Liquid Chromatography Techniques for Sample Preparation

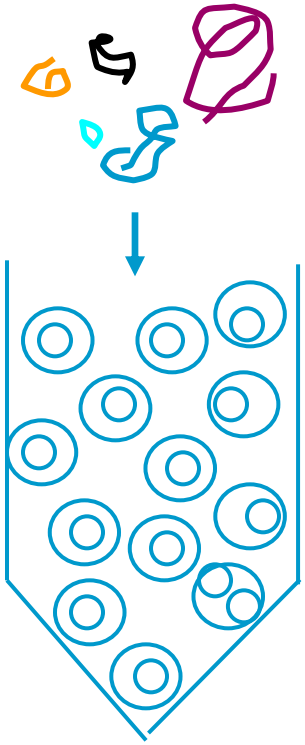
US HUPO 2009

February 22, 2009

Peptide/Protein Liquid Chromatography

Separation Technique	Principle
Size Exclusion Chromatography (SEC)	Hydrodynamic Volume (size/radius)
Hydrophobic Interaction Chromatography (HIC)	Hydrophobic Interaction under high salt
IMAC (immobilized metal affinity chrom.)	Non-specific affinity
Ion Exchange Chromatography	Charge properties of the molecule
Reversed-Phase Chromatography	Hydrophobic Interaction
Affinity Chromatography	(Bio) Specificity for a defined ligand

Size Exclusion Chromatography (Gel Filtration)



Protein/peptide sample contains molecules of different size

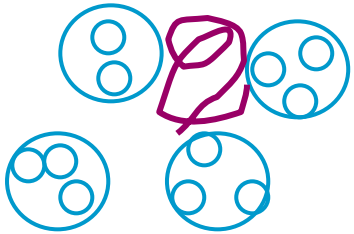
Column contains porous particles

Particles act as molecular sieve

Separation according to size

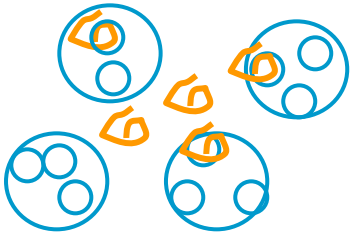
1. Big molecules cannot enter pores

→ fast elution



2. Small molecules enter pores

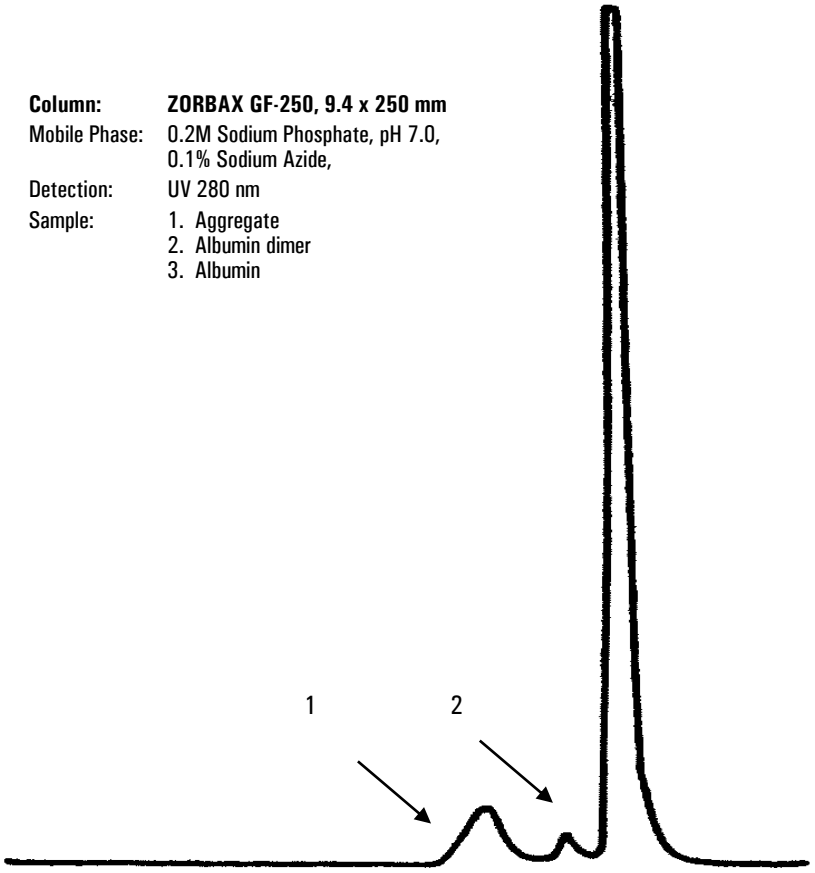
→ later elution



Size Exclusion (SEC) Applications with Proteins

- Commonly used for impurity testing
- MW characterization – good MW accuracy and precision (<2%) over wide MW range (1000 – 10M)
- Expression and folding studies
- Separation of reaction components and products
- Protein purification
- Collection of fractions under non-denaturing conditions - maintains activity

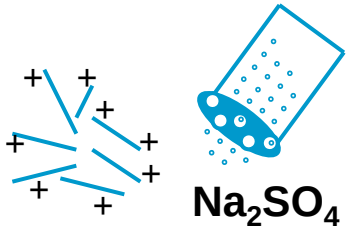
Separation of Albumin Monomer, Dimer and Aggregate



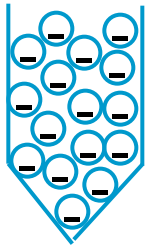
DeLeenheer, A.P. et al. *J. Pharm Sci.*, (1991) 80, 11. Reprinted with publisher's permission

Hydrophobic Interaction Chromatography

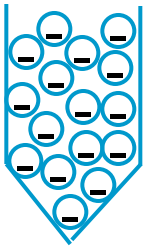
- HIC separates proteins using hydrophobic functional groups such as phenyl, octyl, or butyl
- Hydrophobic amino acid side-chains will bind to the hydrophobic groups on the stationary phase
- High ionic strength buffers (high salt) are used, more hydrophobic proteins will require less salt to promote binding
- Decreasing the salt concentration promotes protein/peptide elution
- HIC is often considered the opposite of IEX chromatography and can be used when proteins will not bind an ion exchanger



High Ionic Strength buffer applied allowing hydrophobic proteins to bind



Elution occurs by applying more and more salt diluting buffer



Decreasing salt concentration will break the hydrophobic interaction and proteins will elute

Samples are separated according to their differing hydrophobicity

IMAC - Immobilized Metal Affinity Chromatography

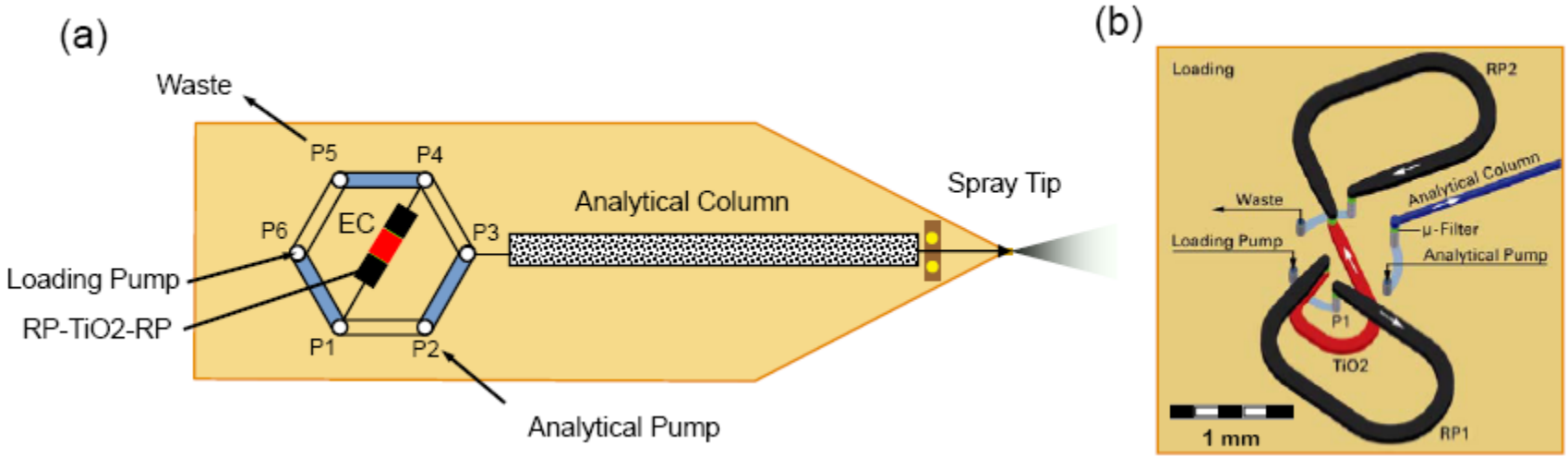
Principle

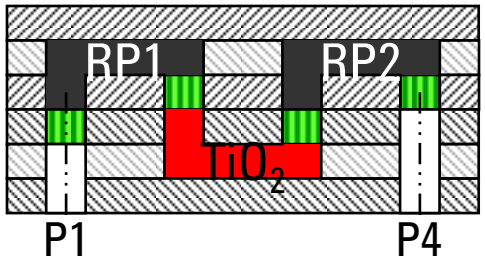
- IMAC is based on covalent binding of amino acids, particularly histidine, to metals
- Proteins with an affinity for metal ions to be retained in a column containing immobilized metal ions, such as cobalt, nickel, copper, zinc, or iron ions
- Naturally occurring proteins often do not have affinity for metal ions and recombinant DNA can be used to introduce metal affinity into a targeted protein
- Common elution methods include changing the pH and/or adding a competition molecule, such as imidazole.

