Fehérjék elválasztástechnikája

Az ábrák több, részben szerzői jogokkal védett műből, oktatási célra lettek kivéve. Továbbmásolásuk, terjesztésük nem megengedett.

TÉMÁK

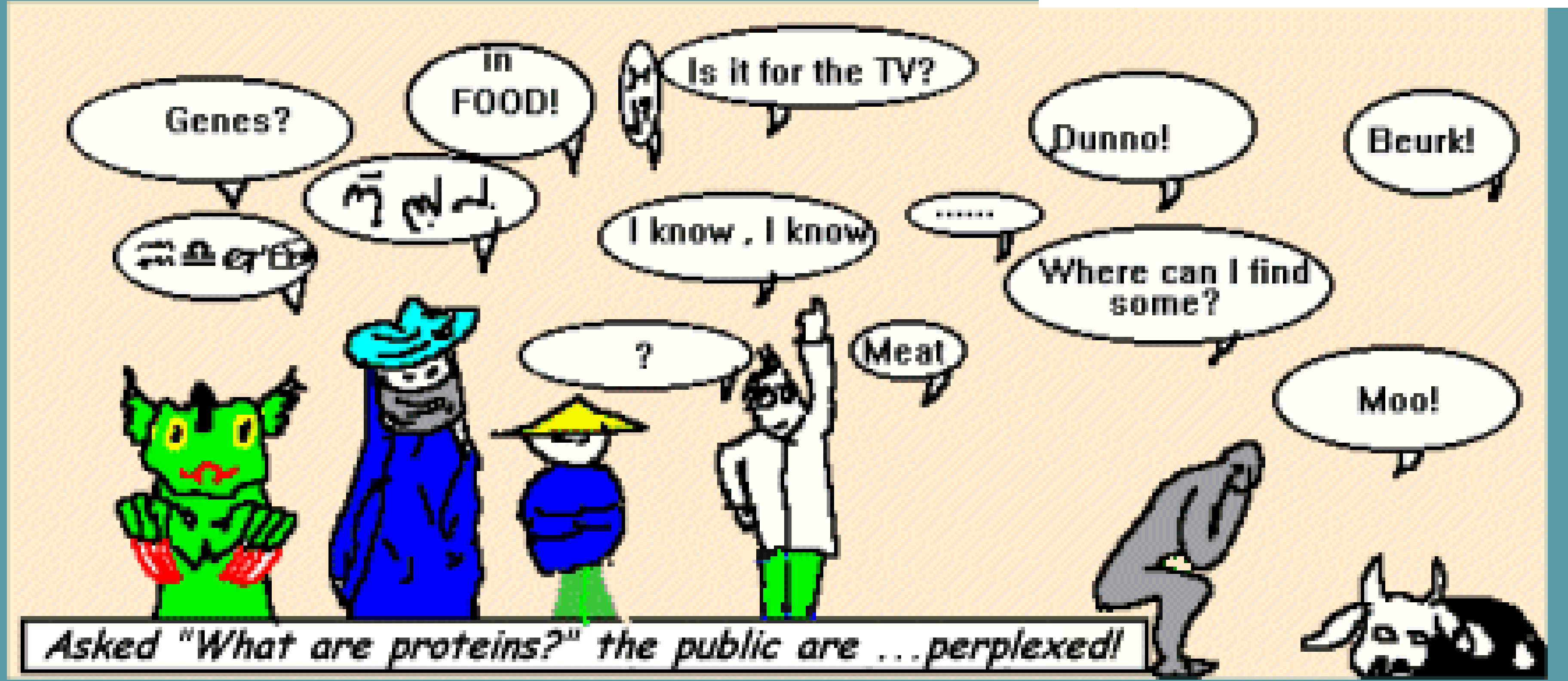
Fehérjék és peptidek kromatográfiája

Elektroforézis és elektrokromatográfia

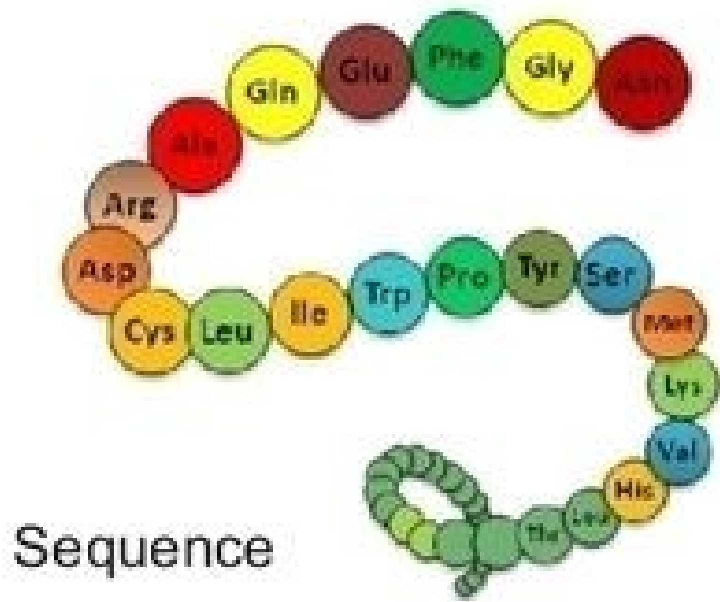
Lab on a chip HPLC

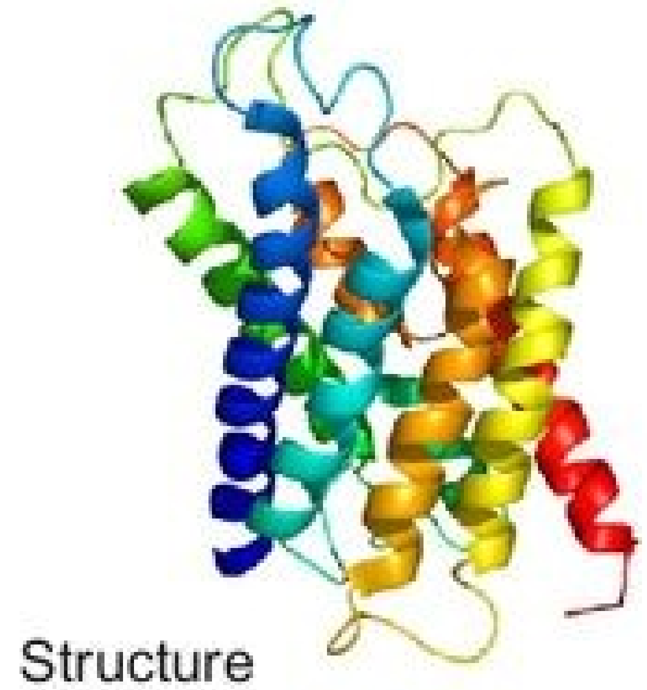
Fehérje és peptid kromatográfia

Gélkromatográfia
Hidrofób kölcsönhatás kromatográfia
Ioncserés kromatográfia
Fordított fázisú kromatográfia
Affinitás kromatográfia

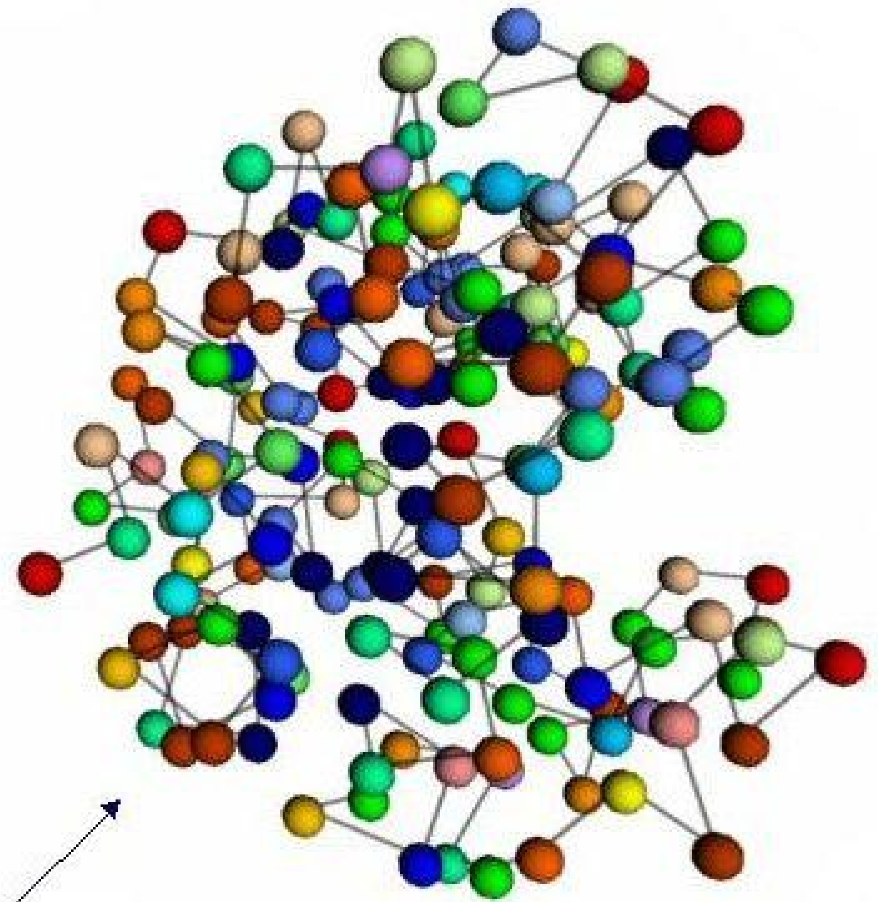


Genes?
in FOOD!
Is it for the TV?
Dunno!
Beurk!
I know , I know
......
Where can I find some?
?
Meat
Moo!
Asked "What are proteins?" the public are ...perplexed!


Gln
Glu
Phe
Gly
Arg
Asp
Cys
Leu
Ile
Trp
Pro
Tyr
Ser
Lys
Val
Sequence

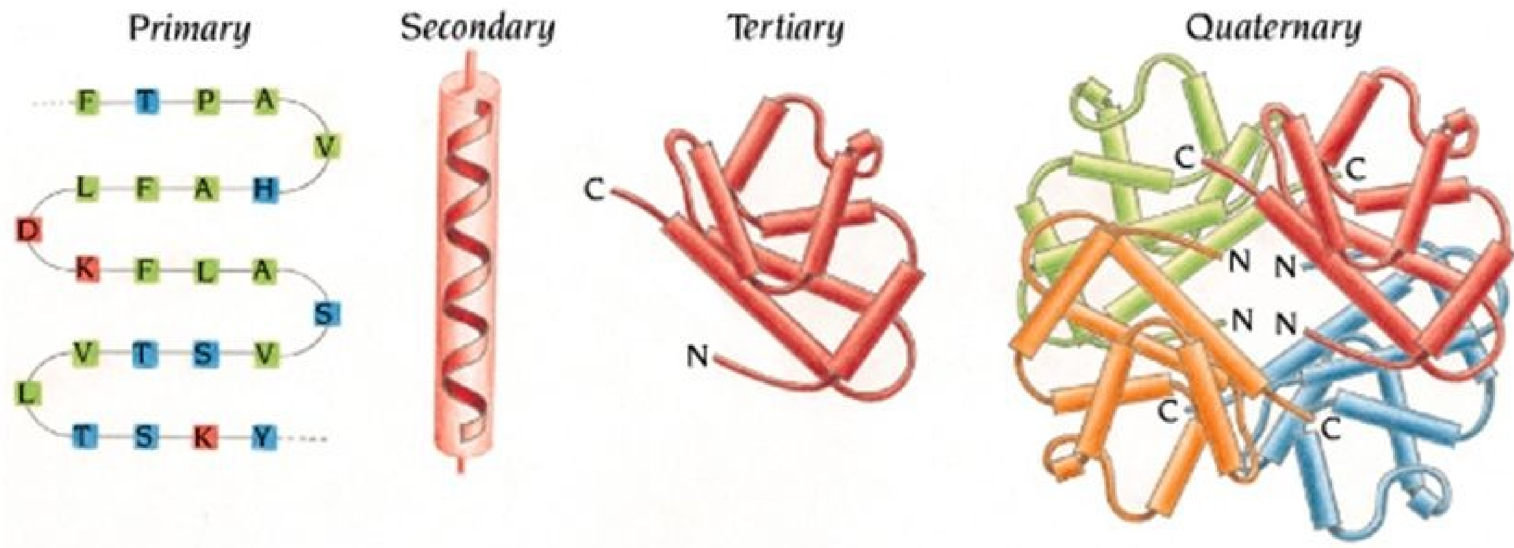

Structure

R T D C Y G N V N R I D T T G
A S C K T A K P E G L S Y C G
V S A S K K I A E R D L Q A M
D R Y K T I I K K V G E K L C
V E P A V I A G I I S R E S H
A G K V L K N G W G D R G N G
F G L M Q V D K R S H K P Q G
T W N G E V H I T Q G T T I L
I N F I K T I Q K K F P S W T
K D Q Q L K G G I S A Y N A G
A G N V R S Y A R M D I G T T
H D D Y A N D V V A R A Q Y Y
K Q H G Y



