EBERHARD KARLS
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MATHEMATISCH-
NATURWISSENSCHAFTLICHE FAKULTÄT

Institut für Physikalische und Theoretische Chemie



Laboratory Techniques and Medical Device Approvals

Bioanalytics: separation and diagnosis

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requirements and literature

- basic knowledge on molecule characteristics/basic chemistry:
  - hydrophobicity, ionization, pK_a
- basic knowledge on interactions between molecules
- basic knowledge on biomolecules (DNA, proteins)

literature recommendations:

- **general analytical chemistry**:

- Harris, Quantitative Chemical Analysis, WH Freeman, Edition 8, New York 2011 (older version available in the internet)
- Skoog, West, Holler, Fundamentals of Analytical Chemistry, Brooks Cole; Edition 9. Belmont 2013

- **capillary electrophoresis:** Lauer, Rozing; High Performance Capillary electrophoresis – A Primer, Agilent Technologies, Waldbronn 2009 (http://www.chem.agilent.com/Library/primers/Public/5990_3777EN.pdf)

- **mass spectrometry**: Gross, Mass Spectrometry, Springer, Edition 2, Heidelberg 2011

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topics in bioanalysis

Bioanalysis comprises:

- DNA/RNA
- proteins and their modifications (phosphorylation, glycosylation,…)
- metabolites
- lipids
- interactions between these classes

used for:

- systems biology (cell culture, mouse models,…)
- genomics, proteomics, metabolomics (glycomics, interactomics,…)
- quality control in biopharma
- biomarker research and validation
- clinical chemistry (immunology, diagnostics,…)
- biotechnology (e.g. white biotechnology with production of chemicals by bacteria like *Corynebacterium glutamicum*)
- forensics
- …

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methods in bioanalysis

preanalytical methods:

- study design, sample preparation, extraction, centrifugation, purification,…

analytical techniques:

- chromatography
- electrophoresis
- mass spectrometry
- microscopy
- immunoassays
- spectroscopy (molecules and atoms)

data evaluation:

- data preprocessing, automation
- chemometrics

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separation techniques and principles

phases	separation principle/equilibrium	method
gas-liquid	• solubility differences for gases in liquids	gas chromatography
gas-solid	• differences in adsorption of gases on solids	gas chromatography
liquid-liquid	• differences in distribution	liquid chromatography
liquid-solid	• differences in adsorption of dissolved solutes onto solids • differences in solubility in liquids	liquid chromatography
external field	**separation principle**	**method**
electric	• different mobility in electric field	electrophoresis

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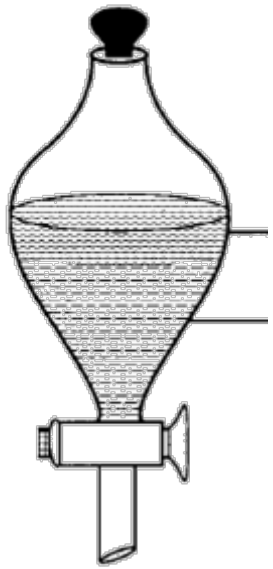
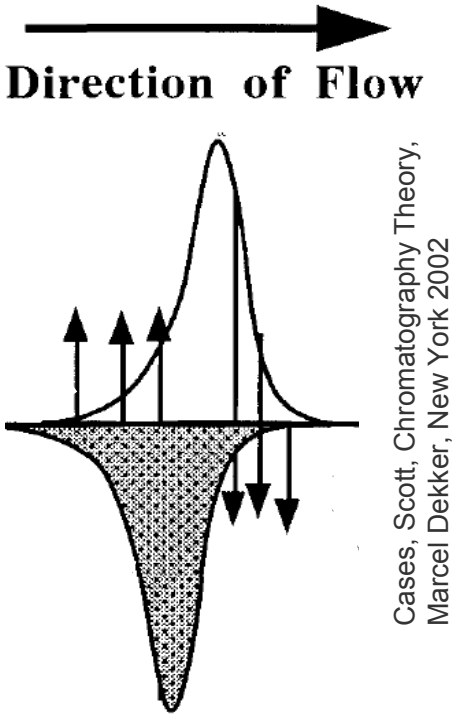
idea of chromatography

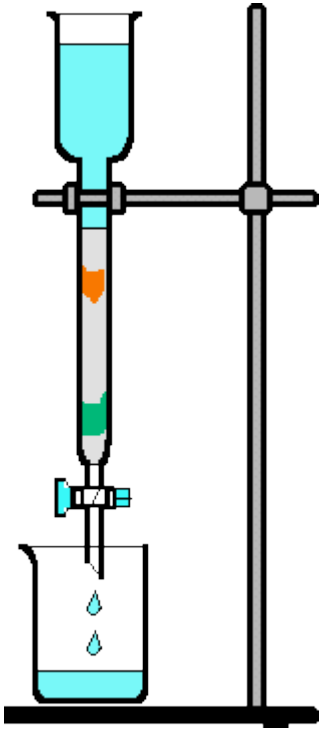
static



Microscopic!



Cases, Scott, Chromatography Theory, Marcel Dekker, New York 2002



http://www.seilnacht.com/versuche/chromat.html

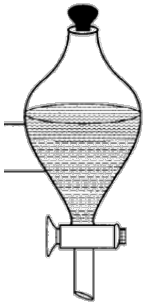
- repetitive equilibration at front and end of the analyte zone („band“)
- movement through column by fresh mobile phase
- retention of analyte in/on stationary phase depending on interaction
- detection at column end to obtain a „chromatogram“ UV,…
- elution time: identity
- band height or area: quantity

idea of chromatography

- chromatography: greek *chroma*: color; *graphein*: to write
- two phases: mobile and stationary phase
- mobile and stationary phase do not mix
- sample is dissolved in the mobile phase
- analytes are transported over the stationary phase
- equilibria between mobile and stationary phase:
  - distribution/partitioning
  - or adsorption onto solid stationary phase
- repetitive equilibration along the chromatographic column

exam! Equilibrium!

distribution constant: $K_c = \frac{c_s}{c_m} = \frac{n_s / V_s}{n_m / V_m}$



c: concentration
n: amount of substance
V: volume
s,m: stationary and mobile phase

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idea of chromatography

types of equilibria:

- adsorption (solid stationary phase) → adsorption chromatography
- distribution (concentration) → normal/reverse phase liquid chromatography
- complexation by e.g. cyclodextrins → chiral chromatography
- ligand binding (antibodies, lectins) → ligand exchange chromatography
- ionic interaction → anion-/cation exchange resins

strong interactions – strong retention – late elution

exam!!!!

or size sieving, size exclusion

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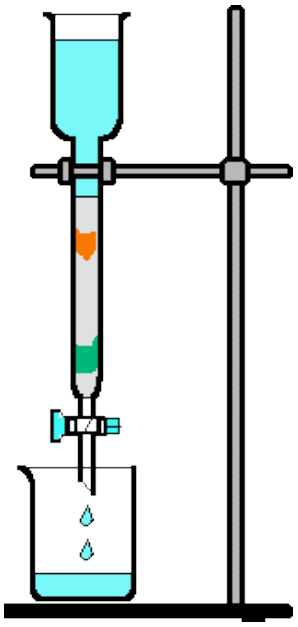
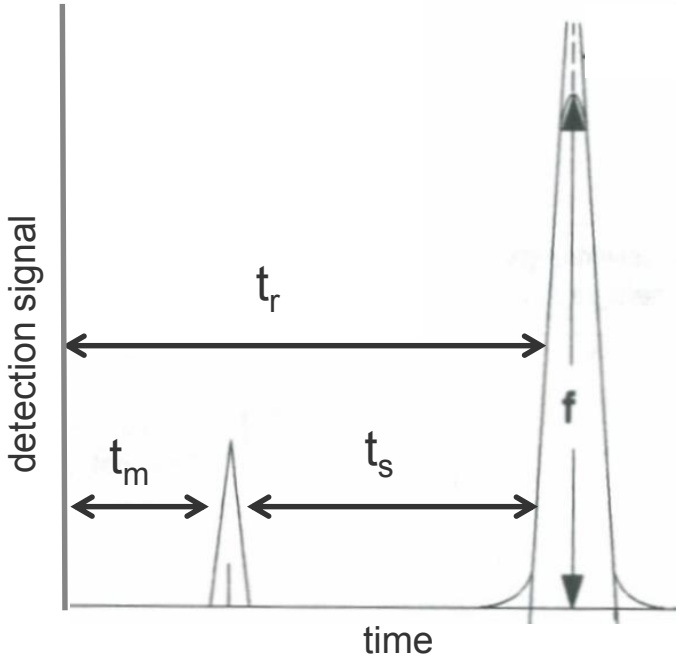
separation process

- non-retarded substance needs time t_m to be transported through column („void volume“/“void time“)
- t_M is a measure of the mean velocity of the mobile phase
- all substances stay in the mobile phase for the time t_m
- retention time t_r for analyte: time from injection to detection
- analyte is retained and thus stays on the stationary phase for the time t_s

Describe tm, ts, tr!! in exam



http://www.seilnacht.com/versuche/chromat.html



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phase equilibria – retention factor

- distribution constant K_c cannot be measured directly
  → use retention time and factor

1) phase equilibrium with retention factor k (dimensionless)

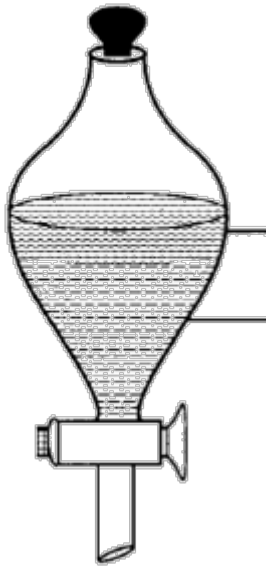
description via time, amount of substance or phase volume:

$$\text{retention factor k} = \frac{\text{time spent in stationary phase}}{\text{time spent in mobile phase}} = \frac{\text{amount of substance in volume of stationary phase}}{\text{amount of substance in volume mobile phase}}$$

understand this!

$$k = \frac{t_r - t_m}{t_m} = \frac{n_s}{n_m} = \frac{c_s V_s}{c_m V_m}$$

calculate out of diagram of slide before



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phase equilibria

2) separation selectivity α

2 substances with k_1 and k_2 are distributed between the two phases g and s

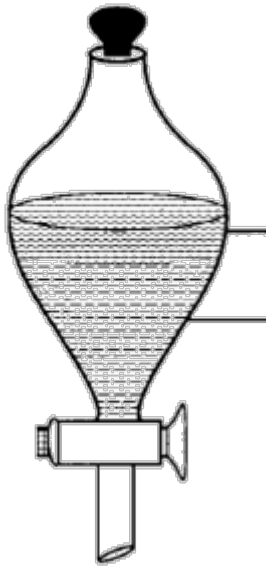
selectivity $$\alpha = \frac{k_2}{k_1} = \frac{n_{2,s}}{n_{2,m}} \frac{n_{1,m}}{n_{1,s}} = \frac{n_{2,s}}{n_{1,s}} \frac{n_{1,m}}{n_{2,m}}$$

high value of alpha, they are very different, long distance between them

is selectivity high or low

look at:
relative amount of each component in one phase
relative amount of the components in each phase

the higher α, the easier is the separation of the two compounds



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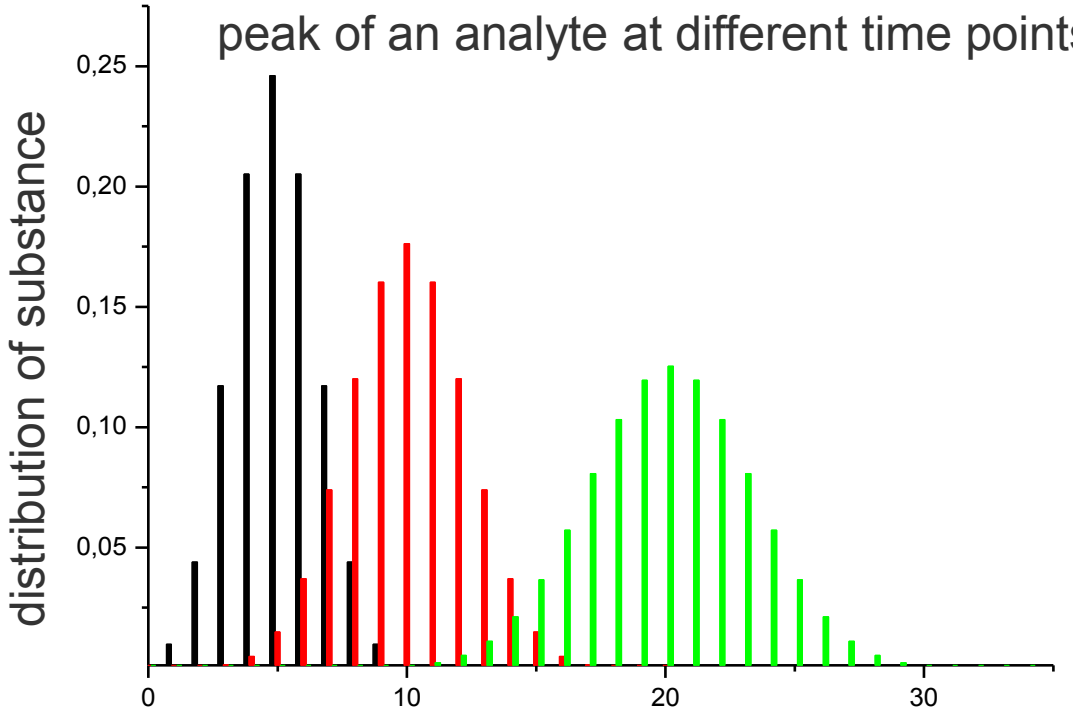
separation in the column



number of equilibration steps
= number of theoretical plates

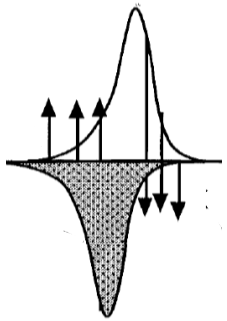
the more the better

transport = „elution“

broadening of the peak

Gaussian peak shape

theoretical plate or column length



$$k = \frac{n_s}{n_m}, \quad \text{here: k=1}$$

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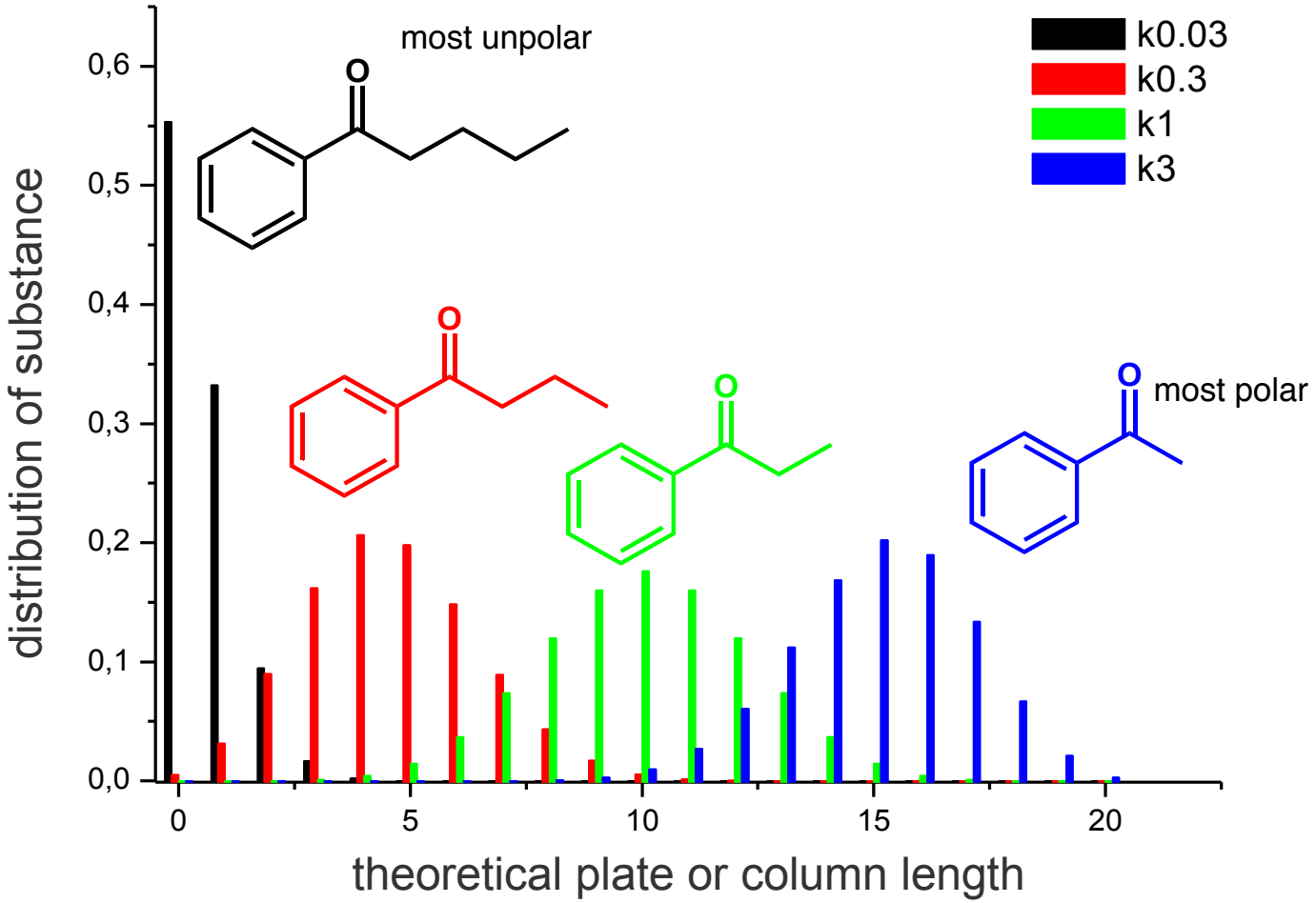
separation in the column

distribution of 4 substances after 20 equilibration steps
„photograph at a certain time point during chromatography“



$$k = \frac{n_s}{n_m}$$

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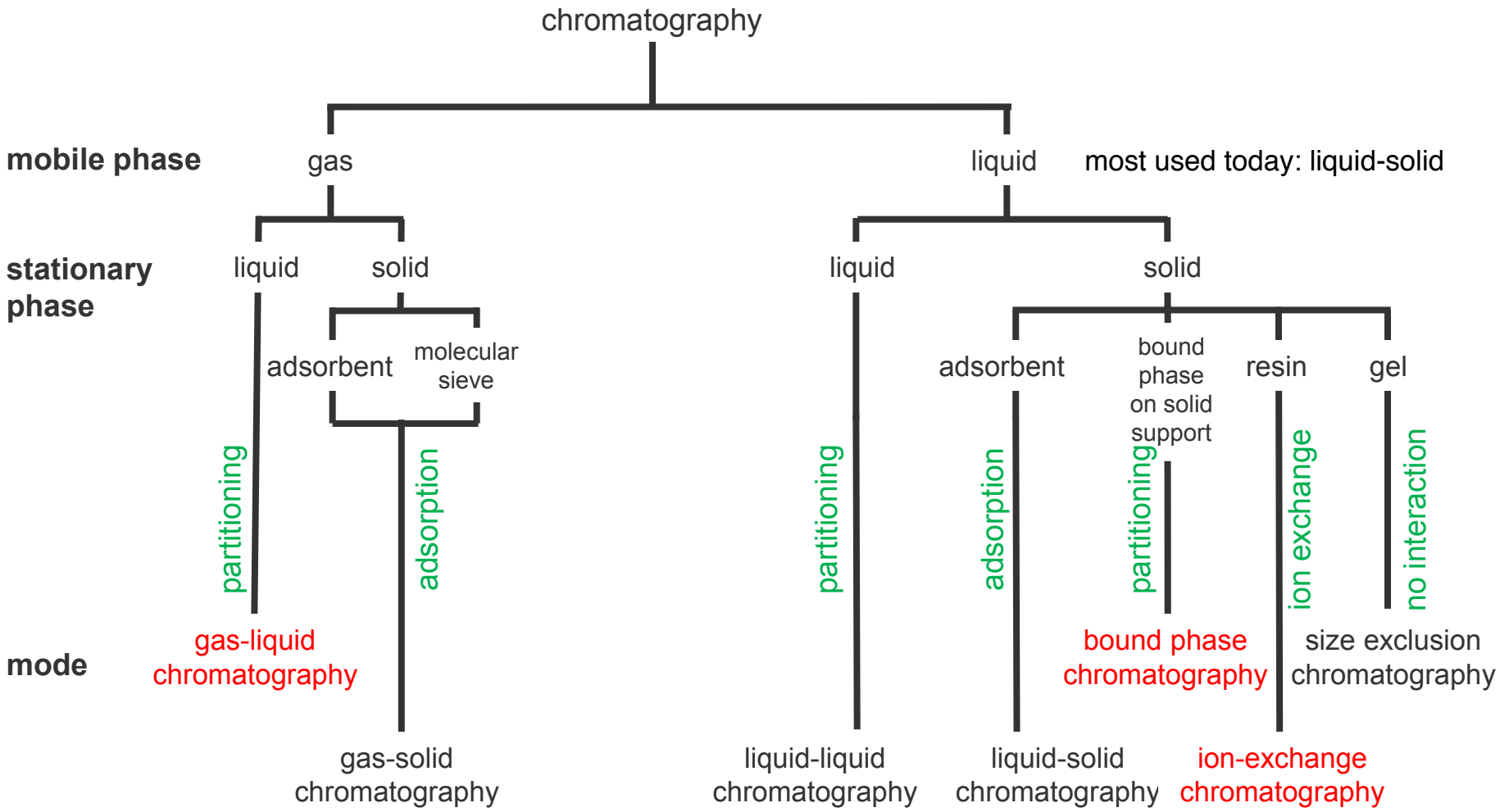
classification of chromatography modes

only red ones are relevant for exam!!!!

classification via phases



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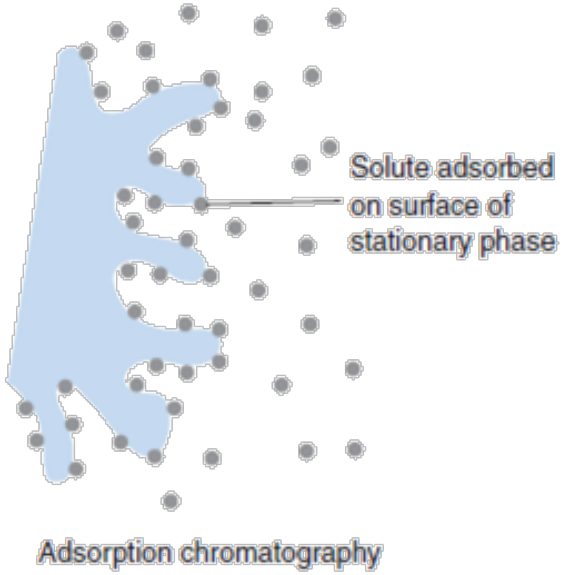
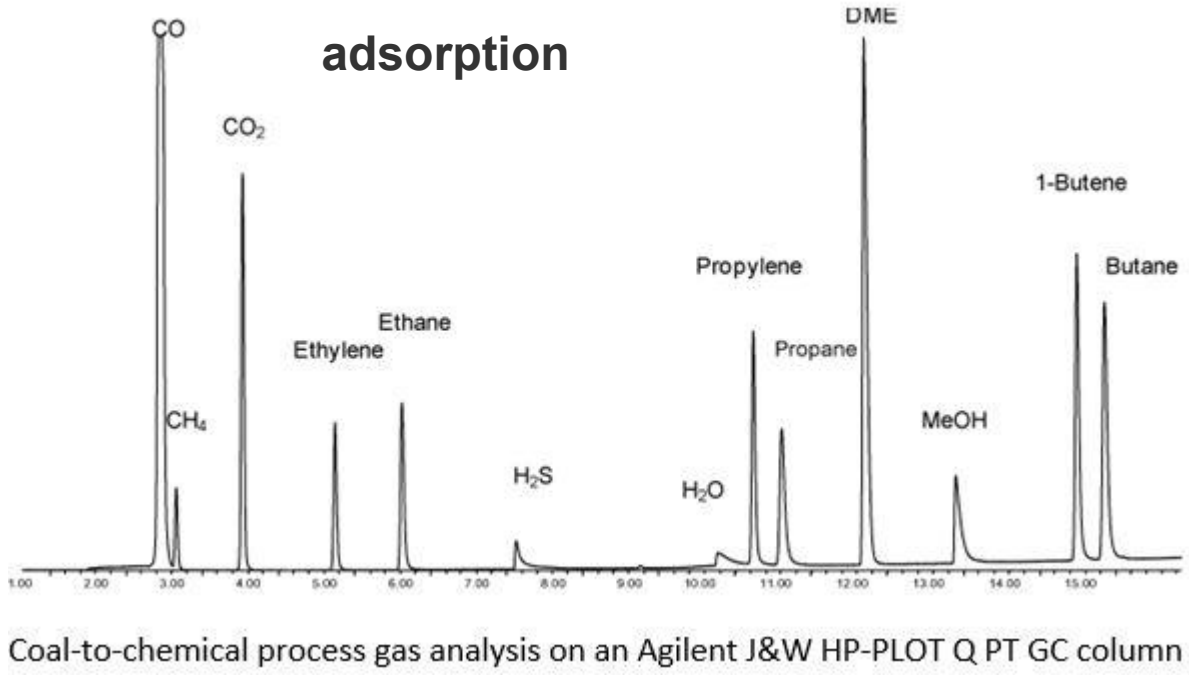
chromatographic separation mechanisms

