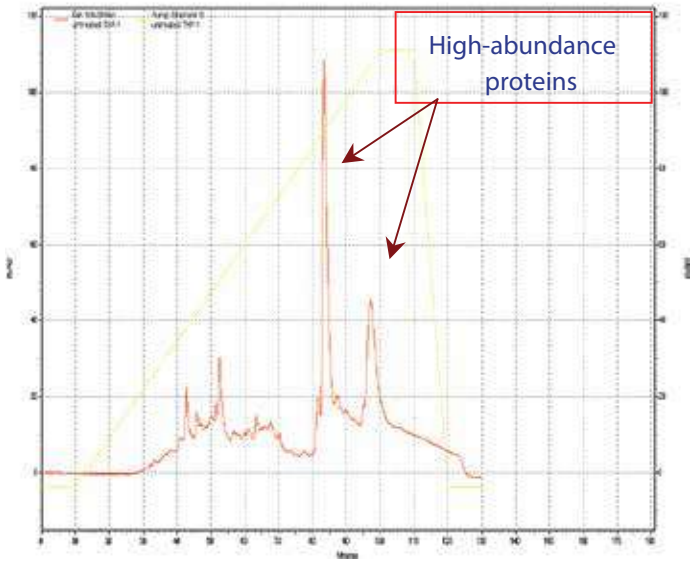
2) Histone H1.5 Phosphorylation Variants



Again, the more highly phosphorylated forms, critical to the cell cycle, can only be detected and quantitated after separation. Phosphorylation proved to proceed in an obligate sequence of residues.
COLUMN: CAT A in CEX-HILIC mode.

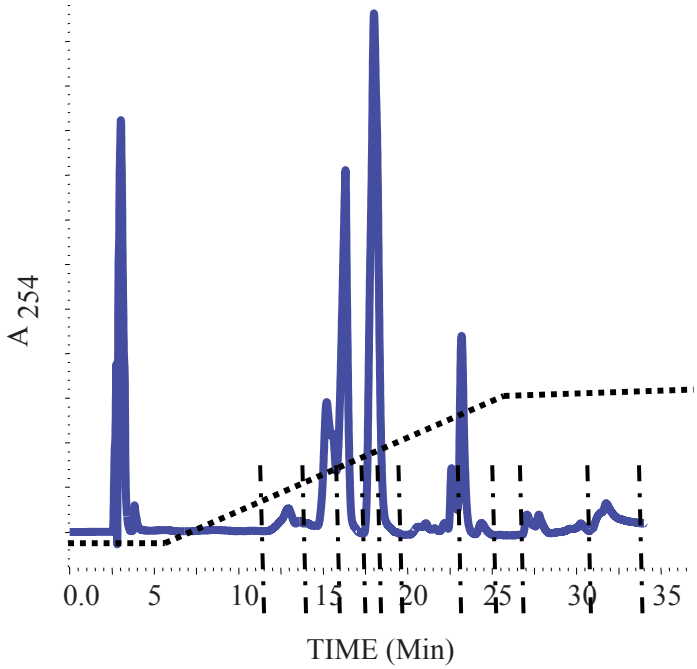
Why fractionate proteins prior to digestion?

1) Many extracts contain a few proteins of much higher abundance than the rest (as in the first example below). Fractionation before digestion permits you to collect those proteins in their own fractions. Their peptides will then not mask low-abundance peptides from the other fractions.
2) Suppose you distribute the proteins uniformly into ten fractions, then digest. The peptides from any protein within its fraction will represent 10x more of the total peptides than would have been the case with digestion of the unfractionated mixture. That greatly increases the chances of identifying more than one peptide from proteins of low abundance. Result: More rugged identifications.



Water-soluble cell lysate.

Mixed-bed IEC of intact proteins from
THP-1 Monocytes (~ $6x10^6$ Cells)
Columns: CAT A/ WAX LP (5 µm; 1000 Å)
Gradient : $NaClO_4$ A_{280}