Primary Sequence 3D Structure

How to Describe Protein Structure



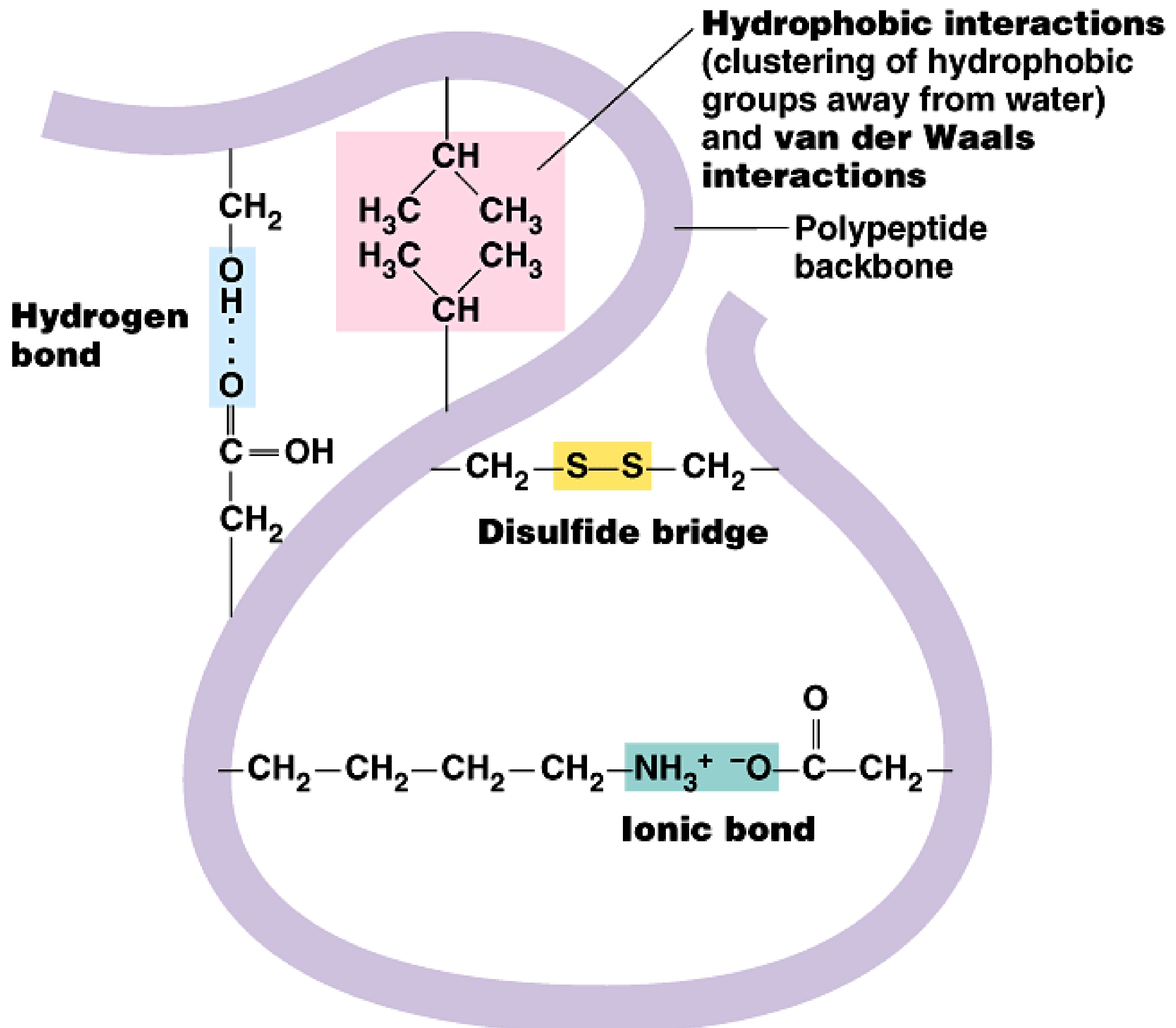
- Primary: amino acid sequence
- Secondary structure: alpha helix, beta sheet and loops
- Tertiary: Phi-Psi angle
- Quaternary: arrangement of several polypeptide chains

Amino Acid Sidechain List

Dual Color Scheme:

1. Name and background shading indicates hydrophobic amino acids (yellow); hydrophilic non-charged amino acids (white); + charged amino acids (blue); − charged amino acids (red); cysteine (green).
2. Atom types indicated as carbon (gray), oxygen (red), nitrogen (blue), and sulfur (yellow)

Name	Amino Acid	Sidechain	Name	Amino Acid	Sidechain	Name	Amino Acid	Sidechain	Name	Amino Acid	Sidechain
Alanine Ala A			Glutamine Gln Q			Leucine Leu L			Serine Ser S		
Arginine Arg R			Glutamic Acid Glu E			Lysine Lys K			Threonine Thr T		
Asparagine Asn N			Glycine Gly G			Methionine Met M			Tryptophan Trp W		
Aspartic Acid Asp D			Histidine His H			Phenylalanine Phe F			Tyrosine Tyr Y		
Cysteine Cys C			Isoleucine Ile I			Proline Pro P			Valine Val V		



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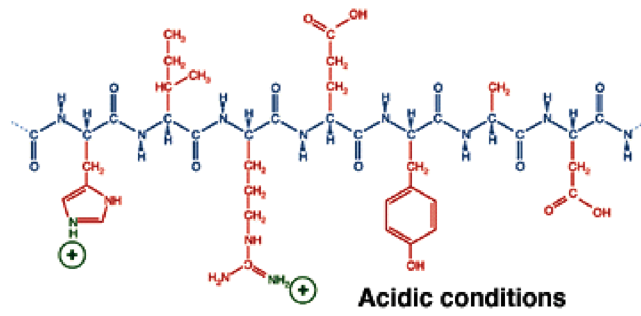
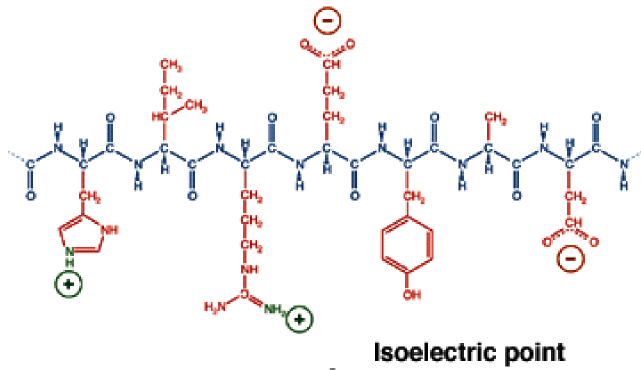
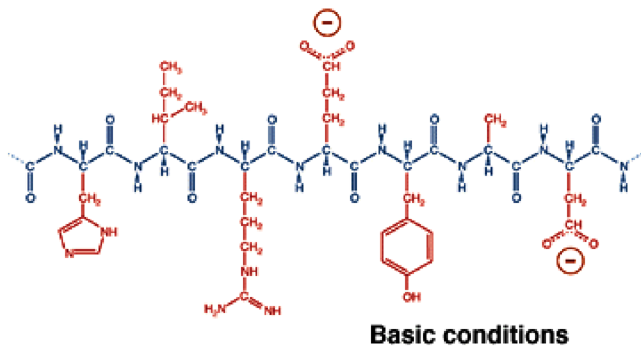
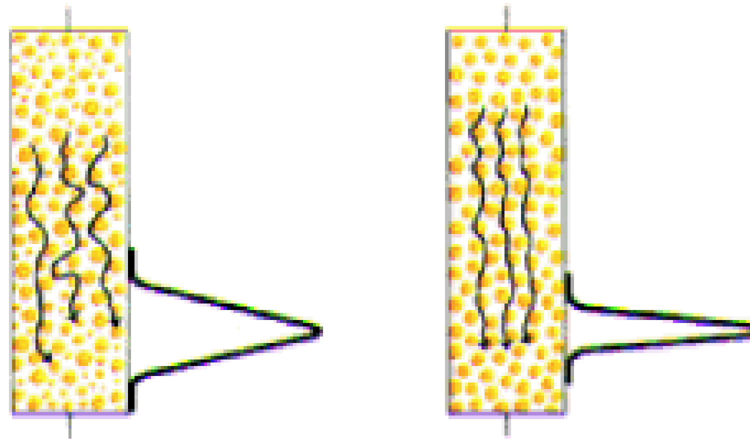

Acidic conditions


Isoelectric point

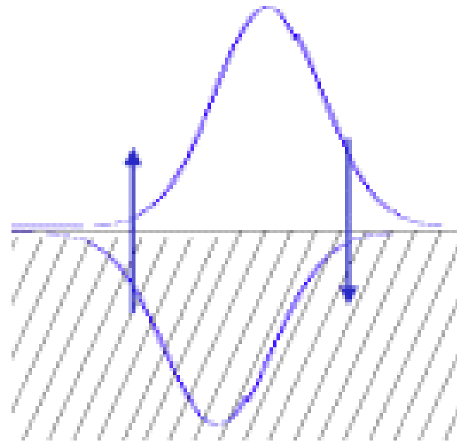

Basic conditions

Eddy diffusion -
Zone broadening caused by bed irregularities.

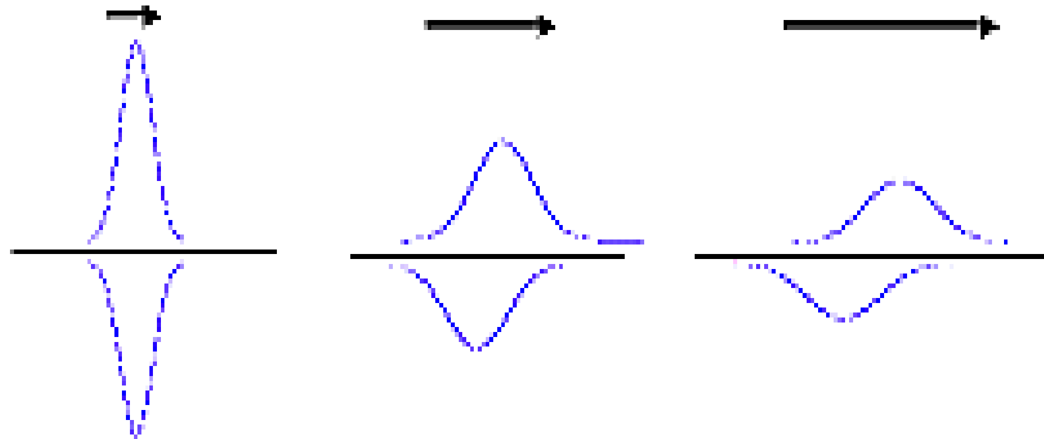


(Az angol helyett magyar feliratú ábrákat a „Feherjekrom-Elvalasztastechn-2012-HGY-CE-MJ.pdf file-változatban találja meg.)

Mass transfer directions in a travelling sample zone.



The flow rate has to be balanced against the mass transfer rate.



Arrow length indicates flow rate

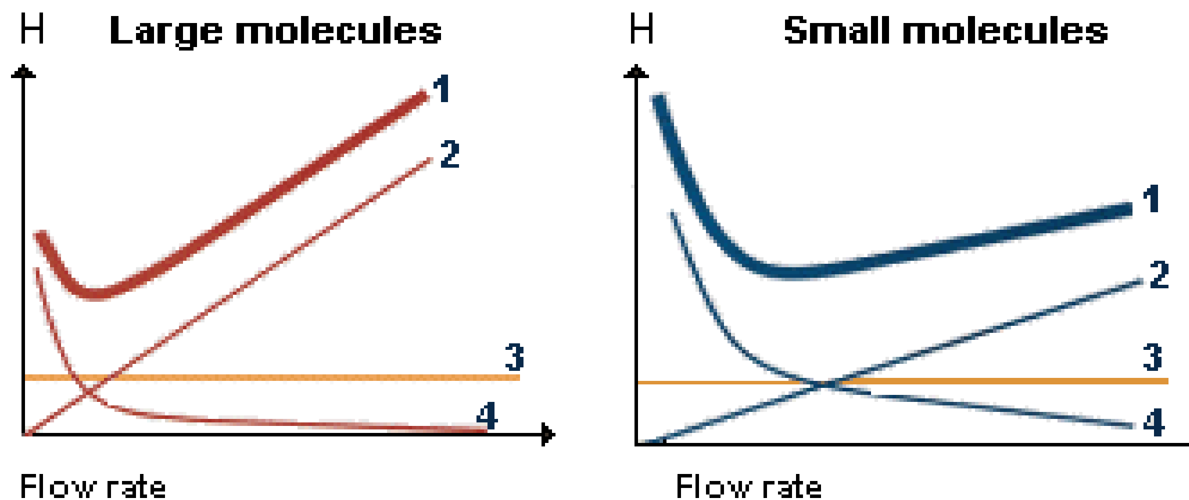


Fig 5.9. The effect of flow rate on zone broadening. Note that optimal flow rate is lower for large molecules than for small ones.

H is the *height of a theoretical plate**.

1 is the sum total zone broadening (2+3+4).

2 is the zone broadening caused by the partitioning mechanism i.e. the mass transfer

3 is the zone broadening caused by *eddy diffusion.*

4 is the zone broadening caused *by axial diffusion.*

---

* *The height of a theoretical plate is measure of chromatographic efficiency inherited from the theory of distillation. H is linearly related to the square of the peak width for a given sample molecule.*

Egy céges ismertető:

Analysis and lab scale isolation of proteins or polypeptides

XXXXX LC Columns contain either well-performing silica or methacrylate-based, ion-exchange, size-exclusion, and hydrophobic interaction, and affinity column chemistries for the separation, lab-scale purification, and characterization of proteins and other biomolecules.

