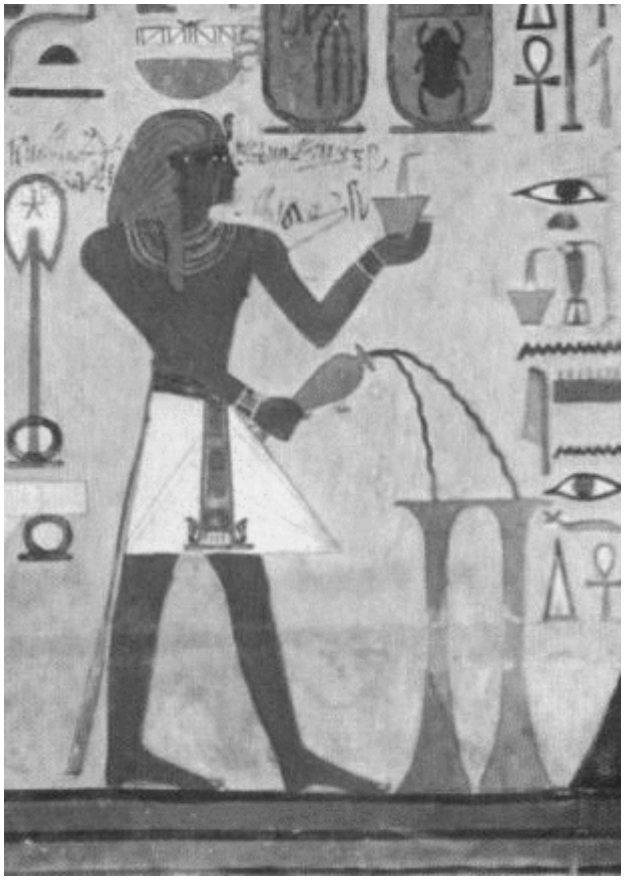
SEPARATION METHODS **B**

Jan Havliš, Ph.D.
Masaryk Uni, Fac Sci



analytical separation

analytical separation methods

: SEC, GPC, HCD and FFF

: GC

: CZE, MEKC, CIEF, ITP, CEC, ACE, NCE and CE-on-chip

separation methods B – **syllabus**

separation of macromolecules (SM)
: definition of macromolecule and its description
: separation using molecular sieves (SEC)
: separation by field-flow (FFF)

gas chromatography
: description of GC as continuous extraction
: special practical aspects of GC
:: injection, detection

electromigration methods (EMM)
: separation by different migration in electromagnetic field
: capillary and slab techniques
: combination with chromatography

separation methods – **overview**

separation principle	method 1) – two phases	method 2) – one phase	
		transport barrier	concentration difference
volatility	distillation		
solubility	zone refining		crystalisation
distribution constant	extraction, distributive chromatography (LL, GL)		
exchange equilibrium	ion exchange and affinity chromatography		
surface activity	adsorption chromatography (LS, GS)		foam fractionation
geometry of molecules		**molecular sieve**	
electromigration			**electrophoresis**

separation of macromolecules

SM history

1556

Agricola : separation of gold using gravity in a flow of water

1870

Lord Rayleigh : basic theory on light scattering on small particles

1940

Debye and **Zimm**; theory on light scattering on large particles

1955

Lindquist and **Storgards** : gel filtration *on starch („molecular sieving“)*

1959

Porath and **Flodin** : gel filtration *on cross-linked dextrans* (Sephadex)
(*GPC*)

1961

Hjertén : use of synthetic gels as stationary phases : *polyacrylamide*

1962

Pedersen : protein separation on small glass spheres (*HDC*)

1964

Hjertén : use of natural gels as stationary phases : *agarose*

1966

Giddings : description of FFF method principles

1969

DiMarzio and **Guttman** : theory of *steric exclusion* for SEC

1970

first commercial instrument using light scattering for mol. mass characterisation

1974

Small : first HDC experiments on non-porous sorbent

1978

Noel : particle separation in empty capillary (*capillary HDC*)

what is that macromolecule?

molecule of M_w >10 000

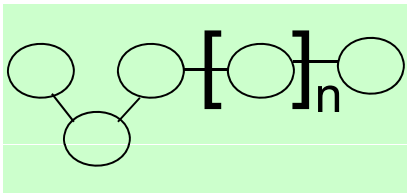
synthetic polymers

monomer, oligomer (10 – 100), polymer

homopolymers (PE, PP, PS, PTFE...)
: one repeated unit (monomer)

$$nM \rightarrow [M]_n$$

linear



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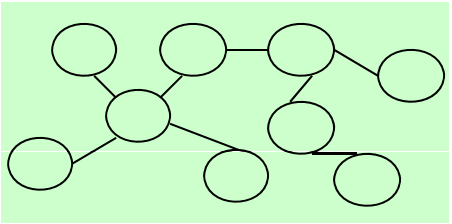
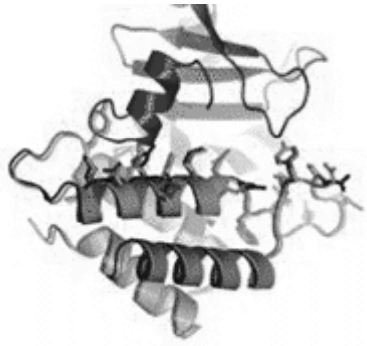


heteropolymers
: more of different units

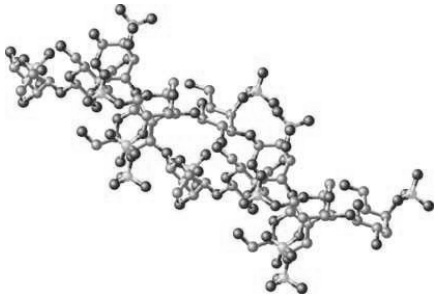
$$nX + mY \rightarrow X_nY_m$$

biological polymers

$M_W \approx 10\,000 – 1\,000\,000$



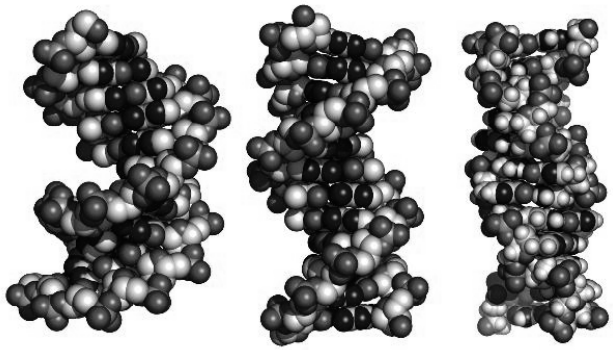
: <u>proteins</u>
peptidic bond, 21 natural amino acids (Se-Met)
complicated **complexes of different** units, e.g. haem + globin



: <u>glycana (polysaccharides, oligosaccharides)</u>
(starch, glycogen, chitin, cellulose, dextrans, pullulans)

: <u>nucleic acids (polynucleotides, oligonucleotides)</u>
nucleotide = phosphate + nucleoside
nucleoside = saccharide + base

DNA – saccharide – deoxyribose
RNA – saccharide – ribose



surface forces *(surface charge, ionic strength of surround)*

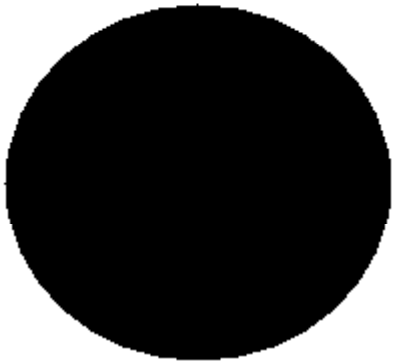
primary ⇒ *secondary, tertiary, ternary structure – native form*

description of macromolecule

macroscopic forms

random coil

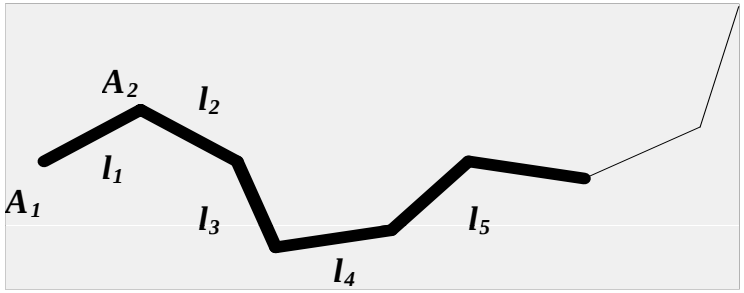
rod



sphere

size of macromolecule

flexible molecule



contour length (**L**)

$$L = n * l$$

n – number of bonds
l – monomer length

end-to-end vector length ($\vec{\mathbf{r}}$,)

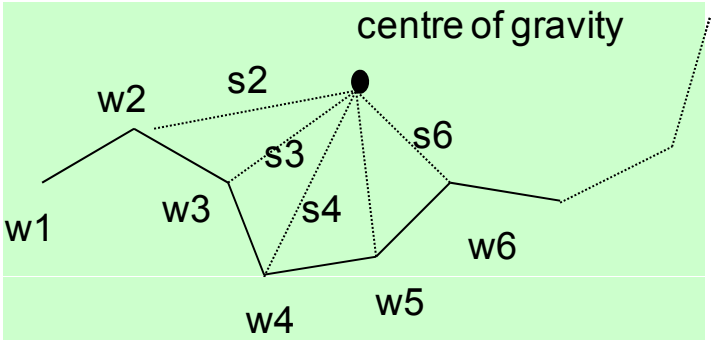
$$\vec{r} = \sum_i \vec{l}_i$$

mean square end-to-end distance (r^2)

$$\left\langle r^2 \right\rangle = \sum_i \sum_j \left\langle \vec{r}_i \cdot \vec{r}_j \right\rangle$$

radius of gyration (s^2)

important quantity
for **light scattering** measurement



$$\left\langle s^2 \right\rangle = \frac{s_i^2}{n}$$

s – distance of unit from centre of gravity

$$\left\langle s^2 \right\rangle = \frac{\left\langle r^2 \right\rangle}{6}$$

if monomer units are identical

relative molecular mass

SM separates mostly according to size = ***f*** (molecular mass, cross section, *etc*)

$$M_r = m * \frac{1}{12} m(^{12}\mathrm{C})$$ SI definition

for macromolecules:

mix of molecules of different molecular mass, differing in number of units = distribution

$$\overline{M_n} = \frac{\sum N_i M_i}{\sum N_i}$$ number average M_r
: measured by osmometry

$$\Rightarrow P = \frac{M_w}{M_n} \geq 1$$ polydispersity
~ distribution

$$\overline{M_w} = \frac{\sum N_i M^2{}_i}{\sum N_i M_i}$$ weight average M_r
: measured by light scattering

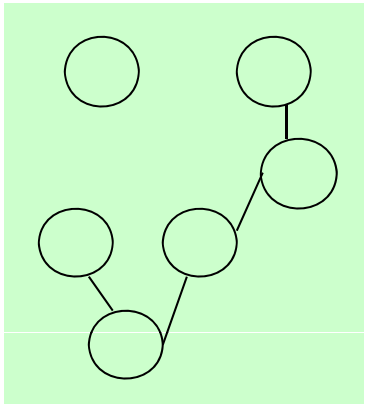
$$\overline{M_z} = \frac{\sum N_i M^3{}_i}{\sum N_i M_i}$$ z-average M_r
: measured by sedimentation analysis

example

what will be the number average, weight average molecular mass and polydispersity of polymer sample?

$$\overline{M_w} = \frac{\sum N_i M^2{}_i}{\sum N_i M_i}$$

average mass



$$\overline{M_w} = \frac{1*1^2 + 1*5^2}{1*1 + 1*5} = 4.33$$

$$\overline{M_n} = \frac{\sum N_i M_i}{\sum N_i}$$

average number of units

$$\overline{M_n} = \frac{1*1 + 1*5}{1+1} = 3$$

$$P = \frac{M_w}{M_n} \geq 1$$

$$P = \frac{4.33}{3} = 1.44$$

basic modes of macromolecule separation

size exclusion chromatography (SEC)
: gel filtration chromatography (GFC)
: gel permeation chromatography (GPC)
: gel filtration (GF)

hydrodynamic chromatography (HC)

flow-field fractionation (FFF)
: sedimentation (SFFF)
: thermal (TFFF)
: electric (EFFF)
: gravity (FFFF)

membrane separation
: ultrafiltration (hydrostatic pressure)
: reversed osmosis (hydrostatic pressure)
: dialysis (concentration gradient)
: electrodialysis (gradient of electric potentials)

separation in force-field
: ultracentrifugation (density gradient)
: mass spectrometry (electromagnetic field, TOF without field)

SEC, size exclusion chromatography

gel permeation chromatography (GPC)
gel filtration chromatography (GFC)