Key Application

- Isolation of phosphorylated proteins or peptides from complex mixtures
- The stationary phase containing an immobilized transition metal is “charged” (Ga(III) or Fe(III)) causing the metals to form tight complexes
- Phosphorylated protein digests are loaded onto the column, the column is washed and then elution occurs either with pH change or imidazole buffer
- Detection of low-abundant phosphopeptides is difficult due to non-phosphorylated high-abundant proteins containing histidine
- Titanium oxide (TiO_2) is another metal-based chromatography method for phosphopeptide analysis

Phosphochip With Titanium Oxide Based Enrichment





Packing material:

RP 1: Zorbax Extend 5 µm, 300Å

TiO_2 : 10 µm spheres

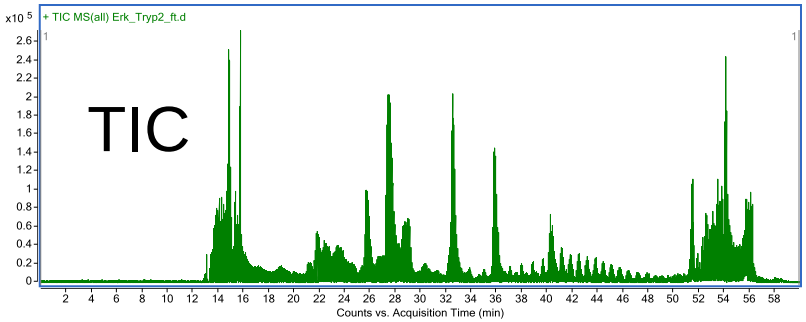
RP 2: Zorbax Extend 5 µm, 300Å

AC : Reprosil-Pur C18-AQ 3 µm

Mohammed, S., Chip-base enrichment and nanoLC-MS/MS analysis of phosphopeptides from whole lysate. JPR, 2008

Phosphochip Analysis of MAP Kinase ERK

Flow-through

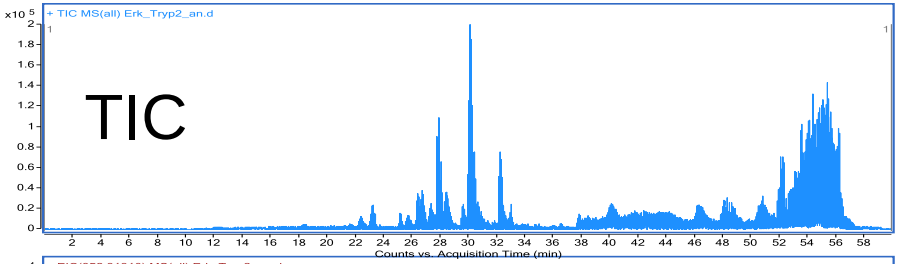


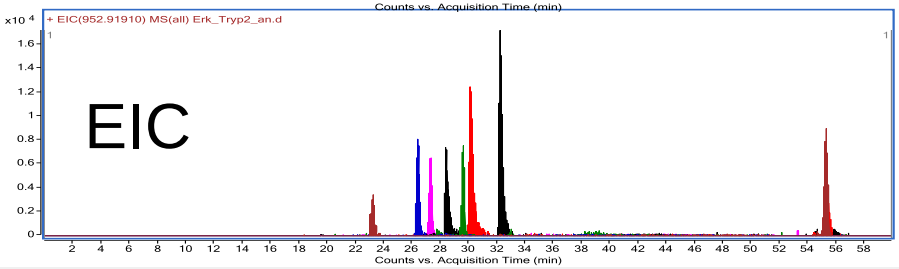
Group (#)	Spectra (#)	Distinct Peptides (#)	Distinct Summed MS/MS Search Score	% AA Coverage	Mean Peptide Spectral Intensity	Database Accession #	Protein Name
1	30	24	400.16	39	2.07e+005	1	rhErk1

#	Score	SPI (%)	Spectrum Intensity	Sequence Map	Modifications	RT (min)	m/z Measured (Da)	MH+ Matched (Da)	MH+ Mass Shift (Da)	MH+ Error (ppm)
1	25.72	95.2	2.90e+005	(R)Y T/Q|L|Q|Y|I|G E/G A Y/G M/V/S/S A/Y D H V R(K)		40.48	870.0756	2608.214	-0.0014	-0.5
2	23.63	97.6	1.59e+005	(R)G S/P G|I|P M/A|A|A|A|A/Q|G G G G G E/P R(R)		25.45	904.9378	1808.871	-0.0025	-1.4
3	22.08	96.0	5.29e+005	(-)M/S|P/I|L/G/Y/W/K(I)		36.08	547.7891	1094.570	0.0006	0.6
4	21.83	56.0	6.05e+004	(K)Y I|A|W|P L/Q/G W Q A/T/F G G G D/H/P P K(S)		43.24	776.0488	2326.140	-0.0085	-3.7
5	19.84	89.2	2.35e+004	(R)A E|I|S/M/L|E|G/A|V|L/D/I/R(Y)		41.01	758.9069	1516.804	0.0026	1.7
6	19.74	93.5	5.06e+004	(R)W Y R A/P/E/I/M/L/N/S K(G)		23.70	503.2617	1507.773	-0.0020	-1.4
7	19.31	97.8	1.91e+005	(R)A/P|E|I|M|L/N/S K(G)		23.49	501.7679	1002.529	-0.0003	-0.3
8	18.93	86.2	2.24e+004	(K)I/S|P/F/E/H/Q T/Y/C Q R(T)	C:Carbamidomethylation	21.61	522.5736	1565.717	-0.0103	-6.6
9	18.34	80.2	1.89e+005	(K)L/T|Q/S/M/A/I/I/R(Y)		25.63	516.7970	1032.587	-0.0003	-0.3
10	18.19	88.1	3.80e+005	(K)I/C|D|F/G/L A/R(I)	C:Carbamidomethylation	26.86	476.2384	951.472	-0.0021	-2.2
11	17.55	89.3	2.02e+006	(R)L/K E L|I|F|Q/E/T A/R(F)		27.56	449.9257	1347.763	-0.0005	-0.4
12	17.19	85.2	4.98e+005	(R)M/L/		47	374.2019	1120.593	-0.0020	-1.8
13	16.31	82.0	1.87e+005	(R)N/Y|		94	525.2842	1049.563	-0.0015	-1.4
14	16.23	88.3	8.54e+004	(R)R T/		36	580.9674	1740.895	-0.0072	-4.2
15	15.23	60.3	2.13e+004	(R)Y I/		59	535.2619	1603.772	-0.0008	-0.5
16	14.59	87.2	6.64e+004	(K)E L|		97	553.7946	1106.584	-0.0021	-1.9
17	13.99	85.2	8.73e+005	(R)F Q|P G V|L|E|A|P(-)		32.20	479.2552	957.504	-0.0009	-0.9
18	13.47	81.0	1.39e+005	(K)G Q|P/F/D/V/G/P R(Y)		23.83	486.7480	972.490	-0.0010	-1.1
19	13.22	82.5	1.56e+005	(K)A/L|D|L/L D/R(M)		28.24	408.2345	815.462	-0.0004	-0.5
20	12.85	81.4	4.60e+004	(R)N/Y|L|Q/S|L/P S|K/T K(V)		21.17	639.8551	1278.705	-0.0023	-1.8
21	12.60	70.1	1.40e+005	(R)I/E|A|I/P/Q/I D K Y L K(S)		33.07	477.6123	1430.825	-0.0030	-2.1
22	11.95	62.3	5.85e+003	(R)Y T|Q|L|Q|Y/I G E/G A Y G m/V S S/A Y D/H V R(K)	m:Oxidized methionine	35.27	875.4174	2608.214	16.0240	11.1
23	11.44	63.1	2.88e+004	(-)m S|P/I/L G/Y W K(I)	m:Oxidized methionine	33.02	555.7836	1094.570	15.9896	-4.8
24	10.65	76.2	3.06e+003	(R)T E|G|V|G/P G V|P G E V E M V K(G)		28.21	792.9000	1584.794	-0.0011	-0.7
25	10.18	55.4	3.77e+003	(K)Y/I|A/M/P L q G W Q A|T F G/G G D H P P K(S)	q:Deamidated Q	45.45	776.3858	2326.140	1.0025	7.9
26	9.82	79.0	5.72e+003	(K)L/V|C/F/K(K)	C:Carbamidomethylation	21.45	333.6839	666.364	-0.0038	-5.7
27	9.20	52.0	6.54e+003	(R)Y T Q L|q/Y/I/G E/G A Y G m V S S A Y D H V R(K)	q:Deamidated Q m:Oxidized methionine	35.42	875.7393	2608.214	16.9897	4.1
28	9.12	59.8	1.06e+004	(R)A/P|E I/M|L/n S K(G)	n:Deamidated	24.32	502.2627	1002.529	0.9893	5.2
29	8.88	52.1	9.22e+003	(R)Y T/Q|L|q Y I/G E/G A Y G M V S S/A Y D H V R(K)	q:Deamidated Q	41.88	870.4075	2608.214	0.9943	3.9
30	8.85	65.6	8.19e+003	(R)T L/R E|I Q|I|L/L/R(F)		33.89	418.9338	1254.789	-0.0024	-1.9

