

Protein chromatography



- ✓ Ion exchange chromatography
- ✓ Affinity chromatography

Jaana Vesterinen

Institute of Biomedicine, University of Helsinki, 2008

Chromatography



- Separation of biomolecules is based on their physicochemical characteristics
 - polarity (solubility, volatility, adsorption) HIC, RP
 - size/mass (diffusion, sedimentation) GF
 - ionic characteristics (charge) IEX
 - shape (ligand binding, affinity) Affinity chr
- based on these properties the molecules differentially separate between the stationary phase and mobile phase

Liquid chromatography (LC)

- **IEX** anion exchange
cation exchange
chromatofocussing
- **Affinity chromatography**
group separations vs. specific interactions
- **HIC** (hydrophobic interaction chr.) /RP
- Gel filtration/size exclusion;
noninteracting & medium resolution

adsorption
chromatogr.

&

high
resolution

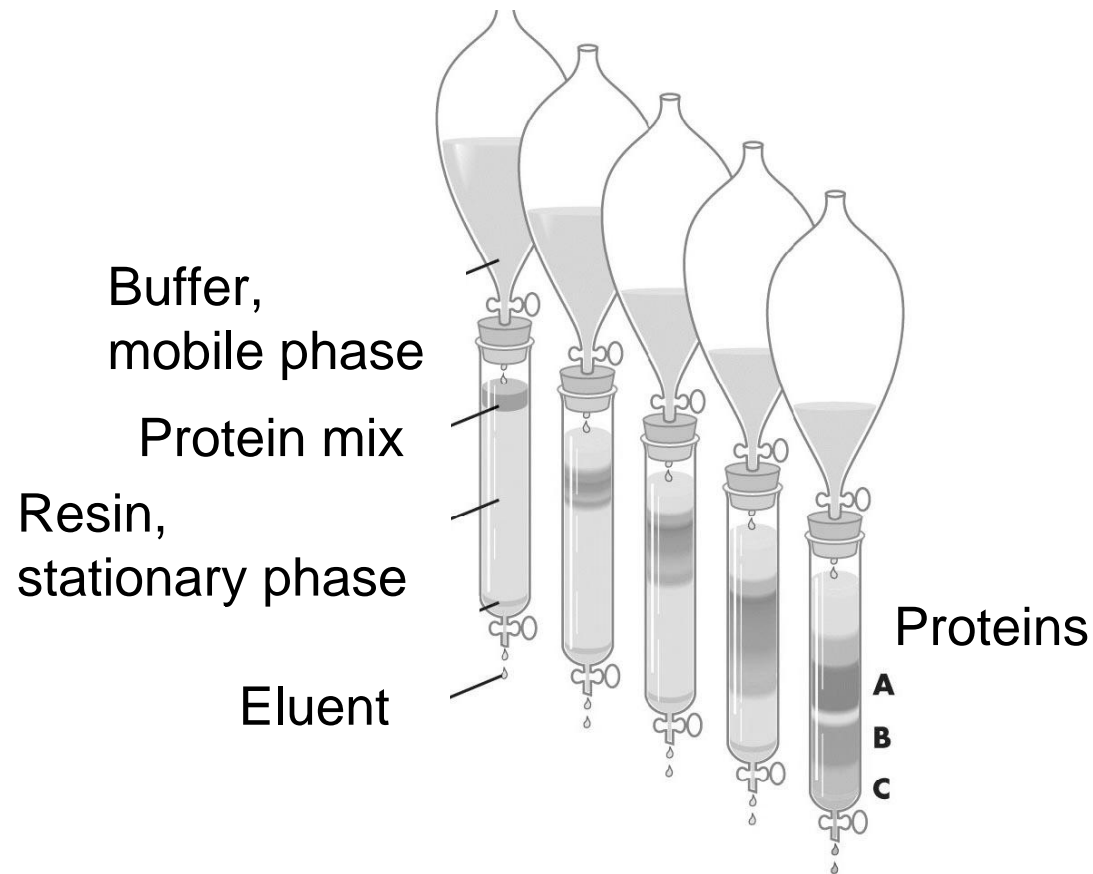
Adsorptive chromatography



- biomolecule adsorbs to the matrix (stationary phase) reversibly
- adsorption is controlled by the mobile phase - elution

eg. IEC: proteins in a low-salt mobile phase may be bound to the matrix, but when the composition of the mobile phase is changed to high-salt, the interaction is reversed and the proteins elute