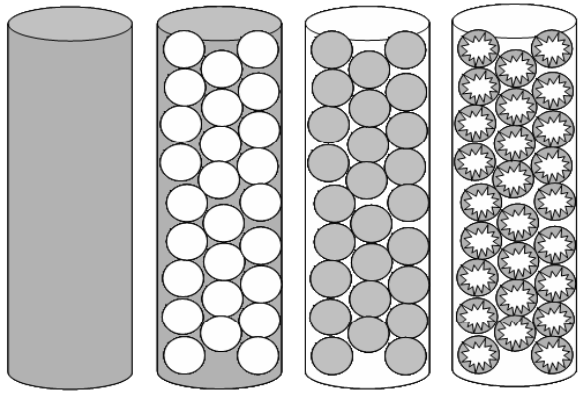
principle: analyte is distributed between MF outside of particles and inside of particles
: sieving effect, steric exclusion
: diffusion
: pressure of carrier liquid – motion of liquid and its flow profile

$$V_R = V_{out} + K'_D * V_{in}$$

V_R – retention volume
K'_D – distribution constant

$$V_{tot} = V_{out} + V_{in} + V_{part}$$

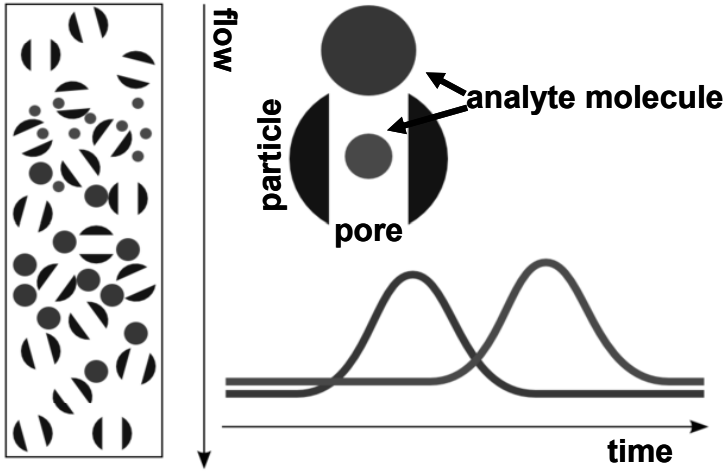
tot – total volume
out – MF outside of particles
in – MF inside of particles
part – volume of particle material



$$V_R = V_{out} + K'_{AV} * (V_{tot} - V_{out})$$ where $$(V_{tot} - V_{out}) = V_{in} + V_{part}$$

K'_{AV} – elution constant

$$K'_{AV} / K'_D = const.$$



thermodynamic interpretation

$$\Delta G = \Delta H - T\Delta S = -RT \ln(K) \quad \Rightarrow \quad K = e^{-\frac{\Delta H - T\Delta S}{RT}} \approx e^{\frac{\Delta S}{R}} < 1$$

ΔH ~ 0 ⇒ process is **entropically controlled**

$$K'_D = \frac{c_{in}(A)}{c_{out}(A)}$$

c_{in} – analyte concentration inside of particles
c_{out} – analyte concentration outside of particles

$$V_R = k_1 * \log M_W + k_2$$

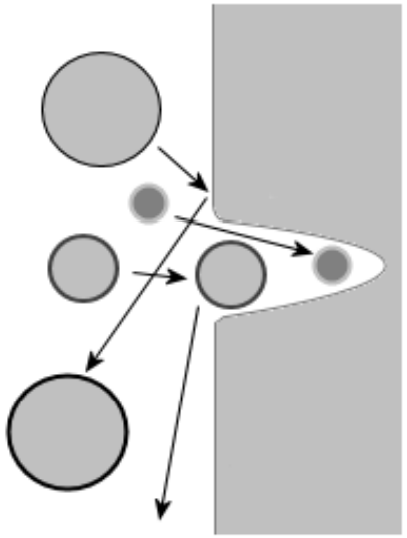
k_1, k_2 – numeric constants

$$V_R = V_{out} + \int_R^{r_{\max}} K'_D(R,r) * \phi(r) dr$$

φ – total pore volume with diameter r to $r+dr$
R – diameter of retained particle

separation is given by **ratio of diameter** of **pore** and **analyte**

sieve model is in many aspects *not exact*:
: flow of liquid out an in pores is different ($F_{out} >> F_{in}$)
: other interactions: adsorption, L-L distribution, electrostatic repulsion ($\Rightarrow K'_D > 1$)



gel LC
SEC

gel LC

mechanical separation of **A** molecules in particles/pores of gel based on their different size

$$K_D = \frac{c_{qS}(A)}{c_M(A)}$$

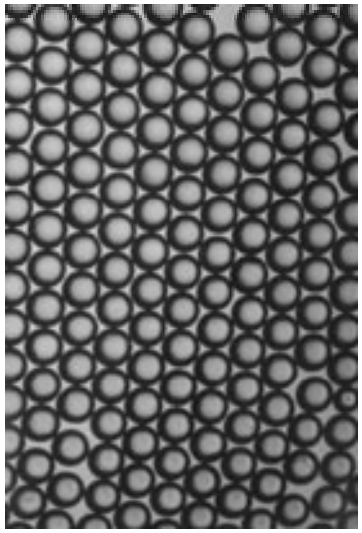
not classic LC, **no** chemical affinity

qS – quazi SF, ***M*** – MF

use of SEC

group separation
: separation of low and high molecular groups
(desalting, extraction agent removal, reaction termination between low molecular mass ligand and biopolymer)

fractionation / purification
: separation of components with significant M_r difference

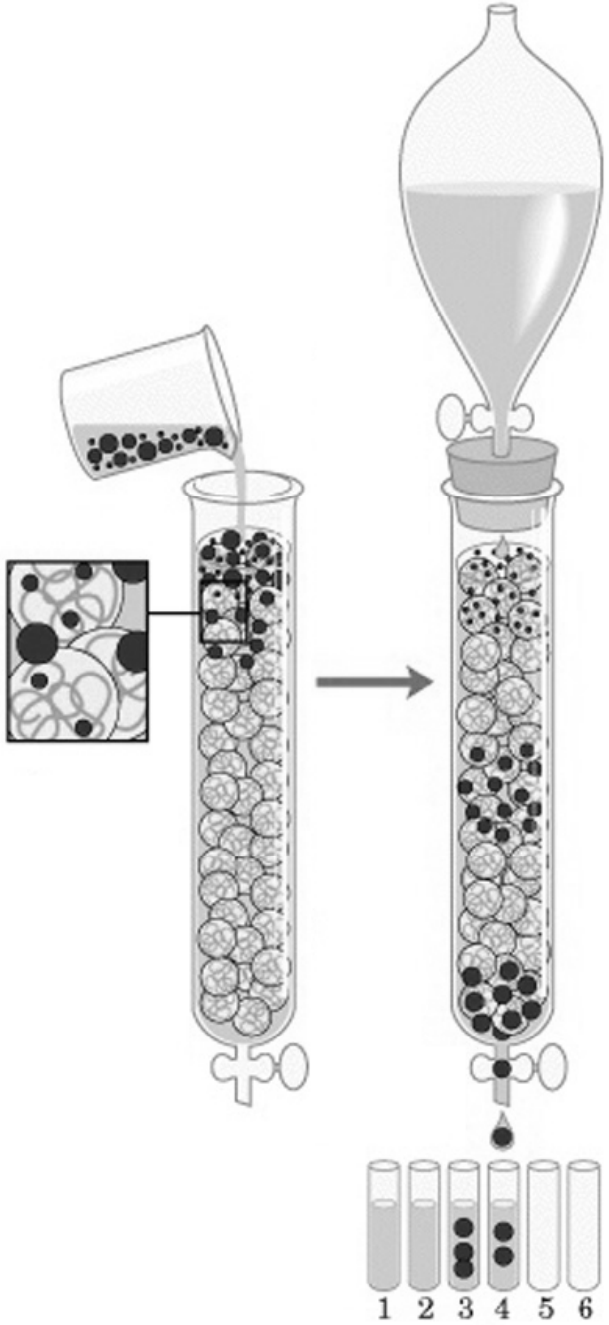


determination of M_r
: comparison with standards (in line increasing M_W)
: polymer polydispersity and distribution

analysis of ligand-biopolymer binding
: emerging complex has higher M_r than components
(complex insulin-antibody by diabetics)

concentrating samples of biopolymers
: dry molecular sieves remove solvent – „dry up“ and concentrate sample

proceeding SEC



column filling

: pre-filled columns
: own filling – SF swelling (uniform, without bubbles)

sample introduction

: injecting 1 – 5 % of column volume
: either on column top or through injection adaptor

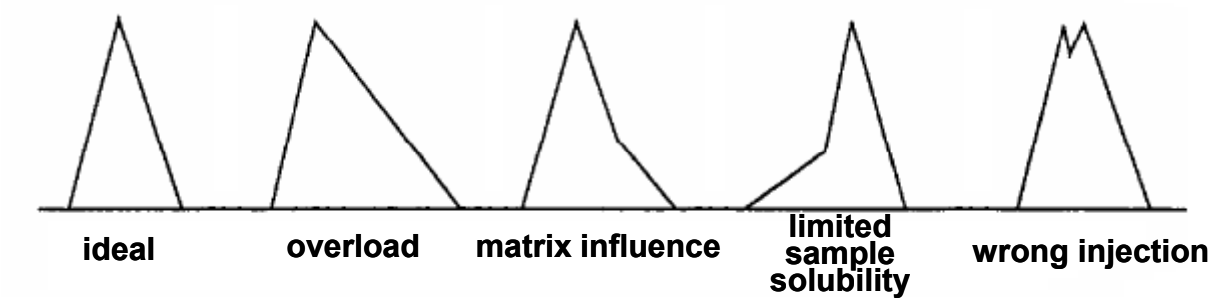
elution

MF not directly influences separation

: solvent viscosity and elution MF ratio < 2
: water – uncharged compounds separation, or buffers
pH and *I* keeps ion interactions minimal

guarding SF

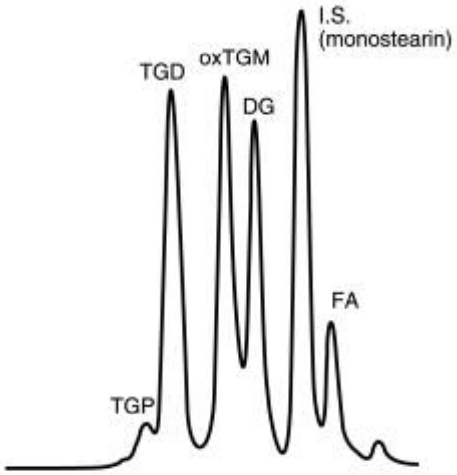
0.02 % sodium azide
0.05 % trichlorobutanol (Chloreton)
0.005 % ethylmercurythiosalicylate (Mertiolate)
0.002 % chlorhexidine

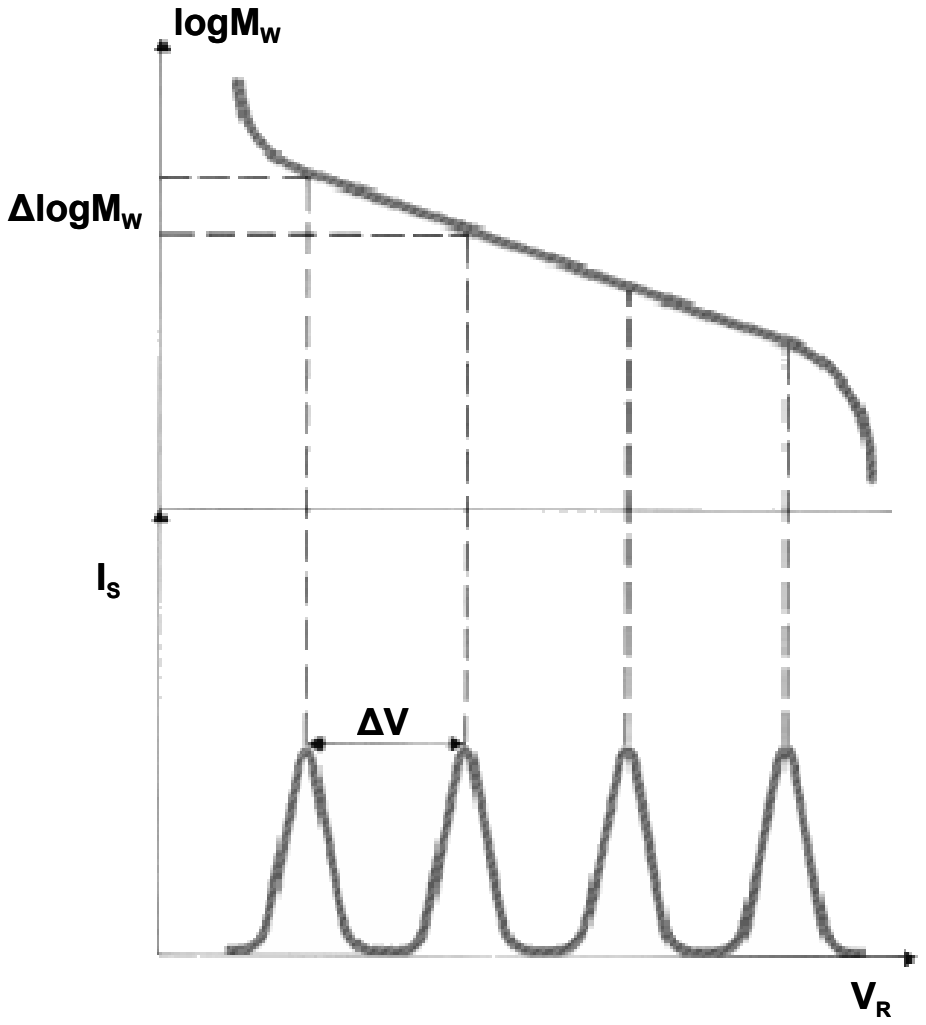


calibration

set of standards

4 – 5 defined native proteins with increasing M_W





absolute calibration

basic parameter defining selectivity – **hydrodynamic volume**

formula for limiting viscosity number of polymer **[η]** derived from Einstein's equation