No Phosphopeptides

Elution





Group (#)	Spectra (#)	Distinct Peptides (#)	Distinct Summed MS/MS Search Score	% AA Coverage	Mean Peptide Spectral Intensity	Database Accession #	Protein Name
1	21	14	216.59	30	9.85e+004	1	rhErk1

#	Score	SPI (%)	Spectrum Intensity	Sequence Map	Modifications	RT (min)	m/z Measured (Da)	MH+ Matched (Da)	MH+ Mass Shift (Da)	MH+ Error (ppm)
1	13.80	66.6	1.70e+005	(R)I/A|D|P E H D H T G F|L|t/E/Y V A t R(W)	t:Phosphorylated T	29.96	777.9932	2172.036	159.9294	-1.4
2	22.77	82.7	7.21e+004	(R)G s/P G I|P m A/A|A/A/A/Q/G G G G G E/P R(R)	m:Oxidized methionine s:Phosphorylated S	22.98	952.9191	1808.871	95.9601	-0.6
3	21.06	92.9	1.29e+005	(K)S D L|V|P R G s P G I|P m A/A/A|A|A|Q|G/G G E/P R(R)	m:Oxidized methionine s:Phosphorylated S	26.18	858.0679	2476.236	95.9530	-3.2
4					s:Phosphorylated S	27.21	944.9240	1808.871	79.9699	1.9
5					s:Phosphorylated S	29.50	852.7387	2476.236	79.9654	-0.4
6					y:Phosphorylated Y	28.70	751.3358	2172.036	79.9572	-4.0
7					t:Phosphorylated T	32.05	751.3358	2172.036	79.9572	-4.0
8	13.30	56.7	7.00e+004	(R)G s P G I|P M A A A/A/A/Q/G G/G G G E/P R(R)	s:Phosphorylated S	27.08	630.2805	1808.871	79.9561	-5.4
9	13.72	61.0	7.39e+003	(K)E R A E I S m/L/E/G/A/V L/D/I R(Y)	m:Oxidized methionine	35.42	606.6565	1801.948	16.0073	6.8
10	12.81	88.4	5.01e+004	(R)R T E G V|G|P G|V|P/G E/V E m V K(G)	m:Oxidized methionine	22.14	586.2969	1740.895	15.9813	-7.8
11	11.49	52.7	8.66e+003	(R)Y T/q L/Q Y/I/G E/G A Y/G/M V/S S A Y D H/V R(K)	q:Deamidated Q	39.53	870.4128	2608.214	1.0102	10.0
12	17.25	78.8	2.48e+004	(R)Y T Q|L|Q/Y/I|G E/G A/Y/G M V/S S A/Y D H V R(K)		39.97	870.0795	2608.214	0.0103	3.9
13	9.94	59.9	2.85e+003	(R)A/P|E/I/M L/N/S K(G)		23.78	501.7689	1002.529	0.0017	1.7
14	10.29	65.8	1.06e+004	(R)N/Y|L|Q/S L/P/S K(T)		24.24	525.2855	1049.563	0.0011	1.1
15	10.55	77.1	1.04e+004	(K)A/L|D|L/L D/R(M)		28.39	408.2339	815.462	-0.0016	-2.0
16	11.39	77.5	2.29e+004	(K)S D/S K/A|L|D|L/L D|R(M)		26.62	616.8255	1232.648	-0.0044	-3.6
17	16.91	96.2	7.86e+004	(R)D L K P S N|L|L|I|N/T/T C D L K(I)	C:Carbamidomethylation	32.78	615.6627	1844.979	-0.0051	-2.8
18	24.40	98.7	8.79e+005	(R)R T E G V|G|P G|V|P/G/E/V/E M/V K/G Q/P F/D/V G P R(Y)		29.85	674.3454	2694.367	-0.0070	-2.6
19	17.98	96.9	1.28e+005	(R)N K K F E L G L E|F|P N|L|P/Y/Y I/D G D/V K(L)		40.03	650.5888	2599.344	-0.0109	-4.2
20	12.87	64.1	3.45e+004	(R)D L K P S N L|L|I/N/T/T C D|L/K/I/C D F/G L A R(I)	C:Carbamidomethylation	39.75	695.1107	2777.432	-0.0115	-4.1

Phosphopeptides

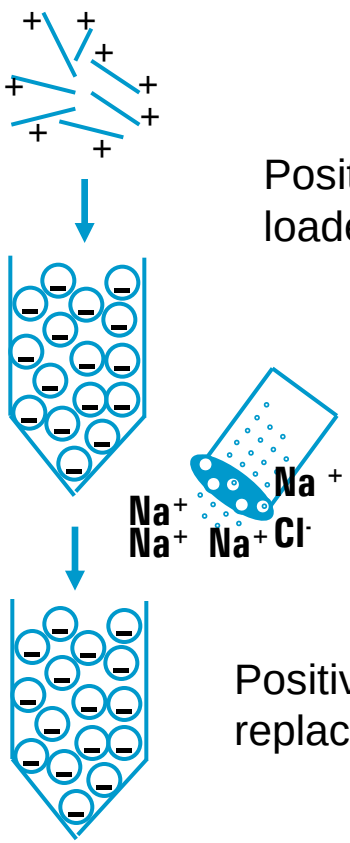
Cation Exchange Chromatography

Principle:
competitive interaction of ions:
charged sample molecule competes with salt ion about fixed charges of stationary phase

Cation exchange:
stationary phase carries negative charge, analyzed peptide molecules are positively charged (at acidic pH)

Functional groups of column are:
Sulfonic acid, sulfomethyl, sulfoethyl, sulfopropyl

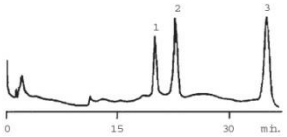
Elution:
by increasing salt concentration or pH change



Positively charged sample is loaded and bound to column

Elution with salt or pH

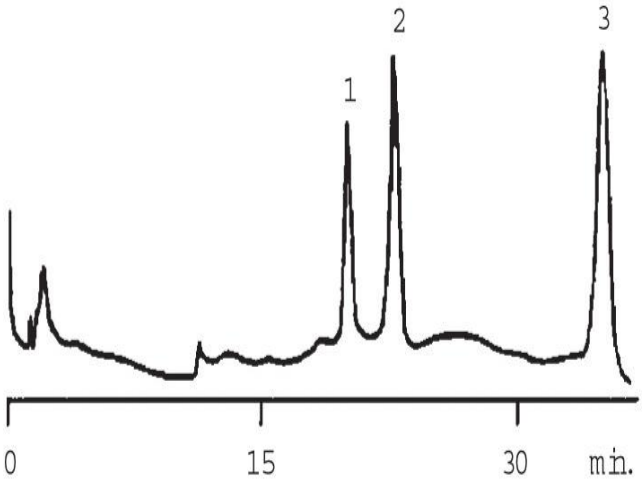
Positively charged salt ions replace bound sample



Samples are separated according to difference in their net charge

Ion Exchange Chromatography of Proteins

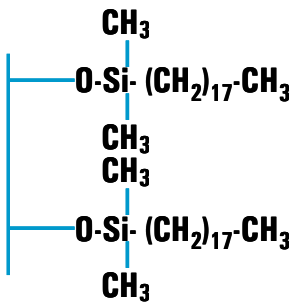
- Ion-exchange chromatography (IEC) discriminates between proteins on the basis of accessible surface charges and their corresponding electrostatic interaction with the column's stationary phase.
- The degree of protein retention is dependent on the strength and number of interactions.
- The 3-D structure of the protein determines which surface residues will be available to contact the column's stationary phase.
- The net charge determines the form of IEC (anion exchange or cation exchange) to be applied.
- Cation exchange is used at pH's below a proteins pI, while anion exchange is used at pH's above a proteins pI.
- Sample pI is a guideline and not absolute. Protein interaction with a column's stationary phase is dependent on the microenvironment of the interaction site.