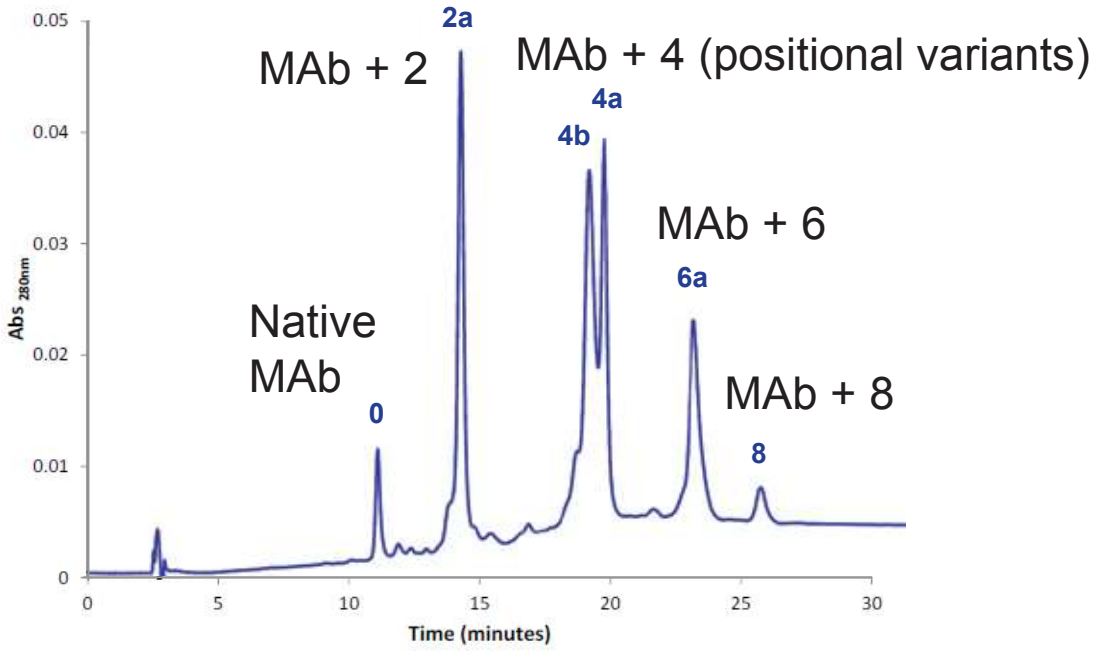
Water-insoluble membrane proteins

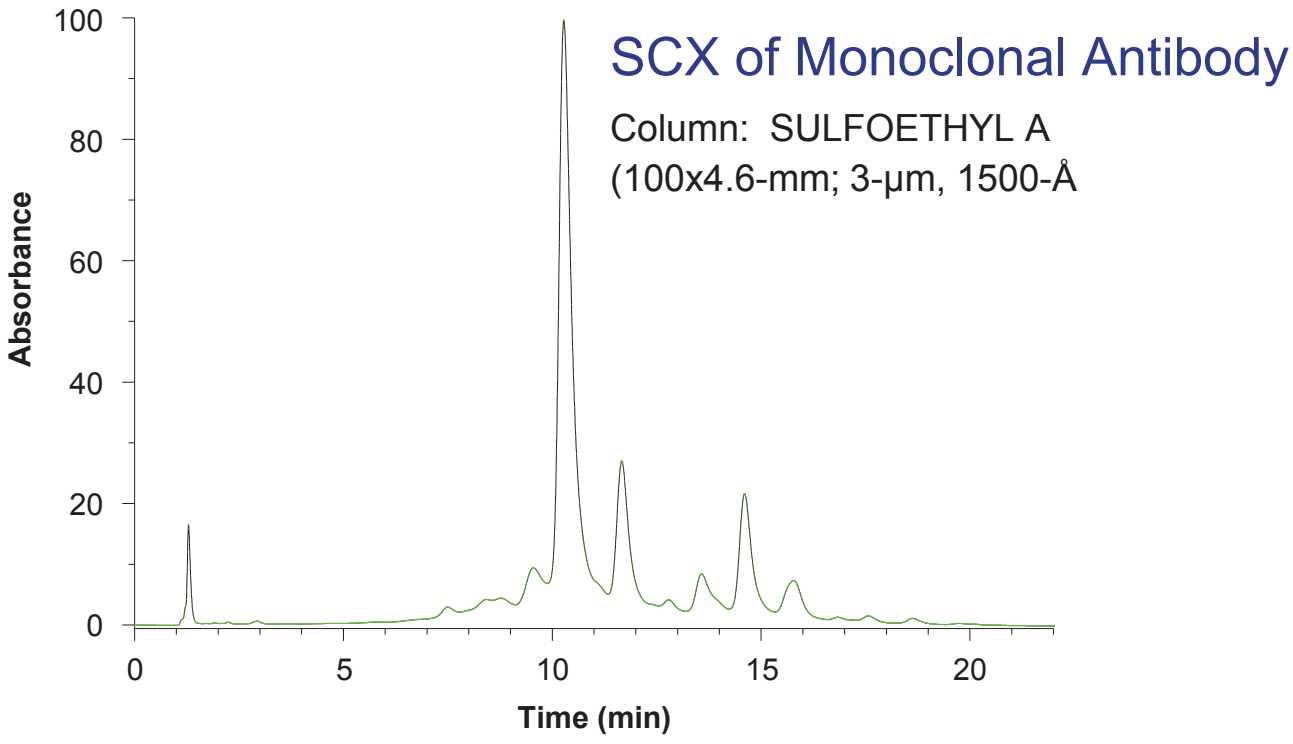
Pellet from mouse brain homogenate,
solubilized with HFIP
Columns: HYDROXYETHYL A
(204HY0503; HILIC mode)
Gradient : 70-40% ACN in Tris.HCl,
pH 6.5, with 40 mM HFIP