GÉLKROMATOGRÁFIA (MÉRETKIZÁRÁSOS KROMATOGRÁFIA)

Gélkromatográfia (méretkizárás)

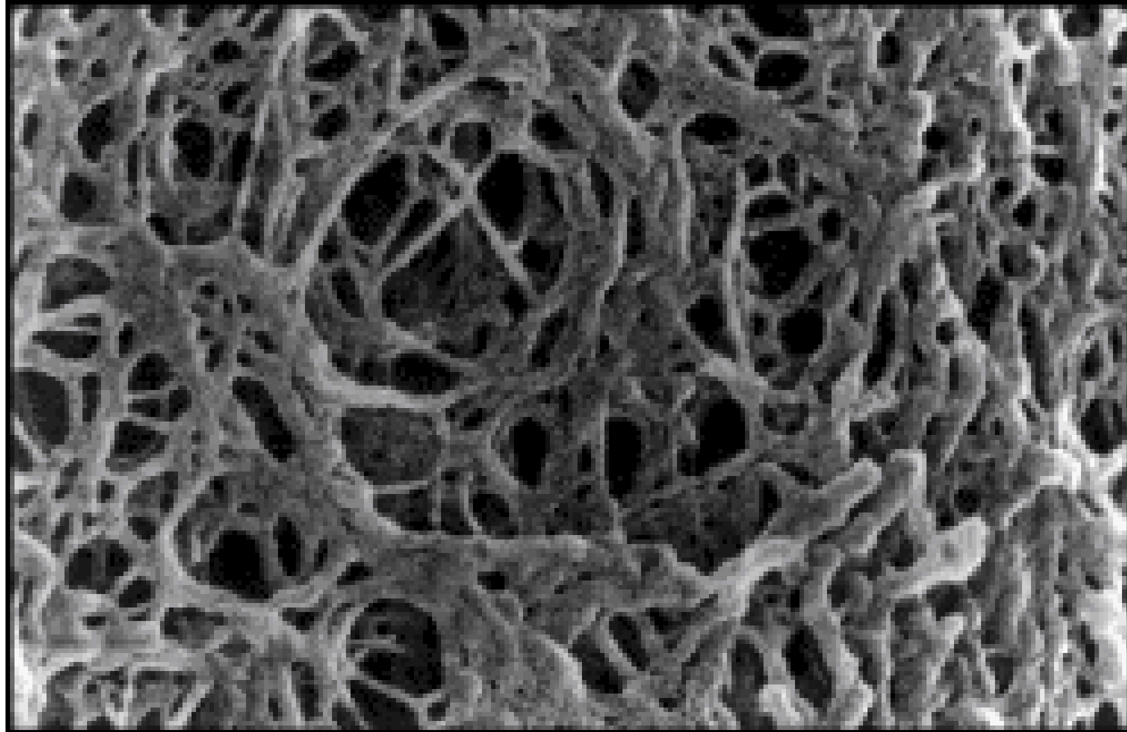
In a column all sample molecules have access to the liquid between the beads . This volume is called *the void volume* in gel filtration and equals **~30%** of the column volume.

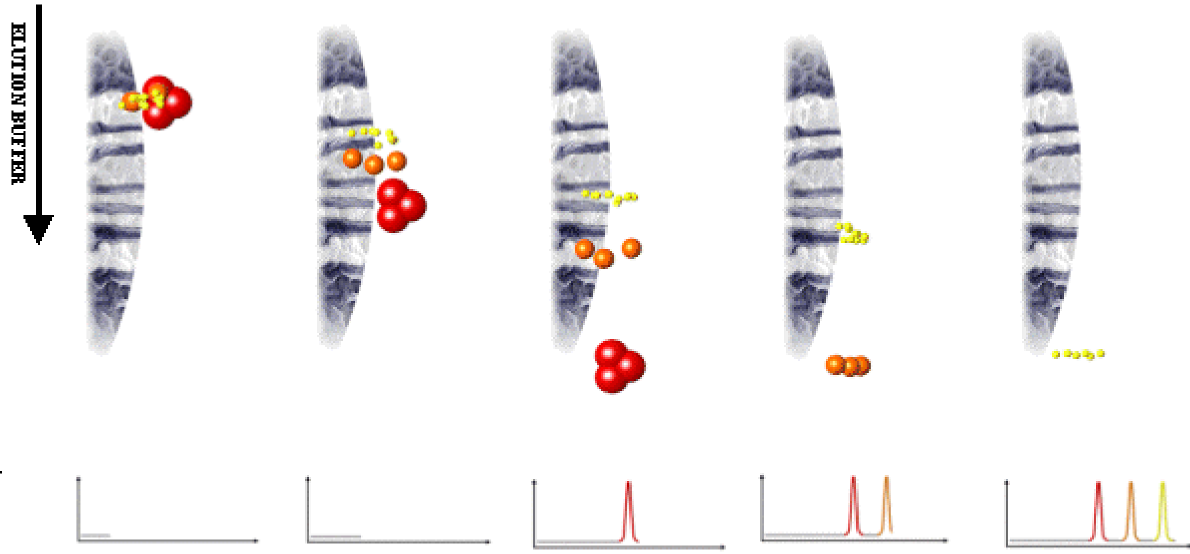
Gel filtration media contain pores allowing the sample molecules to penetrate into the gel filtration beads to different degrees depending on size. Together the volume of these pores form *the pore volume.*

The non-porous part of the beads is called *the backbone* and is inaccessible for the sample molecules. For a good GF matrix the volume of the backbone is around **3-5%** of the column volume of a well- packed column.

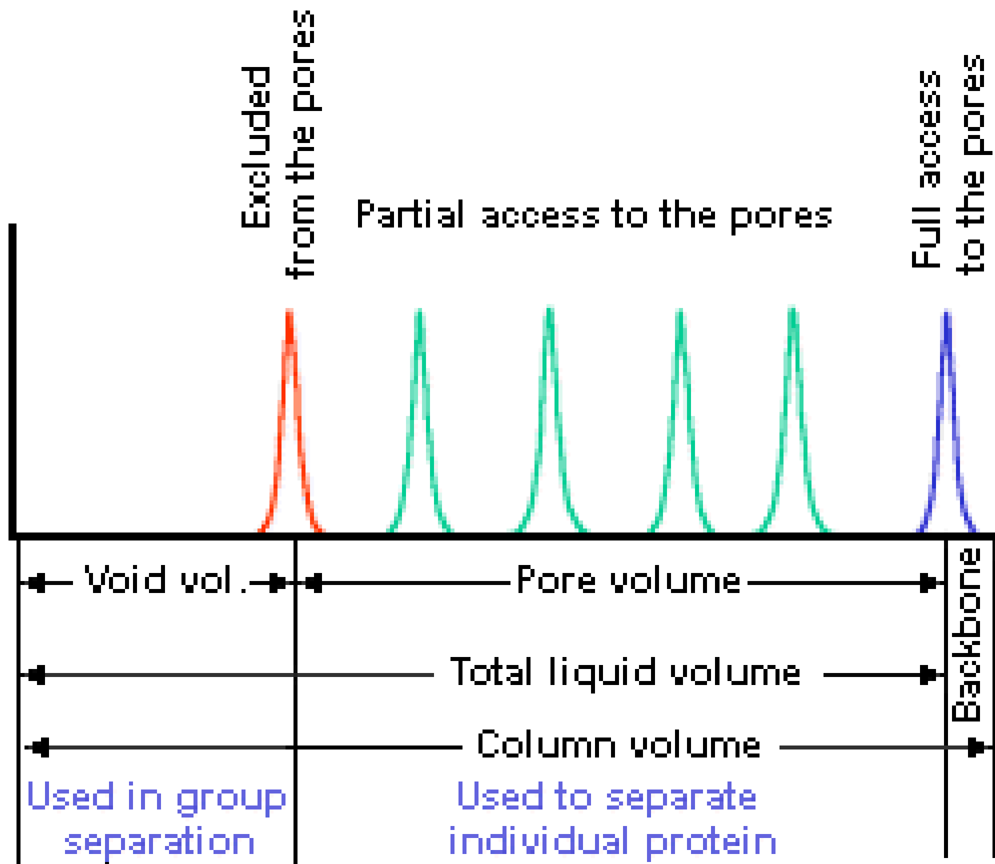
Scanning electron micrograph of an agarose gel. Magnification x 50,000.

Ref. Anders S. Medin, PhD Thesis,
Uppsala University 1995.

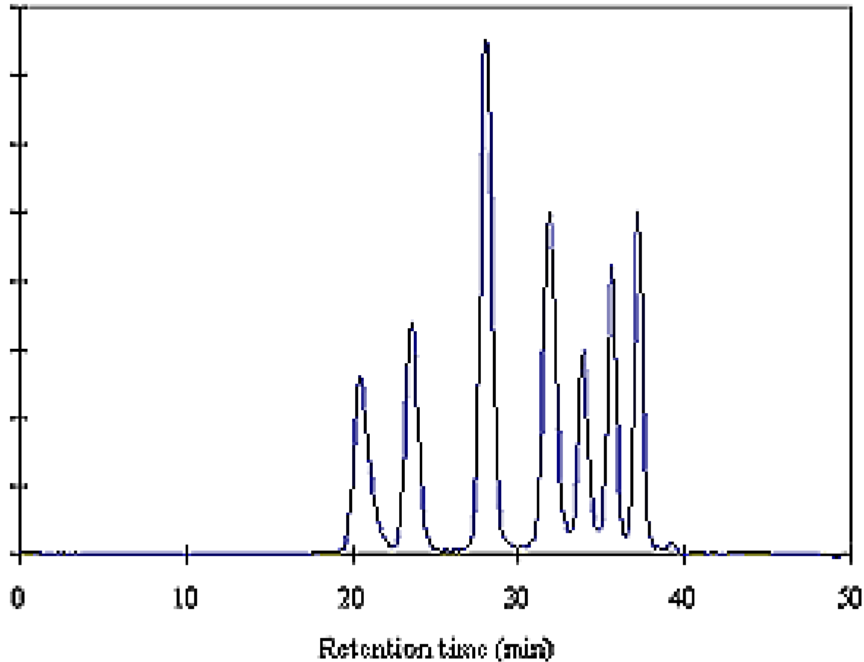



ELUTION BUFFER

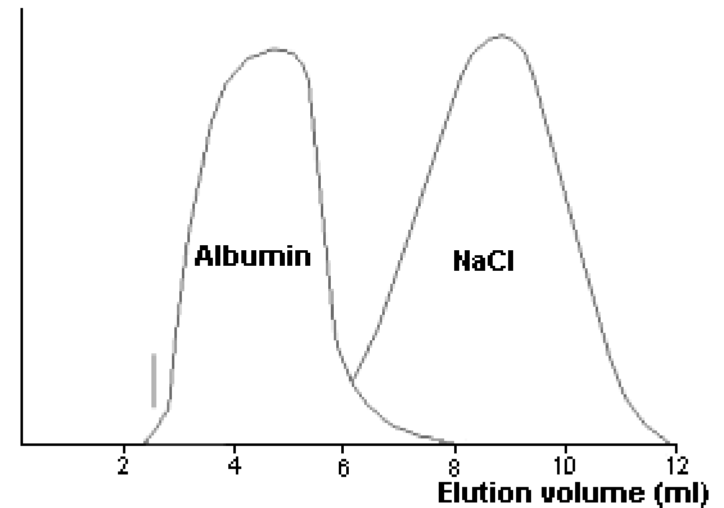
The three categories of accessible volumes are used for different purposes.

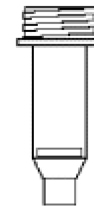


High resolution mode -
High resolution run of Peptides on Superdex Peptide.



Group separation mode -
Desalting albumin on a PD-10 column.





Scetch of a MicroSpin column

Egy céges ismertető:

… packings are based on a 10 μm diol-bonded silica and are available in a variety of pore sizes and column configurations.

The … SEC Columns:

Resolve proteins that differ in molecular weight by a factor of two

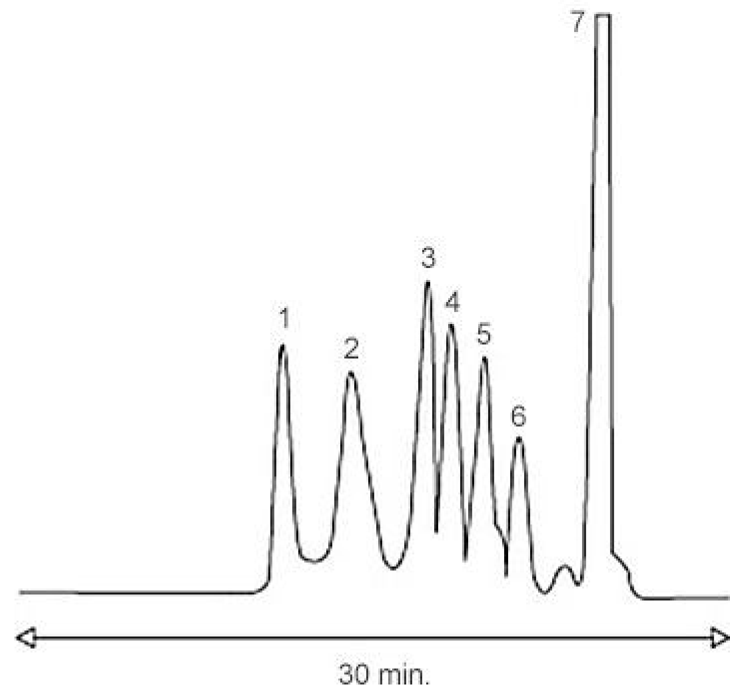
Distinguish proteins differing by as little as 15% in molecular weight

Ideally, there should be no interaction between the stationary phase and the sample molecules. Potential interactions are reduced by adding salts in the 0.1–0.3 M concentration range.