Column:	SynChropak SCX, 4.6 x 100 mm, 6.5 µm
Mobile Phase:	A: 0.02 M tris, pH 7
	B: 0.02 M tris in 0.5M sodium acetate, pH 7
Gradient:	0 – 100% B in 30 min.
Flow Rate:	1.0 mL/min
Detection:	UV 254 nm
Sample:	Basic proteins
	1. RNA polymerase
	2. Chymotrypsinogen
	3. Lysozyme

Reversed Phase Chromatography

- Reversed phase: historical term: “nonpolar hydrocarbon chains are attached to polar groups”
- Stationary phase: silica gels with hydrocarbon chains between 1 and 18 carbon atoms (C1 to C18)
- For peptides: C18 phases are most popular

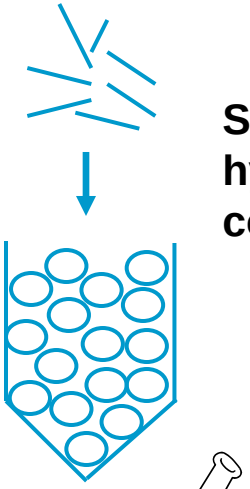


- Mobile phase: organic solvent; ion pair reagent

Elutropic force →

Water > Methanol > Acetonitrile > n-Propanol > THF

← Polarity



Sample retains on nonpolar hydrocarbon chains of column stationary phase

Elution by increasing hydrophobicity:

Concentration of organic solvent increases to release compounds

Proteins and peptides are separated according to their polarity

Fraction collection

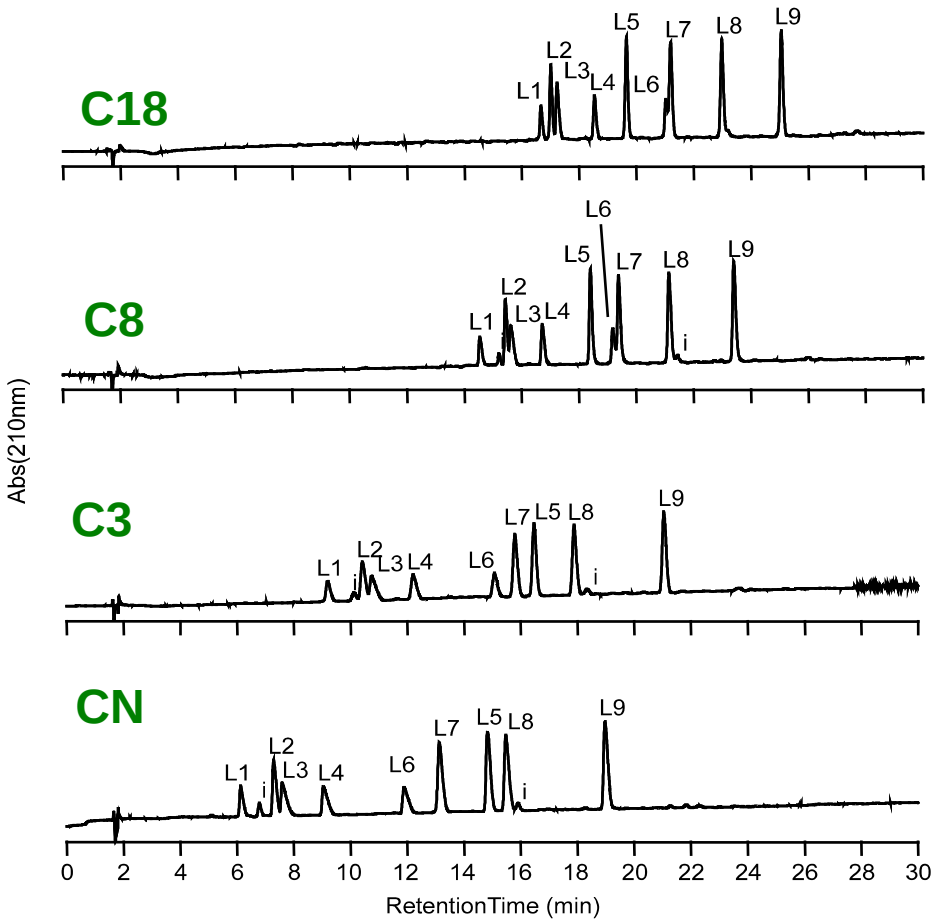
Reversed Phase Columns for Separations of Proteins and Peptides

Requirements

- Wide pore for unrestricted access to bonded phase (especially for intact proteins)
- LC/MS compatible bonded phases at low and high pH – low bleed, high performance
- Multiple bonded phases for method optimization (differing selectivities)
- Many configurations for LC/MS compatibility, small sample sizes and 2-D HPLC for proteomics

Solvent	Application	Comments
0.05-5% TFA in Water	General	Effective solubilization of many samples
6 M Guanidine, buffered at pH 6-8	General	Very good for many proteins and peptides
5-80% Acetic Acid or Formic Acid; 0.1-0.5M Perchloric Acid	Peptides	Frequently used to extract peptides from tissues, precipitating many proteins and cellular debris
6 M Urea/ 5% Acetic aid	Hydrophobic Peptides, Proteins	Useful for membrane proteins, fragments, aggregating systems
Water-Miscible Organic Solvents: Acetonitrile, Methanol, THF, Dioxane, DMSO; +/- TFA; +/- Water	Hydrophobic Peptides, Polypeptides	Limit injection volume to avoid problems; add water, as possible, to improve volume tolerance; acidify with TFA as required

Comparison of Small Peptide Selectivity Differences on Bonded Phases



Conditions:

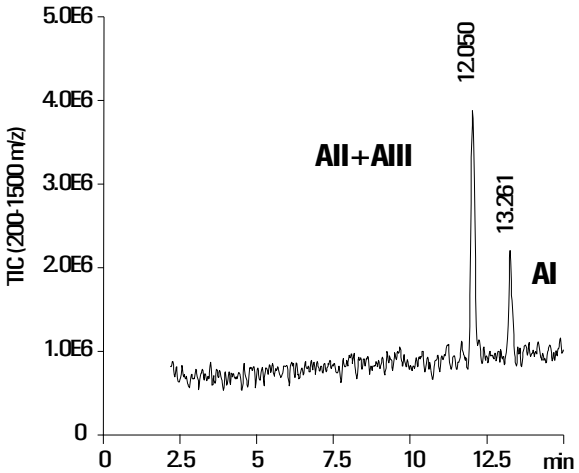
Columns: ZORBAX 300SB, 4.6 x 150 mm, 5 µm
Mobile Phase: Gradient, 0 - 26% B in 30min.
A = 0.1% TFA in Water
B = 0.1% TFA in Acetonitrile
Temperature: 40°C
Sample: 2 µg of each peptide
Flow Rate: 1.0 mL / min.
Detection: UV-210nm

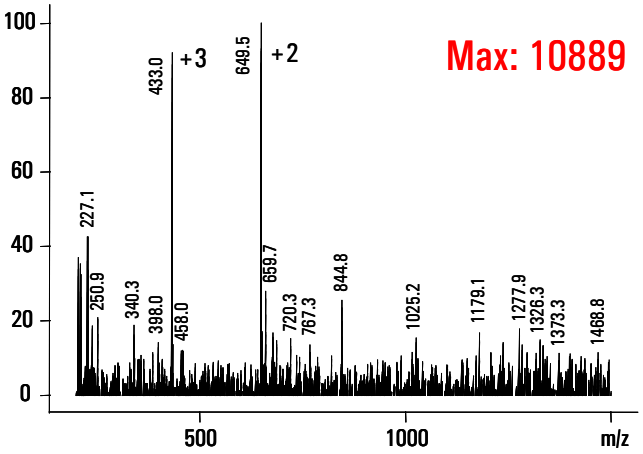
Evaluate High pH for Improved Selectivity and Resolution

- Changing pH of the mobile phase from low pH to high pH can change selectivity and retention
- Special columns are required to work at high pH – many manufacturer's have columns specific for high pH
- Ideal for analysis of proteins and peptides at mid and high pH
- Ammonium hydroxide is an excellent mobile phase additive for LC and LC/MS (can use instead of TFA)

LC/MS of Peptides: Low vs. High pH

Column: ZORBAX Extend-C18, 2.1 x 150 mm, 5 μm Flow Rate: 0.2 mL/min Temperature: 35 C
Mobile Phase Gradient: 15-50% B in 15 min. A: 0.1% TFA in water B: 0.085% TFA in 80% ACN
LC/MS: Pos. Ion ESI – Vf 70V, Vcap 4.5 kV, N_2-35 psi, 12L/min., 325 C Sample: Angiotensin I, II, III 2.5 μL (50 pmol each)



