Protein mass spectrometry and proteomics

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Spring Semester 2025

EPFL

Course outline

- 1. Introduction

Introduction to protein analysis and proteomics; Reminders in mass spectrometry; Why proteomics and mass spectrometry?; Ionization sources, analysers, and detectors used in proteomics; Latest generation of mass spectrometers used in proteomics

- 2. Proteomic strategy and workflows

Bottom-up versus top-down strategies; Data-dependent acquisition (DDA) and data-independent acquisition (DIA) approaches; Sample preparation

- 3. Separations techniques in proteomics

Gel electrophoresis; Isoelectric focusing; Liquid chromatography (RP, IEX)

- 4. Quantitative proteomic workflows

Label-free methods; Labelling-based techniques; Other quantitative techniques

- 5. Proteomic bioinformatics

Databases; Identification of protein; Quantification of proteins; Bioinformatics tools; Practical examples

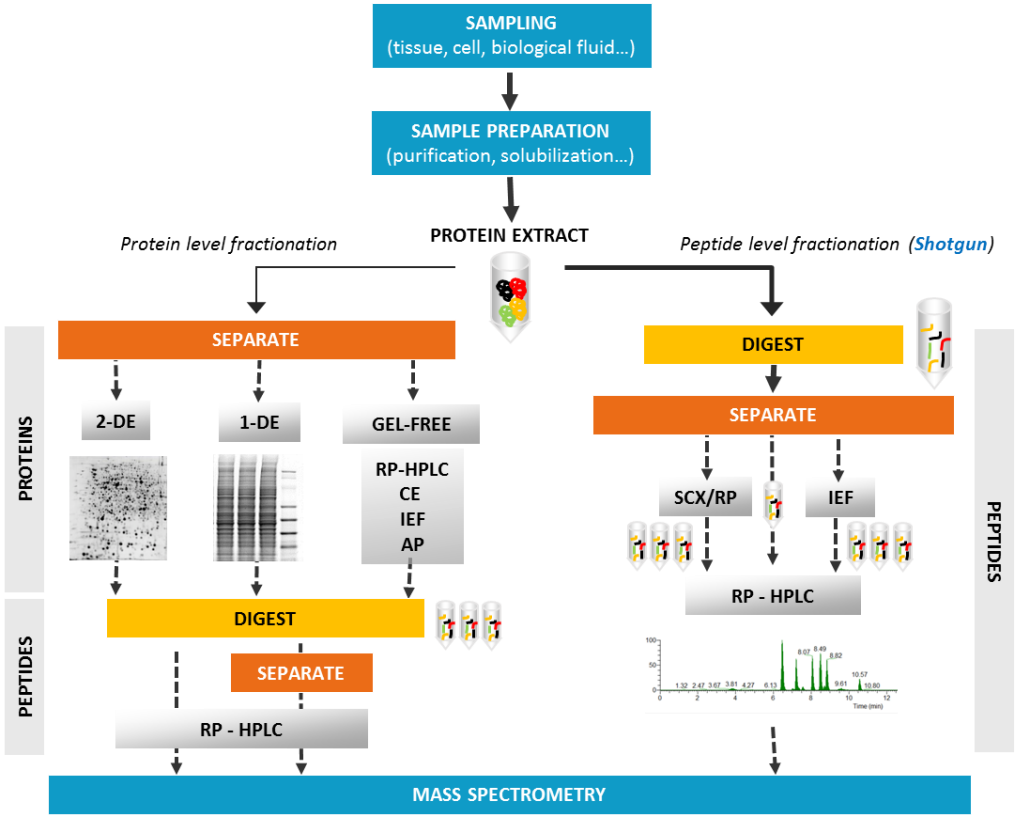
- 6. Applications to biology and clinical research

What strategy?; Experimental design; Biomarker discovery; Industrialized and population proteomics; Forensics; Targeted mass spectrometry-based approaches; Other biological applications of mass spectrometry; Advanced innovations (single-cells, 4D proteomics, multi-omics) and emerging technologies; Limitations and ethical consideration; Lab visit

Course outline

- 3. Separations techniques in proteomics

Gel electrophoresis; Isoelectric focusing; Liquid chromatography (RP, IEX)



M. Abonnenc and M. Mayr, 2012, *Inflammation and Atherosclerosis*, 249-266, DOI: 10.1007/978-3-7091-0338-8_13

Why separation?

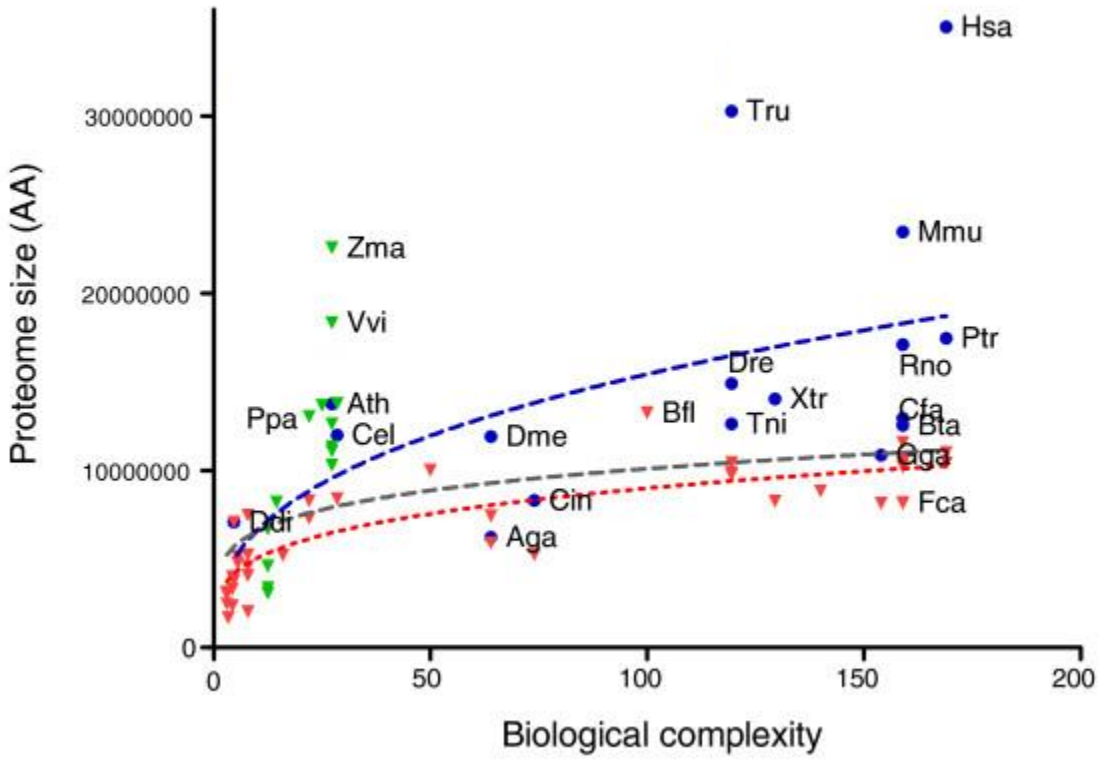
Diversity of sequences

- Arabidopsis ~28000 genes
- Human ~22000 genes
- Worm ~19000 genes
- Yeast ~6000 genes
- *E. Coli* ~5000 genes

Size of proteomes are much larger!

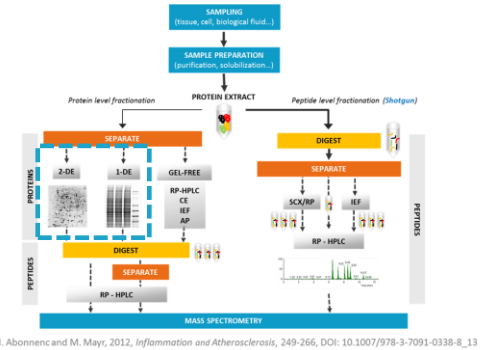
Heterogeneity in protein length, properties, localization…

Dynamic ranges of concentrations



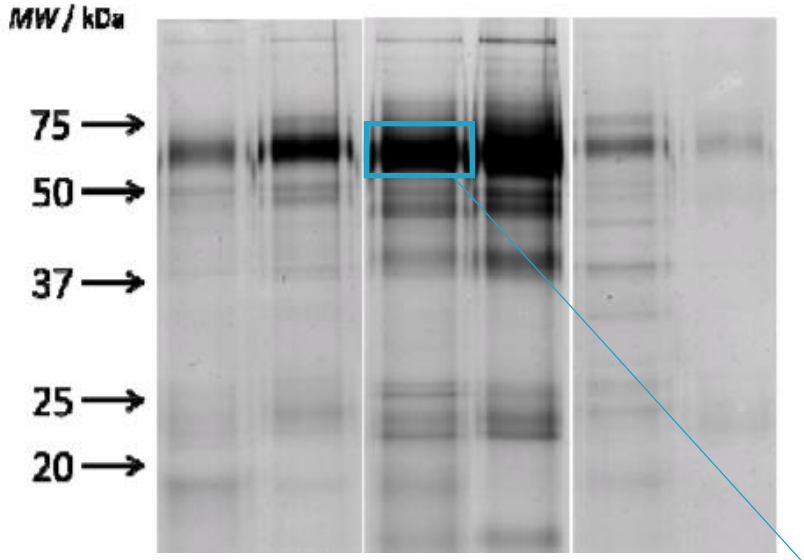
doi: 10.1186/gb-2011-12-12-r120

3.1. Gel electrophoresis



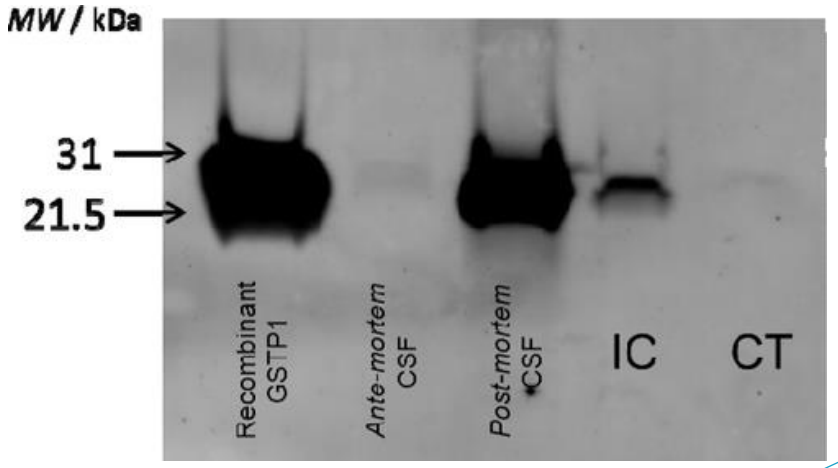
M. Abonnenc and M. Mayr, 2012, *Inflammation and Atherosclerosis*, 249-266, DOI: 10.1007/978-3-7091-0338-8_13

1D gel



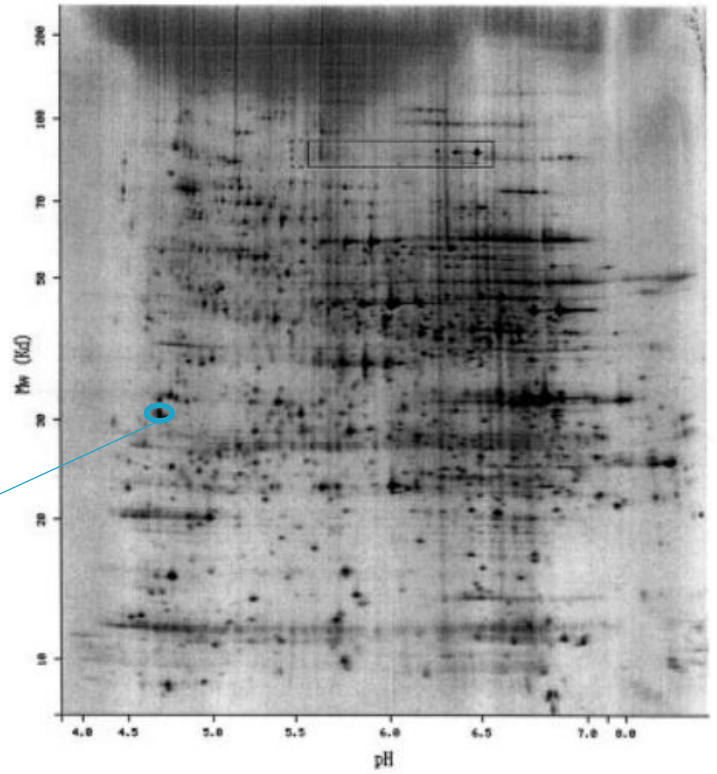
doi: 10.1021/pr101123t

Immunoblotting



doi: 10.1021/pr101123t

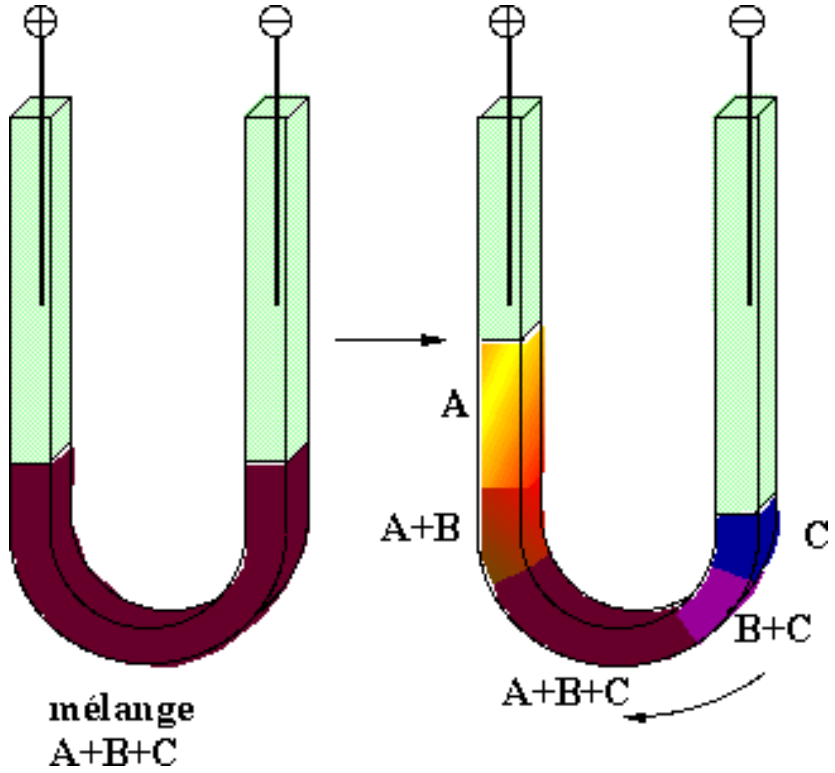
2D gel



Nucleic Acids Res. 1996; 24(1): 180–181

Mass spectrometry

Electrophoresis



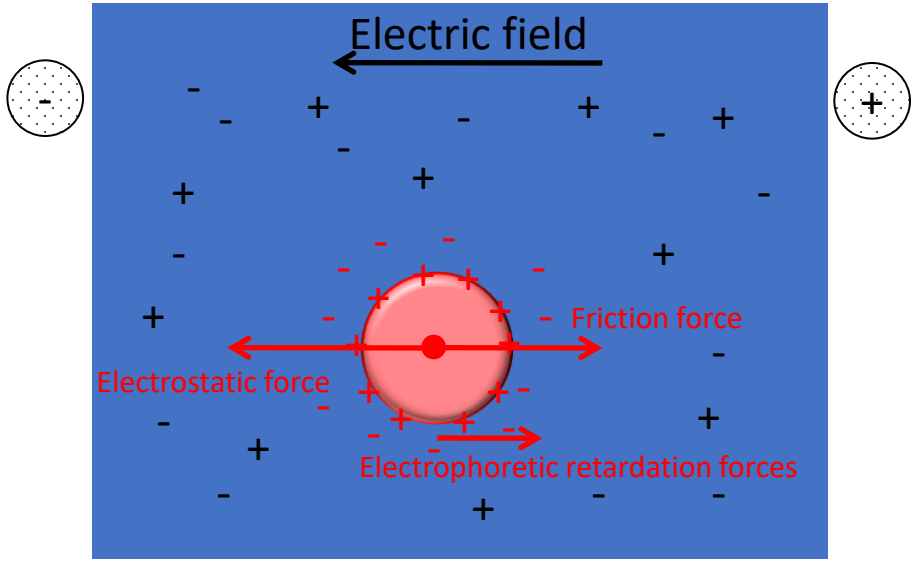
Protein separation
of human serum

The Nobel Prize in Chemistry
1948

Arne Wilhelm Kaurin Tiselius

"for his research on electrophoresis and adsorption analysis, especially for his discoveries concerning the complex nature of the serum proteins"

Electrophoretic separation



http://en.wikipedia.org/wiki/Electrophoresis

Electrophoresis is the motion of particles in an electric field

1) Electric field exerts electrostatic force on the particle through the carried charge

2) Hydrodynamic friction force affects all bodies moving in viscous fluids

3) Electrophoretic retardation forces are applied to the ions in the diffuse layer

Two main modes of electrophoretic separation are used for peptides and proteins:

Zone electrophoresis is based on the differences in the electrophoretic mobilities of charged species (analytes)

Isoelectric focusing is an electrophoretic technique for the separation of amphoteric analytes according to their isoelectric points

Electrophoresis set-ups

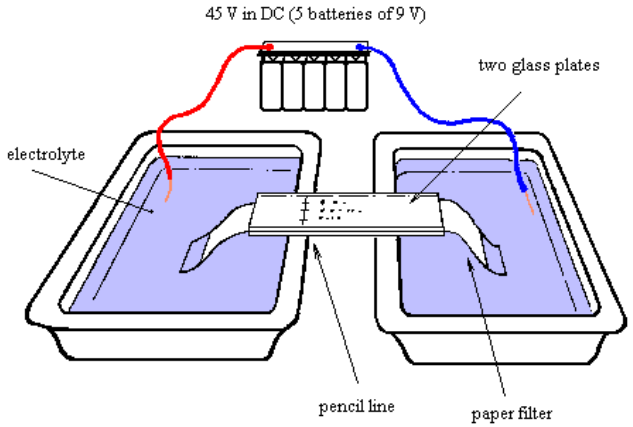
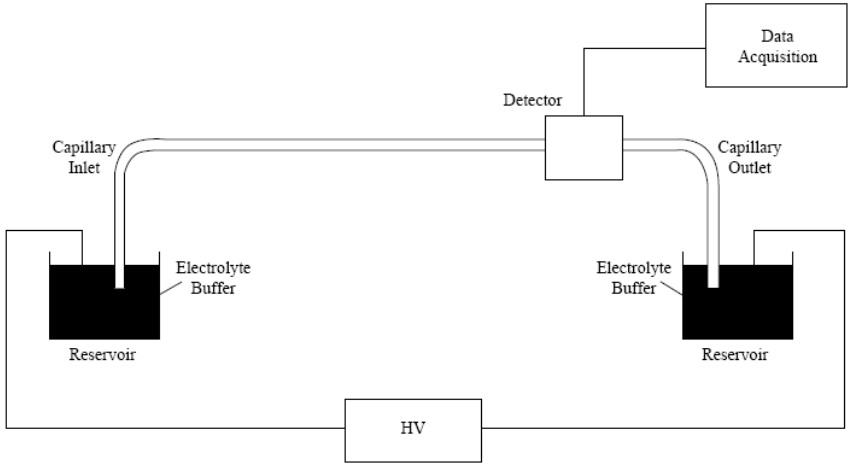
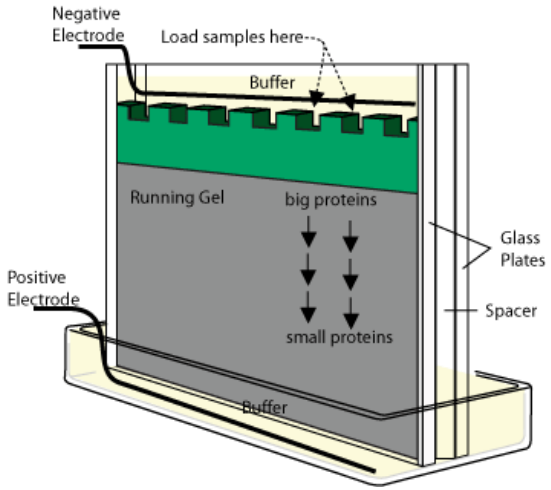


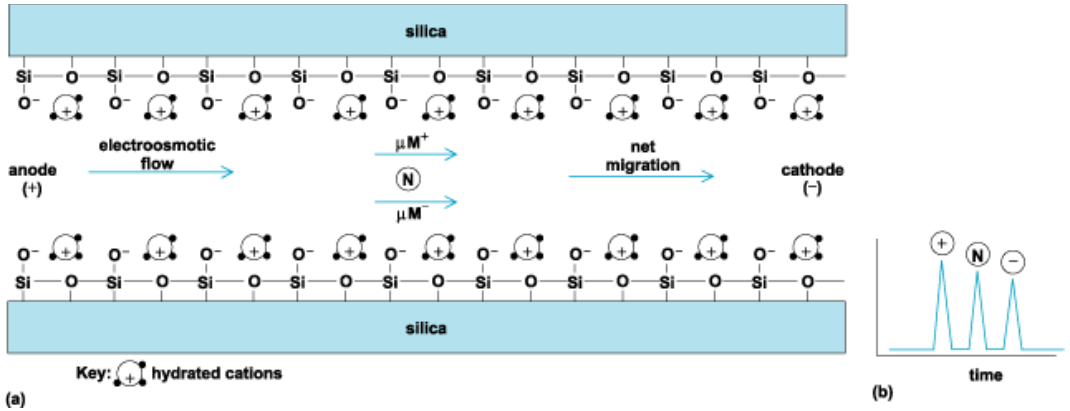
Figure 21 - Apparatus for paper electrophoresis.



Capillary zone electrophoresis set up.



SDS-PAGE electrophoresis set up.



Capillary zone electrophoresis. (a) Separation mechanism showing electrophoretic mobility of the positive ion (μM+) and negative ion (μM−); N is a neutral molecule. (b) Migration order of the ions.

Electropherogram of BSA

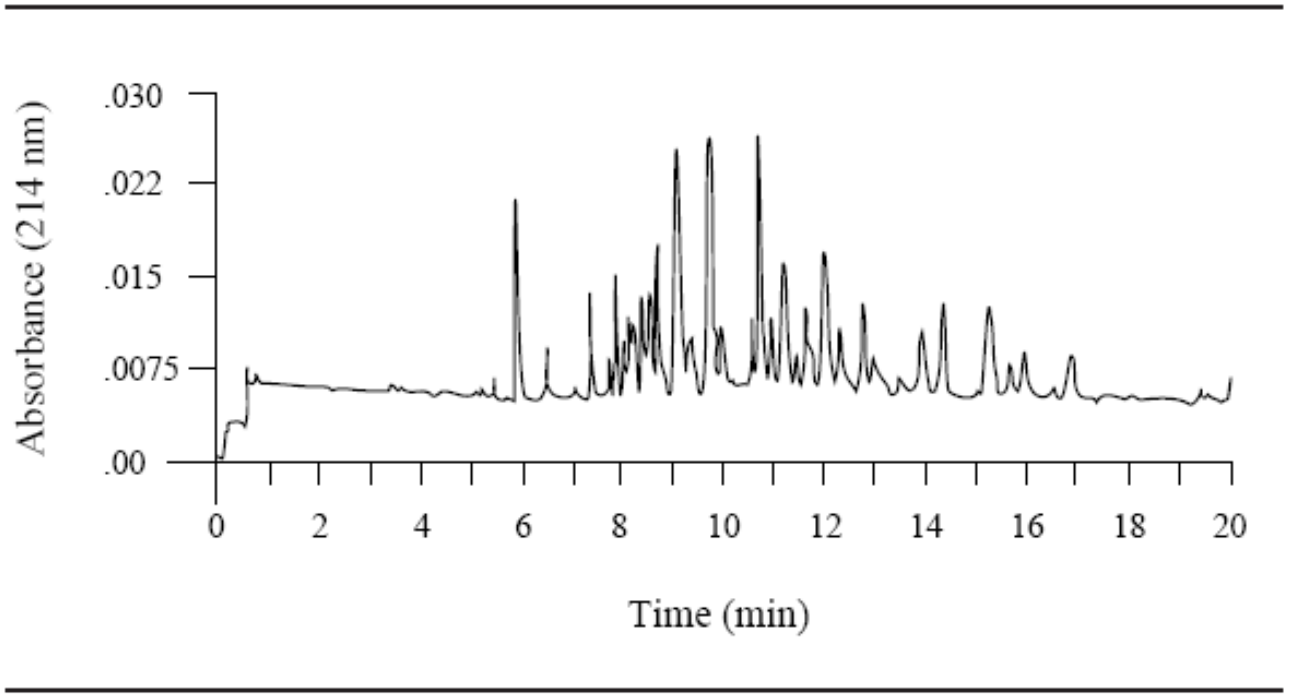


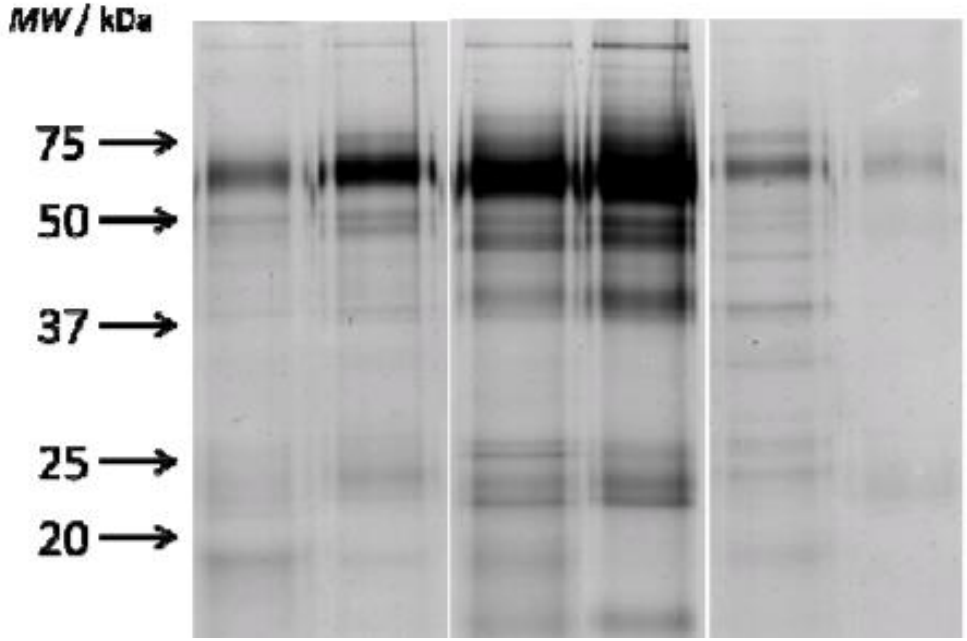
Figure 6. CZE Separation of a Tryptic Digest of Reduced, Denatured and Alkylated Bovine Serum Albumin. Buffer, 21 mM sodium phosphate (monobasic), 1.5 M urea, pH 2.5; capillary, 59 cm. Courtesy of R. Rush, A. Cohen, and B. Karger, Northeastern University.

The migration time (t_m) is the time it takes a solute to move (from the beginning of the capillary to the detector window in capillary zone electrophoresis (CZE) for instance).

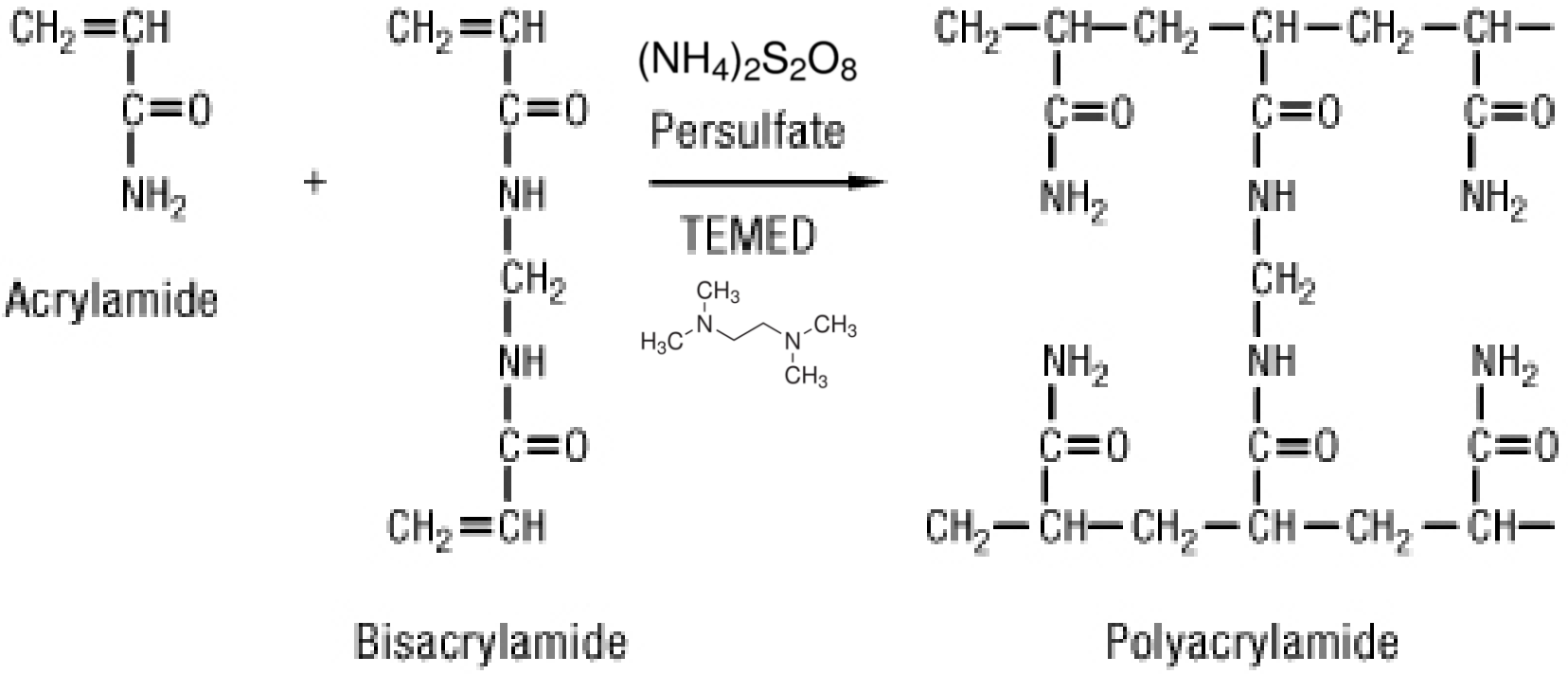
Reprinted from “Introduction to capillary electrophoresis”, Beckman Coulter

Gel electrophoresis

https://www.thermofisher.com



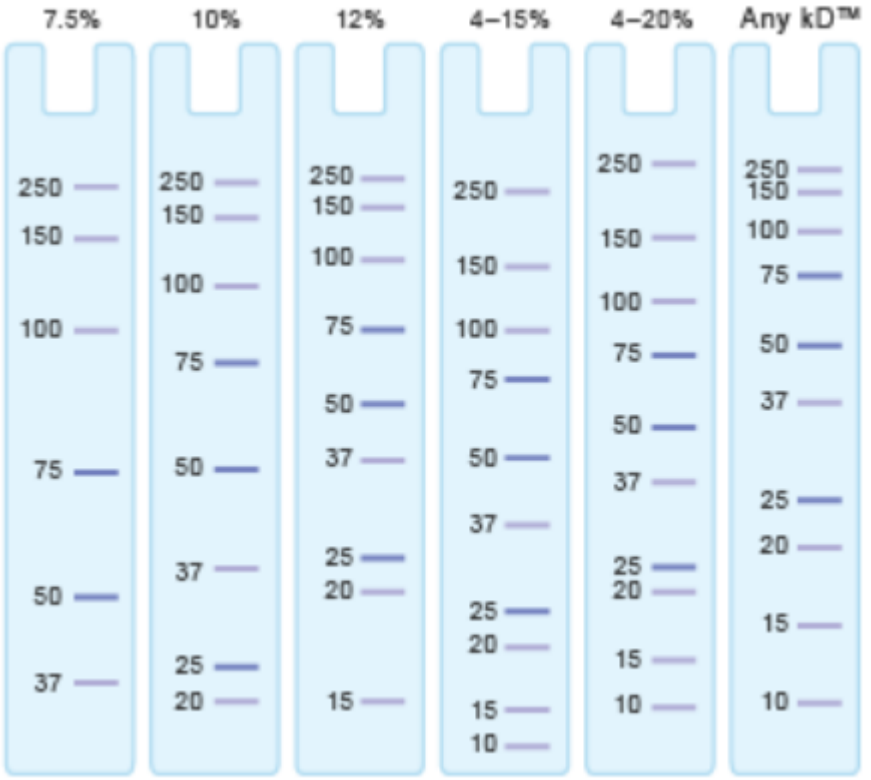
Polyacrylamide gel polymerization



https://www.thermofisher.com

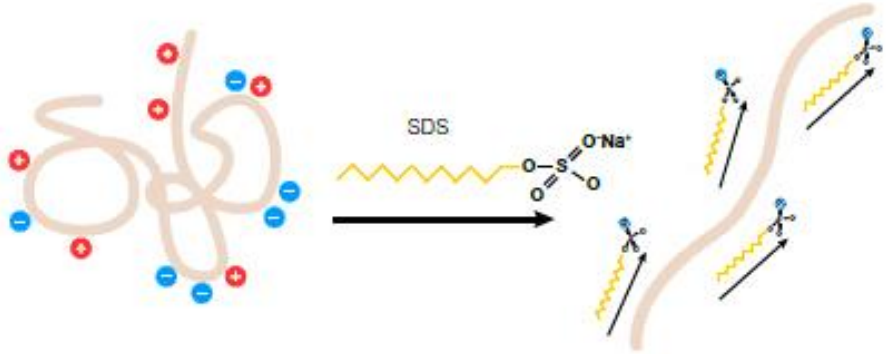
Polyacrylamide gel choice/options

Protein size	Gel acrylamide percentage
4–40 kDa	20%
12–45 kDa	15%
10–70 kDa	12.5%
15–100 kDa	10%
25–200 kDa	8%

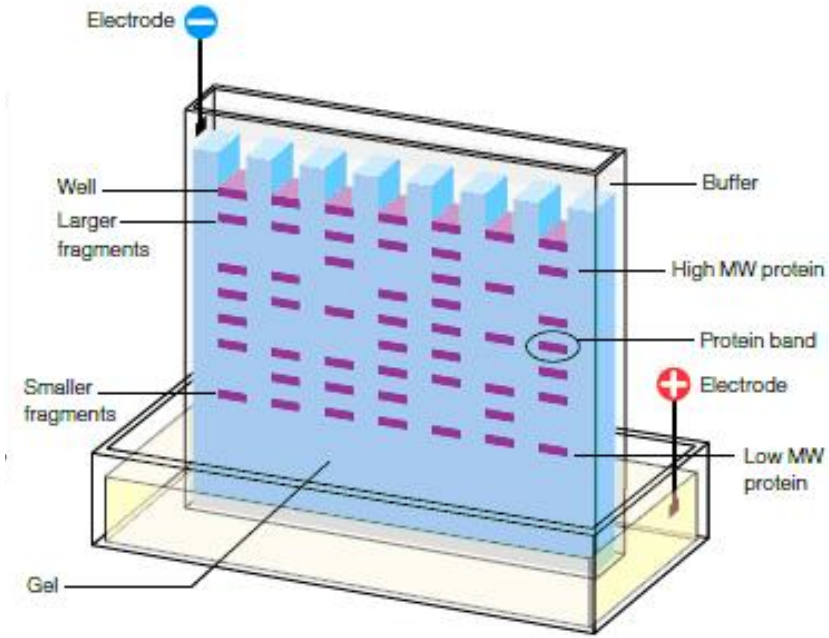


http://www.bio-rad.com

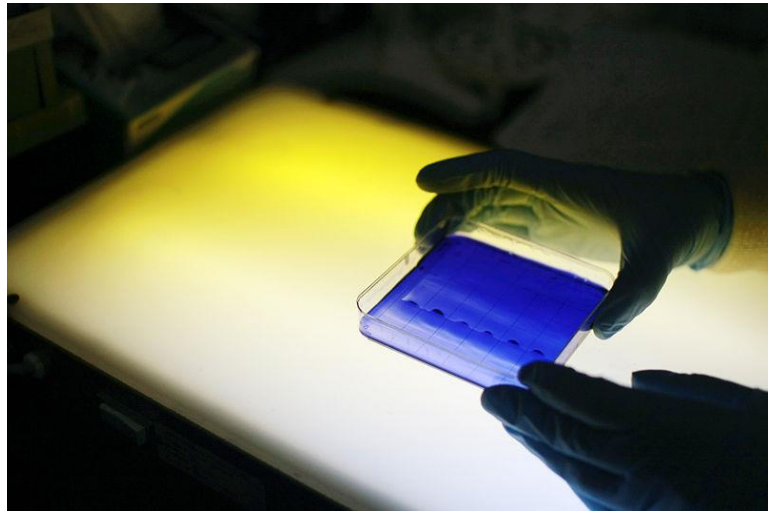
1D SDS PAGE



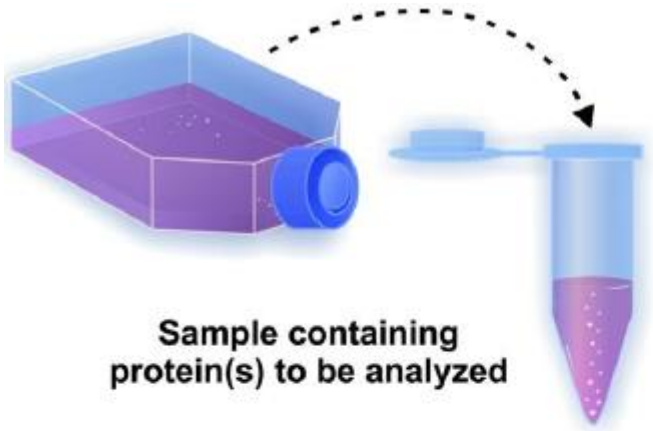
bio-rad.com



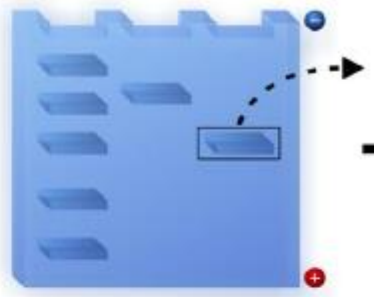
http://www.bio-rad.com



1D SDS PAGE with mass spectrometry



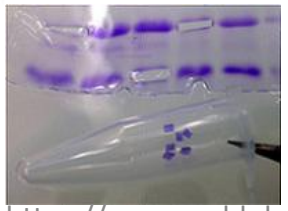
Sample containing protein(s) to be analyzed