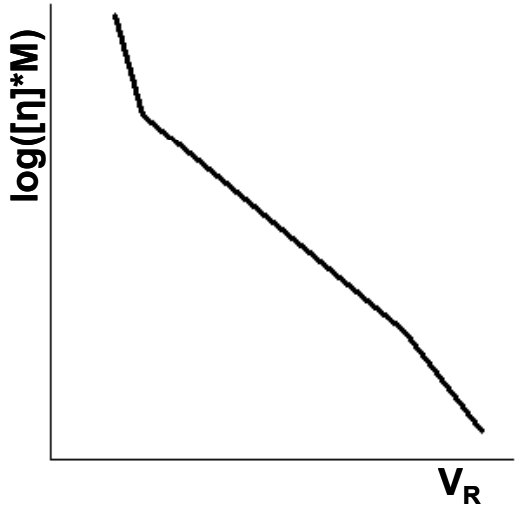
$$[\eta] = \lim_{\rho \to 0} \frac{\eta/\eta^* - 1}{\rho} = \frac{k * V_R}{M} \Rightarrow [\eta] * M = k * V_R$$

independent on macromolecule structure

$$[\eta] = KM^{\alpha} \Rightarrow [\eta](A) * M(A) = [\eta](S) * M(S) = f(V_R)$$

Mark-Houwink's equation

A – analyte, **S** – standard



$$K_A M_A^{\alpha_A+1} = K_S M_S^{\alpha_S+1}$$

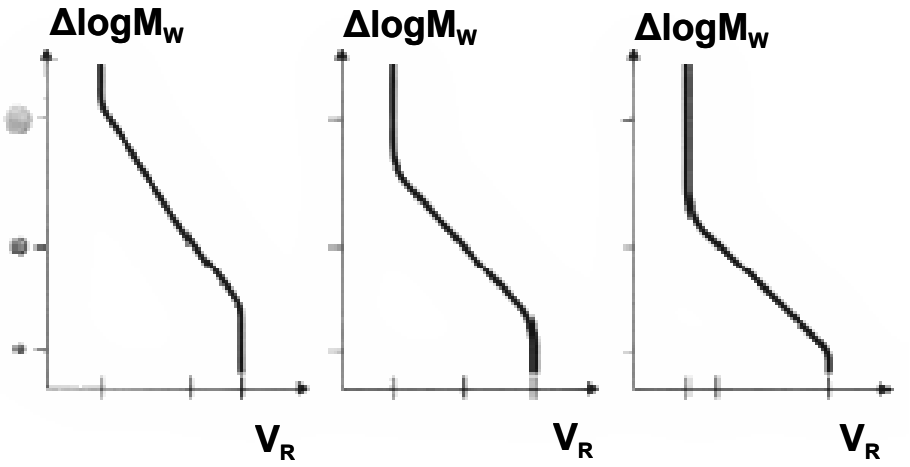
$$M_A = \left(\frac{K_S M_s^{\alpha_S+1}}{K_A} \right)^{\frac{1}{\alpha_A+1}}$$

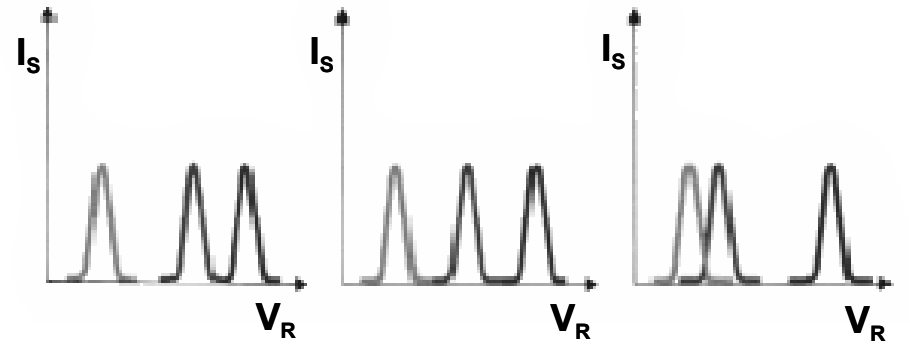
$$\log([\eta] * M) = f(V_R)$$

[η] – by viscosimetry

selectivity in relation to pore size distribution

increasing pore size distribution





separation column

: classical tubular columns
material – mostly soft gels

: **inert** gel matrix (towards analyte and elution solutions)

: long-term **chemical stability** (at different pH and temperature)

: **mechanical stability** (resistance towards high pressure)

: **small** amount of **ionised** groups

: suitable **particle size** (5 – 250 µm)
small particles – high resolution, low rate
large particles – fast separation, low resolution

fractionation range (FR)
M_r range, in which the compounds are separated

elimination limit (EL)
upper limit of fractionation range

column fillings

agarose

large pores, acidic character
elution: polar and non-polar solvents

FR > 200 000
Sepharose

polyacrylamide

low amount of polar groups; low resolution
elution: polar and mild non-polar solvents

FR = 1000 – 3 000 000
Sephacryl, Bio-Gel P

mixed SF: agarose-acrylamide
chemical very resistant

FR = 1000 – 23 000 000
Bio-Gel A, Ultrogel

dextran

strong adsorption effects
elution: polar and non-polar solvents

FR < 10 000
Sephadex

styrene-DVB

strong hydrophobic interactions
elution: non-polar solvents

FR = 400 – 14 000
Bio-Beads, Styragel

methacrylate

hydroxymethylmetacrylate + ethylendimethylmethacrylate
elution: polar and non-polar solvents

Spheron

glycomethacrylate
elution: polar and non-polar solvents

Separon

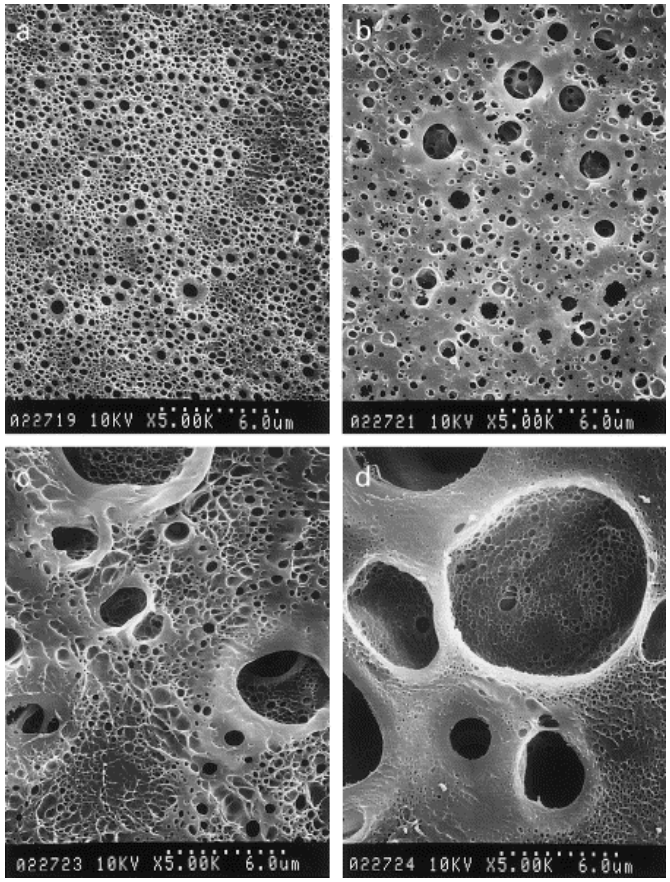
vinylacetate

Merckogel OP-PVA

silica

strong hydrophilic interactions, mildly acidic
elution: polar solvents

Bio-Glass, Porasil, Spherosil



detectors

: detection of separated compounds

: determining molecular mass and polydispersity

absorption photometric detector

: polymers mostly do not contain own chromophores $\Rightarrow$ indirect detection

refractometric detector

: universal

fluorimetric (fluorescence) detector

: own fluorophores (within proteins Trp, Tyr, Phe), or derivatisation

viscosimetric detector

$M_v \in (M_n, M_w)$, $M_v \approx M_w$

$$[\eta] = KM^{\alpha} = \lim_{\rho \to 0} \frac{\eta / \eta^* - 1}{\rho}$$

Mark-Houwink's equation

[η] – limiting viscosity number [m^3/kg]
η* – solvent viscosity
K, α – Mark-Houwink's constants (for globular macromolecules α = 0)

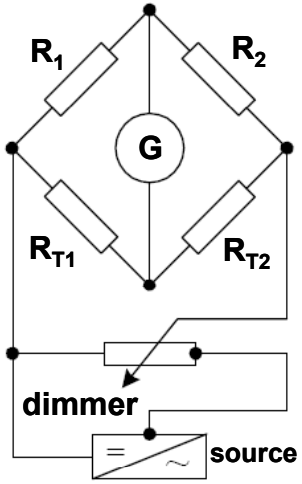
osmometric detector

vapour pressure osmometry (VPO)

: uses **Raoult's law**

: fast, low sample consumption, temperature interval 25 – 130 °C

: M_r = 40 – 35 000, no volatile compounds



T = *const.*, saturated vapours of solvent

1) R_{T1} and R_{T2} – droplet of solvent, $\Delta T_{1,2} = 0$, U = 0

2) R_{T1} – droplet of solvent, R_{T2} – droplet of sample (solvent + analyte)

adding droplet of sample ↓ solvent vapour tension ⇒ condensation of solvent vapours into the droplet ⇒ release of condensation heat ⇒ ↑ temperature of sample droplet, thus also of thermistor, also of solution tension pressure ⇒ Wheatstone bridge unweighing

solvent vapour condensation stops when sample vapour pressure is in equilibrium with pure solvent vapour pressure due to higher temperature

measured voltage, proportional to the difference of temperatures of both thermistors, is proportional to molar concentration of compound in sample

thermal losses ⇒ calibration on standard of known M_r value

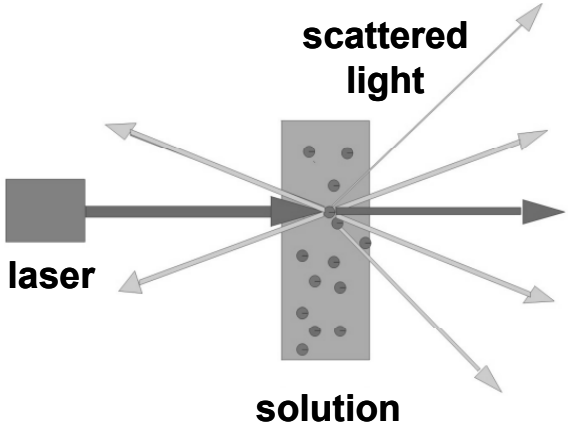
light scattering detector

static light scattering
scattering of light beam on particles of suspension or colloid solution

interaction of light beam electric vector with electron shell ⇒ periodic oscillations

intensity, polarisation and angular distribution of scattered light
depends on size and shape of scattering particles

dynamic light scattering
studies time fluctuations of scattered light on moving particles
: information on diffusion coefficient



light scattering on small particles

macromolecules
particle diameter (d) < λ/20 (Rayleigh scattering)

$$\alpha = \frac{c(\partial n / \partial c)_{\mu} * \overline{n}_0}{2\pi * N}$$

c – concentration
N – number of particles; scattering centres
$\overline{\mathbf{n_0}}$ – refractive index of solvent
$(\partial \mathbf{n}/\partial \mathbf{c})_{\mu}$ – particle refractive index changes at constant **μ**

⇒ particles – secondary source of scattered light of the same wavelength

$$\frac{i_s}{I_0} = \frac{8\pi^2 * V * \alpha^2}{\lambda_0^4 * r^2} * N * \left(1 + \cos^2\theta\right)$$

intensity ratio of scattered ($\mathbf{i_s}$) and original light $\mathbf{I_0}$ (non-polarised)