- adsorption
  - describable via adsorption isotherms (gas-solid, liquid-solid)
  - mostly used for gas analysis in gas chromatography





Coal-to-chemical process gas analysis on an Agilent J&W HP-PLOT Q PT GC column

Harris, Quantitative Chemical Analysis

http://www.analytics-shop.com/de/chromatographie/gc-kapillarsaulen/gc-saulen-saulen-fur-die-gaschromatographie.html

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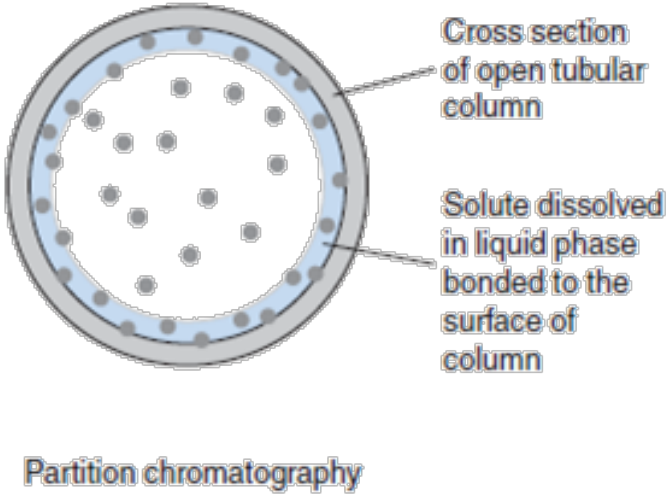
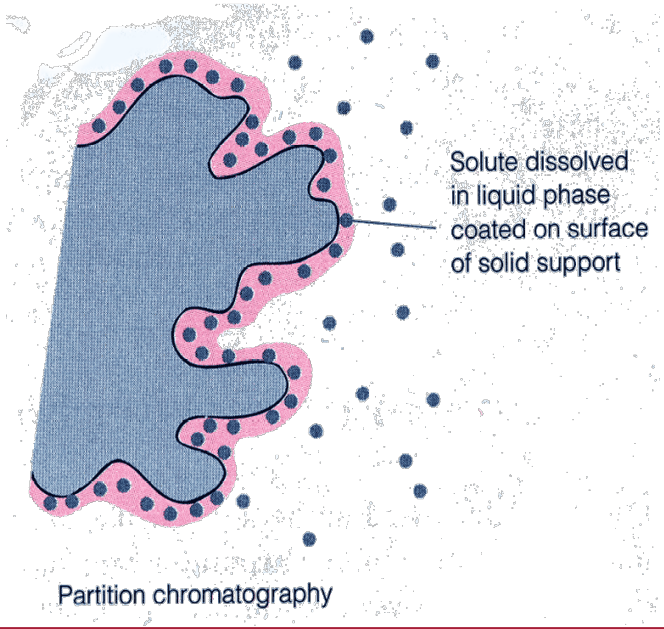
chromatographic separation mechanisms

- distribution/partitioning

exam!

  - liquid film or stationary phase bound onto solid support (mostly silica gel)
  - distribution/partitioning between two phases
  - normal phase NPLC: polar stationary phase, nonpolar mobile phase (pentane, hexane,…)
  - reversed phase RPLC: nonpolar stationary phase (silanol-groups of silica gel derivatized with dichlorosilane and nonpolar alkyl chains) → mostly C_4, C_{18}, polar mobile phase (water, acetonitrile, methanol)





Harris, Quantitative Chemical Analysis

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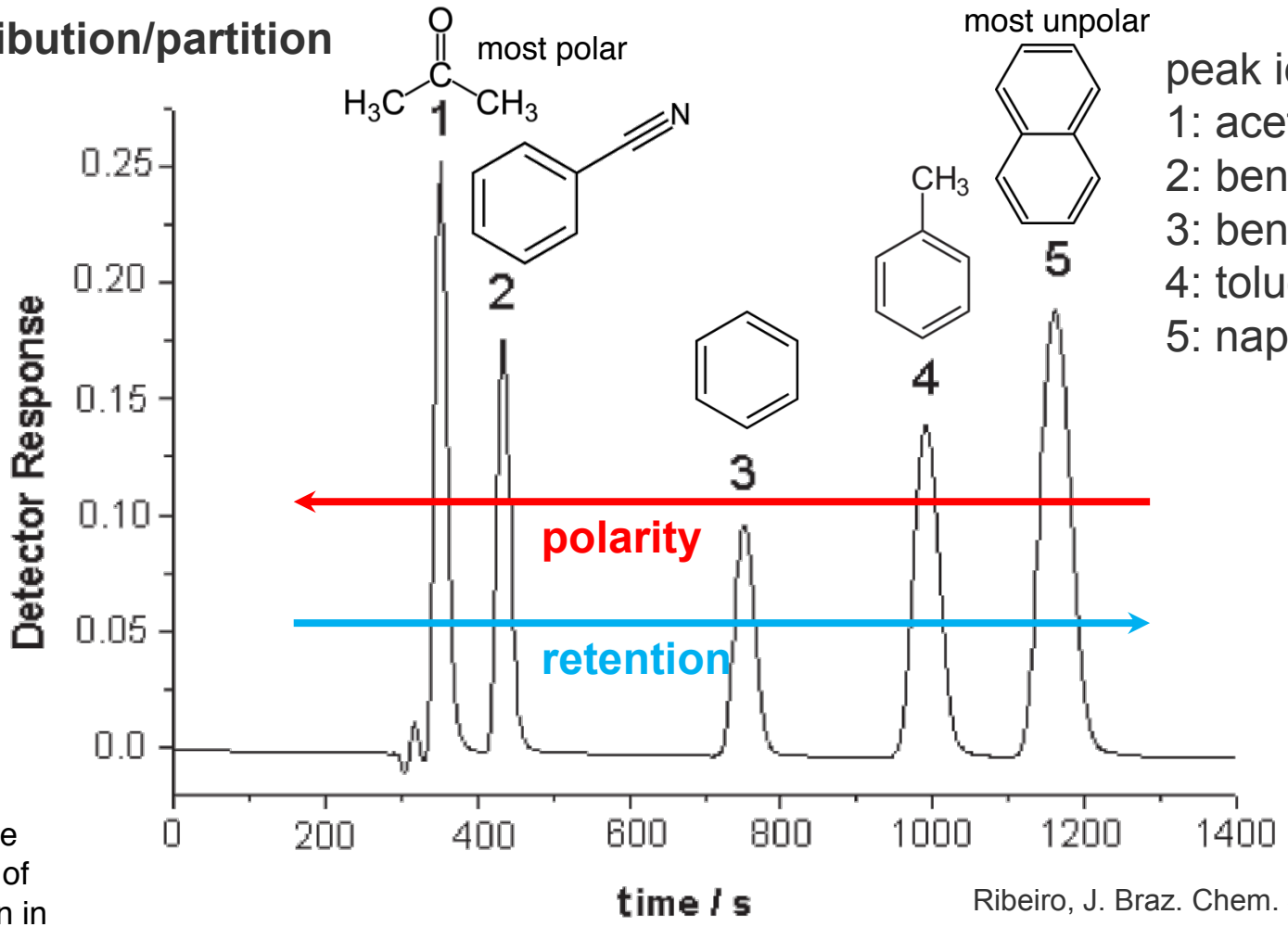
chromatographic separation mechanisms

retention in RPLC based on polarity; high retention for low polarity
C_{18} stationary phase; elution mostly with acetonitrile/water as mobile phase

distribution/partition



peak identification:
1: acetone
2: benzonitrile
3: benzene
4: toluene
5: naphthalene

mnemonic:
RPLC:
polar analytes
in polar
mobile phase

define order of delution in exam

Ribeiro, J. Braz. Chem. Soc. 2004, 15, 300-306

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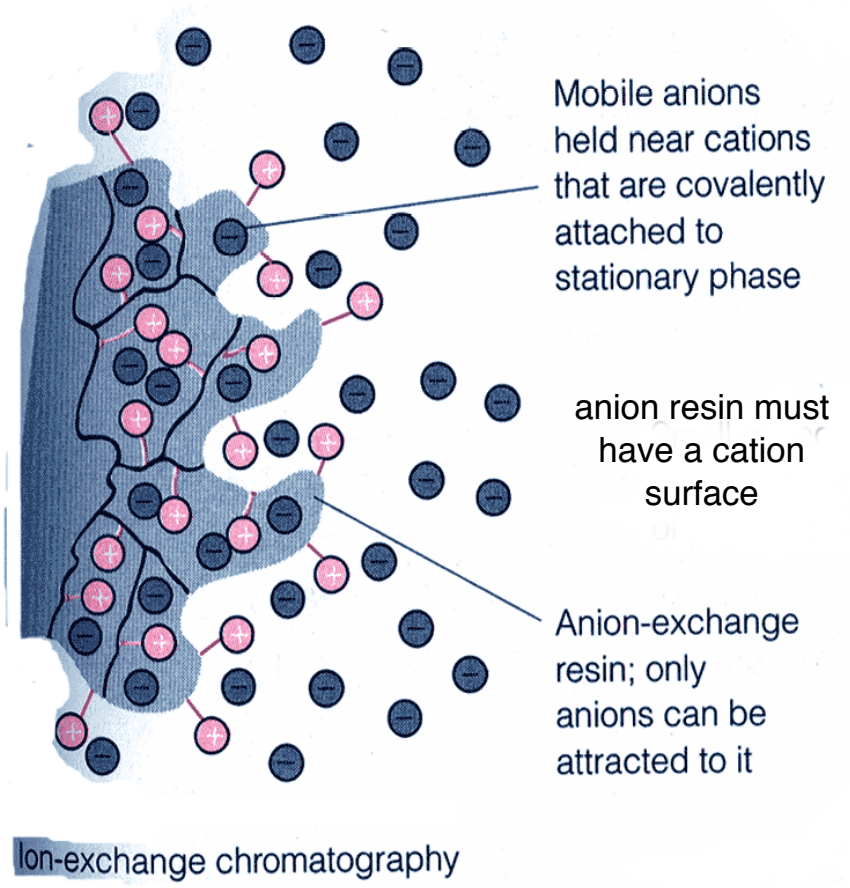
chromatographic separation mechanisms

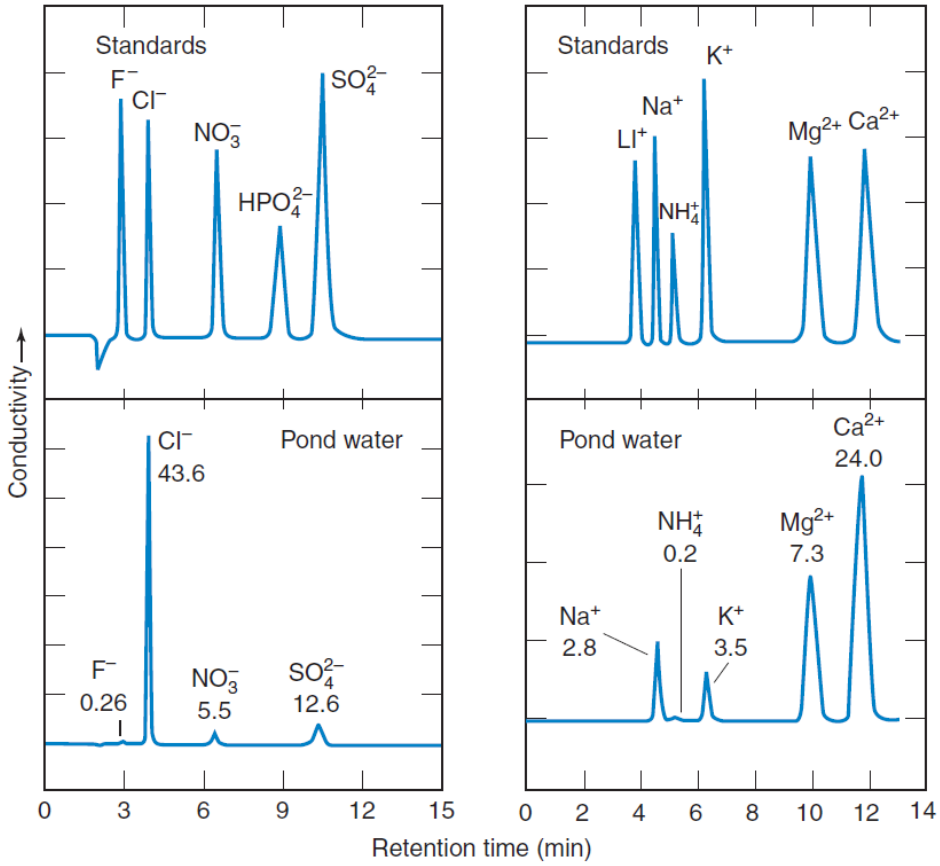
anion exchange chromatography or cation exchange chromatography



Harris, Quantitative Chemical Analysis

ion exchange/ionic interaction

high retention for ions of higher charge, decreased hydrated radius, and increased polarizability



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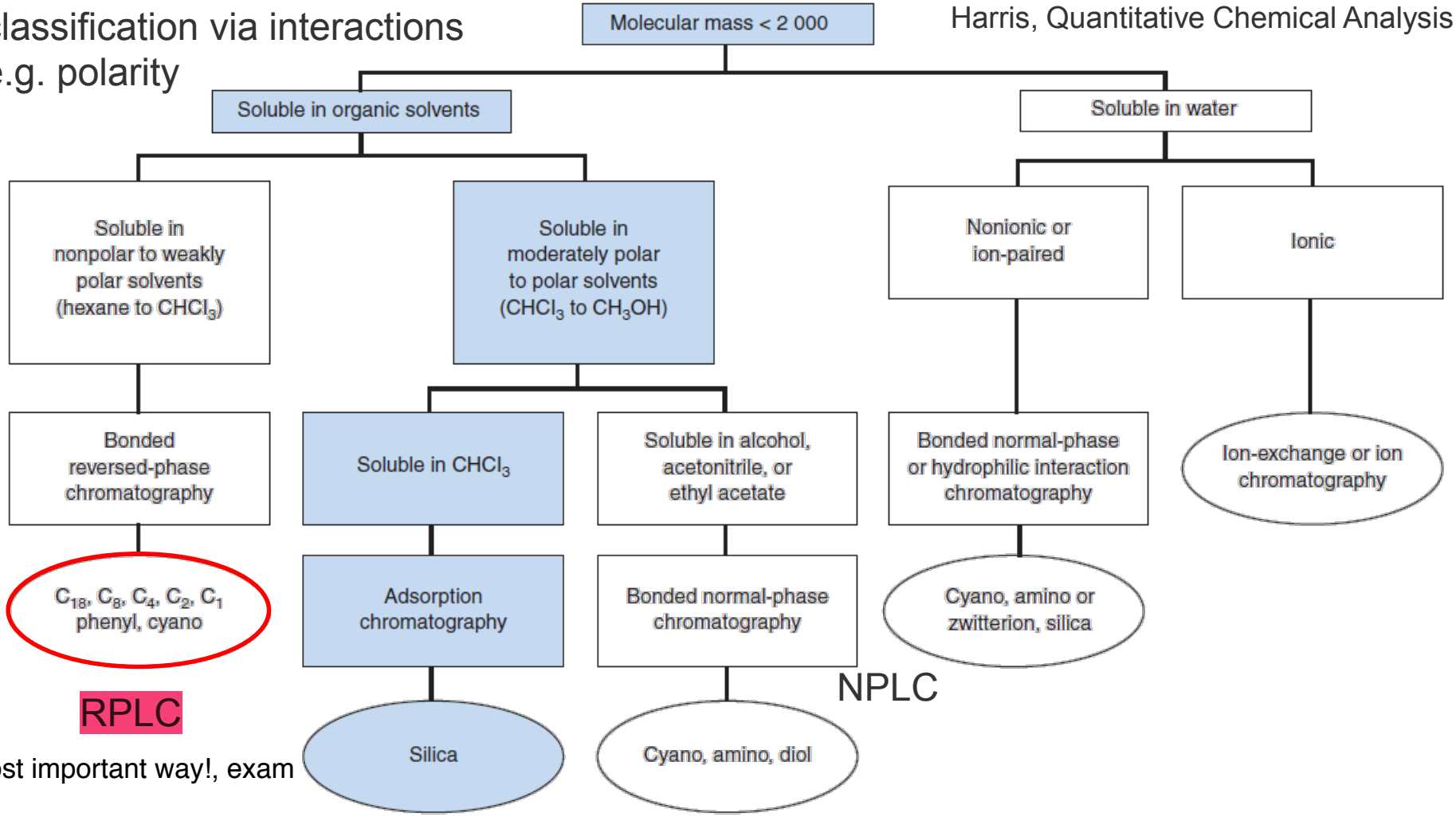
stationary phases in chromatography

classification via interactions
e.g. polarity

Harris, Quantitative Chemical Analysis



RPLC

NPLC

most important way!, exam

RPLC with C_{18} stationary phase for most applications today: proteomics, metabolomics, environmental analysis,…

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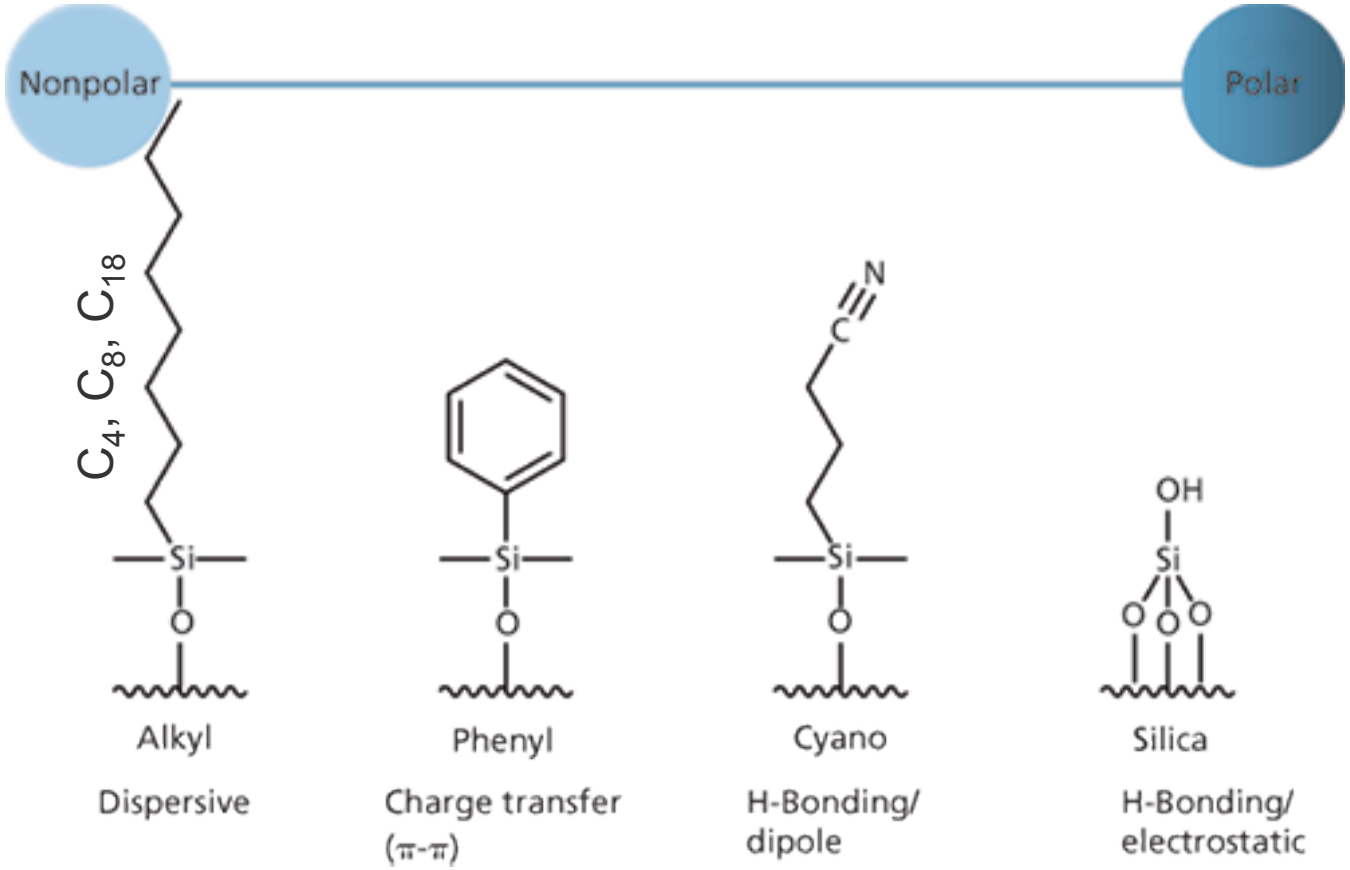
stationary phases in chromatography

stationary phases from RPLC to NPLC



http://www.chromatographyonline.com/choosing-right-hplc-stationary-phase-0

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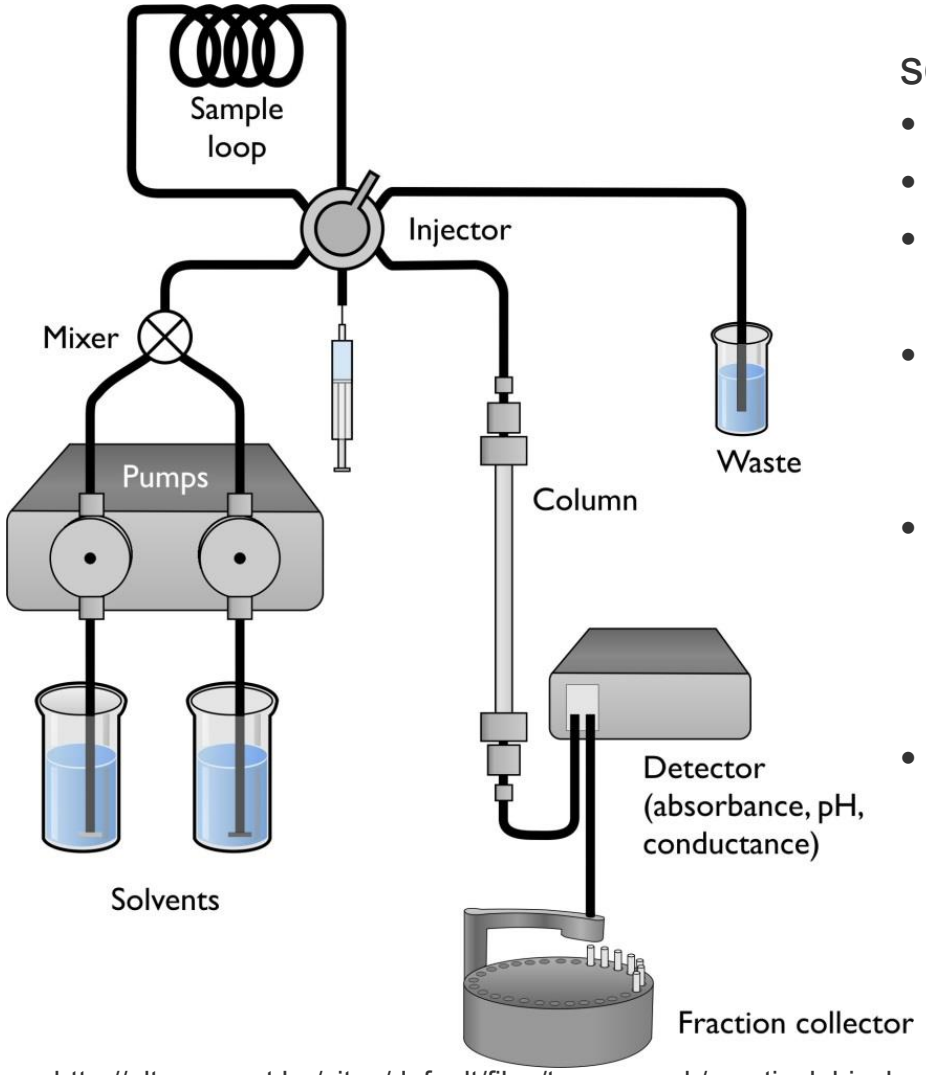
instrumentation in LC



http://elte.prompt.hu/sites/default/files/tananyagok/practical_biochemistry/ch06s05.html

separation column:

- 5-25 cm
- inner diameter 3-5 mm, mostly 4.6 mm
- packing 3, 5, 10 µm particles, mostly 5 µm
  trend to go to smaller particles
- theoretical plates ~ 40 000 - 70 000 per m (10 000 per column)
- pre-column: preseparation of matrix components to save the expensive analytical column
- temperature control

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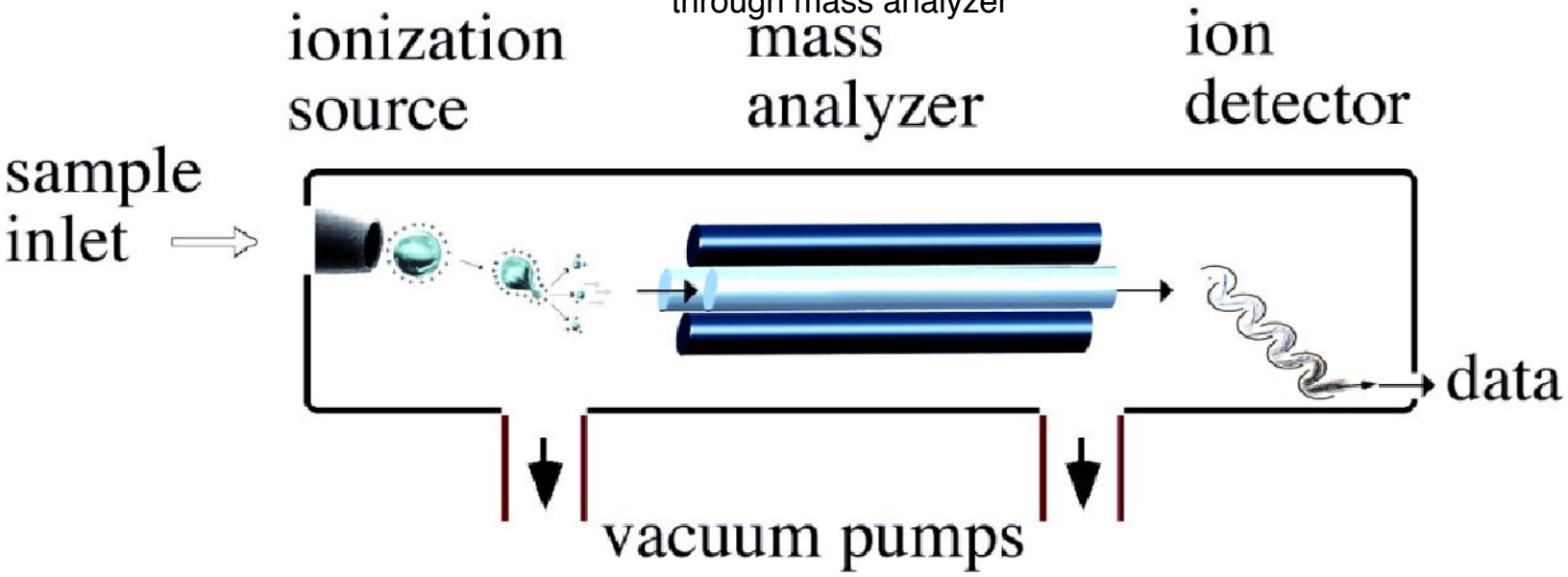
Mass spectrometry

works only with gaseous compounds!

- mass spectrometry to analyze atoms, molecules or their fragments
- gaseous compounds from liquid phase are ionized
- ions are accelerated in the electric field
- separation according to mass to charge ratio m/z
- mass spectrum shows number of detected ions for each m/z

large compounds takes longer to go through mass analyzer



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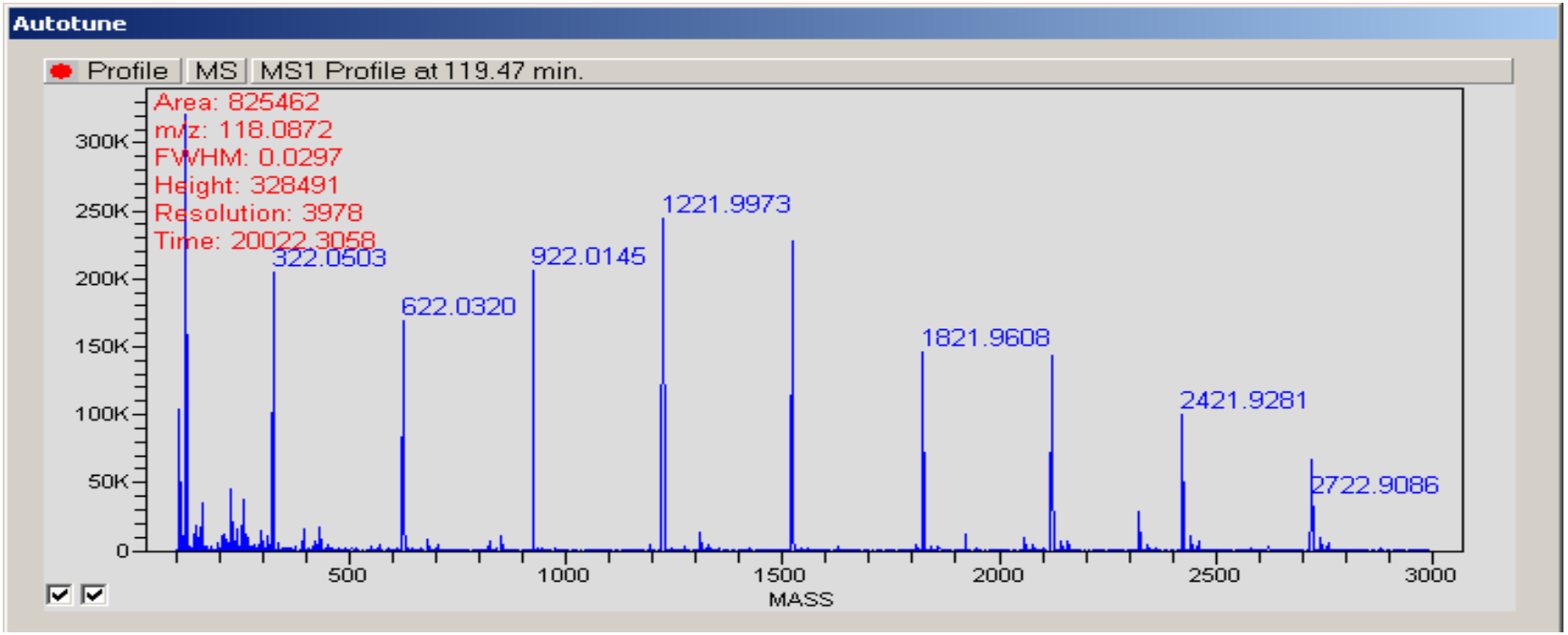
Mass spectrometry

mass spectrum gives abundance of ions of different m/z



m/z

„m over z“

mass to charge ratio

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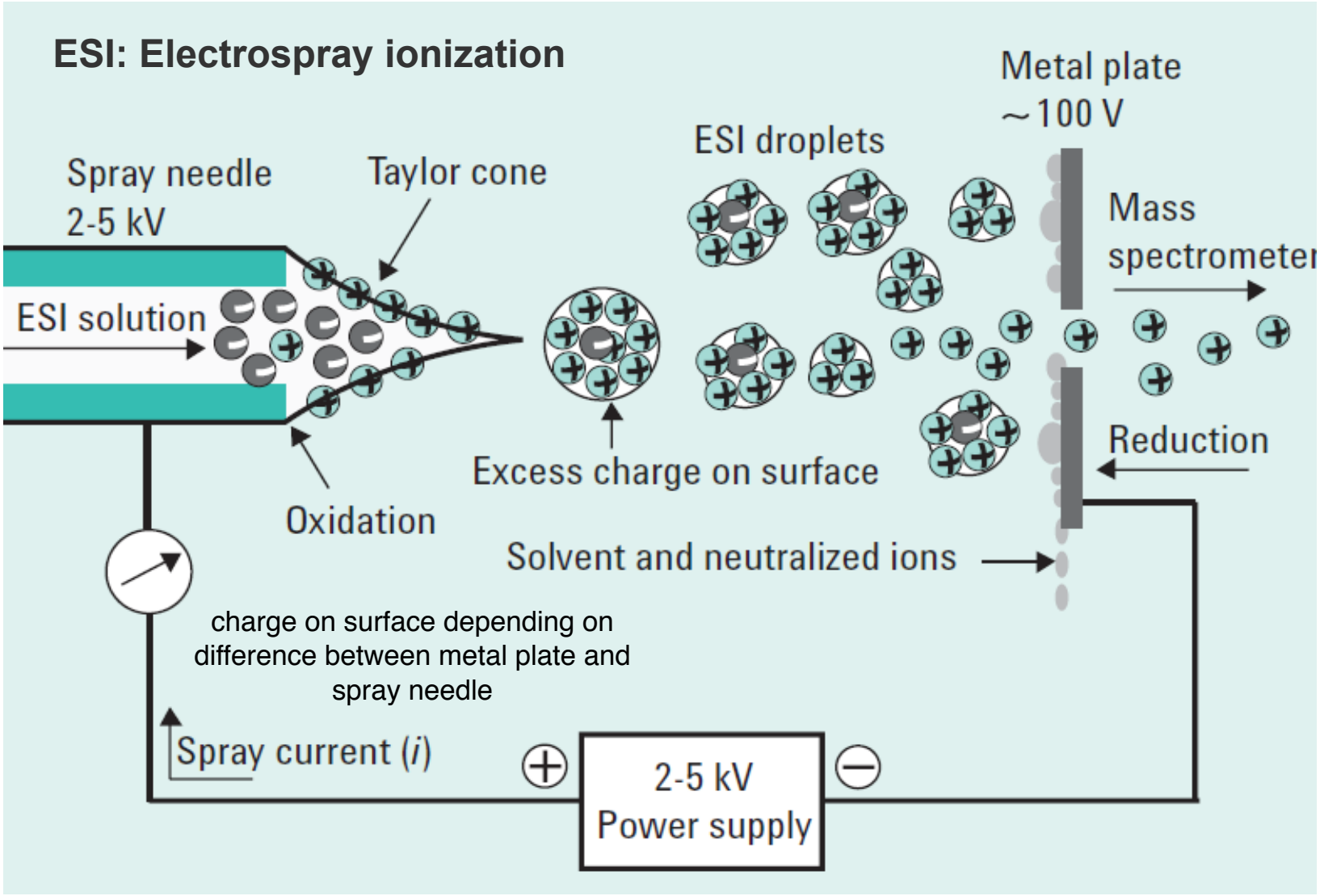
Mass spectrometry



Agilent, High Performance Capillary electrophoresis – A Primer, 2009

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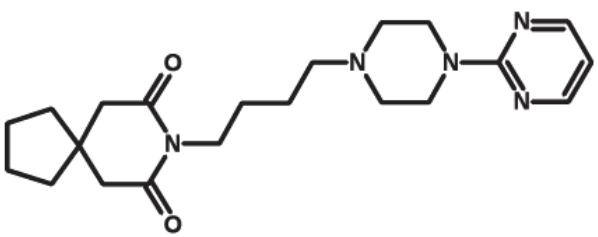
LC-MS application: metabolism



Buspirone

Buspiron: anxiolytic drug
metabolized in the liver by Cytochrome P-450

drug development:

- demonstration of metabolic stability
- body half life
- bioavailability
- toxicity
- side effects

→ identification and quantitation of metabolites

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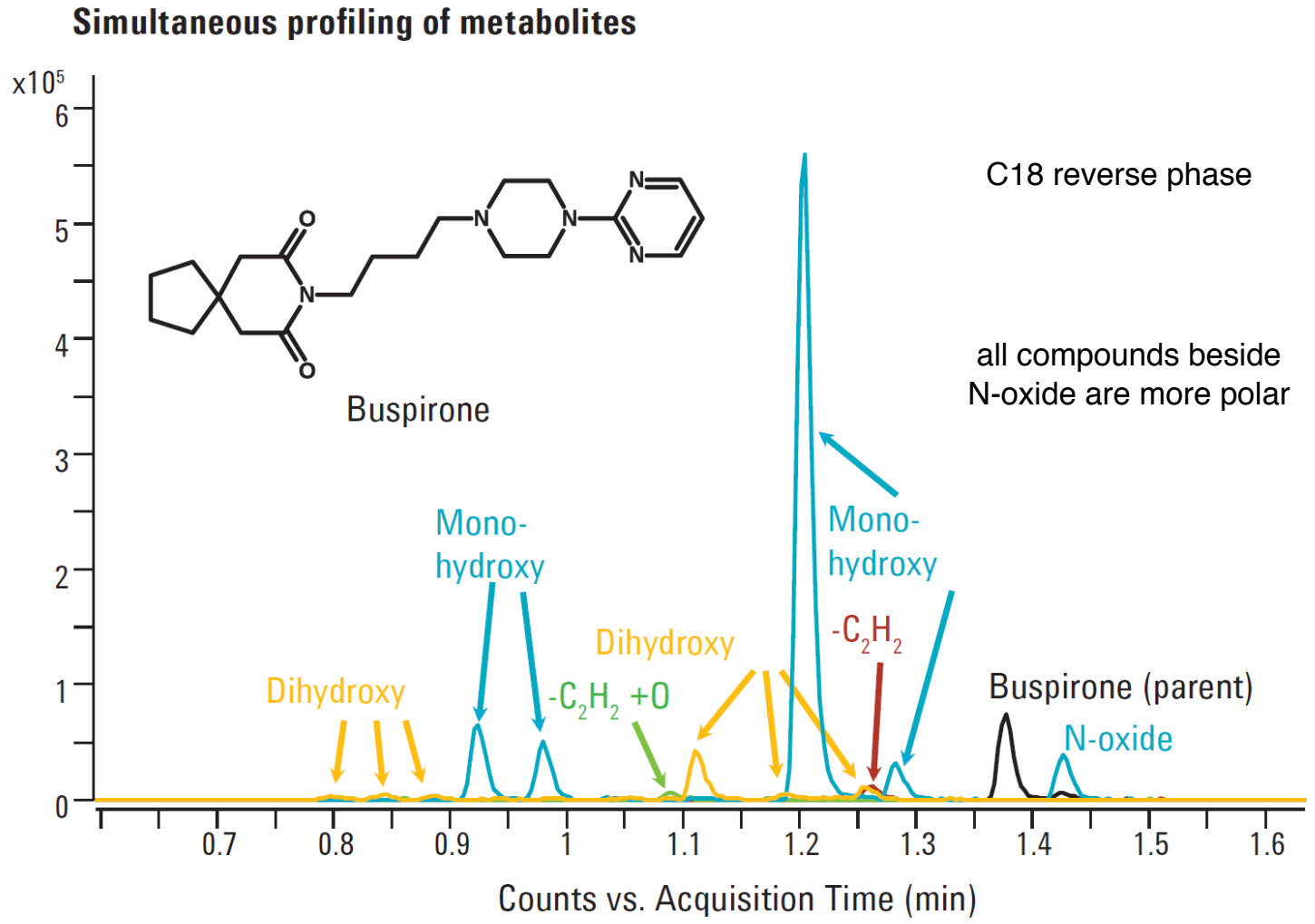
LC-MS application: metabolism

metabolite profiling after 5 min incubation in rat liver preparation



Agilent Application note 5990-6867EN

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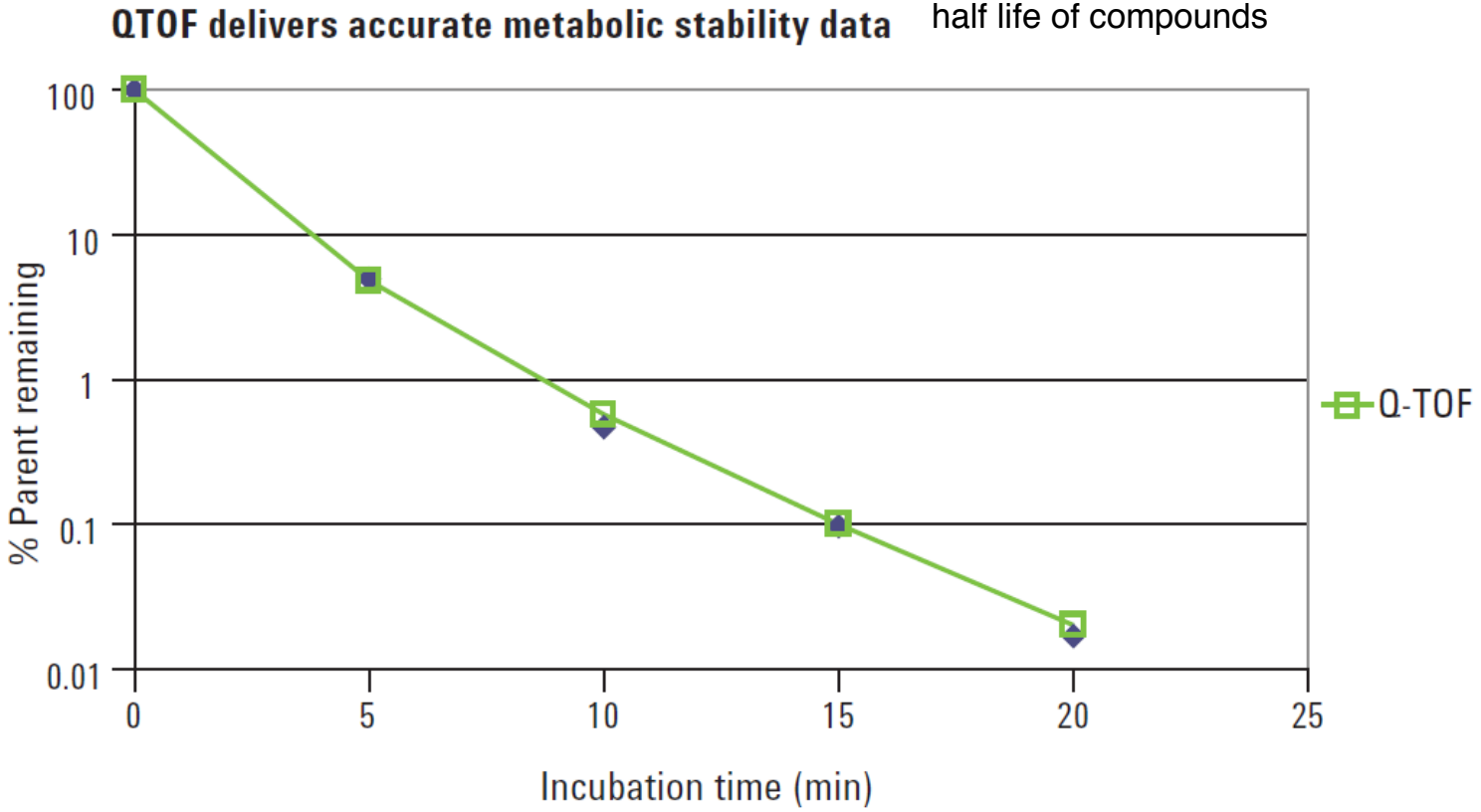
LC-MS application: metabolism

half life of compounds



Time	0 min	5 min	10 min	15 min	20 min
QTOF (%)	100	4.88	0.57	0.100	0.020

Agilent Application note 5990-6867EN

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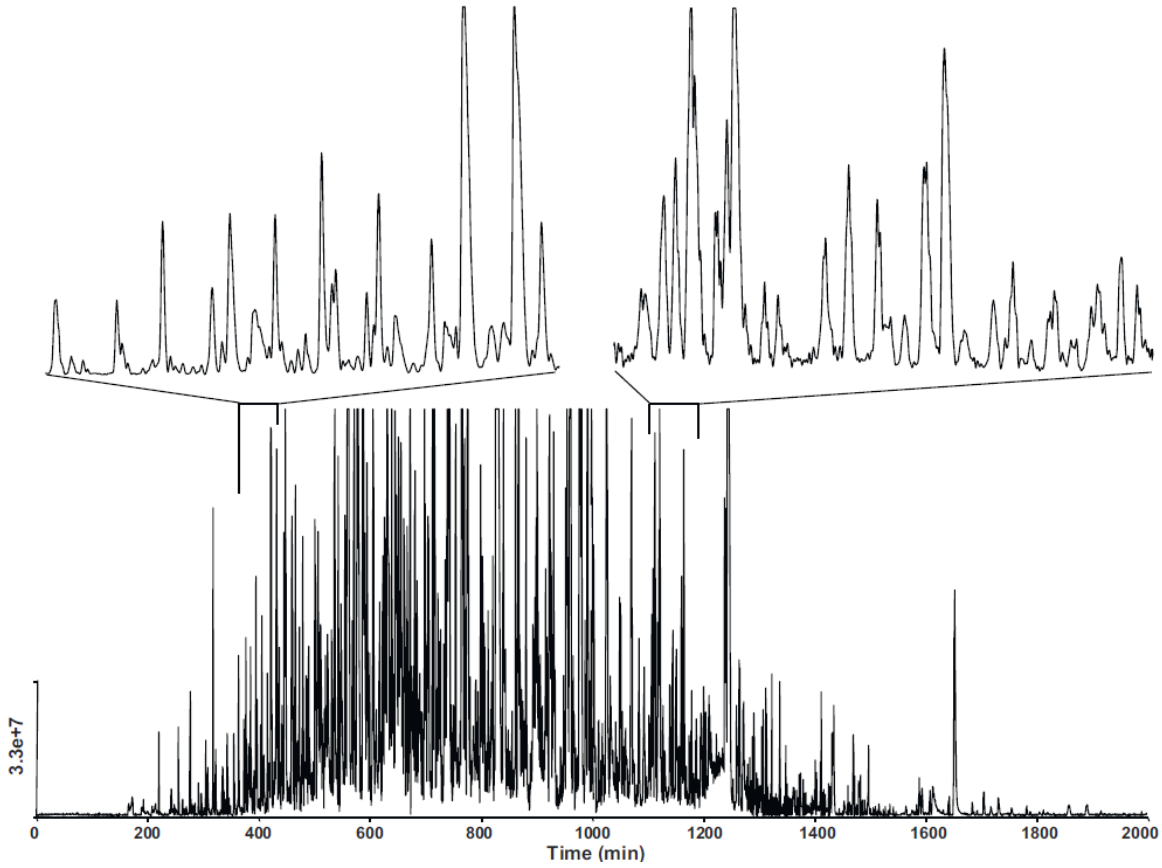
example: LC-MS in proteomics



12 h
12 000 peptides
→ 2000 proteins

Xie, J. Chromatogr. A 2012, 1261, 78– 90

Achieving a chromatographic separation peak capacity of 1500 using a 200 cm × 50 µm i.d. capillary containing 3 µm porous C_{18} particles operated at ~1400 bar for a cellular global tryptic digest of *Shewanella oneidensis*. Detection via a 11.4 T FTICR MS (80-min gradient); flow rate of mobile phase of only 0.12 cm/s

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electrophoresis - basics

dynamic

How does an ion move in an electrolyte upon application of an external electric field (voltage application)?
Which forces are relevant?