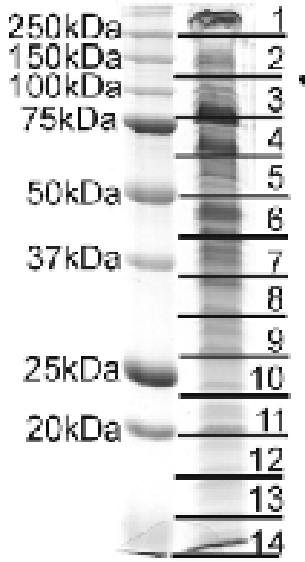
Separation by electrophoresis

1D gel separation
Staining

Band cut
De- staining



https://www.embl.de



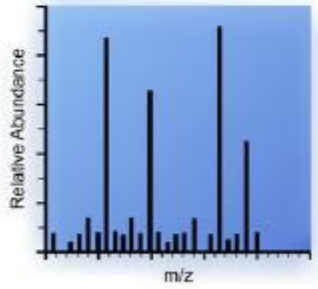
tryptic digest

RP-LC

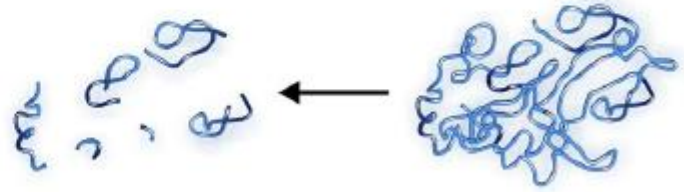
MS ↔ MS/MS

identification
(Mascot)

https://www.biochem.mpg.de



MS analysis
and data evaluation

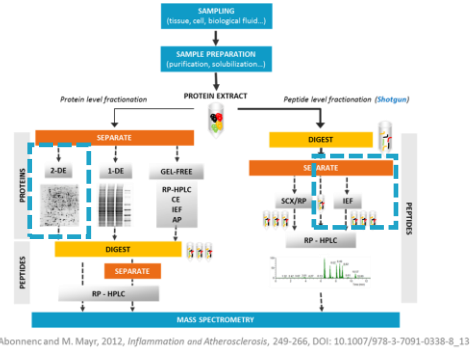


Proteolytic
fragmentation

(*i.e.*, in-gel digestion)

doi: 10.3390/biom3040923

3.2. Isoelectric focusing

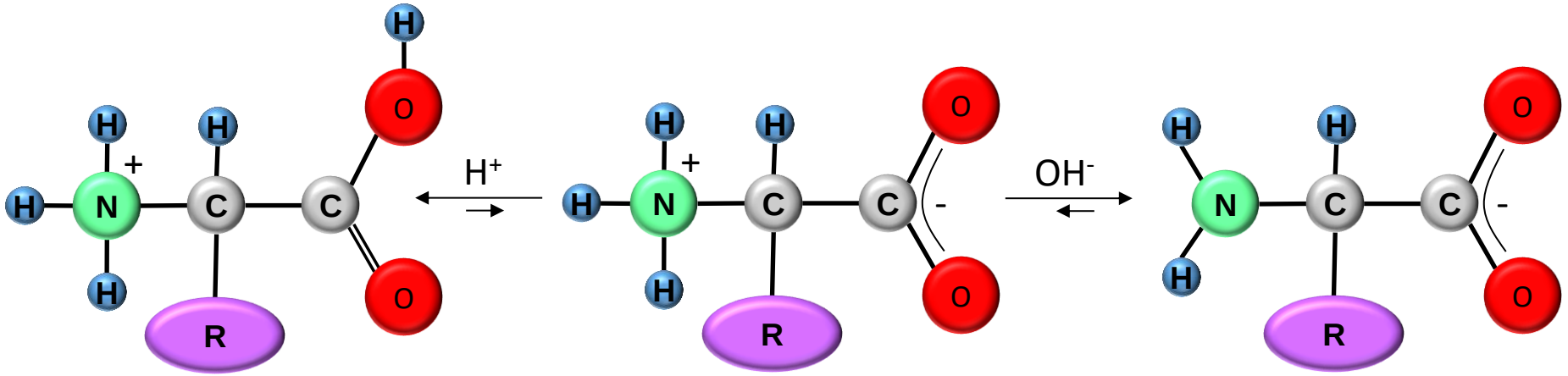


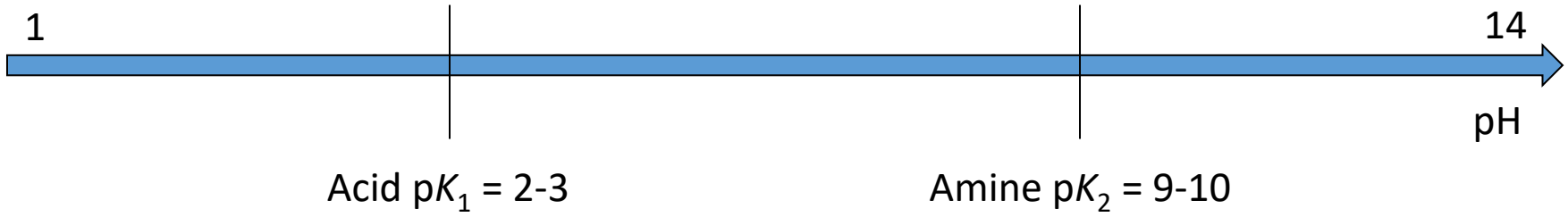
M. Abonnenc and M. Mayr, 2012, *Inflammation and Atherosclerosis*, 249-266, DOI: 10.1007/978-3-7091-0338-8_13

- **Theoretical aspects of isoelectric focusing (IEF)**
  - □ Amphoteric properties of amino acids, peptides, and proteins
  - □ Description of IEF
- **IEF protein/peptide separation for proteomics**
  - □ Capillary IEF (CIEF)
  - □ Immobilized-pH gradient (IPG) IEF
  - □ Electrophoretic prefractionation methods
- **Applications**
  - □ Peptide and protein identification
  - □ Post-translational modifications (PTMs)

Amino acids properties

In water, amino acids are amphoteric

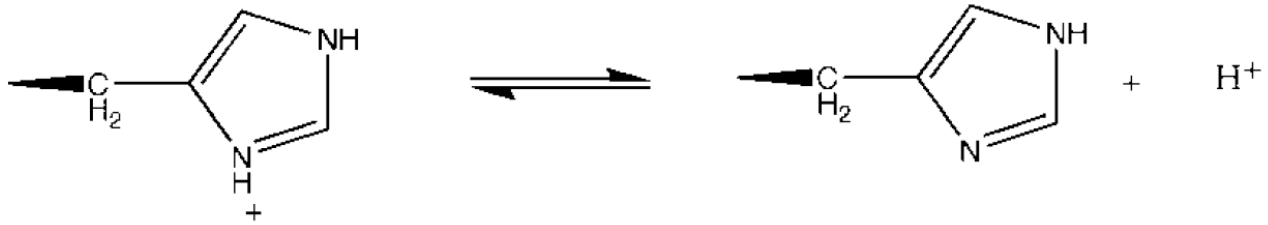




The isoelectric point (p*I*) is the pH where the molecule displays a zero net electric charge

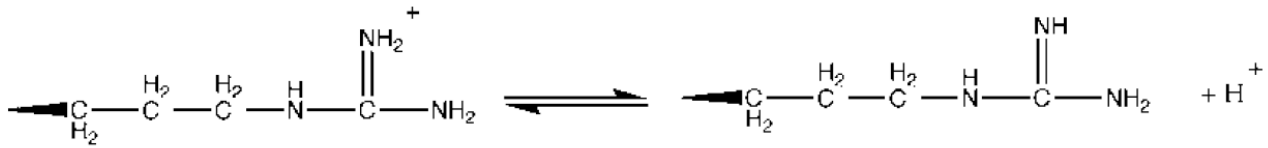
Positively charged lateral chains

Histidine



Lysine

Arginine

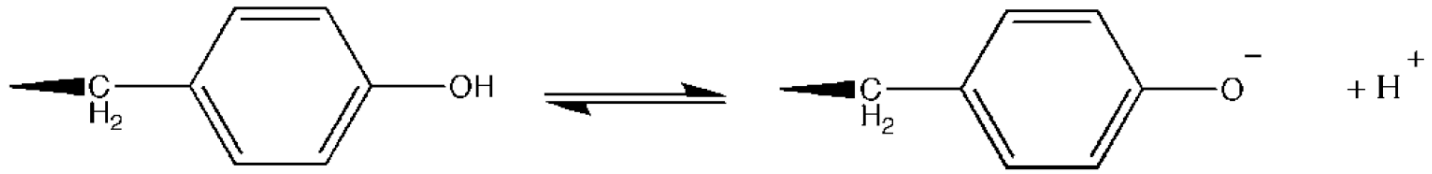


Reprinted from Niels Lion, Summer 2006, Bioanalytics and Analytical Sensors, EPFL.

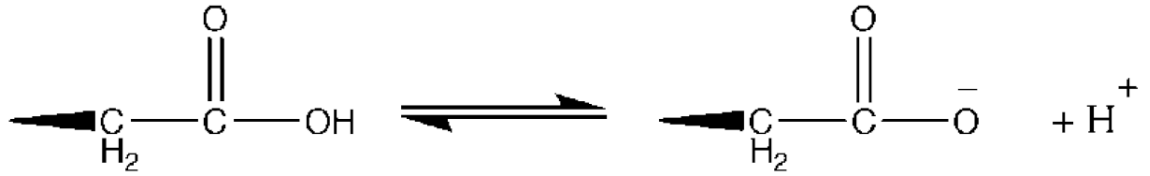
Negatively charged lateral chains

Cysteine

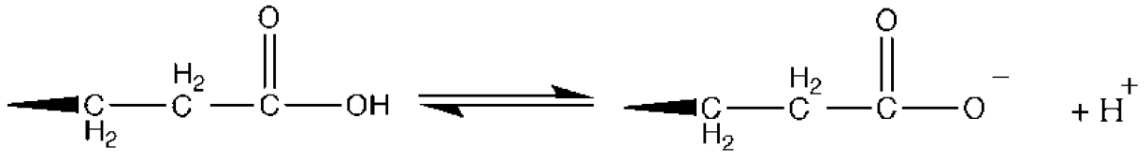
Tyrosine



Aspartic acid



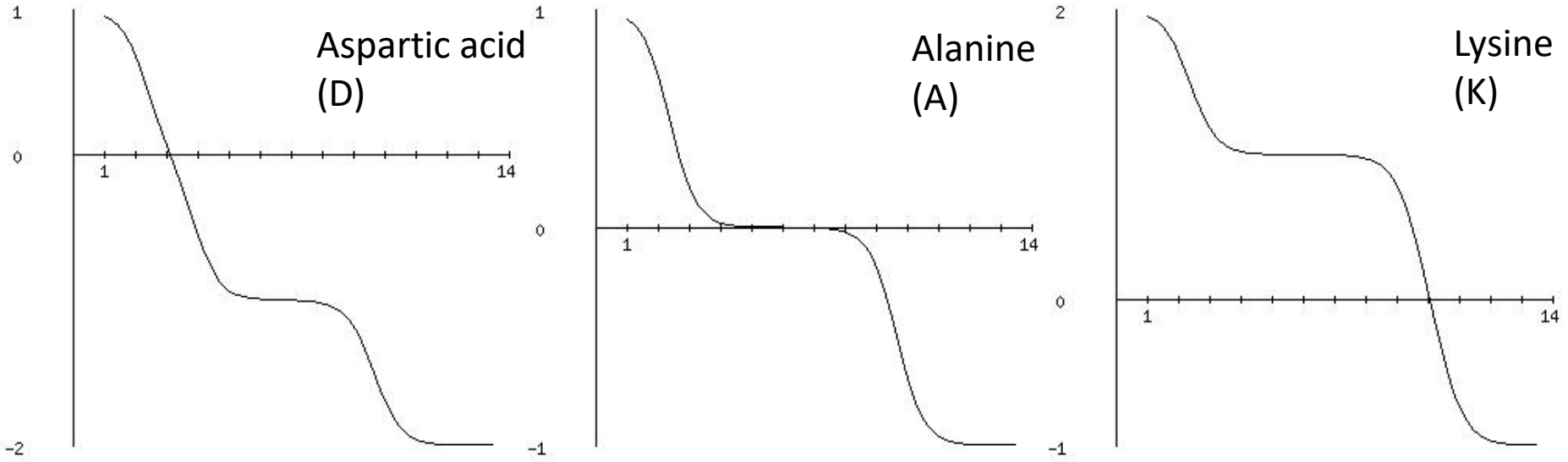
Glutamic acid



Reprinted from Niels Lion, Summer 2006, Bioanalytics and Analytical Sensors, EPFL.

Amino acids properties

Titration curve is the representation of the net electric charge as a function of the pH
Basic or acidic lateral chains influence the total charges, p*I*, and slope at p*I*



Negatively-charged lateral chains:
cysteine, tyrosine, aspartic acid, glutamic acid

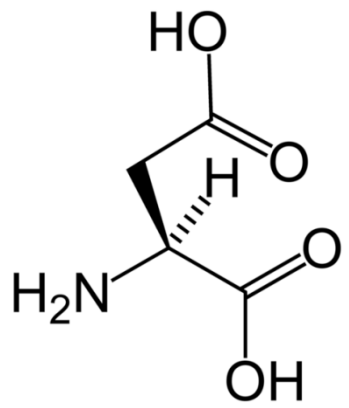
Positively-charged lateral chains:
histidine, lysine, and arginine

Computed from http://www.iut-arles.up.univ-mrs.fr/w3bb/d_abim/compo-p.html

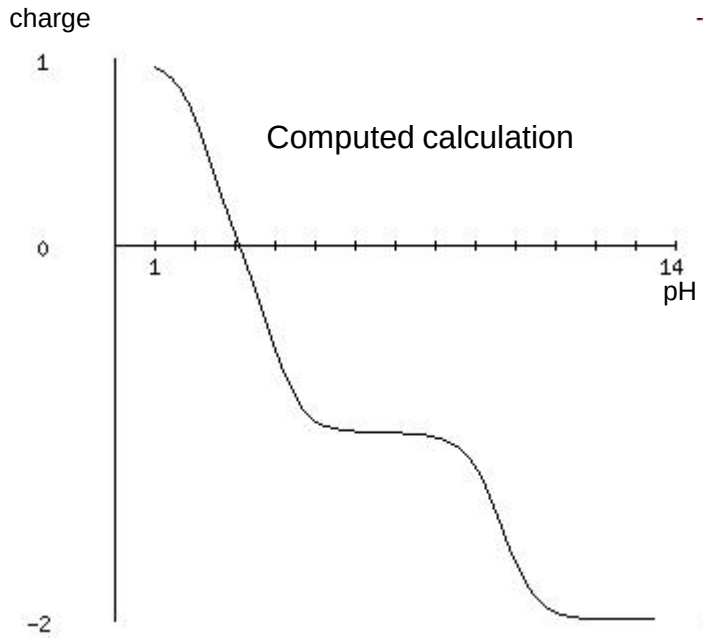
Amino acids, their p*I* and their p*K*s

Name	Code 1 letter	Code 3 letters	Molar mass (g.mol-1)	p*I*	pK_1 (-COOH)	pK_2 (-NH2)	pK_R (-R)
Alanine	A	Ala	89.09	6.11	2.35	9.87	
Arginine	R	Arg	174.2	10.76	1.82	8.99	12.48
Asparagine	N	Asn	132.12	5.41	2.14	8.72	
Aspartic acid	D	Asp	133.1	2.85	1.99	9.9	3.9
Cysteine	C	Cys	121.16	5.05	1.92	10.7	8.18
Glutamic acid	E	Glu	147.13	3.15	2.1	9.47	4.07
Glutamine	Q	Gln	146.15	5.65	2.17	9.13	
Glycine	G	Gly	75.07	6.06	2.35	9.78	
Histidine	H	His	155.16	7.6	1.8	9.33	6.04
Isoleucine	I	Ile	131.17	6.05	2.32	9.76	
Leucine	L	Leu	131.17	6.01	2.33	9.74	
Lysine	K	Lys	146.19	9.6	2.16	9.06	10.54
Methionine	M	Met	149.21	5.74	2.13	9.28	
Phenylalanine	F	Phe	165.19	5.49	2.2	9.31	
Proline	P	Pro	115.13	6.3	1.95	10.64	
Serine	S	Ser	105.09	5.68	2.19	9.21	
Threonine	T	Thr	119.12	5.6	2.09	9.1	
Tryptophane	W	Trp	204.23	5.89	2.46	9.41	
Tyrosine	Y	Tyr	181.19	5.64	2.2	9.21	10.46
Valine	V	Val	117.15	6	2.39	9.74	

Q1: What is the charge depending on the pH?



Aspartic Acid
pK_1 = 1.99
pK_2 = 9.9
pK_R = 3.9



Rapid evaluation

pH	COOH (1)	NH2 (2)	COOH (R)	Total charge
1	0	+	0	+
3	-	+	0	0
5	-	+	-	-
11	-	0	-	-

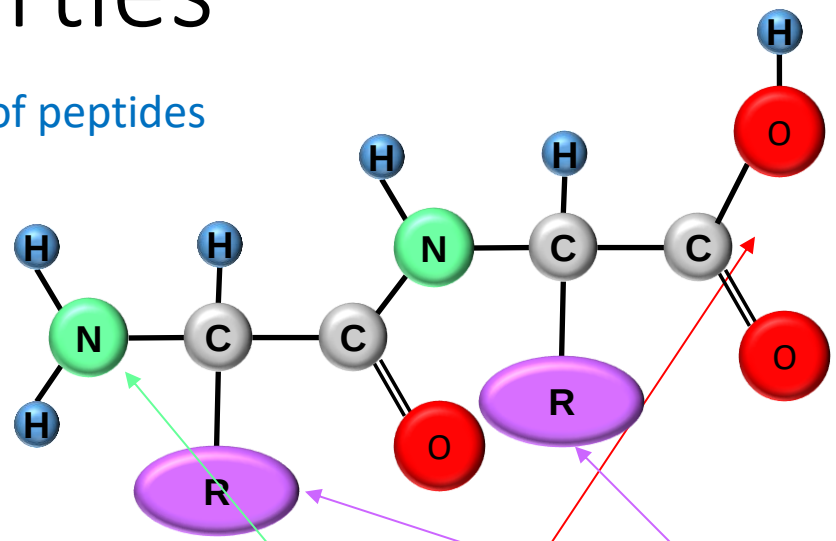
$pI \approx (pK_1 + pK_R) / 2$

http://www.iut-arles.up.univ-mrs.fr/w3bb/d_abim/compo-p.html

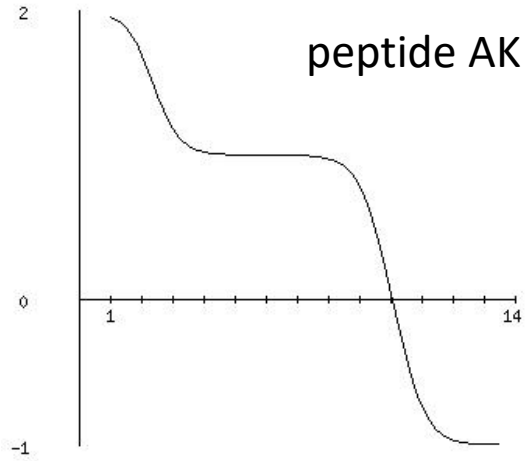
You can try with other amino acids…

Peptide properties

Amphoteric properties of peptides



Potential charges on N-ter, C-ter, and lateral chains (R)



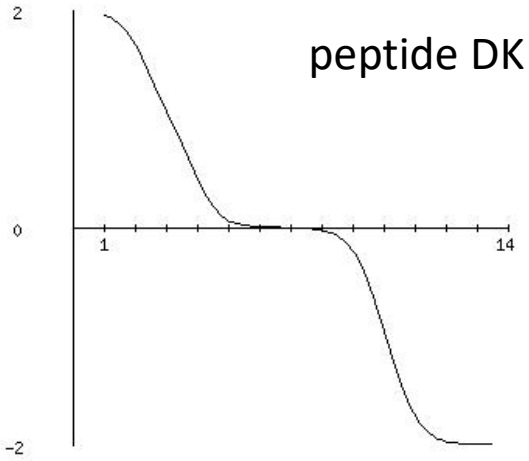
peptide AK

N-ter and basic lateral chain

C-ter

N-ter and basic lateral chain

C-ter and acidic lateral chain



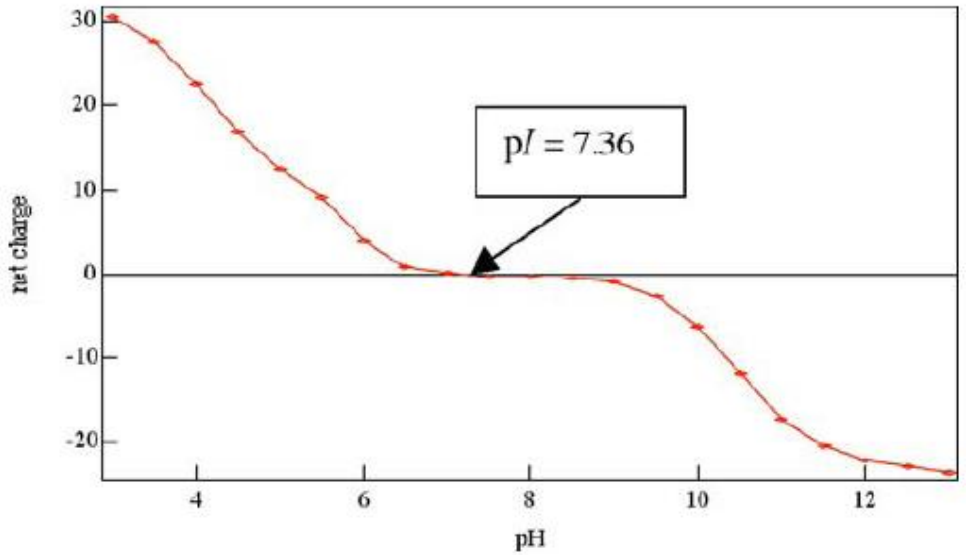
peptide DK

Computed from http://www.iut-arles.up.univ-mrs.fr/w3bb/d_abim/compo-p.html

Charge of polypeptides

The net charge of a protein containing *i* positively and *j* negatively charged groups is

$$\text{net charge} = \sum_{\substack{\text{positively} \\ \text{charged}}} \frac{n_i}{\frac{K_i}{10^{-pH}} + 1} - \sum_{\substack{\text{negatively} \\ \text{charged}}} \frac{n_j}{\frac{10^{-pH}}{K_j} + 1}$$



Net charge versus pH for horse heart myoglobin

Isabelle L. Di Maïo, Thesis EPFL, no 3064 (2004)

Effect of charge and pH

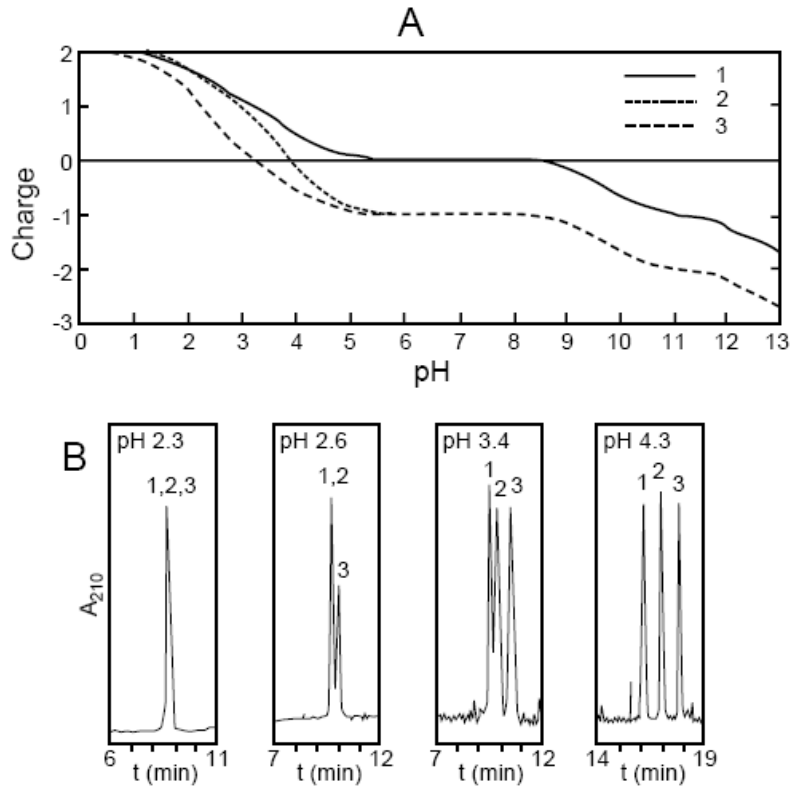
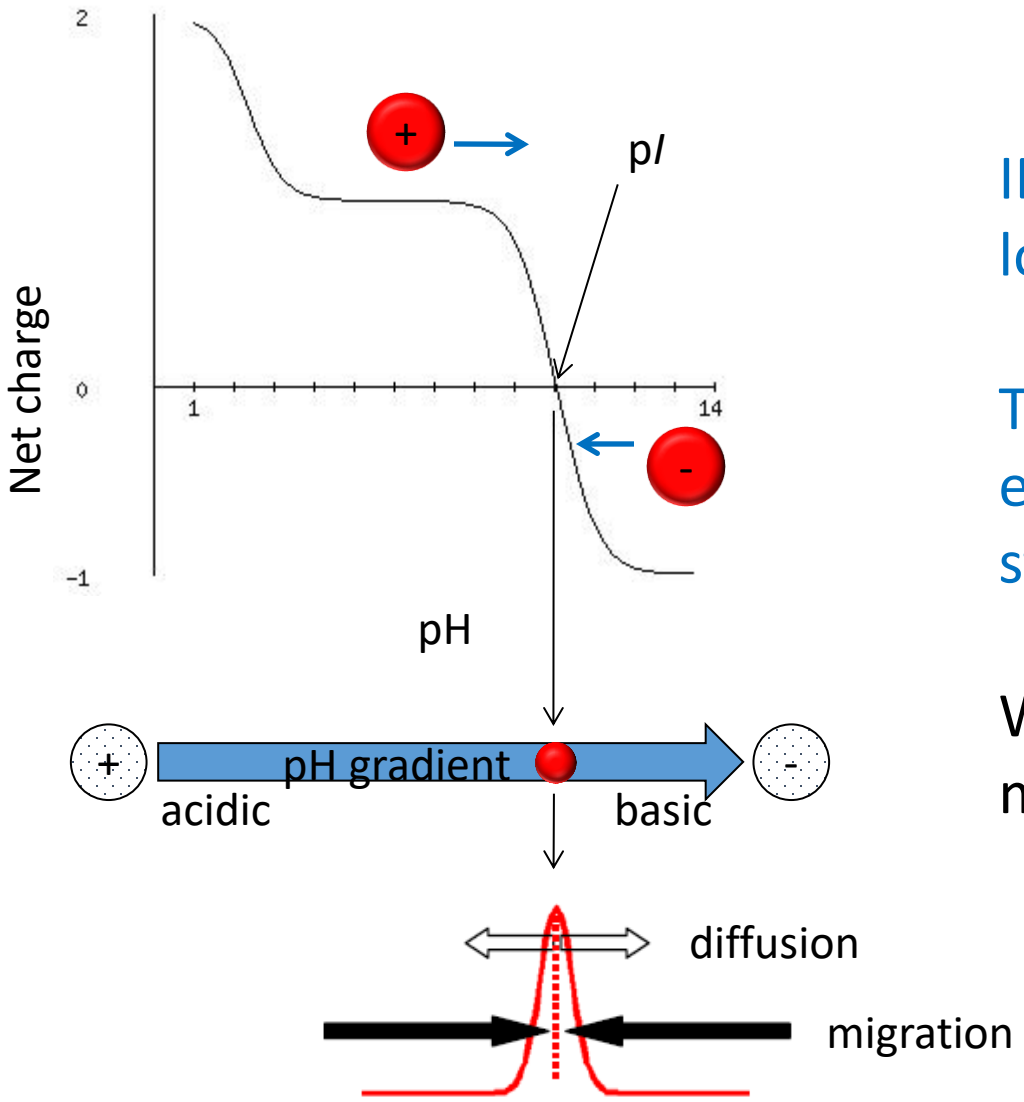


***Figure 1-3**. (A) Calculated charge versus pH profiles and (B) electropherograms at varying pH for the synthetic model peptides (Leu^{27}, Asn^{28}) GRF(22-32)-OH, 2, and (Leu^{27}, β–Asp^{28})-GRF(22-32)-OH, 3. The pH of the 25-mM Na_2HPO_4/H_3PO_4 running buffer is shown in the upper left corner of each panel. Reprinted with permission from Bongers* et al., J. Liq. Chromatogr. 15, *1115 (1992).*

Reprinted from “Introduction to capillary electrophoresis”, Beckman Coulter

Isoelectric focusing (IEF)



IEF is run in a pH gradient where the pH is low at the anode and high at the cathode

The analyte will migrate so long as its net electric charge is not zero to reach a steady-state position

When the analyte is at p*I*, it will stop migrating in the electric field

Generation of pH gradient

To separate biomolecules in a highly resolved and reproducible way, the pH gradient generated in IEF should be time stable with a constant conductivity and buffer capacity

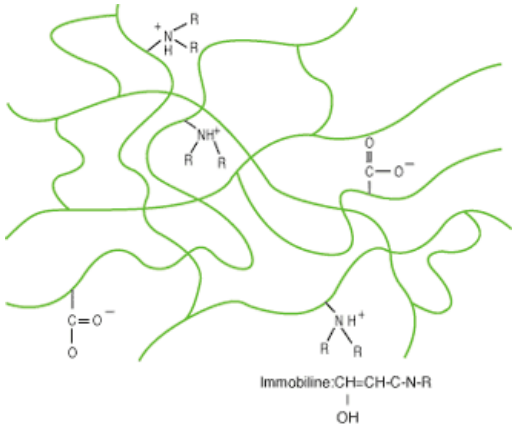
Two ways:

- pH gradients formed in the electric field by amphoteric buffers, the carrier ampholytes (CA)

With 2 fundamental properties of CA:

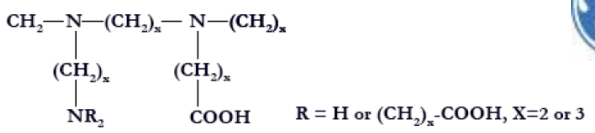
- to be amphoteric so that they could also reach a steady state position during the separation
- to be “carrier” of the current (a good conducting species) and of the pH (a good buffering species)

- immobilized pH gradients, in which the buffering groups are part of the gel



Carrier ampholytes (CA)

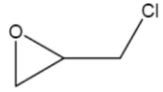
CA: Ampholines™



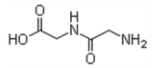
GE Healthcare

CA: Pharmalyte™

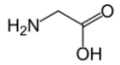
Pharmalyte™ carrier ampholytes, prepared by the co-polymerization of glycine, glycylglycine, amines, and epichlorohydrin, are available in five broad-range and four narrow-range pH intervals. Each interval contains numerous ampholytes with a high buffering capacity per pH unit. Pharmalyte™ carrier ampholytes form an extremely stable linear pH gradient exhibiting even conductivity across the gel.



Epichlorohydrin

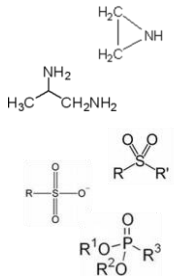


glycylglycine



glycine

CA: Servalyt™



SERVALYT™ ampholytes are a mixture of synthetically derived species of average molecular weight distribution from 400 to 1000 dalton

SERVA
serving scientists

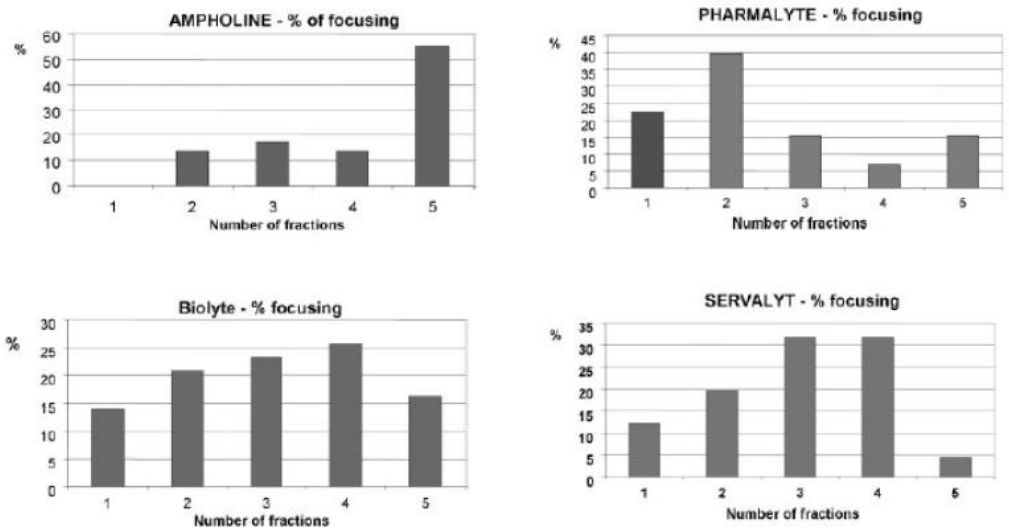
CA: Bio-Lyte®

Not much information available…

BIO-RAD

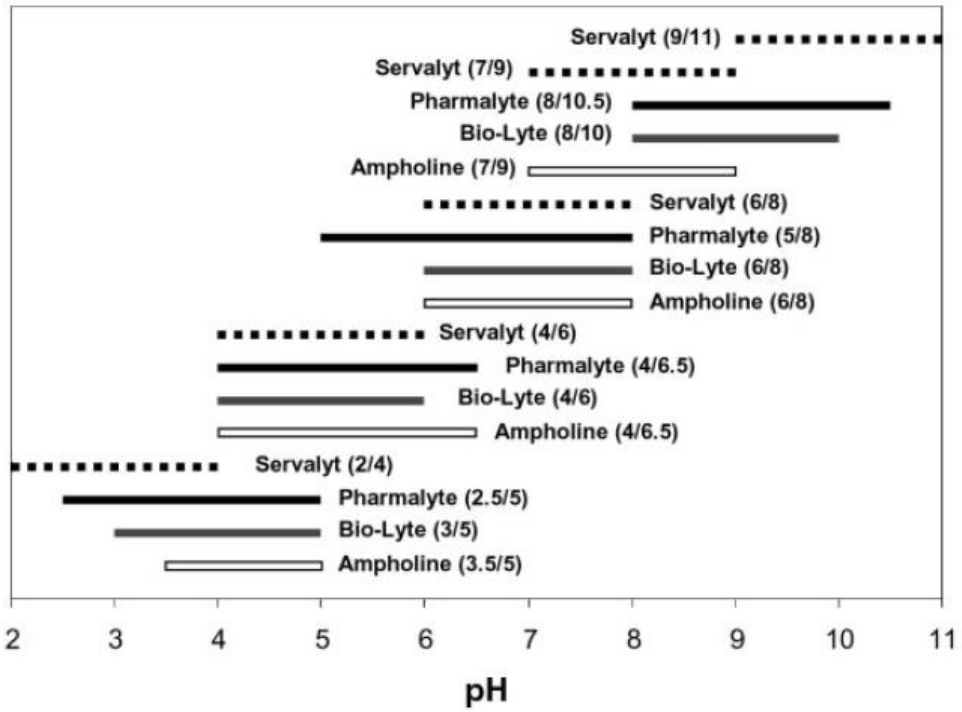
Bio-Lyte 3/10 Ampholyte
Bio-Lyte 4/6 Ampholyte
Bio-Lyte 5/7 Ampholyte
Bio-Lyte 6/8 Ampholyte
Bio-Lyte 5/8 Ampholyte
Bio-Lyte 3/5 Ampholyte
Bio-Lyte 7/9 Ampholyte
Bio-Lyte 8/10 Ampholyte

CA properties



Electrophoresis 2006, *27*, 3919–3934

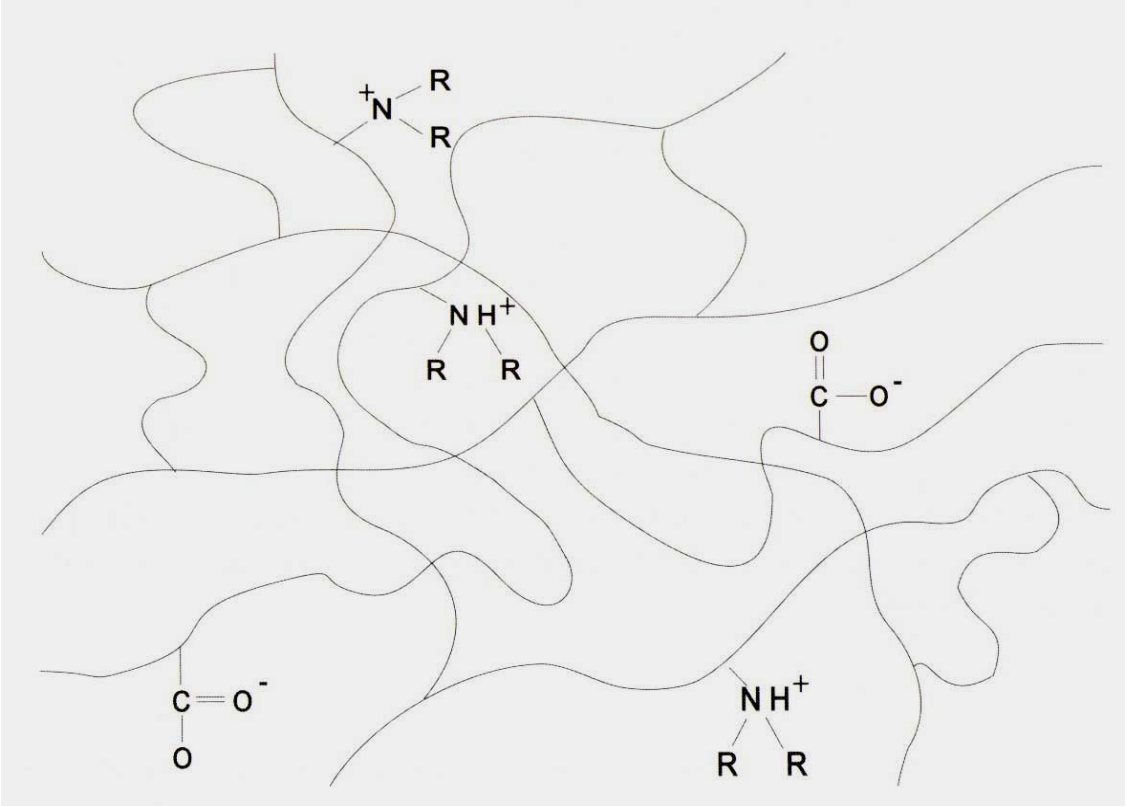
Percentage of species focusing in either a single fraction (1) or over the entire pH gradient (5)



Electrophoresis 2007, *28,* 3799–3810

Electrophoresis 2006, *27,* 4849–4858, *Electrophoresis* 2007, *28,* 715–723, *Electrophoresis* 2007, *28,* 1488–1494, *Electrophoresis* 2007, *28,* 3156–3162

Immobilized pH gradient (IPG) gels



Electrophoresis in Practice, Reiner Westermeir, 1997, Second Edition, VCH, Weinheim

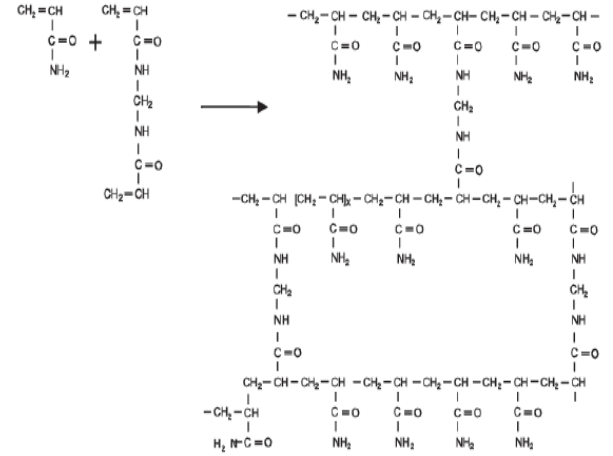
The Immobilines™ are acrylamide derivatives with the general chemical formula:

$$CH2=CH–CO–NH–R$$

where R contains either a carboxylic acid (p*K* 3.6, 4.4 and 4.6) or an amino group (p*K* 6.2, 7.0, 8.5 and 9.3) incorporated in the polyacrylamide gel during polymerization

their concentrations and dissociation constants defined the pH

their incorporation amount and their buffer capacity act on the conductivity of the gel



Polymerization reaction of acrylamide and methylenebisacrylamide

GE Healthcare

Immobilized pH gradient (IPG) gels

To be able to buffer at a precise pH value, at least two different Immobilines are necessary, an acid and a base

A pH gradient is obtained by continuous change in the ratio of Immobilines. The principle is that of an acid base titration and the pH value at each stage is defined by the Henderson-Hasselbalch equation:

$$\mathrm{pH} = \mathrm{p}K_{\mathrm{B}} + \log \frac{C_{\mathrm{B}} - C_{\mathrm{A}}}{C_{\mathrm{A}}}$$

when the buffering Immobiline is a base.

$$\mathrm{pH} = \mathrm{p}K_{\mathrm{A}} + \log \frac{C_{\mathrm{B}}}{C_{\mathrm{A}} - C_{\mathrm{B}}}$$

when the buffering Immobiline is an acid.