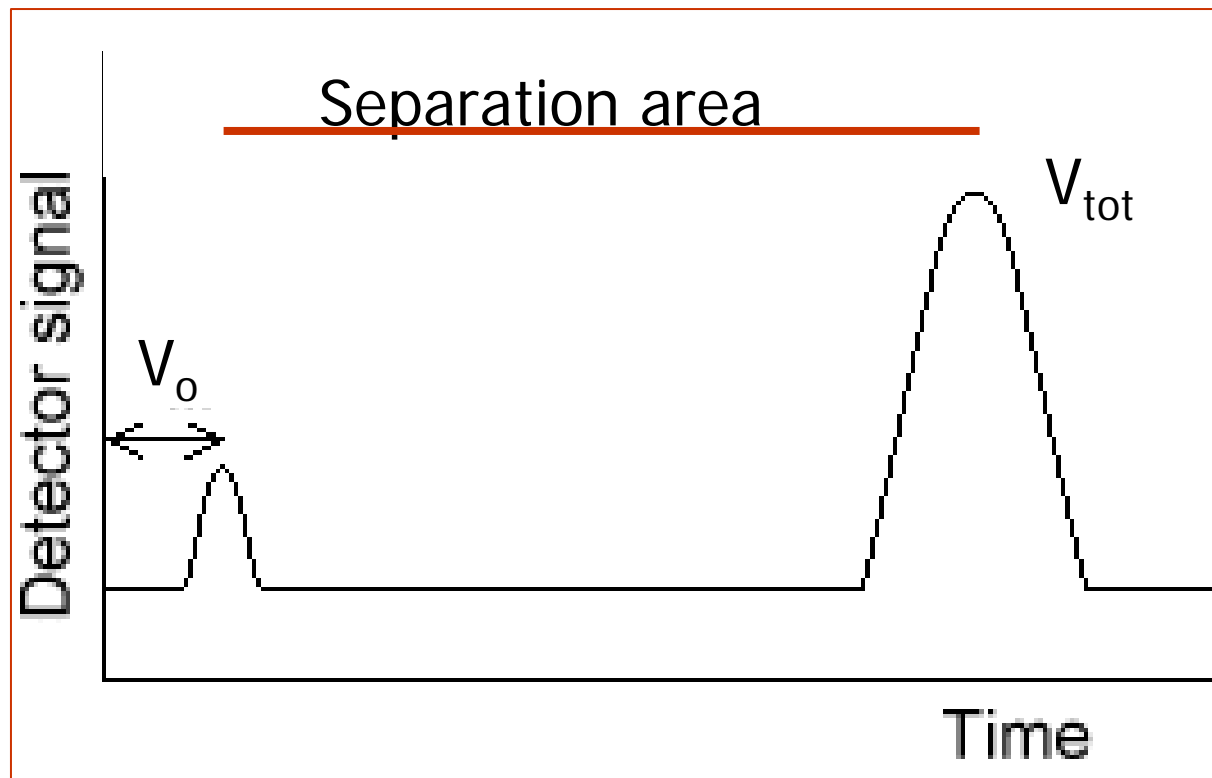
Liquid chromatography, basics



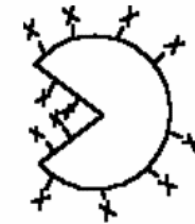
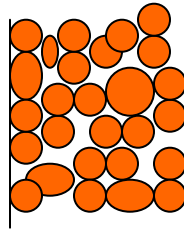
Chromatography
steps:

1. Equilibration
2. Injection
3. Elution
4. Washing

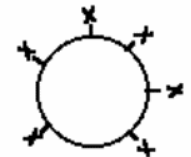
Chromatogram, basics



Matrix



Porous gel



Non porous gel

Porous

cellulose
sugar polymers (agarose, dextran)
polymers (acrylamide, styrene)
silica coated with polymers

particle size, ~5 μm

pore size eg. 100-200 \AA ●

- > chemical and physical stability
- temperature <80 °C vs >200°C)
- pressure porous < nonporous
- pH porous < nonporous
- capacity porous > nonporous

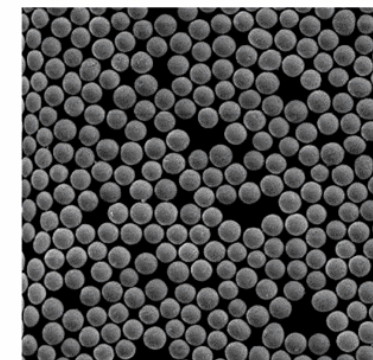
Nonporous

styrene
acrylates
zirkon
monolith

(ProSwift, Dionex)

~2 μm

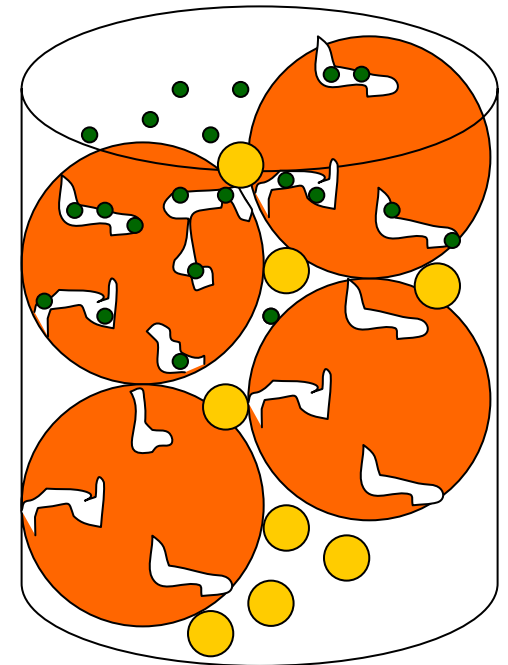
Monodisperse, Spherical and Nonporous Zirconia Particles



6 μm 4000X

Diffusion and porous matrixes

- Size exclusion chromatography is based on diffusion and separation based on size, effective size
 - Small molecules diffuse into smaller pores and travel a longer way, therefore elute last, close to V_{tot}
 - Large molecules do not fit into pores and elute first, close to V_0
- In affinity and IE chromatography the functional groups are attached to the particle surfaces inside the pores



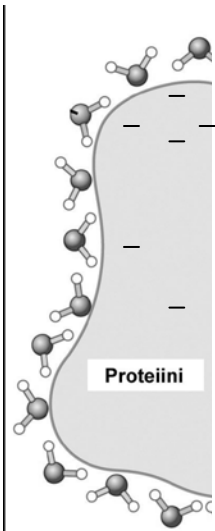
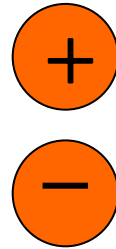
Mobile phase