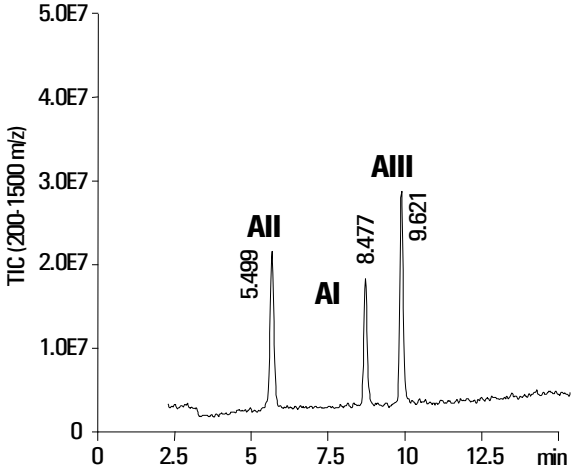


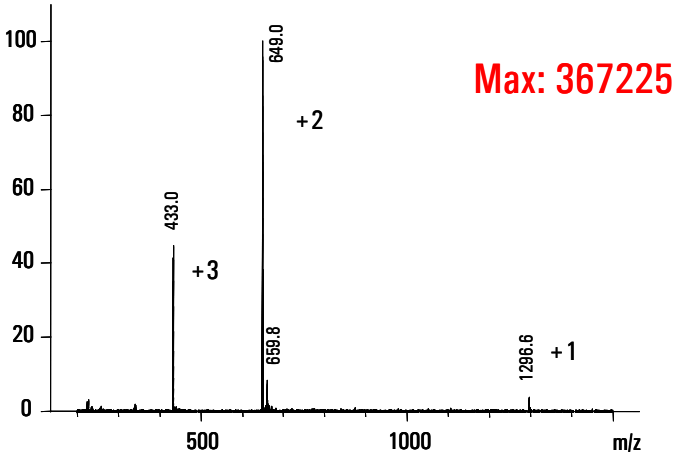
• Acidic conditions can not resolve all three Angiotensins.

LC/MS of Peptides at High pH

Column: ZORBAX Extend-C18, 2.1 x 150 mm, 5 µm Flow Rate: 0.2 mL/min Temperature: 35 C
Mobile Phase Gradient: 15-50% B in 15 min. A: 10 mM NH_4OH in water B: 10 mM NH_4OH in 80% ACN
LC/MS: Pos. Ion ESI – Vf 70V, Vcap 4.5 kV, N_2-35 psi, 12L/min., 325 C Sample: Angiotensin I, II, III, 2.5 µL (50 pmol each)

Signal x10



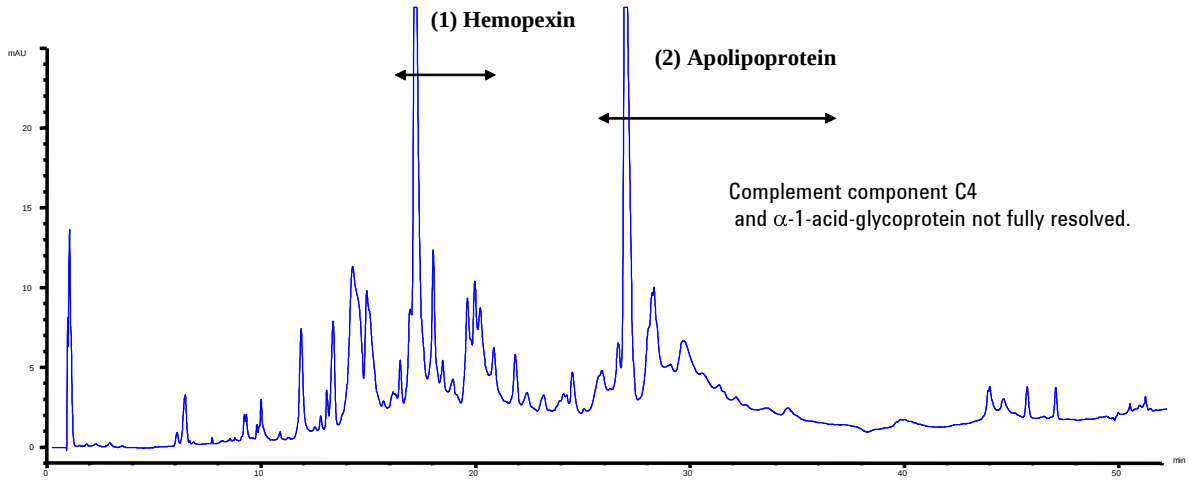


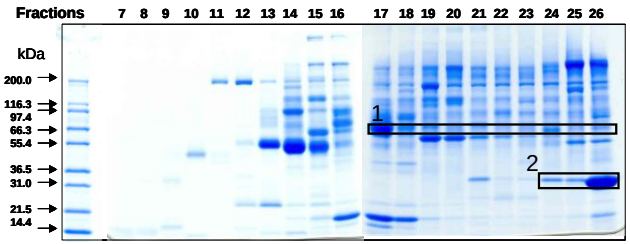
At high pH all 3 angiotensins are resolved and the mass spectrum shows greater signal and improved spectral clarity.

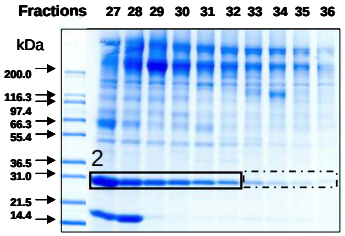
Disadvantages of Traditional Reverse Phase Techniques for Protein Fractionation

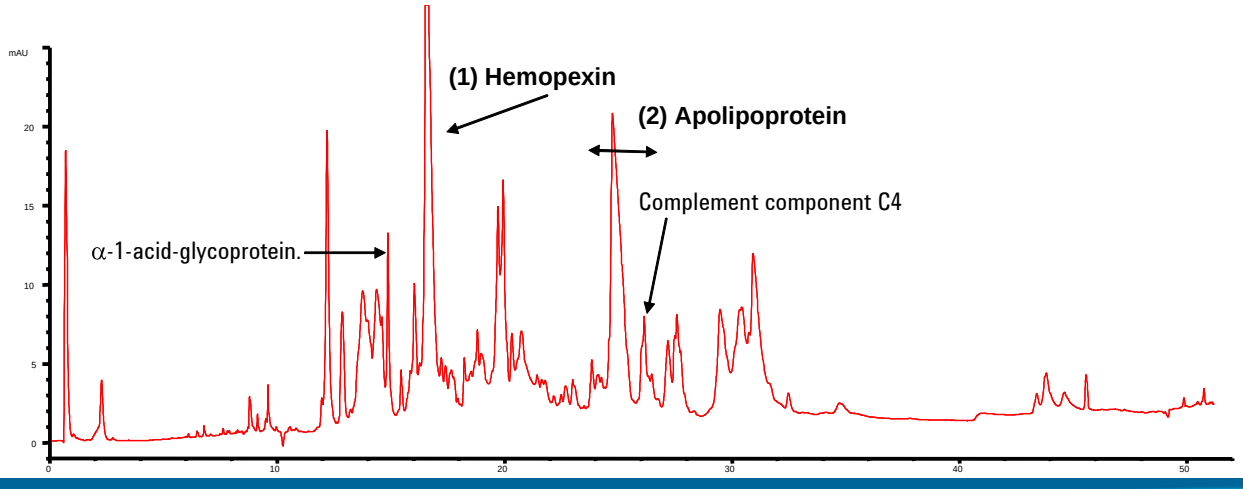
- **Recovery –** typical reverse phase can range from 30-80% recovery
- **Reproducibility –** due to poor recovery, the reproducibility often suffers from carryover
- **Capacity –** limited in ability to load ***AND*** resolve proteins
- **Is it possible with reverse phase to:**
  - Extremely high recoveries
  - High column loads
  - Improved protein resolution
- **What does it take:**
  - Column Packing Materials - strong effect on separation characteristics- resolution, selectivity, reproducibility, load and recovery
  - Gradient – improves recovery and enhances higher abundant protein resolution
  - Temperature- Improves protein separations and aids in recovery

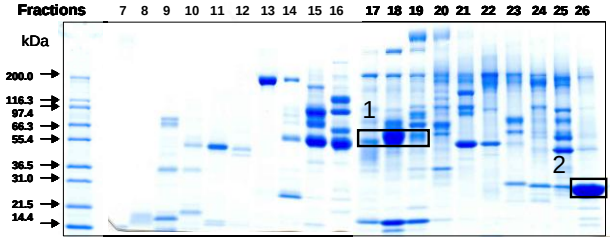
Macroporous Reverse Phase Column (mRP) for Protein Fractionation