C_A and C_B are the molar concentration of the acid, and basic Immobiline

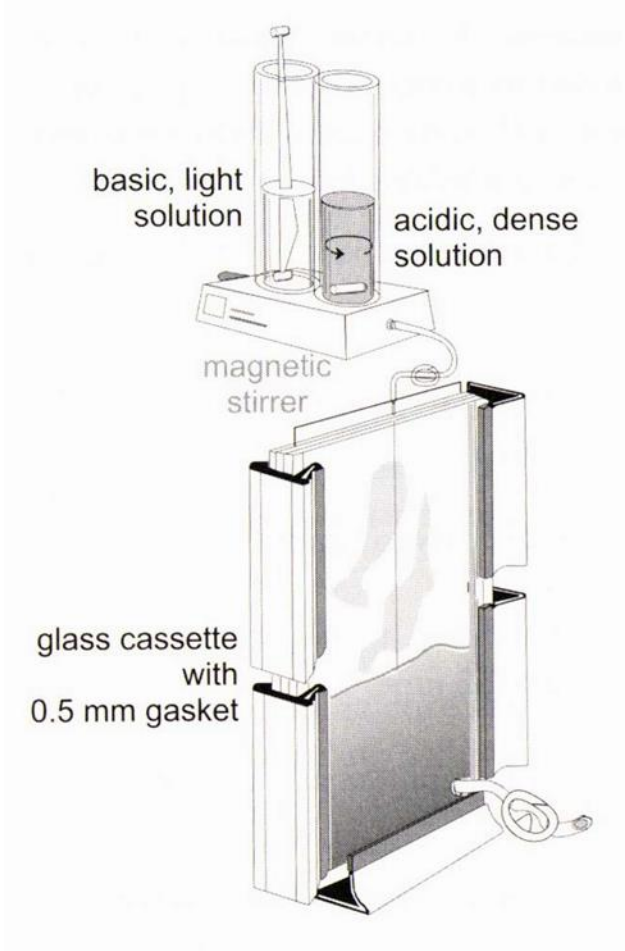
Electrophoresis in Practice, Reiner Westermeir, 1997, Second Edition, VCH, Weinheim

Immobilines™ and IPG gel preparation

Available Immobilines™ allow the generation of any narrow pH gradient between pH 3 and 10

	H_2O		Polyacrylamide gel T% = 5%, C% = 3%		Polyacrylamide gel T% = 5%, C% = 3% glycerol 25% (w/v)	
	10°C	25°C	10°C	25°C	10°C	25°C
Acid						
Immobiline p*K* 3.6	3.57	3.58	-	-	3.68 ± 0.02	3.75 ± 0.02
Immobiline p*K* 4.4	4.39	4.39	4.30 ± 0.02	4.36 ± 0.02	4.40 ± 0.03	4.47 ± 0.03
Immobiline p*K* 4.6	4.60	4.61	4.51 ± 0.02	4.61 ± 0.02	4.61 ± 0.02	4.71 ± 0.03
Base						
Immobiline p*K* 6.2	6.41	6.23	6.21 ± 0.05	6.15 ± 0.03	6.32 ± 0.08	6.24 ± 0.07
Immobiline p*K* 7.0	7.12	6.97	7.06 ± 0.07	6.96 ± 0.05	7.08 ± 0.07	6.95 ± 0.06
Immobiline p*K* 8.5	8.96	8.53	8.50 ± 0.06	8.38 ± 0.06	8.66 ± 0.09	8.45 ± 0.07
Immobiline p*K* 9.3	9.64	9.28	9.59 ± 0.08	9.31 ± 0.07	9.57 ± 0.06	9.30 ± 0.05

T is the total acrylamide concentration and C is the degree of cross-linking



Immobiline = 0.2 M

Acidic solution is usually made denser by adding glycerol so that the layers in the molds do not mix.
The mixing of the less dense dilute solution with the viscous solution takes place in the mixing chamber using a magnetic stirrer bar.

The gel is usually 0.5 mm thick

But we buy them commercial…

IEF peptide separation for proteomics: Capillary IEF (CIEF)

Separation carried out in capillaries:

- Limited convection because small cross-section, electric power and heating

- Resolution to 0.01 p*I* units

- Concentration of analytes by a factor of 2 or 3 orders of magnitude

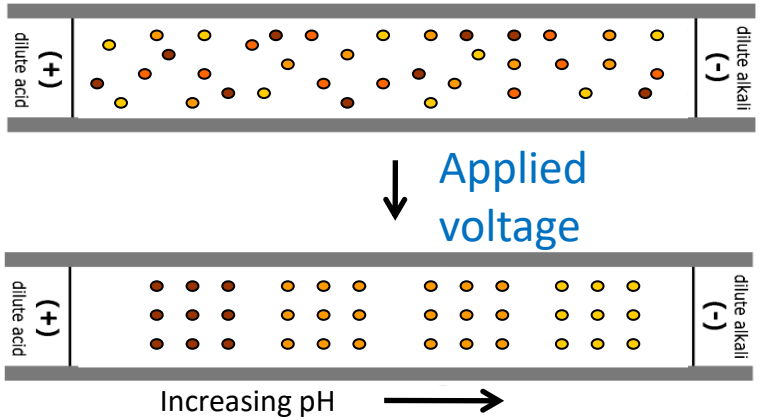
- Rapid focusing (min range)

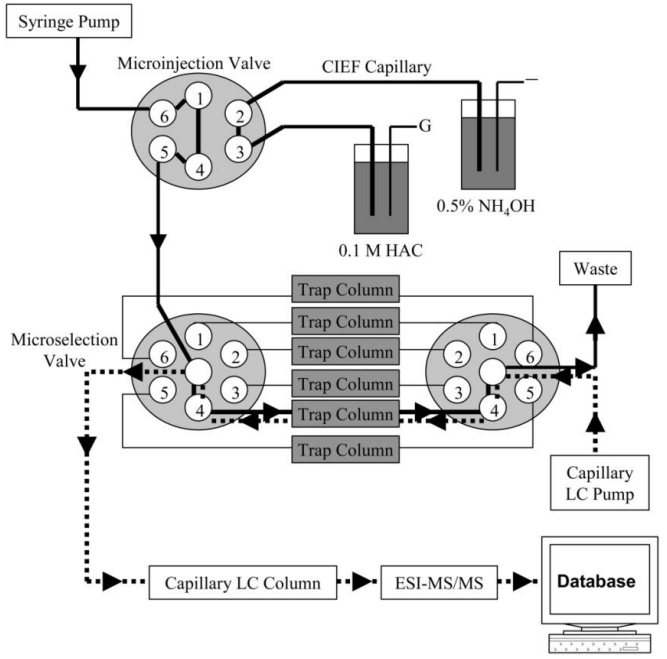
No widespread use in proteomics because:

- Presence of carrier ampholytes (UV and MS)

- Need for p*I* markers to estimate the p*I*

- Low loading achievable in capillary electrophoresis and precipitation





Kašička, Electrophoresis. 2008, 29, 179-206
Simpson & Smith, Electrophoresis. 2005, 29, 1291-1305
Shimura et al., Anal.Chem. 2000, 4747-4757
Shen et al., Anal. Chem. 2000, 72, 2154-2159

Chen et al., Anal. Chem. 2003, 75, 3145-3152

IEF peptide separation for proteomics: Capillary IEF (CIEF)

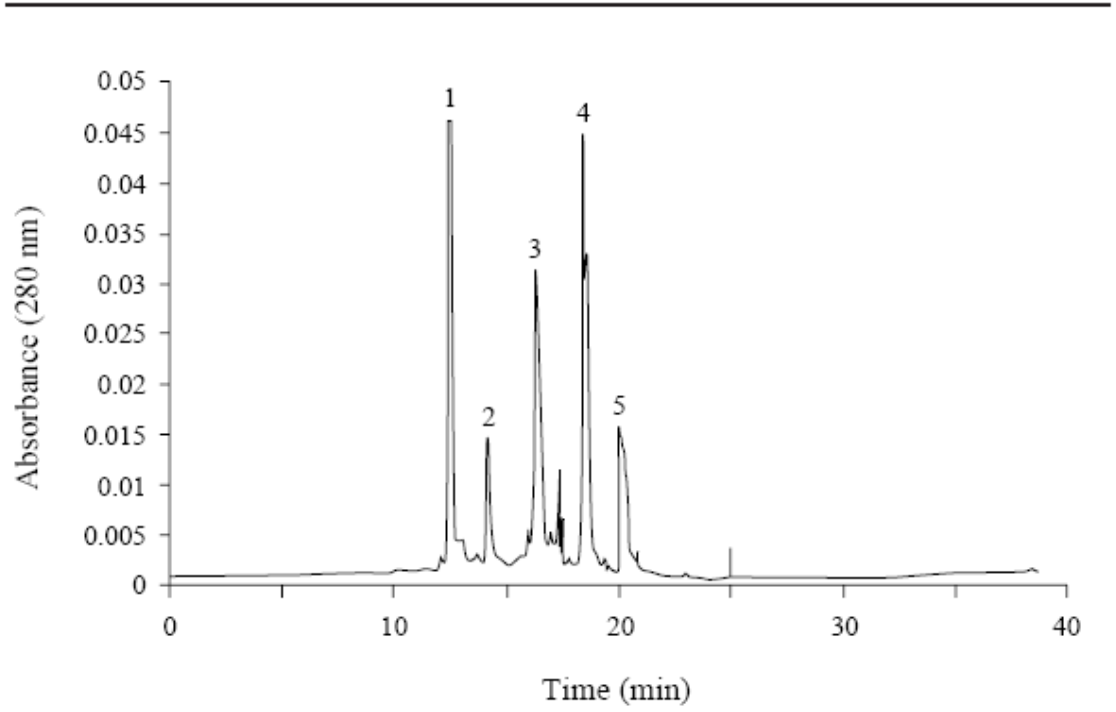
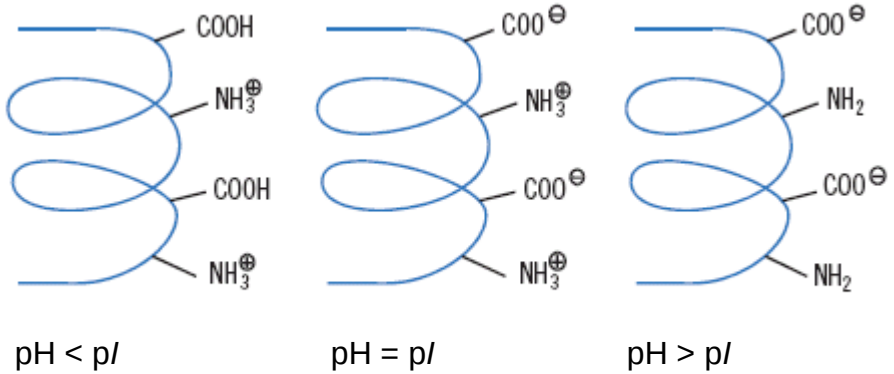


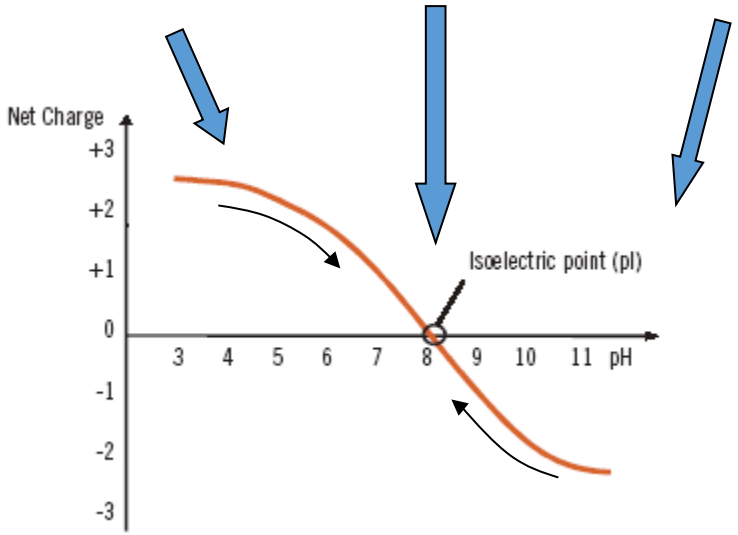
Figure 8. Separation of a Protein Mixture by IEF. Ampholyte pH range, 3.5-10; catholyte, 50 mM sodium hydroxide; analyte, 150 mM phosphoric acid; voltage, 25 kV; mobilization (catholyte), 50 mM sodium chloride, 50 mM sodium hydroxide. Courtesy of R. Nelson and B. Karger, Northeastern University.

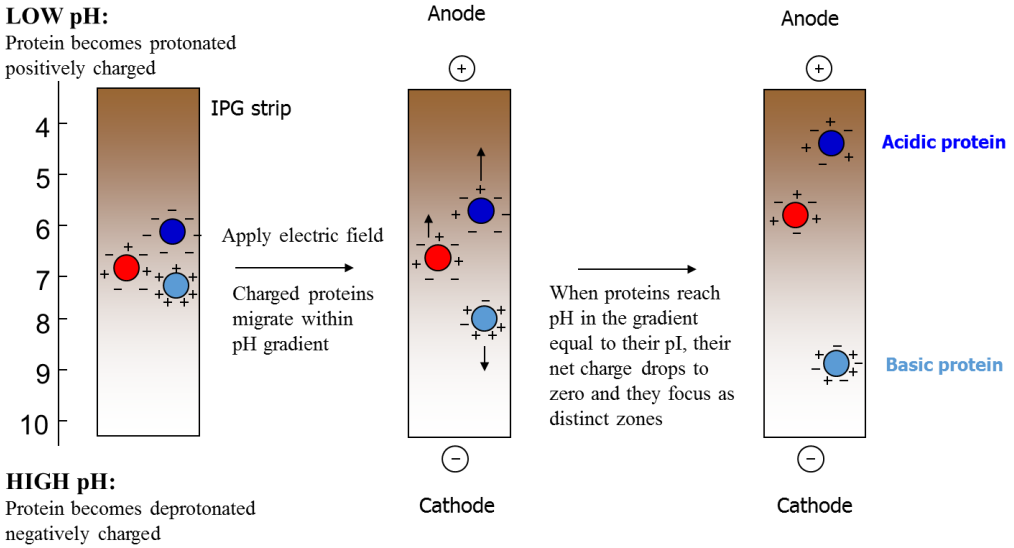
Reprinted from "Introduction to capillary electrophoresis", Beckman Coulter

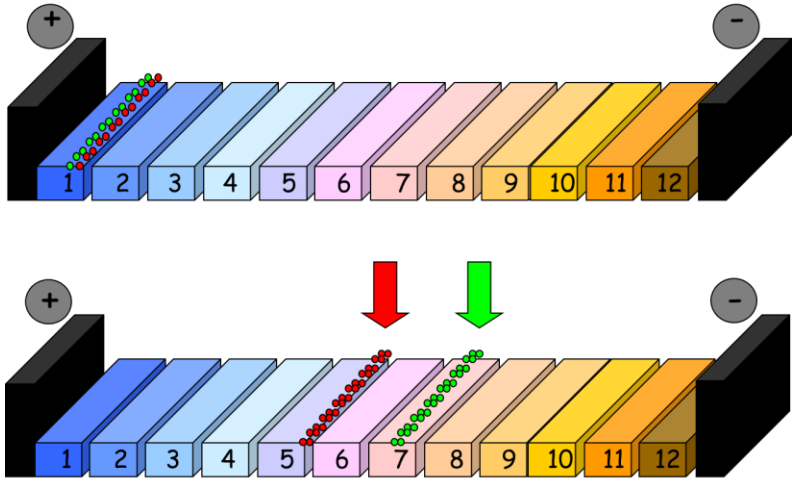
IEF peptide separation for proteomics: IPG IEF, first dimension of protein 2D-PAGE



pH < p*I* pH = p*I* pH > p*I*





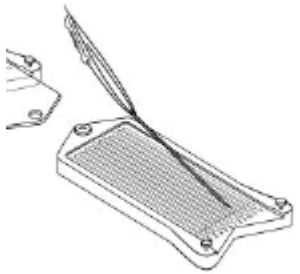


IPG IEF in the lab (1)

IEF performed with IPG strips

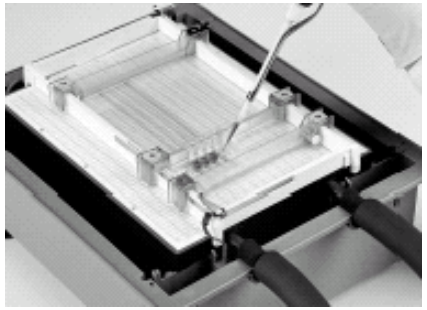
Dry IPG strip

pH gradient immobilized in polyacrylamide gel 5 %T, plastic backing
IPG strips home-made or commercial
Different strip lenth and pH ranges available



Strip rehydration

e.g. Strip length. 18 cm, 450 ul of rehydration solution

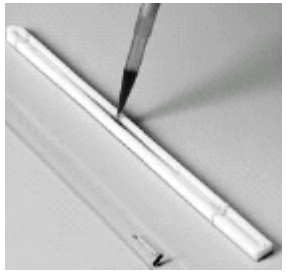


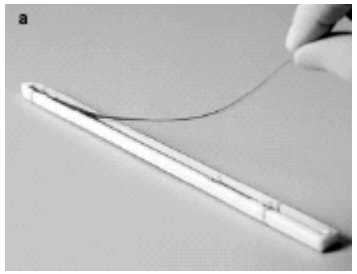
Sample application via sample cups

e.g. Strip length. 18 cm, load 60ug of protein, maximal volume 100 ul (ideal for concentrated sample and plasma)- Cathode loading

Strip rehydration and sample application

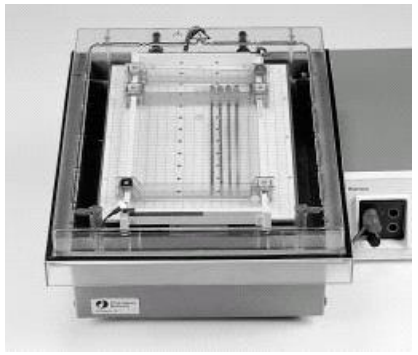
e.g. Strip length. 18 cm, load 60ug of protein, maximal volume 450 ul (ideal for diluted samples)





From handbook 2-D Electrophoresis, Principles and Methods, Amersham Biosciences

IPG IEF in the lab (2)



Multiphor II

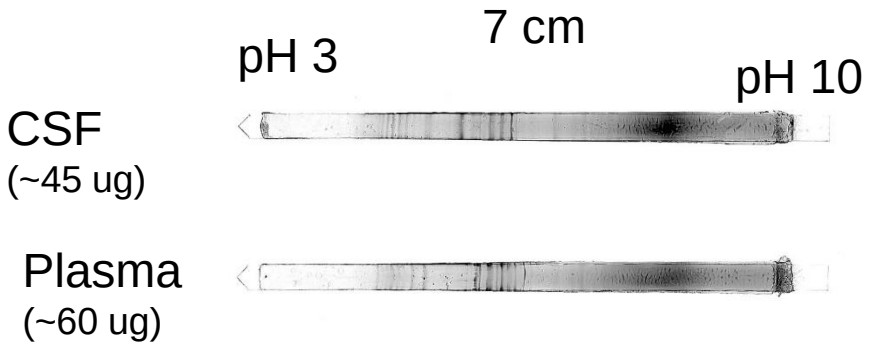
Migration in electric field

e.g. Entry phase 300 V,
stepwise increase up to 5000 V
In total ~100 kVh
duration ~ 8 h
constant temperature 15 °C

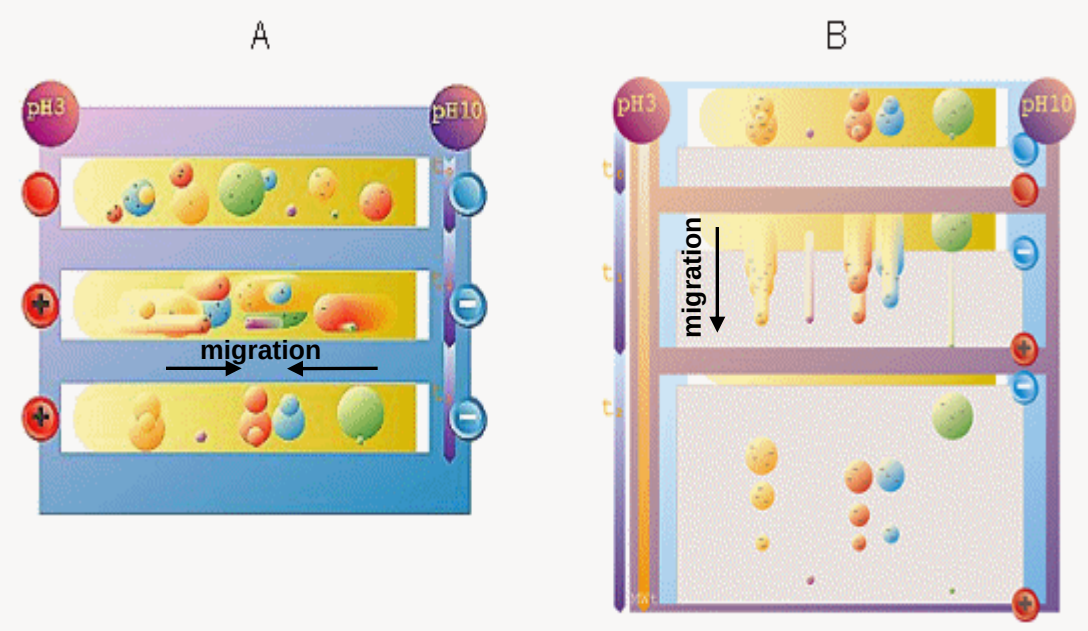
IPGphor

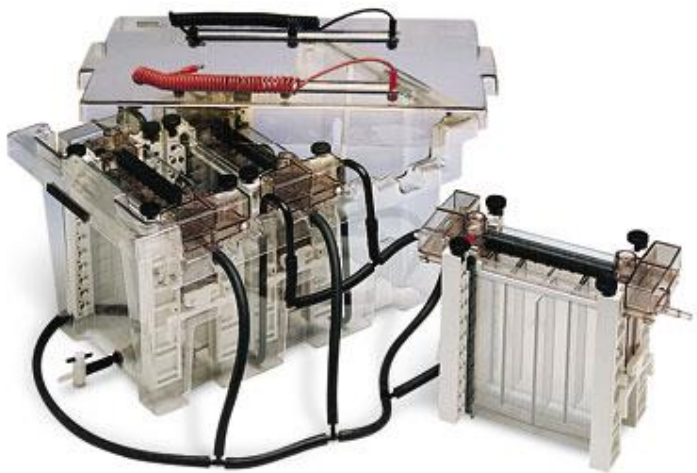
From handbook 2-D Electrophoresis,
Principles and Methods, Amersham Biosciences

IPG strips, silver stained

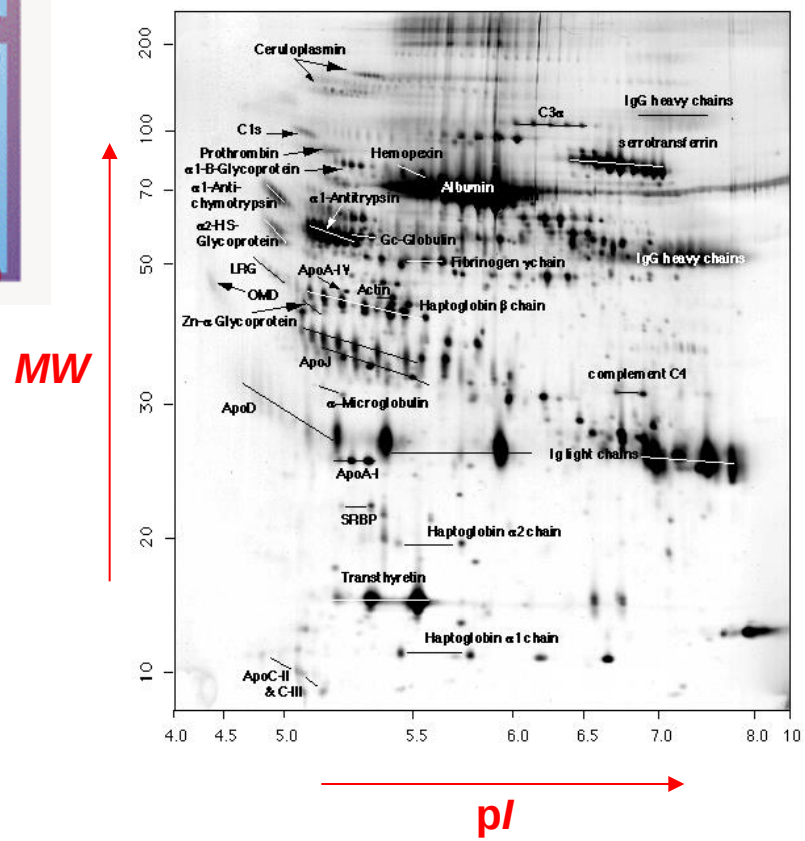


2D electrophoresis

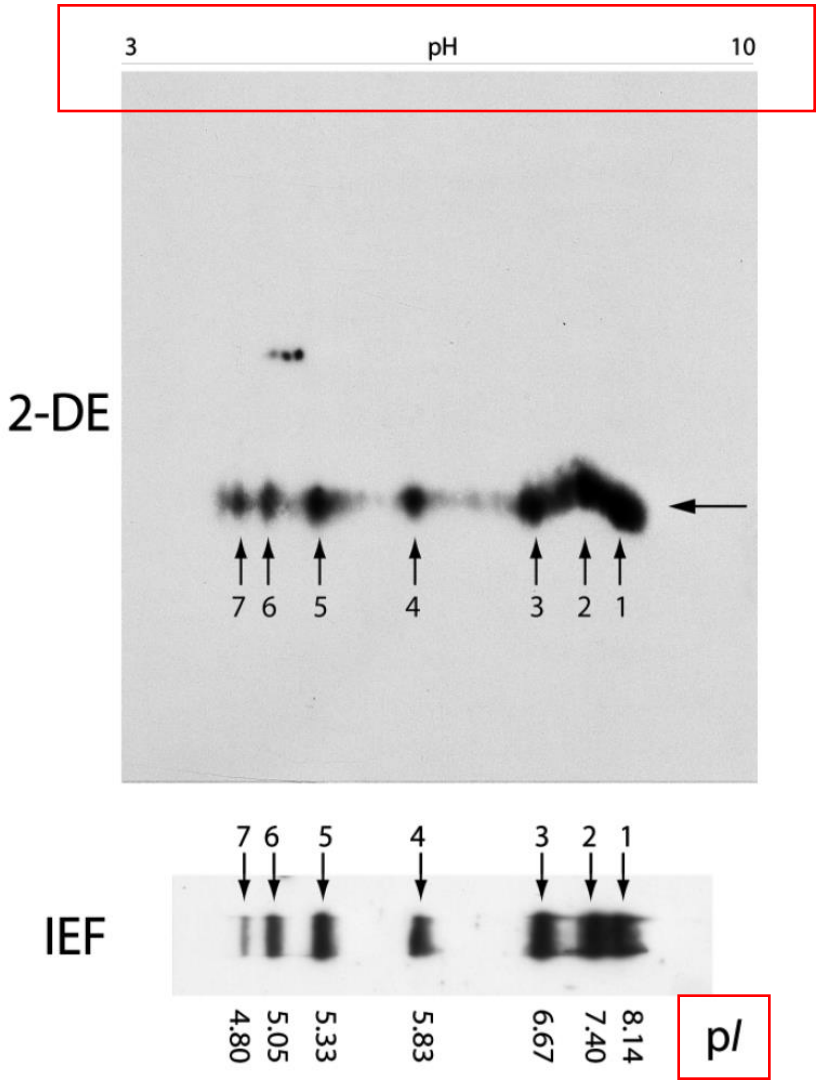




Human cerebrospinal fluid



Example of prostaglandin D2 synthase in CSF



Separation of isoforms

Figure. Comparison of prostaglandin D2 synthase (PGDS) posttranslational modification (PTM) patterns obtained with 2-DE- and IEF-immunoblotting techniques. Fortyfive micrograms of protein was loaded on an IPG strip (pH 3–10 NL, 7 cm) in the 2-DE method. SDS-PAGE dimension was performed on a 12% T vertical gel. Proteins were electroblotted onto a PVDF membrane. Two micrograms of protein was loaded on an IPG strip (pH 3–10 NL, 7 cm) in the IEF method. Proteins were transferred onto a PVDF membrane by passive diffusion. Human PGDS isoforms were detected with a rabbit polyclonal antibody and ECL reagents

The PTM changes, characterized by a shift from acidic to more basic isoforms, are most probably related to a deglycosylation process

Electrophoresis 2005, *26,* 4563–4570

Electrophoretic prefractionation methods

- Rotofor
- IsoPrime
- ZOOM IEF fractionator
- Free flow electrophoresis (FFE)
- **Shotgun IEF**
- **OFFGEL electrophoresis (OGE)**

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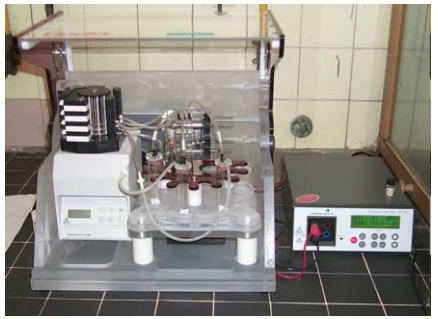
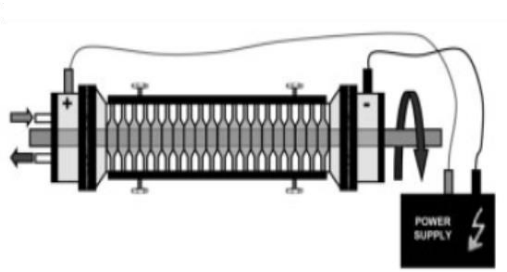
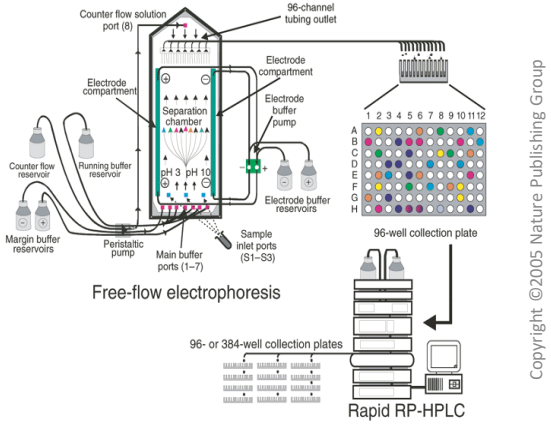




Righetti, Electrophoresis 2007, *28,* 3799–3810



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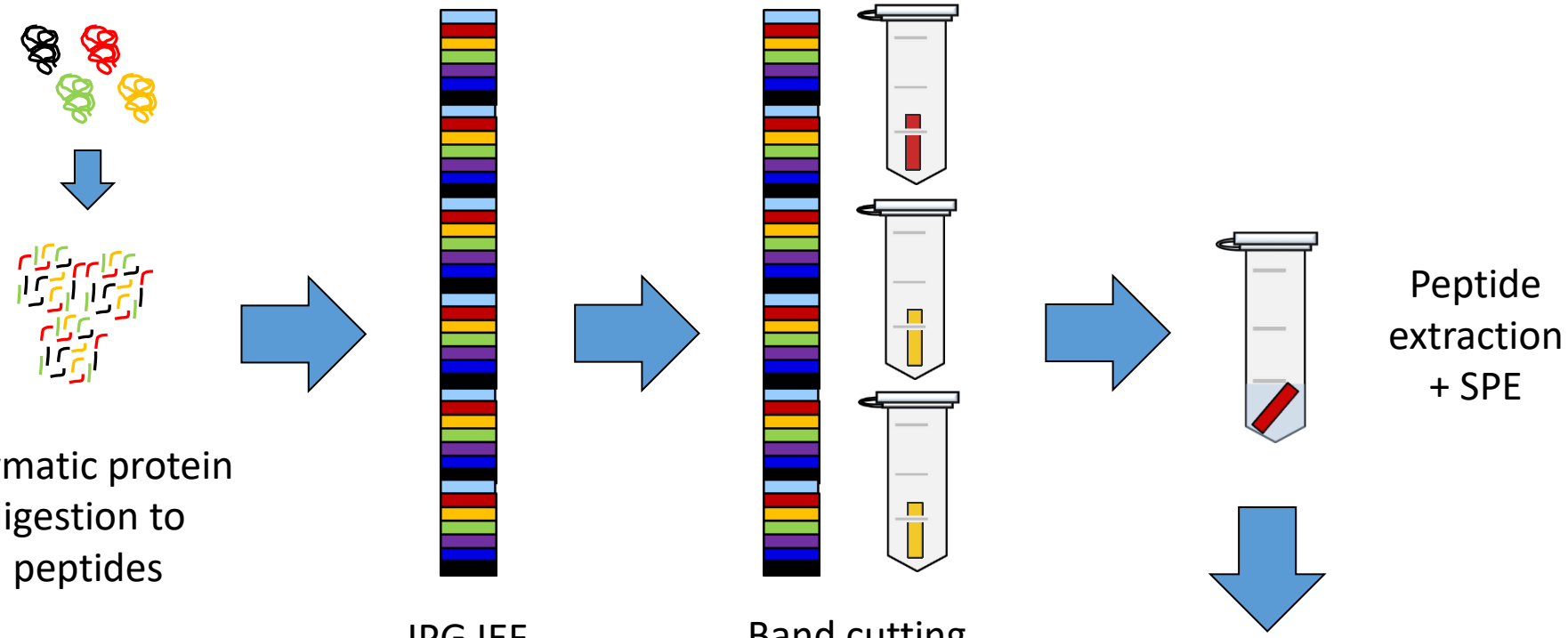
Moritz and Simpson, Nature Methods 2005, 2, 863 - 873

Copyright © 2011 Bio-Rad Laboratories, Inc.

https://www.youtube.com/watch?v=umQpk-ryqEk

Immobilized pH gradient (IPG) shotgun IEF



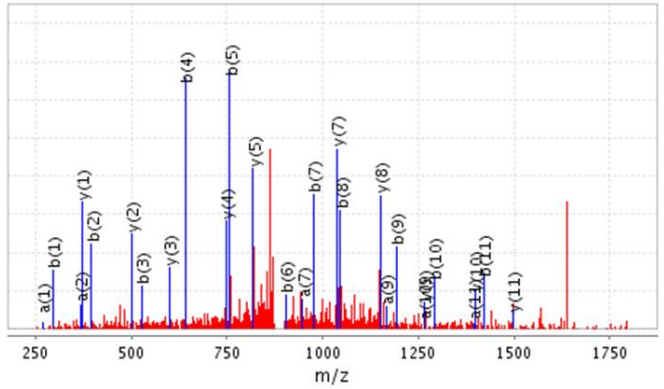
Enzymatic protein digestion to peptides

IPG IEF

Band cutting

Peptide extraction + SPE

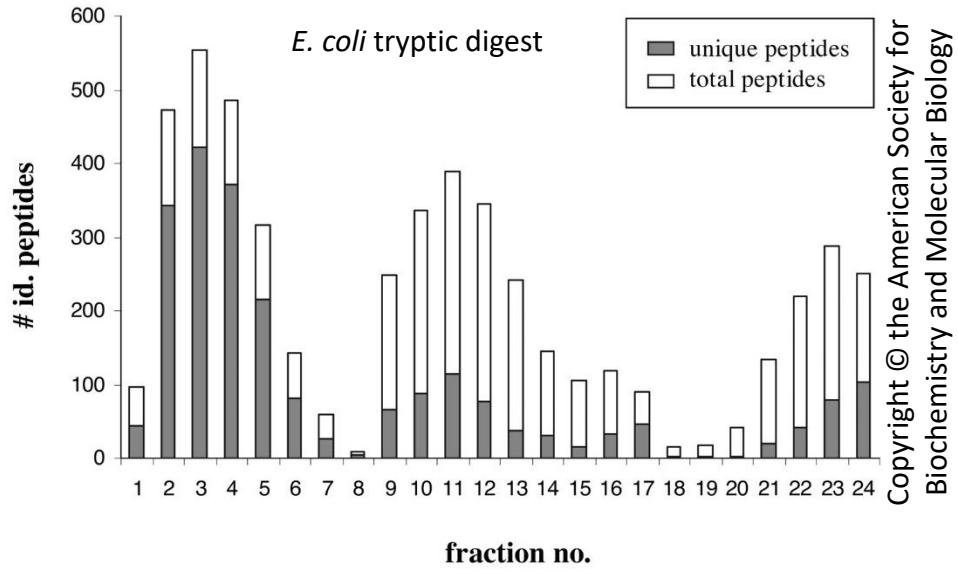
Database searching (and p*I*-based data filtering)



LC MS/MS analysis

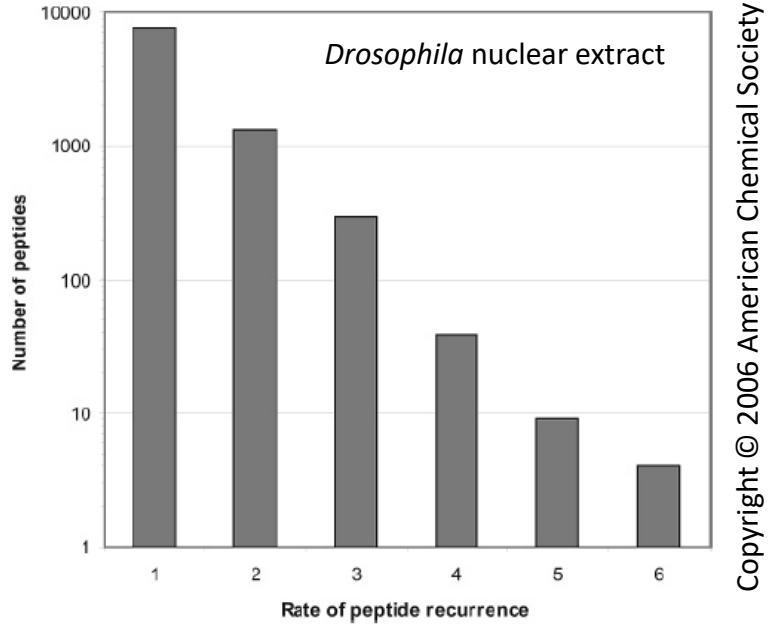
Cargile et al., J. Biomol. Tech., 2005, 16(3), 181–189

Separation efficiency



Horth et al., Mol. Cell. Proteomics 2006, 5, 1968-1974

log scale



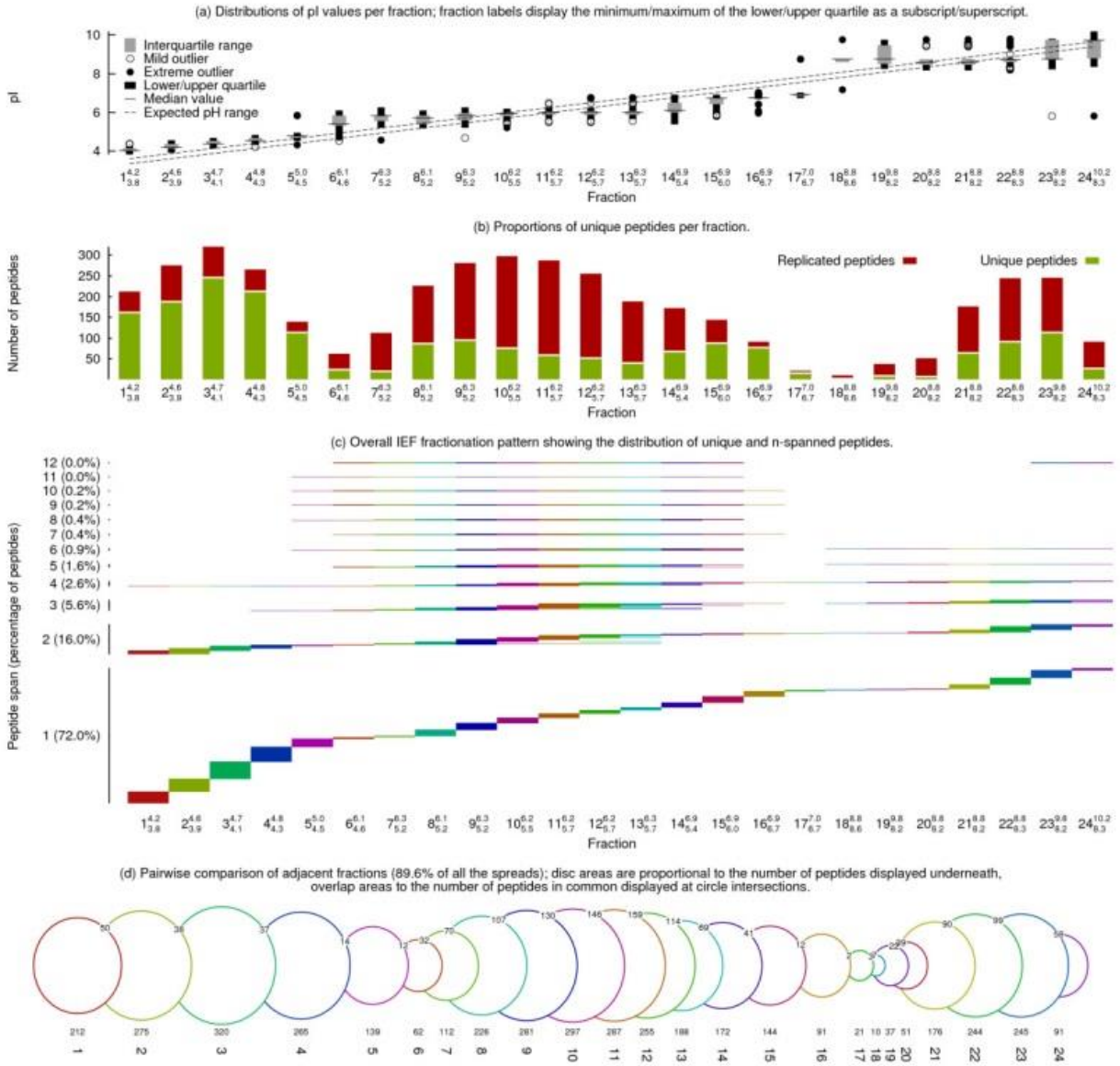
Krijgsveld et al., J. Proteome Res. 2006, 5, 1721-1730

- Good reproducibility of IPG gels
- High resolving power (steep slope between fraction and uniqueness)
- High loading capacity (0.1-1 mg)

- Small redundancy between IEF fractions (13 cm strip, 20 fractions)
- 96% of all peptides were found in two fraction at the maximum (82% in a single fraction)
- 16 h rehydratation + 2-4 h focusing