V – unit volume
$\boldsymbol{\lambda_0}$ – wavelength
r – distance from particle
θ – angle measured from main light beam

number of scattering centres **N** in case of identical macromolecules
(monodisperse sample)

$$N = \frac{c * N_A}{M}$$

N_A – Avogadro's number
M – molecular mass

$$\Rightarrow \frac{i_s}{I_0} = \frac{2\pi^2 * \bar{n}_0^{\,2} * (\partial n / \partial c)^2 * V * c * M}{\lambda_0^4 * r^2 * N_A} * (1 + \cos^2\theta)$$

$$R_\theta = \frac{i_s * r^2}{I_0 * V * (1 + \cos^2\theta)}$$

Rayleigh's radius

+

$$K = \frac{2\pi^2 * \bar{n}_0^{\,2} * (\partial n / \partial c)^2}{\lambda_0^4 * N_A}$$

summing constants into one, K

$$\Rightarrow \frac{K * c}{R_\theta} = \frac{1}{M}$$

in polydisperse sample, **M** is substituted

$$M_w = \frac{\sum c_i * M_i}{\sum c_i}$$

inter-molecular interactions and non-zero concentrations taken in account (Debye):

$$\frac{K*c}{R_\theta} = \frac{1}{M} + 2A_2*c + 3A_3*c^2 + \ldots$$

A_2, **A_3**… – virial coefficients; mostly **A_3** and higher are omitted

A_2 – phys.-chem. measure of thermodynamic solvent quality for given macromolecules

good solvent **$A_2 > 0$** : macromolecule expands

bad solvent **$A_2 < 0$** : macromolecule shrinks

θ-solvent **$A_2 = 0$** : macromolecule preserves its volume

light scattering on large particles

macromolecules
particle diameter (d) > λ/20 (Debye scattering)

: large particles ⇒ **phase shift of light scattering** from different parts of molecules
: phase difference is dependent on angle θ; for θ = 0 is the difference 0
: **beam interference** ⇒ angular distribution of scattered light intensity P(θ)

$$P(\theta) = \frac{I_s}{I_{s(\theta=0)}} \Rightarrow P(\theta) = 1 - \frac{16\pi^2 \langle s^2 \rangle}{3\lambda_0^{\,2}} * \sin^2\left(\frac{\theta}{2}\right)$$

Zimm's equation

use of P(θ) parameter to express scattering

$$\frac{K*c}{R_\theta} = \left[\frac{1}{P(\theta)}\right] * \left[\frac{1}{M} + 2A_2 * c\right] \Rightarrow$$

if **$(1-x)^{-1} \approx (1+x)$**

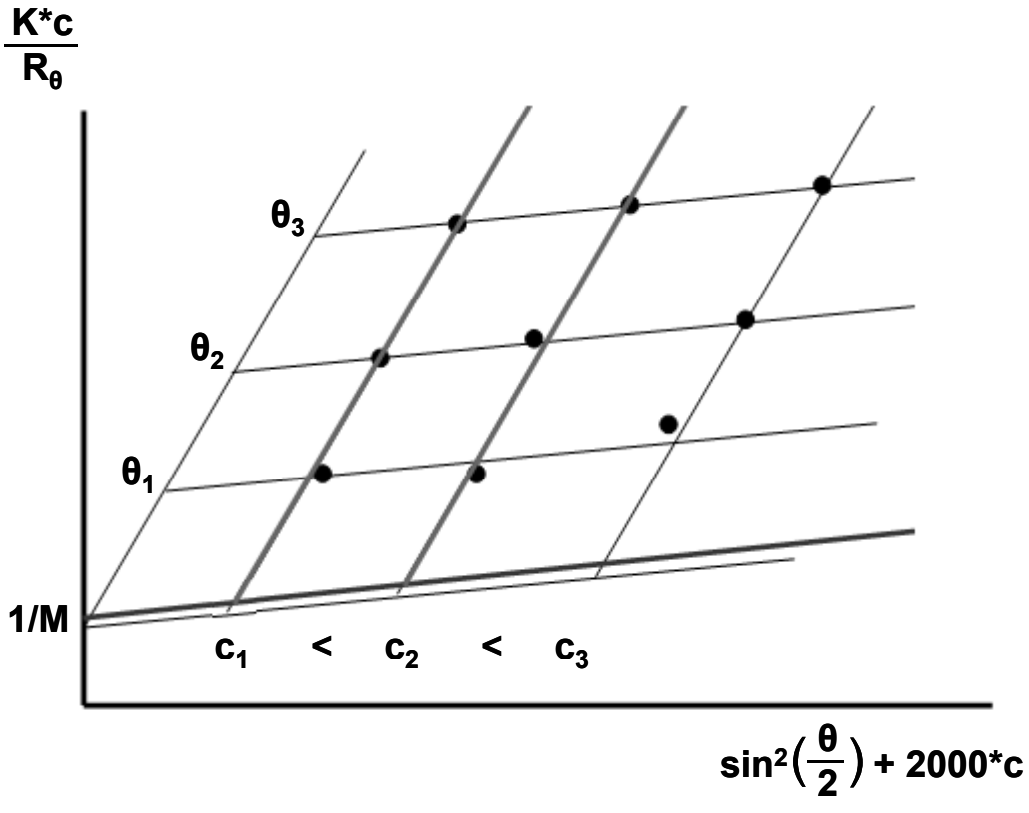
$$\Rightarrow \frac{K*c}{R_\theta} = \left[1 + \frac{16\pi^2 \langle s^2 \rangle}{3\lambda_0^{\,2}} * \sin^2\left(\frac{\theta}{2}\right)\right] * \left[\frac{1}{M} + 2A_2 * c\right]$$

experimental bases for calculation of **gyration radius**

multiple angle laser light scattering

(MALLS)

Zimm's graph



M_W – double extrapolation to **y**-axis

$$\frac{K*c}{R_\theta} = f(\sin^2\frac{\theta}{2} + K_S*c)$$

K_s – arbitrary constant;
graphically separates diagram lines

different concentrations **c** of sample
laser – **λ_0** source of **I_0** intensity
refractometer (also as *concentration detector*) – $\overline{\mathbf{n}_0}$ and **$(\partial n/\partial c)_\mu$** (see constant **K**)
i_S – scattered light intensity in different angles **θ** in known distance **r** from cuvette

θ → **0** (c = *const.*) blue lines, from blue slope we extract *gyration radius* ⟨**s^2**⟩

c → **0**, slope ~ **A_2**, interception **$1/M_W$** red line

low angle laser light scattering

(LALLS)

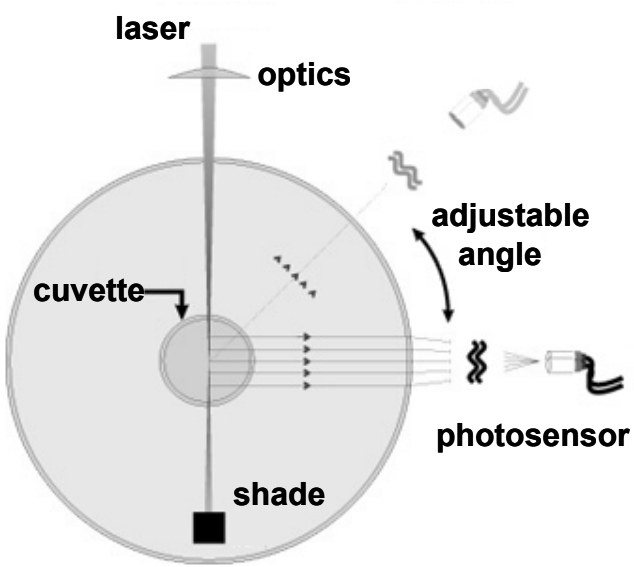
at small angles θ (< 7 °) $\sin^2(\theta/2) \sim 0 \Rightarrow P(\theta) \rightarrow 1$

then $$\frac{K * c}{R_\theta} = \frac{1}{M} + 2A_2 c$$

for $M_W > 10^7$ or within associated systems this approximation fails

---

instrumentation



advantage:
: absolute technique, no calibration needed M_W, A_2
for $\langle s^2 \rangle$ – standards necessary
: fast
: connectible with separation technique (GPC, FFF)

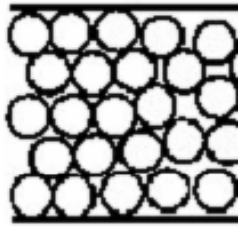
disadvantages:
: *dust* – demanding high solution purity

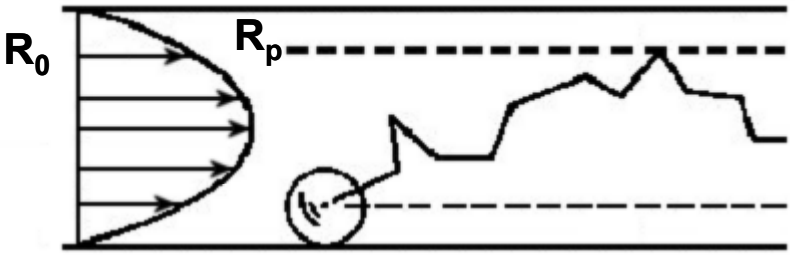
HC, hydrodynamic chromatography

principle: combination of *steric exclusion* with *surface* (colloid) *interaction* sample-filling, eventually *solute retardation behind streamlines of laminar flow with profile* (**wall effect**)

non-porous material

sample moves with MF flow →

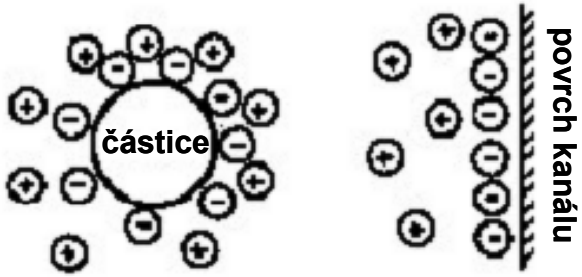




gravity centre of large macromolecule cannot reach the channel wall (R_p) ⇒ cannot move in slower flow near to it (*wall effect*; *given by laminar flow profile* R_0)

⇒ heavier (larger) molecules run through channel faster than smaller ones

other influences:
: electric double-layer
: van der Wals interactions



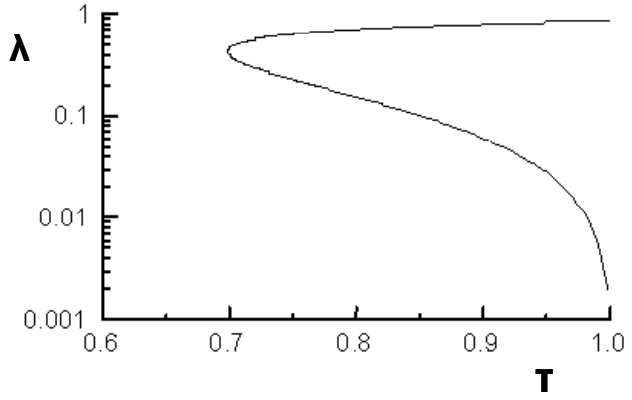
⇒ sample moves in channel *hydrodynamically* or *electrically*

separation description

$$\tau_i = \frac{t_i}{t_M} = \frac{1}{1 + B\lambda_i - C\lambda_i^2}$$

τ – polymer retention factor
t_i a **t_M** – retention time of polymer and unretained component
λ – ratio between macromolecule radius and flow channel half-height
B and **C** – constants dependent on channel symmetry, **C** also on retention model

calibration



λ = *f* (τ) and thus on M_W
in case of tubular micro-capillary use and C → 2.3

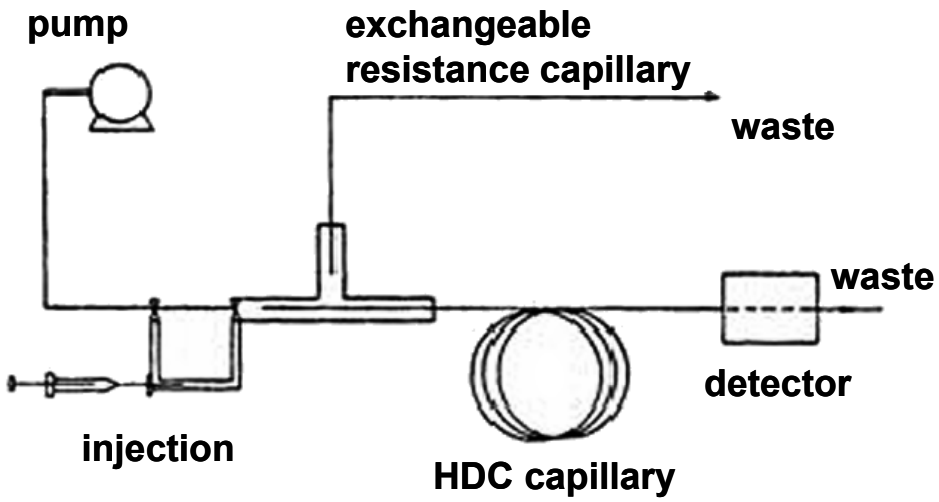
porous material

pores of filling : 50 – 50 000 nm
sample : larger molecules

capillary fractionation

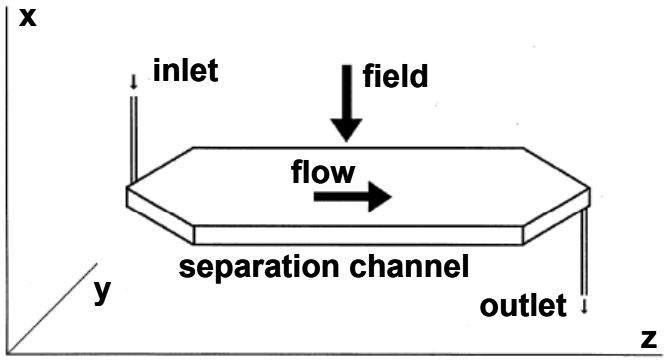
(CHDF, *capillary hydrodynamic fractionation*)