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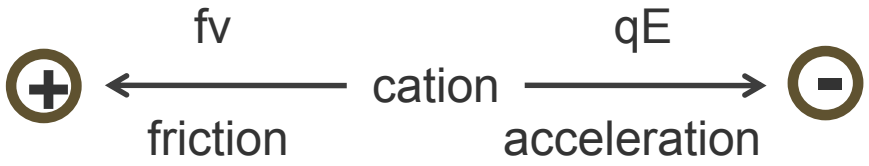
electrophoresis – basics: model

„electrophoresis is the movement of a charged particle in an electric field“

an ion of charge q experiences the acceleration force qE in the electric field E and the friction force fv with the friction coefficient f:

f: friction coefficient
v: analyte velocity



q: ion charge
E: electric field strength

Hückel equation $v = \frac{qE}{6\pi\eta r}$ → v = μE

μ: electrophoretic mobility

Stokes equation for spherical particles: f = 6πηr

r: radius
η: viscosity

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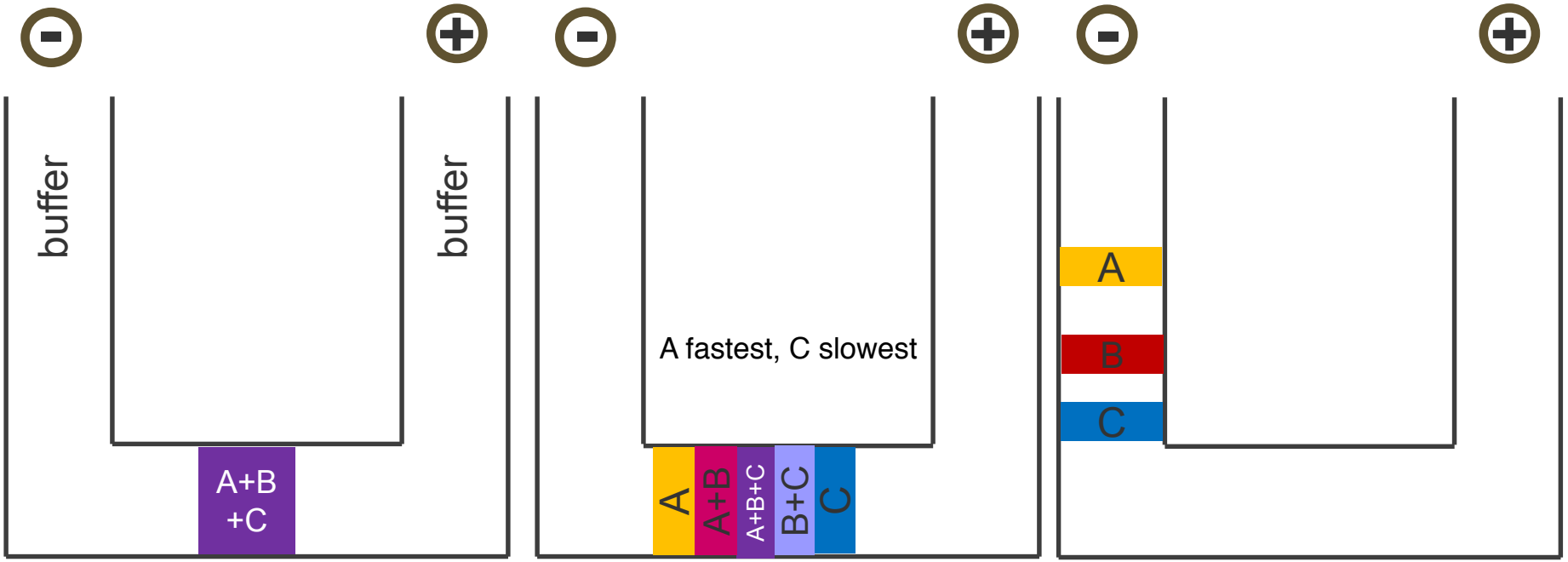
electrophoresis – basics: model

small sample plug in homogeneous electrolyte solution and application of voltage



after some time full separation of cationic analytes

electrophoretic mobility: $\mu_A > \mu_B > \mu_C$

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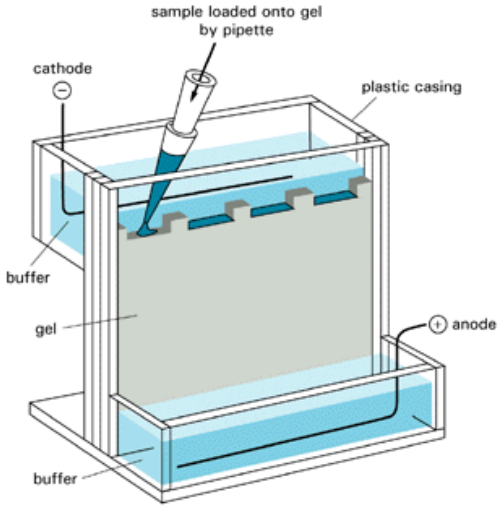
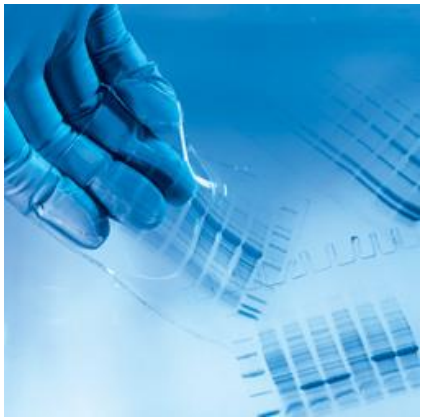
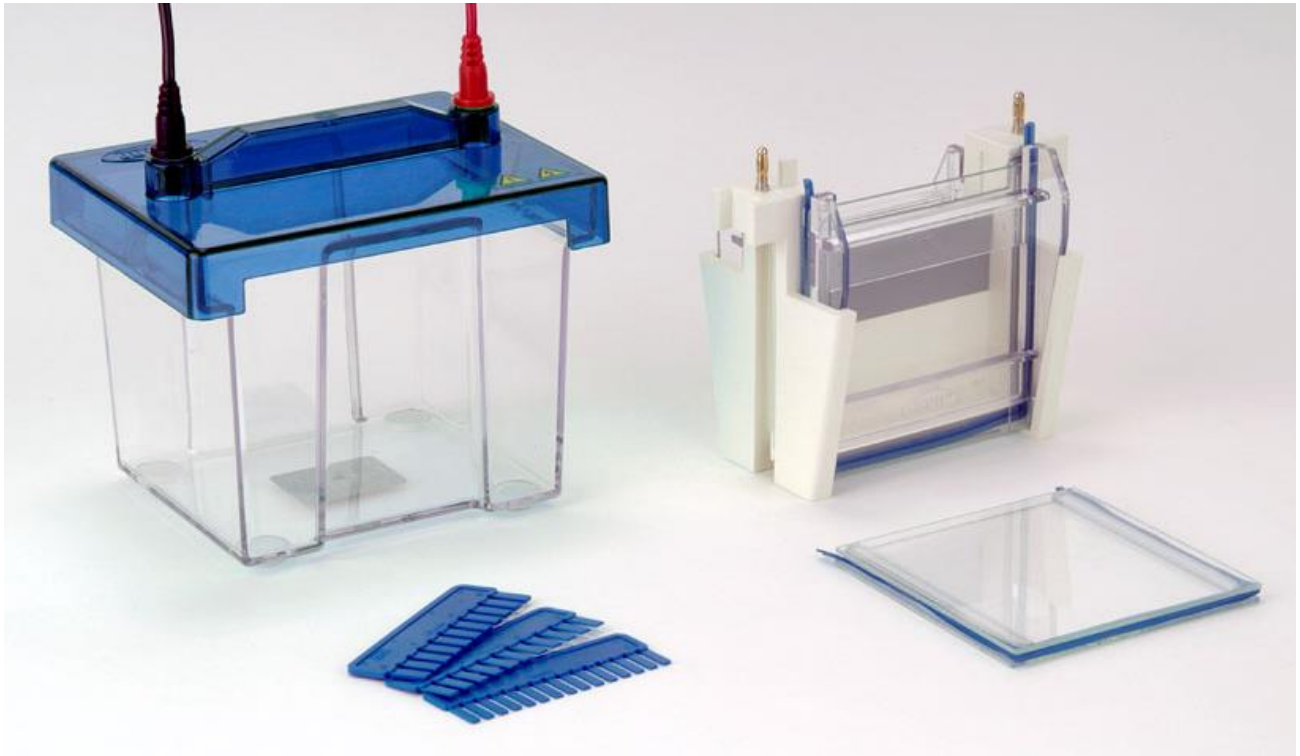
instrumental: gel electrophoresis



http://www.imb-jena.de/~rake/Bioinformatics_WEB/gifs/sds_page2.gif



http://www.piercenet.com/method/overview-electrophoresis



http://www.uniequip.com/de/img/vertical_pace_devices.jpg

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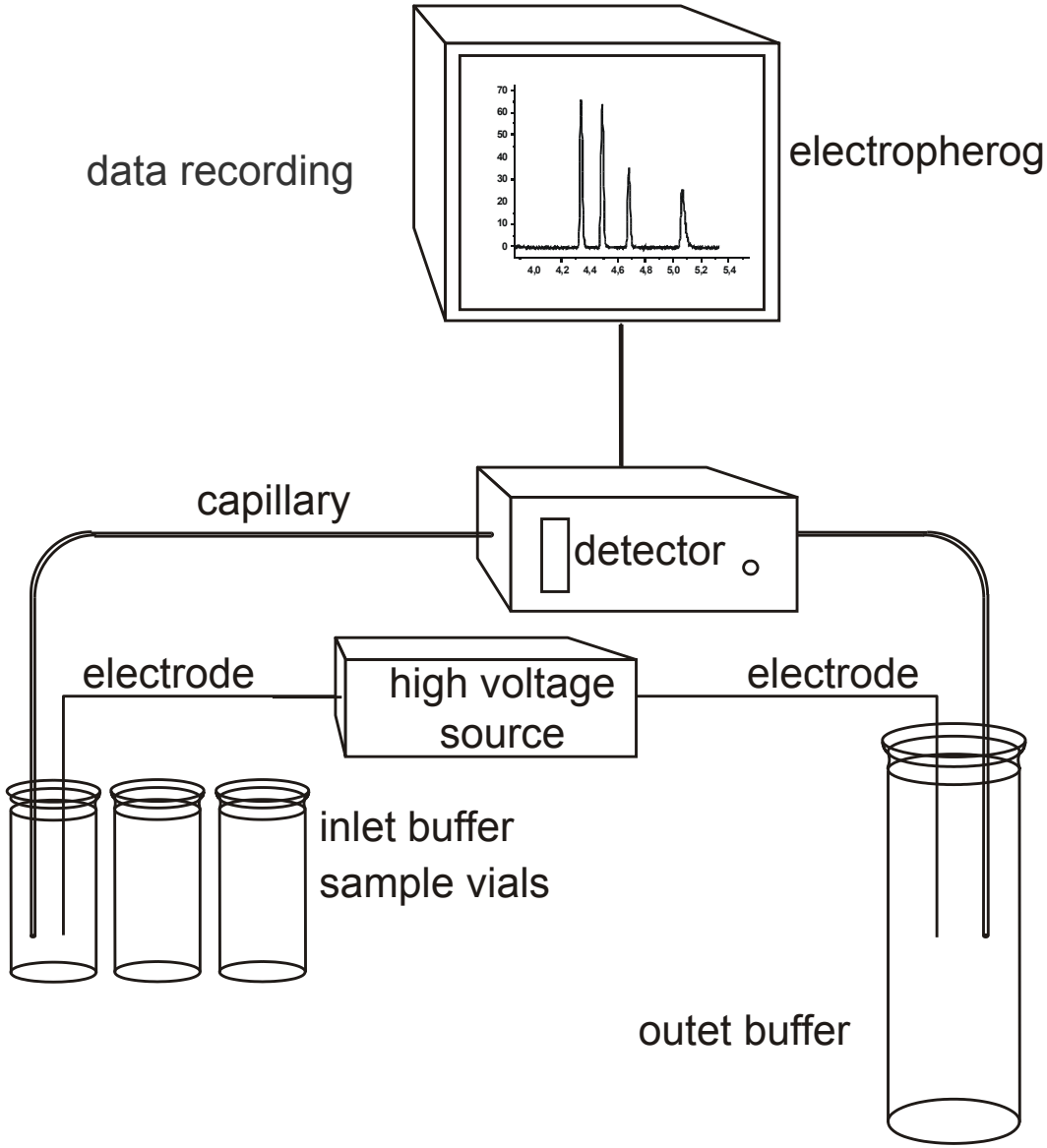
instrumental: capillary electrophoresis



Capillaries
fused silica with polyimide coating
L_{ges}=10-100 cm
i.d. = 10-100 µm
o.d. = 365 µm

Voltage
U up to +/- 30 kV

on-column or post-column detection

temperature control

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electrophoresis – basics: model

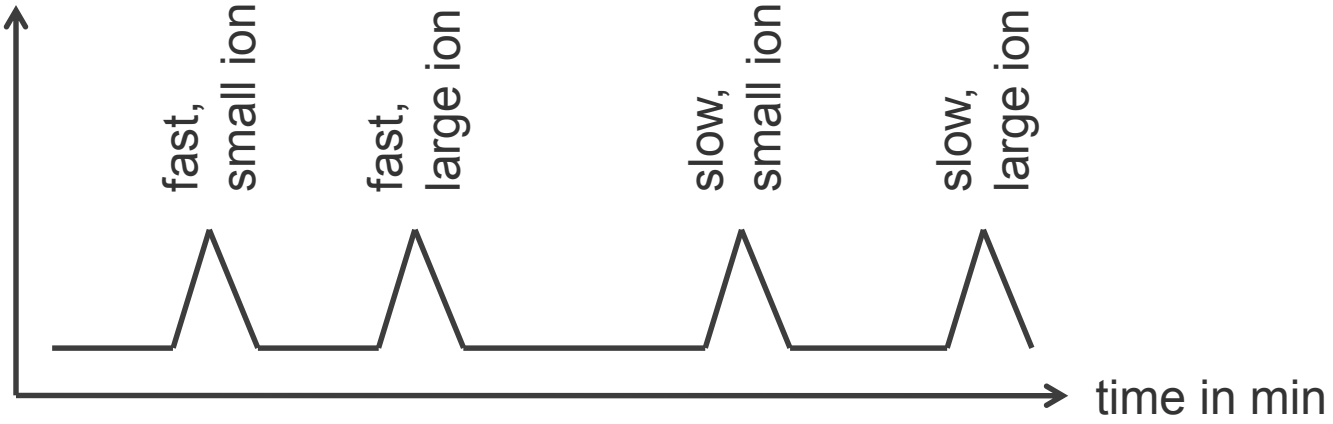
factors influencing the separation:

Exam!

analyte properties
- analyte charge
- analyte size (hydrodynamic radius)
- shape
- diffusion coefficient

external parameters
- pH of the electrolyte
- viscosity
- applied voltage
- capillary length

fast migration of highly charged, small ions



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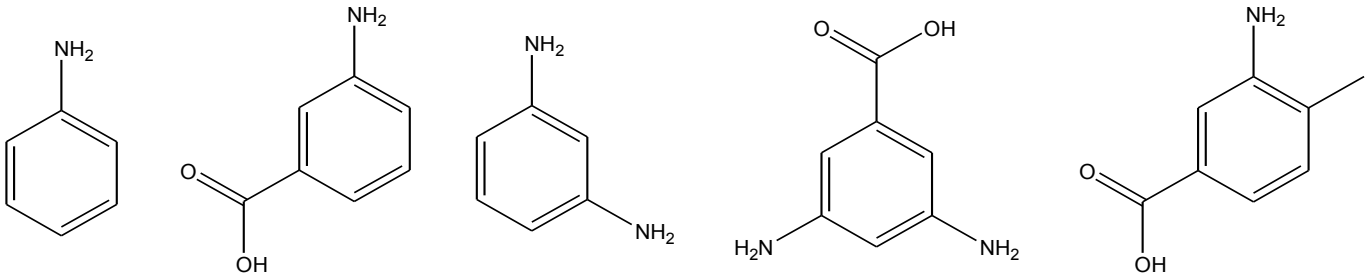
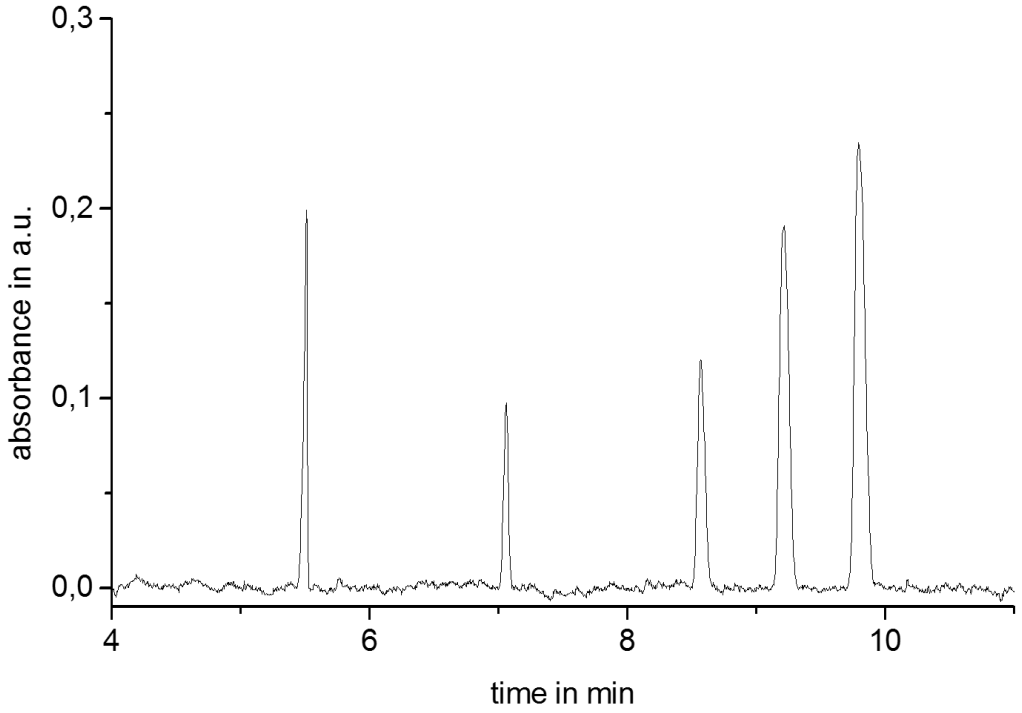
electrophoresis – basics: model

separation of aromatic amino acids:
at pH 2





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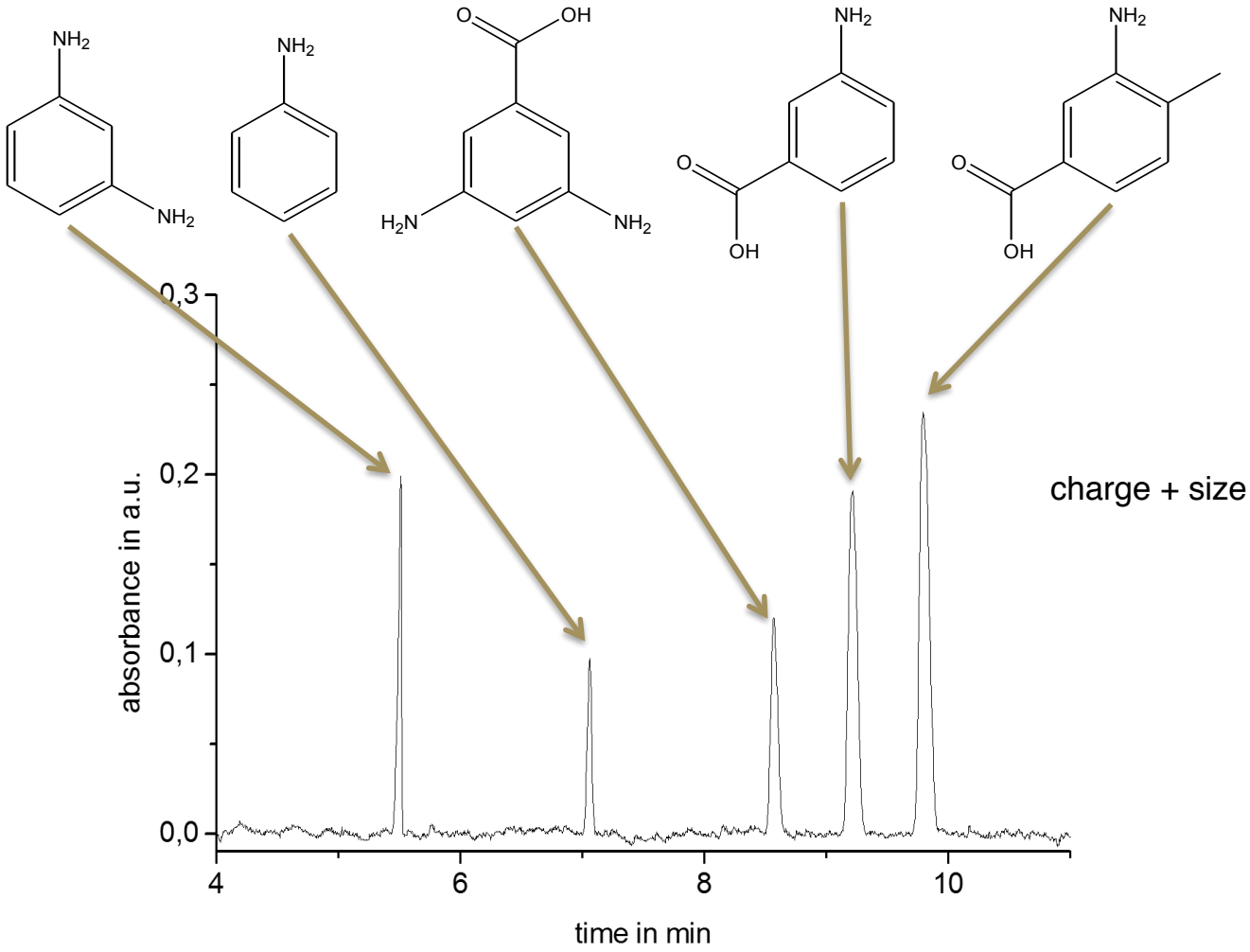
electrophoresis – basics: model

separation of aromatic amino acids:
at pH 2: separation according to charge and thus number of amine functional groups

exam!



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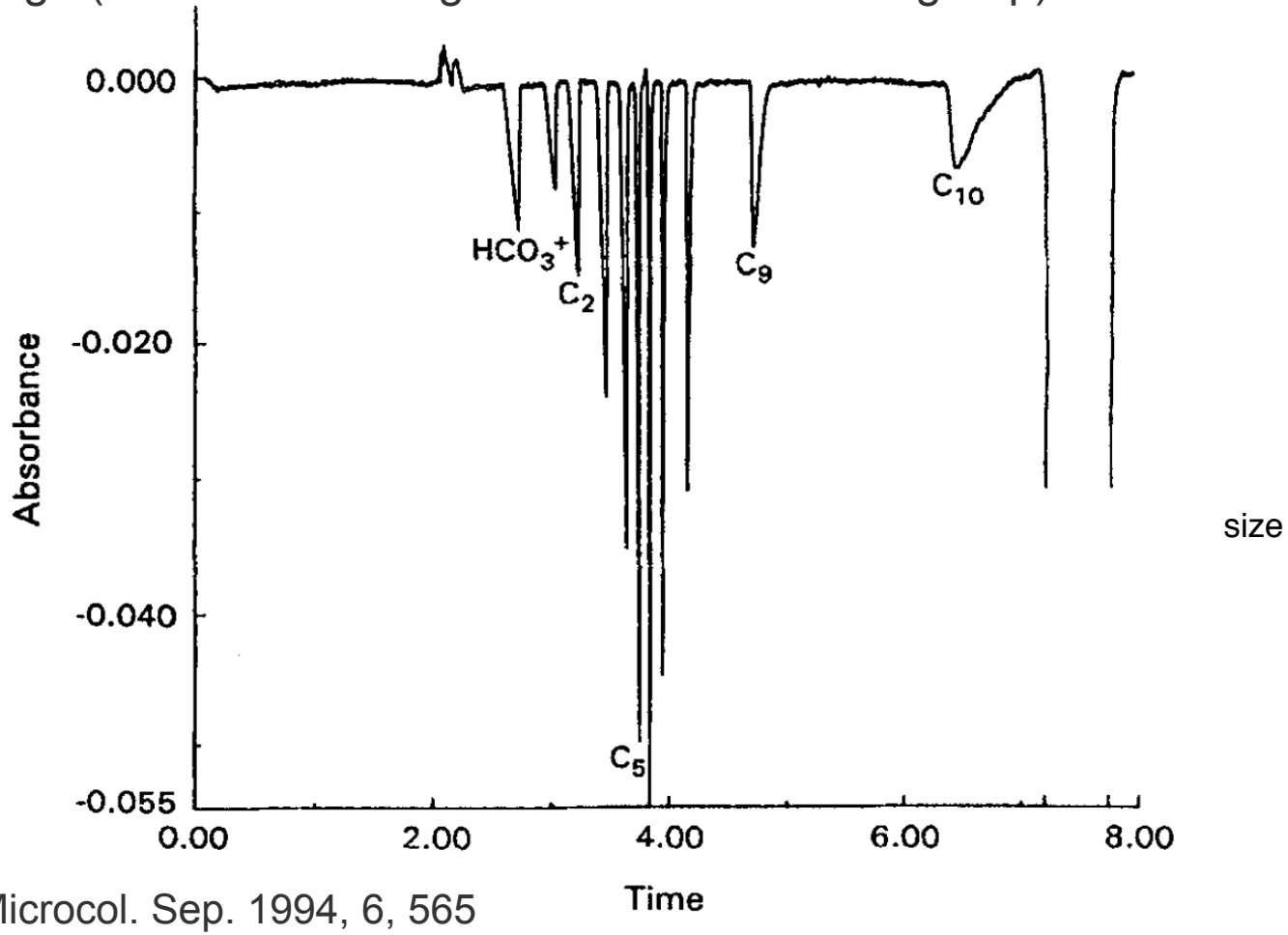
electrophoresis – basics: model

separation of homologues of aliphatic fatty acids:
The fatty acids only differ in the length of the alkyl chain and thus in size, but not in charge (all with one charge on the $–COO^-$ head group).