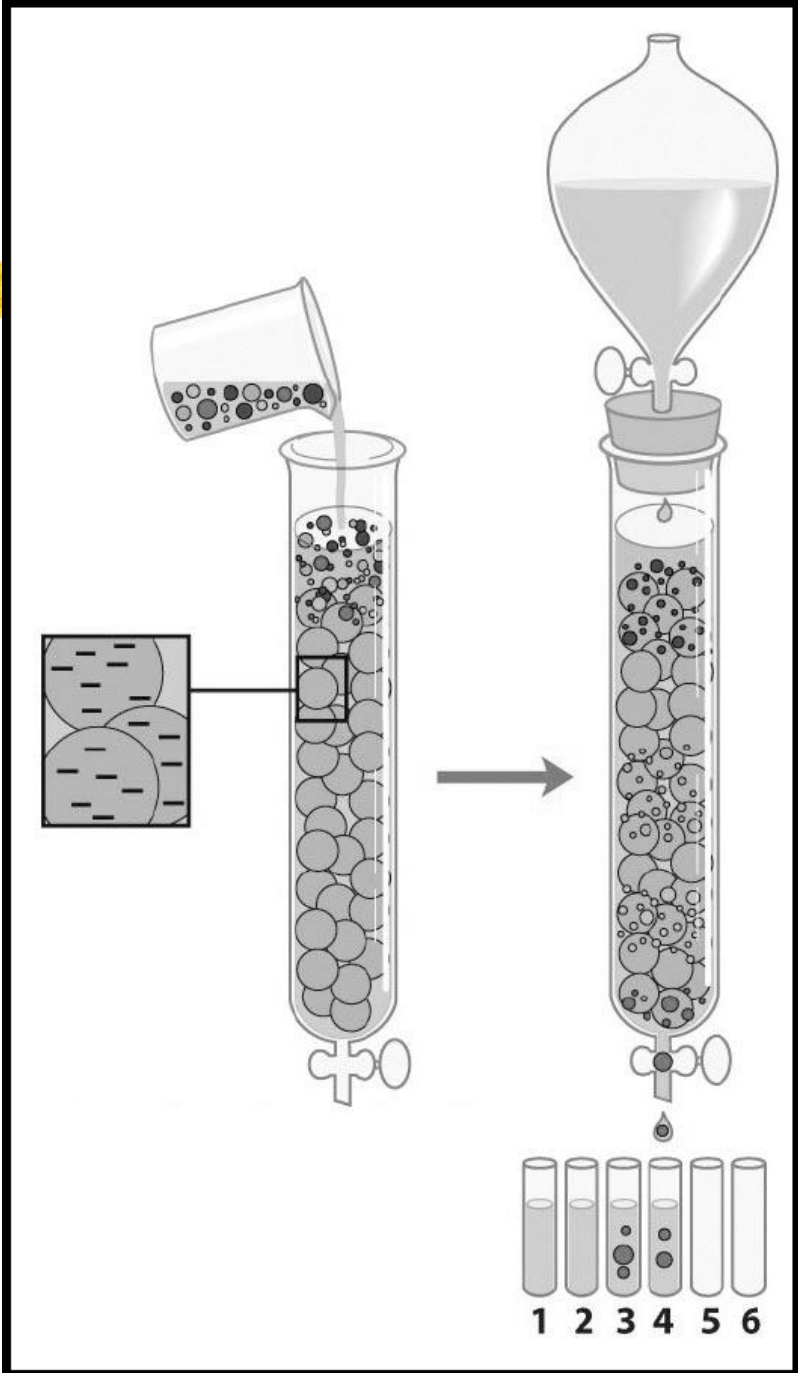
- Composition
- Type of elution
 - isocratic vs gradient elution (gradient shape)
- Flow rate
 - depends on the matrix
 - Affects resolution; in porous matrixes should be slow enough to allow diffusion to pore cavities, in nonporous matrixes higher flow rates may be used
-> UPLC

Ion exchange chromatography

- Based on ionic interactions
- Anion exchange
- Cation exchange



local vs net charge



IEC in practise



1. Choose the matrix according to your target protein
 2. Equilibrate (low salt, 20 mM)
 3. Inject protein sample (in low salt), balance
 4. Apply gradient (increasing salt) to elute proteins
- Obey buffer instructions:
AEC- cationic buffers, CEC- anionic buffers
 - Nonionic detergents (!)
 - Elution:
increasing salt gradient (0-1M NaCl in 20 mM buffer)
or pH gradient (ampholytes in chromatofocussing)
type of gradient: linear gradient /step wise/shape of gradient

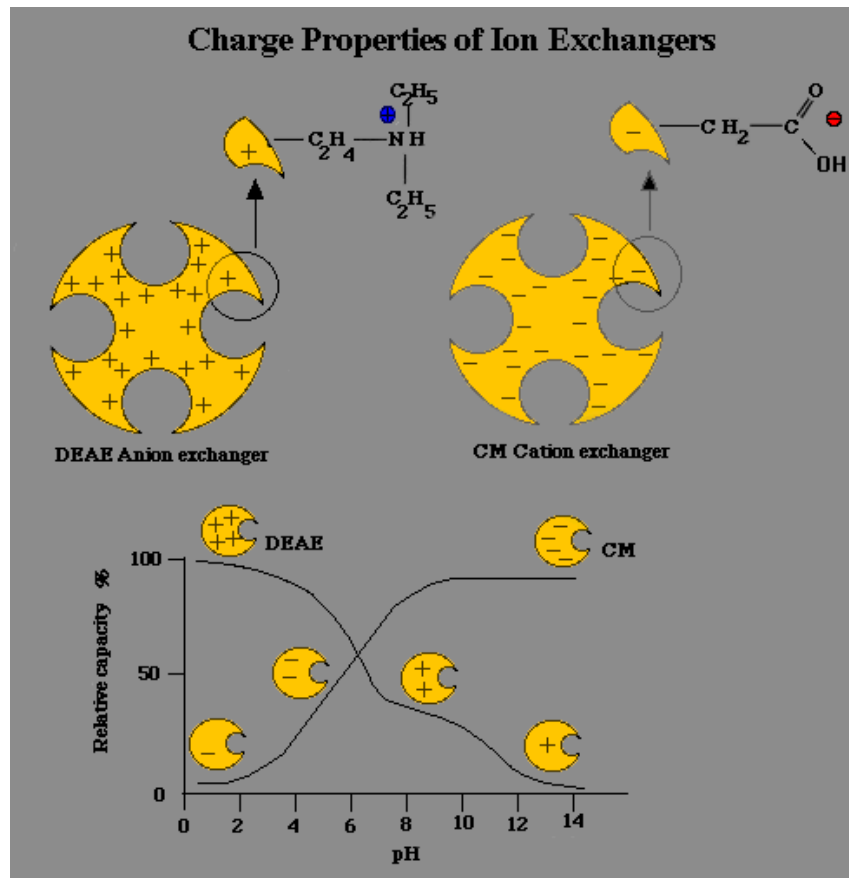
Examples of buffers in IEC

Shoko America, Inc. (Shodex)

pH	IEC <u>QA-825</u>, <u>DEAE-825</u>, AXpak <u>WA-624</u>	IEC <u>SP-825</u>, <u>CM-825</u>
6	20mM Piperazine HCl	20mM Sodium malonate
7	20mM Bis-Tris propane HCl	20mM Sodium phosphate
7.5	20mM Tris HCl	20mM Sodium phosphate
8	20mM Tris HCl	20mM HEPES
9	20mM Ethanolamine HCl	
10	1,3-Diaminopropane HCl	