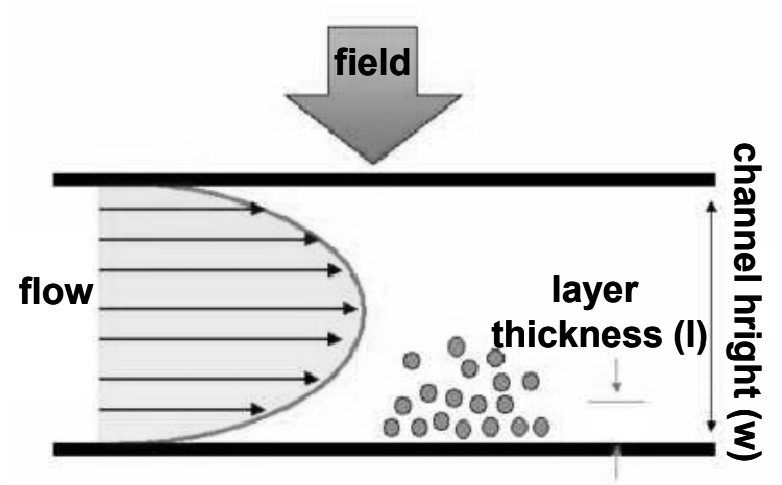
other influences in account:
: colloidal forces
: non-linear inertial forces depending of flow-rate gradient and position
(*lift forces*; *tubular pinch effect*)



FFF, flow-field fractionation

principle:
physical field inflicts some property of analyte and creates concentration gradient ***∂c/∂x***
⇒ **concentration profile c(x)** across channel is **specific** for given analyte





$$J = W * c - D * \nabla c$$

J – flow of analyte
W – transport rate of analyte

$$W = v + U$$

v – portion given by liquid flow
U – portion given by field
c – concentration of analyte
D – diffusion coefficient (2nd Fick's law)

c is not constant in axis of field application (x)

$$J_x = W_x * c(x) - D * \frac{\partial c}{\partial x}$$

$$\lambda = l / w$$

$$v_x = 0 \Rightarrow W_x = U_x = -ax^n$$

$$c(x) = c_0 * e^{\int_0^x \left(\frac{U_x}{D}\right) dx}$$

n – either 0 or 1
0 – constant flow
1 – depends on position in channel

brownian elution mode

$n = 0$ $\quad U_x * t \approx \sqrt{2D * t}$

parameters influencing separation:
: analyte properties
(field-analyte interaction parameter, diffusion coefficient)
: strength of applied field

$$c(x) = c_0 * e^{-\frac{|U_x|}{D} * x}$$

field-analyte interaction parameter
: effective mass $\mathbf{m_{ef}}$

$$m = V_{part} * (\rho_{part} - \rho_{liq})$$

$$R = \frac{6k * T}{F * w}$$

retention ratio
is function of **λ**

k – Boltzmann constant
T – absolute temperature
F = g*m$_{ef}$; **g** – gravity acceleration
w – height of separation channel

steric elution mode

$n = 0$ $\quad U_x * t >> \sqrt{2D * t}$

particles create a layer near to channel wall
concentration of analyte **extra muris = 0**

$$R = \frac{6r_p}{w}$$

$\mathbf{r_p}$ – particle radius

focustion elution mode

$n = 1$ $\quad U_x = -a(x - s)$

particles create a layer near to channel wall
concentration of analyte **extra muris = 0**

$$c(x) = c_0 * e^{-\frac{a}{2D} * (x - s)}$$

s – position, where resulting force inflicting analyte is = 0; position of zone centre from channel wall

$$R = \frac{6s}{w}$$

importance of **hydrodynamic force**:
its influencing: *liquid flow profile*
channel profile

use of FFF

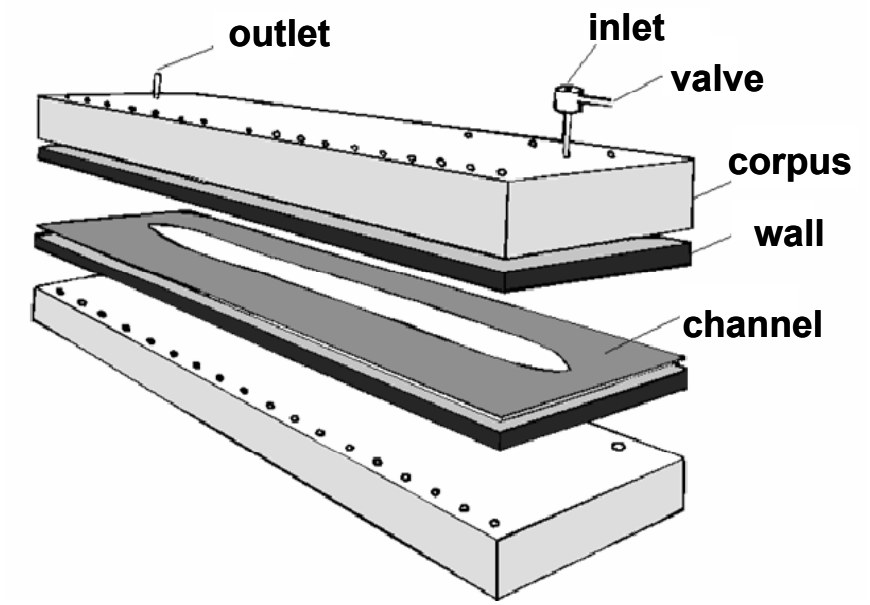
: no SF (one-phase chromatography) ⇒ no interactions with active surface

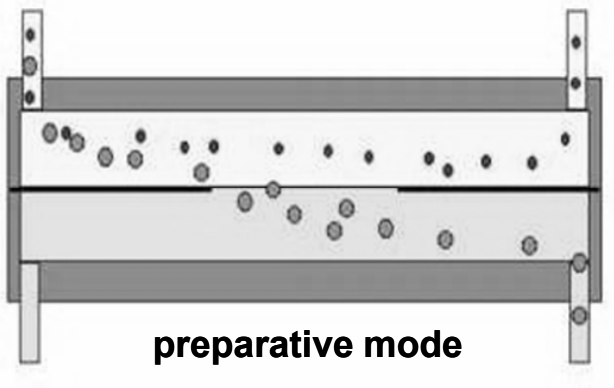
: MF is carrier liquid, influences separation indirectly only

: variables influencing separation may be changed continuously in wide range

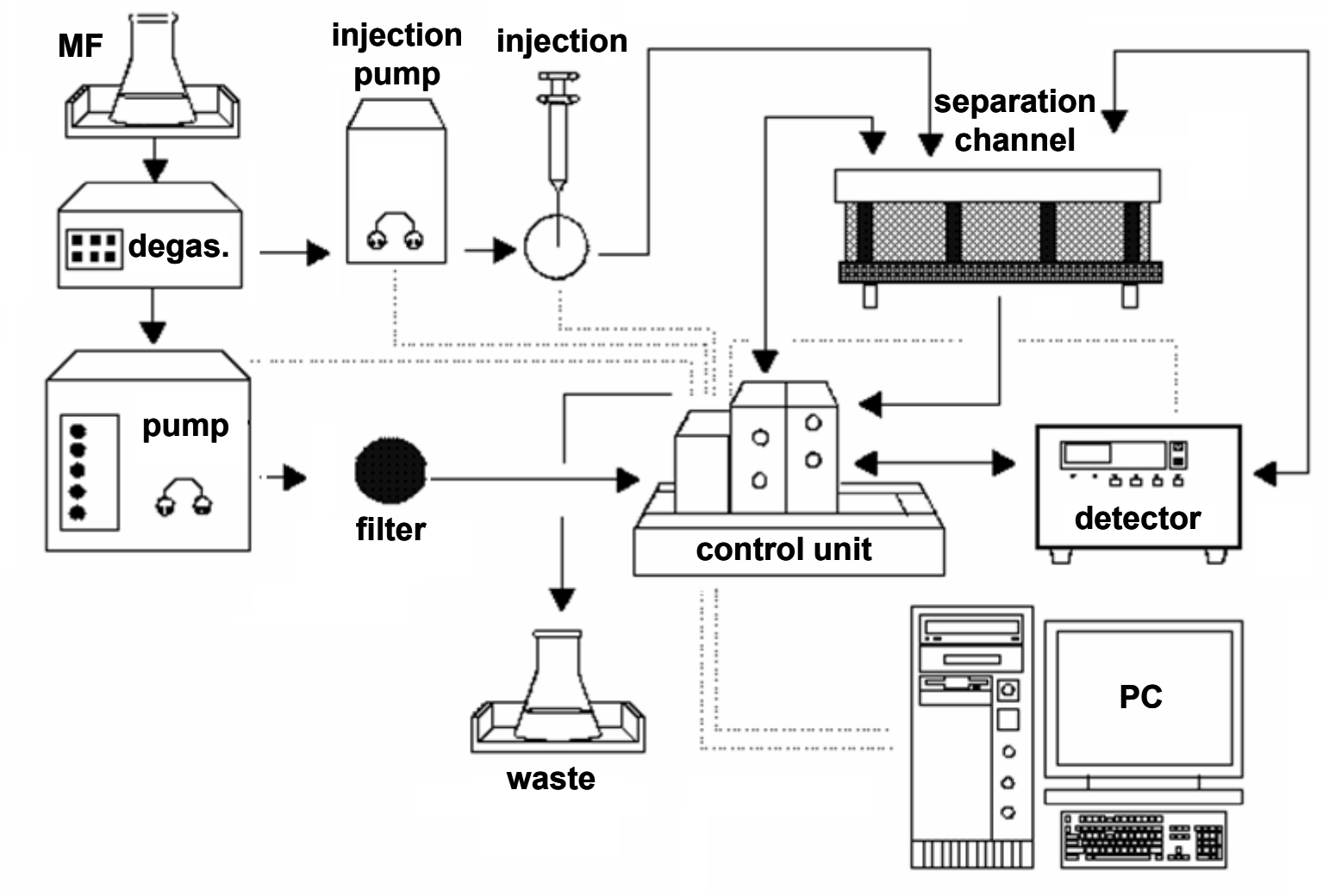
separation of macromolecules and particles **10^3 – 10^{15} Da**

proceeding FFF





instrumentation



pumps

: wide range of adjustable flow-rates
: no need for high pressure, but for pulseless flow !!!
: with constant pressure and flow (reciprocal, peristaltic)

injection device

similar to LC

: septum
: multi-way valve
: linear injectors (infusion)

detectors

similar to SEC

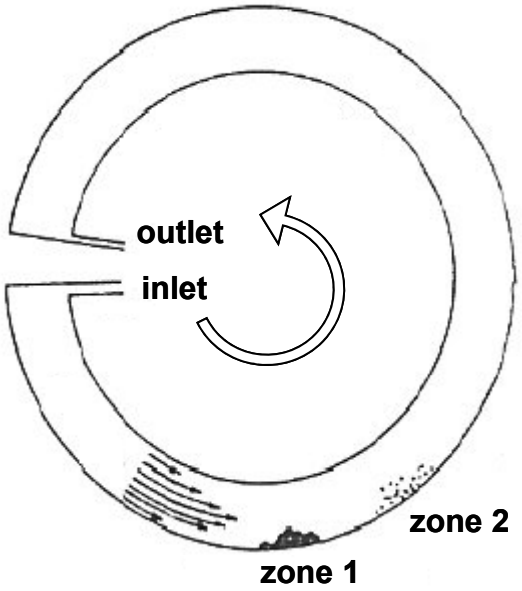
: refractometer
: photometer – absorption, fluorescence, optical rotation, scattering
: other – viscosimeter, densitometer, osmometer...