PROTEINS IDENTIFIED (partial list):
Troponin I
Na/K ATPase α-1 chain
ADP/ATP carrier protein (isoforms T1 & T2)
Calpexin (pp90)
Neurofilament Triplet L

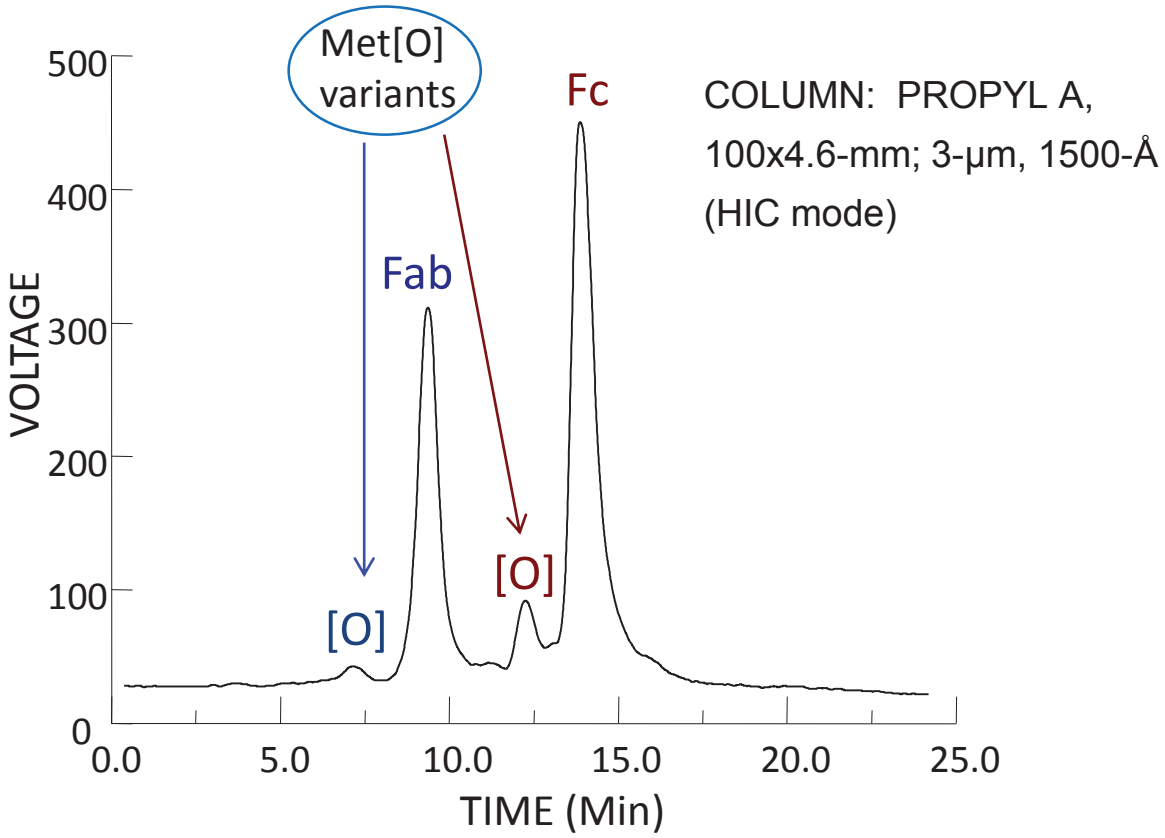
QUALITY CONTROL APPLICATIONS

ADC: Antibody conjugated to 2, 4, 6 or 8 molecules of a drug candidate Absorbance
COLUMN: PROPYLA, 100x4.6-mm; 3-µm, 1500-Å (HIC mode)