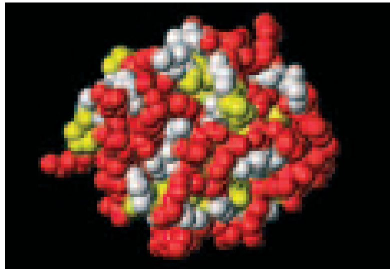
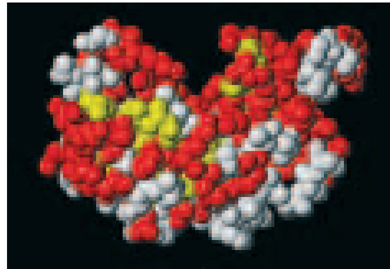
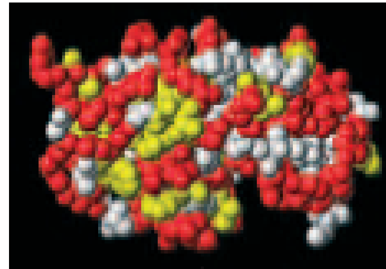
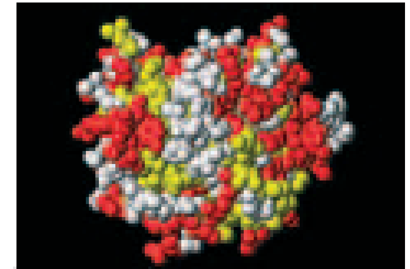
Fast Molecular Weight Screening of Protein Samples

Sample: 50 µL Protein Standards, 5 mg/mL
Column: (2) Protein-Pak™ 300SW, 7.8 x 300 mm
Buffer: 0.1 M K_2HPO_+, pH 7.0
Flow rate: 1.0 mL/min
Detection: 280 nm

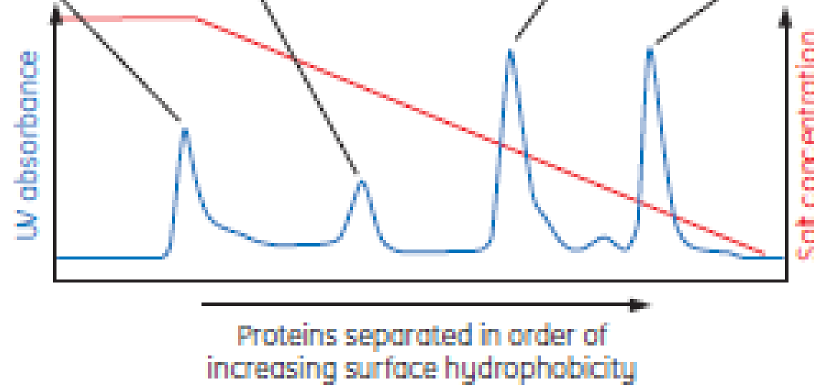
1. Blue Dextran
2. Ferritin
3. BSA
4. Ovalbumin
5. Trypsin inhibitor
6. Ribonuclease A
7. Guanosine



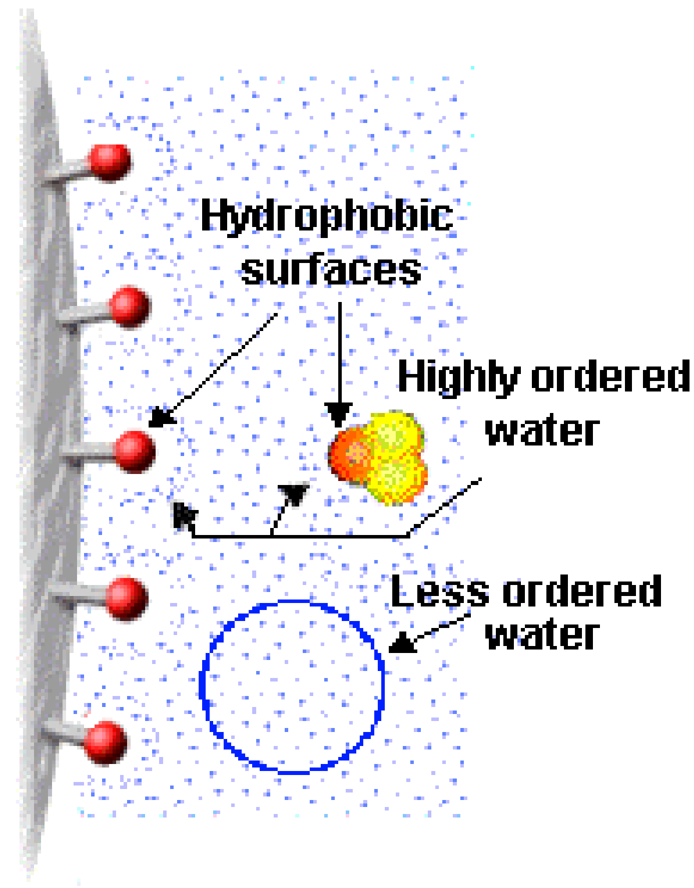
HIDROFÓB KÖLCSÖNHATÁSI KROMATOGRÁFIA (HIC: hydrophobic interaction chromatography)

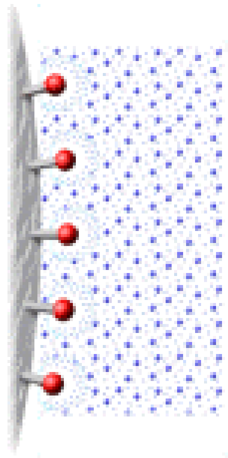
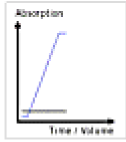
Cytochrome c

RNAse A

Lysozyme

α-chymotrypsin


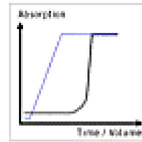
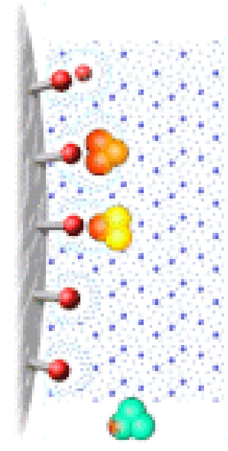
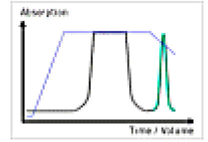
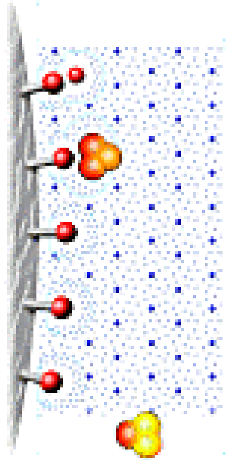
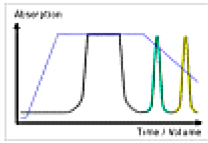
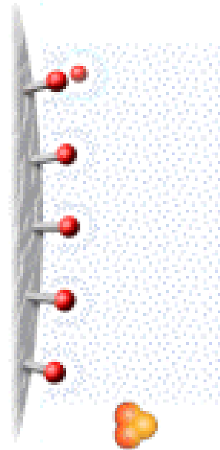
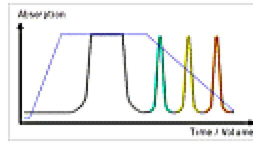
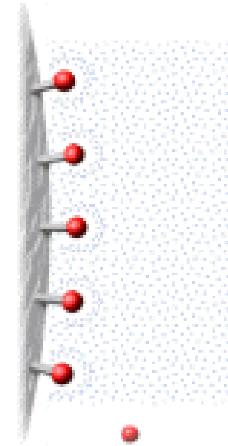
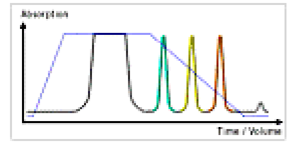
Yellow: hydrophobic residues


UV absorbance
Salt concentration
Proteins separated in order of
increasing surface hydrophobicity

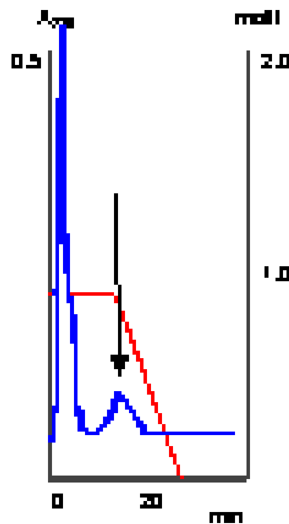
HIC deals with the relation between water shells around hydrophobic surfaces, bulk water clusters and salts enhancing hydrophobic interaction.

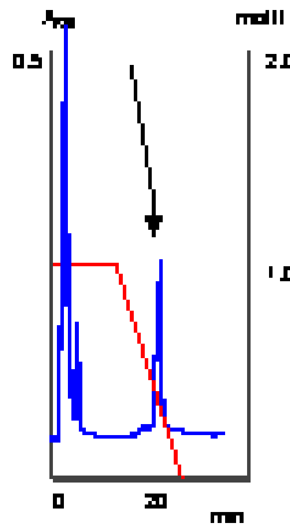


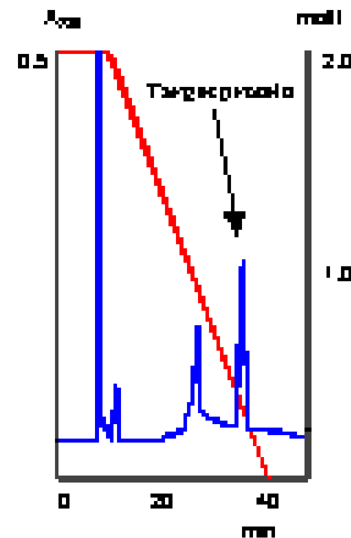


Absorption
Time / Volume


Absorption
Time / Volume


Absorption
Time / Volume


Absorption
Time / Volume


Absorption
Time / Volume


Absorption
Time / Volume

0.8 M $(NH_4)_2SO_4$ | 1 M $(NH_4)_2SO_4$ | 2 M $(NH_4)_2SO_4$







Too low | **Optimal** | **Too high**

The start level of the gradient concentration is quite important for the results. In the left chromatogram the salt concentration was not enough to fully bind the target protein (arrow).
In the center chromatogram the target protein elutes within the gradient as a sharp peak. From a purification point of view the start level of the gradient concentration in the right chromatogram is less advantageous, since sample contaminants also bind an elute within the gradient.
For standard protein purification by HIC, eluent pH is normally ignored as an optimising parameter.

Egy céges ismertető:

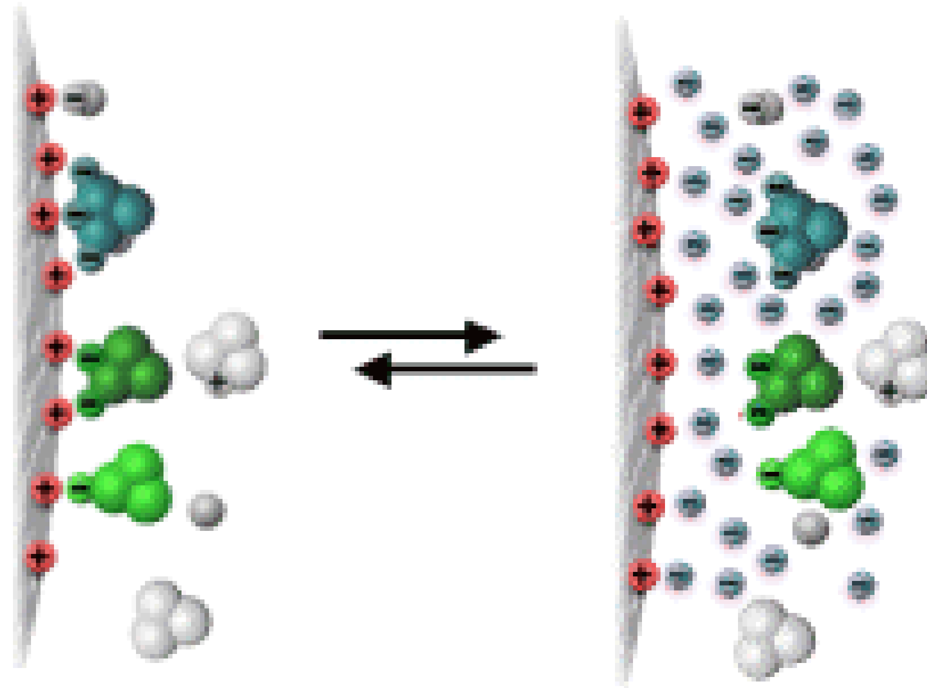
… HIC Columns contain non-porous, polymethacrylate-based particles (2.5 μm) functionalized with a butyl ligand coating

Ideally suited for hydrophobic-based separations for protein characterization using non-denaturing conditions.

Help deliver fast, efficient separations using non-porous particles to address high-throughput needs.

IONCSERÉS FEHÉRJE-KROMATOGRÁFIA

Ioncserés fehérjekromatográfia



Charged sample molecules adsorb to ion exchangers of the opposite sign. The interaction is a dynamic equilibrium that can be influenced by pH or salt concentration.

Varying the pH is a powerful way of influencing the net charges of the sample molecules and is therefore normally used to control the selectivity (elution order and distance between eluted peaks).

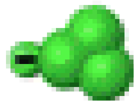
Adding a competing ion will not influence the selectivity, but provide a means of desorbing the sample molecules in order of increasing net charges

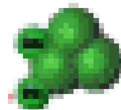
Most IEX experiments use a neutral monovalent salt such as NaCl as the desorbing agent, mainly because NaCl has little or no effect on the running pH.

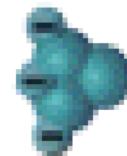
The higher the net charge, the stronger the adsorption and the higher the salt concentration needed to desorb the sample molecule.