SdFFF, sedimentation flow-field fractionation

: the oldest technique
: effective force = natural gravity or centrifugal force
: rotation 20000 r.p.m. (injection in steady state)

$$\lambda = 6RT \Big/ \pi * d_p^3 * G * w * \Delta q$$

G – gravity (g) or centrifugal acceleration
Δq – density difference between particles and solvent
d_p – particle diameter



GFFF : > 1 μm
SdFFF (G = 10^5 * g) : 10^6 Da or > 10 nm

DNA, proteoglycans, river water colloids, viruses and silicagel SF for HPLC

ThFFF, thermal flow-field fractionation

separation channel – two metallic (cupric) blocks

the upper one is electrically heated, the lower one is water cooled

$\Rightarrow$ gradient 20 – 1000 °C/cm

: distance teflon foil: 50 – 250 µm

temperature gradient causes slower flow at colder wall (non-isoviscose liquid)

$$\lambda = \left(w * \frac{\alpha}{T} * \frac{\partial T}{\partial x} \right)^{-1}$$

$\mathbf{D_T}$ = thermal diffusion coefficient

α – thermal diffusion factor = $\mathbf{D_T * T / D}$

TFFF : to describe thermal diffusion

EFFF, electric flow-field fractionation

walls – semipermeable cellulose membranes

high voltage gradient; low absolute voltage – low current ⇒ low heating

$$\lambda = D/\mu_e * E * w$$

μ_e – electrophoretic mobility
E – electric field intensity

EFFF : proteins with different isoelectric point

FFFF, flow-field flow fractionation

external field – solvent flow orthogonal to flow of basic media

tube of semipermeable material ⇒ solvent intrusion, not of analyte

$$\lambda = RT * V_0 / 3\pi * N * \eta * V_c * w^2 * d$$

V_0 – channel volume
η – viscosity
V_c – volumetric orthogonal flow
d – effective Stokes diameter

FFFF : > 1 nm

gas chromatography

GC history

1941

Synge and **Martin** : theoretic base for GC:
„...*very refined separations of volatile substances should be possible in a column in which permanent gas is made to flow over gel impregnated with a non-volatile solvent. . ....*“

1952

James and **Martin** : practical introduction of GC; separation of volatile fatty a.

1963

GC-MS – first hyphenated technique

1980

capillary columns in GC – distinctive separation improvement

in principal the same as for LC separation

difference: *gas* is **compressible** (liquid not)

equilibrium on column

$$A\,(g) + SF\,(s) \leftrightarrow A\text{-}SF\,(s)$$

$$K_D = \frac{c_S(A) * \gamma_S(A)}{c_M(A) * \gamma_M(A)}$$

$$c_S(A) = \frac{K_D}{R*T} * p(A)$$

Raoult‘s law

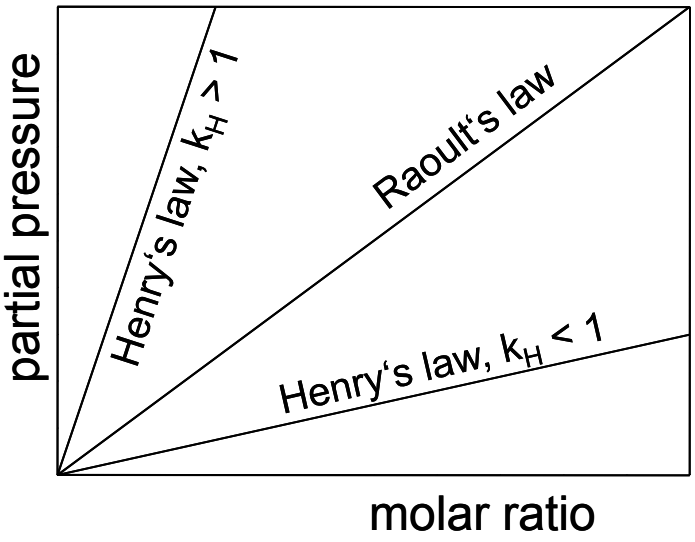
$$p(A) = p^0(A) * x(A)$$

x(A) – molar ratio of **A** in mixture
p^0(A) – pressure of saturated vapours of **A**

Henry isotherm

$$c_S(A) = k_H * p(A)$$

low concentrations of **A**, non-ideal solution
k_H – Henry‘s constant
p(A) – partial pressure of **A** over mixture



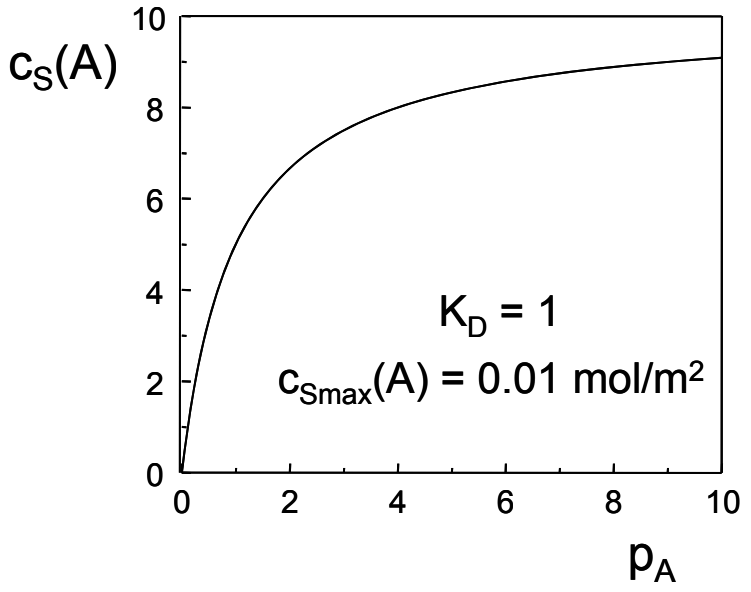
relation between
Raoult‘s and Henry‘s laws

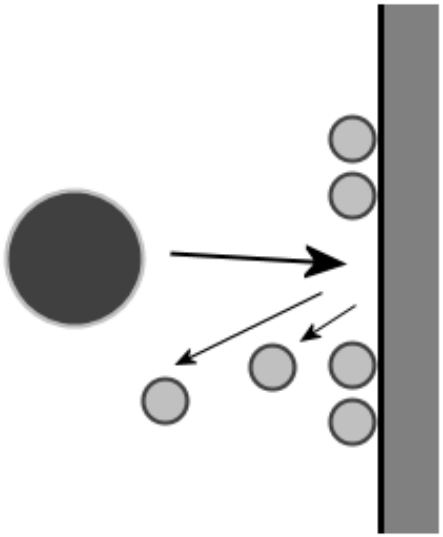
Langmuir isotherm

$$c_S(A) = c_{S\max}(A) * \frac{K_D * p(A)}{1 + K_D * p(A)}$$

c_{Smax} – maximal bound concentration

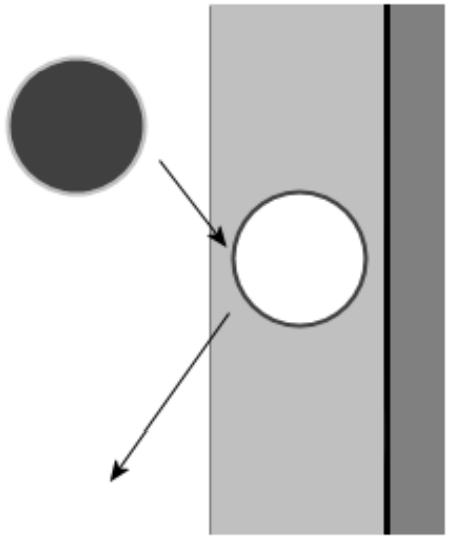
graphical presentation
of Langmuir isotherm





adsorption GC
GSC

distribution GC
GLC



adsorption (distribution) GC

distribution chromatography (GLC)

vapour tension of analyte (**A**) over liquid phase

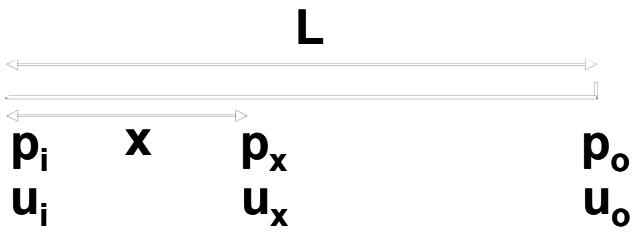
adsorption chromatography (GSC)

different adsorption of molecule **A** onto SF surface with active centres

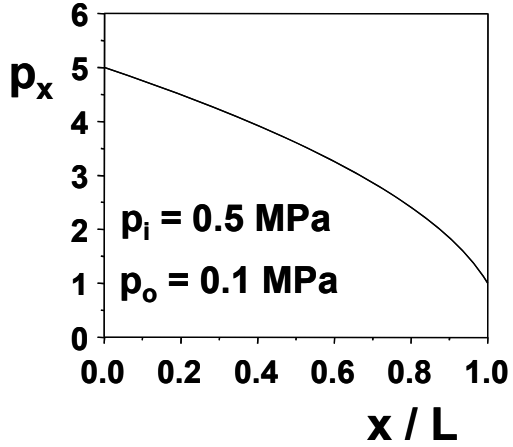
$$K_R = \frac{c_S(A)}{c_M(A)}$$

S – SF, ***M*** – MF

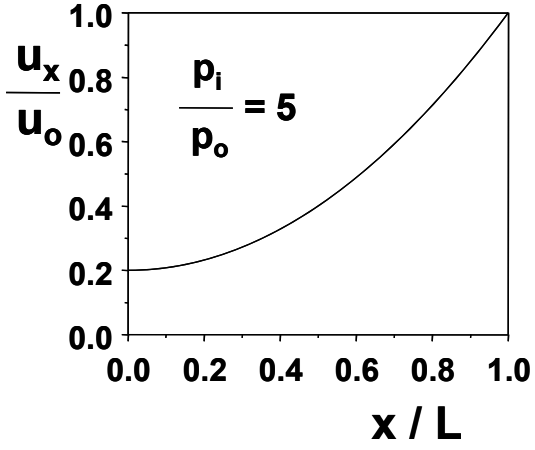
linear flow rate of carrier gas (MF)



L – column length
p – gas pressure
u – linear flow rate
indices: **i** – on inlet
x – in point **x** of length
o – on outlet



pressure gradient profile
on column



value profile
of linear flow rate

average linear MF flow rate

$$\bar{u} = \frac{B_0 * (p_i - p_o)}{\eta * \varepsilon * L}$$

B_0 – specific permeability of column [m^2]
(p_i-p_o) – pressure gradient [Pa]
η – dynamic viscosity [Pa.s]
ε – sorbent inner porosity
L – column length [m]

compressibility factor

$$\bar{u} = j * u_o$$

$$j = \frac{3}{2} * \frac{\left(\frac{p_i}{p_o}\right)^2 - 1}{\left(\frac{p_i}{p_o}\right)^3 - 1}$$

retention quantities

retention volume / time of *i*-th analyte $\mathbf{V_{R,i}}$ [ml], $\mathbf{t_{R,i}}$ [min]

$$V_{R,i} = F_M * t_{R,i}$$

void volume / time of column $\mathbf{V_m}$ [ml], $\mathbf{t_m}$ [min]

$$V_m = F_M * t_m = V_M$$

reduced retention volume / time $\mathbf{V'_{R,i}}$ [ml], $\mathbf{t'_{R,i}}$ [min]

$$t'_{R,i} = t_{R,i} - t_m$$

$$V'_{R,i} = F_M * t'_{R,i}$$

$$V'_{R,i} = V_{R,i} - V_m$$

net retention volume $\mathbf{V_N}$ [min]

$$V_N = F_M * t'_{R,i} * j = V'_{R,i} * j$$

$\mathbf{V'_{R,i}}$ corrected to carrier gas compressibility

specific volume $\mathbf{V_h}$ [ml/g] or $\mathbf{V_p}$ [ml/m^2]

$$V_p = \frac{273.15 * V_N}{S * T_k}$$

$$V_h = \frac{273.15 * V_N}{w_L * T_k}$$

$\mathbf{V_N}$ related to 1 g or 1 m^2 SF and to 0 °C

temperature influence

$$T_k > T_{boil} \wedge T_{inj} \geq T_k \wedge T_d > T_k$$

T_{inj} – injection head temperature
T_k – column thermostat temperature
T_d – detector temperature