Gutnikov, J. Microcol. Sep. 1994, 6, 565

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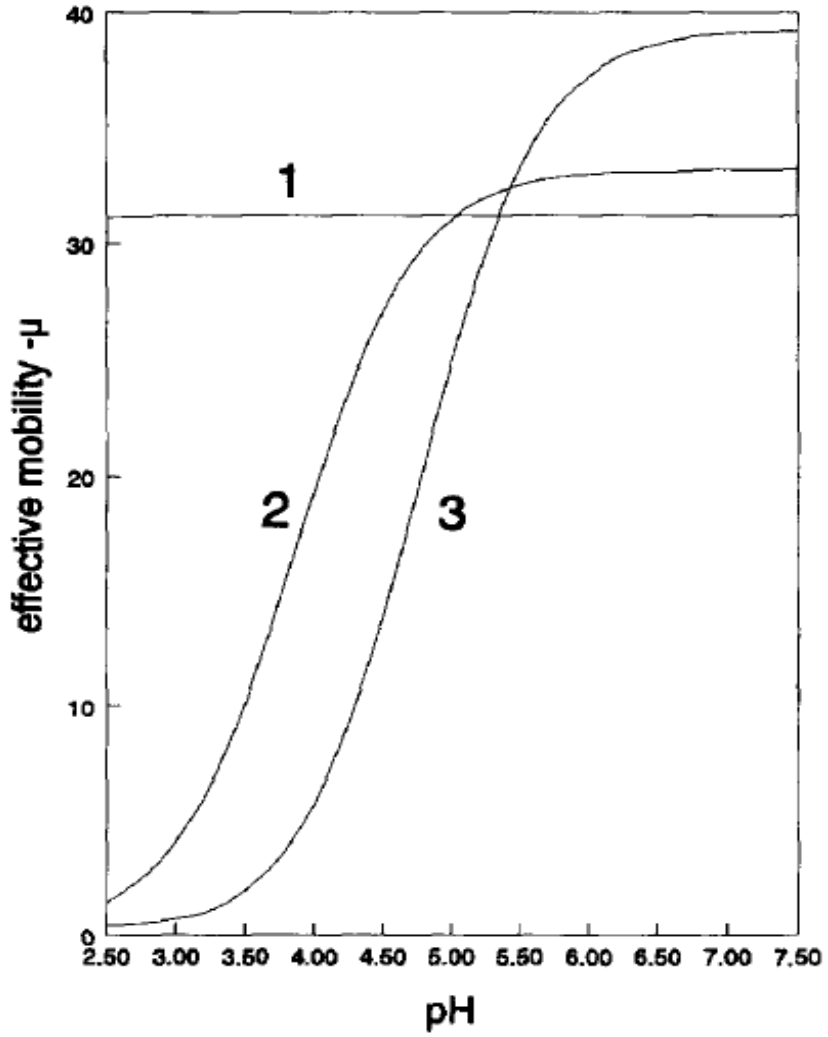
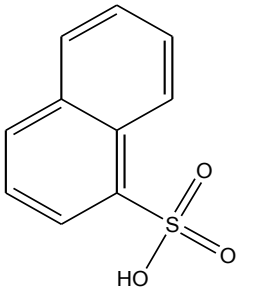
electrophoresis – basics: model

Can you explain the pH-dependence of mobilities of these molecules?

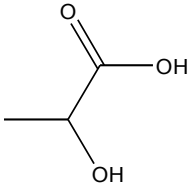


separation at different pH



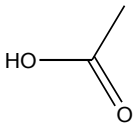
naphthalene sulfonic acid

1: naphthalene sulfonic acid



lactic acid

2: lactic acid



acetic acid

3: acetic acid

Reijenga et al., J. Chromatogr. A 1996, 744, 147-153

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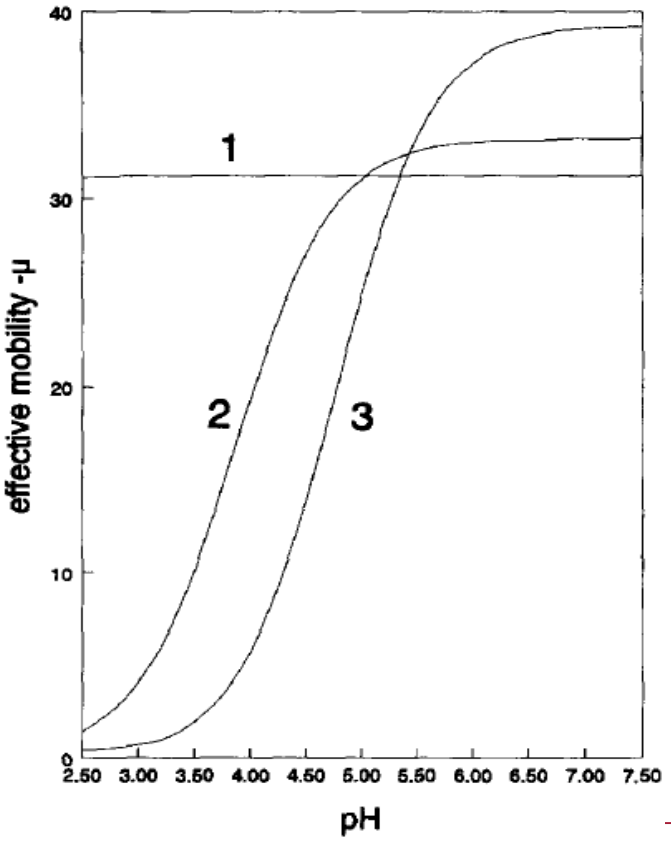
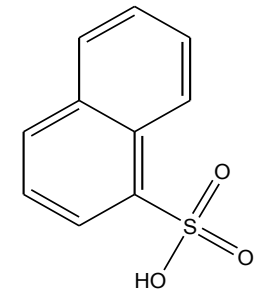
electrophoresis – basics: model

exam

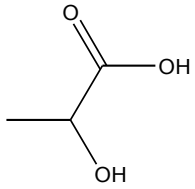
Sulfonic acids have a very low pK_a and are always fully charged in aqueous solution. Their electrophoretic mobility is independent of the pH-value. For lactic and acetic acid, the plot of effective electrophoretic mobility vs. pH shows their titration curve: at low pH they are protonated and not charged, at pH = pK_s they are charged by 50%; and fully deprotonated with charge number = 1 at high pH.





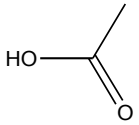
naphthalene sulfonic acid

1: naphthaline sulfonic acid



lactic acid

2: lactic acid



acetic acid

3: acetic acid

Reijenga et al., J. Chromatogr. A 1996, 744, 147-153

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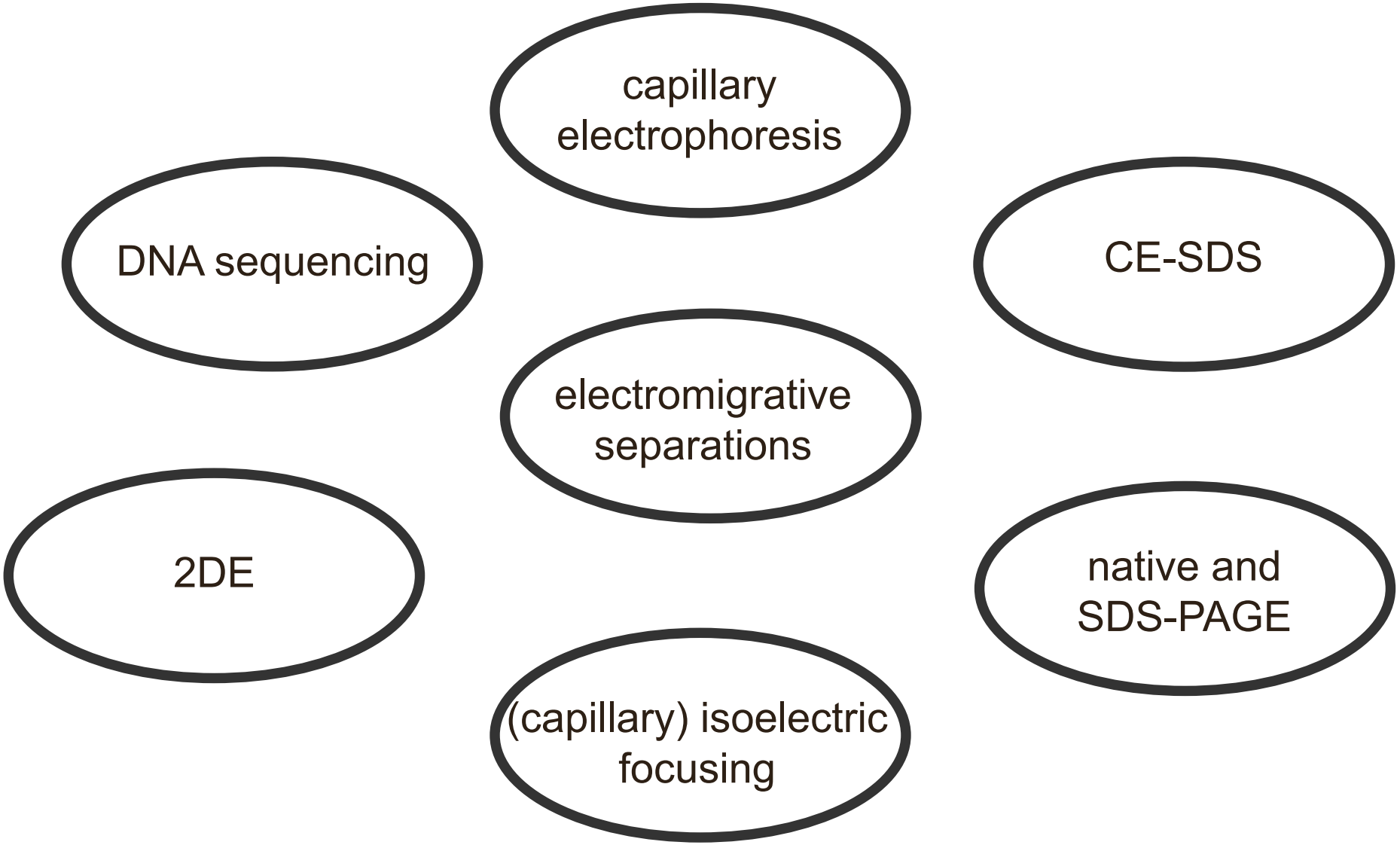
modes of electromigrative separation techniques



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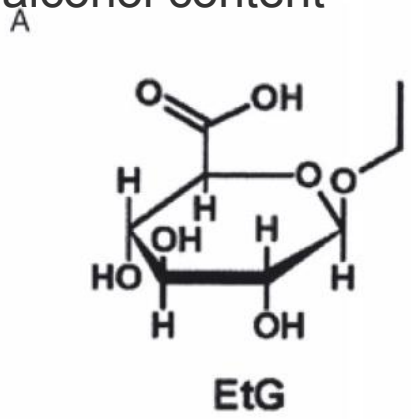
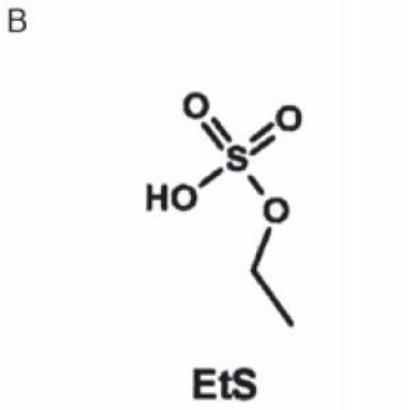
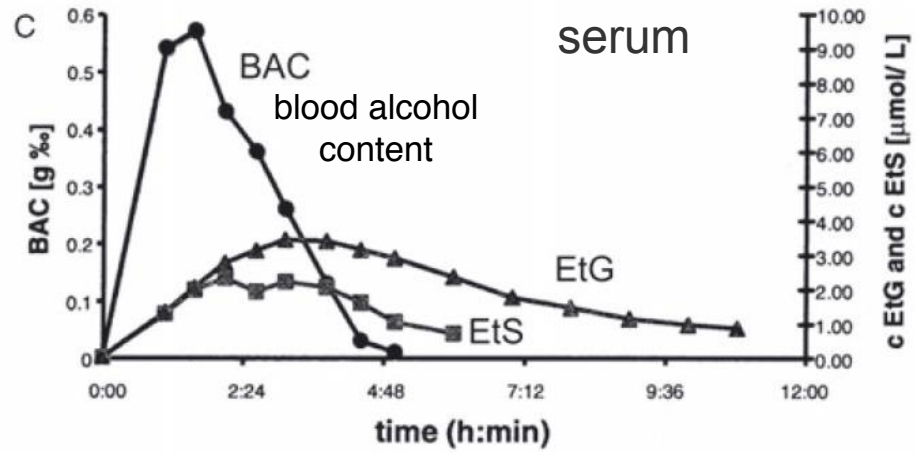
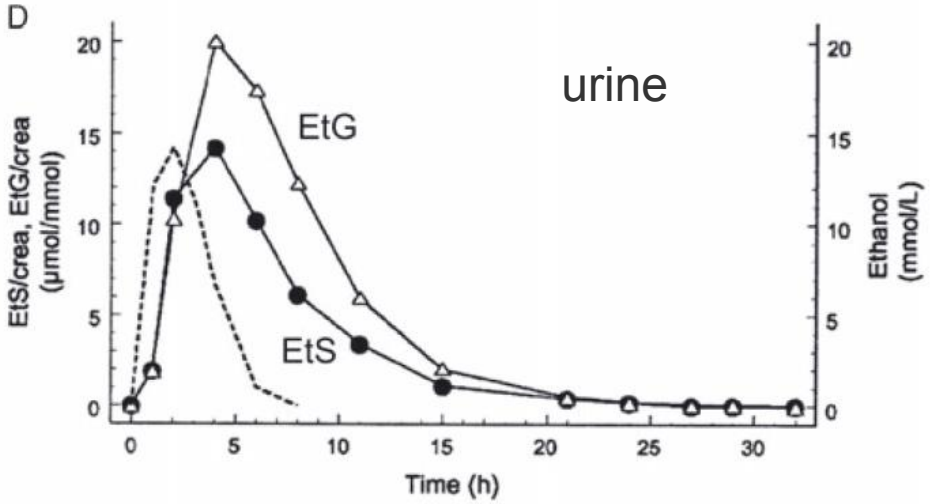
example: ethyl sulfate as alcohol marker

EtG and EtS data obtained after ingestion of about 0.5 g ethanol per kg body mass and analysis of (C) serum and (D) urine using LC-MS/MS
BAC: blood alcohol content









Caslavska, J. Sep. Sci. 2013, 36, 75–95

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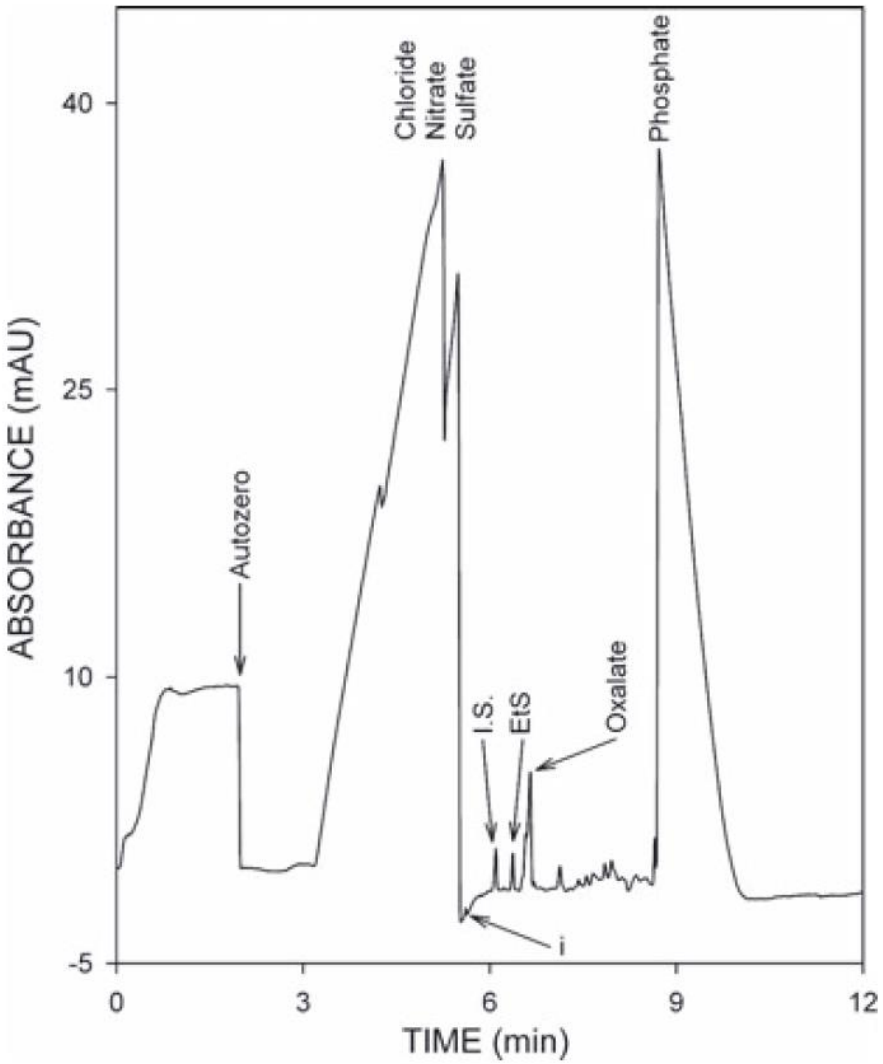
example: ethyl sulfate as alcohol marker



- ethyl sulfate is a strong acid (pK_a < -3), fully charged in aqueous buffers
- separation at very low pH = 2.2
- then only ethyl sulfate and inorganic/small organic ions are still charged
- high selectivity as most matrix is positively charged or neutral
- polarity: inlet negative, outlet positive; -10 kV applied voltage
- ethyl sulfate without chromophore→ detection via indirect detection (replacement of absorbing buffer component)

Caslavska, J. Sep. Sci. 2013, 36, 75–95

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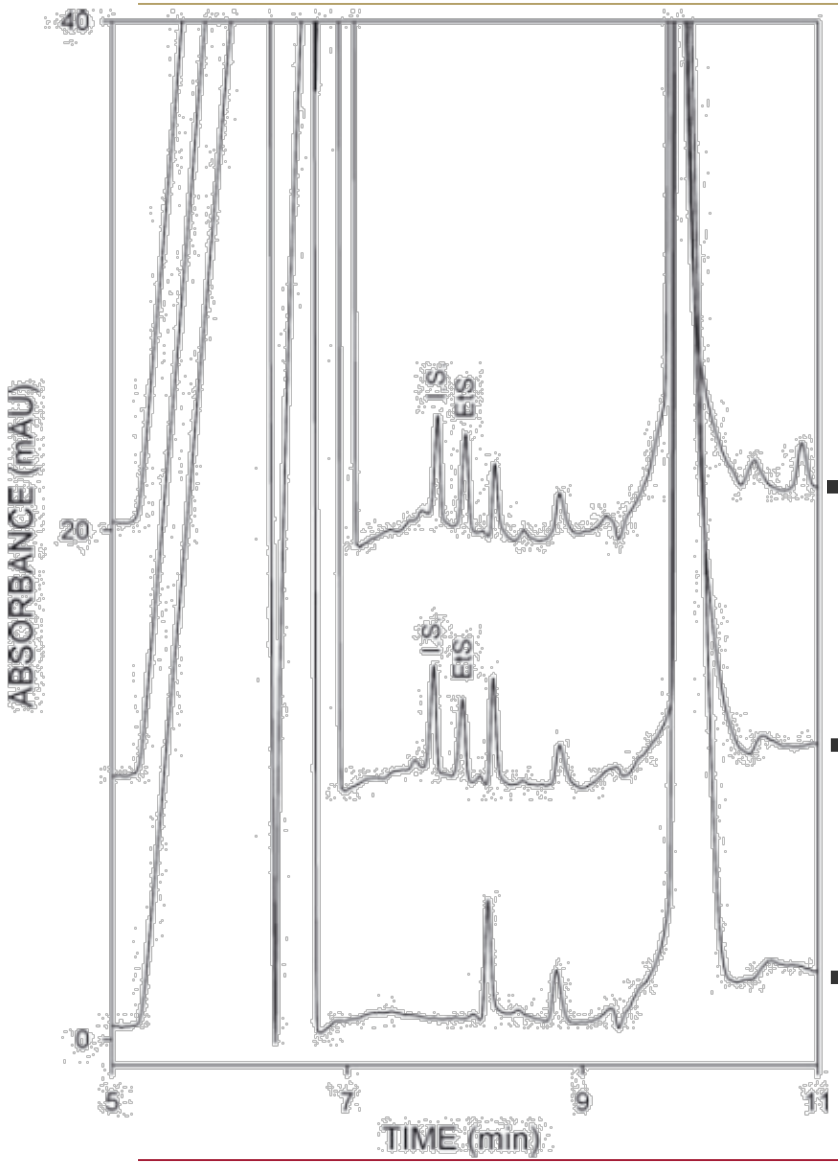
ethyl sulfate, ethyl glucuronide



analysis of serum extracts

real sample with an EtS serum concentration of 1.70 mg/L

blank serum spiked with EtS (1.5 mg/L) and internal standard I.S.