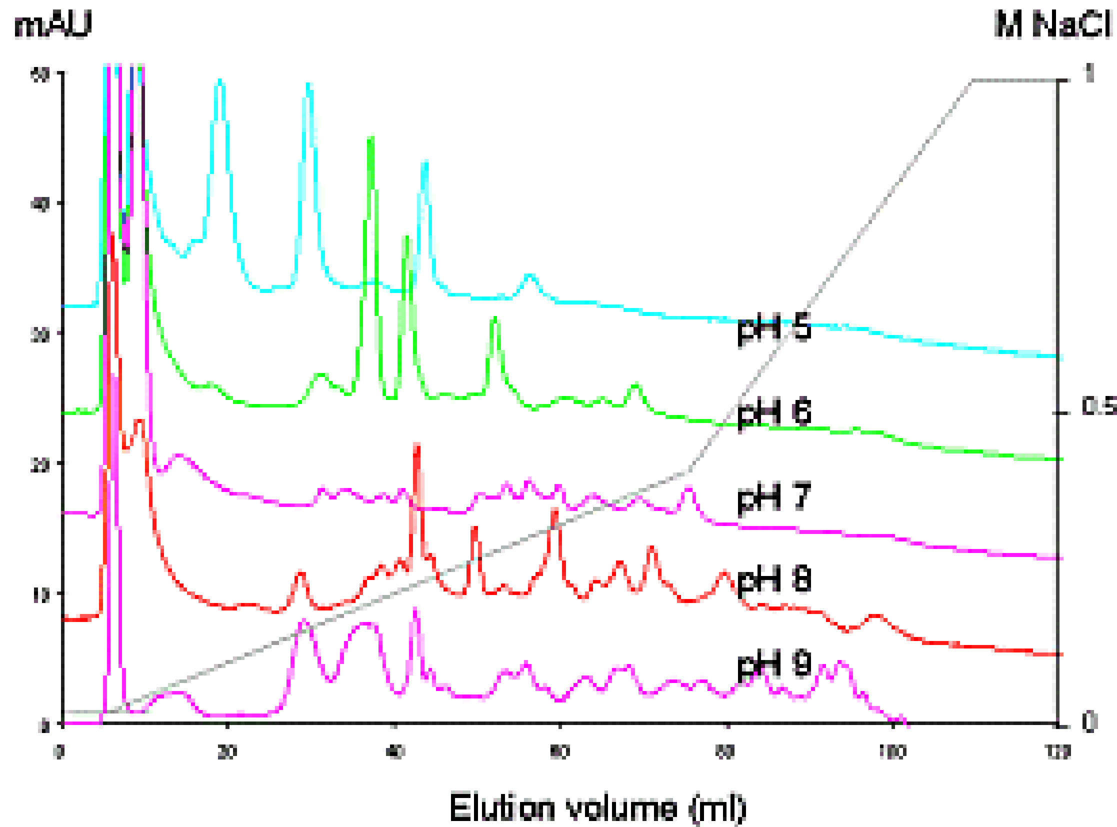
The most frequently used elution mode in high resolution applications of IEX is salt concentration *gradient elution*.

Elution order:









Automatic pH scouting performed on ÄKTAexplorer 100.
Sample: 2 mg crude pancreatin.
Column: RESOURCE Q; 6 ml

Egy céges ismertető:

… packing materials are based on rigid, hydrophilic, polymethacrylate particles with large 1000 Å pores. The naturally hydrophilic polymer reduces non-specific adsorption, resulting in quantitative recovery of protein mass and bioactivity. These packings are compatible with buffers in the pH range 2-12, and will withstand exposure to caustic solutions.

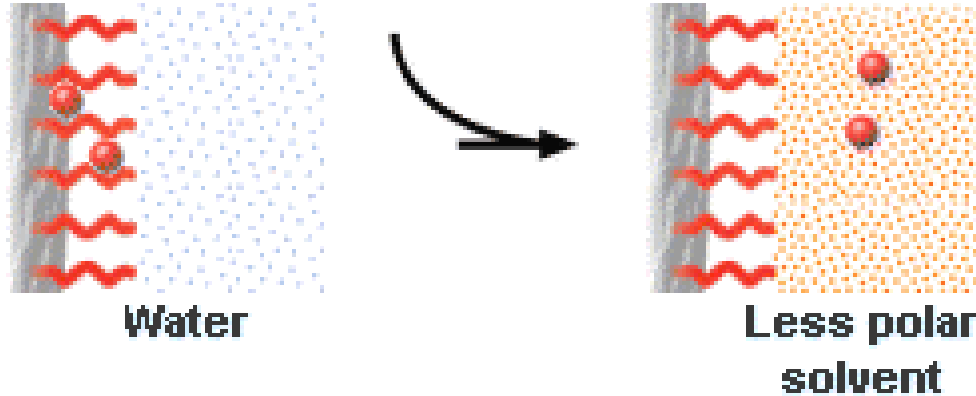
… ion exchangers are available with a:

strong anion exchanger or
weak anion exchanger or
strong cation exchanger or
weak cation exchanger

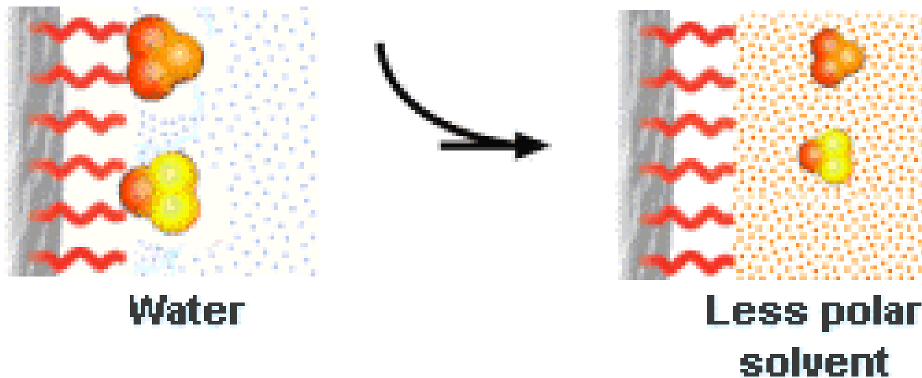
functional group

FORDÍTOTT FÁZISÚ KROMATOGRÁFIA (RP-HPLC)

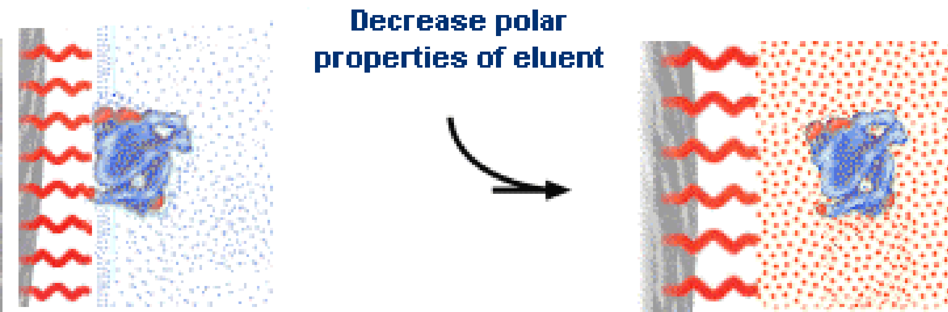
RP-HPLC



Organic molecules are "embraced" by the carbon chains of the stationary phase.



Unlike the typical organic target molecule peptides and proteins *adsorb* to the stationary phase often by multi-point attachment.



A decrease in the polar properties of the mobile phase will weaken the hydrophobic interaction.

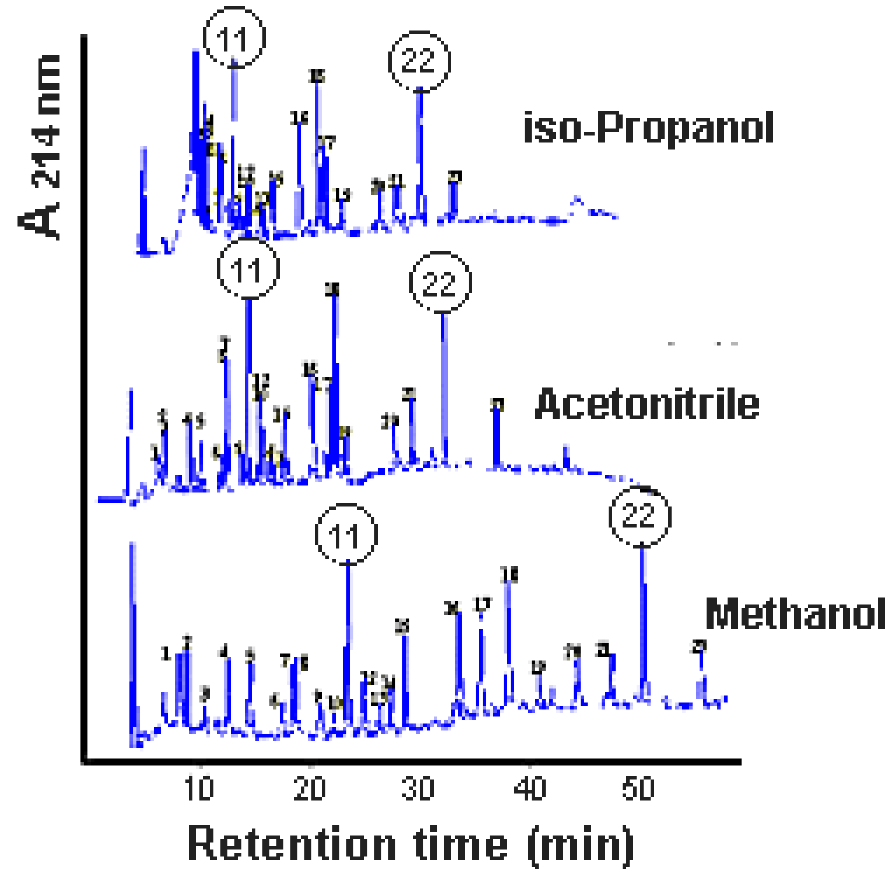
Protein tertiary and quaternary structures depend to a large extent on hydrophobic interaction as a stabilising force.
RPC eluents are designed to weaken hydrophobic interactions and are thus potential denaturants.

RPC of proteins is therefore a delicate balance between desorption and denaturation and care must be taken to satisfy this balance or the protein may be irreversibly destroyed.
(Not a problem with most peptides, since no intramolecular hydrophobic interactions.)

Reversed phase chromatography in practice

Use of Reversed phase chromatography	
High resolution mode (gradient elution)	**Group separation mode (step elution)**
Separates peptides, proteins and oligonucleotides according to net hydrophobicity.	Concentrates dilute oligonucleotide and peptide samples.
Suitable for intermediate steps and polishing in multi-step purification protocols.	Suitable for so called solid phase extraction.
Main technique for the purification of synthetic peptides.	Suitable for desalting of peptide and oligonucleotide samples.
Main technique for the analysis of peptides and for peptide mapping.	

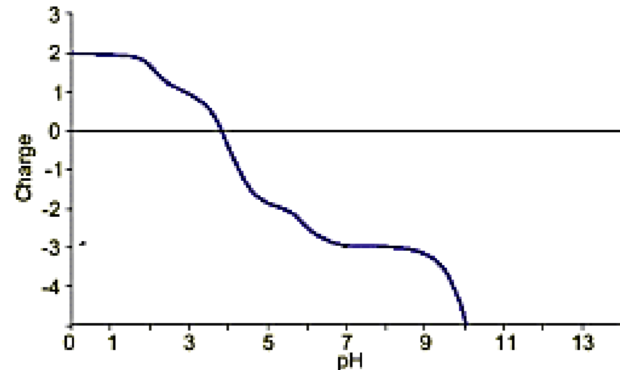
For proteins and peptides gradient elution is typical.
As seen, the eluents shown
differ in eluting strength rather than in their influence on selectivity.



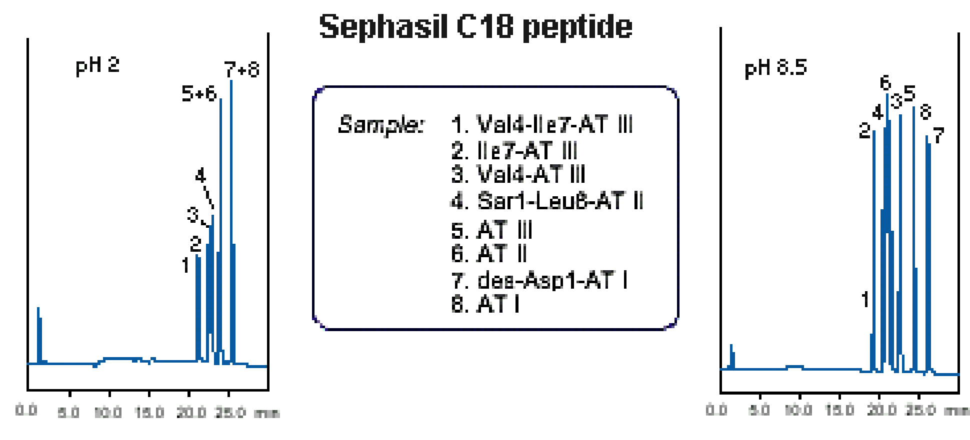
Ref. Aguilar,M-I. and Hearn, M. Meth. Enzymol.vol.270;3-26; 1995

ACN has a very good UV transparency and contributes rather little to the eluent viscosity and thus to the back pressure over the column.
For peptide and protein separations ACN is the by far most commonly used organic modifier and iso-propanol is turned to only when required by the sample stability.

H-Ile-Ser-Pro-Asp-Gly-His-Glu-Tyr-Ile-Tyr(PO_3)-Val-Asp-Pro-Met-Gln-Leu-Pro-Tyr-OH



Protein and peptide net charges vary with ambient pH, a fact that influences their hydrophobic properties and thus their chromatographic behavior in RPC

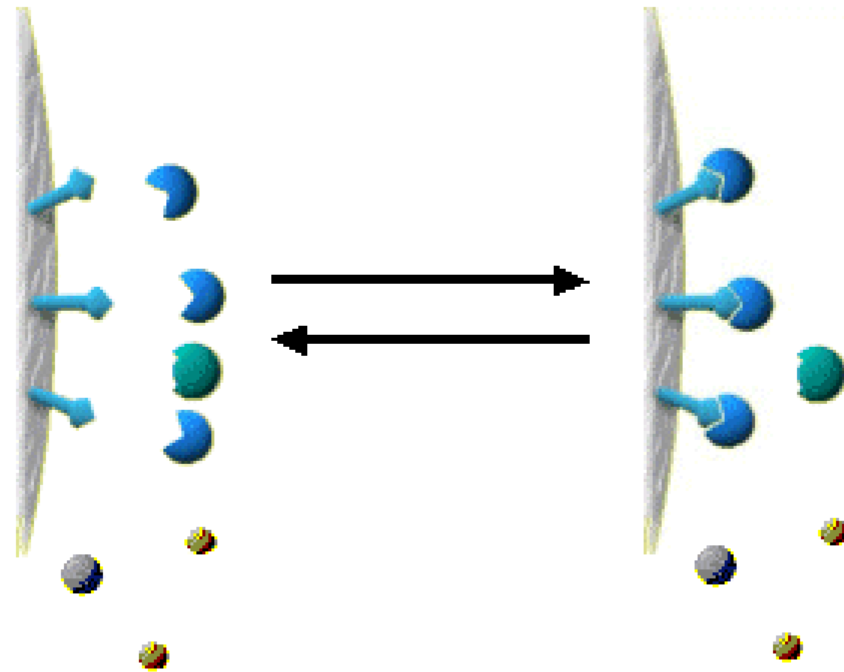


Peptide net hydrophobicity is influenced by pH and when run at different pH values, peptide elution positions may change considerably.