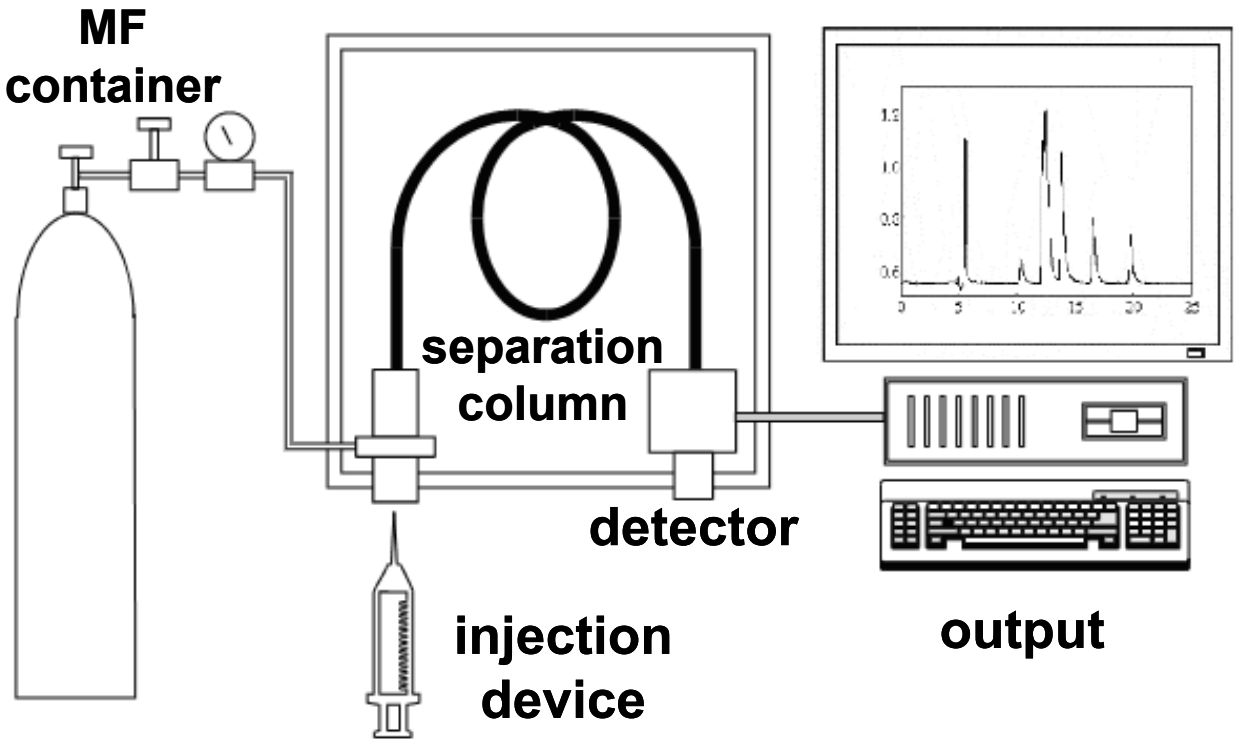
↑ T_k leads to faster analysis

↑ T_k demands ↑ MF pressure on column inlet for keeping **u** through column

isothermic analysis: T_k = *const.*

analysis with temperature gradient: $T_{k2} - T_{k1} > 0$

GC arrangement

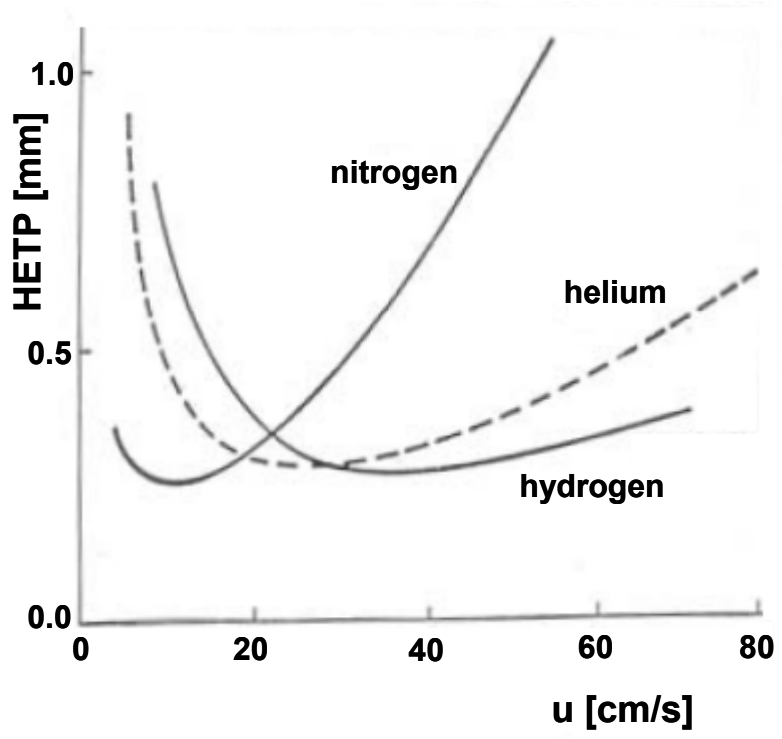


MF delivery

gas : 0.5 ml/min – 400 ml/min (HP-GC 1200 ml/min)
: pressure containers : pressure up to 400 kPa (HP-GC 1 MPa)
: compressor : pressure and flow control
: electrolyser : thermostating

carrier gas advanced flow control
(AFC)

carrier gas advanced pressure control
(APC)



carrier gas

N_2 (nitrogen)

+ cheap, safe

– low thermal conductivity

H_2 (hydrogen)

+ high thermal conductivity, low viscosity

– high diffusivity, explosive

He (helium)

+ combines advantages of N_2 and H_2

– expensive

Ar (argon)

especially for ECD

must be *chemically inert* – always necessary to remove **humidity** and **O_2**

purity – pre-set guard column with molecular sieve

injection device

loading of **A** onto column
: more difficult than by LC

tubular columns: 1 – 20 µl
capillary columns: ~ 1 nl

inject *small volume* and *quickly*
: slowly and large volume (overload) ⇒ broad zones and resolution loss

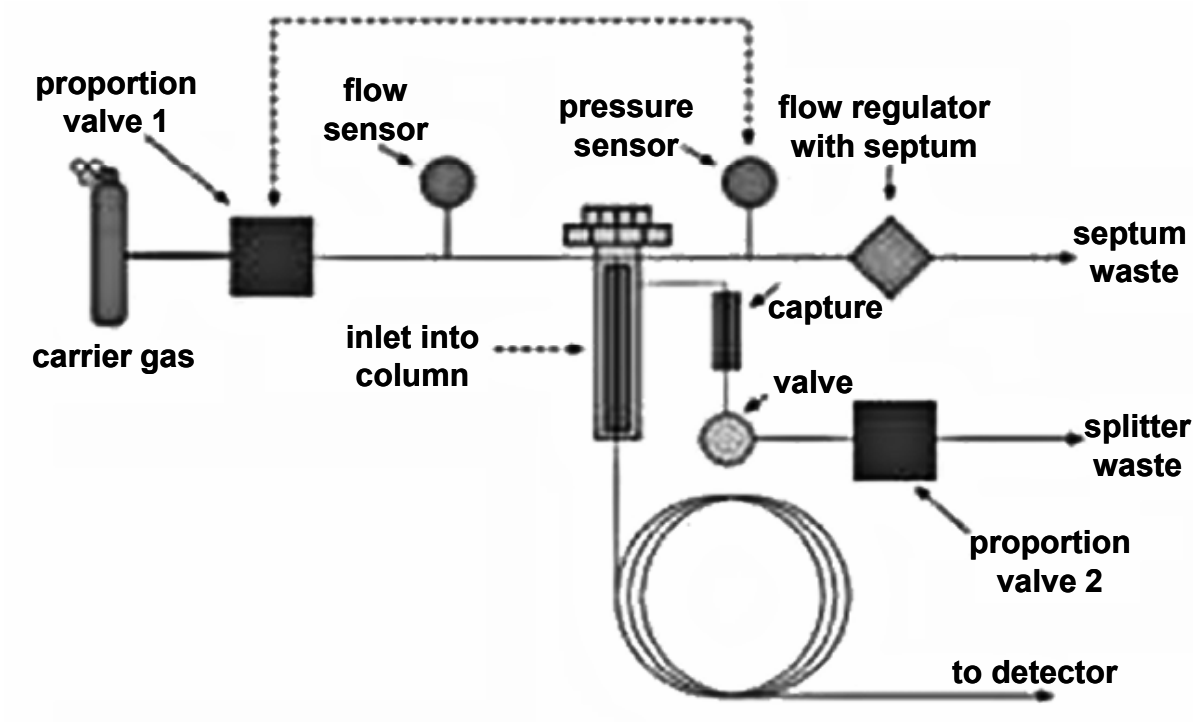
sample evaporation

necessity to transform liquid and solid samples
into gaseous state

heated space on the beginning of the column

<u>volatility increment</u>
chemical derivatisation: *silylation* (N,O-bis(trimetkylsilyl)acetamide)
silanisation (dimethylchlorsilane)
and *acetylation* (acetanhydride)

splitless injection



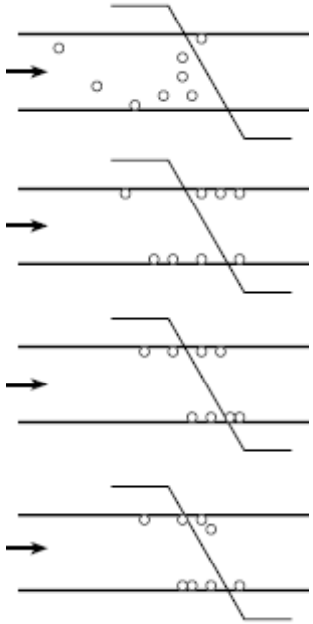
: with closed valve pressurise using proportion valve 1: flow sensor = 5 ml/min, pressure sensor = 70 kPa

: septum flow set to 2 ml/min ⇒ slow flow of 3 ml/min onto column

: sample introduced into injector and is carried onto column

: after certain time without splitting (10 – 40 s /optimum 20 s/, *splitless time*), which happens after injection, the valve is open and rest of the sample is washed out

it demands sample **reconcentration**
: prevents zone broadening



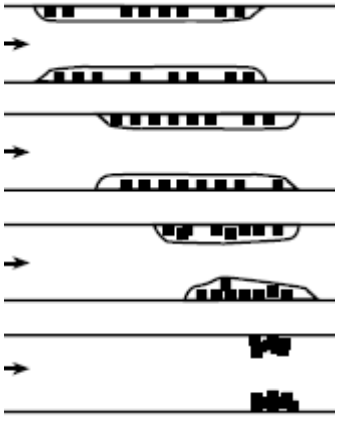
cold trapping
: first few centimetres of column has negative temperature gradient
(~ 250 °C /injection/ >> 40 °C capture region; *ca* < 150 °C than T_{boil})

⇒ mobility of components with high T_{boil} is zero
⇒ their reconcentration

solvent effect
: first few centimetres of column has negative temperature gradient
(~ 250 °C /injection/ >> capture region is *ca* 20 °C bellow solvent T_{boil})

⇒ sample components with low T_{boil} condensate with solvent

from the created thin film, the solvent is slowly evaporating
⇒ reconcentration of components with low T_{boil}



split injection

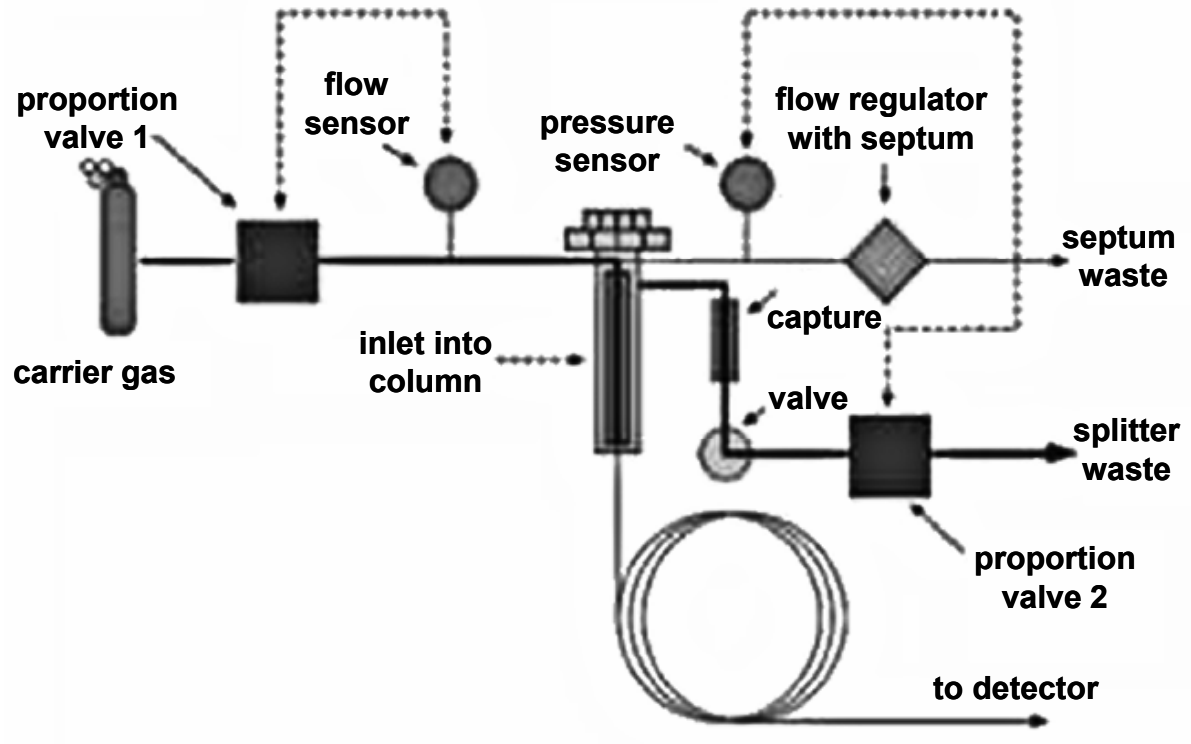
splitter allows: easy injection of *small volume*
: is related to sharp zone entering onto column and column capacity

$$S = \frac{F_M}{F_S + F_M}$$

S – degree of sample splitting,
$\mathbf{F_M}$ – column flow rate, $\mathbf{F_S}$ – splitter flow rate
(proportion valve 2)

disadvantages:
: unsuitable for trace analysis
: depends of splitter geometry

today the most used way of injection



: pressurise using proportion valve 1: flow sensor = 103 ml/min, pressure sensor = 70 kPa