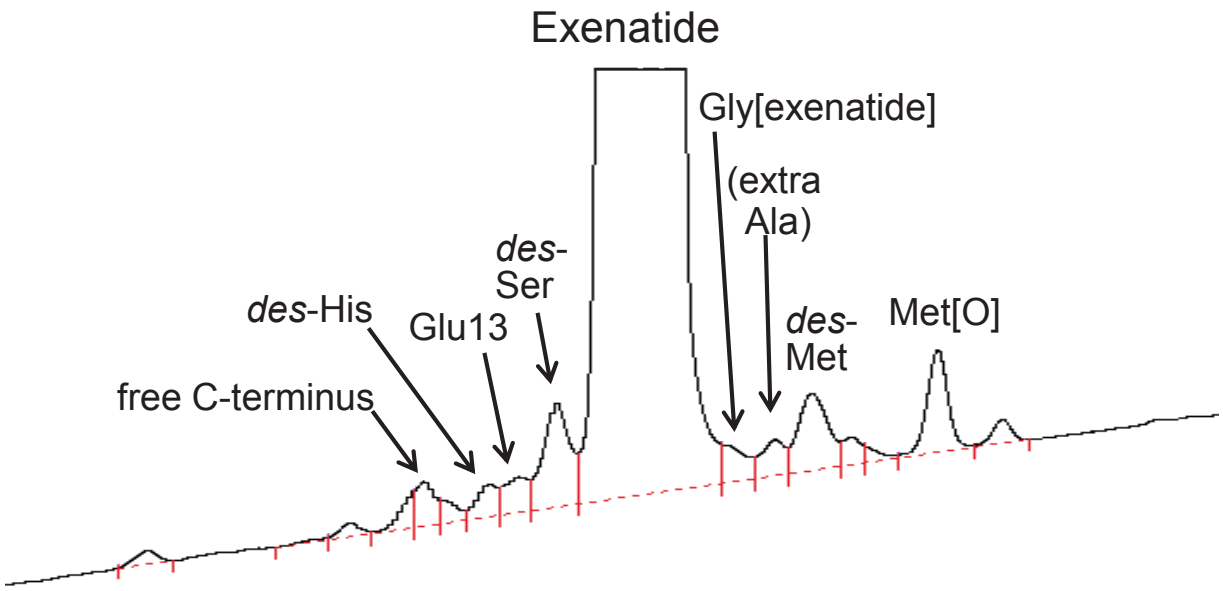


Separation of Oxidation Variants



SCX of Exenatide Variants

COLUMN: SULFOETHYL A (two columns in series)



Top-Down Proteomics

The reversed-phase columns used for bottom-up proteomics of peptides don't work with many proteins. Some don't elute at all while others elute in peaks 15 minutes wide. Progress in topdown proteomics requires alternative modes of chromatography.Examples:

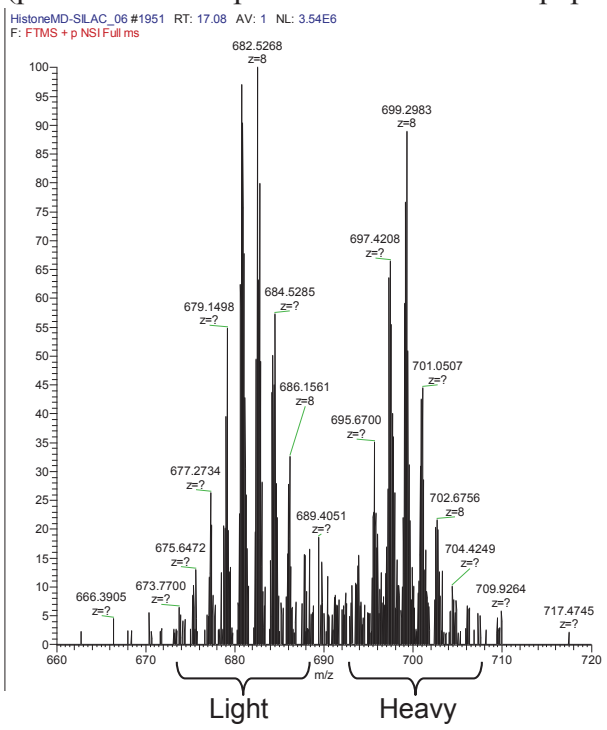
WCX-HILIC of Histone isoforms: Histone H3(2-51)

Over 700 variants of H3 have been identified with various combinations of 17 types of posttranslational modifications (PTM's). Most of the PTM's are in residues 2-51. H3 is typically analyzed via that fragment from Glu-C digestion ("middle-down" proteomics). Success in this identification requires good separation of the variants by chromatography prior to MS. This is accomplished with capillaries of our WCX material, CAT A. Inclusion of 60-70% ACN superimposes hydrophilic interaction and hence sensitivity to variations in polarity (e.g.,methylation) as well as charge. WCX materials lose their (-) charge below pH 4. WCX-HILIC can then be performed with a totally volatile mobile phase with a decreasing gradient of ACN (tuning down the hydrophilic interaction) and pH (uncharging the CAT A). The proteins can then be eluted directly to MS.