The pH change from 2 to 6.5 actually rearranges the elution order of the angiotensin derivatives of the figure quite considerably.

AFFINITÁS KROMATOGRÁFIA

Affinitás kromatográfia

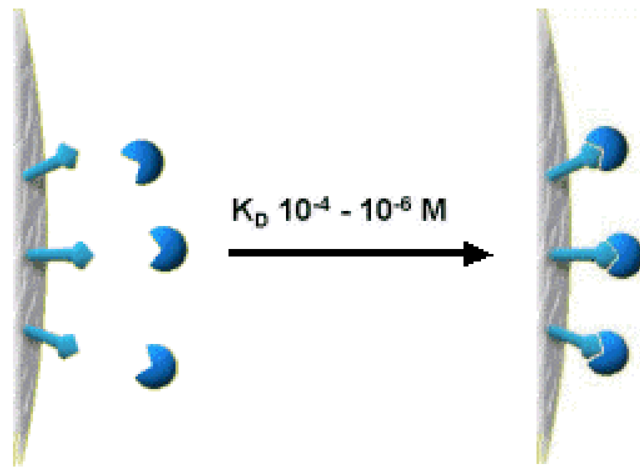


Affinity chromatography relies upon a reversible highly specific binding reaction.

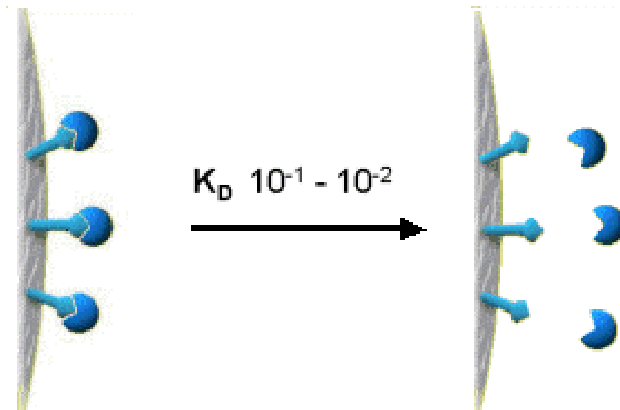
Ligands used for group-specific affinity chromatography have a much wider applicability and affinity media for this purpose are consequently commercially available.

The table below lists examples of commonly used ligands of this type.

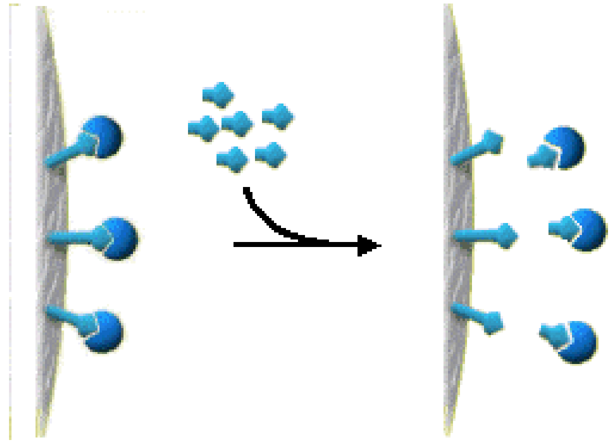
Group-specific ligand	Specificity
Protein A	Fc region of IgG
Protein G	Fc region of IgG
Concanavalin A	Glucopyranosyl and Mannopyranosyl groups
Cibacron Blue	Broad range of enzymes, serum albumin
Procion Red	NADP+ dependent enzymes
Lysine	Plasminogen, ribosomal RNA
Arginine	Serine proteases
Benzamidine	Serine proteases
Calmodulin	Proteins regulated by calmodulin
Heparin	Coagulation factors, lipoproteins, lipases, hormones, steroid receptors, protein synthesis factors, Nucleic acid-binding enzymes
Transition metal ions	Proteins and peptides which contain accessible Histidine



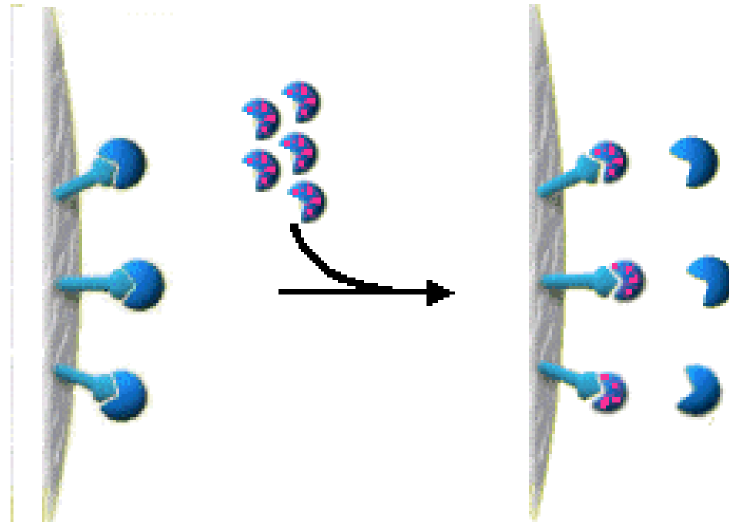
K_D values for good binding are typically in the range of 10^{-4} - 10^{-6} M.



Elution (desorption): K_D values suitable for elution are typically in the range of 10-1 - 10-2.



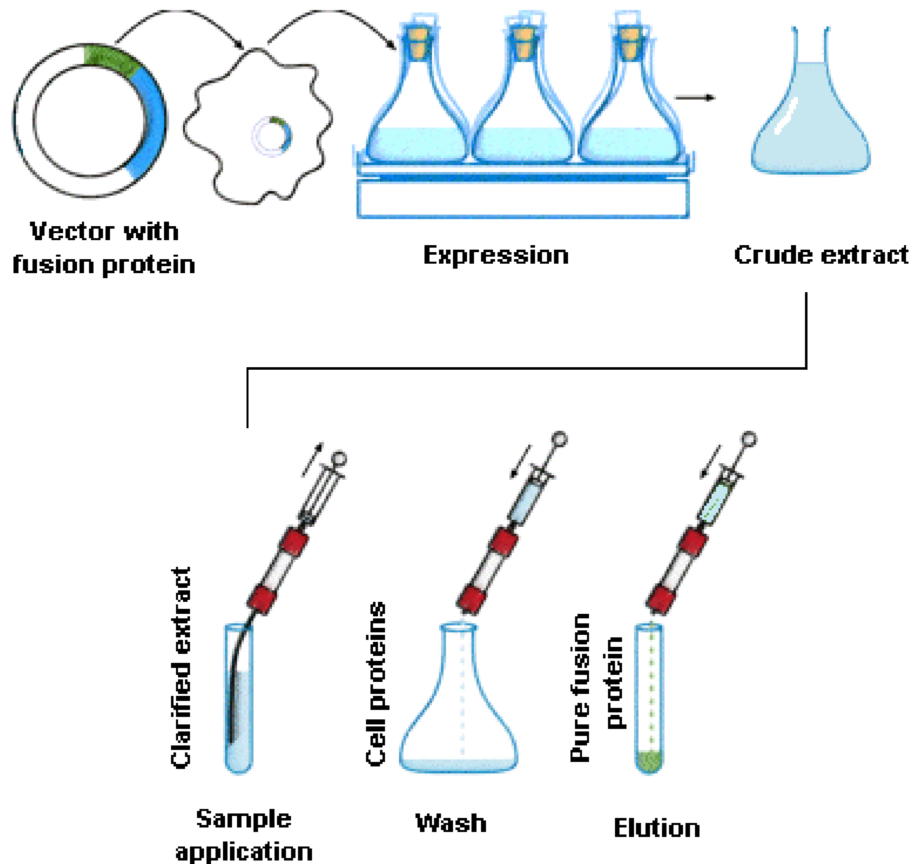
Elution by displacement, free ligand: Free ligand is added to displace the target from the matrix-bound ligand.



Elution by displacement, free target analogue: An analogue is added to displace the target from the matrix-bound ligand.

Affinity chromatography applied to recombinant proteins

The purification of recombinant proteins may be drastically simplified by fusing the gene for an affinity tag (or handle) with the gene for the recombinant. The host will then express the recombinant protein tagged with the affinity handle and affinity chromatography techniques can be applied to isolate and purify this so-called *fusion protein* (Fig). Though not always necessary, the affinity tag can be removed by special cleave-off enzymes after the purification.



Egy céges ismertető:

… affinity epoxy-activated packing consists of 40 µm, 500Å pore size particles that have a hydrophilic bonding layer with a glycidoxypropyl functionality, resulting in a seven atom spacer arm.

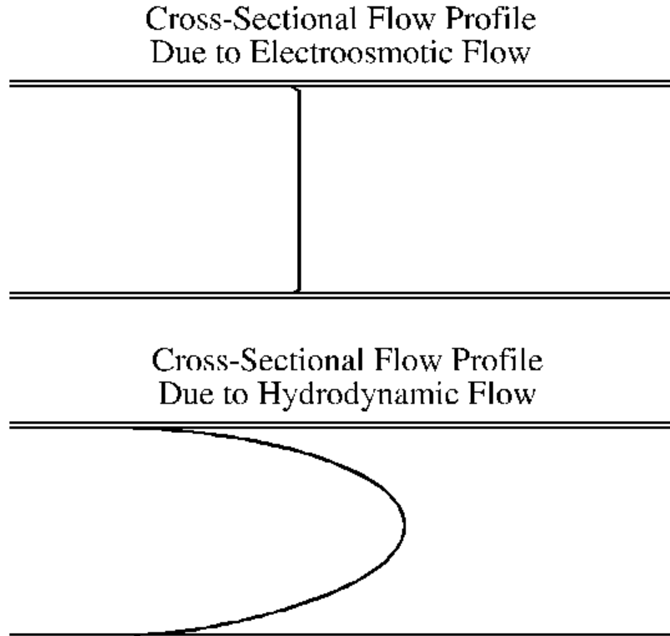
The epoxy-activated surface can immobilize a wide range of ligands via a covalent linkage with amino, hydroxyl or sulfhydryl groups using simple coupling procedures.

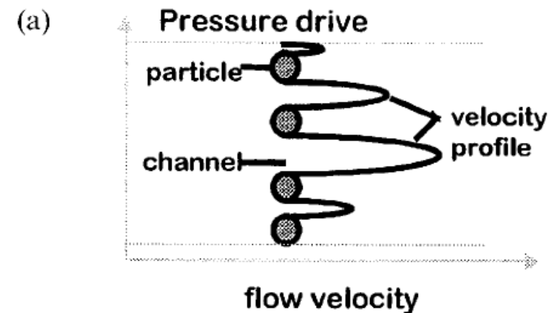
ELEKTRO-KROMATOGRÁFIA

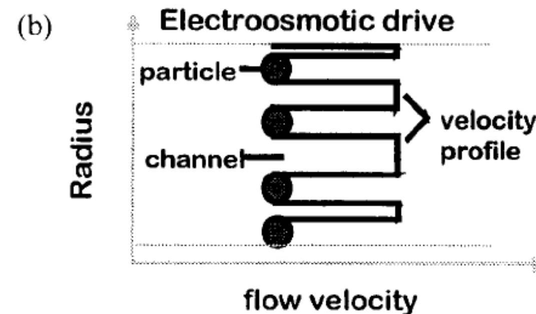
Capillary ElectroChromatography (CEC)

- Use EOF to "electro-pump" mobile phase through packed micro-column
  - analyze charged and neutrals similar to HPLC
  - stationary phases selected according to HPLC and UPLC innovations

- EOF provides superior flow profile
  - square flow profile results in less band broadening, higher plate count, better resolution

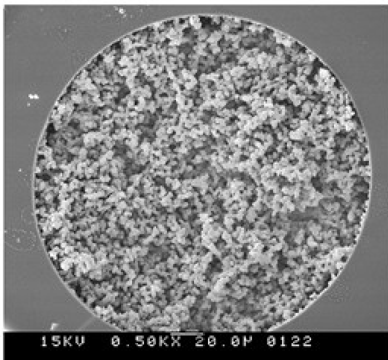


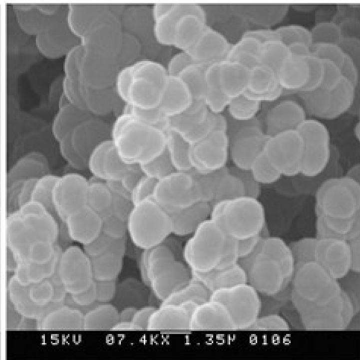


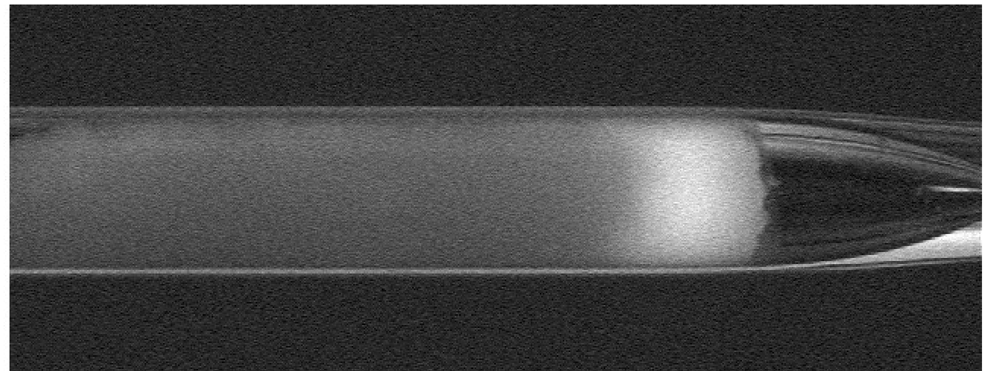


Porous monoliths as micro-column packings

•Monoliths in columns: continuous porous separation medium
- ease of preparation
- no need for end frits
- versatile surface modification
- no interparticular voids (less eddy diffusion?)
- good peak capacity (high surface area)
- fixed bed stationary phase may be more stable