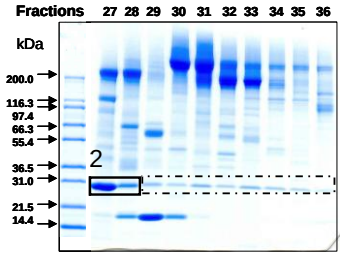




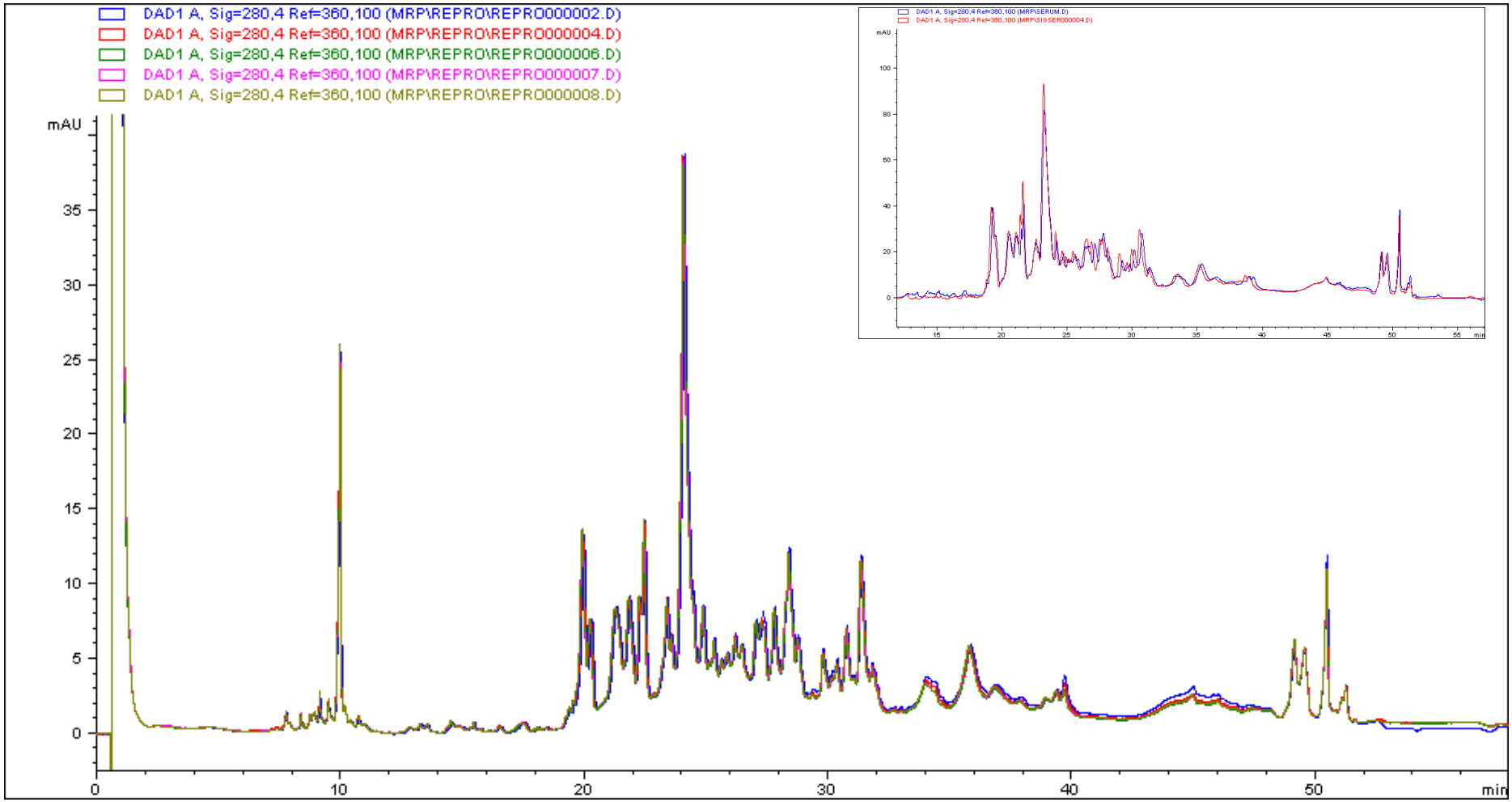




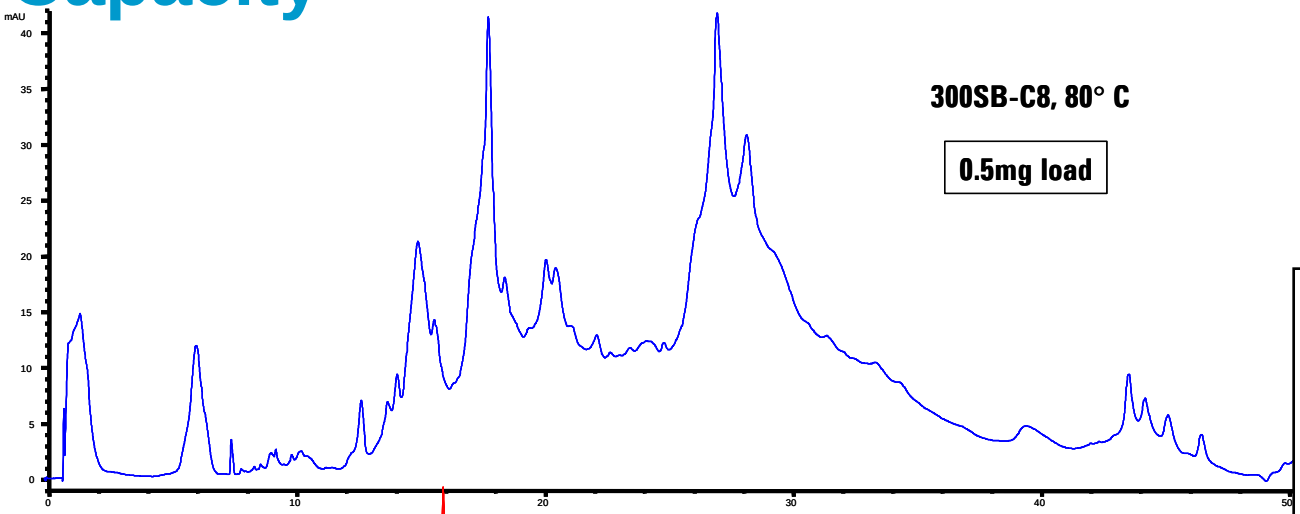


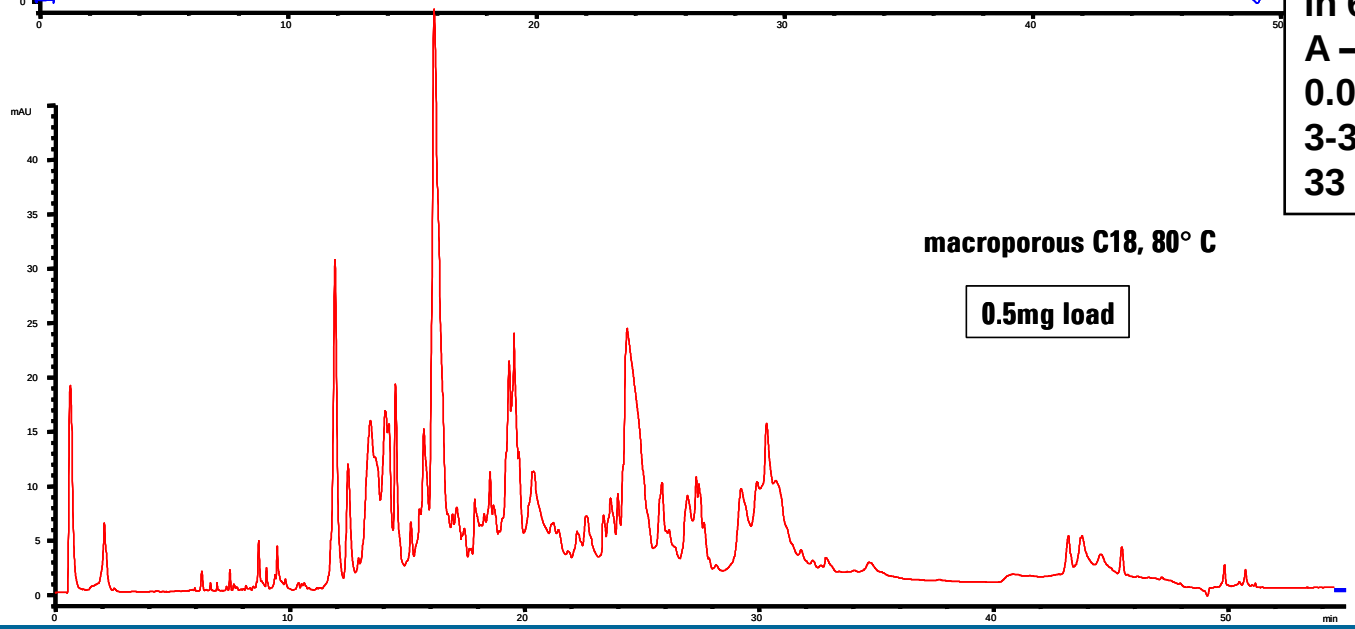


Macro Reverse Phase Reproducibility



Macroporous Reverse Phase Resolution and Capacity





Conditions: mRP-C18, 4.6 mm ID x 50 mm; 0.75 mL/min.
Sample: Immunodepleted Human serum (500 ug Protein) in 6M urea/1% HOAc
A – 0.1% TFA in water, B – 0.08% TFA in AcN
3-30%B in 6 min, 30-55%B in 33 min, 55-100%B in 10 min