IEC matrixes

- strong vs weak ion exchangers
- anion EC: positive matrix
 - DEAE diethyl aminoethyl (W)
 - QAE quaternary aminoethyl
 - Q quaternary amine
- cation EC:negative matrix
 - CM carboxymethyl (W)
 - SP sulphopropyl
 - S sulphonate



Titration curve of a protein

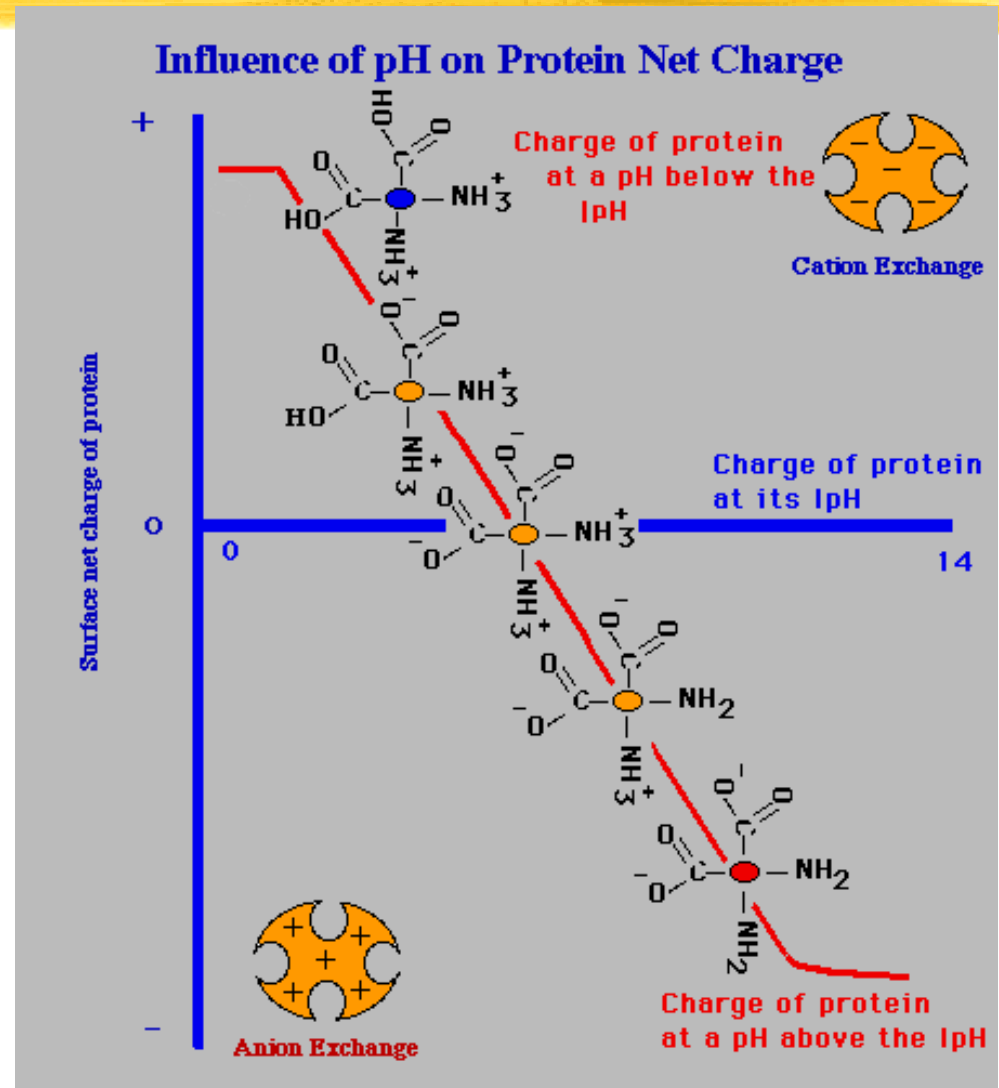
- charge of a protein is pH dependent !

amino acids with ionizable side chains:

Arg, His, Lys
Asp, Glu
Cys, Tyr

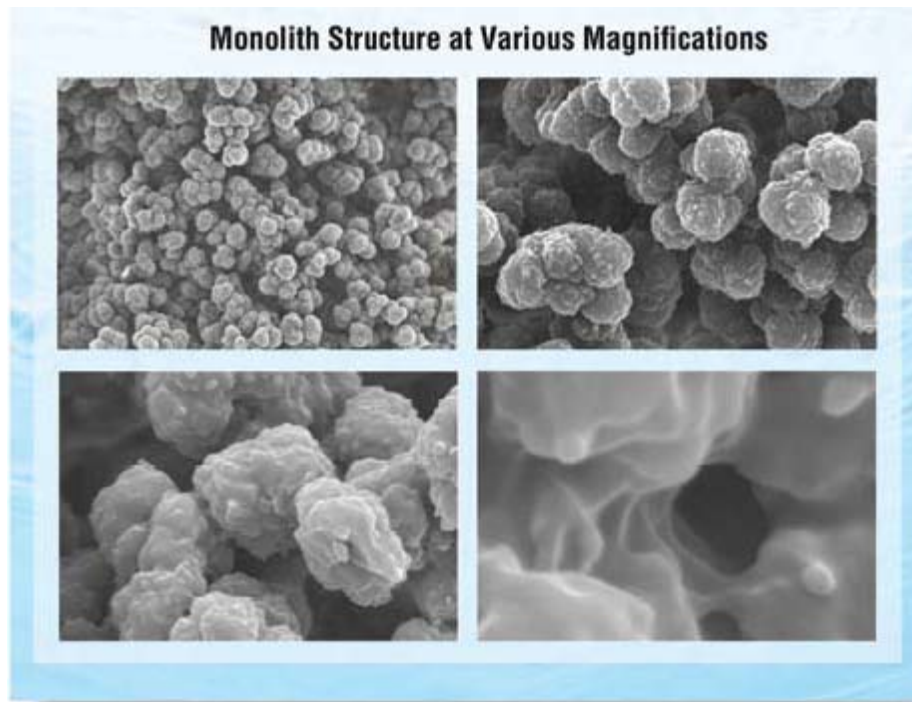
- pI = isoelectric point

if $\text{pH} > \text{pI}$ use anion EC
if $\text{pH} < \text{pI}$ use cation EC



Example: Dionex, ProSwift matrixes

(www.dionex.com)



- monolith matrix
- nonporous, cavities
- combine the stability of nonporous matrixes and capacity of porous matrixes
- optimal mass transfer – little diffusion...
peak shape and reproducibility good

ProSwift, IEC

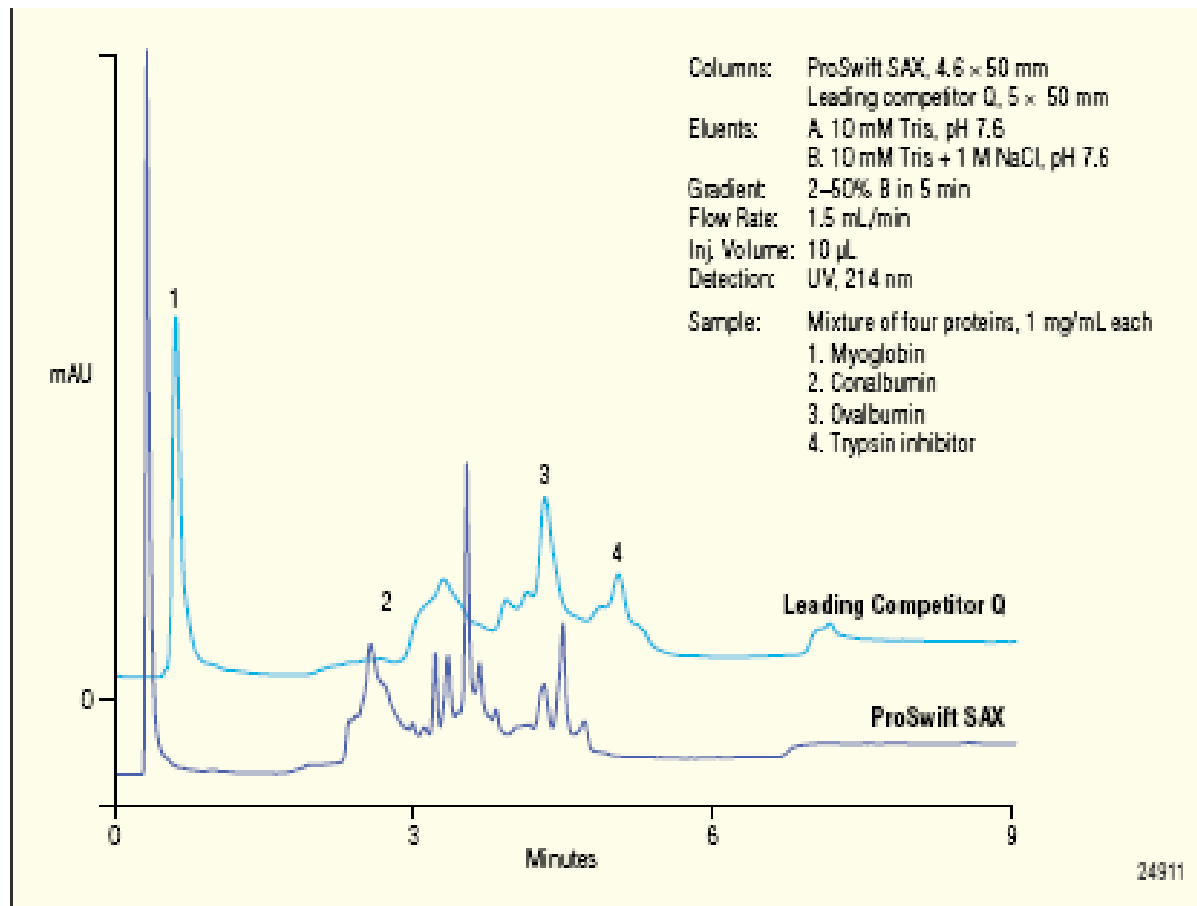


Figure 1. Comparison of ProSwift SAX-1S and leading competitor—separation of a protein mixture.