Full MS spectra (= intact mass) of histone H3 fragments: The two peak clusters represent light and heavy (= isotopically heavy Arg & Lys residues) SILAC histone H3 tails differing by 1 methylgroup (= 14 Da). The 2-µm CAT A material [RIGHT] affords sharp peaks with half as many unresolved variants complicating the mass spectrum as with the 3-µm material's results [LEFT].

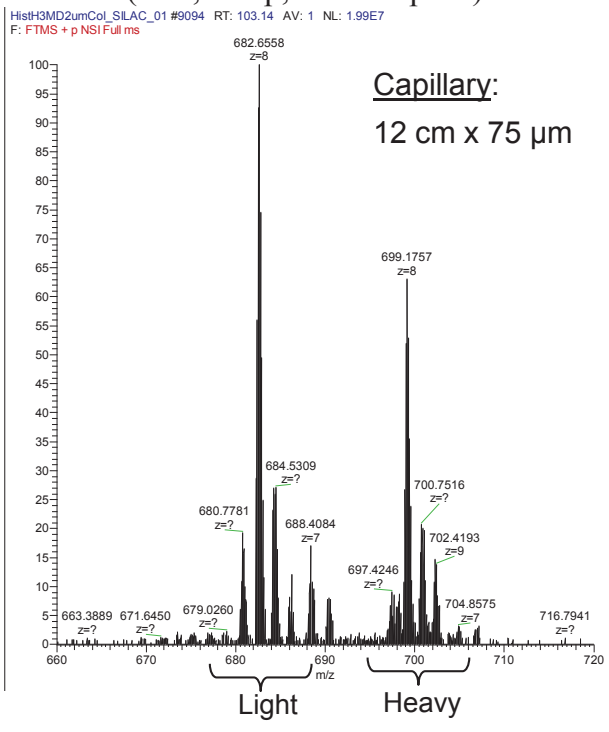
3 µm, 1500-Å CAT A

(peak not well-separated from other H3 peptides)



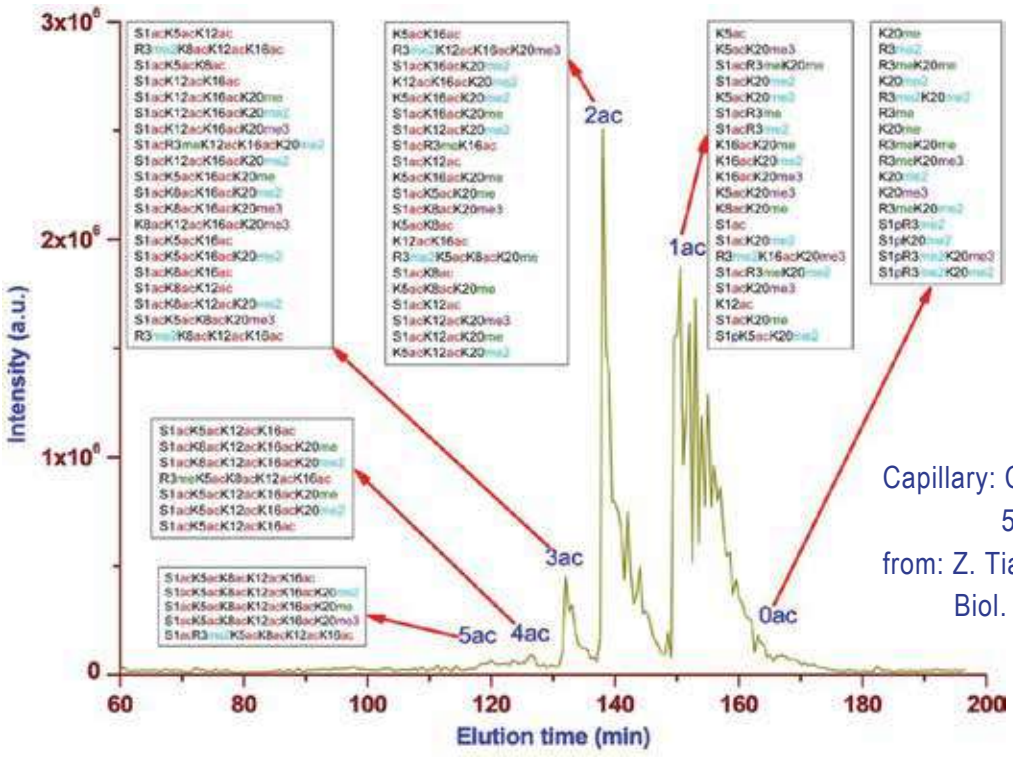
2 µm, 1000-Å CAT A

(nice, sharp, isolated peak)