Cargile et al., Electrophoresis 2004, 25, 936–945

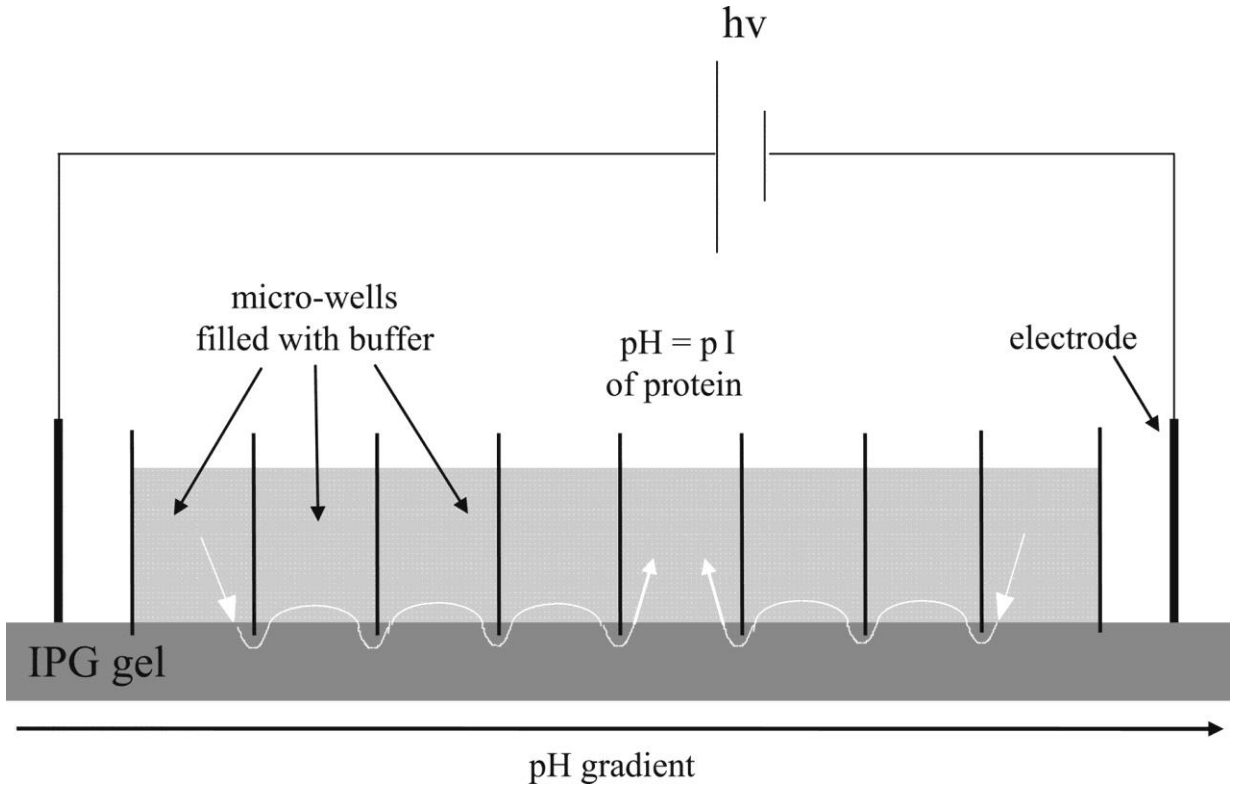
Separation visualization



- Peptide p*I* distribution per fraction
- Percentage of unique peptides per fraction
- Distribution of unique and *n*-spanned peptides
- Identification spread of peptides across adjacent fractions

Azulay et al., BMC Bioinformatics 2010, 11, 371

Off-gel electrophoresis (OGE)

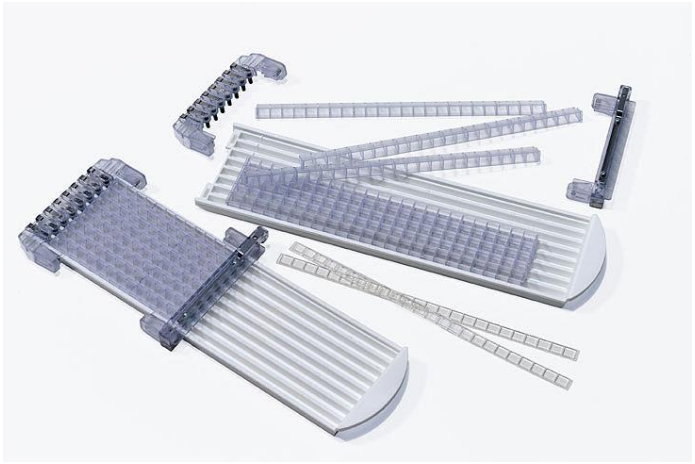


Horth et al., Mol. Cell. Proteomics 2006, 5, 1968-1974

Ros et al., Proteomics 2002, 2, 151–156
Michel et al., Electrophoresis 2003, 24, 3–11

Off-gel in practice

CONFIGURATIONS:
- Peptides or proteins
- 12 or 24 wells (13 or 24 IPG strips)
- High and low resolution

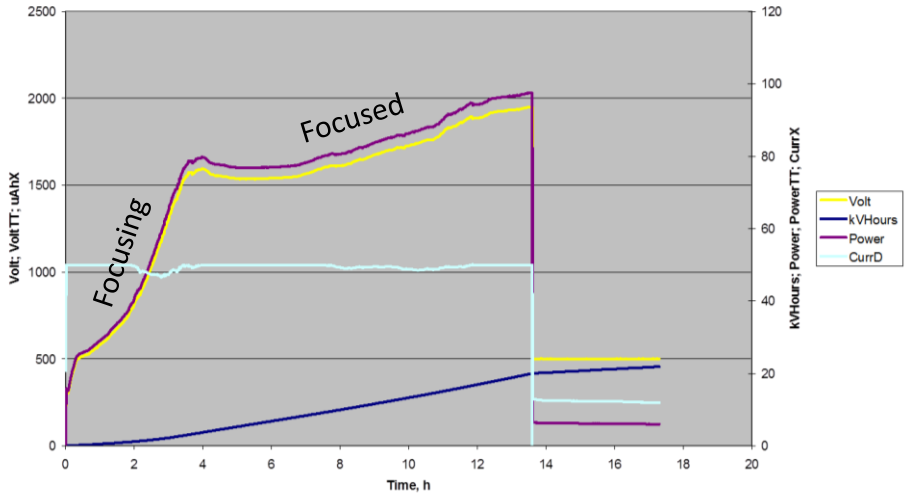


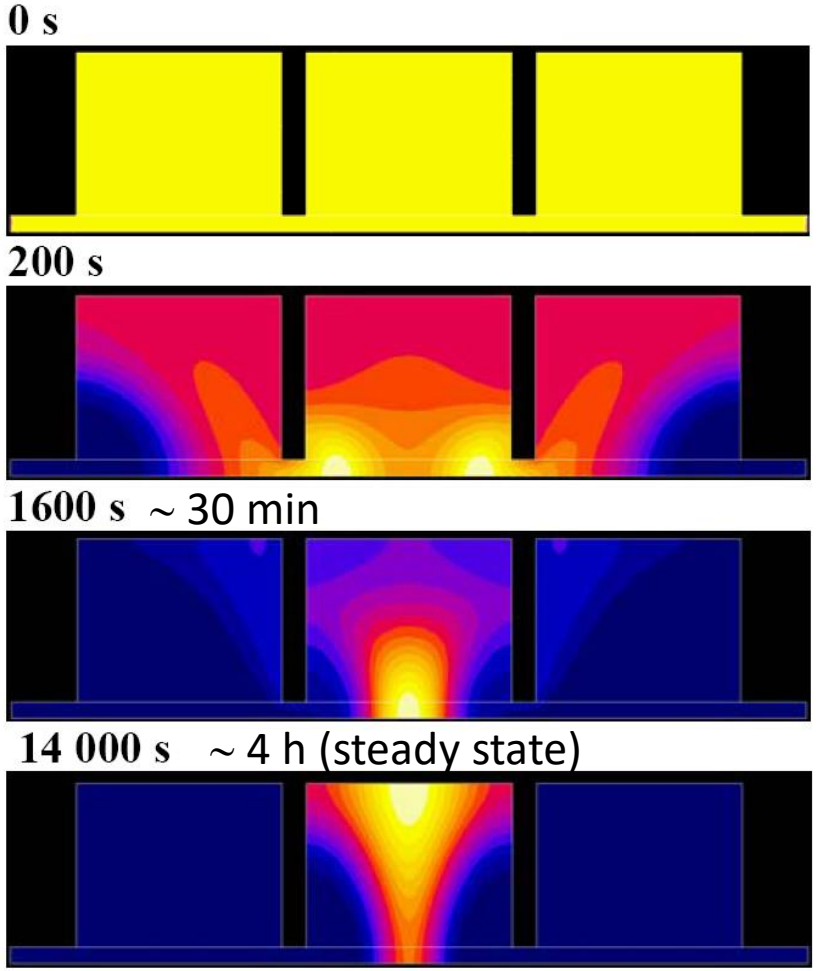
PROCEDURES:
- Assemble the IPG strips, frames and electrodes
- IPG strip rehydration (15- 30 min)
- Sample loading in the wells
- Mount the electrodes
- Apply the voltage (limit current)

Focusing efficiency of Off-gel

Numerical studies to characterize off-gel IEF in a 3-compartement device:

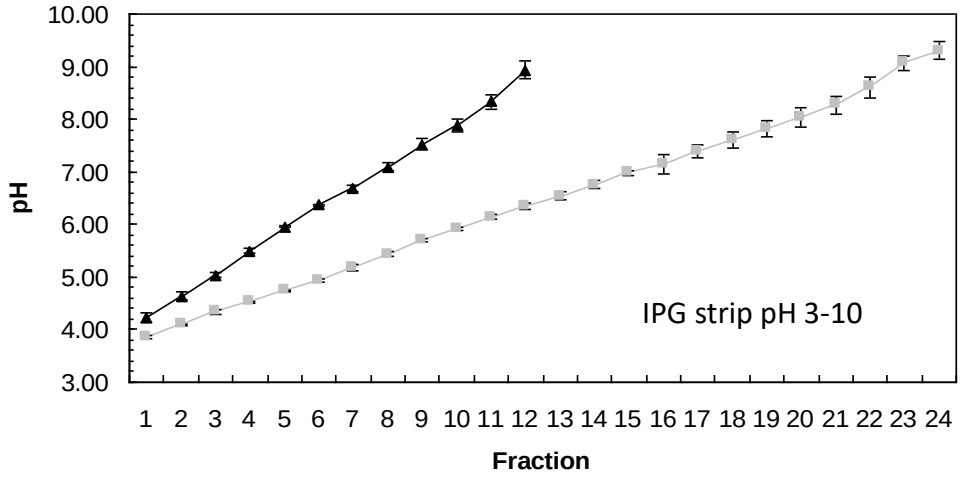
- Electric field vectors: the electric field arriving from the gel enters the well on the left end side, and occupies the full volume of the well
- Concentration isovalues of angiotensin II: focusing time depends on slope at p*I*; however diffusion ("vertical focusing") reduce the difference





Lam et al., J. Proteome Res. 2007, 6, 1666 - 1676

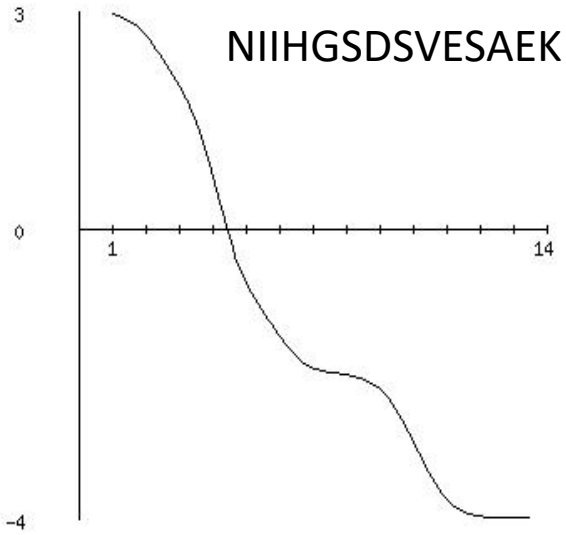
Focusing efficiency of Off-gel



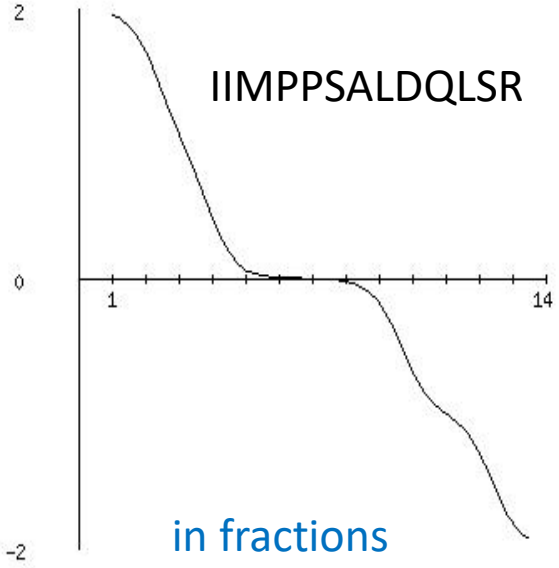
pH measurements without any sample:

- 24 wells: RSD from 0.2 to 2.7% (mean 1.2%) => mean ΔpH = 0.24 between wells
- 12 wells: RSD from 0.3 to 2.4% (mean 1.3%) => mean ΔpH = 0.43 between wells

Charge derivative dz/dpH at p*I*: experimental data with off-gel IEF



in fraction 5

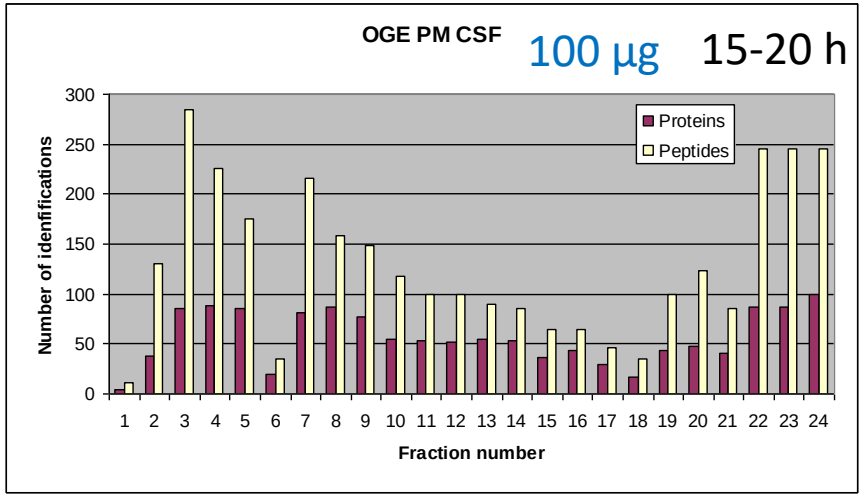


in fractions 8, 9, 10, 11 and 12

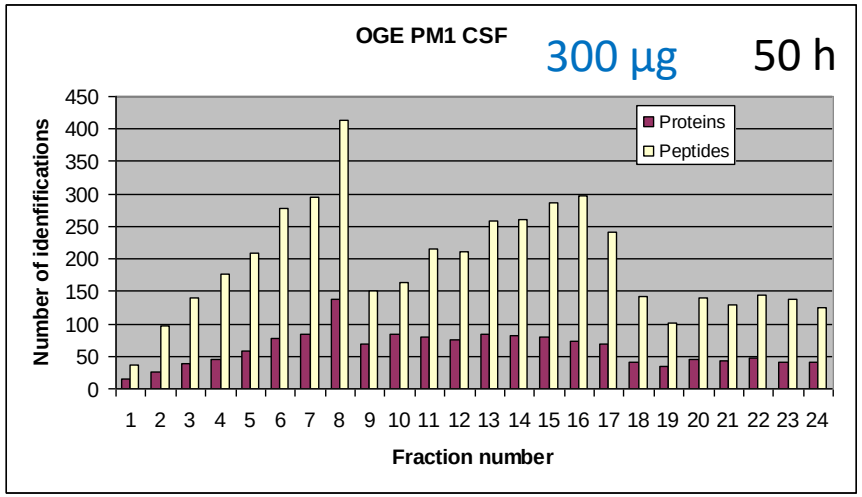
Focusing efficiency of Off-gel

Influence of salts

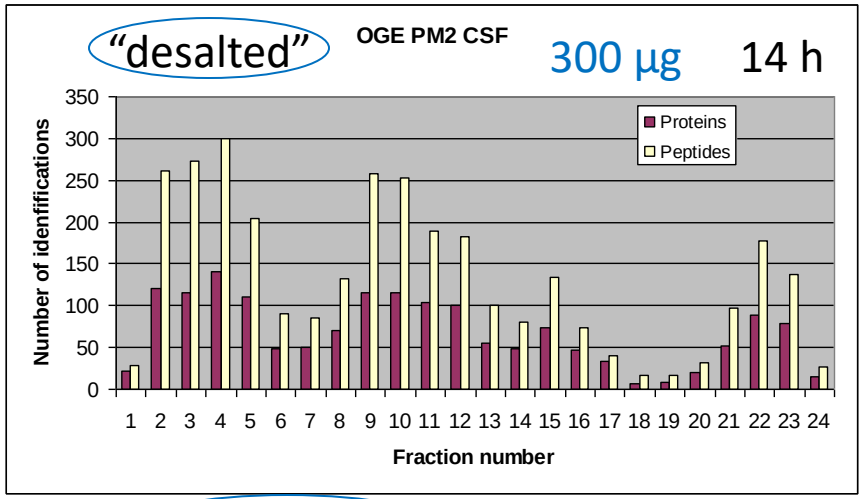
Digests of *post-mortem* cerebrospinal fluid separated by OGE (TOF/TOF MS)



364 proteins (3590 peptides)



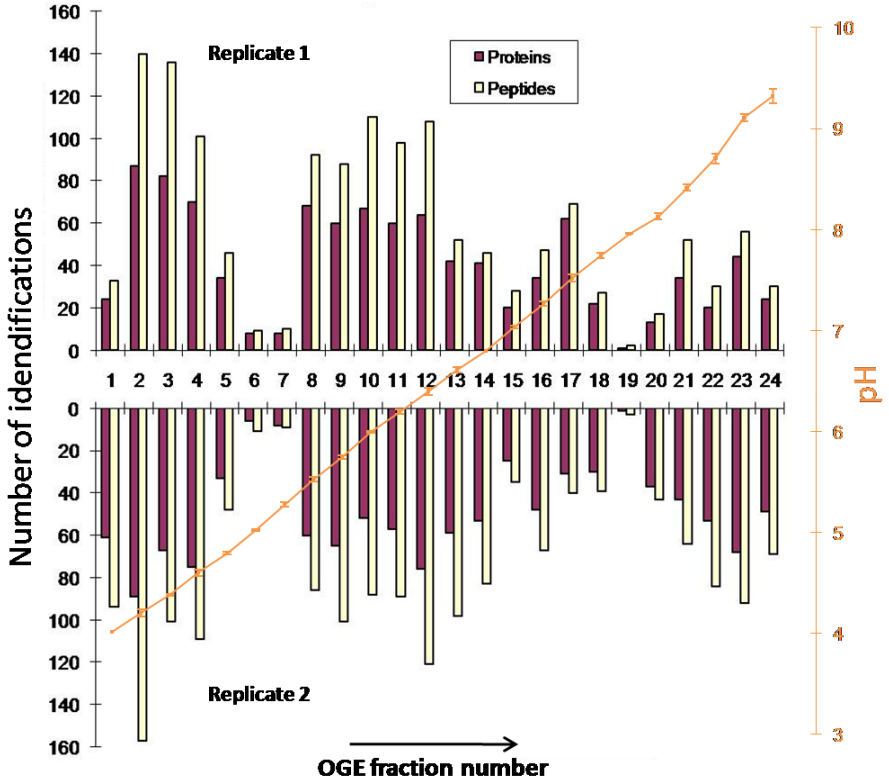
273 proteins (5186 peptides)



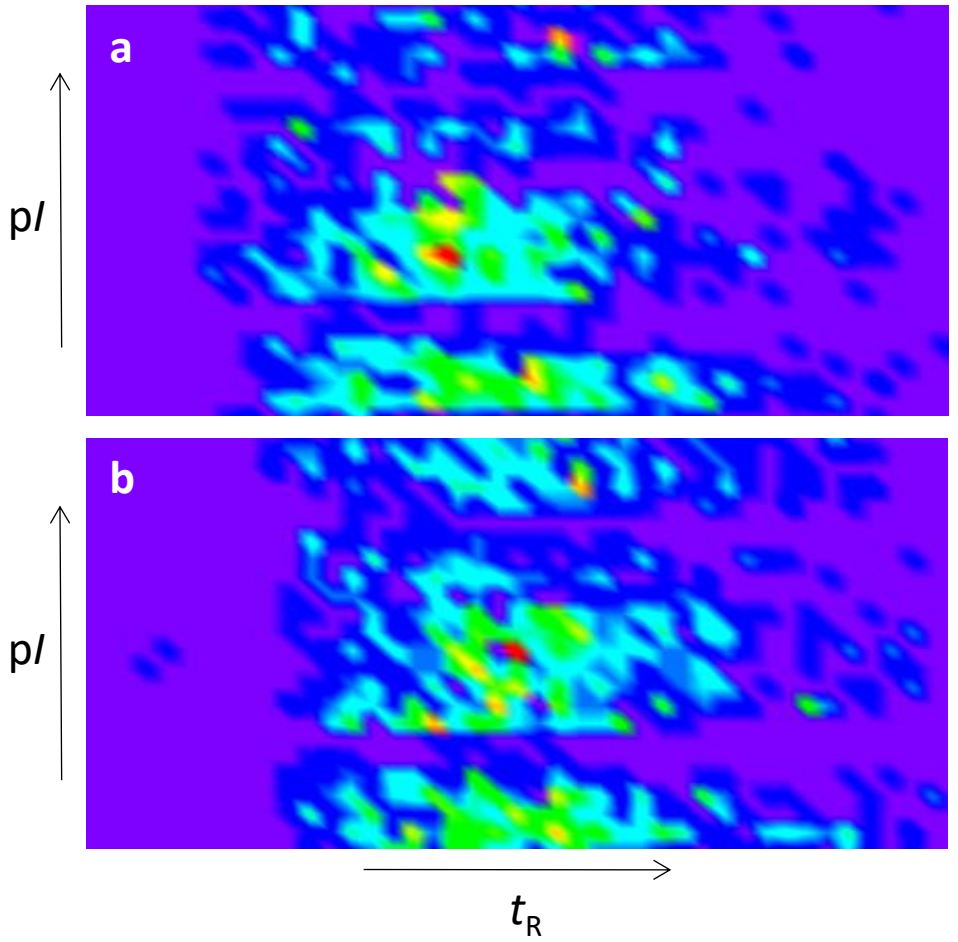
582 proteins (4019 peptides)

Reproducibility and orthogonality with RP-LC

Cerebrospinal fluid



Dayon et al., Chimia 2010, 64, 132-135



Dayon et al., Anal. Chem. 2010, 82, 848-858

- Reproducibility
- Orthogonality

Off-gel IEF reproducibility

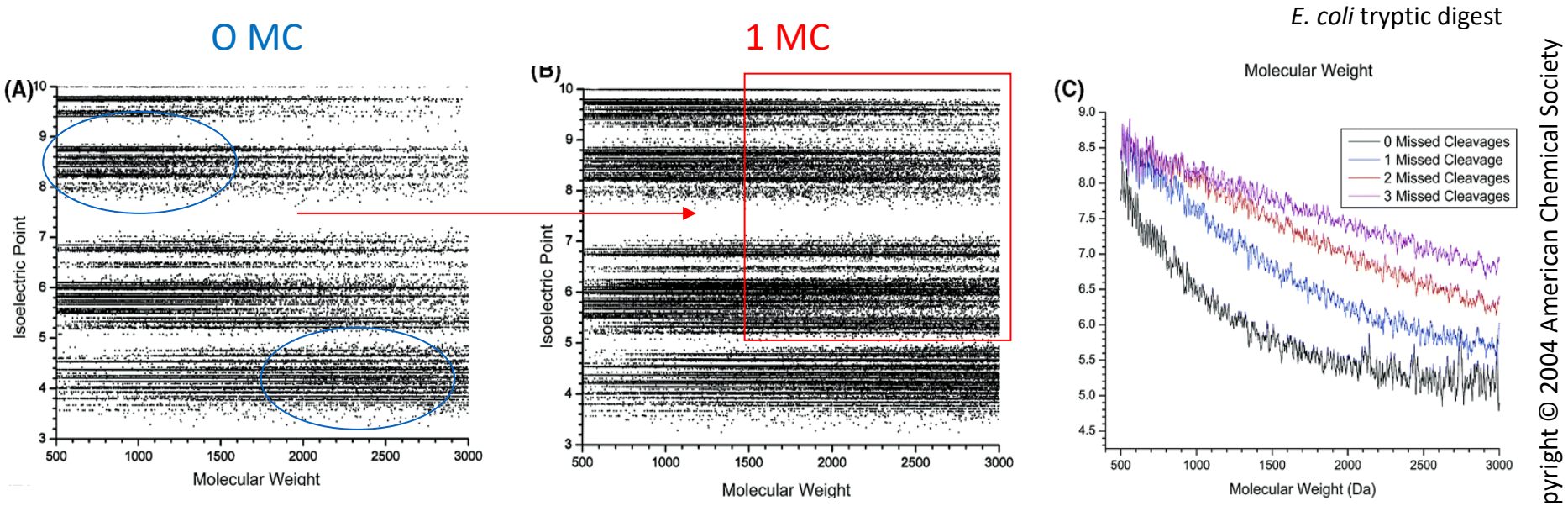
	Fraction number	1	2	3	4	5	6	7	8	9	10	11	12
	Average pH	4.02	4.21	4.38	4.60	4.80	5.03	5.28	5.53	5.75	6.00	6.20	6.40
	pH RSD / %	0.9	0.2	0.6	0.3	0.1	0.4	0.4	0.4	0.1	0.4	0.6	0.4
TOF/TOF	Unique peptides (sample 1)	31	134	135	97	43	9	10	92	88	110	98	108
TOF/TOF	Unique peptides (sample 2)	86	150	99	108	48	11	9	84	99	87	89	118
TOF/TOF	Common peptides / %	27	59	43	55	57	54	33	49	60	54	51	50
LTQ-OT	Unique peptides (sample 1)	210	376	373	330	213	65	80	316	336	350	363	359
LTQ-OT	Unique peptides (sample 2)	206	344	256	333	185	55	57	274	307	312	297	334
LTQ-OT	Common peptides / %	40	57	44	52	52	56	54	55	53	52	52	55

	Fraction number	13	14	15	16	17	18	19	20	21	22	23	24
	Average pH	6.61	6.80	7.04	7.27	7.52	7.75	7.96	8.13	8.42	8.71	9.11	9.33
	pH RSD / %	0.0	0.2	0.3	0.5	0.1	0.4	0.3	0.6	0.6	0.4	0.8	1.6
TOF/TOF	Unique peptides (sample 1)	52	46	26	43	68	25	2	16	48	26	55	27
TOF/TOF	Unique peptides (sample 2)	97	81	35	64	40	35	3	42	59	77	88	65
TOF/TOF	Common peptides / %	26	27	25	35	35	30	67	21	34	27	42	26
LTQ-OT	Unique peptides (sample 1)	300	342	226	277	337	119	22	164	312	247	307	264
LTQ-OT	Unique peptides (sample 2)	301	288	248	247	274	79	19	139	246	270	238	201
LTQ-OT	Common peptides / %	55	56	48	55	53	46	41	54	38	45	49	50

Dayon et al., Anal. Chem. 2010, 82, 848-858

- 80% of the peptides in a unique fraction
- 49% in the same off-gel fraction between sample 1 and sample 2 (DDA mode)
- 54% of the peptides were commonly identified in sample 1 and sample 2

Application of peptide IEF: MS data validation/filtering through p*I*



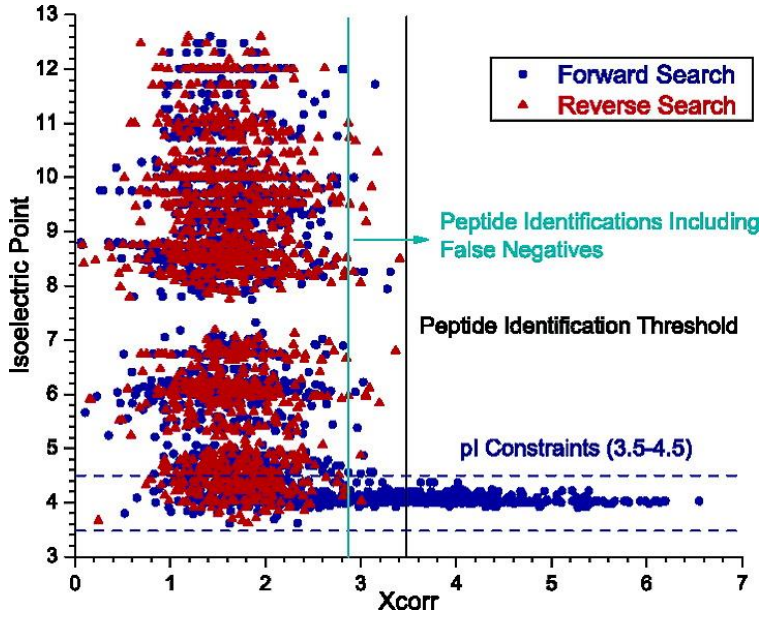
Cargile et al., J. Proteome Res. 2004, 3, 112-119

⇒ data is spread out over the entire mass/p*I* plot

⇒ MC lead to an increase in the absolute number of peptides, but also an increase in the medium to high molecular weight range of the middle to high p*I* (6.0-9.5)

⇒ as the peptide increase in mass, the average p*I* decreases (fig. c)
⇒ as the number of MC increases, the average p*I* increases (fig. c)

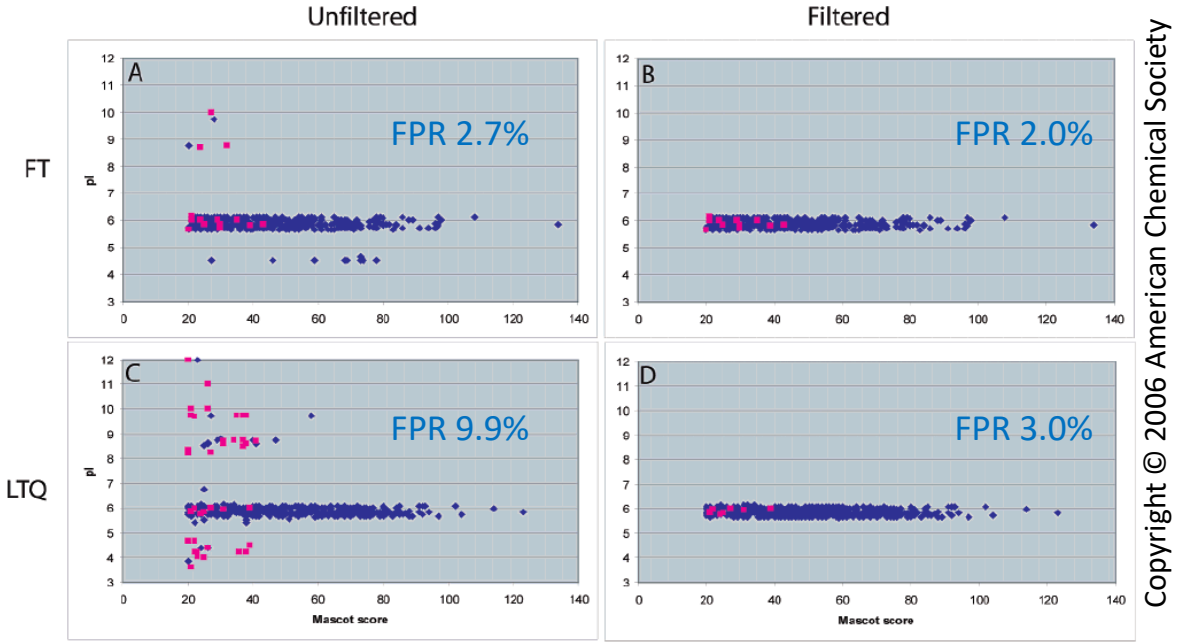
Application of peptide IEF: MS data validation/filtering through p*I*



Cargile et al., J. Proteome Res. 2004, 3, 1082-1085

Reducing of false positive rate by filtering of peptides based on p*I*

=> Importance of p*I* calculation algorithms



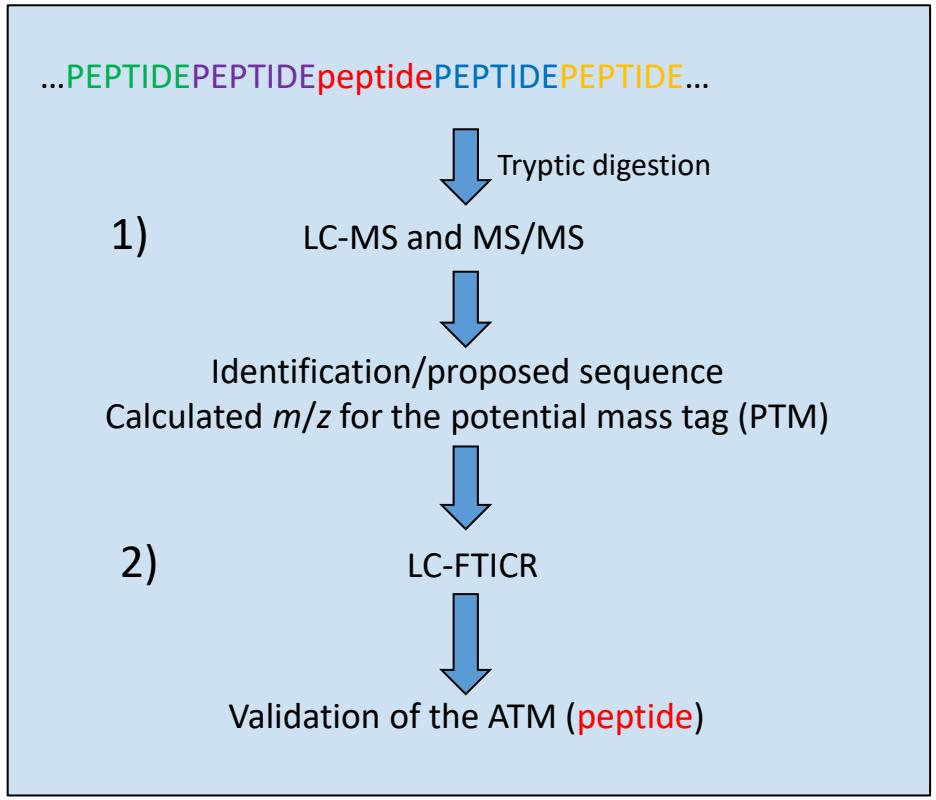
Krijgsveld et al., J. Proteome Res. 2006, 5, 1721-1730

Application of peptide IEF: p*I* and mass accuracy

Accurate mass tags (AMT)

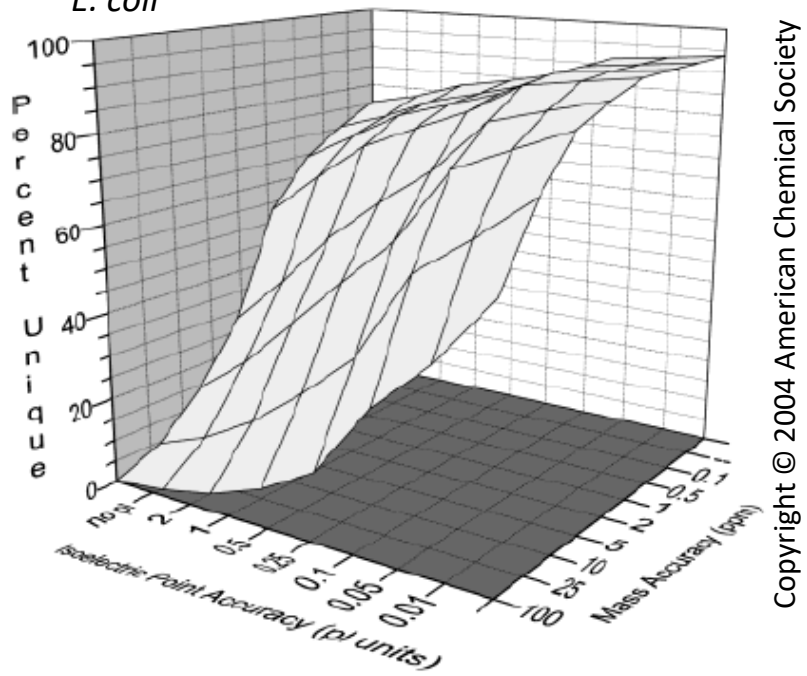
1) Shotgun LC MS/MS
2) Find unique peptides (no isobars) at 1 ppm accuracy) representing a protein

=> Screening of comparable samples by LC FT-ICR-MS looking at AMT



Smith et al., Proteomics 2002, 2, 513–523

E. coli



Cargile et al., Anal. Chem. 2004, 76, 267-275

Find complement to mass measurement to discriminate information:

- retention time in LC
- p*I* …

Application of peptide IEF: IEF and chemical tagging for phosphoproteomics

Anal. Chem. **2007**, *79*, 2007−2014

Selective Enrichment and Fractionation of Phosphopeptides from Peptide Mixtures by Isoelectric Focusing after Methyl Esterification

Chong-Feng Xu,† Huaibin Wang,‡ Daming Li,§ Xiang-Peng Kong,‡ and Thomas A. Neubert*†

Electrophoresis 2007, *28*, 2044–2052

Chien-Wen Hung[1]
Dieter Kübler[2]
Wolf D. Lehmann[1]

[1]Central Spectroscopy, German Cancer Research Center, Heidelberg, Germany
[2]Biomolecular Interactions Group, German Cancer Research Center, Heidelberg, Germany

Research Article

p*I*-based phosphopeptide enrichment combined with nanoESI-MS

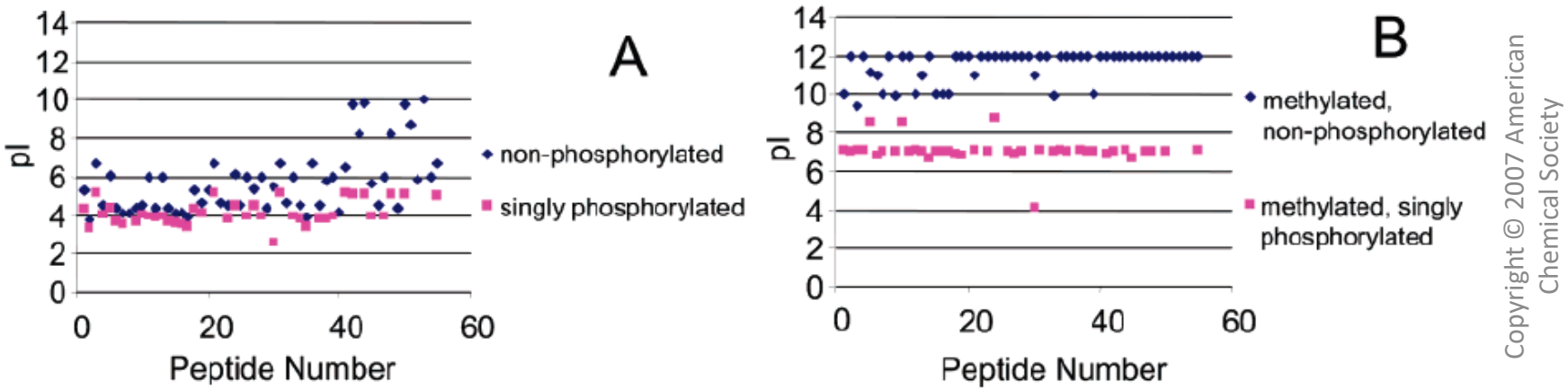
Journal of Proteome Research 2007, 6, 1153-1157

research articles Journal of proteome research

Isolation of Phosphopeptides by p*I*-Difference-Based Electrophoresis

Yingda Xu, Robert Sprung, Sung Won Kwon, Sung Chan Kim, and Yingming Zhao*

Application of peptide IEF: IEF and chemical tagging for phosphoproteomics



Xu et al., J. Proteome Res. 2007, 6, 1153-1157

p*I* values of all peptides increase after esterification of acidic groups, However, methylated phosphopeptides have much lower p*I*s than methylated non-phosphopeptides.