ProSwift, IEC, phosphorylation

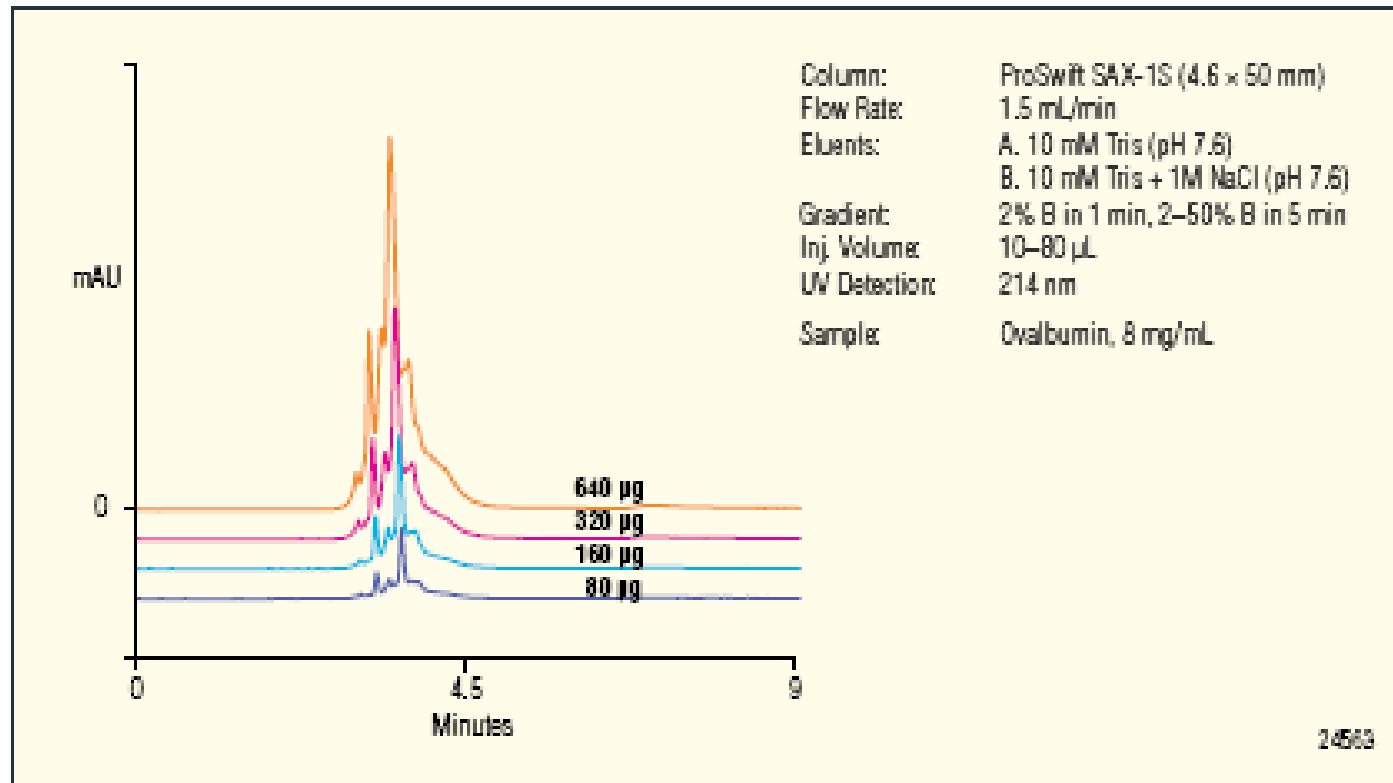


Figure 3. Separation of phosphorylation variants of ovalbumin using the ProSwift SAX-1S 4.6 × 50 mm column.

UPLC



- Ultra Performance Liquid Chromatography (UPLC) is a chromatographic separations technique first introduced at Pittcon 2004 with the Waters ACQUITY UPLC System
- Now available also from other companies
- Nonporous sub two-micron particles give chromatographic run times that are up to 9X shorter than HPLC systems, up to 2X better peak capacity or resolution, 3X better routine sensitivity, and, generally speaking, more information from a single run than anything today's HPLC systems can provide.
- IEC, RP
- Dionex: ultra fast fraction collection

IEC



++++++

- concentrates the sample
- sample volume not restricting
- gentle
- high resolution
(strong vs weak)