Affinity Chromatography

Principle:

Bio-specific technique that relies upon inherent biological properties such as shape (enzyme:substrate), confirmation changes, areas of molecule (antigen:antibody)

Immunoaffinity (example):

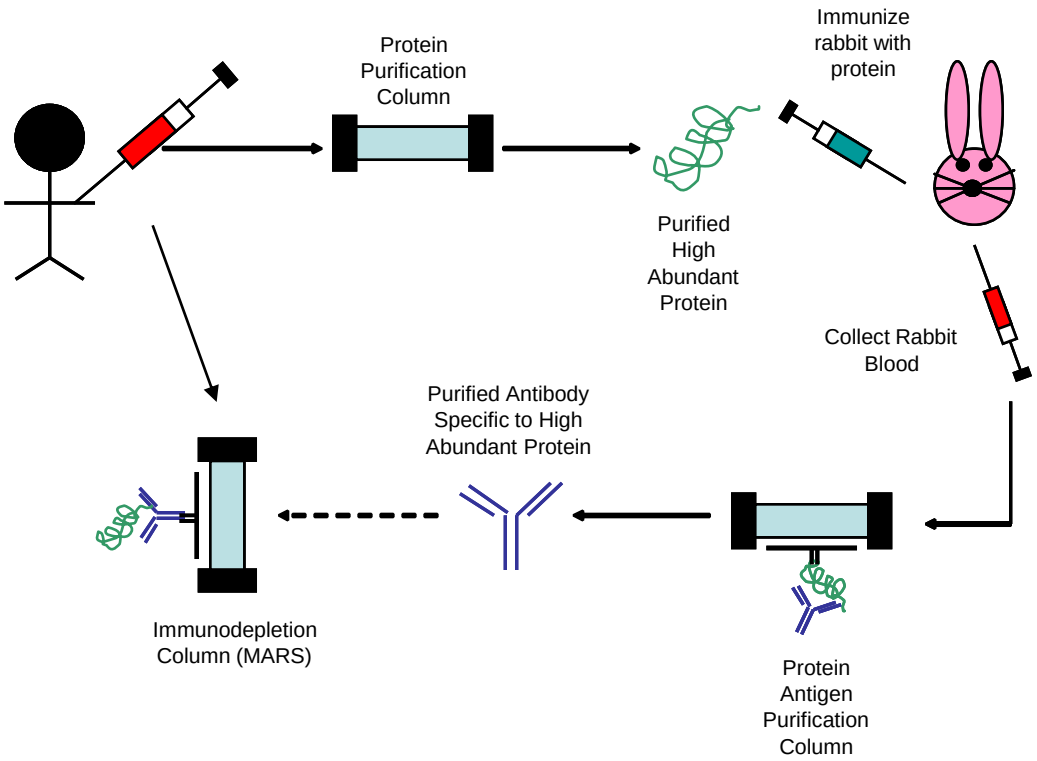
antibody bound to bead with proper orientation, optimize buffer to minimize non-specific interaction

Matrix Design:

Hydrophilic, large pores, rigid, inert and stable

Elution:

use specific elution if possible by competitive action or non-specific elution with buffers



Applications for Affinity Chromatography

- Depletion of High-Abundant Proteins
- Affinity purification of selected protein or peptides
- Validation of protein biomarker

Antibody Based

Pros

•Selectivity

•Robust

•Specificity

Cons

•Cost

•Purity of antigen

•Slow

Dye Based

Pros

•Cost

•Robust

Cons

•Reproducibility

•Specificity

•Selectivity

HPLC and LC/MS of Proteins for Proteomics

Proteins (mixtures of ~ 10^4 to 10^5 different proteins) are present at low – high levels and one protein results in up to 70 tryptic peptide fragments

- Requires Multi-D techniques to obtain information on all proteins present – use 1 or more different chromatographic techniques to reduce complexity before LC/MS analysis
- High Sensitivity LC/MS

MS for identification

- Typically 10 – 30 % sequence coverage is enough for a significant hit in protein data base search
- If protein mixture is too complex, MS/MS information is lost through co-elution of too many tryptic peptides -> less sequence coverage or even no protein identification

HPLC and LC/MS for Protein Fractionation

Advantages

- Recovery (always improving)
- Resolution of proteins
- Reproducibility
- Visible – one can see the “proteome”
- Automation

Limitations

- Limits visibility of PTM proteome - (IMAC & TiO_2)
- Low abundant proteins are masked by high abundant proteins

2-D-HPLC for Proteomics

Advantages

- Most sensitive for low abundance proteins
- Easier automation
  - proteins stay in liquid
  - fraction collection
  - sample preparation
- Flexibility
  - separation technique
  - Chemistry
- Time
- Application for most types of proteins
- Concentrates sample
- Direct coupling to MS

Limitations

- Less resolution
- Less comparative data
- Digestion prior to separation

Applications

- Targeted (functional) proteomics
- Identify as many proteins as possible
- Protein expression profiling
- Mapping of protein modifications
- Protein-network mapping

2D/Multidimensional HPLC Principle

- **Two or more modes of HPLC with independent physical characteristics**
  - Ion Exchange/Affinity/SEC
  - Reverse Phase
- **OFF-Line or ON-Line**
  - ON-line more automatable
  - OFF-line allows more solvent flexibility, cleaner samples and possibly more sample capacity

OFF-Line

- Run first dimension and collect peaks
- Inject fractions on second dimension or reverse phase/MS
- Run 2D, then spot to or inject into MS

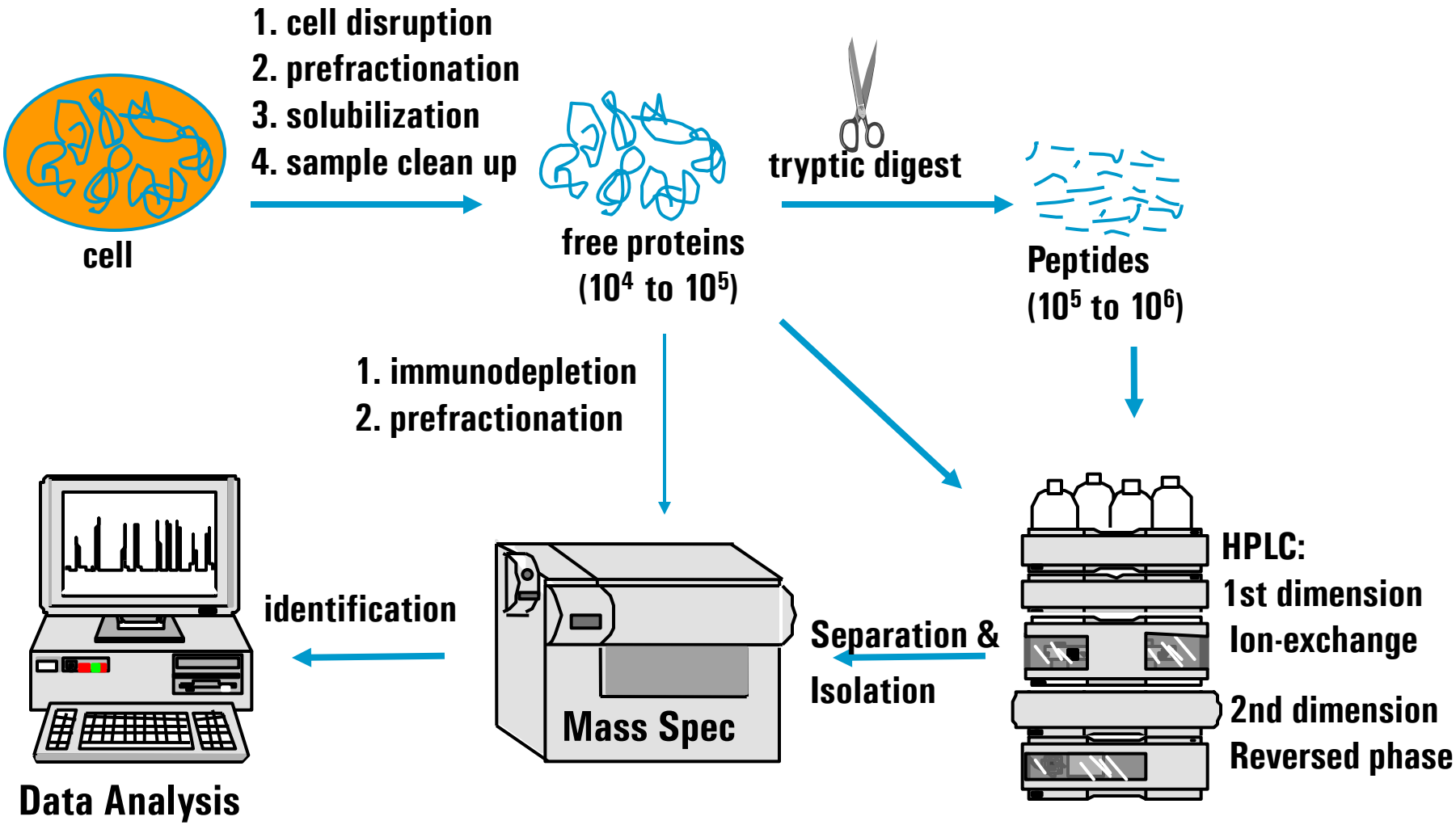
ON-Line

- Automate both separations on a single instrument with direct interface to MS

Chromatographic Methods for Single and Multidimensional HPLC of Peptides/Proteins