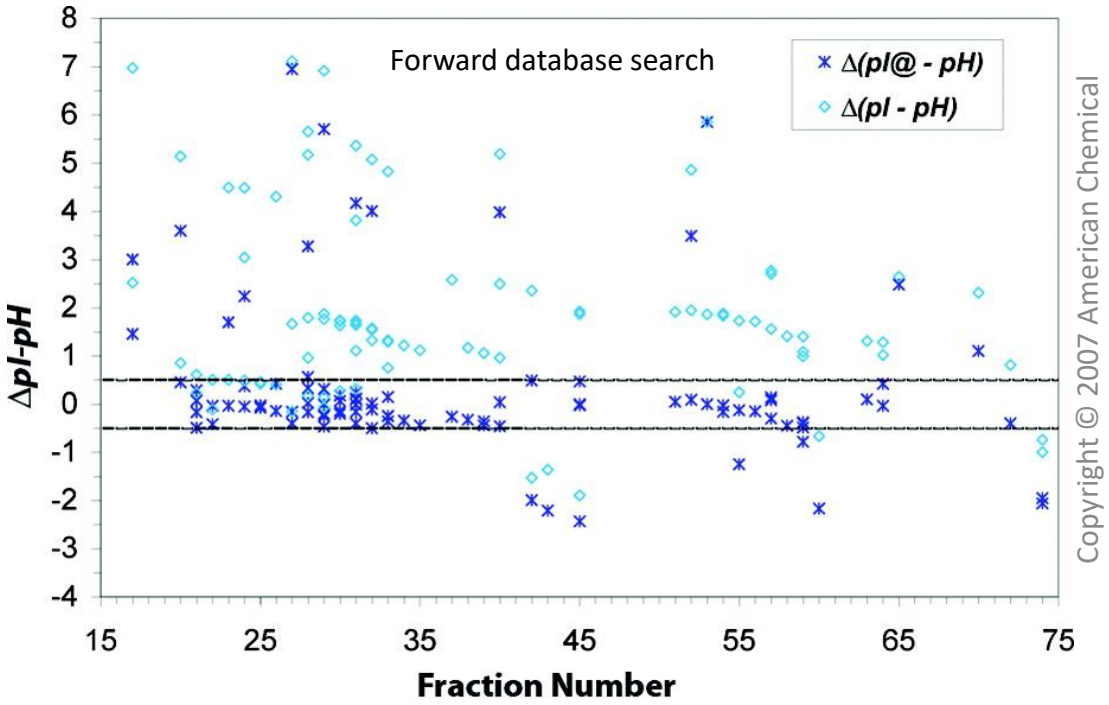
Table 1. Prediction of the pIs for Phosphopeptides Using pIMethylation and Scansite

protein	seq. no.	sequence	MW+H w/o methylation	MW+H methylation	pI (w/o methylation) Scansite	pI (w/o methylation) pIMethylation	pI (methylation) pIMethylation	pI (methylation) observed pI[a] (pH 6.3−8.3 gel)
β-casien	33−48	FQpSEEQQQTEDELQDK	2061.83	2159.94	3.37	3.34	7.00	6.7−7.1
	1−25	RELEELNVPGEIVEpSLpSpSpSEESITR	3122.27	3234.40	2.51	1.66	1.68	6.7−7.9
α-S1-casein	106−119	VPQLEIVPNpSAEER	1660.80	1716.86	3.69	3.67	6.97	6.7−7.1
	104−119	YLGEYLIVPNpSAEER[c]	1832.83	1888.89	3.69	3.67	7.00	6.3−6.7
	43−58	DIGpSEpSTEDQAMEDIK	1927.69	2025.80	2.78	2.33	3.84	≤6.3−6.7[b]
	104−119	YKVPQLEIVPNpSAEER	1951.95	2008.01	4.26	4.25	8.61	7.9−8.3
	59−79	QMEAEpSIpSpSpSEEIVPNpSVEQK	2720.91	2805.00	1.93	1.02	1.02	n/a
α-S2-casein	138−149	TVDMEpSTEVFTK	1466.61	1522.67	3.60	3.58	6.66	6.7−7.1
	138−150	TVDMEpSTEVFTKK	1594.70	1650.76	4.14	4.13	8.14	7.5−7.9[b]
	1−24	KNTMEHVpSpSpSEESIIpSQETYKQEK	3132.20	3216.29	3.59	3.52	5.75	≤6.3−6.7[b]

[a] Observed pI was the pH fraction that contained the majority of phosphopeptides in the 2-h focusing experiment. [b] Phosphopeptides that were observed only in the 1-h focusing experiment. [c] Alternative splice form of this peptide.

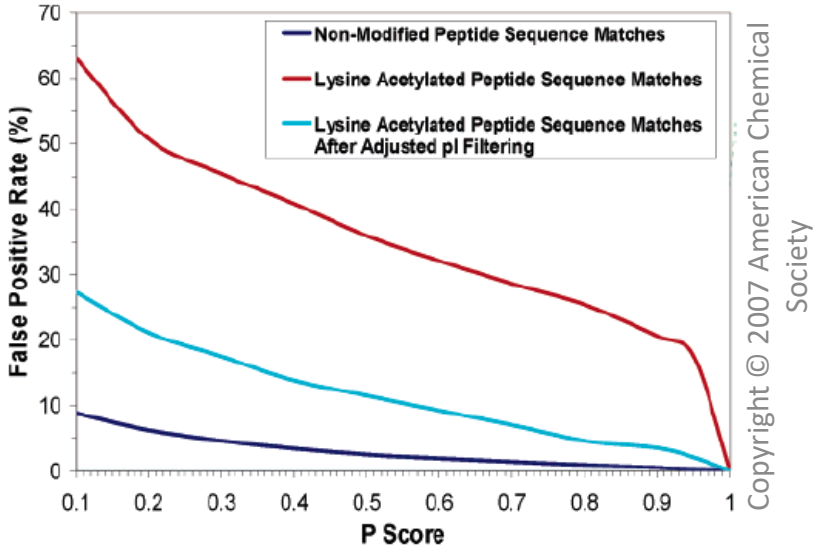
Xu et al., Anal. Chem. 2007, 79, 2007-2014

Application of peptide IEF: p*I* of acetylated peptides



Xie et al., J. Proteome Res. 2007, 6, 2019-2026

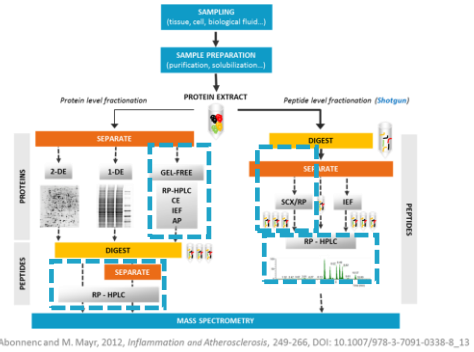
p*I* of acetylated peptides



Xie et al., J. Proteome Res. 2007, 6, 2019-2026

Improving the identification of PTMs which cause a shift to p*I* of peptides

3.3. Liquid chromatography (LC) in protein profiling

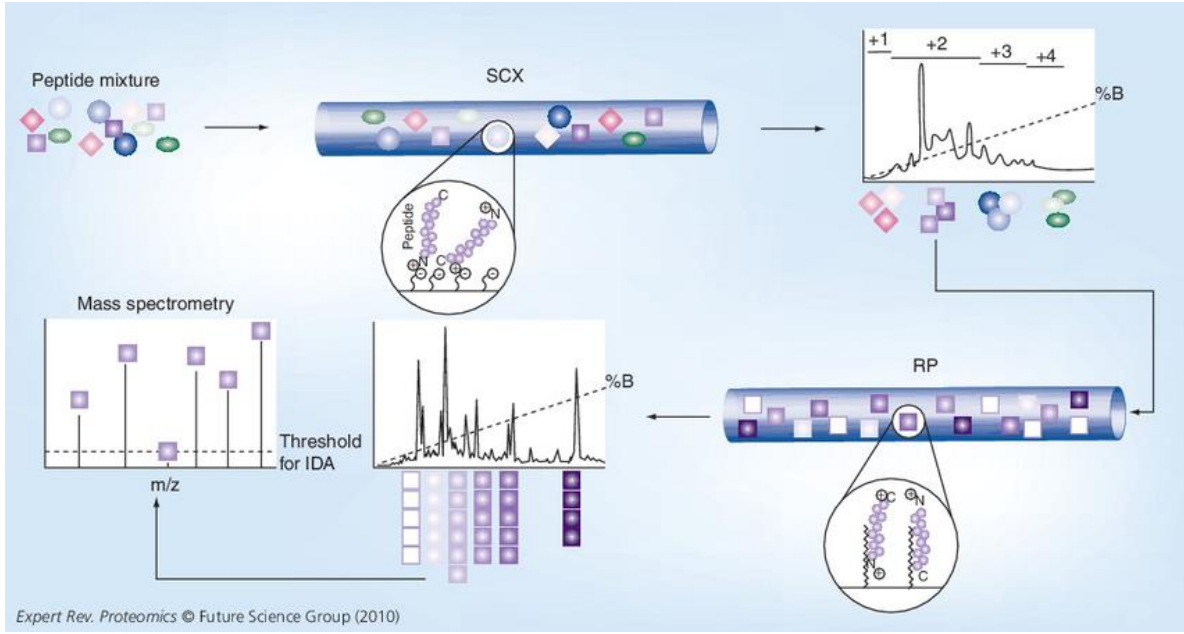


M. Abonnenc and M. Mayr, 2012, *Inflammation and Atherosclerosis*, 249-266, DOI: 10.1007/978-3-7091-0338-8_13

- Chromatography: generalities
  - Definitions
  - Principles of chromatography
  - Theory and chromatographic performance

- Liquid chromatography (LC)
  - Adsorption
  - Partition
  - Size Exclusion
  - Ion exchange
  - Affinity

- High performance liquid chromatography (HPLC)



DOI: 10.1586/epr.10.46

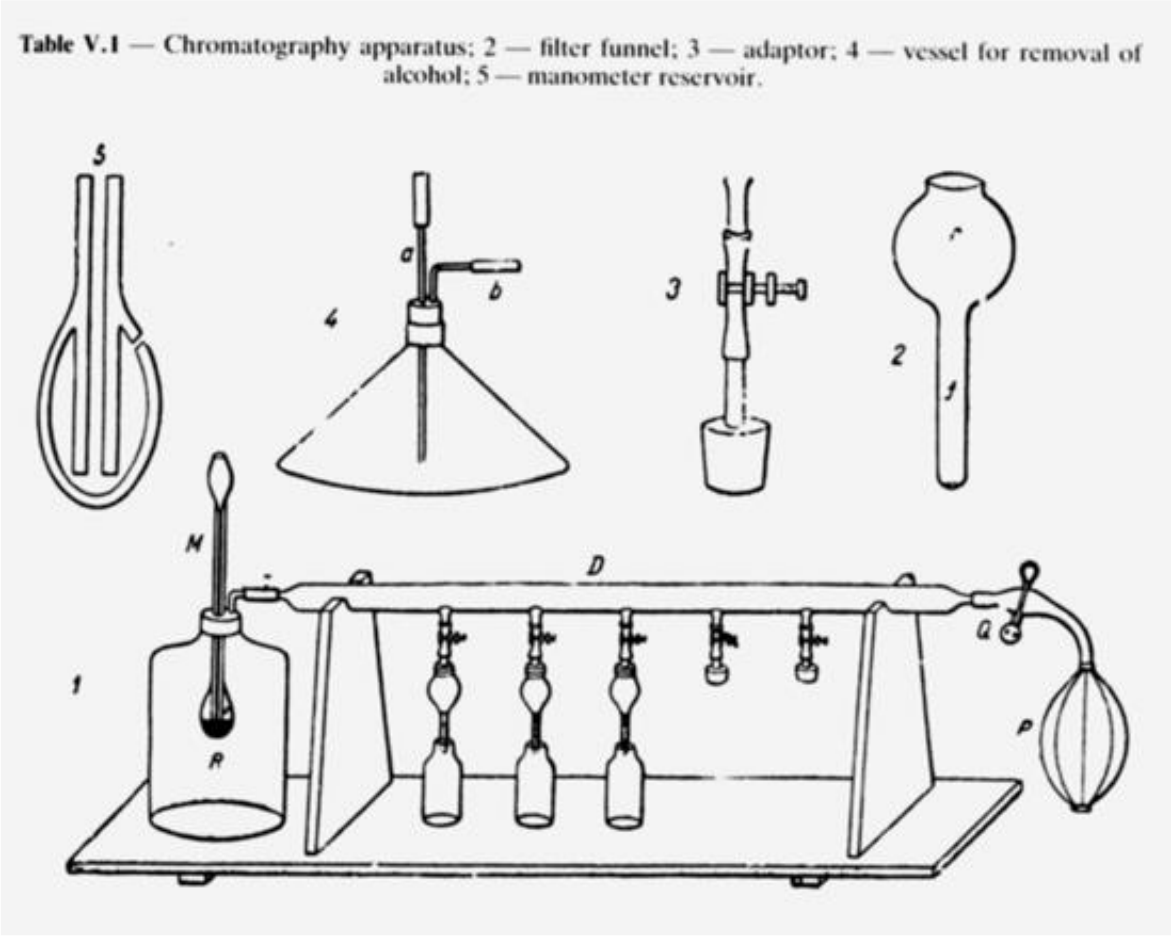
- Applications of liquid chromatography in biological research
  - Affinity chromatography
  - Multi dimensional separation

Definition of chromatography

- Chromatography is a separation technique which involves the interaction of one or more solutes with two phases: a stationary phase and a mobile phase.

- Sample components are carried by the mobile through the bed stationary phase based on various interactions such as:
  - Adsorption
  - Partition
  - Size Exclusion
  - Ion exchange
  - Affinity

History of chromatography



Tswett-Apparatus (1905)

Michail Semjonowitsch Tswett

He used liquid-adsorption column chromatography with calcium carbonate as adsorbent and petrol ether / ethanol mixtures as eluent to separate chlorophylls and carotenoids

History of chromatography

In 1952, **Archer John Porter Martin** and **Richard Laurence Millington Synge** were awarded the Chemistry Nobel Prize for their invention of partition chromatography

In 1968, High Performance Liquid Chromatography (HPLC) is implemented

In 1979, chiral compounds are separated by HPLC

151. A NEW FORM OF CHROMATOGRAM EMPLOYING TWO LIQUID PHASES

1. A THEORY OF CHROMATOGRAPHY

2. APPLICATION TO THE MICRO-DETERMINATION OF THE HIGHER MONOAMINO-ACIDS IN PROTEINS

By A. J. P. MARTIN and R. L. M. SYNGE

From the Wool Industries Research Association, Torridon, Headingley, Leeds

(*Received 19 November 1941*)

Introduction

In most forms of counter-current extraction column the very small drop required for the rapid attainment of equilibrium, and hence for high efficiencies, cannot be used owing to the difficulty of preventing it moving in the wrong direction. In the case of a solid, however, for any reasonable size of particle a filter will prevent movement in any undesired direction. Consideration of such facts led us to try absorbing water in silica gel etc., and then using the water-saturated solid as one phase of a chromatogram, the other being some fluid immiscible with water, the silica acting merely as mechanical support. Separations in a chromatogram of this type thus depend upon differences in the partition between two liquid phases of the substances to be separated, and not, as in all previously described chromatograms, on differences in adsorption between liquid and solid phases.

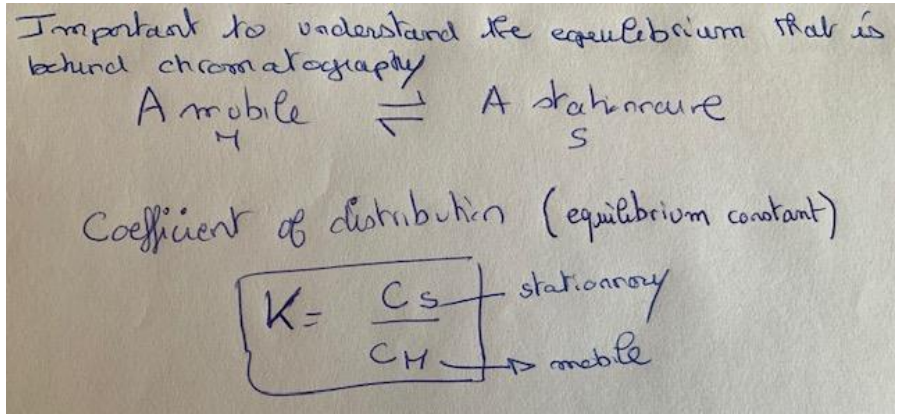
The difficulties of using chromatograms are very greatly lessened when the substances to be separated are coloured, or if colourless can be made visible. Various methods have been used for this [cf. Zechmeister & Cholnoky, 1936; Cook, 1941], though none of these was suitable for our problems. As the substances which we desired to separate were acids, and water was one of our phases, we were able to obtain visual evidence of the presence of any of these acids by adding a suitable indicator to the water with which the gel was saturated.

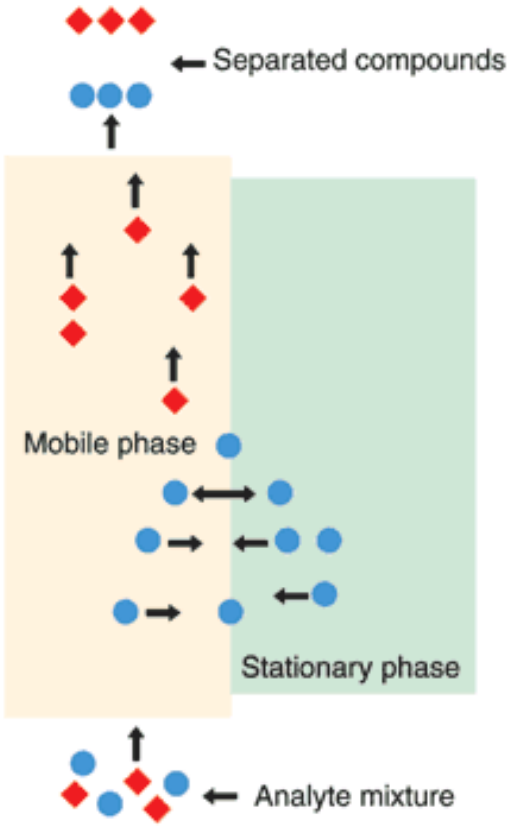
In the present paper we present an approximate theory of chromatographic separations, and describe an application of the new chromatogram to the micro-determination of the higher monoamino-acids in protein hydrolysates. This method is based on the partition of acetamino-acids between chloroform and water phases, and supersedes the macro-method described by us [Martin & Synge, 1941, 1], being rapid and economical both of materials and of apparatus.

Work is in progress, using ethyl acetate as the less polar phase in the chromatogram, on the separation of the acetyl derivatives of most of the other naturally occurring amino-acids, and the method promises also to be of use in analogous separations of simple peptides.

We wish to stress, however, that the possible field of usefulness of the new chromatogram is by no means confined to protein chemistry. By employing suitable phase pairs, many other substances should be separable. Where water is suitable as one of the phases, an indicator may be used to render visible the separation of organic acids or bases. Even where this is not possible, as with

Basis of chromatography





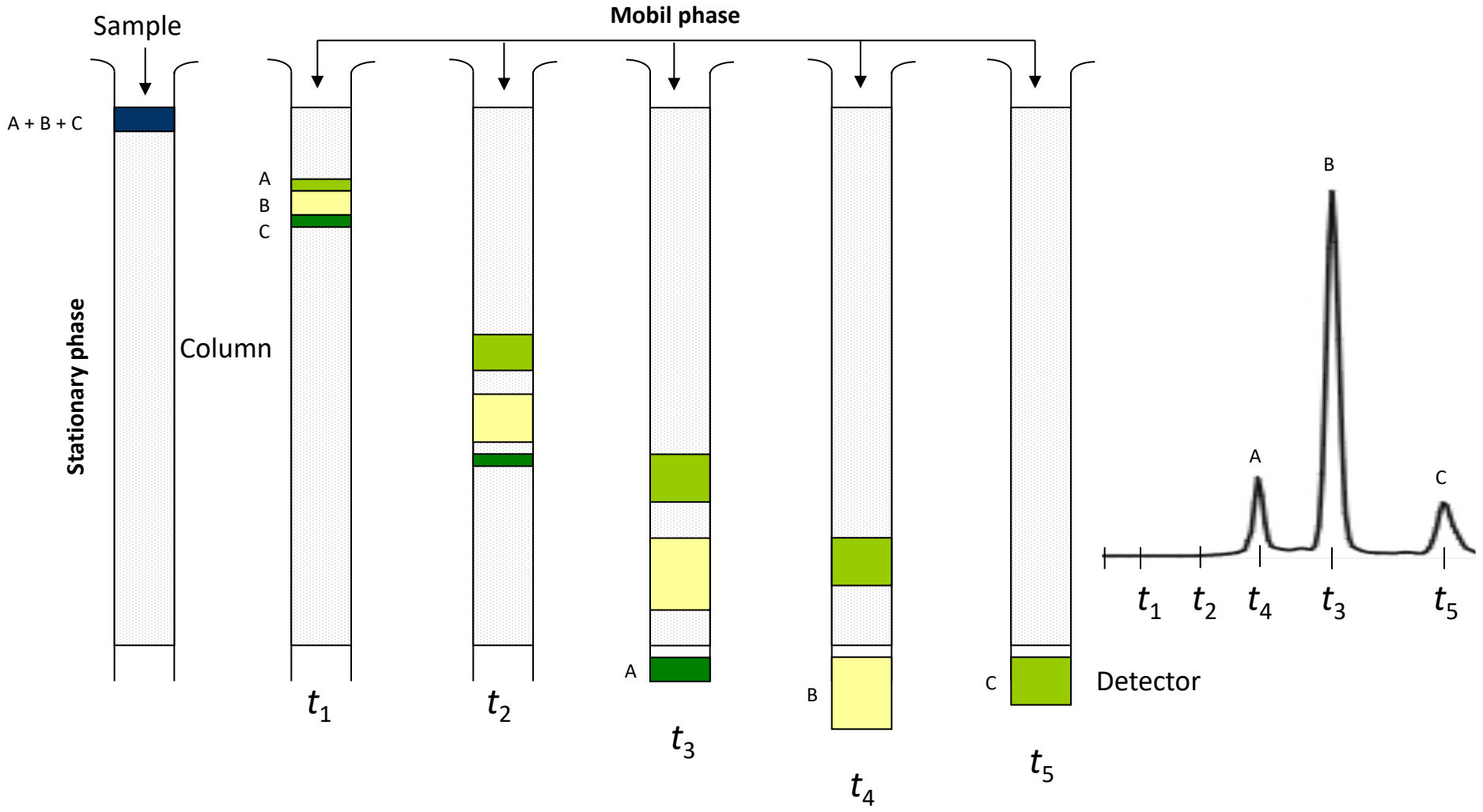
- The circle compound ● is highly soluble in the stationary phase and partitions into it easily, which retards its flow through the system

- The ♦ compound is less soluble, or repelled by the stationary phase, and this remains more mobile, quickly passing through the system

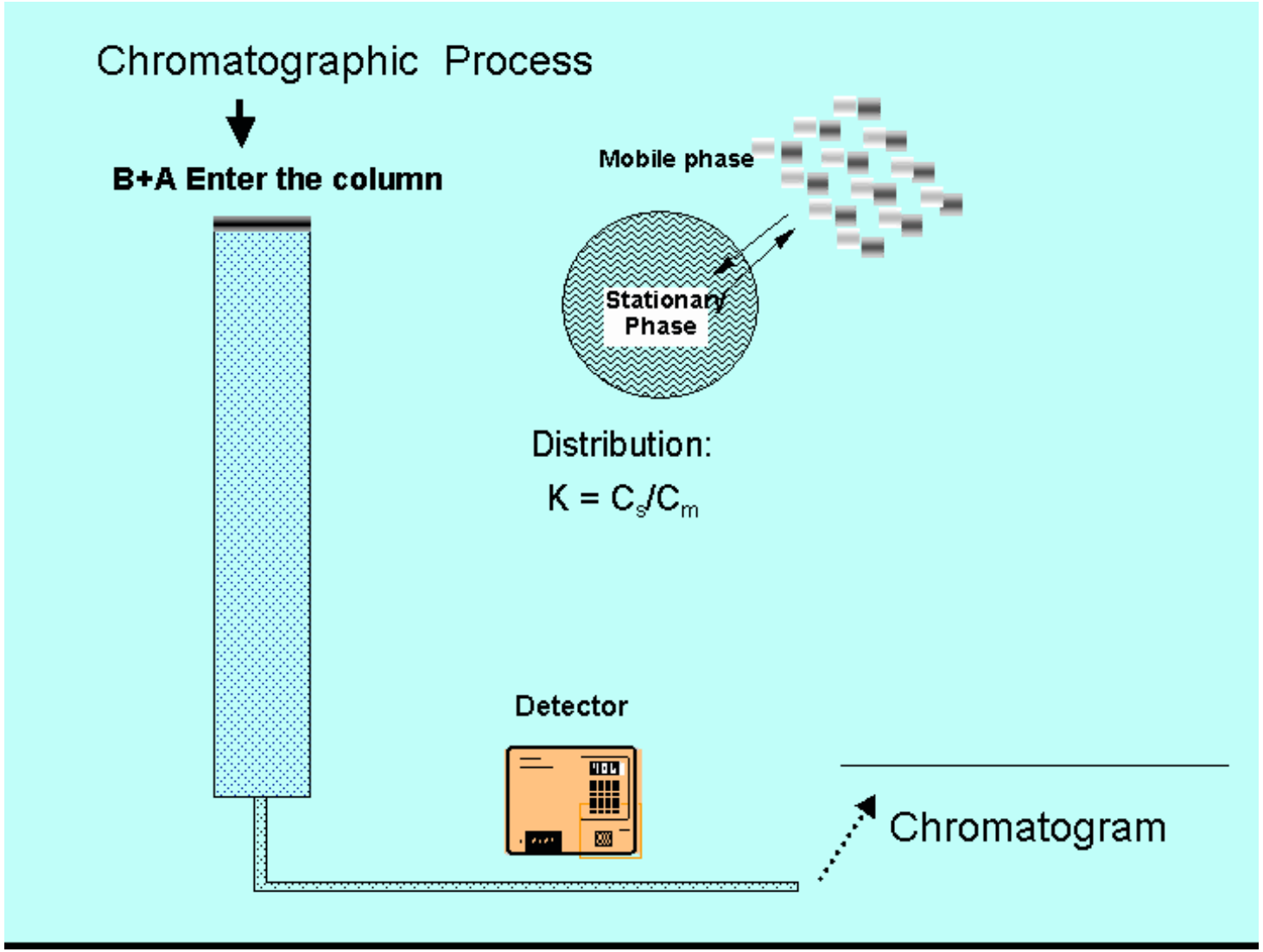
- This allows efficient separation of the two compounds and is the basis for all chromatography

pubs.acs.org/journals/chromatography/chap1.html

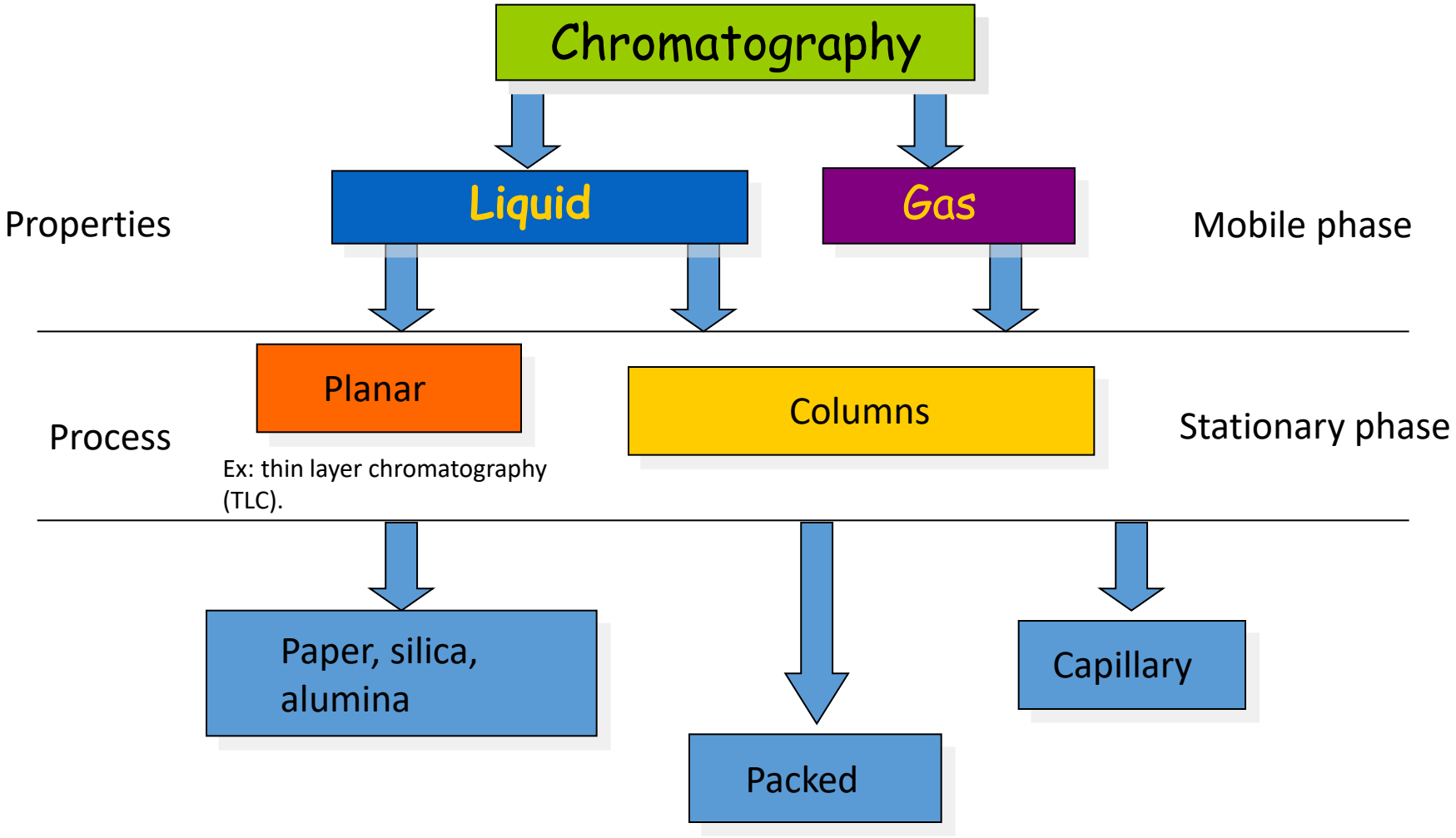
Basis of chromatography



Basis of chromatography



Different types of chromatography



Chromatography performances and parameters

There are various parameters used to determine how well our chromatography is behaving.

- Selectivity
- Efficiency
- Resolution

- Retention time

Time taken to reach the detector after injection

- Selectivity

Measurement of the difference in retention between components on the same column

- Efficiency

Measurement of band-broadening, function of peak width and retention

- Resolution

Extent of separation between 2 peaks (how well they are resolved)
Includes both selectivity and efficiency

- Capacity

Amount of sample that a chromatography system can separate

Retention time (t_R)

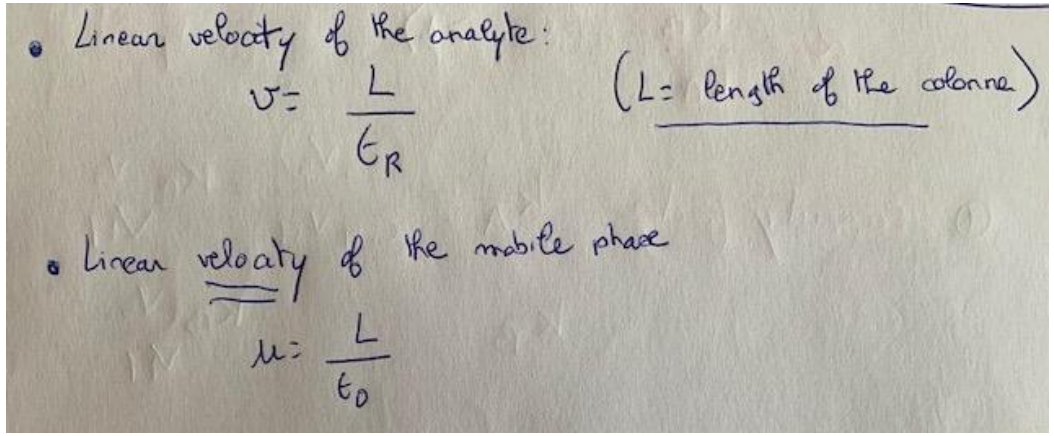
- Time taken to reach detector after injection (t_R)

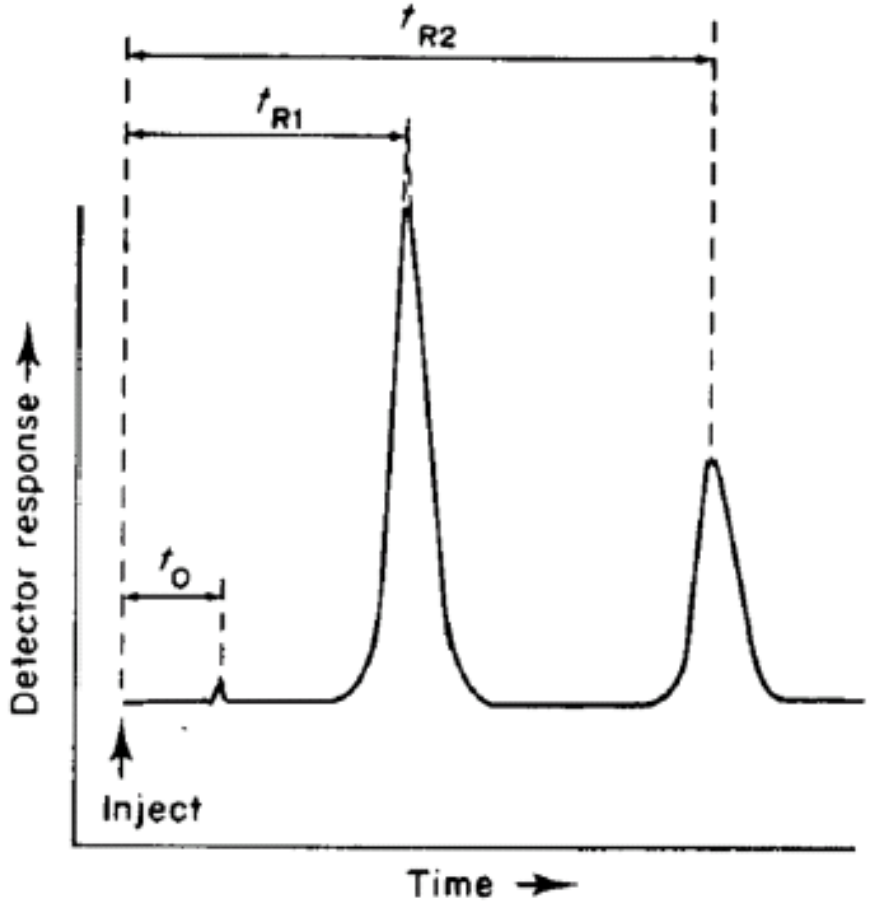
- Measured at peak apex

- Factors that affect t_R include:

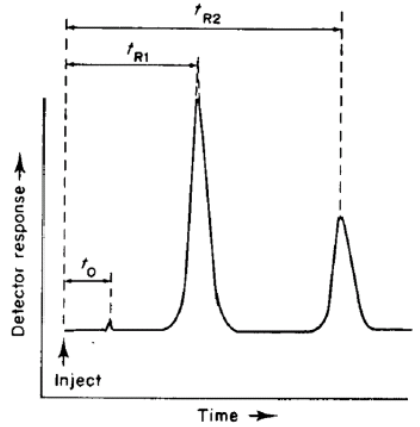
  - velocity (flow rate) of mobile phase
  - chromatographic retention

- Time spent in the stationary phase $t_R' = t_R - t_0$





Selectivity (α)



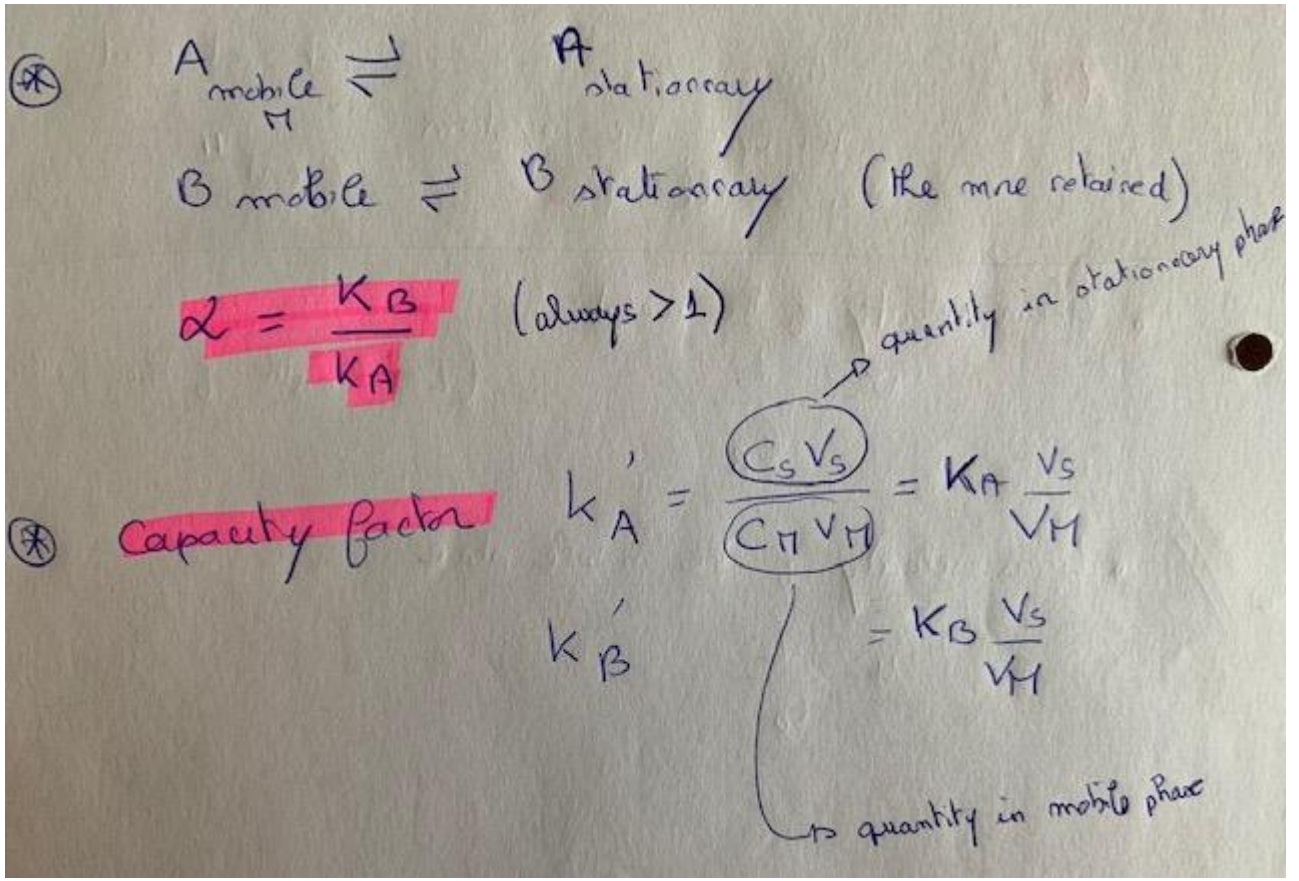
• Measurement of the difference in retention between two components in the sample:

$$\alpha = \frac{t_{R2} - t_0}{t_{R1} - t_0} = \frac{k'_2}{k'_1} = \frac{t'_{R2}}{t'_{R1}}$$

• t_{R1} and t_{R2} are retention times for compounds 1, 2

• k'_1, k'_2 = capacity factors for compounds 1, 2

• Separation factor doesn't account for peak widths



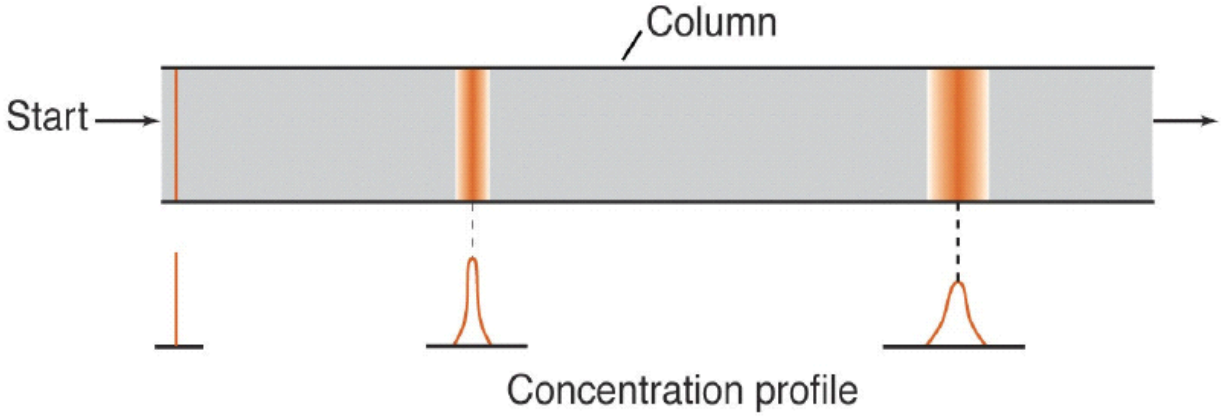
Efficiency and band broadening

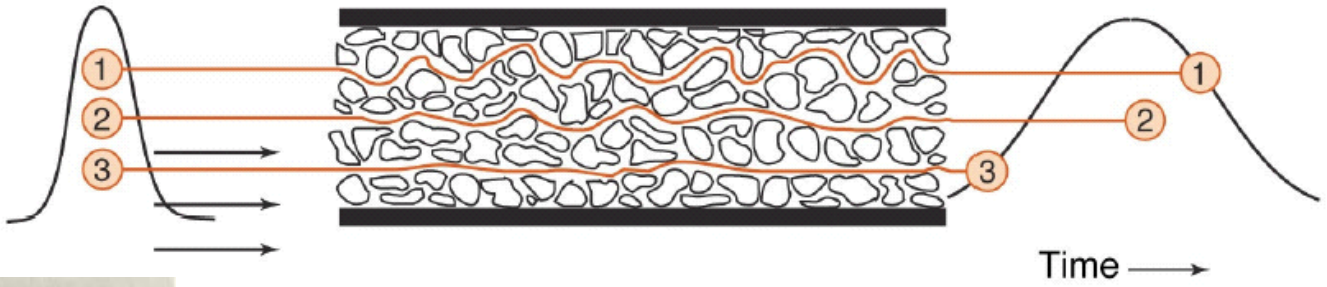
After injection, a narrow band is broadened during its movement through the column by:

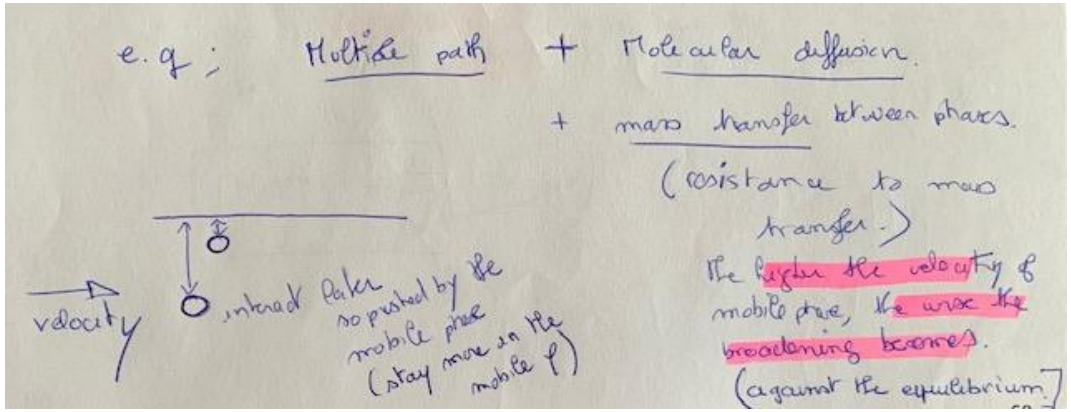
-multiple path of analyte

-Longitudinal diffusion

-mass transfer between phases





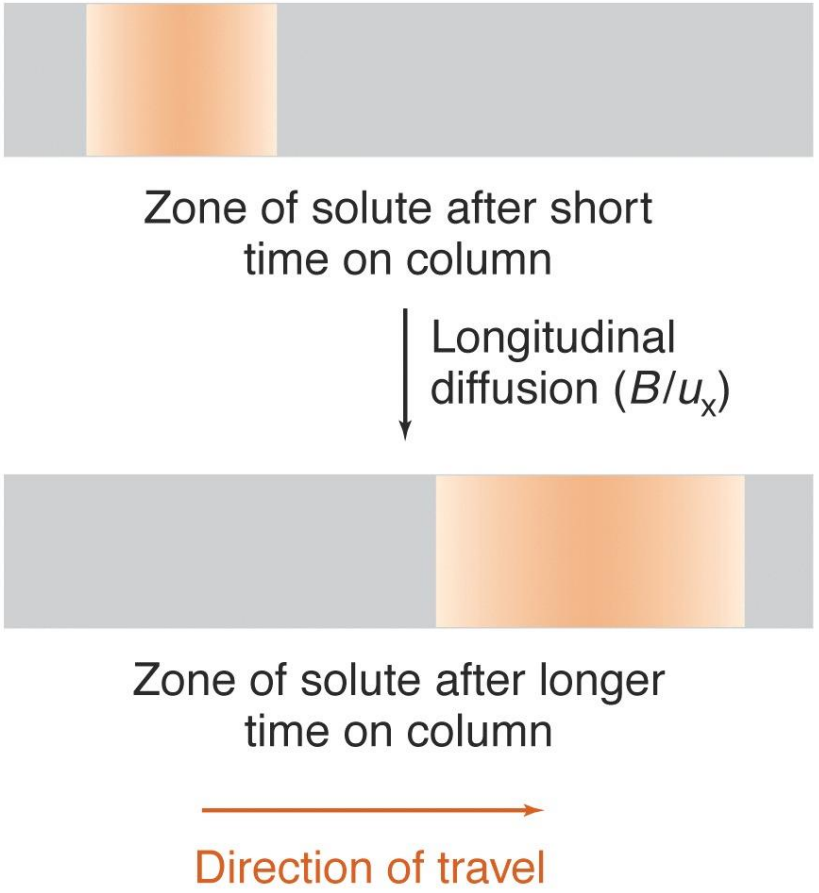


The higher the column band broadening, the smaller the number of components that can be separated.