blank serum

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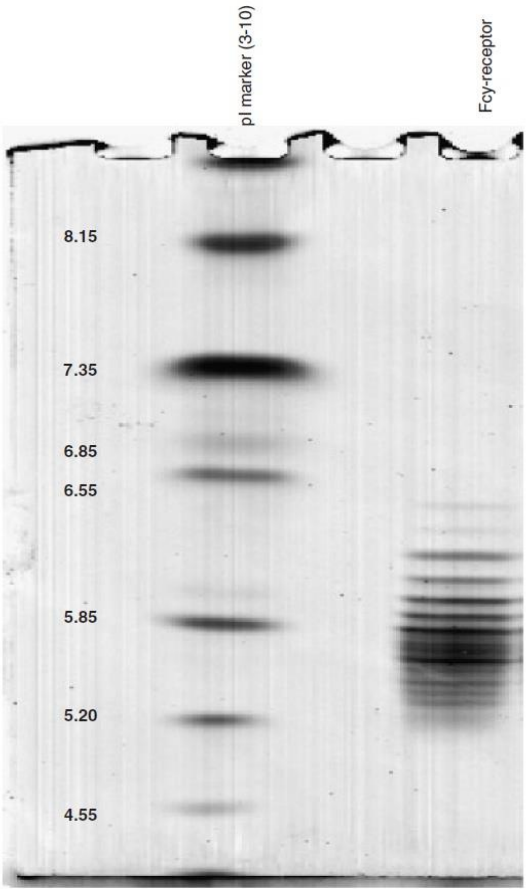
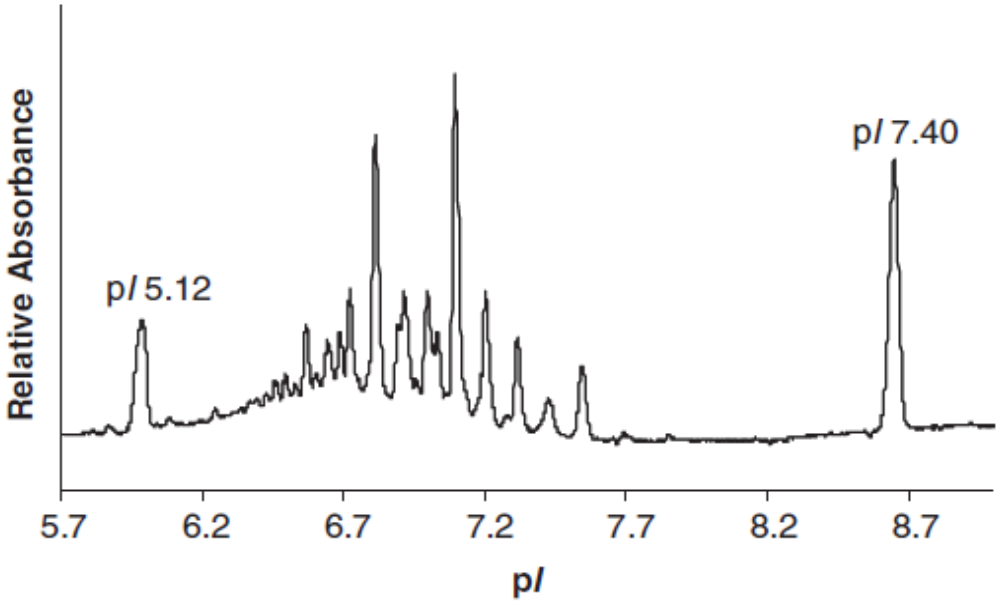
isoelectric focusing

formats:

- „slab gel“
- capillaries

pI = pH were proteins are neutral, isoelectrical point





charge heterogeneity of the $F_c\gamma$- receptor fusion protein

Z. Sosic et al. Electrophoresis 2008, 29, 4368-4376

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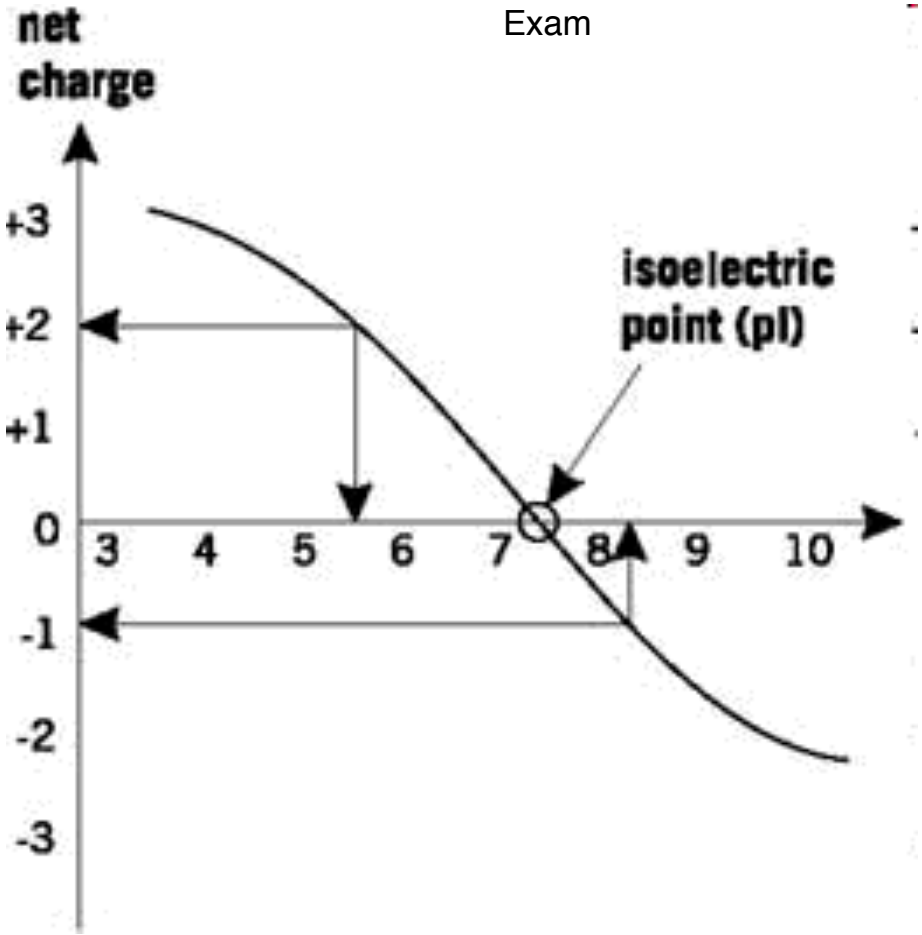
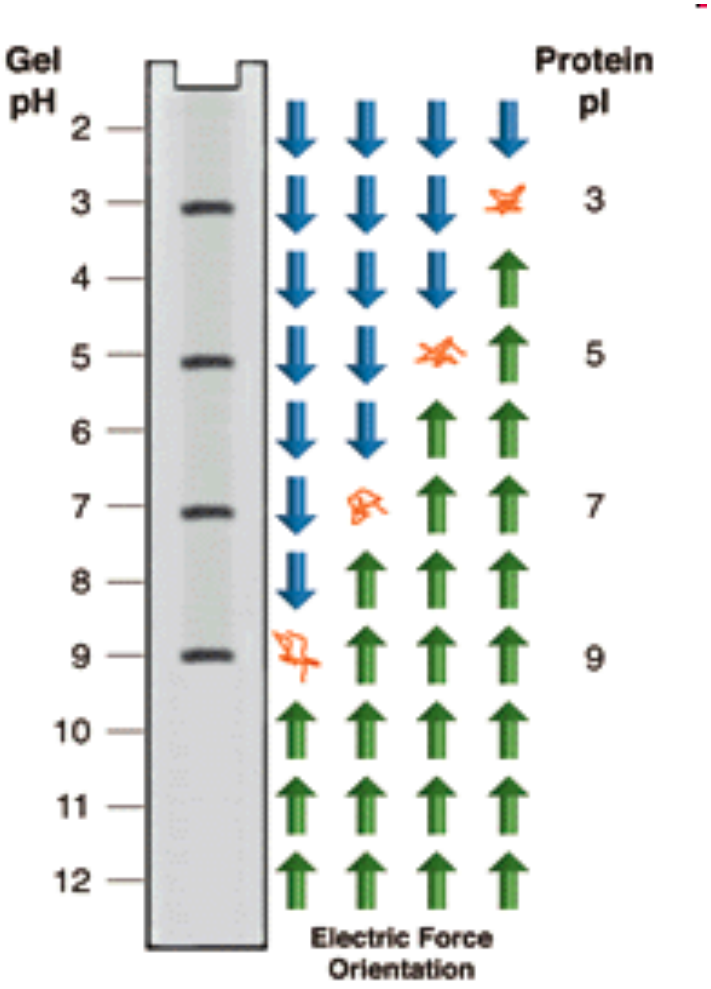
isoelectric focusing

Exam



http://www.gelifesciences.com/aptrix/upp00919.nsf/Content/elpho_applications~elpho_applications_1d_protein_analysis~elpho_ief_separation



http://nationaldiagnostics.com/article_info.php/articles_id/65

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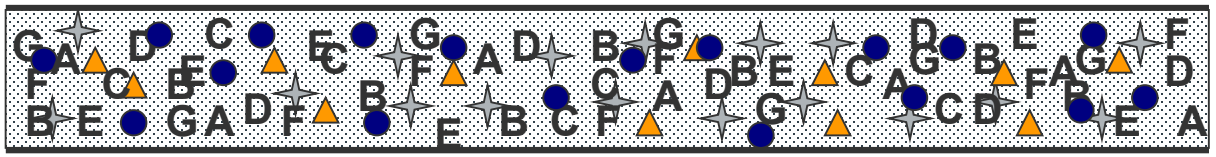
isoelectric focusing

general procedure

sample injection

catholyte
NaOH

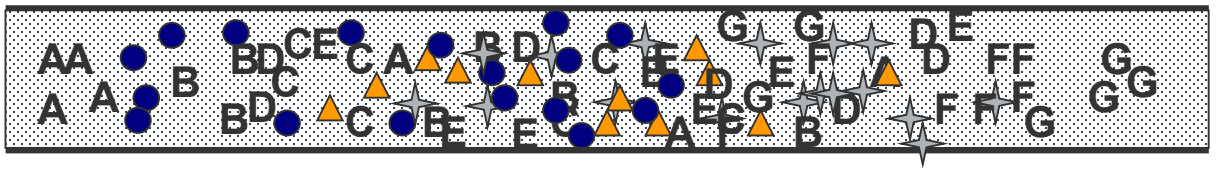


anolyte
H_3PO_4



transient state

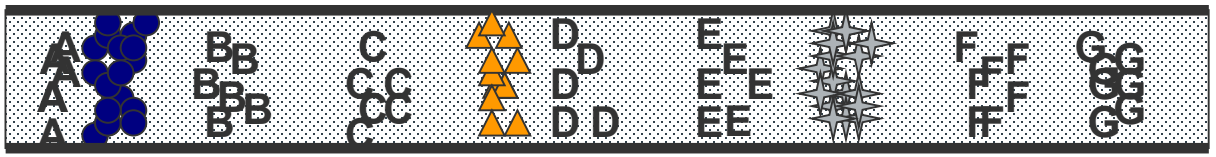
catholyte
NaOH



anolyte
H_3PO_4

stationary state

catholyte
NaOH



anolyte
H_3PO_4

preconcentration and focusing

A,B,C,D,E,F,G: ampholytes

●, ▲, ✦: analytes, markers

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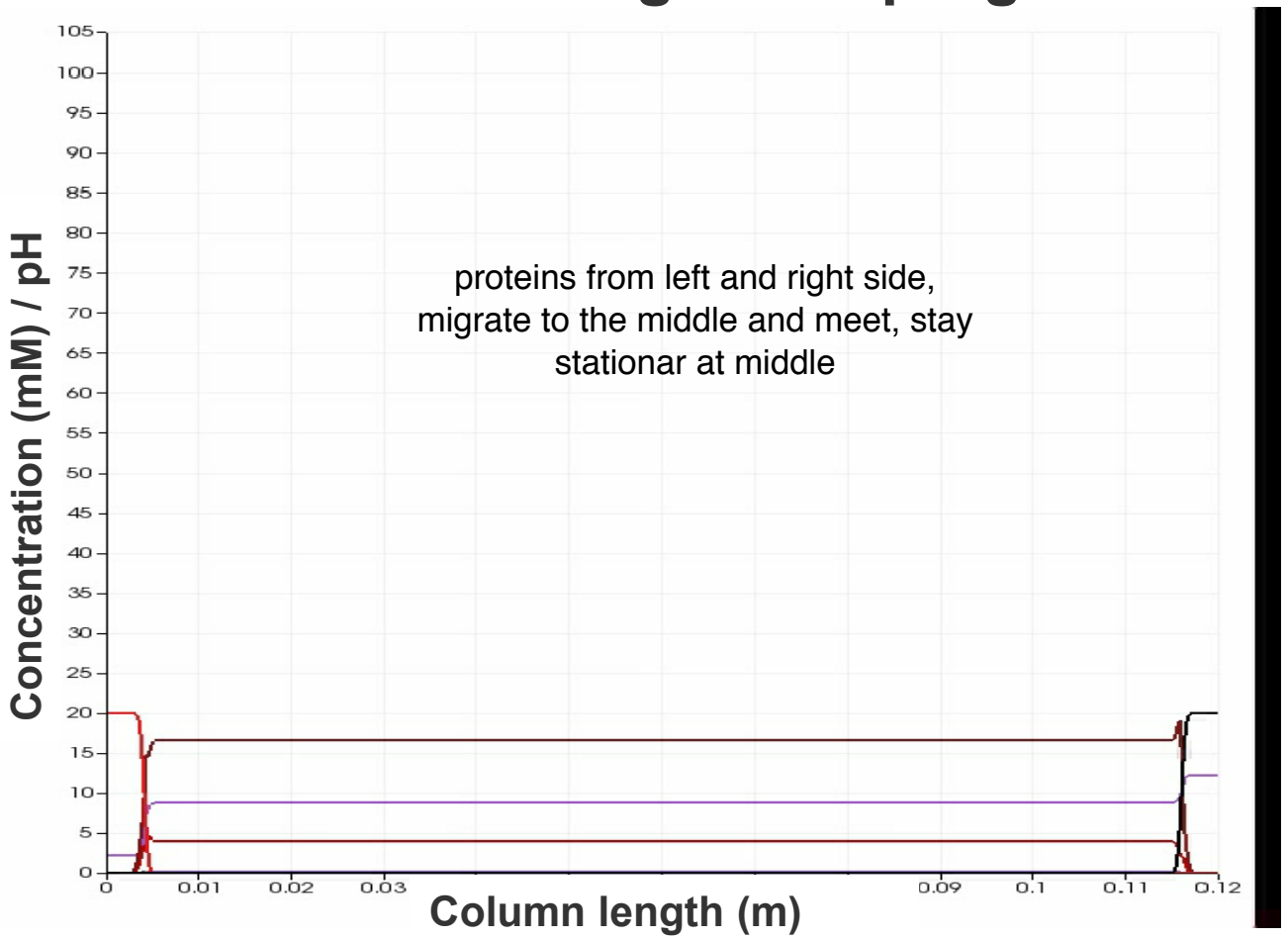
isoelectric focusing

Numerical modeling of the pH gradient



P. A. Kler et al., Comput. Mech. 2013, 51, 171-185

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isoelectric focusing

sample and buffers:
- sample in ampholyte mixture filling the whole capillary
- inlet: catholyte - strong base (mostly NaOH) – high pH
- outlet: anolyte - strong acid (mostly H_3PO_4) – low pH
- or: immobilized pI gradients

Exam

transient state:
- migration of OH^- and H^+ into/through capillary
- → acid-base-reaction with ampholytes/analytes
- migration of all components until their pI is reached → focusing

stationary state:
- after ca. 20 min
- no further migration
- self-sharpening

mobilization
- pressure or pH (exchange of catholyte NaOH by acetic acid)

or whole column detection
or second dimension PAGE in 2DE

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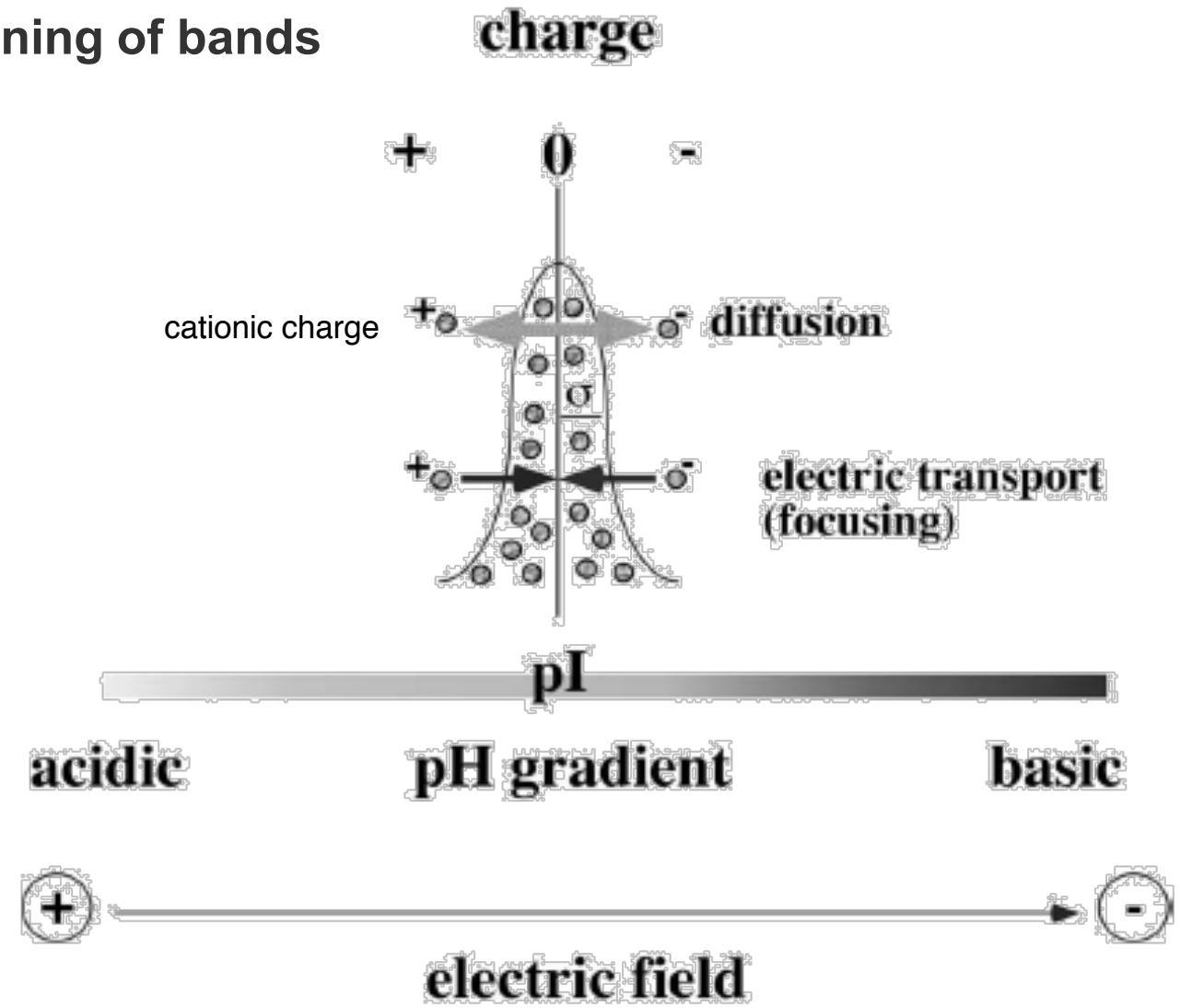
Explain isoelectric focusing with this pic

isoelectric focusing

self-sharpening of bands



http://arxiv.org/ftp/arxiv/papers/0909/0909.4158.pdf

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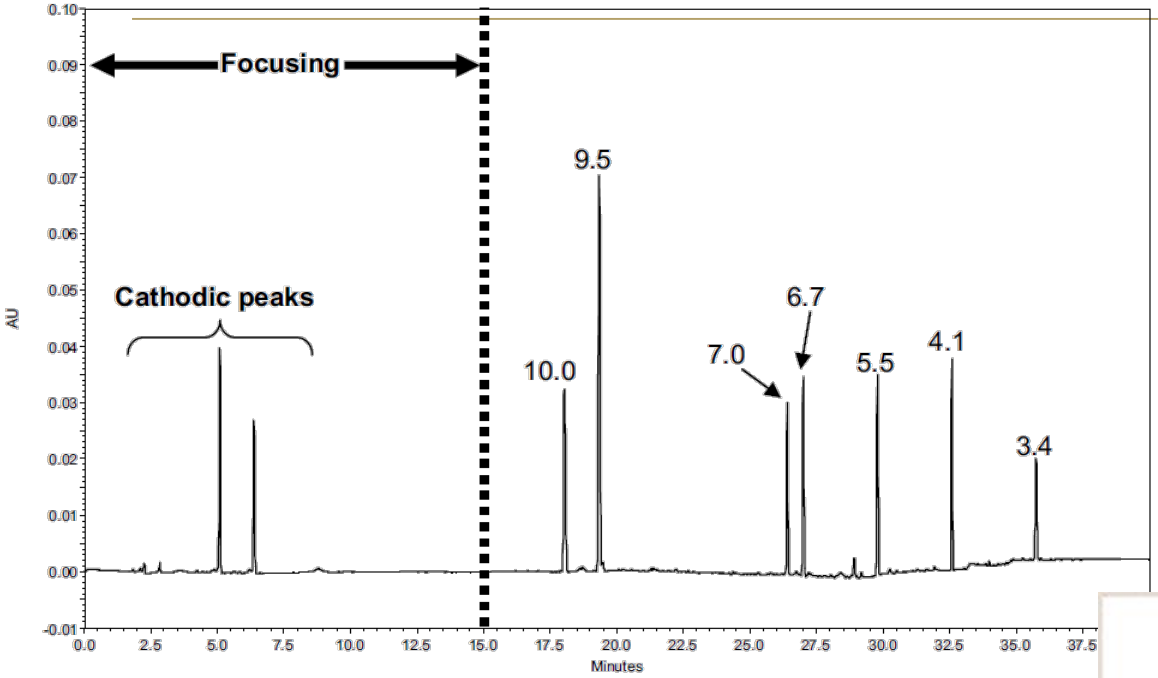
EBERHARD KARLS
UNIVERSITÄT
TÜBINGEN

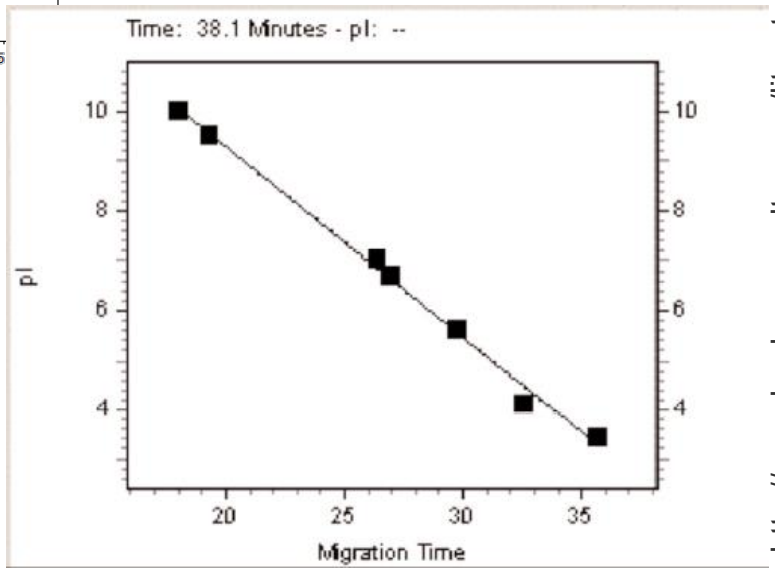
isoelectric focusing



pI determination:
cIEF of pI Marker
Pharmalyte 3-10
15 min focusing at 25 kV,
catholyte NaOH, anolyte: H_3PO_4,
then mobilization to detector

pI Marker	pI value
Trp-Tyr-Lys-Lys	10.0
Trp-Tyr-Tyr-Tyr-Lys-Lys	9.5
Trp-Glu-His-Arg	7.0
Trp-Glu-His-His	6.7
Trp-Glu-His	5.5
Trp-Asp-Asp-Arg	4.1
Trp-Asp-Asp-Asp	3.4

pI calibration



http://www.beckmancoulter.com/literature/Bioresearch/A-11634A.pdf

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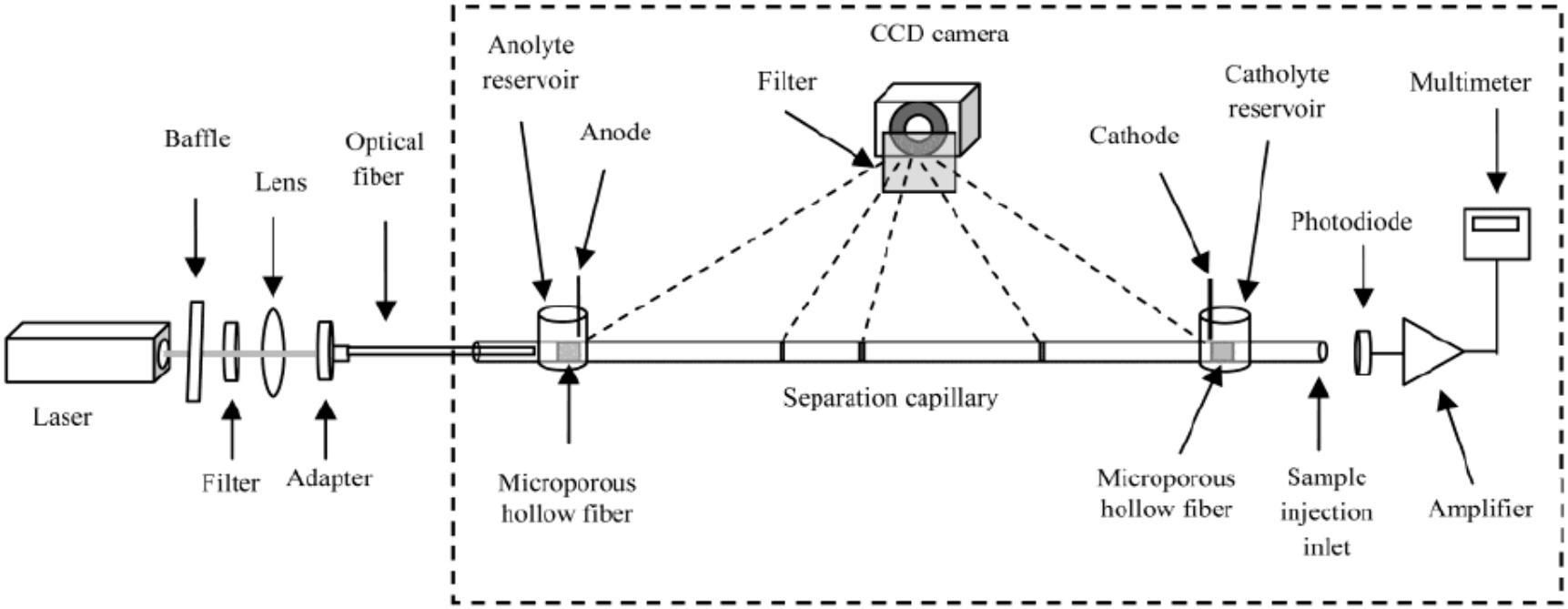
isoelectric focusing