HPLC CHIP

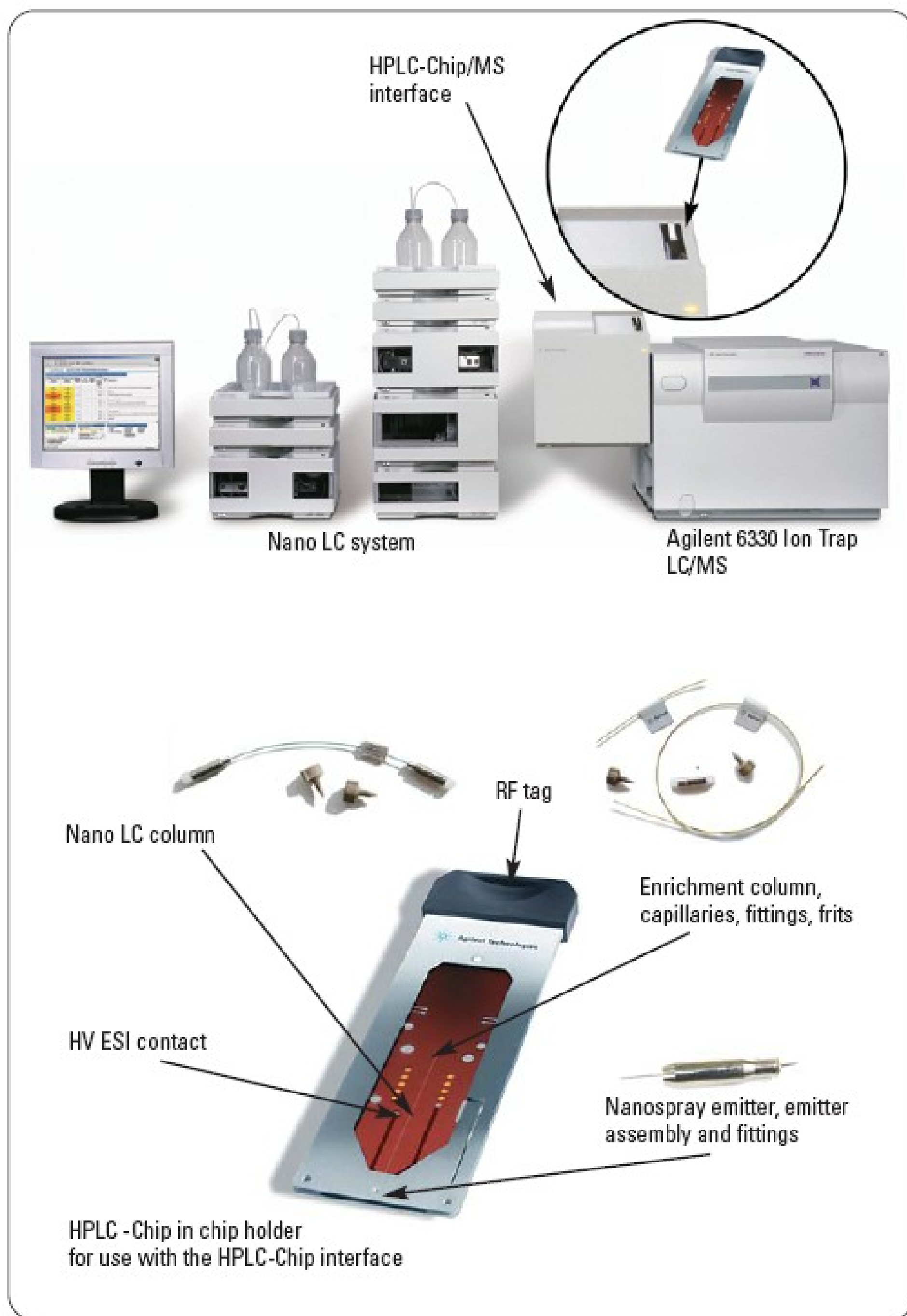


Figure 1
Agilent HPLC-Chip/MS system.

HPLC-Chip (G4240-65001):

- A 40-nL enrichment column packed with ZORBAX 80 SBC18, 5-µm particle size
- A 0.075 x 43 mm analytical column packed with ZORBAX 80 SB-C18, 5-µm particle size.
- All connections between the two columns and between the analytical column and the nanospray emitter
- The nanospray emitter (10-µm ID).

The HPLC-Chip is inserted into the HPLC-Chip/MS interface (HPLC-Chip cube). This interface provides all fluid connections to the Agilent 1200 Series nanoflow LC system and ensures efficient coupling of the nanospray emitter to the Agilent 6330 Ion trap LC/MS.

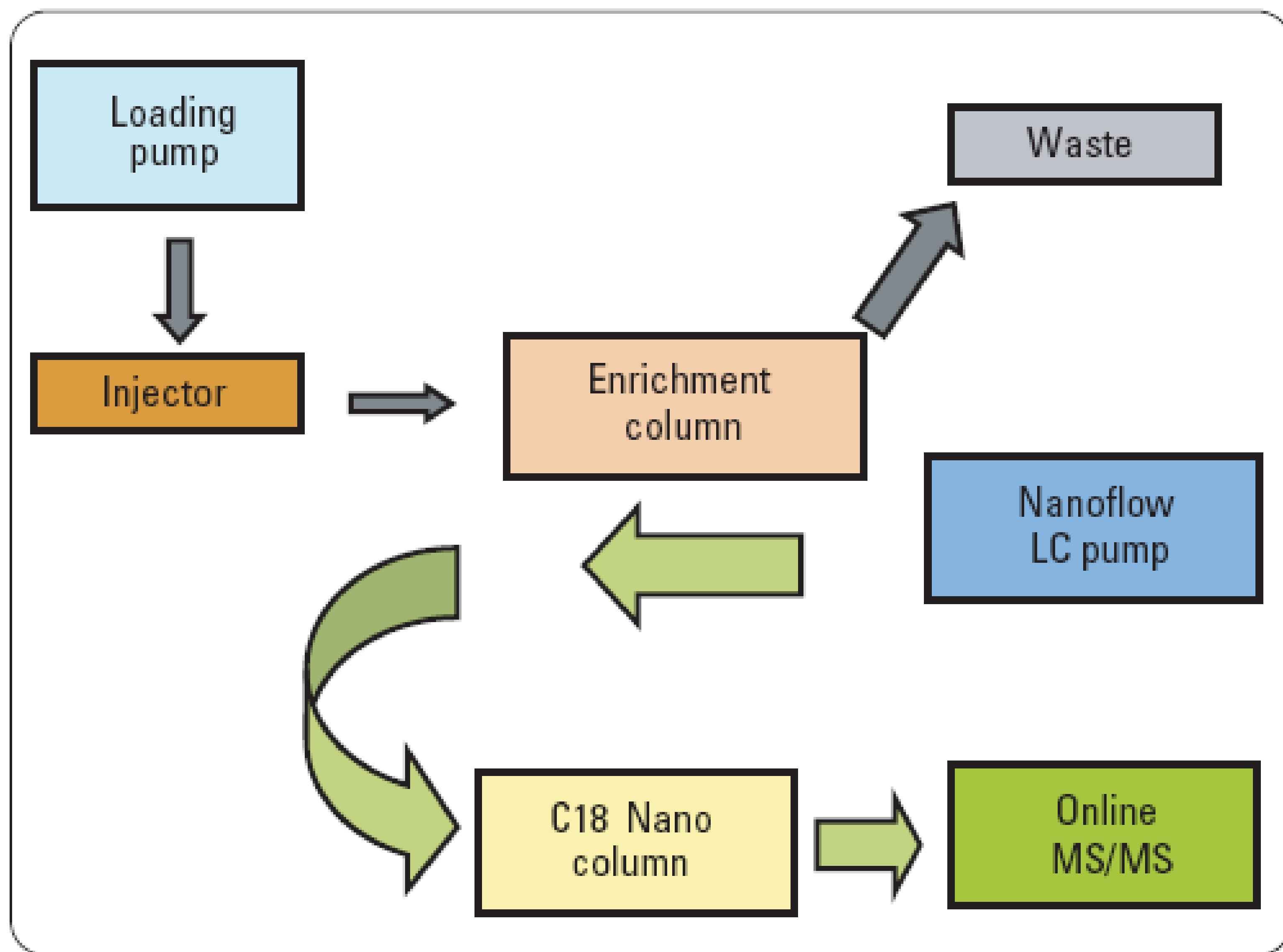


Figure 1
Flow diagram for a conventional nanoflow LC/MS system with sample enrichment.

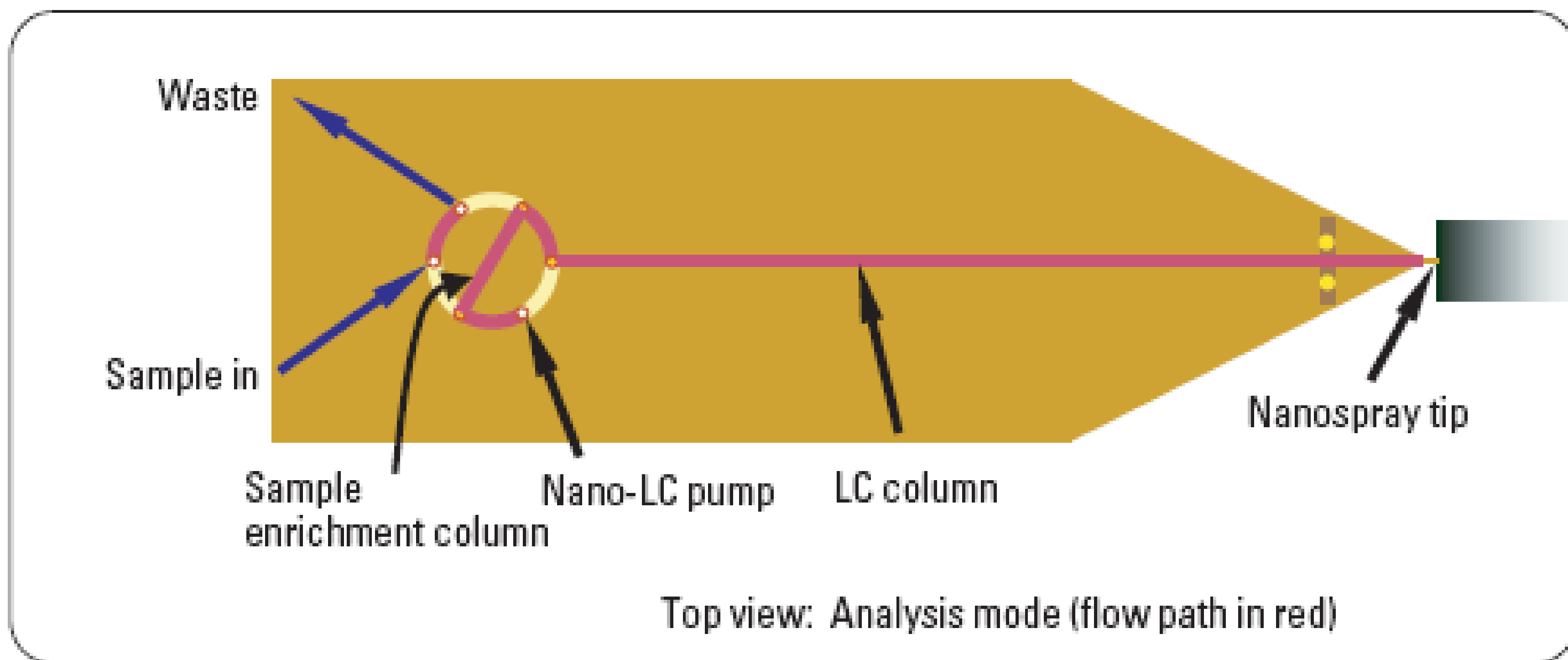


Figure 2
Diagram of the HPLC-Chip.

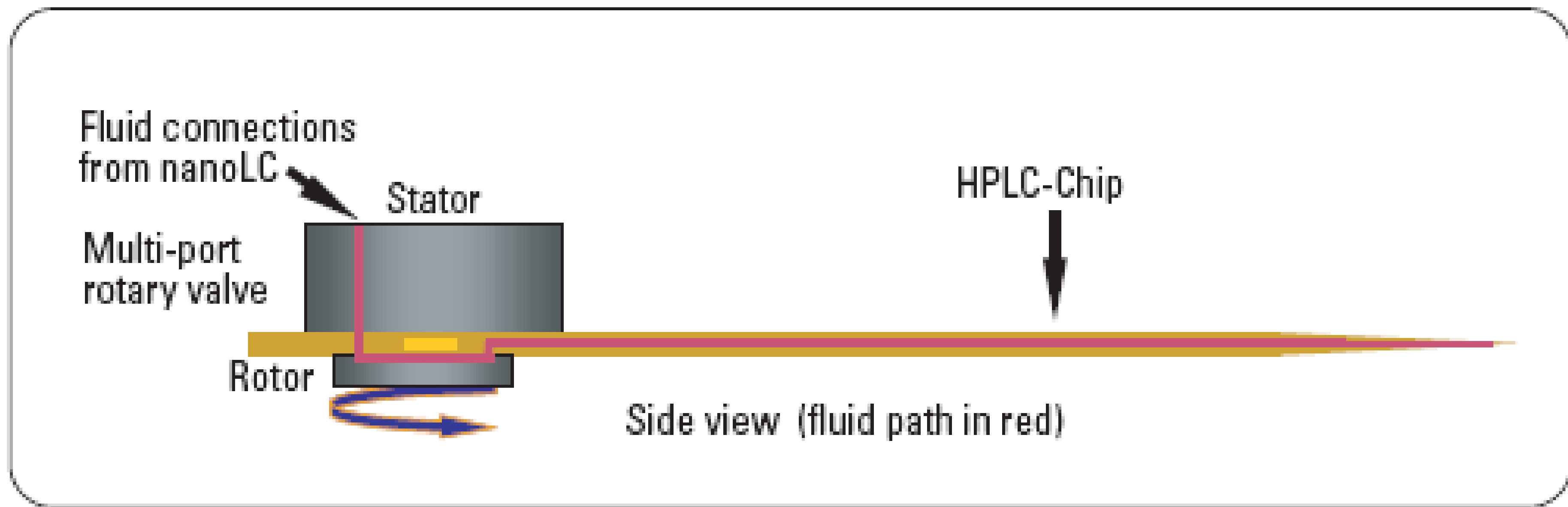


Figure 4
The microvalve in the HPLC-Chip/MS interface docks to an HPLC-Chip.