The sharpness of the peak is an indication of how good, or efficient a column is.

Efficiency and band broadening

- The peak width is an indication of peak sharpness and, thus, an indication of the column efficiency
- The narrower the peaks, the better the ability to separate components
- Efficiency is a measurement of the band-broadening of a column. It is a function of peak width and retention volume (or retention time)



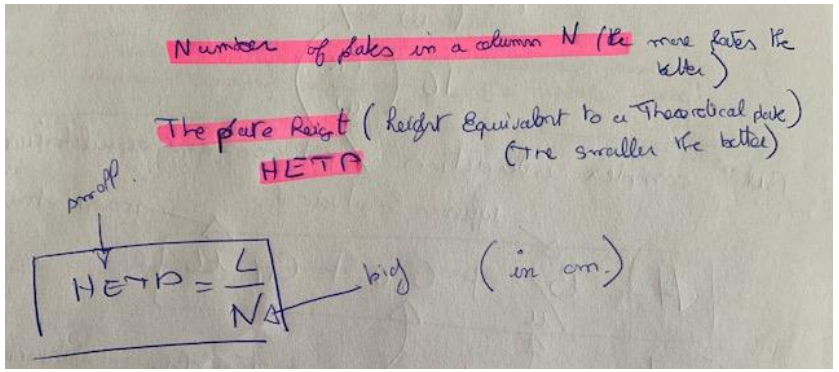
Efficiency and number of theoretical plates (*N*)

- It is primarily controlled by: column length, flow rate, particle size

- Flow rate is the only parameter which can be changed from run to run on the same column.

- The plate number ***N*** depends on column length ***L*** : the longer the column, the larger the plate number. Therefore, the plate height term ***h*** has been introduced to measure the efficiency of a column. ***h = L/N***

- The lower the plate height and the higher the plate number, the more efficient the chromatographic column

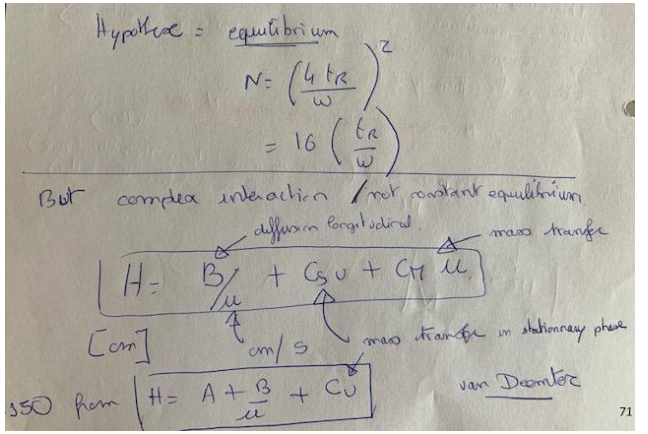


Theoretical plate model

- The chromatographic column is divided into a number of separate layers, called [theoretical plates]
- Separate partitioning of the sample between the stationary and mobile phase occur in these "plates“
- The analyte moves down the column by transfer of equilibrated mobile phase from one plate to the next
- This model serves as a way of measuring column efficiency by stating:
  - The number of theoretical plates in a column, *N* (the more plates the better)
  - The plate height (the smaller the better)
  - where *w* is the peak width at baseline
  - Columns behave as if they have different numbers of plates for different solutes in a mixture

$$HETP = \frac{L}{N}$$

$$N = \left(\frac{4\,t_R}{w}\right)^2$$

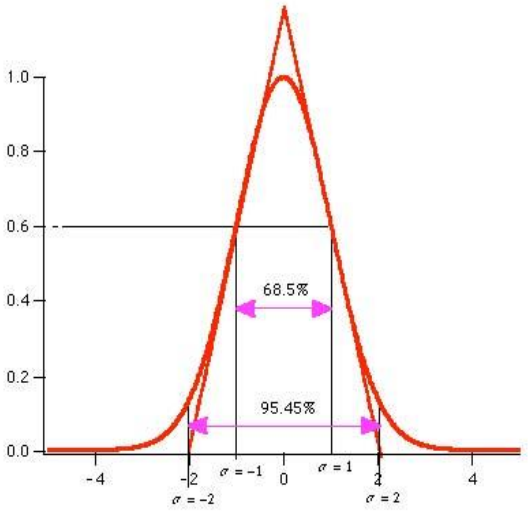


Resolution

- Although the selectivity factor α describes the separation of band centres, it does not take into account peak widths
- The resolution measures how well species have been separated
- The resolution *R* of two species, A and B, is defined as

$$R = \frac{[\, t_{R2} - t_{R1} \,]}{\frac{1}{2}(w_1 + w_2)}$$

where t_R is the retention time and *w* is the peak width at baseline.



Resolution

- Resolution is the difference in retention (time or volume) divided by the average base peak width

- *R* is a measure of two separate parameters – selectivity and efficiency

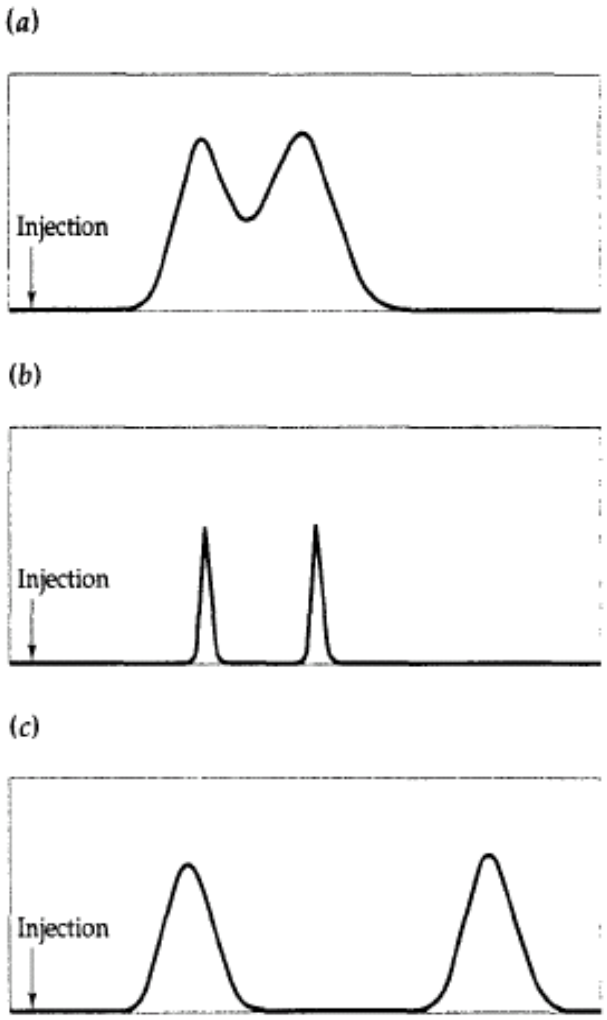
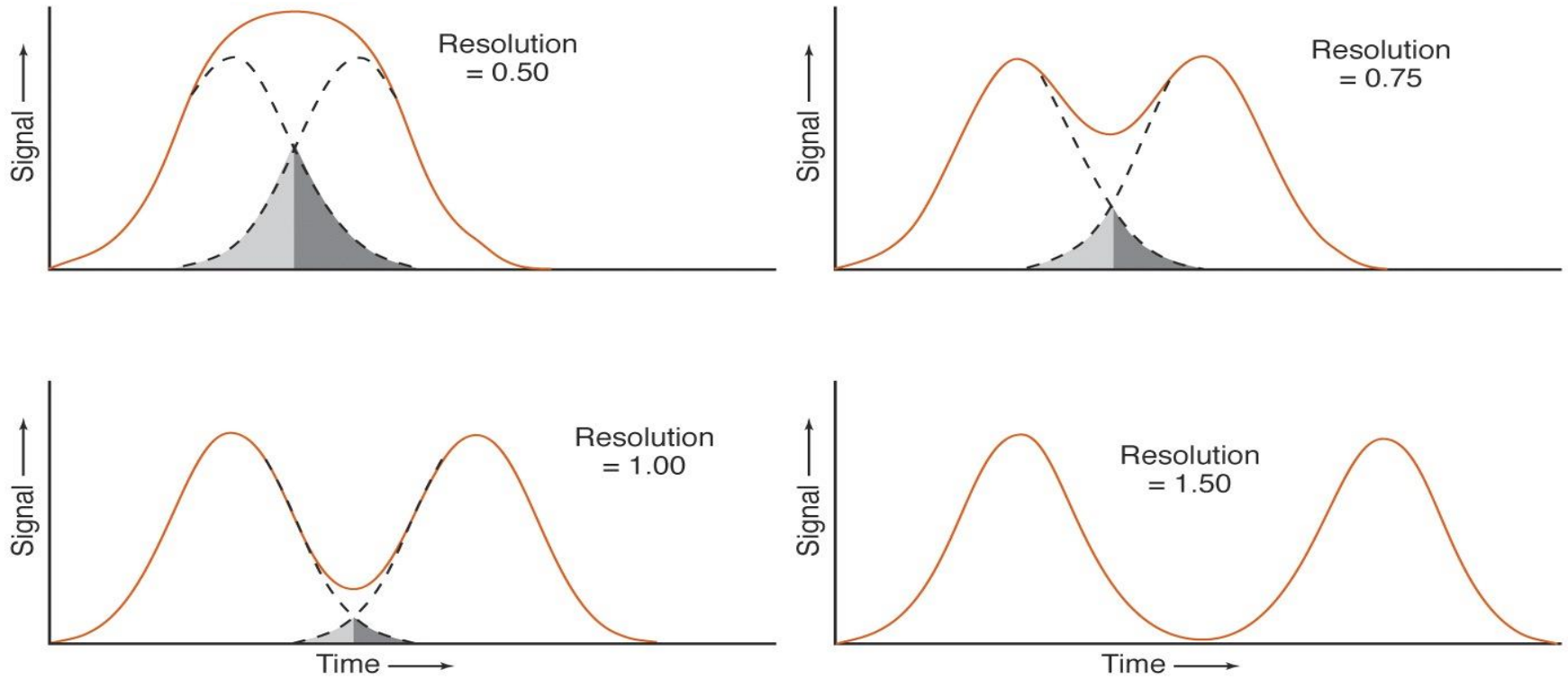


FIGURE 13.5 ▶
Improving resolution.

(a) The two partially resolved components can be resolved more completely by (b) sharpening the zones, (c) spreading them further apart, or both. Assume the flow rate is the same for all three chromatograms and the samples are identical. The conditions of (c) are not as good as those in (b); it takes longer to obtain a separation of the same quality.

Note that the sensitivity of the vertical scale in the middle chromatogram is decreased. If the vertical scales were all the same, the peak heights of (b) would be significantly larger than those of (a) or (c). However, the peak areas in all three would be equal since the quantities of analytes are equal.

Resolution


Signal
Resolution
= 0.50
Signal
Resolution
= 0.75
Signal
Resolution
= 1.00
Time
Signal
Resolution
= 1.50
Time

Factors affecting resolution

1. Column length
2. Column diameter
3. Flow-rate
4. Uniformity of column packing
5. Uniformity of stationary phase (packing material)
6. Sample size
7. Suitability of stationary phase
8. Suitability of mobile phase
9. Pressure
10. Gradient elution

Capacity

- Determines the size of column and system requirements for a given sample amount
- Saturation or equilibrium capacity – maximum amount of sample that will bind using the given mobile phase
- Measured by mixing a known amount of stationary phase with an excess of the analyte and measuring the bound versus free analyte
- There are also other methods for expressing column capacity *e.g.* dynamic capacity (which takes into account the flow on the column)
- When the capacity of the column is exceeded, asymmetric peaks are observed

Asymmetric peaks

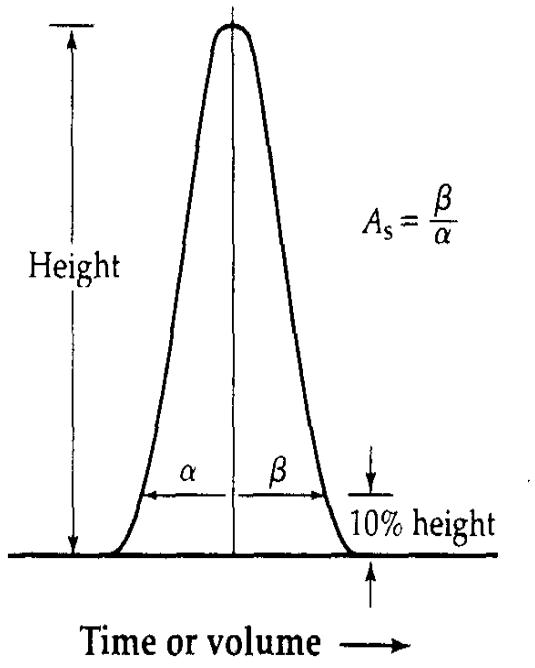


FIGURE 13.21 ▲
Definition of the peak asymmetry factor A_s.

- Chromatographic parameters assume symmetric, Gaussian peaks
- Symmetric: $A_s = 1.0$
- Tailing: $A_s > 1.0$
- Fronting: $A_s < 1.0$

  Fronting peaks are rare

Asymmetric peaks

Tailing is usually due to overloading the column by adding too much sample

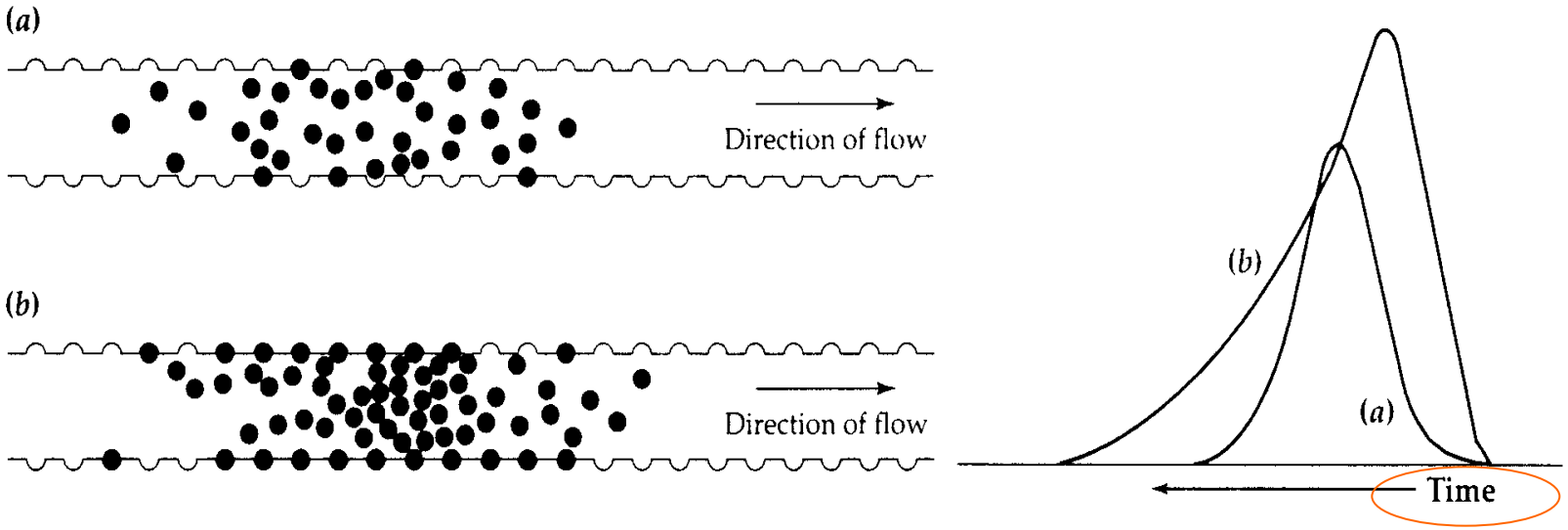
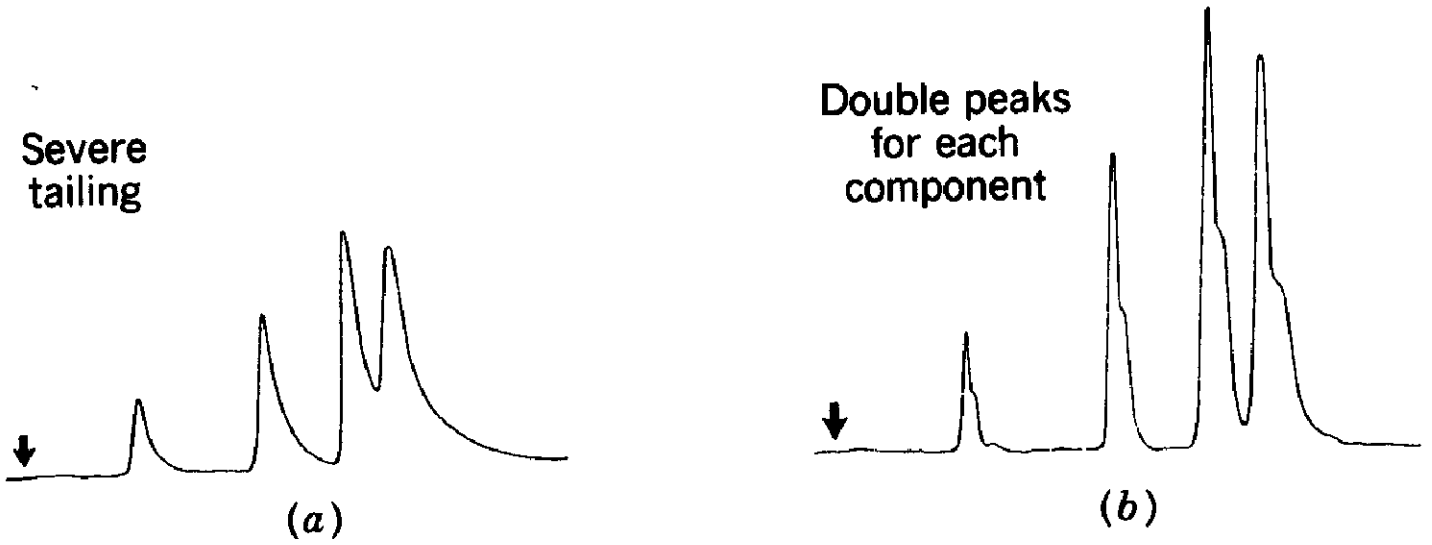


FIGURE 13.19 ▲
Illustration of the effects of overloading a stationary phase in chromatography.

chem.unk.edu/chem475/ch13slides.pp

Asymmetric peaks

Tailing and double peaks can also result from column void, a plugged inlet frit, and/or irreversible adsorption of analytes on the column



Q2: Questions?

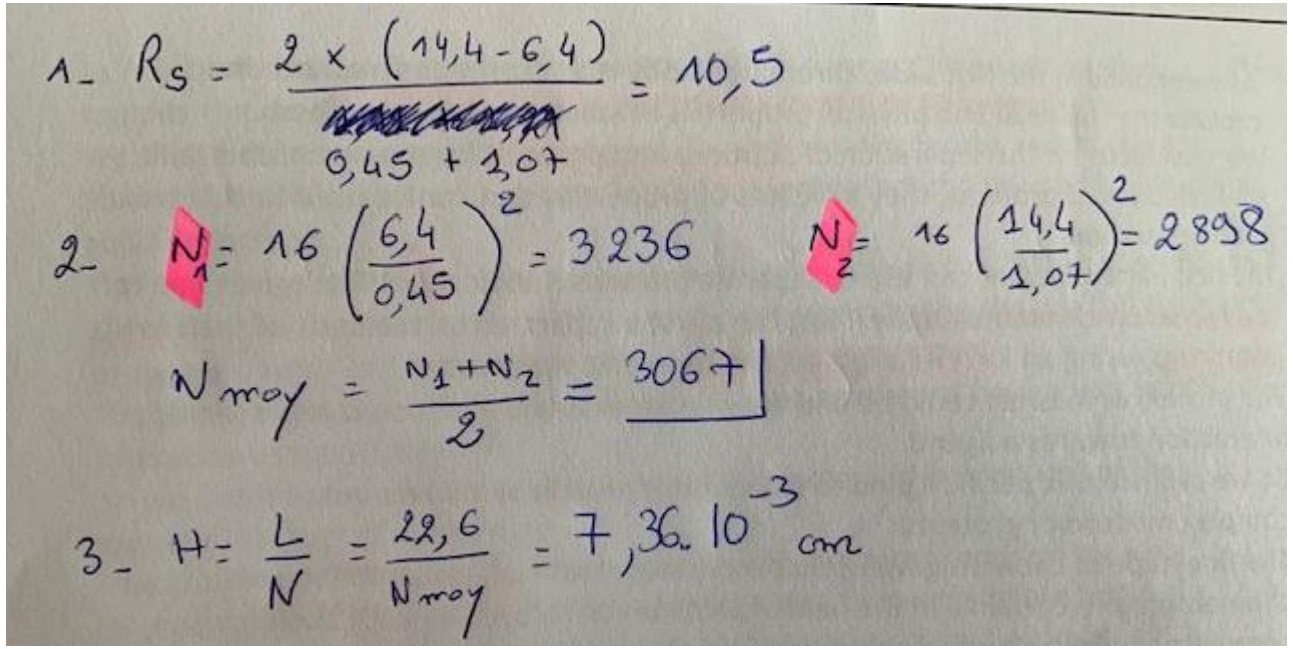
- What are some measures of chromatographic performance?
- What is resolution and what factors can affect it?
- What happens if too much sample is loaded on a column?

Exercise: Substances A and B have retention times of 6.4 and 14.4 min, respectively, on a 22.6 cm column. An unretained sample of air passed through the column in 1.30 min. The widths of the peak bases were 0.45 and 1.07 min. Calculate the:

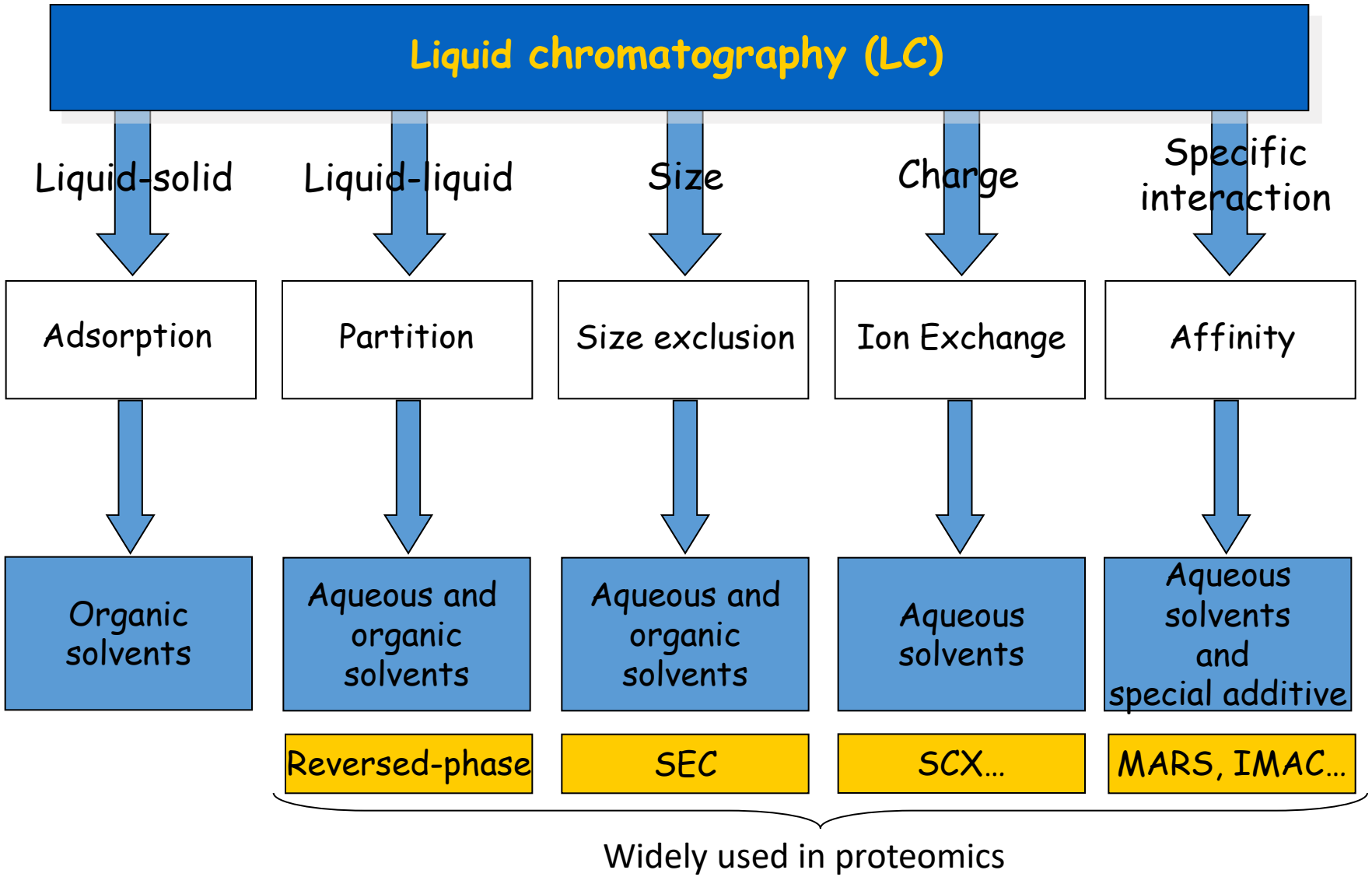
1.The column resolution

2.The average number of plates

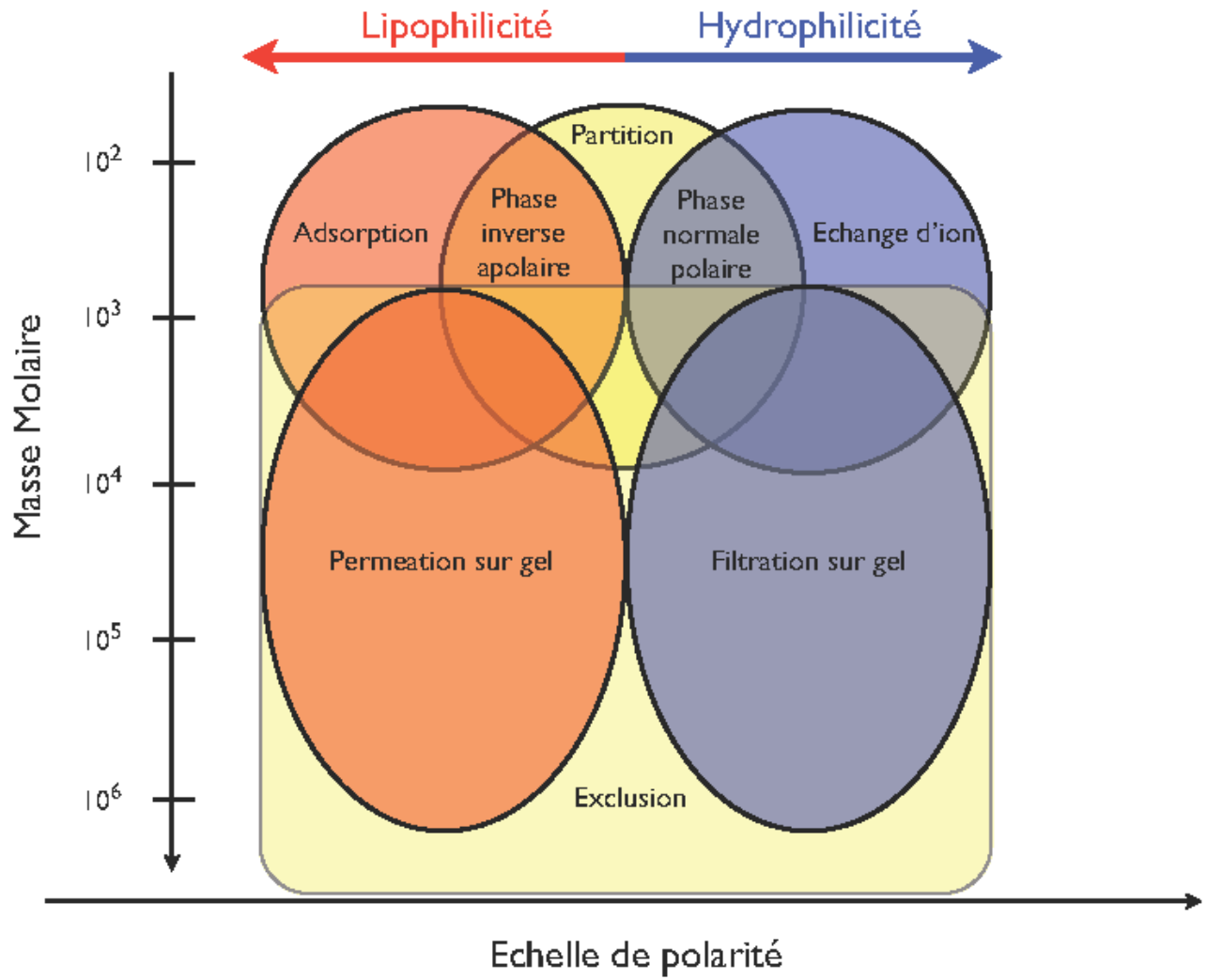
3.The plate height



Liquid chromatography (LC)



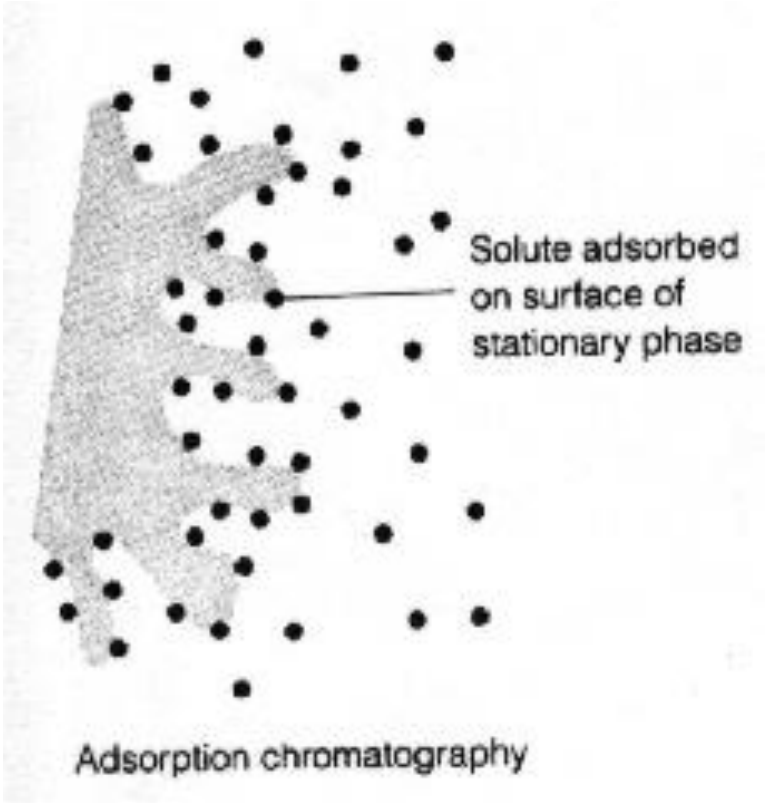
Liquid chromatography (LC)



http://lepa.epfl.ch/ICP3_lecture_notes/Separation_Methods/Cours_2005/5_HPLC_SEC.pdf

Adsorption chromatography

Adsorption chromatography is probably one of the oldest types of chromatography around. It utilizes a mobile liquid that is adsorbed onto the surface of a stationary solid phase

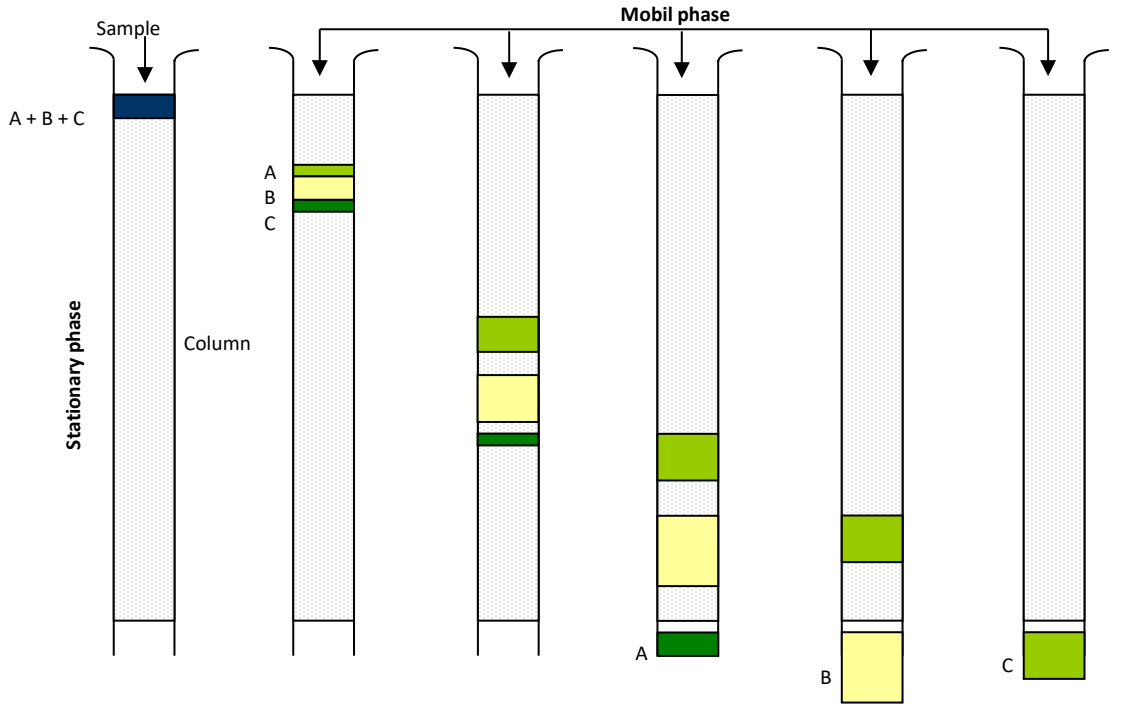


Adsorption chromatography

Adsorption chromatography is based mainly on differences between the adsorption affinities of the sample components for the surface of an active solid

Adsorption chromatography

Normal-phase chromatography is based on an elution procedure in which the stationary phase is more polar then the mobile phase. This term is used in liquid chromatography to emphasize the contrast to reversed-phase chromatography.



http://www.iupac.org/goldbook/N04214.pdf

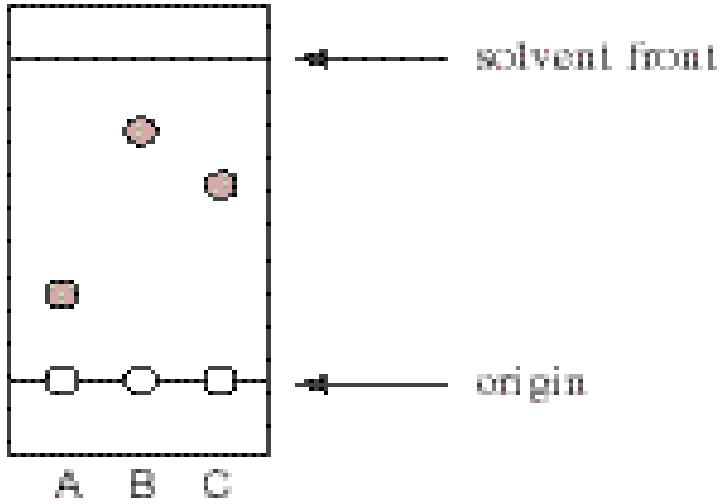
The most used polar adsorbent are:

- Silica (acidic)
- Alumina (basic)

Adsorption chromatography

Column chromatography

Thin layer chromatography (TLC)