Capillary:
12 cm x 75 µm

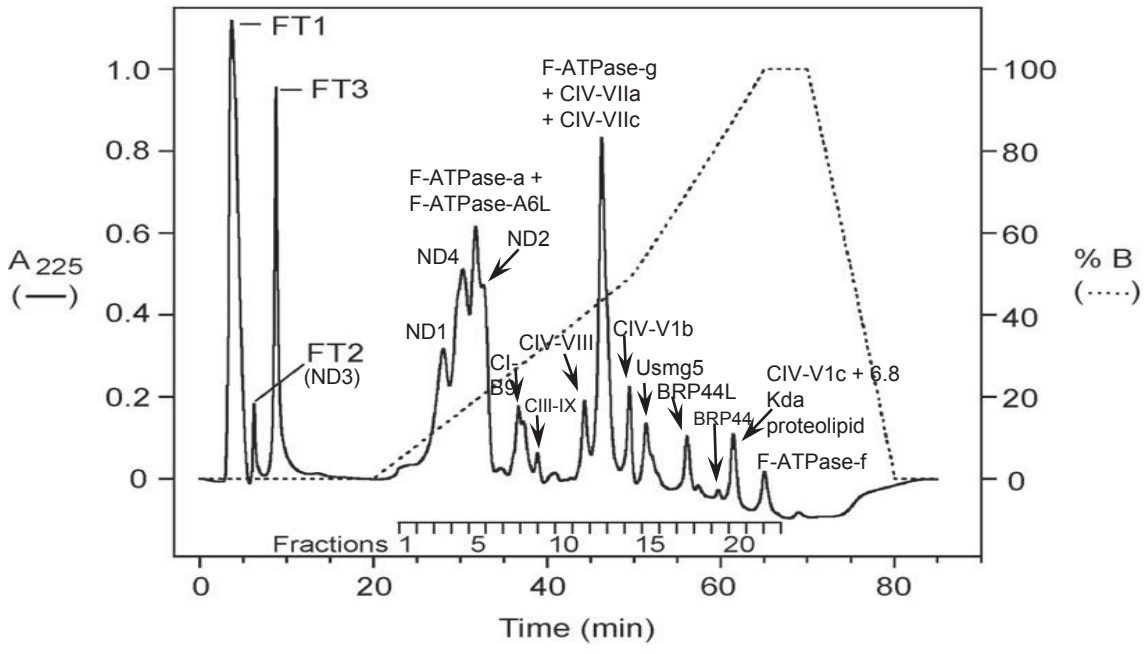
WCX-HILIC of Histone isoforms: Histone H4



Capillary: CAT A, 5-µm, 1000-Å; 50 cm x 100 µm
from: Z. Tian et al., Genome Biol. 13 (2012): R86

Histone H4 features acetylation on Lys. The more highly acetylated isoforms have less (+) charge and elute earlier in WCX-HILIC. The example above shows the elution positions of histone H4 isoforms with numerous PTM's in addition to acetylation on Lys.

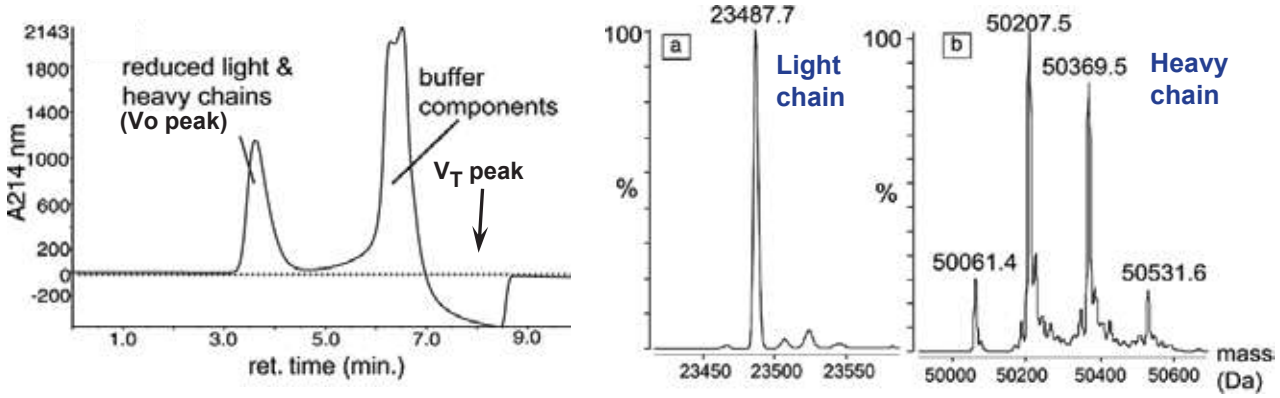
HILIC-MS of Mitochondrial Membrane Proteins