- no salt in binding phase
- sample eluted in high salt

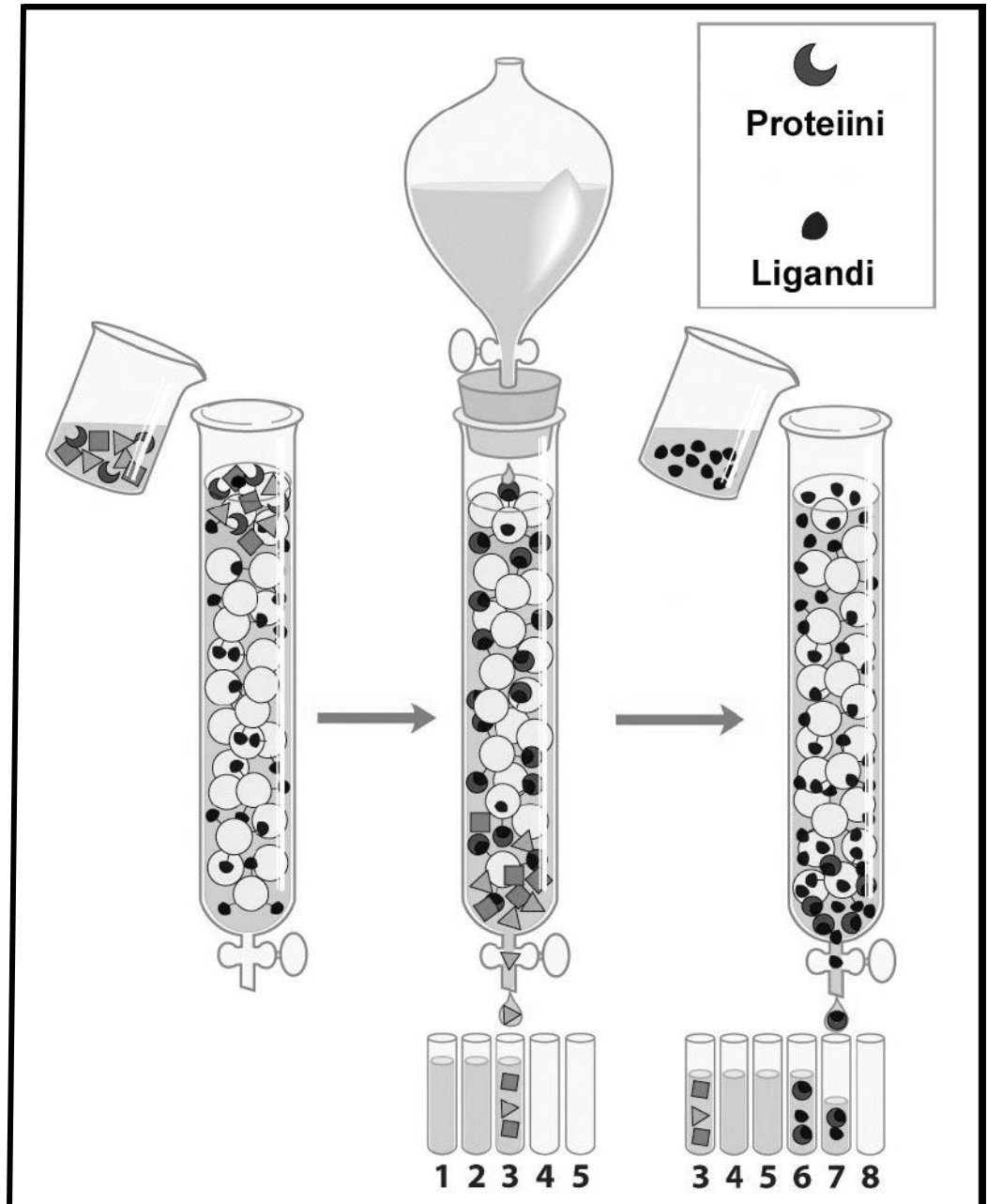
Affinity chromatography



- Based on specific interactions
- group affinity vs specific affinity

- elution nonspecific vs specific
- effective purification in one step
- concentrating
- IMAC often used for recombinant protein purification

1. Protein mix into column
2. Washing of non-binding proteins away
3. Specific elution by ligand of target protein



IMAC

Immobilized metal ion affinity chromatography

- single step chromatography
- for Zn-finger proteins and recombinant proteins with 6x His-tag

PDC-Sepharose (www.affiland.com)

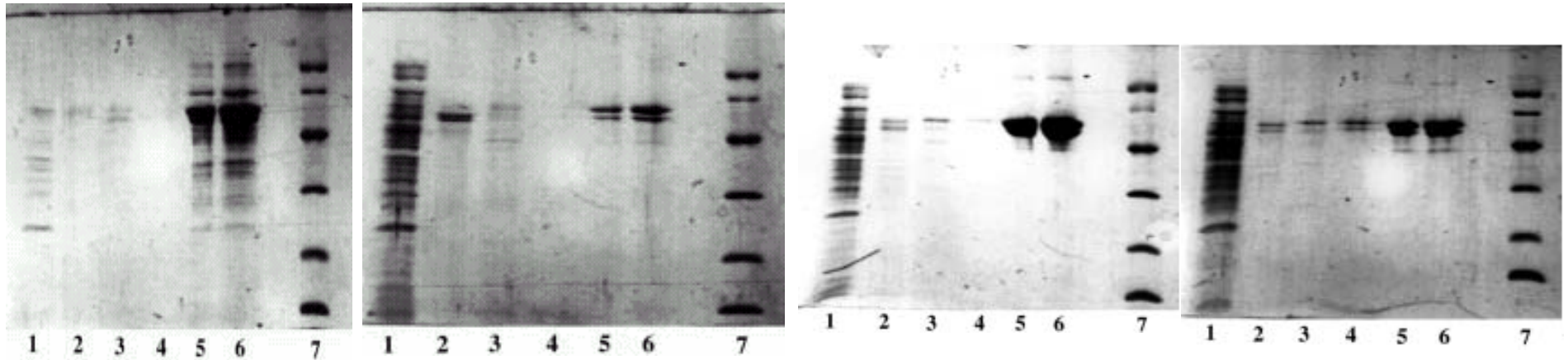
- pentadentate chelator coupled to Sepharose[®] CL-4B forms complexes with polyvalent metal ions Cu^{2+} , Ni^{2+} , Zn^{2+} , and Co^{2+}
- stable metal ion-chelator complex
- five coordination sites occupied by the chelator
- one site free for binding of his-containing biomolecules
- can be eluted with chaotropic buffers such as guanidine hydrochloride 6M pH 8.0, urea 8M pH 8.0 or non-ionic detergents

... PDC-Sepharose

Example 1. Purification of 6x His-tagged HSP 60 expressed in *E. Coli* from crude clarified lysate

E. Baise, Laboratoire de Biochimie B6, Sart Tilman 4000 Liège (Belgium).

Sample volume loaded onto each column: 500 μ l (conc. in HSP 60 : 5 mg/ml).



Cu-PDC

Ni-PDC

Zn-PDC

Co-PDC

Lane 1: flow through + 50 mM Pi, 150mM NaCl, pH 8

Lane 2: wash, same buffer; Lane 3: 50 mM Tris-Ac, 150 mM NaCl, pH8; Lane 4: 50 mM Tris-Ac, 150 mM NaCl, pH6

Lane 5,6: elution with 50mM Na-Acetate, 150 mM NaCl, pH 4

... PDC-Sepharose

Example 2: Purification of alpha macroglobulin

Gel volume: 25 ml Ni²⁺PDC

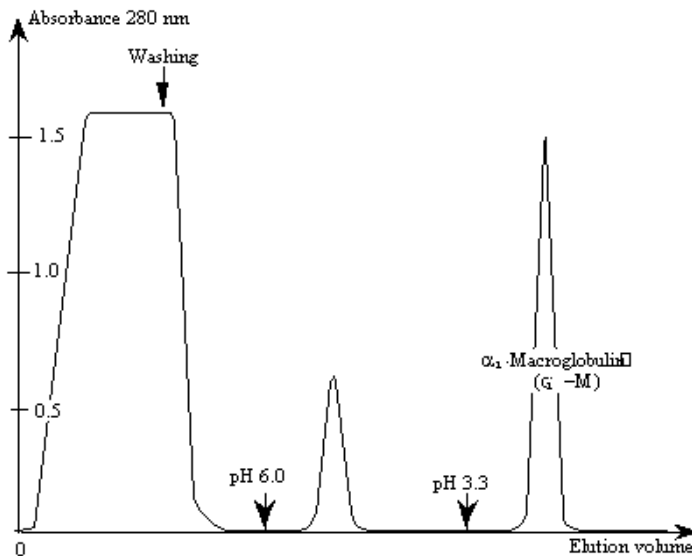
Sample: 100 ml serum.