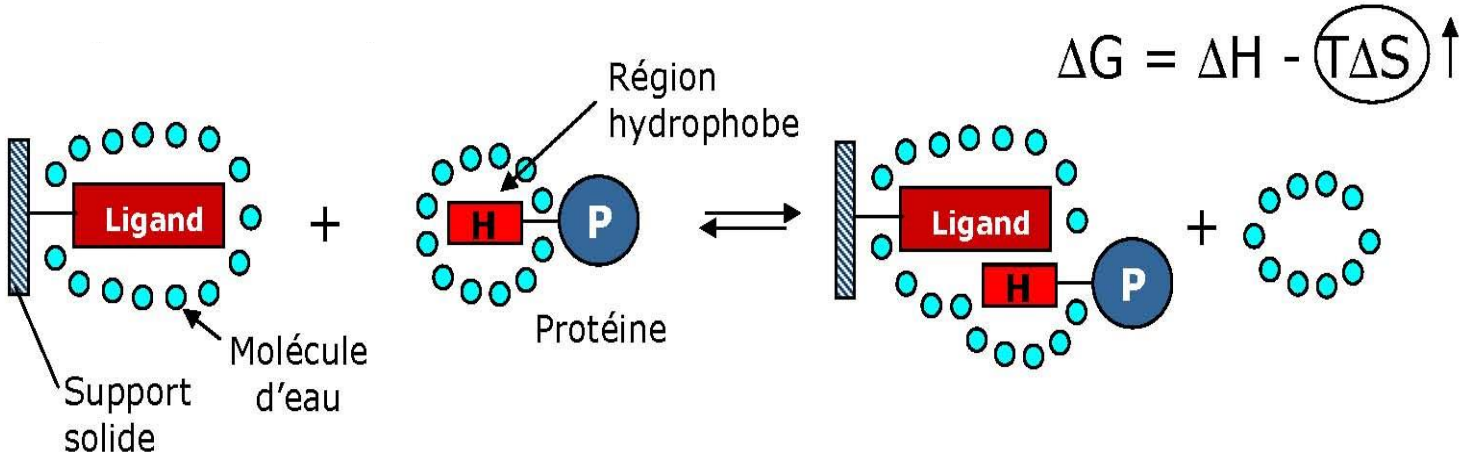
Reversed-phase (RP) chromatography

- Historically has been named “reversed-phase HPLC“ because unlike “normal phase” chromatography it uses non-polar stationary phase and polar mobile phase

- RP-HPLC has quickly grown in its number of applications, and now it is the most widely used HPLC mode

- RP-LC exploits the differences of hydrophobicity of proteins and peptides

Reversed-phase (RP) chromatography

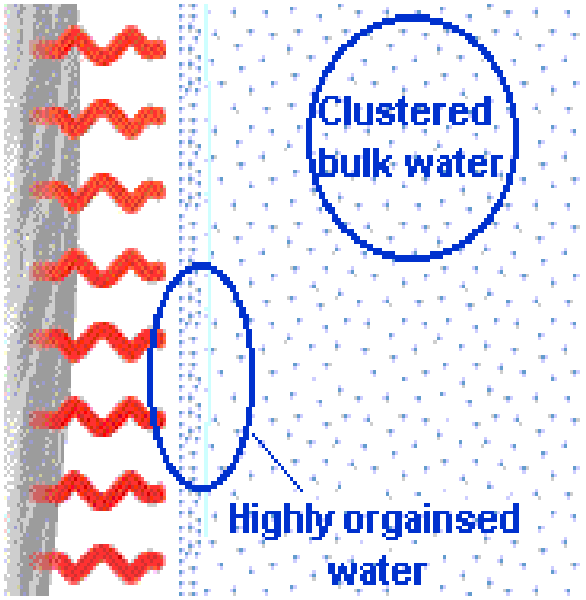


Water molecules at the ligand surface and the hydrophobic protein region are more organized than in solution

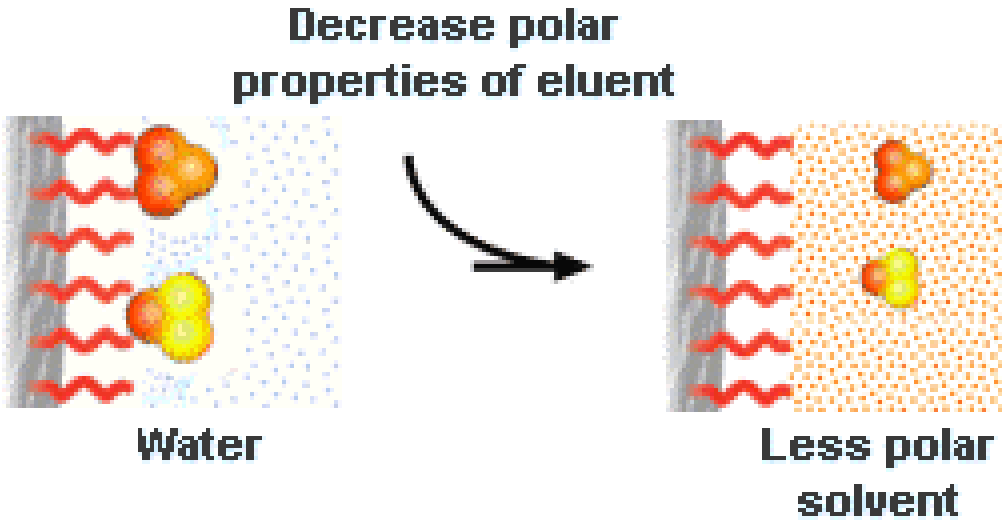
The displacement of the water molecules around the ligand surface and the hydrophobic protein region induces an increase of the entropy, that is thermodynamically favorable

http://www.esi.umontreal.ca/~badiaa/CHM3102_chromato3a.pdf

Reversed-phase (RP) chromatography

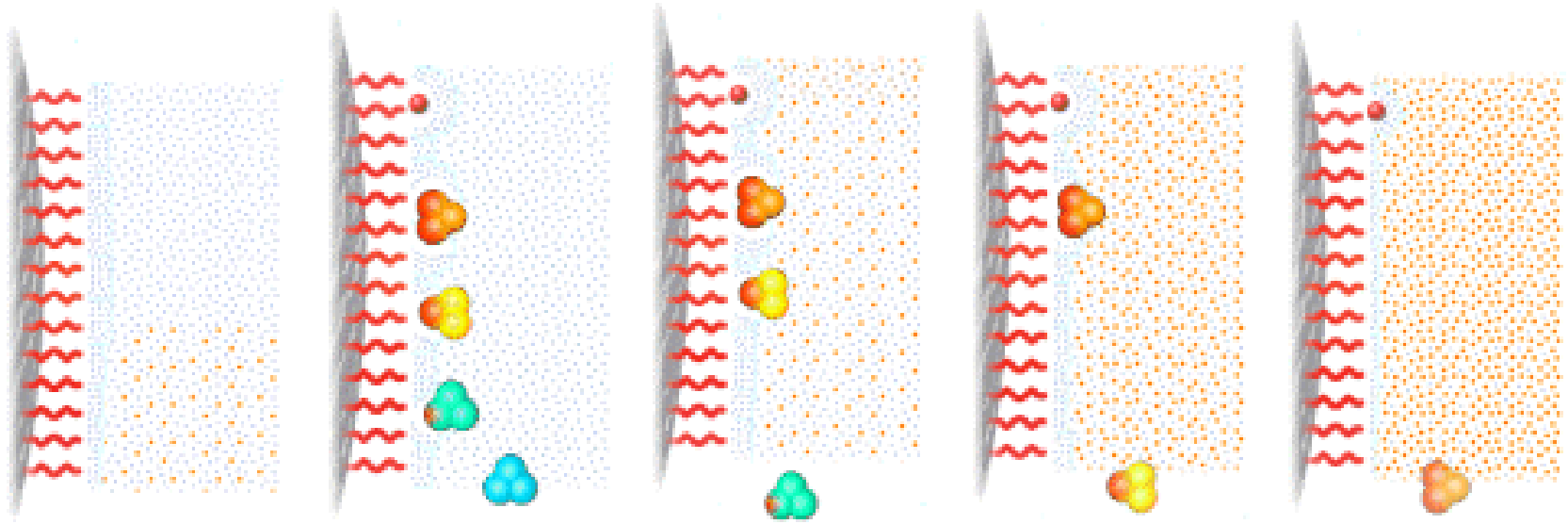


- Proteins are retained by the solid phase (silica carrying hydrophobic groups (C4, C8, C18)) according to their hydrophobicity



Reversed-phase (RP) chromatography

- When solvent is very polar all proteins will be captured (solvent polarity drives hydrophobic interactions)
- As polarity decreases, proteins are eluted from the column according to hydrophobicity (least hydrophobic first)

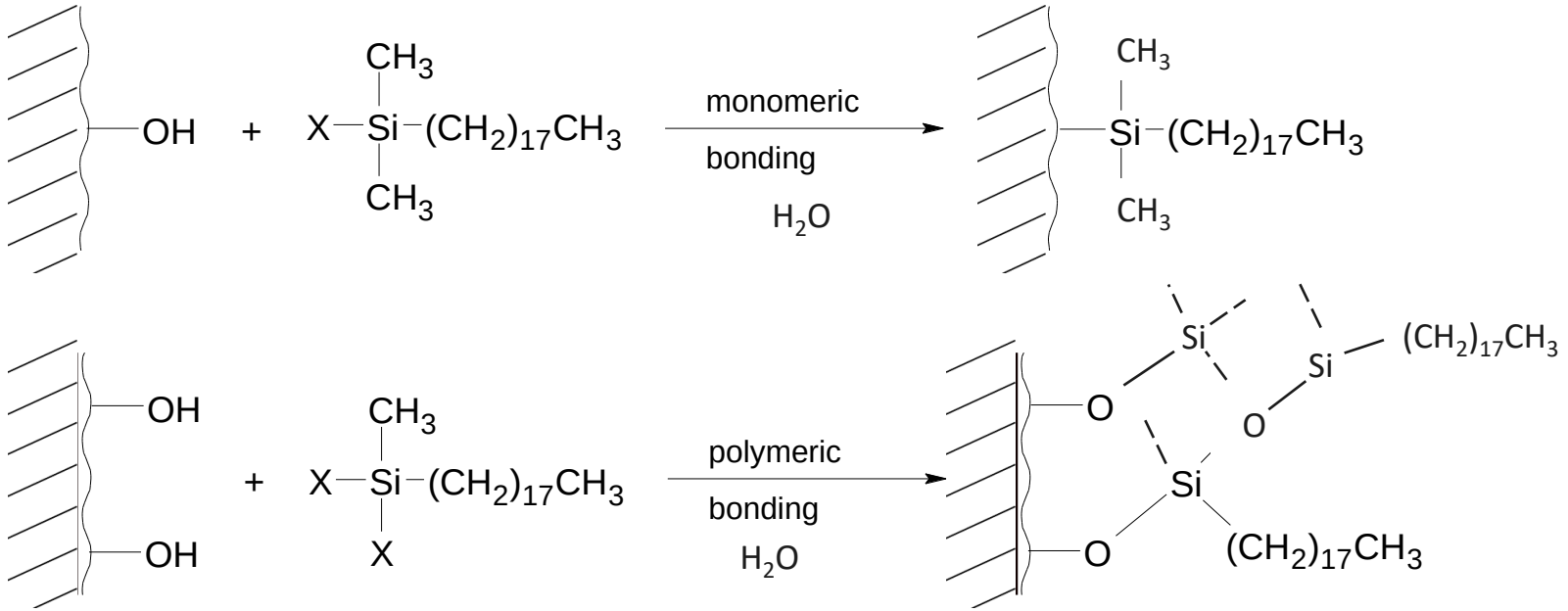


Reversed-phase (RP) chromatography

Organic modifier	**Suitable for:**	**UV cut-off**	**Viscosity**	**Comments**
Methanol	Org. molecules	210	Medium- low	May destabilize protein structure
Ethanol	Org. molecules	205	Medium- low	May destabilize protein structure
Isopropanol	Proteins Peptides	210	High	Least effect on protein structure
Acetonitrile (CH_3CN)	Org. molecules Proteins Peptides	190	Low	Less effect on Protein structure

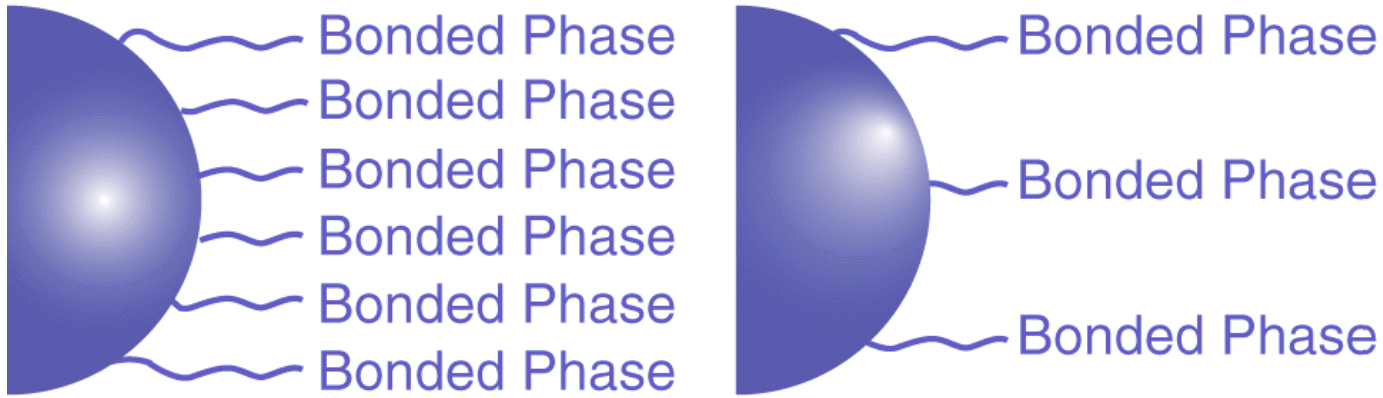
Bonding type

- Monomeric bonding offers increased mass transfer rates, higher column efficiency, and faster column equilibration
- Polymeric bonding offers increased column stability, particularly when highly aqueous mobile phases are used
- Polymeric bonding also enables the column to accept higher sample loading



Carbon load

- Higher carbon loads generally offer greater resolution and longer run times
- Low carbon loads shorten run times and many show a different selectivity

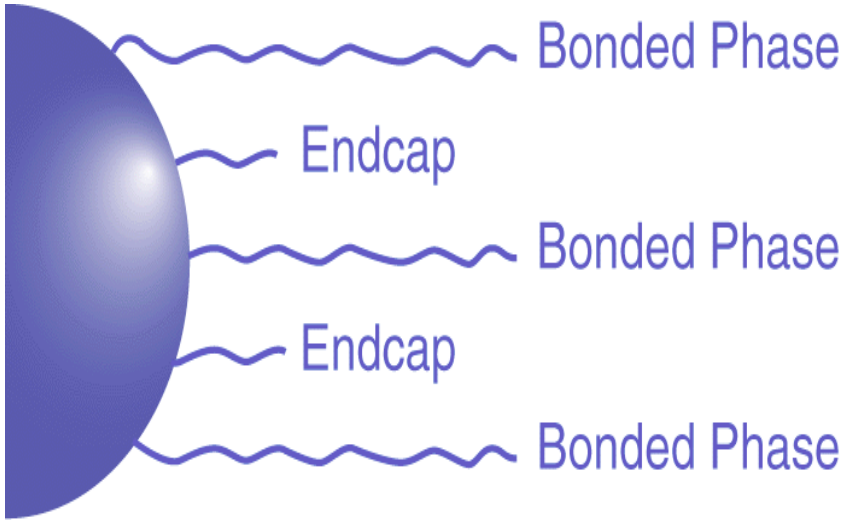


Endcapping

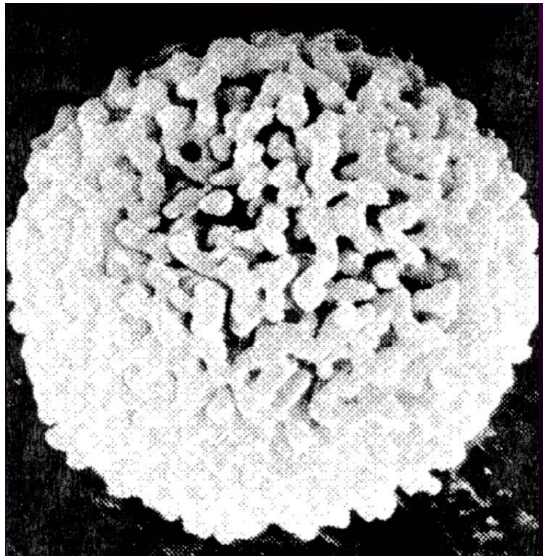
• Definition: blocking remaining free silanols of silica following attachment of alkyl chains

• Endcapping reduces peak-tailing of polar solutes that interact excessively with the otherwise exposed, mostly acidic silanols

• Non-endcapped packings provide a different selectivity than do endcapped packings, especially for such polar samples



Particles

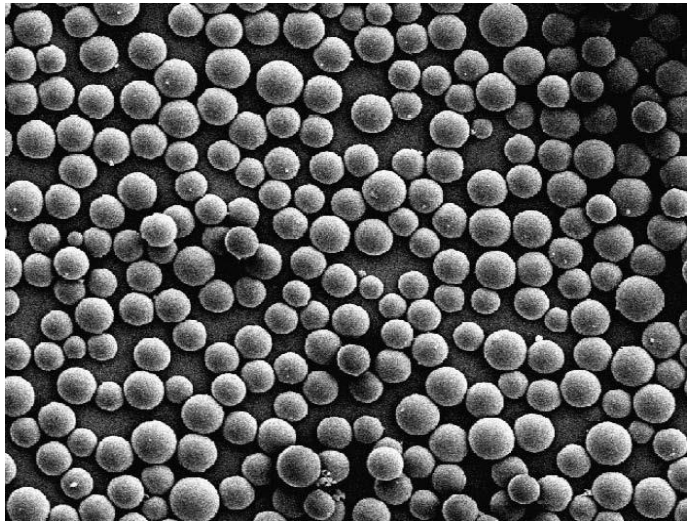


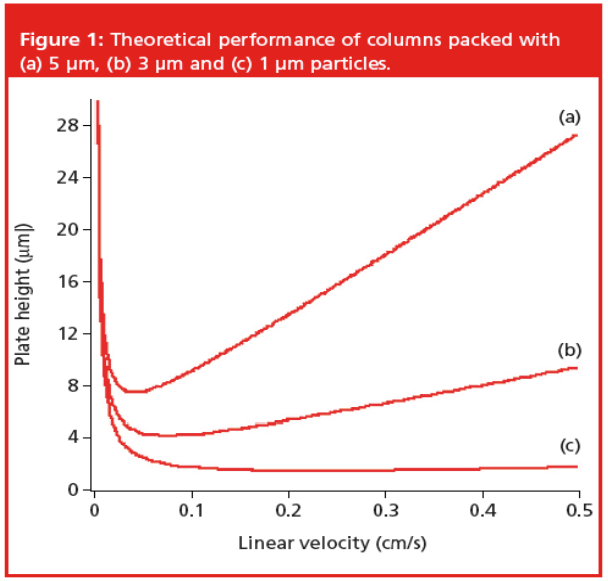
Size (smaller particles give higher efficiency but require higher operating pressures)

- usually 3-10 mm for analytical HPLC separations
- 10-40 mm for preparative HPLC
- 40-150 mm for low pressure or large scale preparations

Pore (pore size defines an ability of the analyte molecules to penetrate inside the particle and interact with its inner surface) General guide:

- Choose a pore size of 150 Å or less for sample *MW* < 2000
- Choose a pore size of 300 Å or greater for sample *MW* > 2000

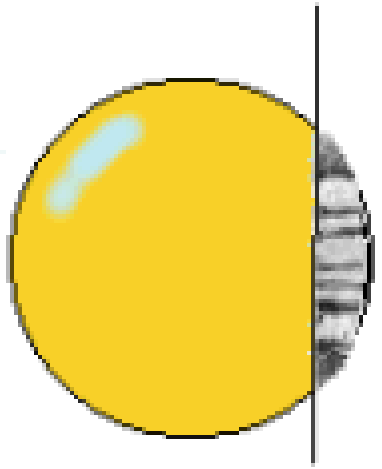




Van Deemter curves

Gel filtration - size exclusion chromatography (SEC)

- Separates proteins according to their size and shape
- Separation depends upon differences in the ability of different proteins to gain access to pores in the resin beads



Resin bead

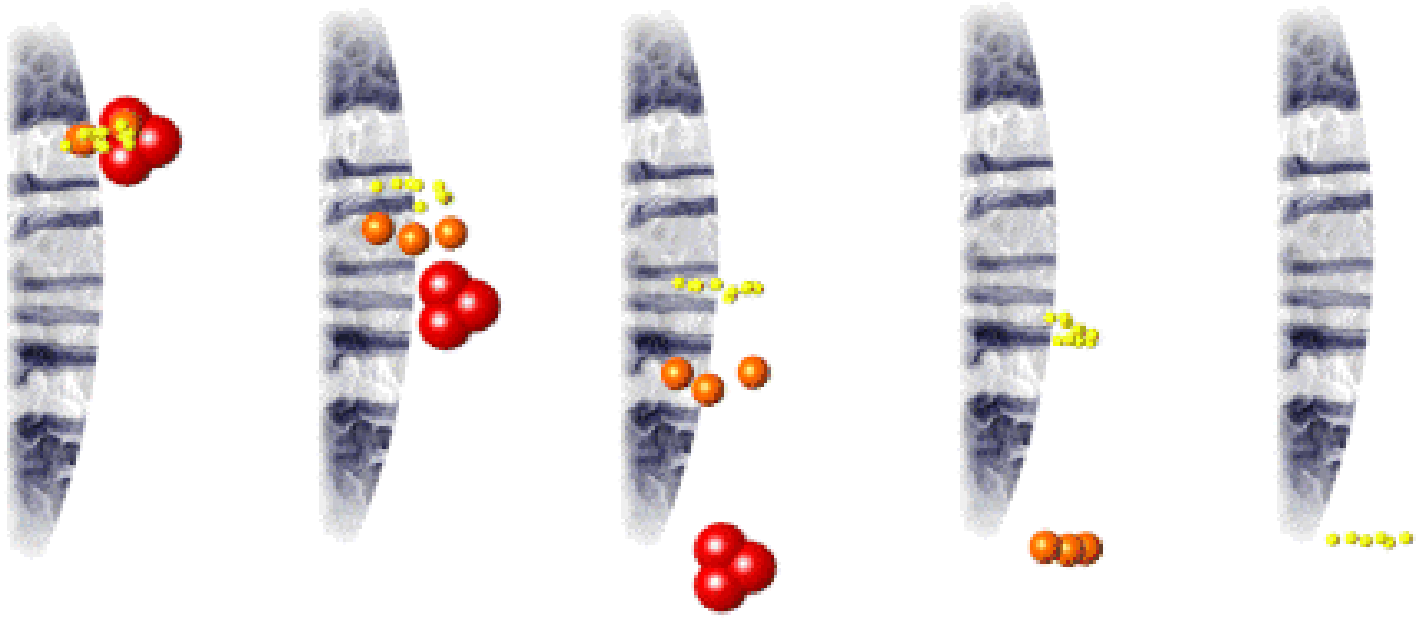
Large protein – no access to pores

Medium-sized protein – partial access to pores

Small protein – full access to pores

Gel filtration - size exclusion chromatography (SEC)

- Larger molecules have a lower volume to diffuse through, so they are eluted more rapidly than smaller molecules

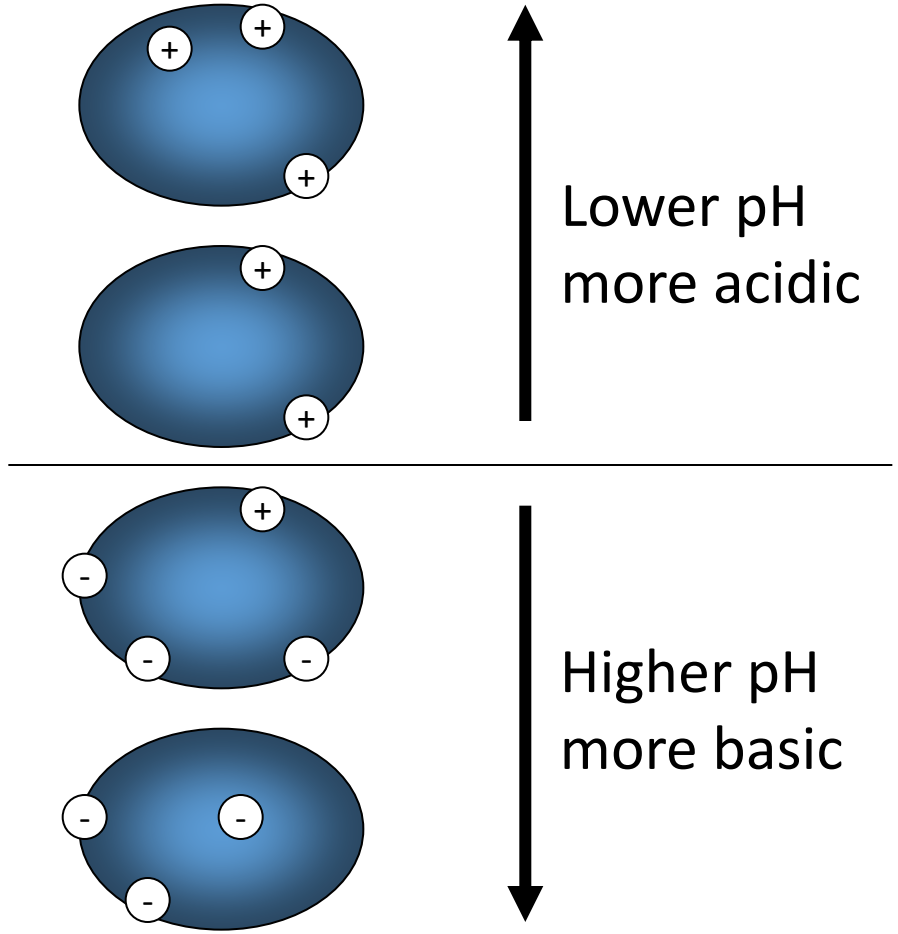


Ion exchange chromatography (IEC)

- is a process that allows the separation of ions and polar molecules based on their charge properties
- IEC retains analyte molecules based on coulombic (ionic) interactions
- The stationary phase surface displays ionic functional groups that interact with analyte ions of opposite charge
- Cation exchange chromatography retains positively charged cations because the stationary phase displays a negatively charged functional group such as a phosphonic acid
- Anion exchange chromatography retains negatively charged anions using positively charged functional group such as a quaternary ammonium cation

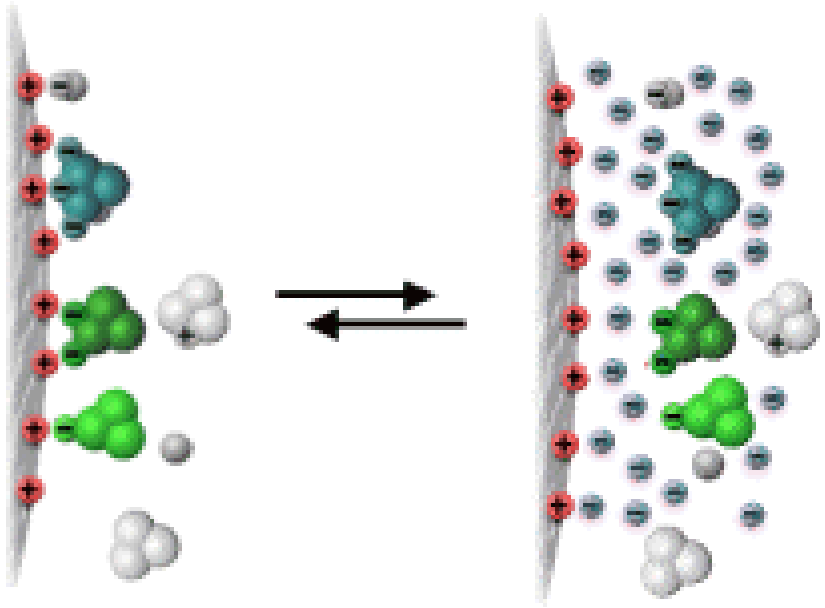
Ion exchange chromatography (IEC)

- Based on the net surface charge of the protein.
- This will depend on the protein's isoelectric point and the pH of the solution (buffer) used for the chromatography.



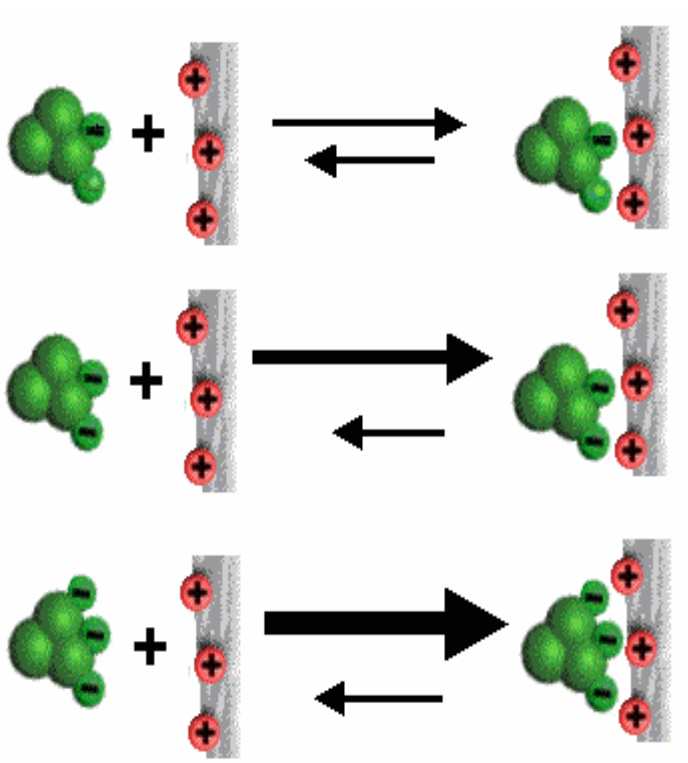
Ion exchange chromatography (IEC)

- In this example, negatively charged proteins bind to a positively charged resin
- They can be eluted by adding an excess of salt – the anion competes with the protein for positive charges on the solid phase thus displacing it



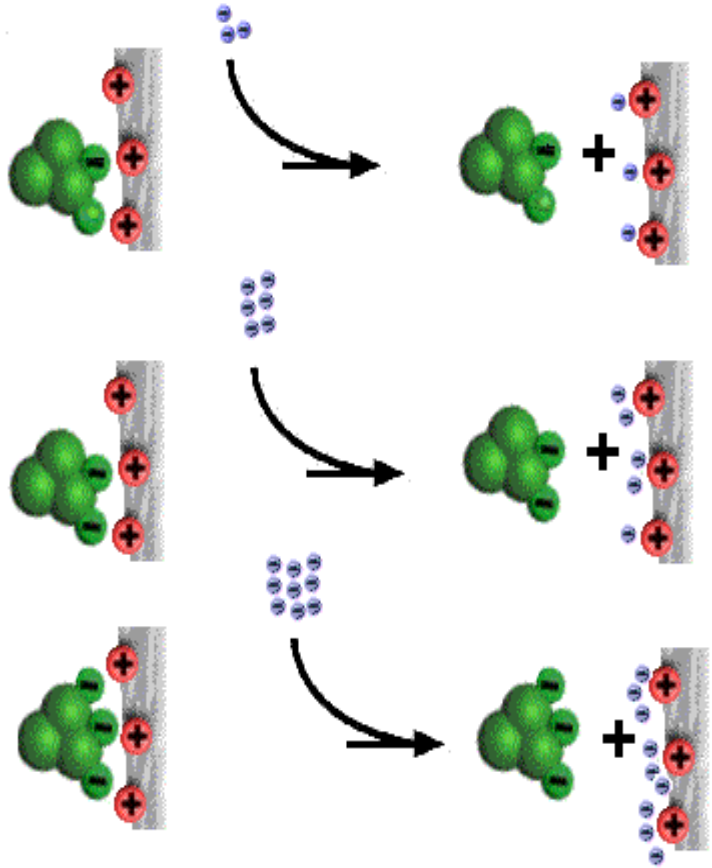
Ion exchange chromatography (IEC)

- Proteins that carry more negative charges have higher affinity for the positively charged surface.



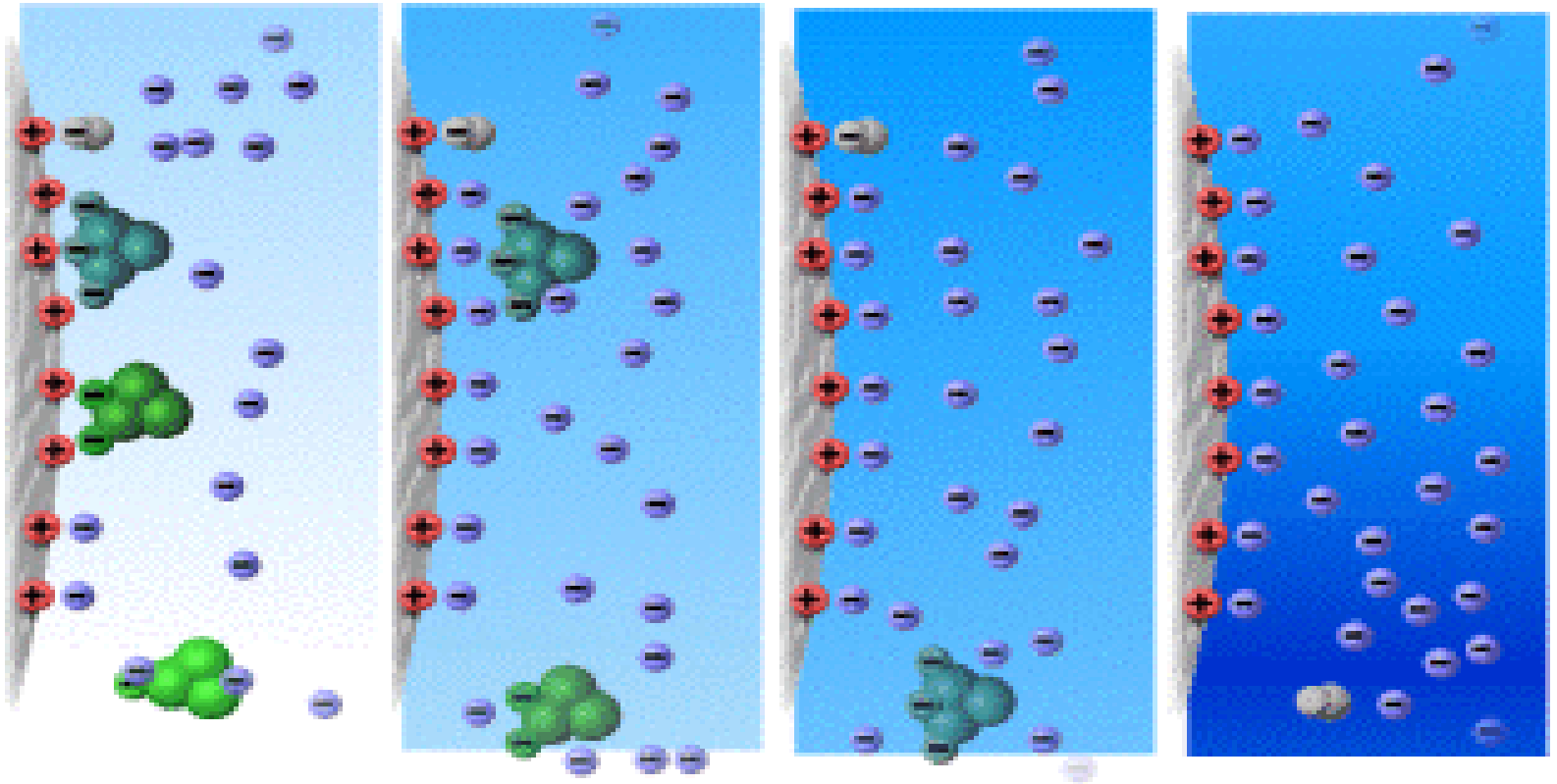
Ion exchange chromatography (IEC)

- Lower salt concentrations are required to elute the less charged proteins from the column.



Ion exchange chromatography (IEC)

- Gradient elution (increasing salt concentration) allows sequential elution of proteins according to their surface charge.