Type of Chromatography	Mechanism	Proteins and Steps Used For	Used in 2 or Multi-D
Ion exchange	Charge	All/Fractionation, Initial separations, preceding RP	Yes
Reversed phase	Hydrophobic/non-polar interaction	All/1-D, 2-D and Multi-D Separations prior to MS	Yes
Size exclusion/gel filtration	Molecular size	All/Fractionation	Yes
IMAC- immobilized metal affinity	Non-specific affinity to metal ions	Histidine containing and Phosphoropeptides for fractionation	Yes
Affinity	Bio-specificity for a defined ligand	Targeted proteins by ligand used for fractionation	Yes
Hydrophobic Interaction	Hydrophobic (high salt) interaction	All	?

Typical Proteomics 2D-LC/MS -Workflow



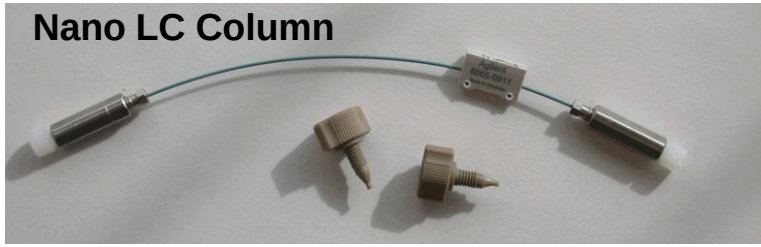
Why Use Nanospray LC/MS ?

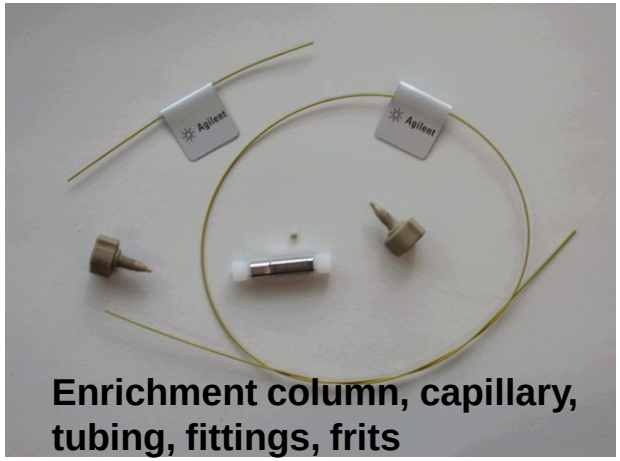
Benefit

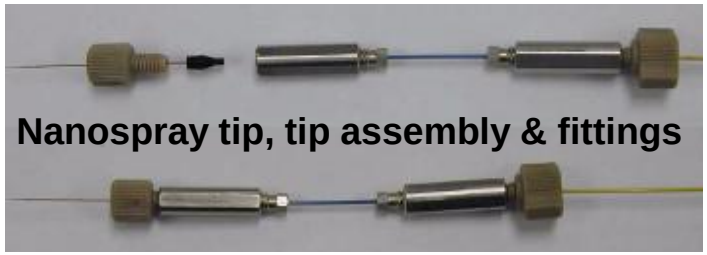
- high sensitivity MS detection

Issues

- challenging to implement and maintain
  - multiple small capillary tubing connections
  - frequent clogging and leaks
  - chromatographic degradation caused by tubing dead volume
- compromised reliability, ease-of-use, robustness and chromatographic performance



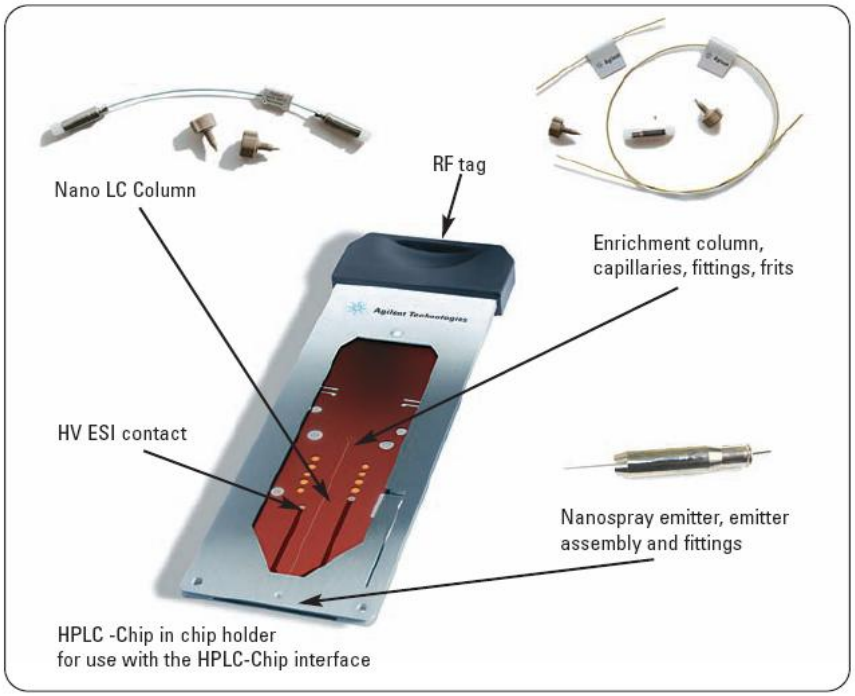


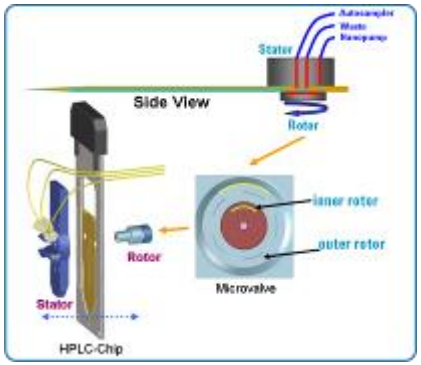


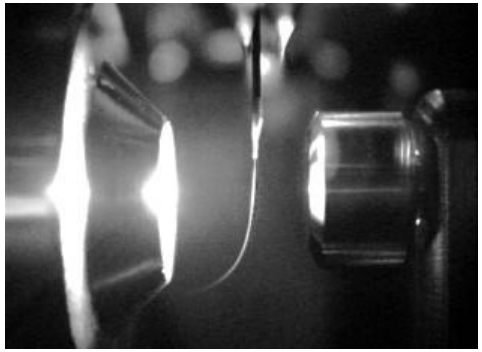
Why Use Microfluidics For Nanospray LC/MS?

Integrate functional components onto a reusable, biocompatible chip

- **enrichment and analytical nanocolumns,**
- **nanospray emitter**
- **fittings and connection capillaries**
- **directly on a reusable biocompatible polymer chip.**







Separation Options for Protein Identification

- 1D - nano or capillary HPLC for simple samples, e.g. single bands
- Online sample enrichment + 1D nano or capillary HPLC for medium complex diluted samples
- 2D - capillary + nano HPLC for complex samples
  e.g. 1. Cation Exchange, 2. Reversed Phase Separation
- Multidimensional - Fractionation columns + 2D HPLC for complex samples, e.g. 1. IMAC, SEC, etc. 2. SCX, 3. Reversed Phase Separation
- Identify with MS

Parting Thoughts . . .

- Ion exchange chromatography, size exclusion chromatography and reversed phase HPLC columns are the most popular choices for the analysis of proteins and peptides.
- A variety of reversed phase columns make optimization of protein separations possible. And a variety of column configurations – length and id make it easy to find the right column for any size sample.
- Movement towards faster analytical separations, smaller sample sizes, and more sensitive detection has increased sample throughput and proteomics applications
- Proteomics applications use the same columns and chromatographic techniques, but with so many proteins present in each sample, pre-fractionation, orthogonal techniques and nano scale analysis are necessary.

Conclusions

- Reducing sample complexity through intelligent sample preparation leads to more positive identification of low abundant proteins
- Separation of proteins prior to sample digestion results in more proteins identified
- Improving the chromatographic separation of peptides results in more proteins identified
- Multidimensional approaches generally require more analysis time which must be taken into consideration