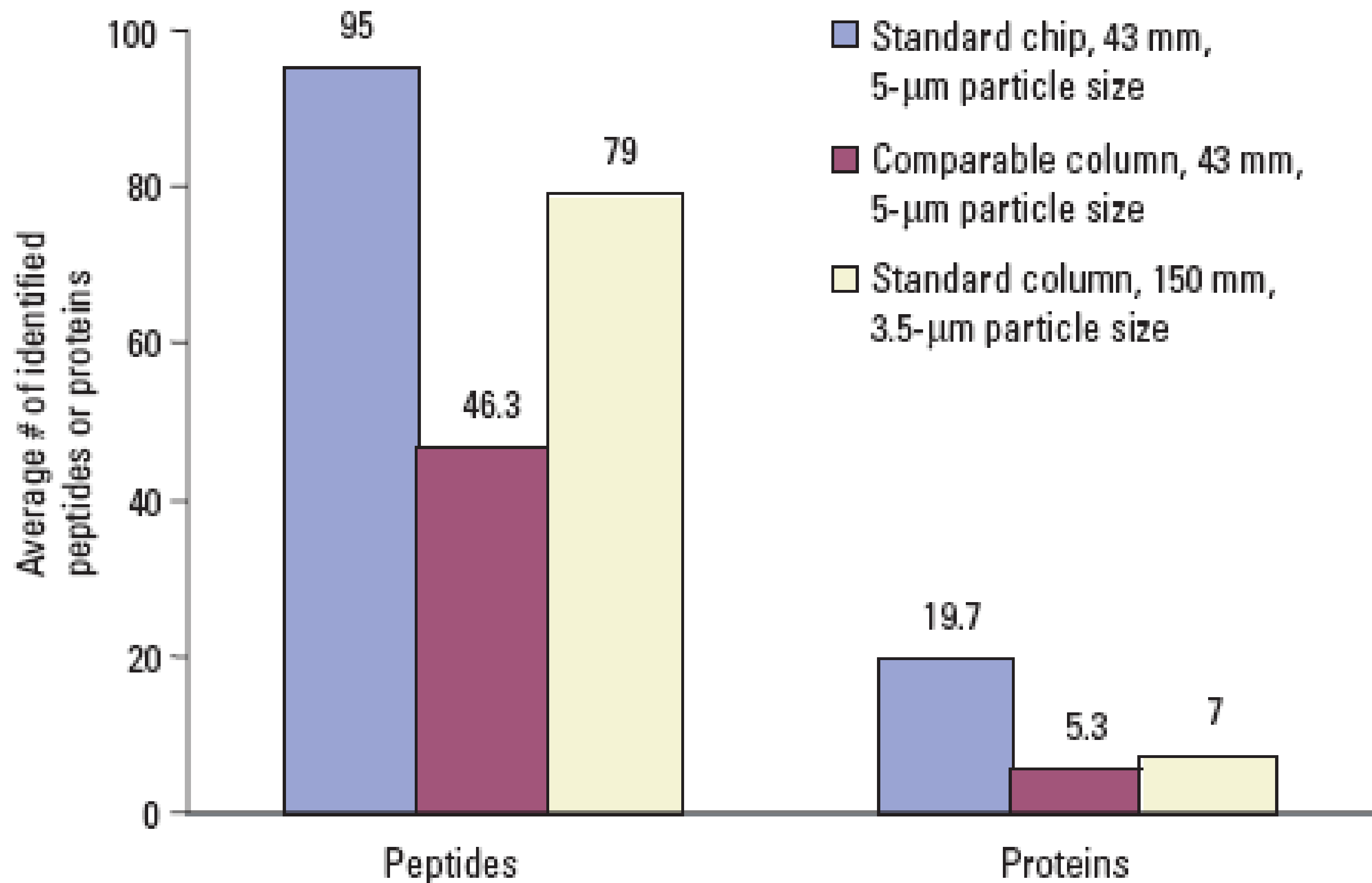


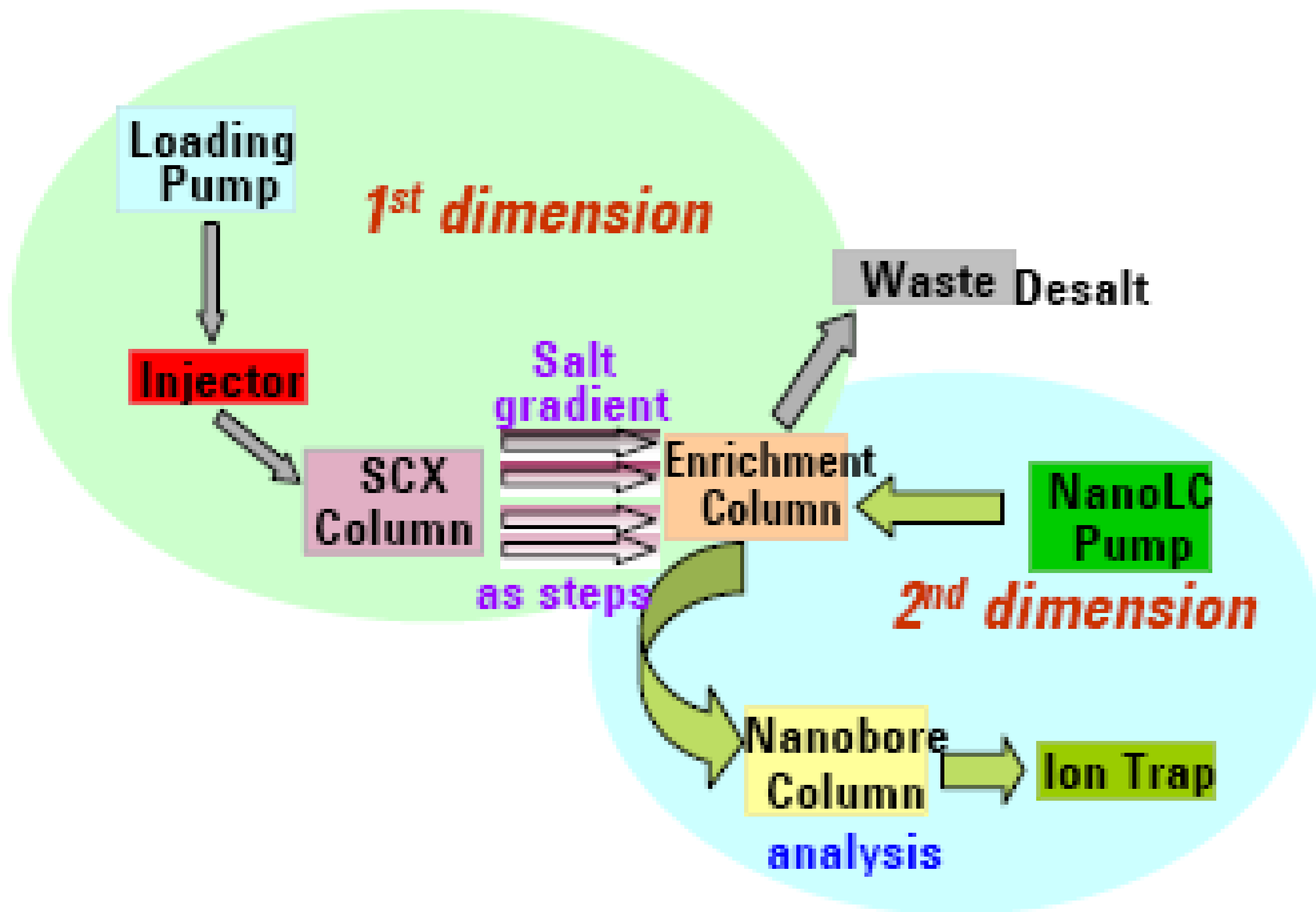
Figure 5
Average number of identified peptides and proteins from yeast gel band using the HPLC-Chip/MS (43 mm) versus conventional LC/MS with nanocolumns (43 mm and 150 mm).

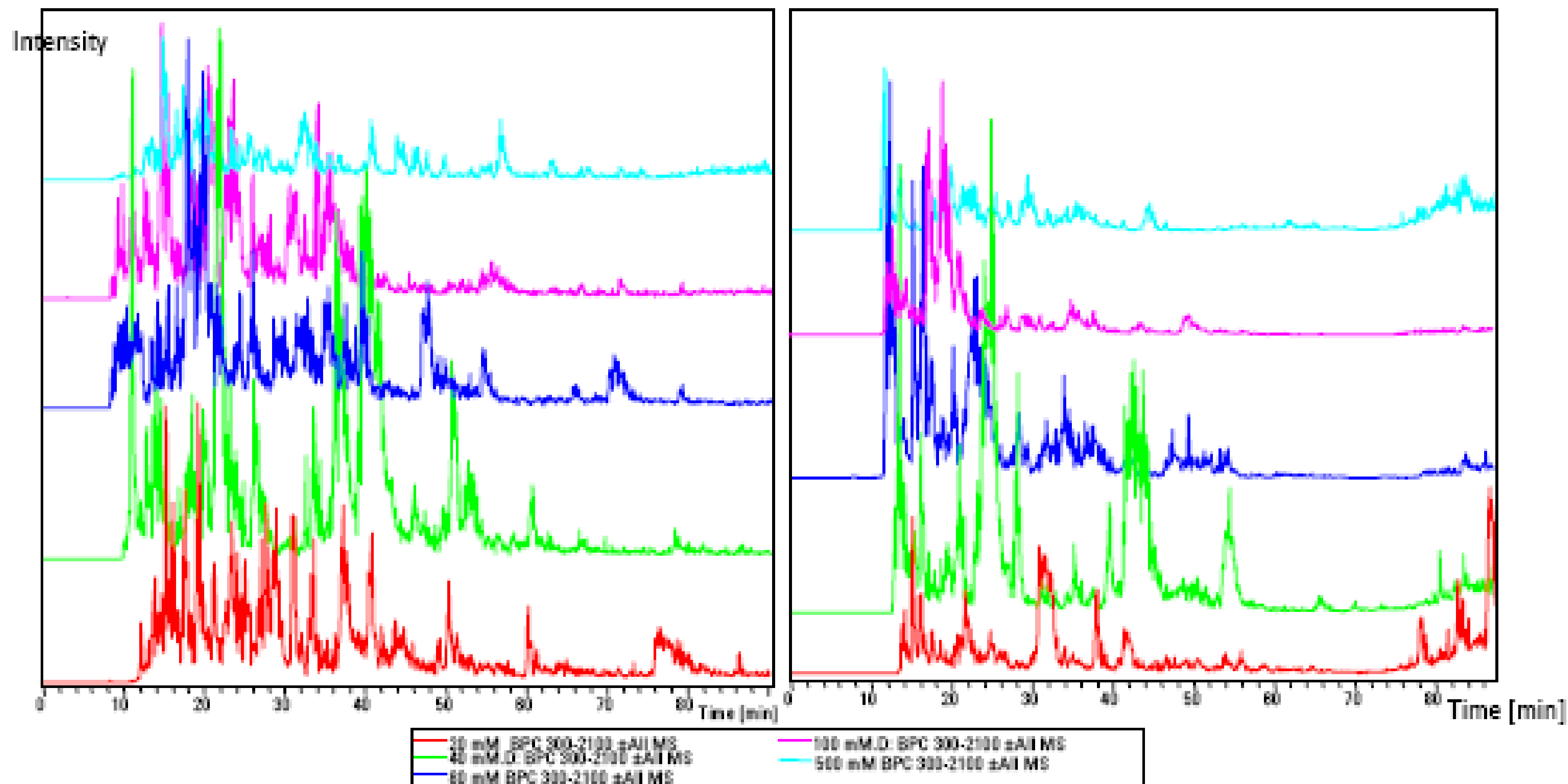
First, since integration of components on the HPLC-Chip eliminated most plumbing connections, dead volumes were reduced. Second, sample adsorption was minimized by the use of biocompatible polyimide and the elimination of troublesome connectors susceptible to sample adsorption. Third, since the electrospray emitter was integrated into the HPLC-Chip, postcolumn peak dispersion was negligible. Overall, the optimized design of the sample pathway **minimized sample loss and reduced dead volume**. These enhancements significantly increased the number of identified peptides and proteins with the HPLC-Chip format.

Two dimensional chromatography

By combining SCX with RP and nanospray iontrap MS/MS a comprehensive and sensitive differential proteome analysis from a complex biological sample was demonstrated.

The sensitive detection of a sub-proteome of a few proteins in a background of a few thousand proteins was successfully demonstrated


Loading Pump
1st dimension
Waste
Desalt
Injector
Salt gradient
SCX Column
Enrichment Column
NanoLC Pump
as steps
2nd dimension
Nanobore Column
Ion Trap
analysis



Base peak chromatograms from selected 2D-SCX-RP fractions (left lactose, right glucose culture)

KÖSZÖNÖM A FIGYELMET