Binding buffer: 50mM phosph, 0.15M NaCl, pH 8.0.

Washing buffer:

1. 50mM Phosph, 0.15M NaCl, pH 8.0.
2. 50mM Phosph, 0.15M NaCl, pH 6.0.

Elution buffer: 50mM NaAcetate pH 3.3.



Summary of PDC

Binding buffer:

50mM Phosph, 0.15M NaCl, pH 8.0

Washing buffer:

50mM Phosph, 0.15M NaCl, pH 8.0

(NaCl 0.15 -1 M, GuHCl 2-6M, urea 4-8M, all pH 8)

Elution buffer:

a) 50mM Phosph, 0.15M NaCl, pH 6.0

b) imidazole 20mM - 500mM pH 7.4

c) gradient of NaH₂PO₄ 50mM pH 7.4 - 4.0

Criteria for chromatography



- needs:
 - active/inactive
 - absolutely pure/rather pure
 - ng/mg
 - selecting the type of chromatography:
 - denaturing/nondenaturing
 - resolution
 - yield (eg. activity/mg of protein)
 - capacity / sensitivity
 - speed
- = efficiency

Multidimensional chromatography



- Novel applications in micro/nanoscale
- Combination with MS techniques used in proteomics
- Results in fractionation of sample and identification of target proteins

- MudPIT technology
- SELDI (Ciphergen)

MudPIT Proteomics



- MudPIT = Multidimensional Protein Identification Technology
- Used for separation and identification of complex protein/peptide mixtures - alternative to 2D electrophoresis
- MudPIT separates peptides in 2D LC, and interfaces with the ion source of a mass spectrometer
- Strong cation exchange (SCX) material back-to-back with reversed phase (RP) material inside fused silica capillaries
- Automated 2D LC proceeds in cycles: an increase in salt concentration "bumps" peptides off of the SCX, and is followed by an increasing hydrophobicity to elute peptides from the RP into the ion source
- Mass spectrometer's data-dependent acquisition isolates peptides as they elute, subjects them to dissociation, and records the fragment ions
- Spectra are matched to database by SEQUEST algorithm. SEQUEST's peptide identifications are assembled and filtered into protein-level information by the DTASelect algorithm.

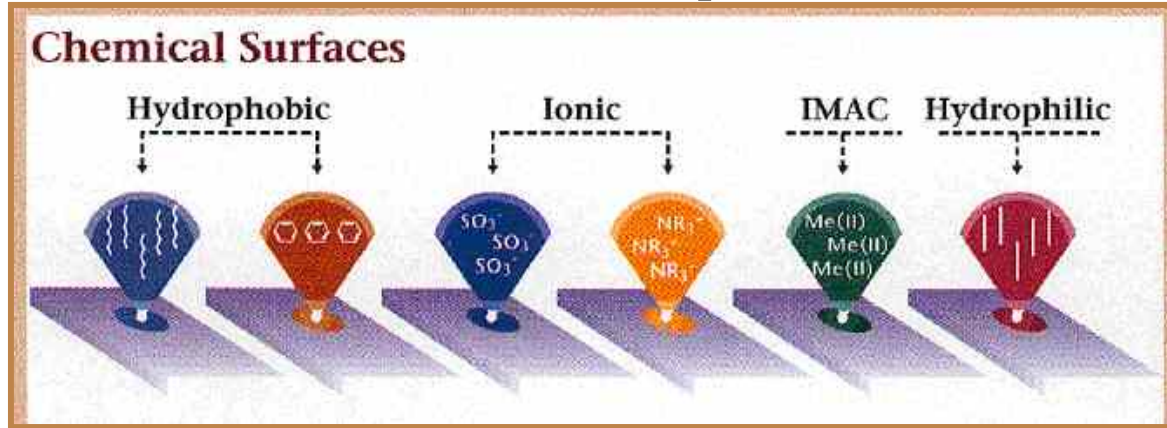
SELDI Mass Spectrometry

Surface-enhanced laser desorption/ionization

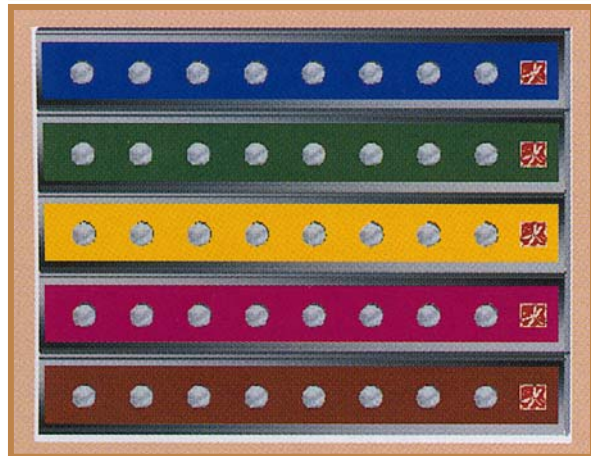
- mass spectrometric technology on a chromatographic chip surface
- used to analyse complex mixtures such as serum, urine, blood
- biomarker discovery
- differentially expressed proteins are determined by comparing protein peak intensity within mass spectra

Proteomics Using Ciphergen's SELDI Technology (Nowadays by BioRad)

Surface **E**nhanced **L**aser **D**esorption **I**onization



←**Surface Chemistries**
Each chip binds a specific set of proteins based on the chromatographic surface of the ProteinChip®.



←**Protein Chips**
Each spot on the chip will contain sera from a control- or toxicant-treated animal. The spots are analyzed separately and a mass spectra is created for each spot representing the proteins bound to the chip surface.