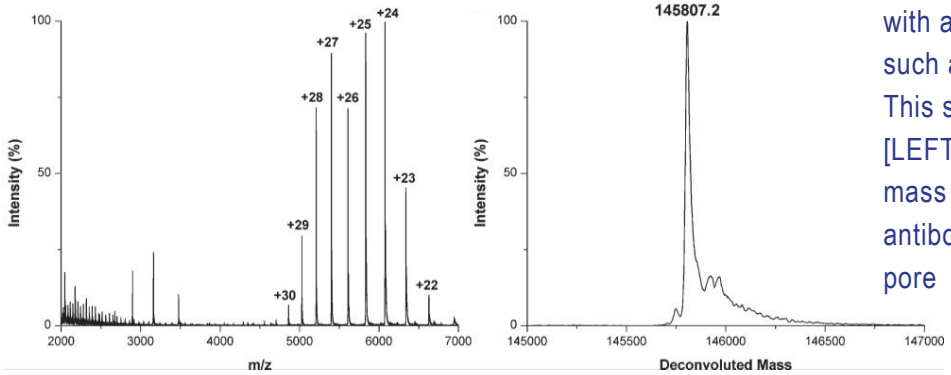


HILIC tends to works well for proteins that don't normally occur in aqueous media, such as membrane proteins, apolipoproteins, etc. In this case, a blend of 63% 2-propanol + 22.5% ACN was used, along with 0.5% hexafluoro-2-propanol and 20 mM NH4-formate (pH 3.7). A decreasing organic gradient was run.
Column: HYDROXYETHYL A, 5 µm, 300 Å; 100x1.0 mm

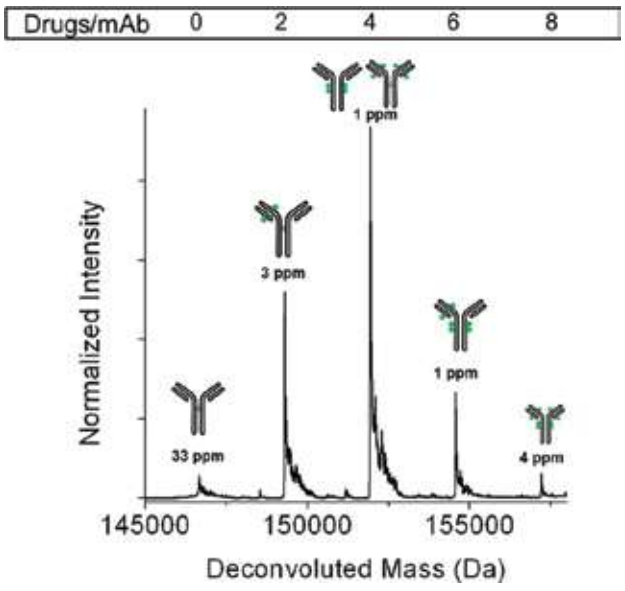
Size-Exclusion Chromatography: Examples with antibodies

SEC can be performed using volatile solvents. Here, antibody light and heavy chains all elute in the total exclusion volume peak (Vo) from a HYDROXYETHYLA column with a pore diameter of 200 Å and a mobile phase of 50 mm formic acid [LEFT]. The Vo peak is sent to a mass spectrometer and deconvoluted mass spectra are obtained for the light and heavy chains [RIGHT]