whole column detection

here: laser-induced fluorescence



Z. Liu, J. Pawliszyn, Anal. Biochem. 2005, 336, 94-101

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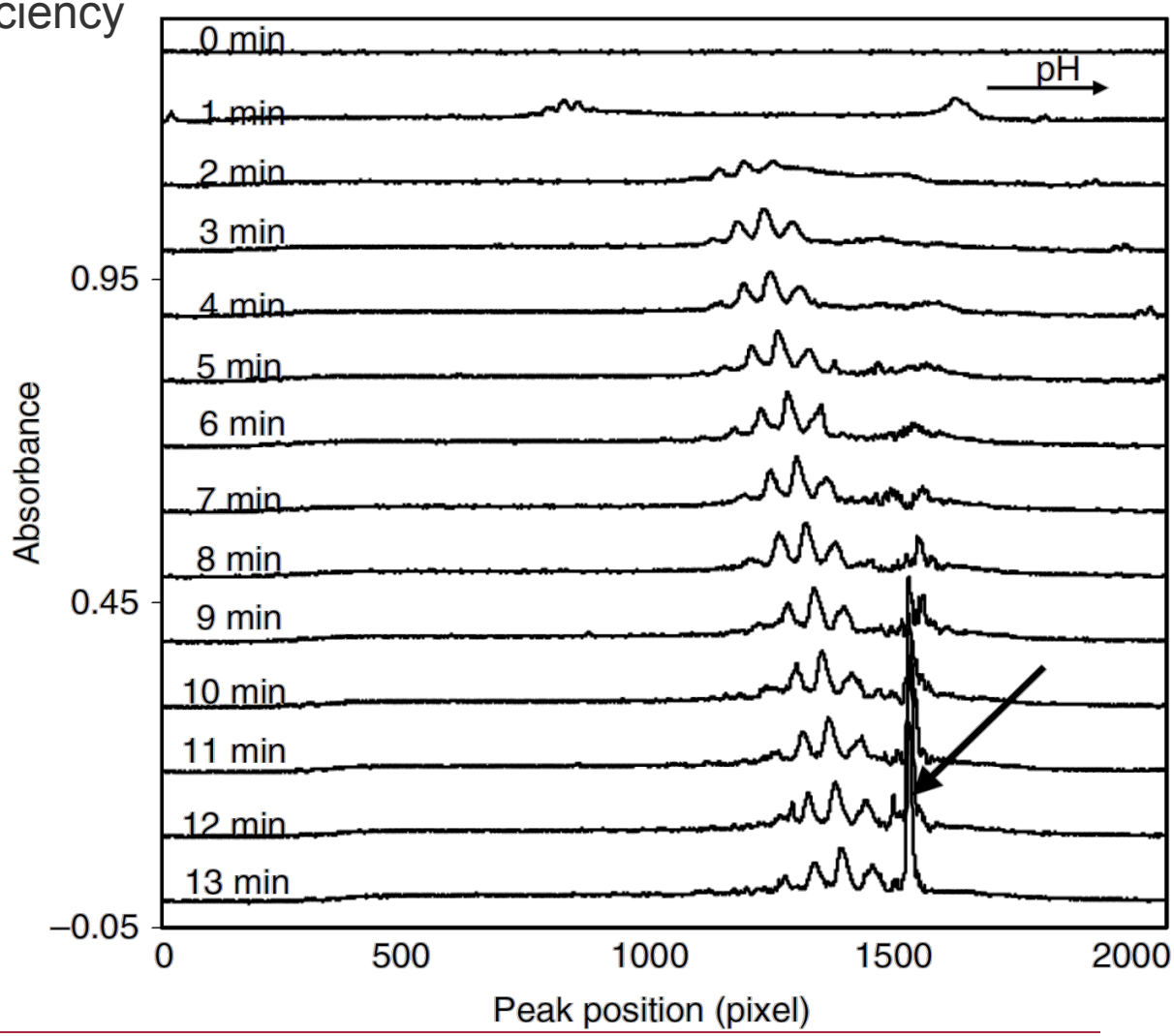
isoelectric focusing

picture of whole capillary → no mobilization necessary
→ high resolution and efficiency
→ high robustness



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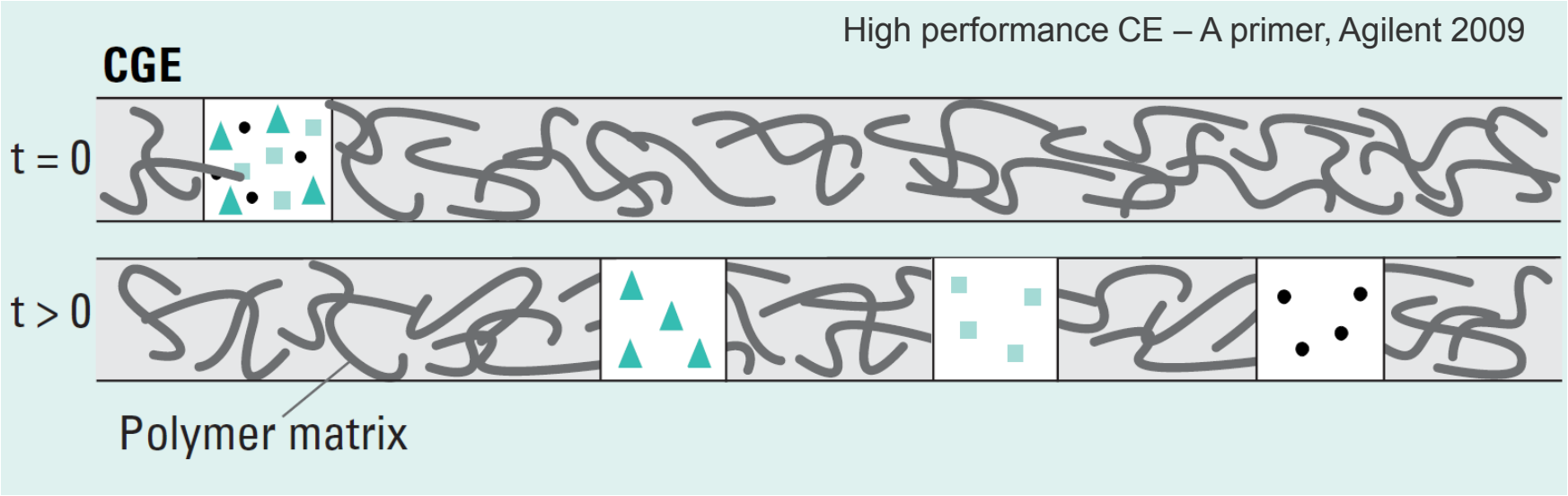
size sieving electrophoresis

High performance CE – A primer, Agilent 2009



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size sieving electrophoresis

CSE or CGE (capillary sieving or gel electrophoresis):
addition of a sieving matrix for size-based separation
chemical gels or physical polymer networks

CSE applicable for:
separation of analytes with identical mass to charge ratio
e.g. DNA with every base being singly phosphorylated or proteins being saturated with charged surfactand SDS (sodium dodecyl sulfate); 1.4 g of SDS is bound per g protein
used for:
- size-based separation of proteins (SDS-PAGE, CE-SDS)
- size-based separation of DNA
- DNA sequencing

fields of application
forensics, clinical diagnostics, protein characterization, DNA sequencing, identification of food

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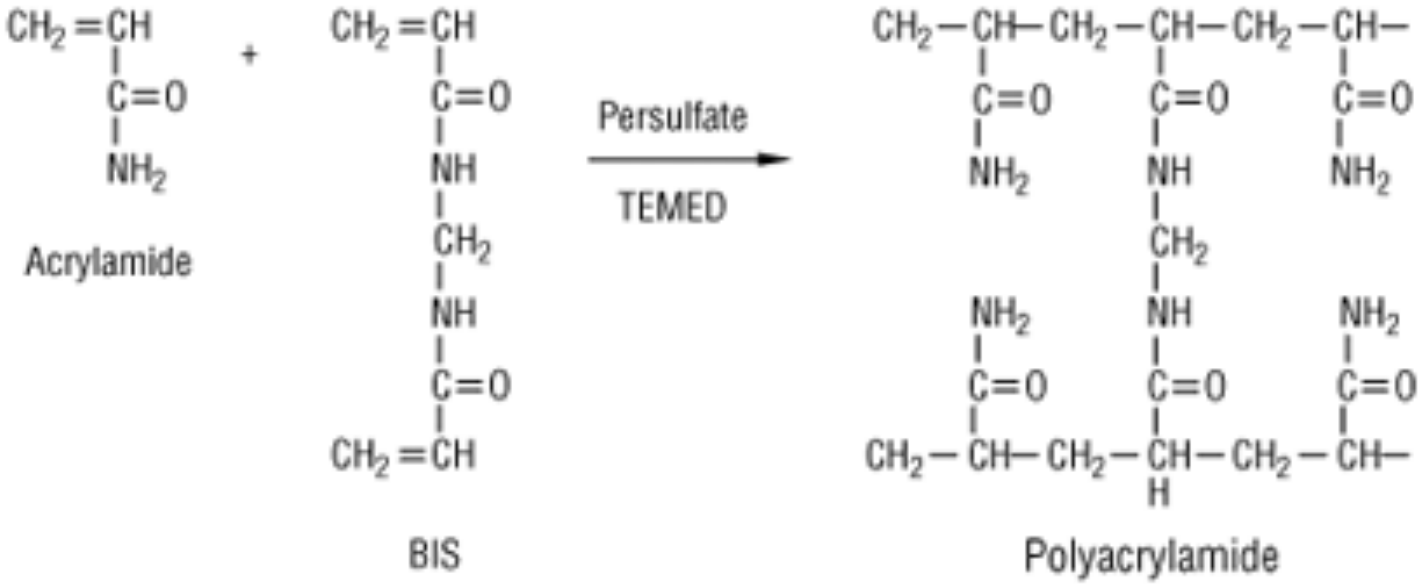
sieving matrices (gels)

a) chemical gels

- for slab gel format
- mostly cross-linked polyacrylamide (acrylamide with bisacrylamide (crosslinker))
- pore size via relative concentrations

  e.g. 7% gel with large, 12% gel with small pores



http://www.piercenet.com/method/overview-electrophoresis#2deminsionalgelelectrophoresis

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sieving matrix in capillaries

b) physical „gels“: polymer network, entangled polymers („intertwined chains“)

- only for capillary format
- made of hydrophilic polymers in the buffer
- forming a network from polymer molecules
- concentration gives mash size of the sieve

linear polyacrylamide (LPA) or polydimethylacrylamide (PDMA)
polyethylenglycol/polyethylenoxide
polyvinylalcohol
alkyl celluloses

nicht auswendig lernen!

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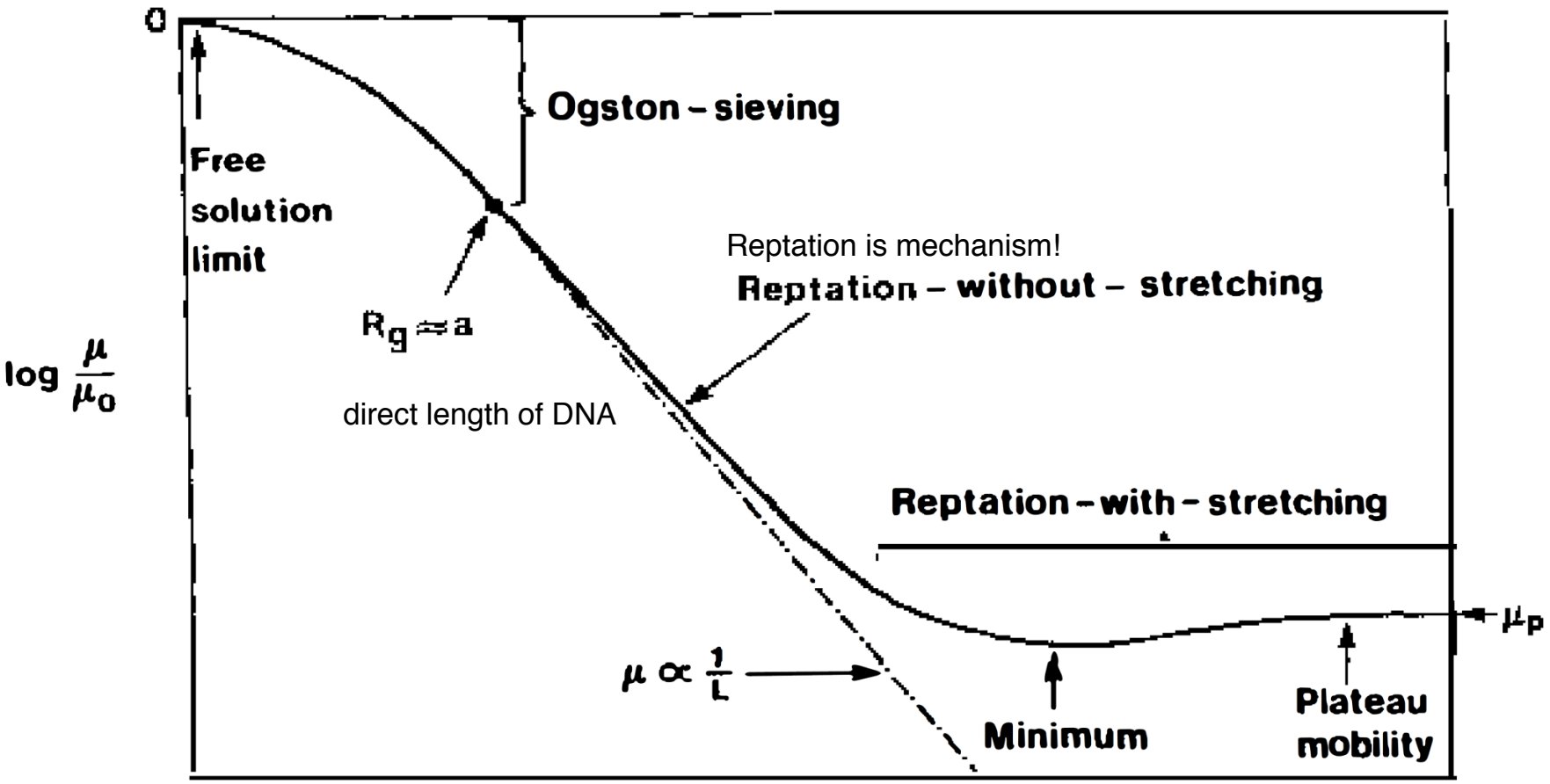
model of size sieving for DNA

describe in exam!!!, z.B. white out labelling



log(DNA length)

J. Noolandi, Annu. Rev. Phys. Chem. 1992, 43, 237-56; G.W. Slater, J. Noolandi, Biopolym. 1989, 28, 1781-91

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model of size sieving for DNA

free migration:
mobility is not size dependent but equal to free migration in solution

Ogston model: the average pore size is about the hydrodynamic radius (for DNA <300 DNA bases)
classical sieving effect: DNA migrates similar to spherical particles through the pores; exponential dependence on the molecular weight: $\mu \sim \exp^{(-MW)}$

reptation regime: analytes are larger than pores and cannot simply penetrate. DNA has to stretch out an move snake-like through the pores; mobility negatively correlated with molecular weight: $\mu \sim 1/MW$

biased reptation regime:
at extremely high field strengths: $\mu \sim 1/MW + bE$, with the parameter b summarizing pore size, analyte charge,…

Plateau mobility: molecules orient in field direction; no more size-based separation

molecular weight

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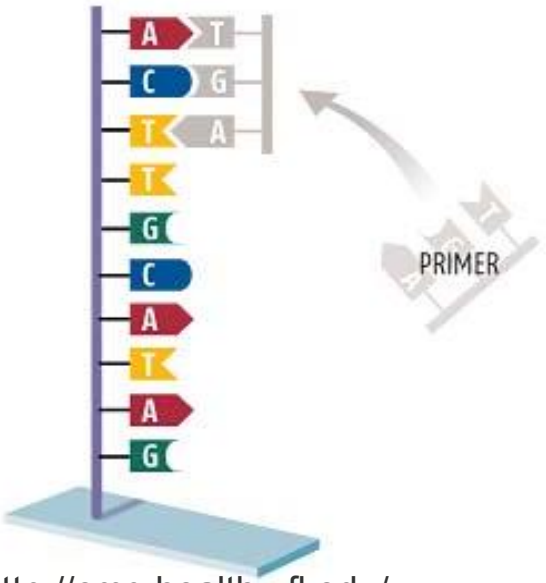
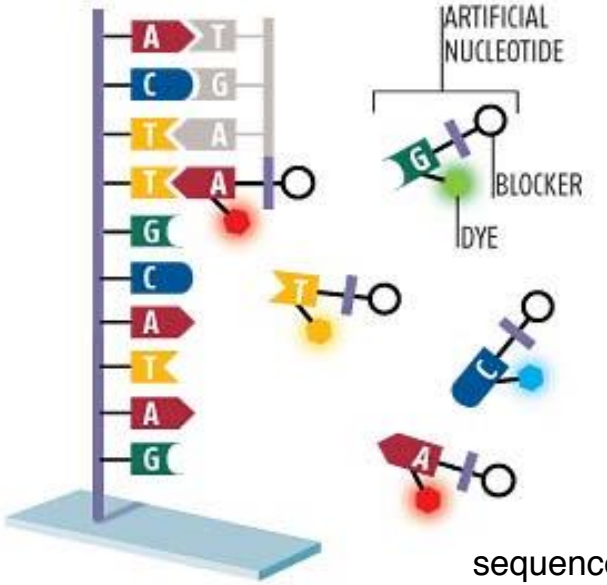
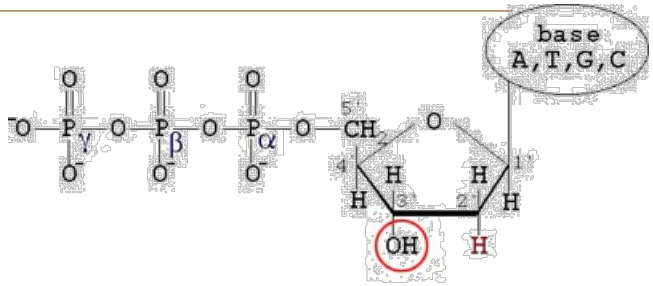
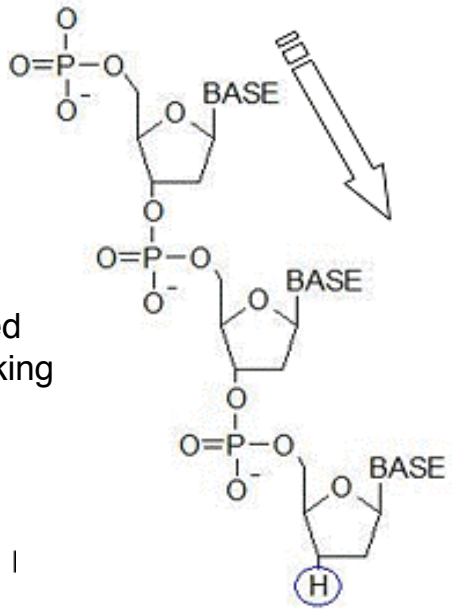
DNA sequencing

four color sequencing









http://cmg.health.ufl.edu/

know for exam what is needed for DNA sequezing

sequence is stopped when one of the blocking coloured is built in

- DNA splicing into two strands
- attachment of small DNA primers
- enzyme DNA polymerase synthesizes complementary strand
- using artifical nucleotides
- addition of fluorescence-labeled blocker nucleotides (dideoxy sugars)
- analysis of new DNA strands