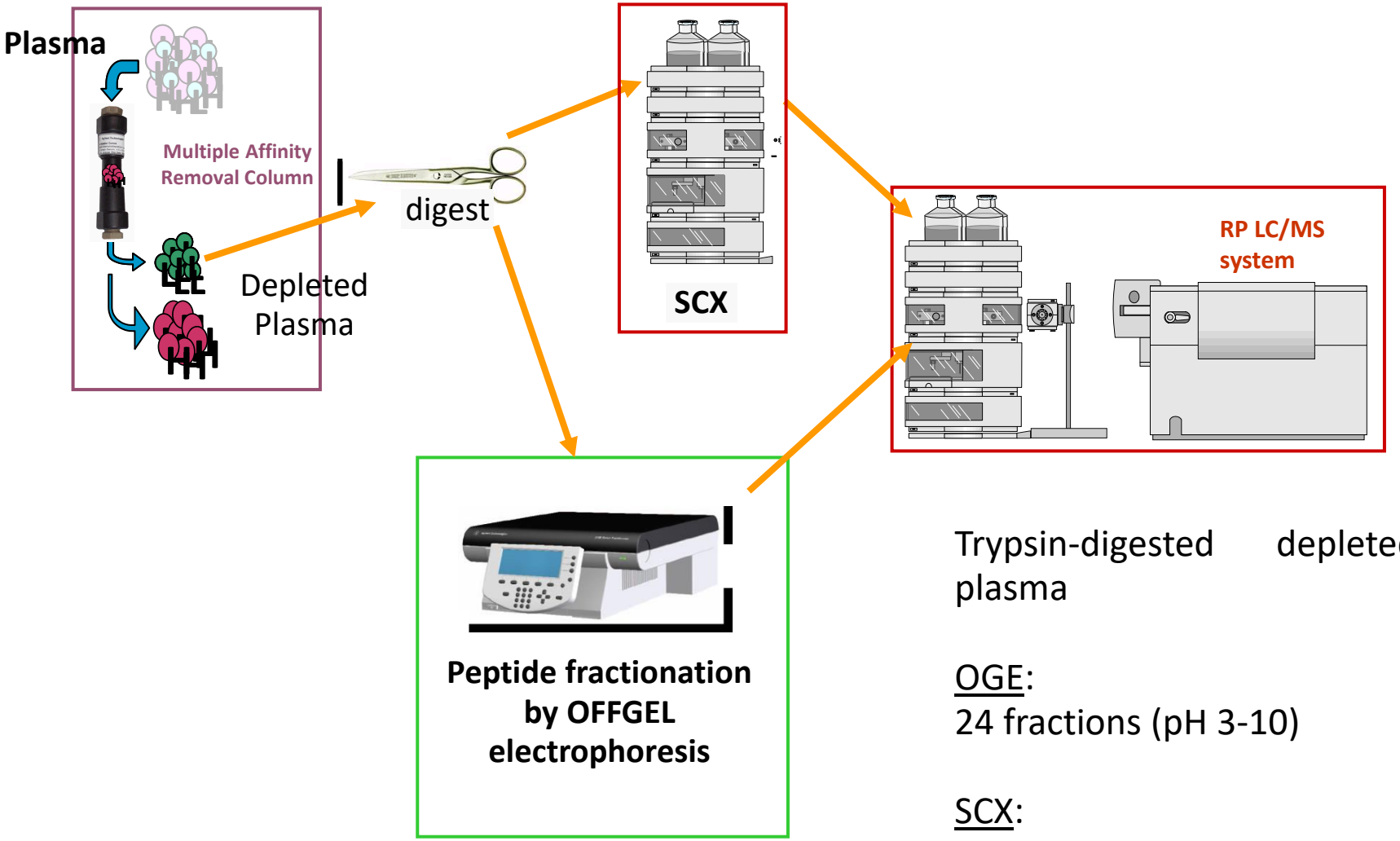
Ion exchange chromatography (IEC)

- Relatively low specificity
- Resin has high binding capacity
- Elution conditions are mild – unlikely to damage proteins

Anion exchangers	Functional group
Diethylaminoethyl (**DEAE**)	-O-CH_2-CH_2-$N^+H(CH_2$-$CH_3)_2$
Quaternary aminoethyl (**QAE**)	-O-CH_2-CH_2-$N^+(C_2H_5)_2$-CH_2-CHOH-CH_3
Quaternary ammonium (**Q**)	-O-CH_2-CHOH-CH_2-O-CH_2-CHOH-CH_2-$N^+(CH_3)_3$

Cation exchangers	Functional group
Carboxymethyl (**CM**)	-O-CH_2-COO^-
Sulfopropyl (**SP**)	-O-CH_2-CHOH-CH_2-O-CH_2-CH_2-CH_2-SO_3^-
Methyl sulfonate (**S**)	-O-CH_2-CHOH-CH_2-CH_2-CHOH-CH_2-SO_3^-

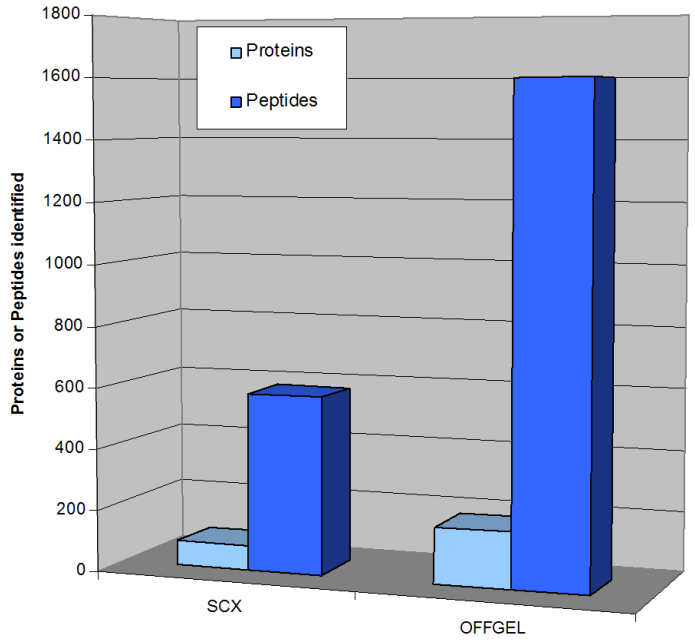
Off-gel IEF *versus* SCX chromatography



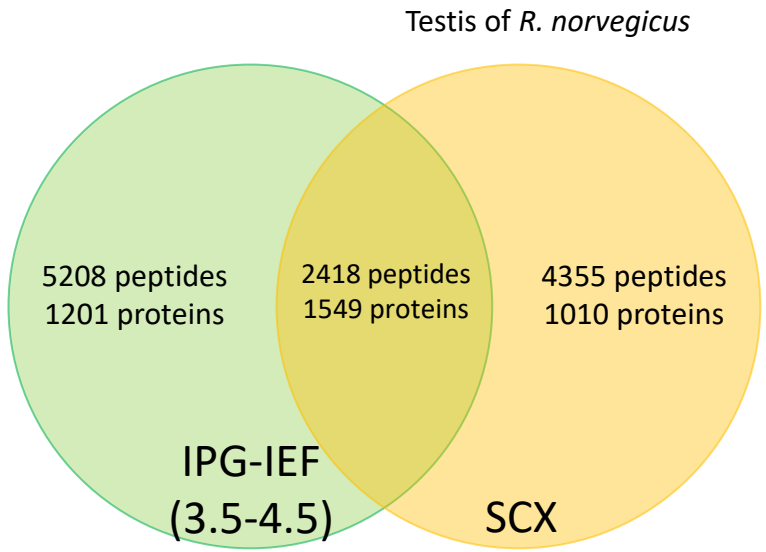
Trypsin-digested depleted plasma

OGE:
24 fractions (pH 3-10)

SCX:
50 fractions

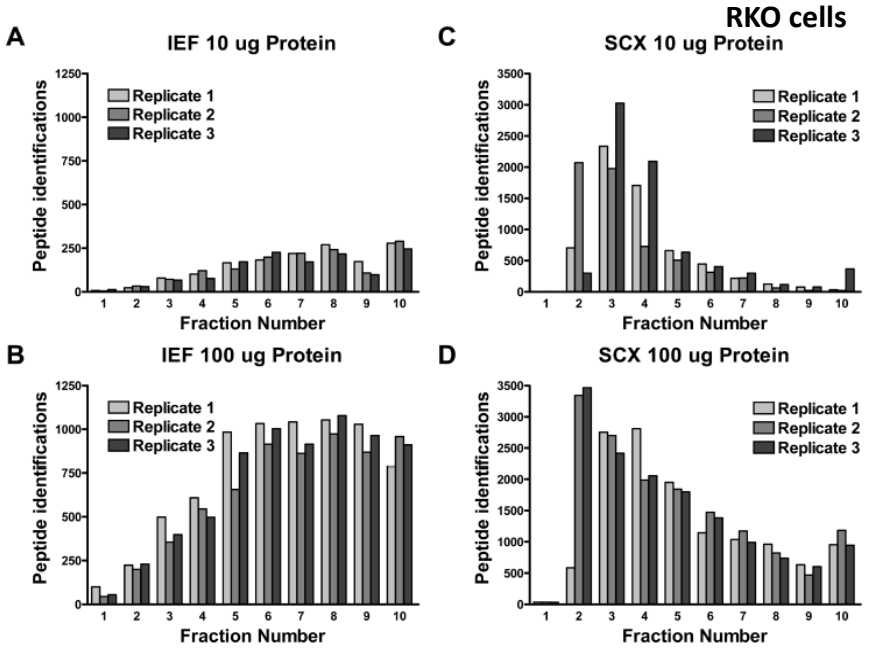


Off-gel IEF *versus* SCX chromatography



Cargile et al., J. Biomol. Tech., 2005, 16(3), 181–189

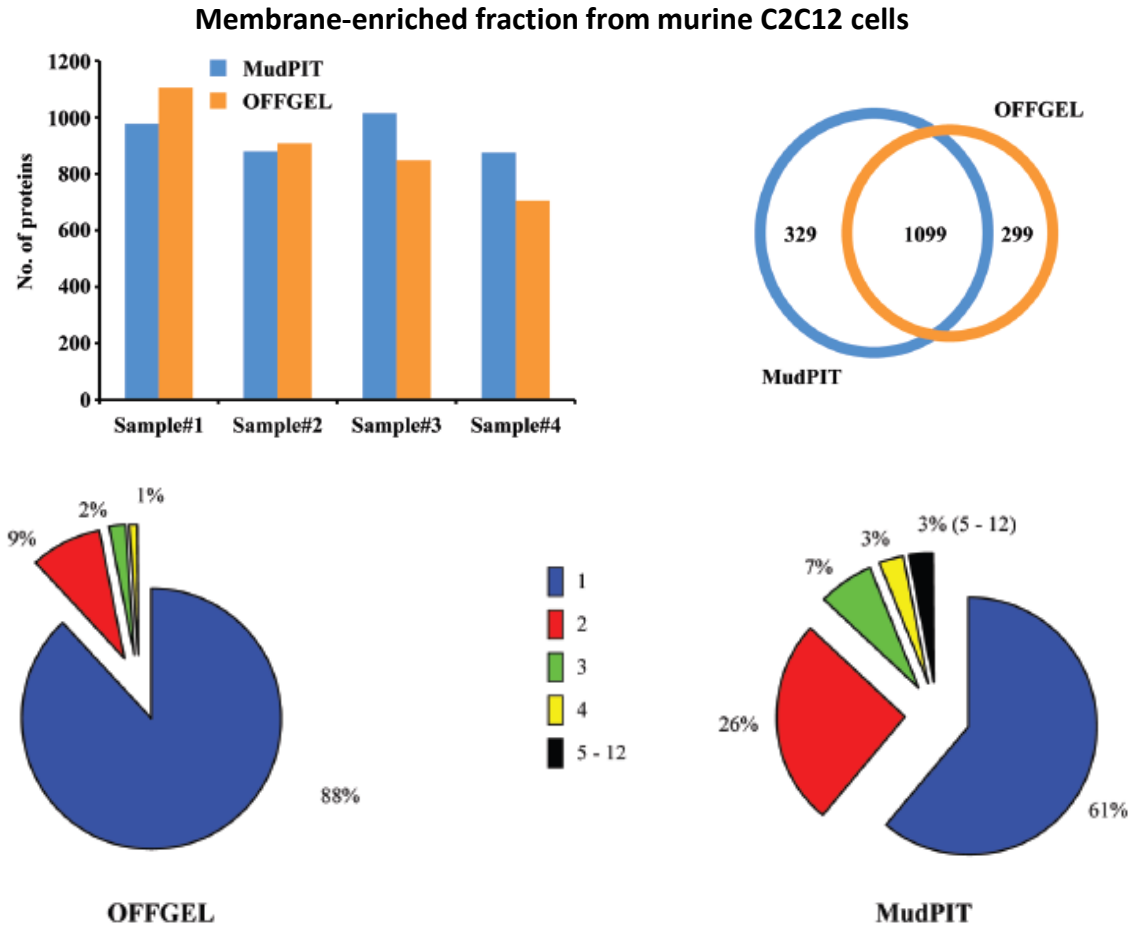
- Comparison of narrow range IPG IEF (pH 3.5-4.5) with off-line strong-cation exchange chromatography
- 13% more proteins with IEF, less fractions to analyze with LC MS (43 *vs.* 128)
- **Complementarities for in depth proteome coverage**



Slebos et al., J. Proteome Res. 2008, 7, 5286-6294

- Greatest number of peptides and proteins with SCX-based platform (twice more for low sample amount (10 µg))
- **Greatest reproducibility with IEF platform**
- **Greatest resolution with IEF platform** (90% of all peptides found in a single fraction)

Off-gel IEF *versus* SCX chromatography



- Comparable in terms of protein identifications (12 *vs.* 12 fractions)
- Off-gel 88% peptides in a single fraction vs. SCX 61% => **superior focusing quality of IEF**
- Sample complexity (off-gel superior to SCX for a more complex sample (HEK cell lysate)), 3143 *vs.* 2242 proteins

- Proteome coverage apparently depends on peptide amounts and sample complexity

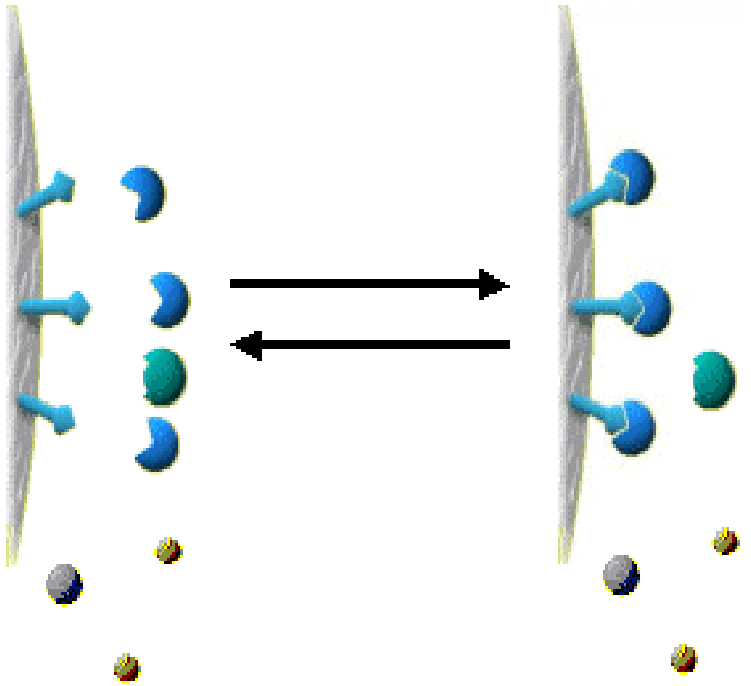
But all agree for:
- Superior resolution
- Better reproducibility
of peptide IEF

Elschenbroich et al., J. Proteome Res. doi: 10.1021/pr900318k

Affinity chromatography

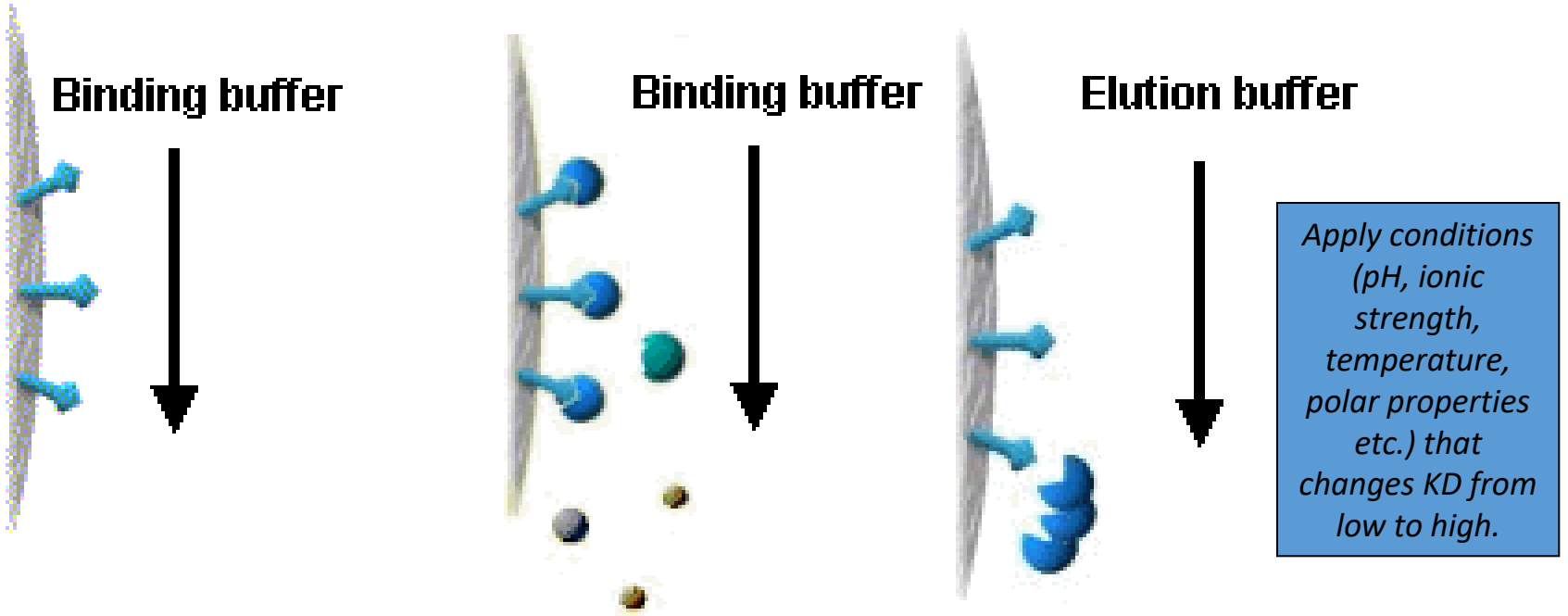
- Makes use of a highly specific interaction between a target protein and a ligand on the solid phase.

- *e.g.*. antibody on column, antigen tag on protein
- *e.g.*. glutathione on column, target protein as GST-fusion



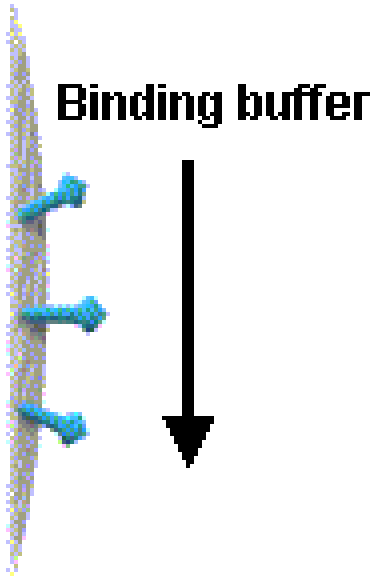
Affinity chromatography

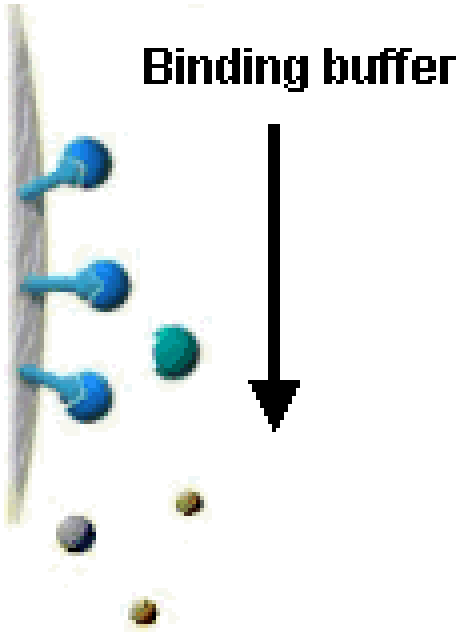
- Affinity resin captures the target with high specificity
- Elution can be performed by mild denaturation



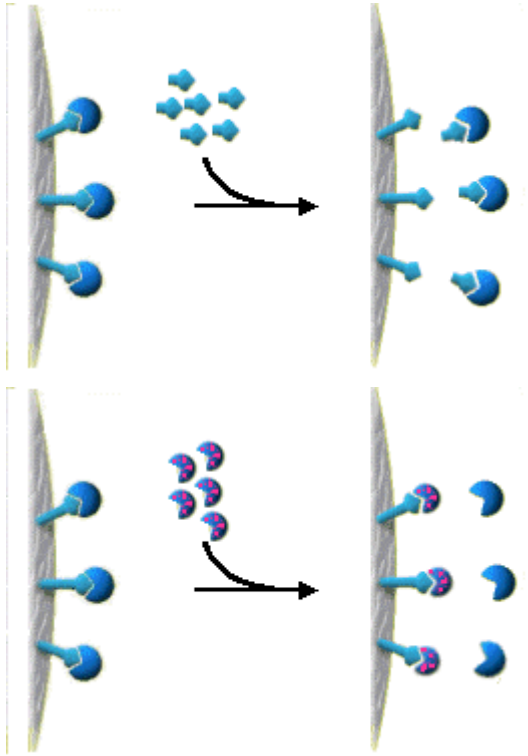
Affinity chromatography

- Affinity resin captures the target with high specificity





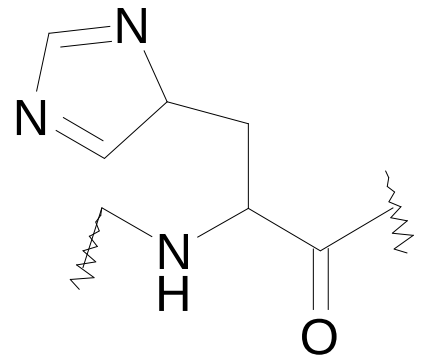
- or by a soluble competitor

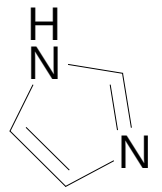


Immobilised metal affinity chromatography (IMAC)

- Immobilised metal chelating group (*e.g.* imidodiacetate) with a bound multivalent transition metal
- The metal ion (Cu^{2+}, Zn^{2+} , Ni^{2+} , Co^{2+} or Fe^{2+}) is bound so that at least one coordination site is available for interaction with proteins or peptides
- Proteins with affinity to metal ions can be bound to the column and subsequently eluted by change of conditions
- Not many naturally occurring proteins have affinity for metal ions so it is very specific
- Technique is mainly used to purify recombinant proteins expressed with N-terminal Histidine–tag (His-Tag)
- Elution is generally performed using imidazole (2), which competes for the metal site with histidine (1) and releases the protein

1





2

Affinity chromatography

- High specificity
- Low capacity
- Denaturing elution can damage protein

Group-specific separations

- Glycoproteins
- IgG antibodies
- Enzymes
- Proteins/peptides with accessible histidines

Mono-specific separations

- Antigens
- Antibodies
- Hormones
- Receptors
- Enzymes
- Tagged recombinants

Three groups of properties of the target molecule are used in affinity chromatography:

1. Specific binding properties based on biological activity like:
- Enzyme active sites
- Receptor binding sites
- Antibody binding sites etc

These are used together with the natural ligand or an analogue of it. Sometimes the analogue has a broader specificity and can be used for group separations

2. Naturally occurring prosthetic groups like:

Polysaccharides etc
Such properties normally allow group separations only

3. Molecules equipped with an affinity tag like:

Glutathione-S-Transferase (GST)
- Oligo histidine etc

This group of properties is used almost exclusively for recombinant fusion proteins

High performance LC (HPLC)

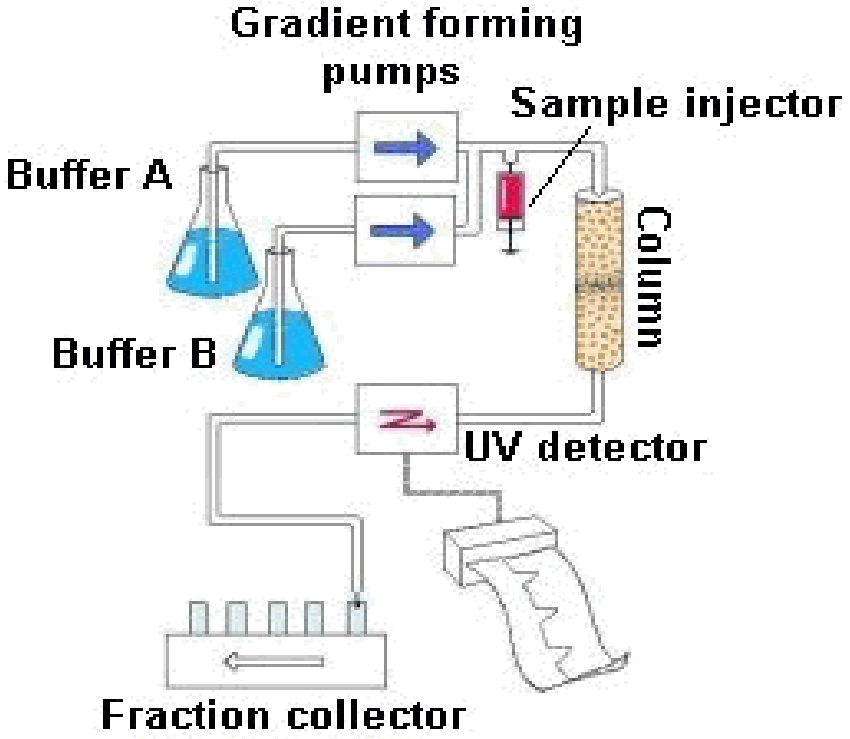
- High Performance Liquid Chromatography (HPLC) is the most widely used analytical technique

- In the column, the mixture is resolved into its components. The amount of resolution is important, and is dependent upon the extent of interaction between the solute components and the stationary phase

- The interaction of the solute with mobile and stationary phases can be manipulated through different choices of both solvents and stationary phases

- As a result, HPLC acquires a high degree of versatility not found in other chromatographic systems and it has the ability to easily separate a wide variety of chemical mixtures

High performance LC (HPLC): Components (1)



1. Solvent Reservoir- Glass bottles or flasks with:
- Tubing to pump
- Filter at tubing entrance to remove particles
- Gas line to purge dissolved gases (or vacuum degasser)
- Solvent

2. Pump
- To aspirate the mobile phase & force it through the column & detector
- Operate in two modes:
  - Isocratic: single solvent
  - Gradient: change solvent stepwise or continuous

3. Injectors
- To introduce sample into column
- Sample loop injector
- Manual injection or automatic injection using autosampler

High performance LC (HPLC): Components (2)

4. Column

- Chosen according to the type chromatography
- Dimensions
- Internal diameter

5. Detectors

- Many types based on physical & chemical processes:
  - UV-vis
  - Refractive Index (RI)
  - Mass spectrometry (MS)
  - Electrochemical (EC)
  - NMR - novel

Injectors

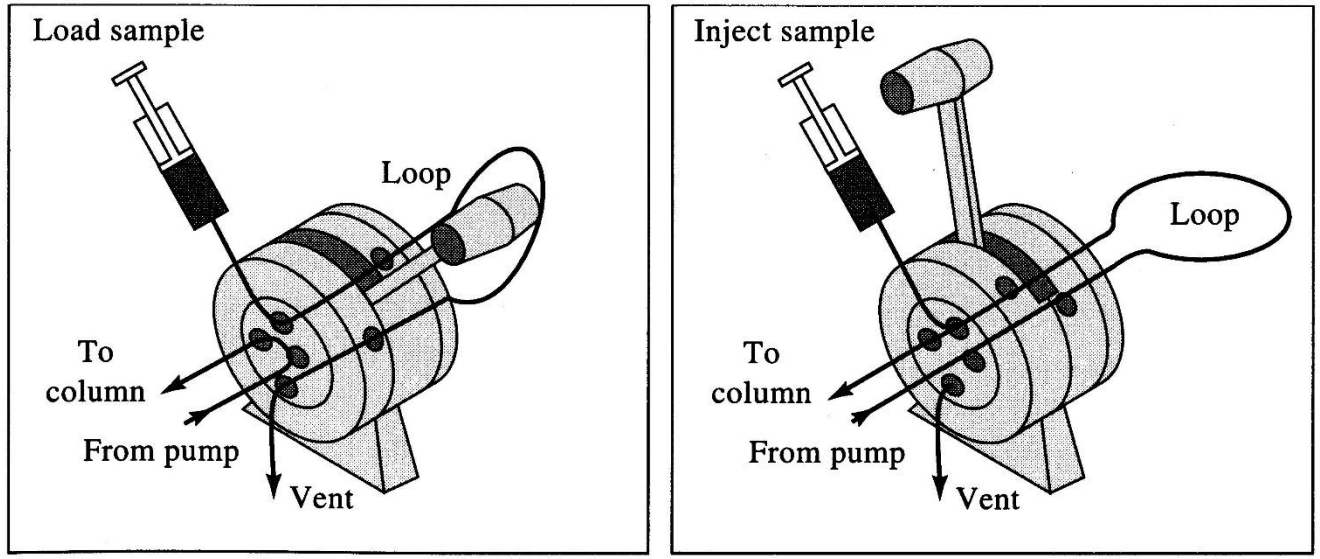


Figure 28-7 A sampling loop for liquid chromatography. *(Courtesy of Beckman Instruments, Fullerton, CA.)* With the valve handle as shown on the left, the loop is filled from the syringe, and the mobile phase flows from pump to column. When the valve is placed in the position on the right, the loop is inserted between the pump and the column so that the mobile phase sweeps the sample onto the column.

Columns

- **Short** (30-50mm) - short run times, low backpressure
- **Long** (250-300mm) - higher resolution, long run times

- **Narrow** (≤ 2.1mm) - higher detector sensitivity
- **Wide** (10-22mm) - high sample loading

Table 1: Nomenclature for HPLC Columns in Cylindrical Formats.

Description	Dimension	Approx. typical flow-rate (velocity 1–10 mm/s)
Open tubular liquid chromatography	< 25 µm i.d.	< 25 nL/min
Nanobore column HPLC	25 µm ≤ i.d. ≤ 100 µm	25–4000 nL/min
Capillary column HPLC	100 µm < i.d. < 1 mm	0.4–200 µL/min
Microbore column HPLC	1 mm ≤ i.d. ≤ 2.1 mm	50–1000 µL/min
Narrow(small)-bore column HPLC	2.1 mm < i.d. < 4 mm	0.3–3.0 mL/min
Normal-bore column HPLC	4 mm ≤ i.d. ≤ 5 mm	1.0–10.0 mL/min
Semipreparative column HPLC	5 mm < i.d. ≤ 10 mm	5.0–40 mL/min
Preparative column HPLC	i.d. > 10 mm	> 20 mL/min

Capillary (cap-) and nano-LC

- Require low flow rates (down to nl/min) either from specialized pumps or with flow splitting
- As internal diameter of the column decrease, the analyte concentration increases (in proportion to the inverse square of the diameter)
- Optimum flow rate decreases (therefore solvent consumption is reduced)
- Similar pressures
- Resolution of components increases
- Improved sensitivity
- Practical considerations:
    - Leaks difficult to find
    - Limited sample capacity
    - More prone to clogging

Detectors

	Detection limit (g/mL)	Used with a gradient
UV absorption	10^{-10}	yes
IR absorption	10^{-6}	yes
Fluorimetry	10^{-11}	yes
Refractiometry	10^{-7}	no
Conductimetry	10^{-8}	no
MS	10^{-10}	yes
Amperometry	10^{-12}	no

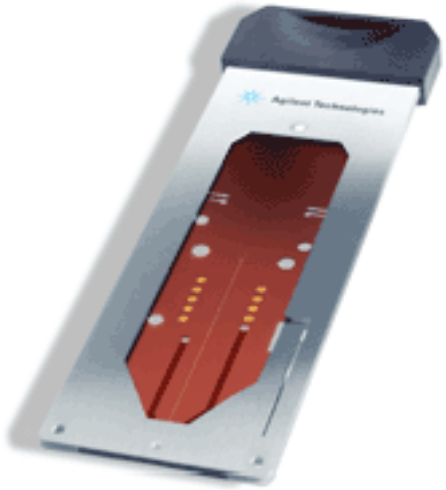
- MS ultimate detector for HPLC system in proteomics
- Separates and detects gaseous ions based on their mass-to-charge ratios (and not mass only!)
- Therefore, we must first produce gaseous ions

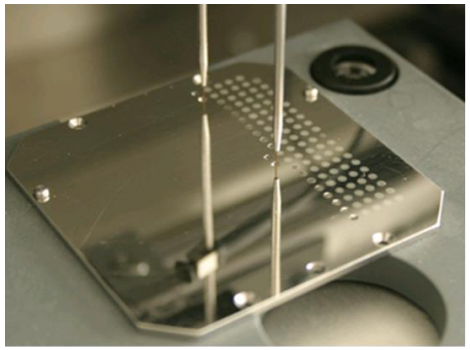
- Two ionisation methods are
  - MALDI (matrix assisted laser desorption ionization)
  - ESI (electrospray ionization) although it's not strictly speaking an ionisation method!

But this you already know…

Q3: MALDI and ESI can be used in on-line off-line LC-MS?

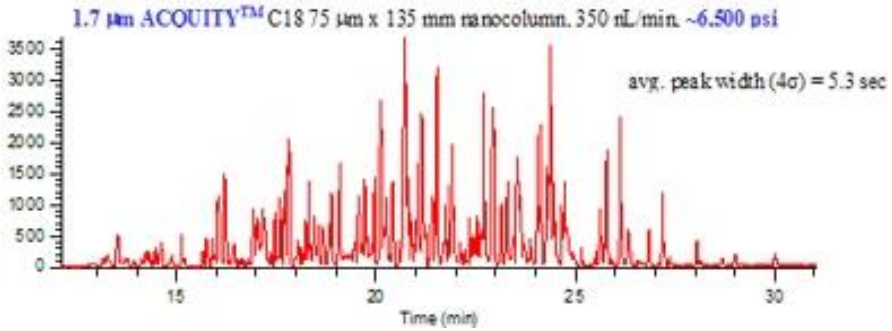
Some set-ups





Into the mass spectrometer

- Peptide + UV absorbing matrix (in 50% CH_3CN, 0.1% TFA)
- The mixture is deposited on a stainless steel target and allowed to crystallise
- The surface of the target is then irradiated (using a UV laser)
- Ions are then extracted into the mass spectrometer (detection)
- MALDI-LC is an offline technique.



20 fmol each of enolase, phosphorylase b, hemoglobin, ADH, BSA

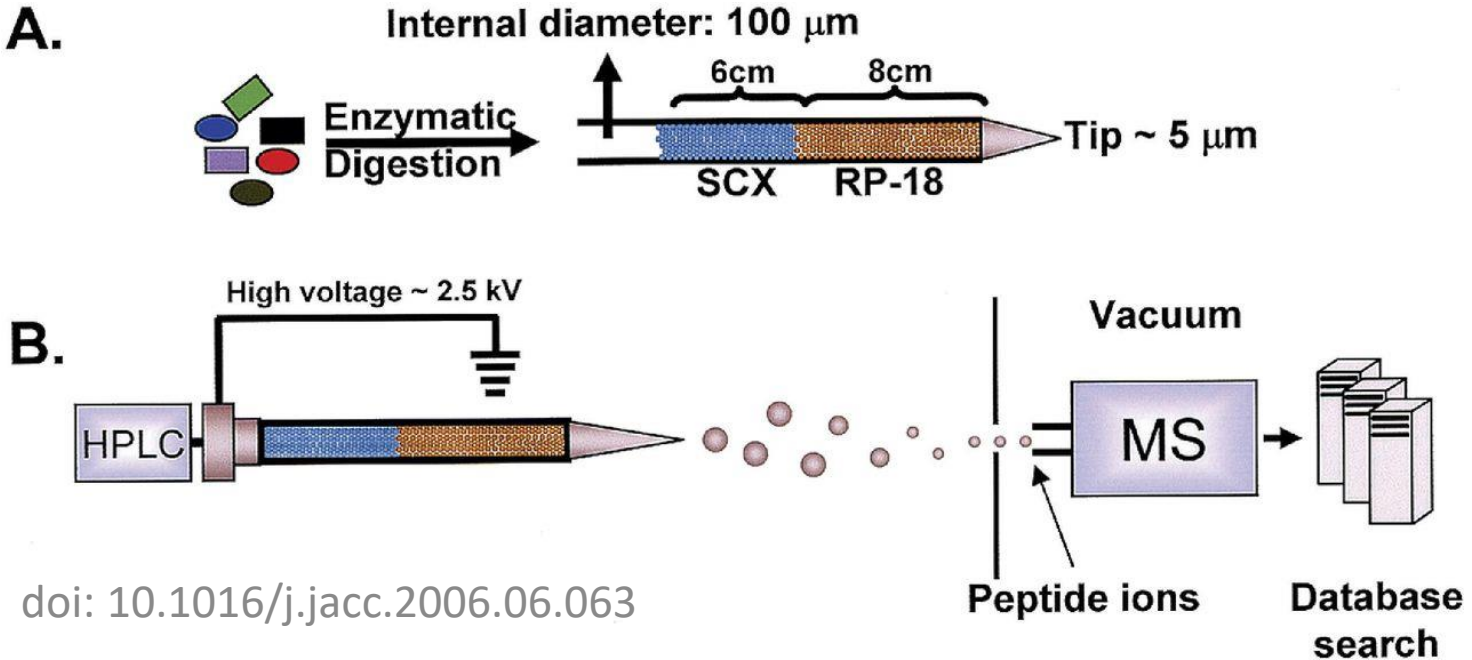
Q4: Questions?

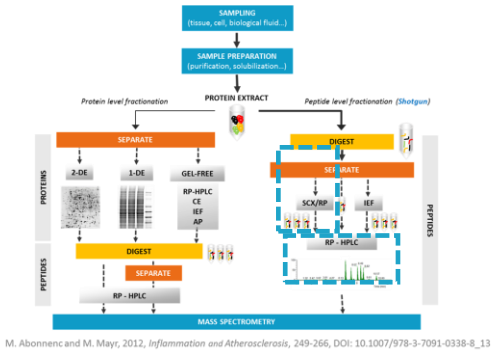
- What are the important properties of chromatographic particles?
- What types of stationary phases can be used for chromatography?
- What is HPLC?
- What detectors are used for HPLC?
- What are the desirable properties for a detector?

Applications of liquid chromatography in biological research

- In proteomics, generally we use a combination of chromatographic techniques in order to fractionate peptides/protein mixtures

- The key to designing a good chromatographic methodology is to maximise the protein-peptide separation by exploiting the physical and chemical properties of the various proteins to be separated





M. Abonnenc and M. Mayr, 2012, *Inflammation and Atherosclerosis*, 249-266, DOI: 10.1007/978-3-7091-0338-8_13

doi: 10.1016/j.jacc.2006.06.063

2D liquid chromatography

- Often, multiple fractionation steps are required to reduce the complexity of biological sample

- If we use more than one LC technique, we will achieve multi-dimensional separation of our proteins/peptides!

•For example, peptides are separated according to their charge (1st dimension), on a strong cation exchange (SCX). The peptides are then loaded onto a C18 RP column and separated according to their hydrophobicity (2nd dimension)

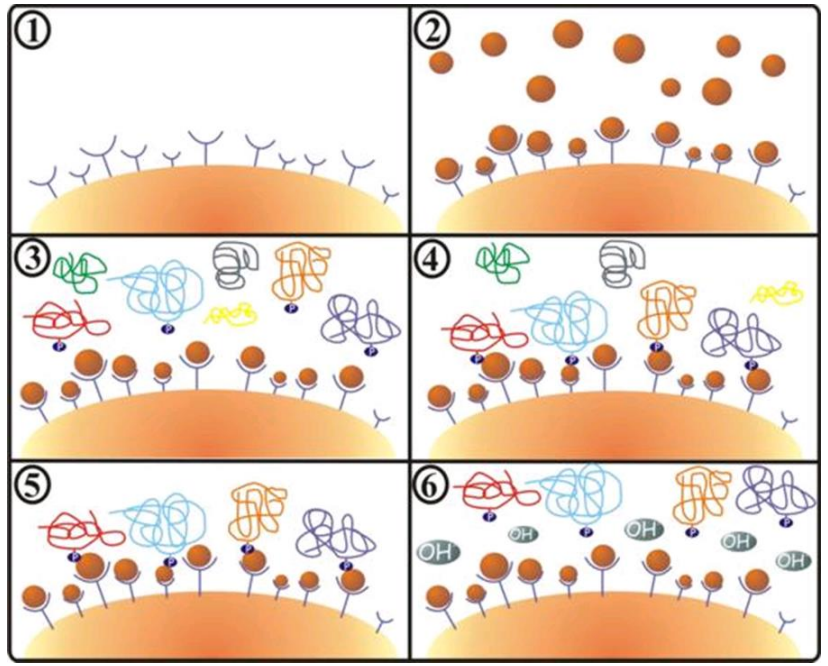
•Or peptides are separated according to their hydrophobicity at basic pH (1st dimension), onto a C18 RP column. The peptides are then loaded onto a C18 RP column and separated according to their hydrophobicity at acidic pH (2nd dimension)

Q5: Why this order?

Applications of liquid chromatography in biological research

IMAC (or titanium dioxide (TiO_2) beads) in phosphoproteomics

- Protein phosphorylation is directly or indirectly involved with most cell processes
- Phosphorylation acts like a switch, turning the function of a protein on or off
- Phosphorylation is regulated by kinases, which transfer a phosphate group from ATP to another protein
- Dephosphorylation is regulated by phosphatases, which remove the phosphate group
- Primarily, the residues on the proteins that are phosphorylated are tyrosine, serine and threonine



(1) Stationary phase with covalently bound chelating-region
(2) Loading with metal ions (e.g., Fe(III))
(3) Removal of excess ions and injection of sample
(4) Phosphorylated peptides are bound under acidic conditions
(5) non-phosphorylated species are removed.
(6) Elution of phospho-peptides by shifting pH (*e.g.,* with ammonium hydroxide)

www.protein-ms.de/METHODS/HPLC/IMAC.htm

Q6: Exercise

- A tryptic hydrolysate of a-casein was analysed by C18 RP LC-ESI-MS.
- Assuming full tryptic digestion (C-terminal to K and R) with no missed cleavages. What is the order of e
(underlined serines) ?

1.Generate peptide sequences

VNELSK ③
② DIG SESTEDQAME DIK
QMEAESI SSSEEIVPNS VEQK ①
④ VPQLEIVPNS AEER

What influences the retention time in RP LC?

Hydrophobicity
Hydrophilic peptides elute first

Put the peptides in the increasing order of hydrophobicity

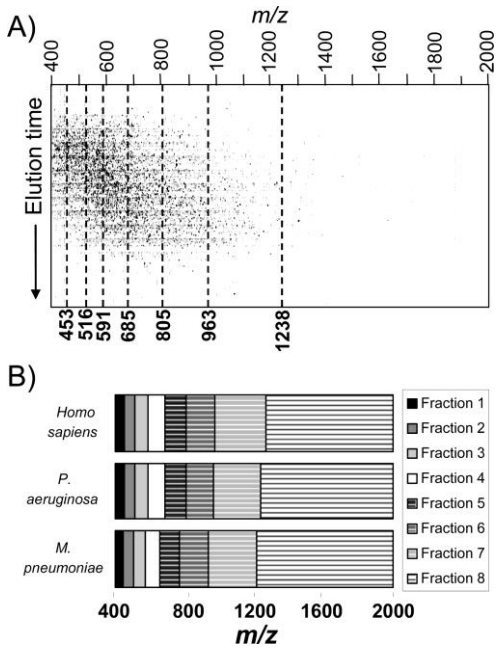
Mol. & Cell. Proteomics, Jan 2006; 5: 172 - 181

Q7: Question?

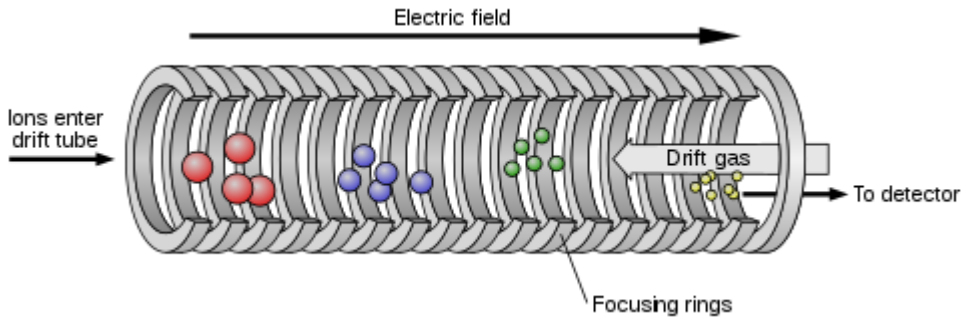
- You’ve just digested your cell lysate and by mistake you’ve added SDS (a negatively charged detergent). How do you propose to rescue your digest before an LC-MS analysis?

Summary

- In proteomics, main separations are performed with electrophoresis or liquid chromatography…
- But the mass spectrometer can also be used to better separate complex mixtures



A. Scherl et al., Anal Chem. 2008;80(4):1182-91.



https://en.wikipedia.org/wiki/Ion-mobility_spectrometry