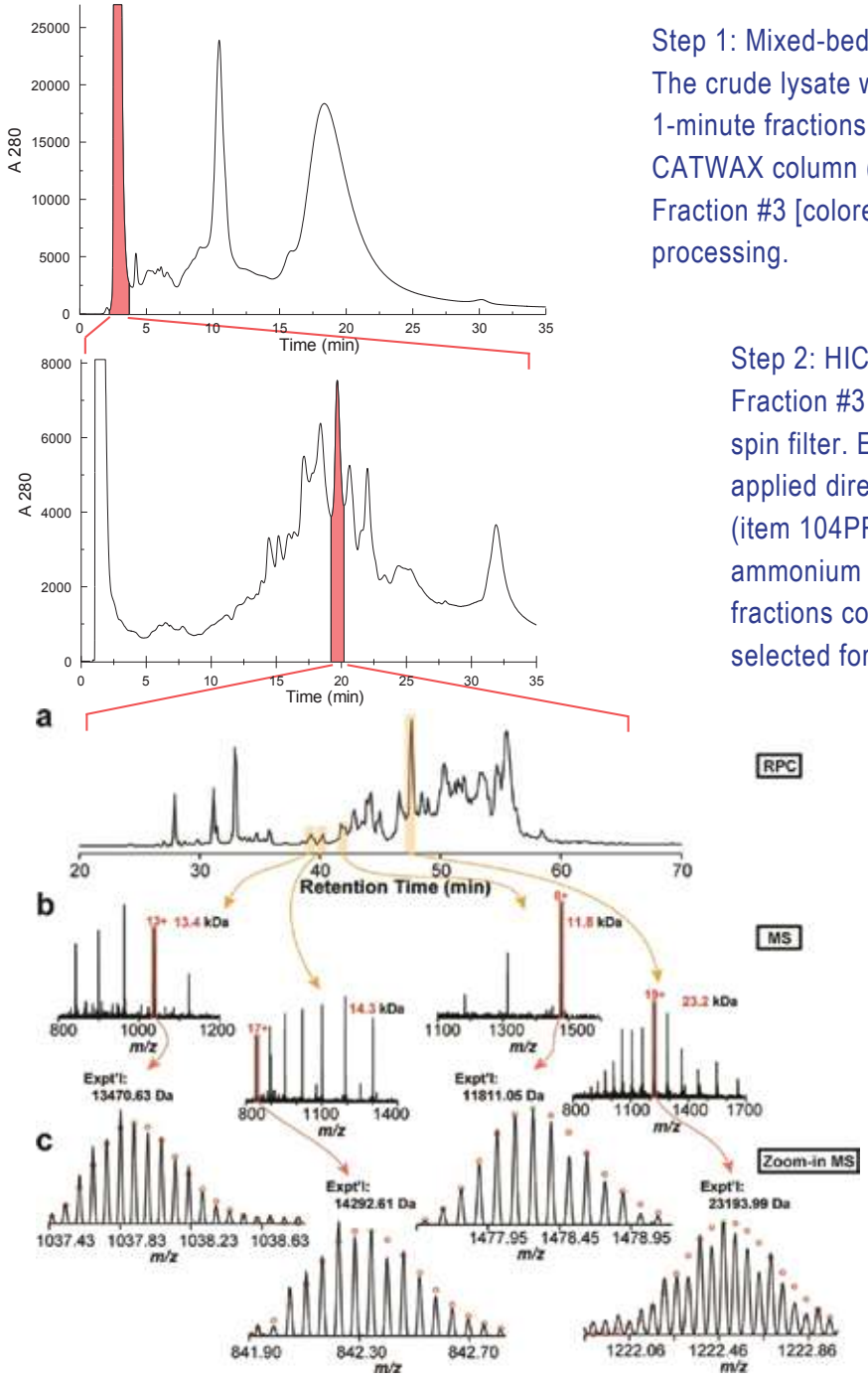
It is also possible to perform SEC with a nondenaturing mobile phase such as 200 mM ammonium acetate. This shows the raw unprocessed [LEFT] and deconvoluted [RIGHT] mass spectrum of an intact antibody molecule. Here, a 300-Å pore SEC column was used.



Here, nondenaturing SEC-MS is used to ascertain the distribution of antibody-drug conjugates (ADC's) with varying numbers of drug molecules conjugated to the antibody. This data was obtained with a 150x0.3-mm capillary of HYDROXYETHYL A (5-µm, 300-Å) for microscale analysis of 2 µg of sample.

3-D Protein Fractionation: An IEX-HIC-RPC Sequence

Sample: HEK 293 cell lysate



Step 1: Mixed-bed IEX
The crude lysate was separated into 35 1-minute fractions with a salt gradient on a CATWAX column (item 204CTWX0510). Fraction #3 [colored] was selected for further processing.

Step 2: HIC
Fraction #3 was concentrated with a 10 kDa spin filter. Extra salt was added and it was applied directly to a PROPYLA column (item 104PR0315). A decreasing gradient of ammonium tartrate was used, again with 35 fractions collected. Fraction #20 [colored] was selected for further processing.

Step 3: RPC
HIC fraction #20 was desalted with a 10 kDa filter and applied to a PLRP-S capillary (100 mm x 100 µm). Proteins were eluted with an ACN gradient in 0.25% formic acid into a Q Exactive Orbitrap mass spectrometer. Representative mass spectra are shown for the indicated peaks, along with zoom-in spectra with unit mass isotopic resolution.

RESULT:

Starting with all 35 HIC fractions from IEX Fraction #3, 640 intact proteins were identified. 201 of these id's were nonredundant (some proteins were identified in more than one HIC fraction). A 3D analysis of all 35 IEX fractions would have required 35x35 = 1225 RPC runs. Many more proteins would have been identified, at the cost of more time and effort. There is a tradeoff involved. It is still worthwhile; omitting the HIC step (with an IEX-RPC sequence only) resulted in just 47 nonredundant id's.

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