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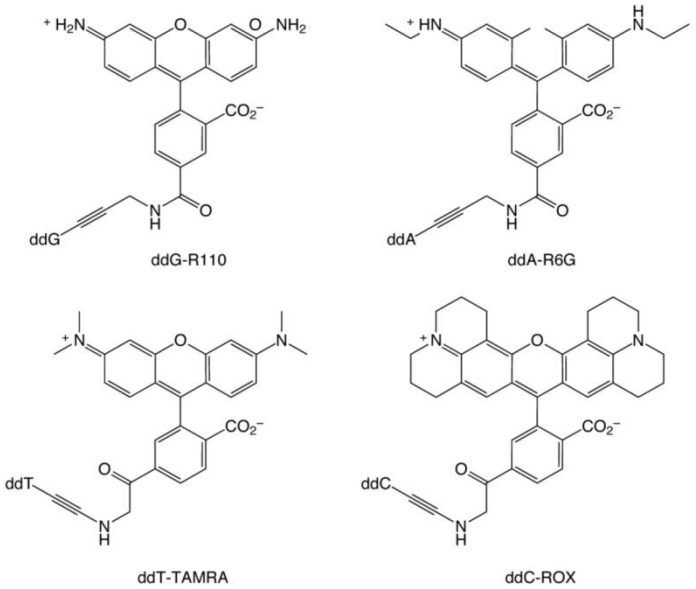
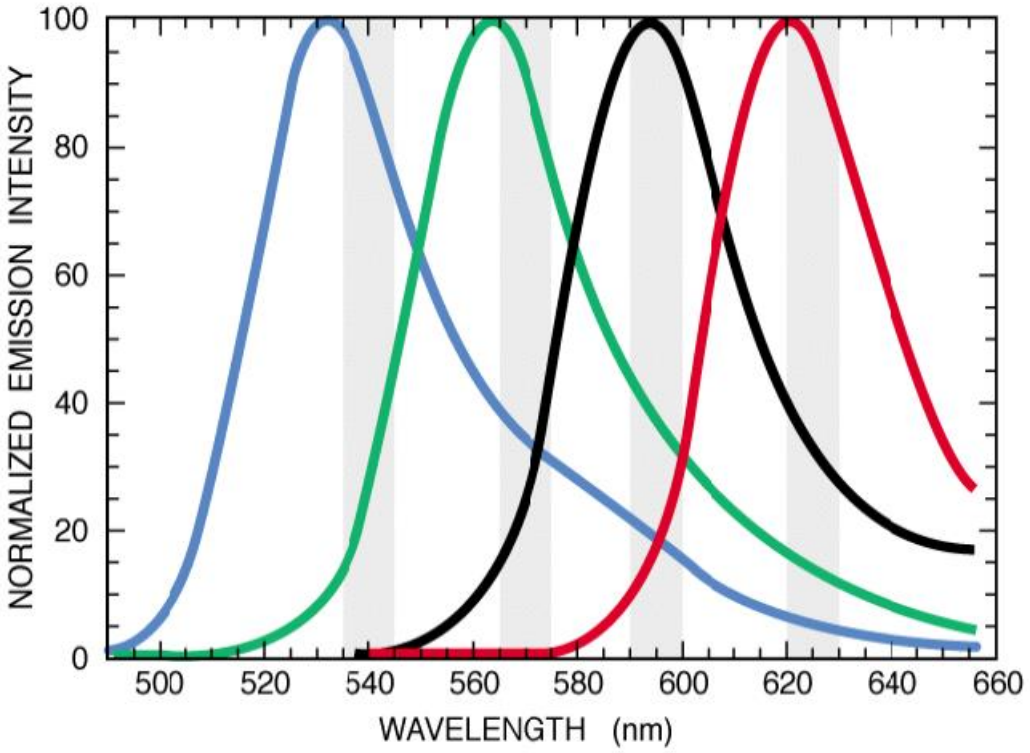
DNA sequencing

rhodamine colorants for blocker nucleotides with low spectral overlap



structures of colorants



spectral overlap of rhodamines and
4 optical filter ranges for detection

http://csb.wfu.edu/brf/ABIguide.pdf

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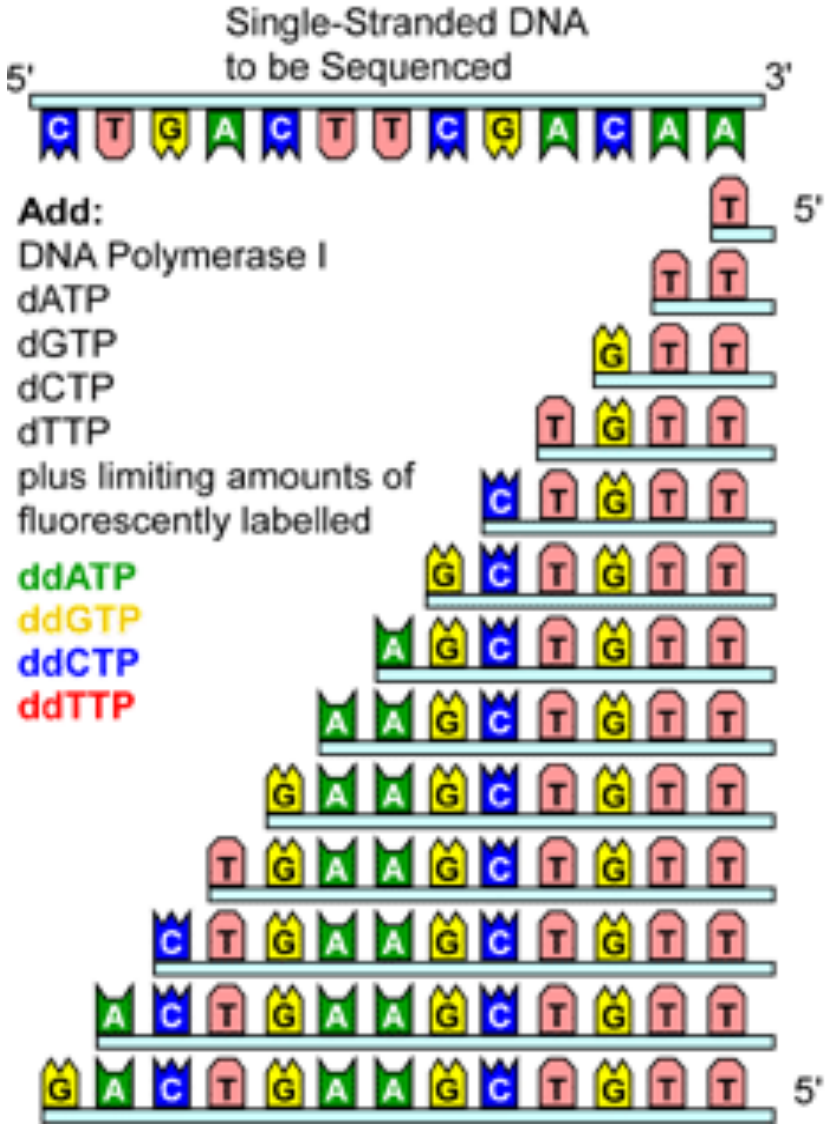
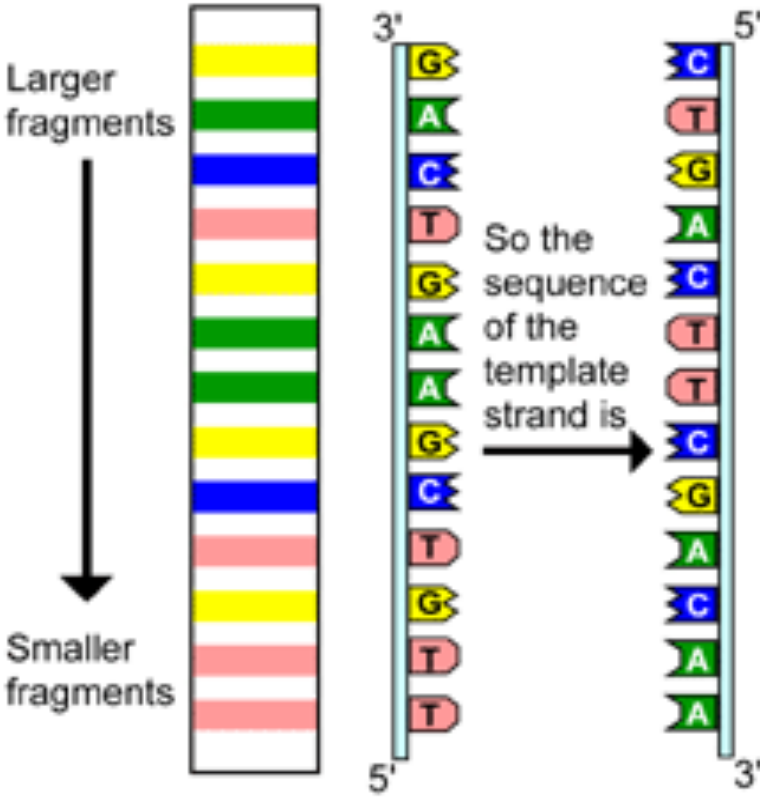
DNA sequencing





http://www.daviddarling.info/encyclopedia/D/DNA_sequencing.html

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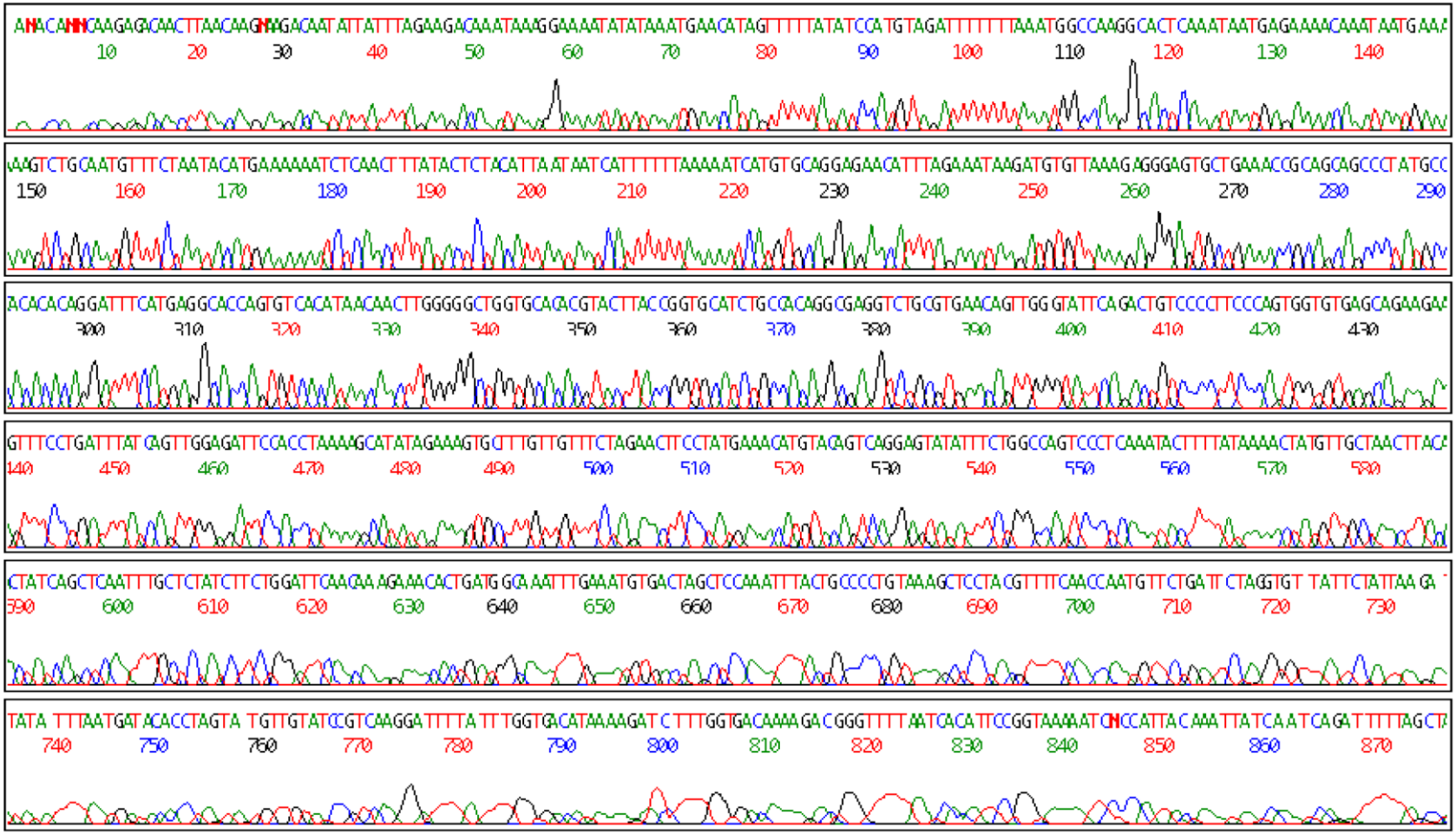
EBERHARD KARLS
UNIVERSITÄT
TÜBINGEN

DNA sequencing

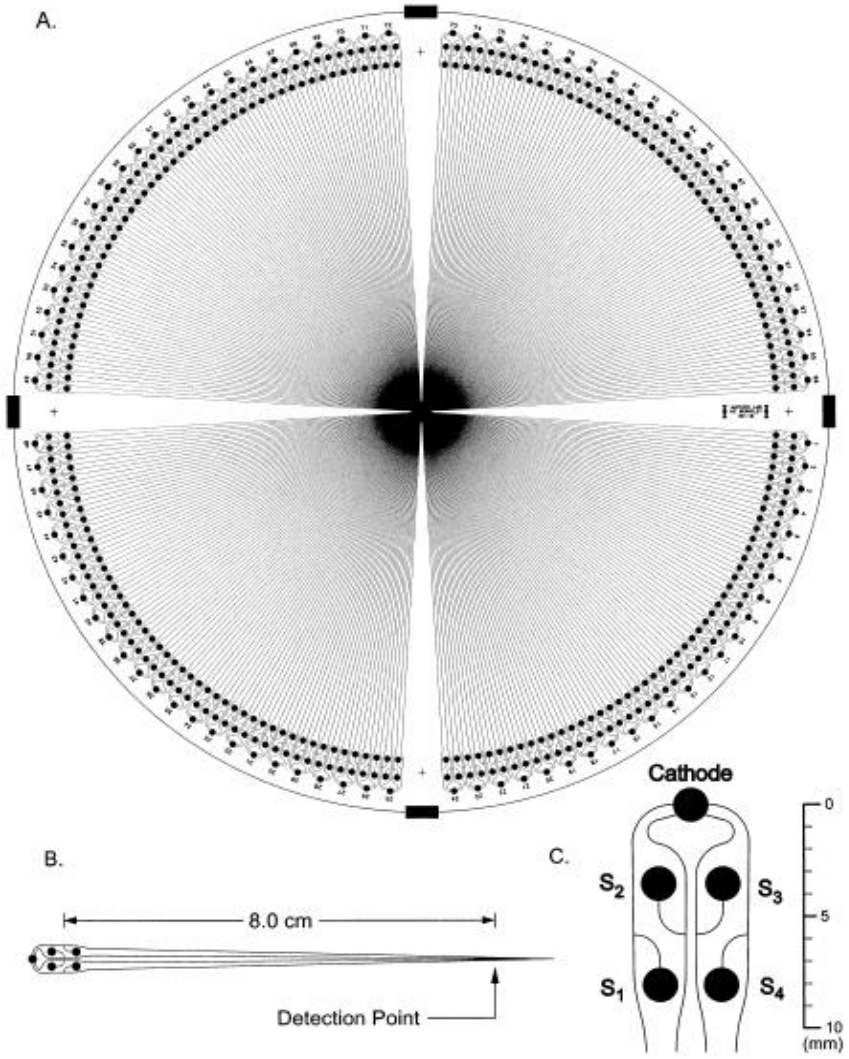
color code for sequencing: 880 base pairs were sequenced



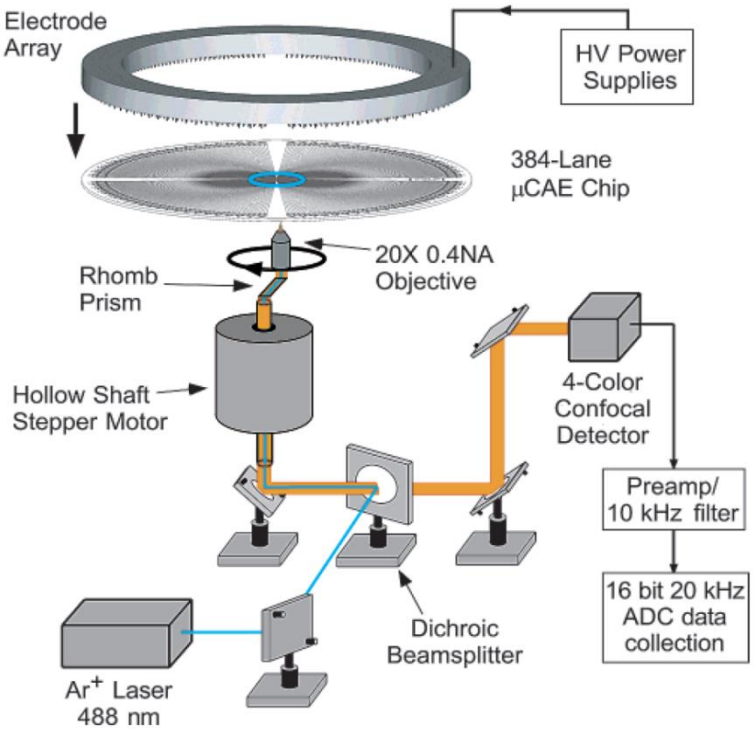
automation: sequencer-instruments with up to 384 parallel capillaries

http://csb.wfu.edu/brf/ABIguide.pdf

example: genomic analysis on chips



200 mm glass slight
384 channels, each 8 cm
4 sections à 24 quartets
PDMA sieving matrix
detection: 4 colors
fluorescence detection



C.A. Emrich, Anal. Chem. 2002, 74, 5076-5083

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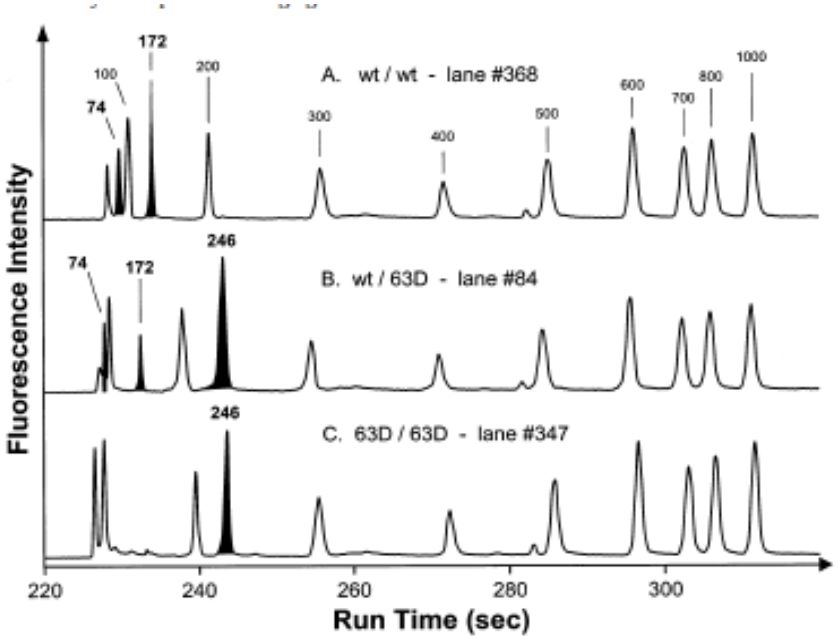
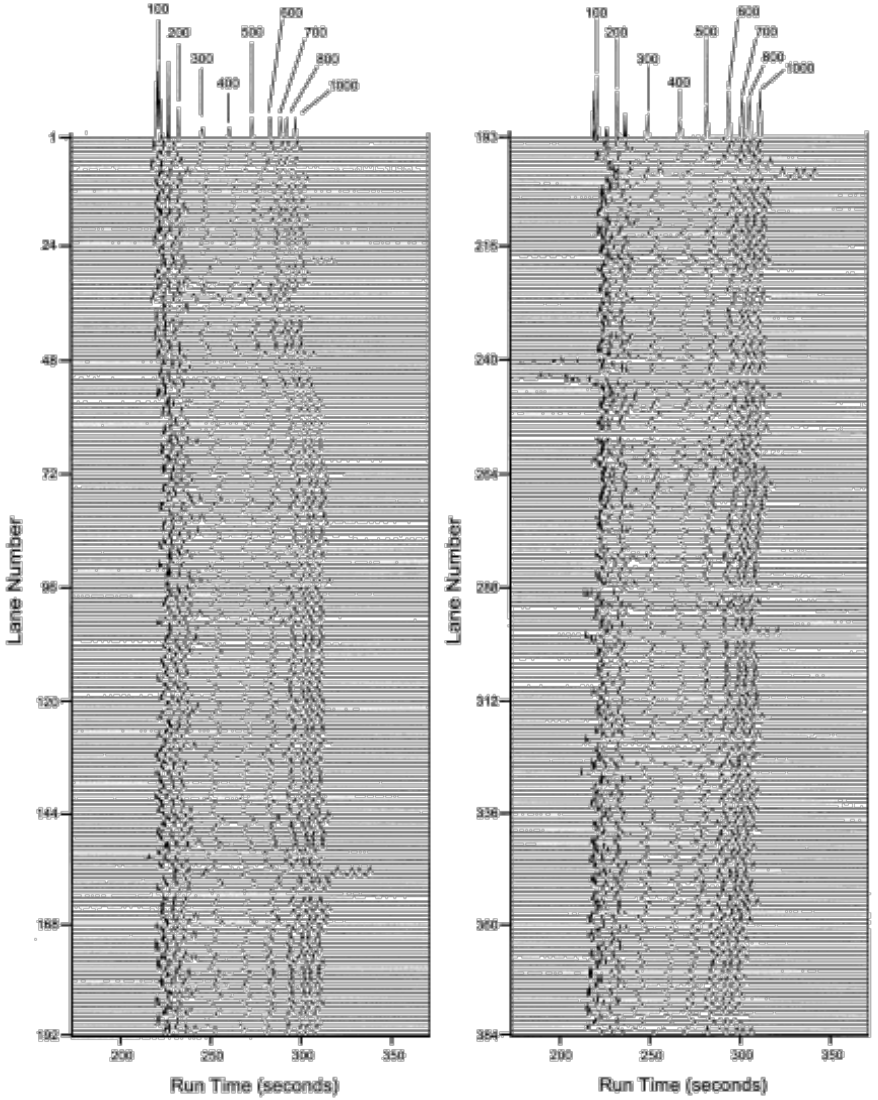
example: genomic analysis on chips





inheritable hemochromatose (HHC) (elevated iron absorption in epithelia celss of gastrointestinal tract) with 3 possible genotypes

HHC genotyping:
397 channels successful (98.7%)
total analysis time only 325 s

analysis of large sample cohorts: e.g. 5000 samples in 15 runs

C.A. Emrich, Anal. Chem. 2002, 74, 5076-5083

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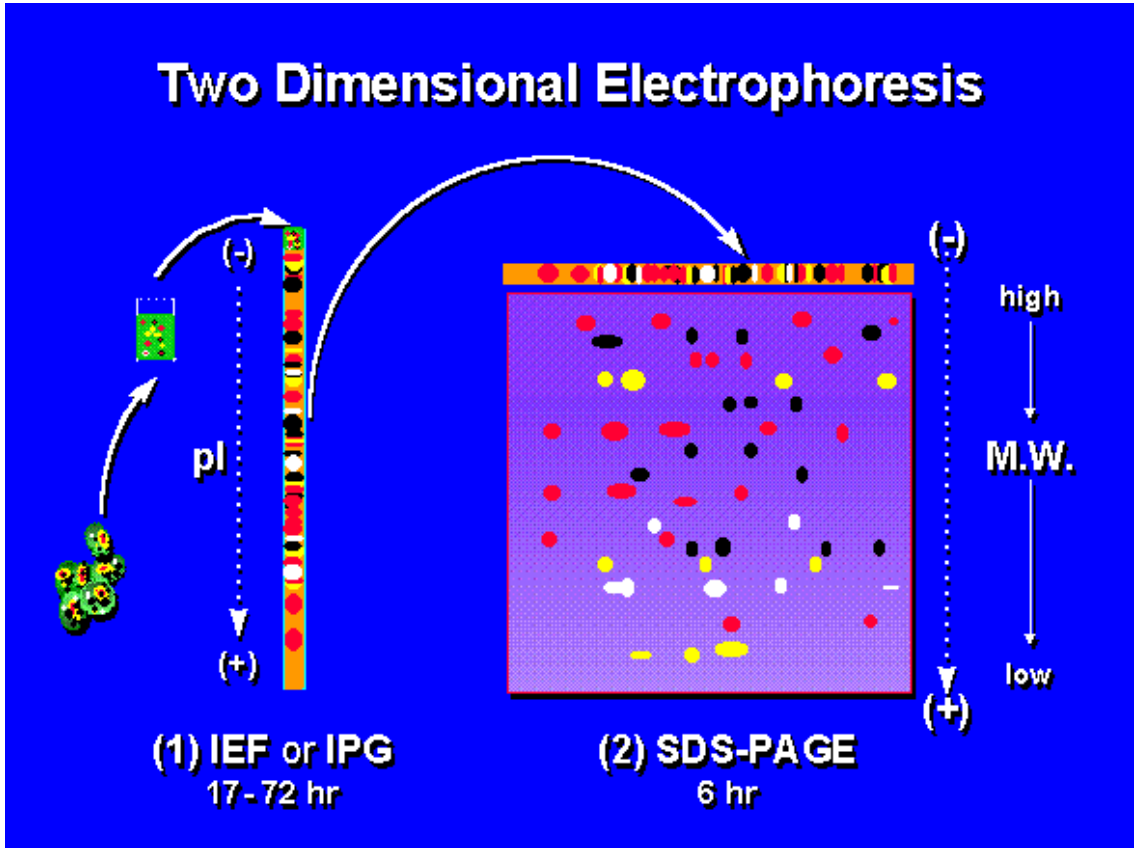
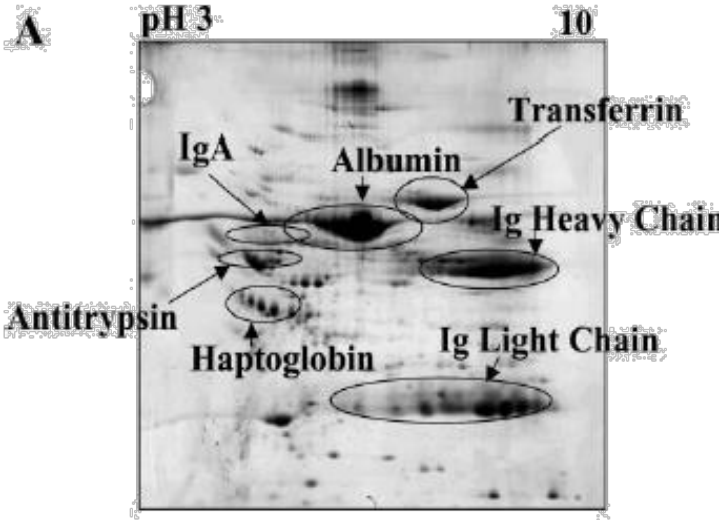
2DE: IEF plus SDS-PAGE

one of the following lectures:





Proteomics 2005, 5, 3386–3396

separation according to pI,
then separated according to charge

http://en.wikibooks.org/wiki/Proteomics/Protein_Separations-_Electrophoresis/Two_Dimensional_Polyacrylamide_Gel_Electrophoresis%282D-PAGE%29

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no multiple choice!!! in exam gauglitz + huhn





Laboratory Techniques and Medical Device Approvals

Bioanalytics: separation and diagnosis

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End

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