: septum flow set to 2 ml/min ⇒ slow flow of 3 ml/min onto column

: pressure sensor sets proportion valve 2 to 100 ml/min ⇒ onto column 1 ml/min ⇒ through inlet MF flow quickly, 101 ml/min

: sample introduced into injector and according to split equation, part goes onto column, part out to waste

on-column injection

: injects precise amount
: suitable for analytes with high T_{boil} – no evaporation during injection

instrumentally demanding – restrict pressure losses within injection

overloads column with liquid (1 µl for 50 cm of column) ⇒ peak broadening
: solution as within splitless injection

: gas entrance to column is *sealed*

: with closed valve pressurise using proportion valve 1: flow sensor = 7 ml/min, pressure sensor = 70 kPa,

: septum flow set to 2 ml/min

: sample introduced into injector and carried onto column by flow rate 5 ml/min

: after certain time without splitting (*splitless time*), which happens after injection, the valve is open and rest of the sample is washed out

separation column

tubular
: analytical
: preparative

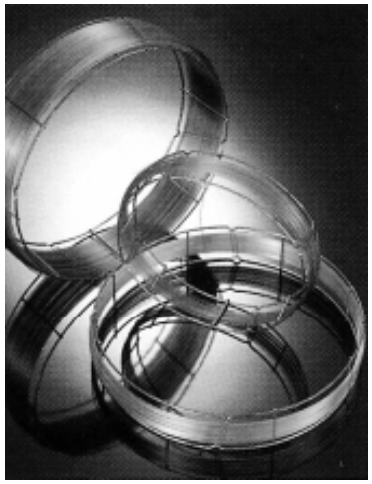
length: 0.5 – 10.0 m
diameter: 1 – 6 mm

length: 2 – 6 m
diameter: > 6 mm

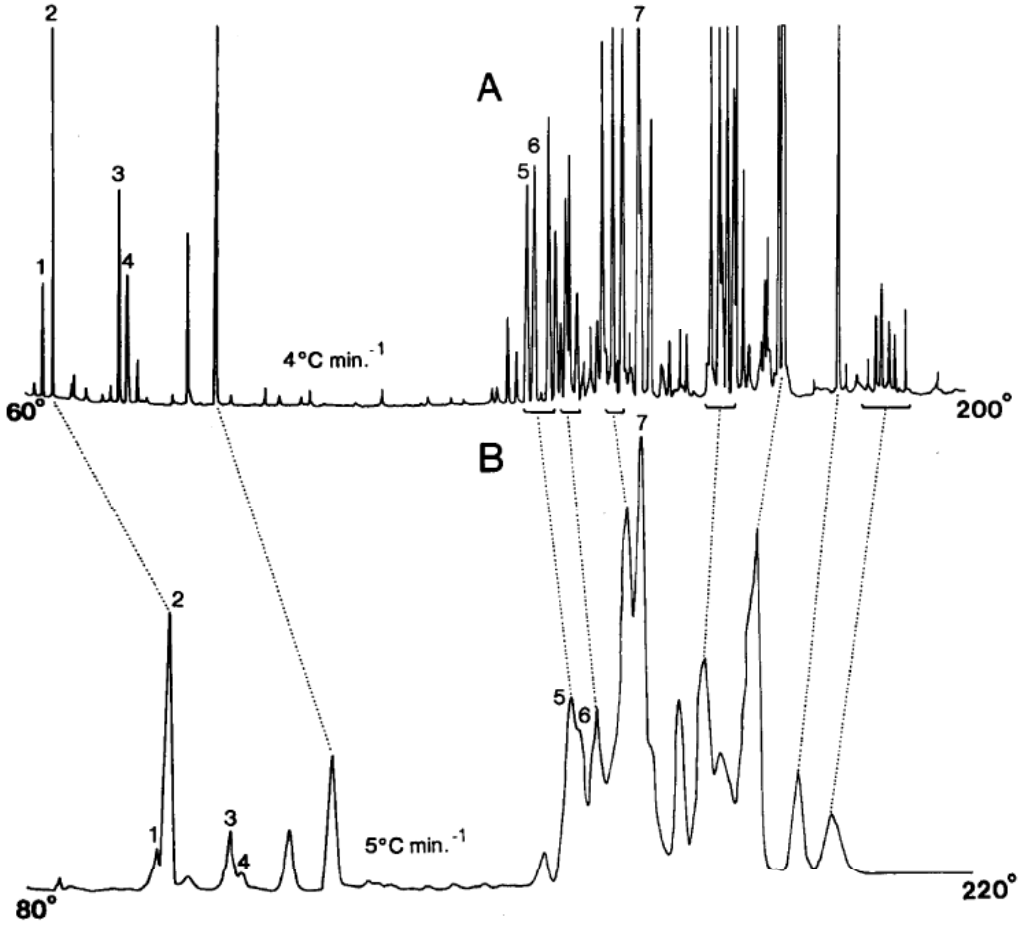
capillary
: open
: filled

length: 10 – 100 m
diameter: 0.1 – 0.5 mm

length: 0.5 – 50.0 m
diameter: 0.3 – 1.0 mm



separation efficiency comparison of different column types



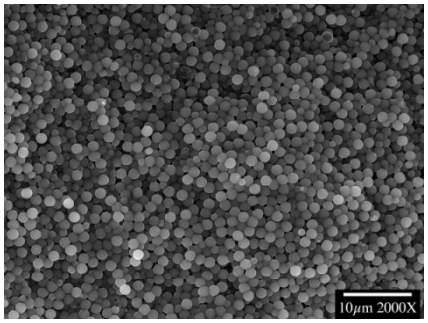
GC separation of calamus oil components
A – 50 m capillary column
B – 4 m tubular column

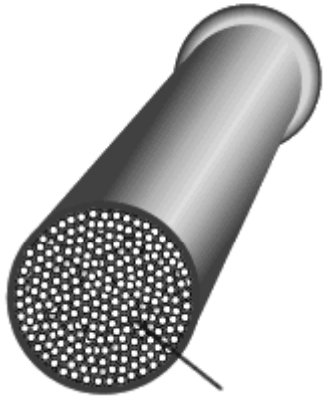
column filling

tubular columns
cover: glass, steel, copper, polymers

carriers
modified infusorial earth
active centres (silanols and siloxanes) ⇒ tailing of more polar components
suppression – *silylation*

adsorbents
: *unspecific* (active carbon)
: *specific* (silicagel, alumina, molecular sieves etc.)

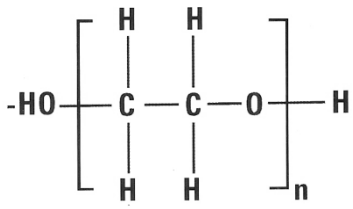




carrier – fine, solid and inert material (spherical silicagel)

serves directly as SF (GSC),
or is covered by thin film of liquid phase (GLC)

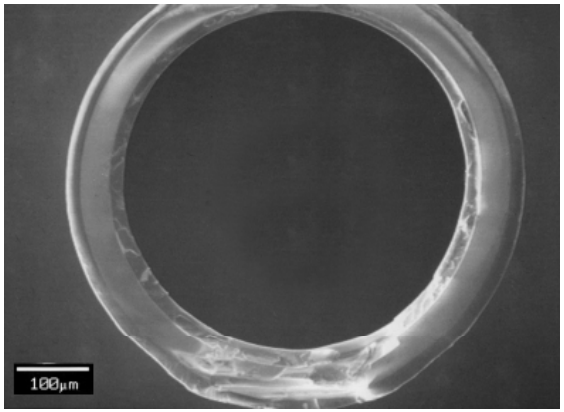
non-polar
: methylated polysiloxane, squalene



mildly polar
: phenylated polysiloxane

strongly polar
: polysiloxane with CH_2-CH_2-CN, -CH=CH-CN, Carbowax 20M (based on PEG)

<u>capillary columns</u>

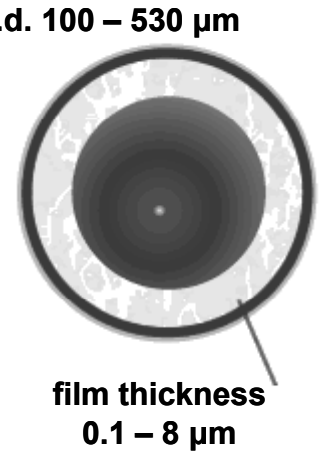


silica

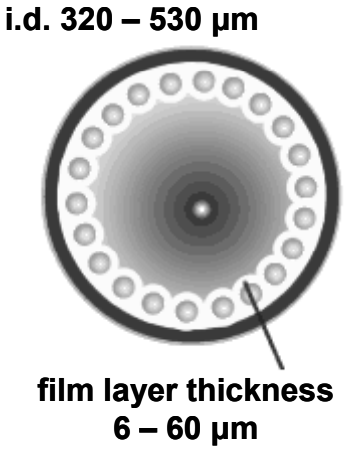
surface enlargement by etching
polyimide cover ⇒ increase of mechanical stability

SF universal non-polar silicon phases or immobilised Carbowax

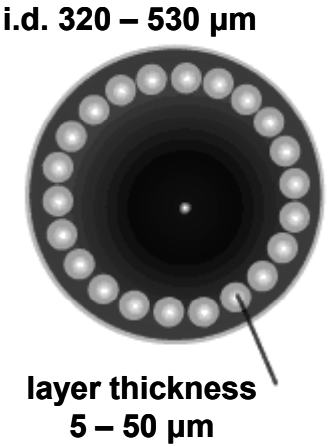
wall-coated open tubular columns
(WCOT)
liquid SF directly on the capillary wall



fused silica open tubular
(FSOT)
thin wall with outer polyimide cover (mechanical stability)

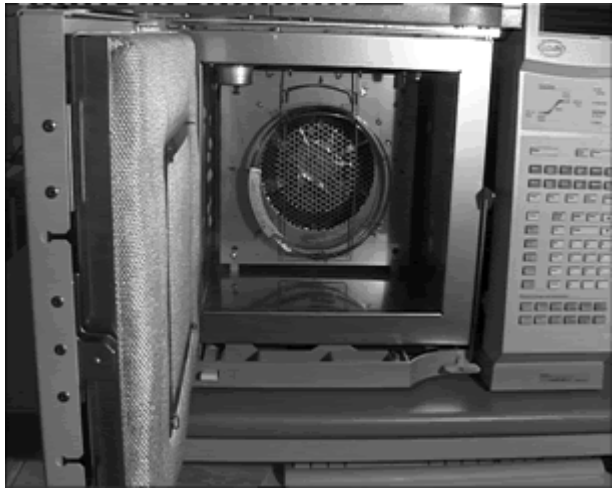


support-coated open tubular columns
(SCOT)
carrier is on capillary wall, SF is on it



porous-layer open tubular columns
(PLOT)
layer of solid active sorbent on an inner capillary wall

column thermostat



<u>importance of temperature of GC</u>

: evaporation of liquid or solid sample
: kinetic aspects of separation

kept with precision of 0.1 °C; thermostat range (T_{lab} + 4 °C) – 450 °C

optimal loading temperatures – T_{boil} of component with highest value + 30 – 50 °C

optimal column temperature ~ T_{boil} of analyte
column temperature ≥ T_{boil} ⇒ t_R = 2 – 30 min

minimal temperature ⇒ better resolution, but higher t_R

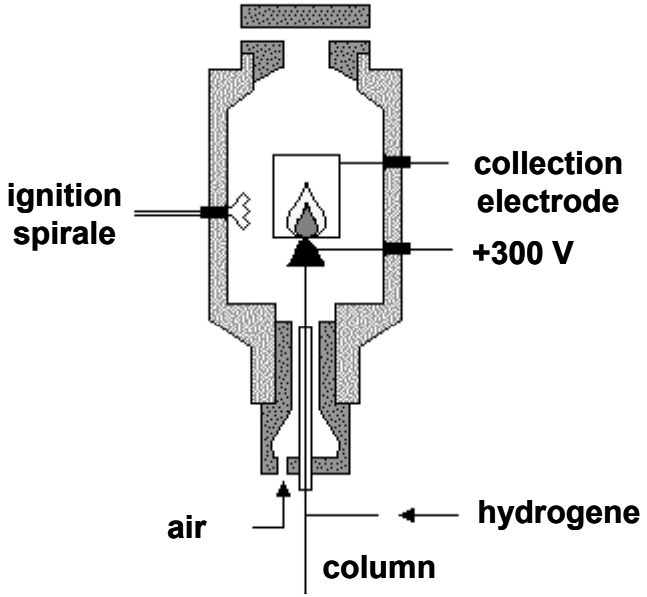
wide range of T_{boil} of separated components ⇒
⇒ *temperature programme / column gradient* (Δ temperature during experiment)
temperature may be increased gradually or in steps

detectors

detected compound is volatile, in gaseous state

concentration dependent detector (CDD)
: non-destructive, dilution with carrier gas decreases sensitivity

mass dependent detector (MDD)
: destructive, carrier gas interferes not, depends on introduction rate into detector

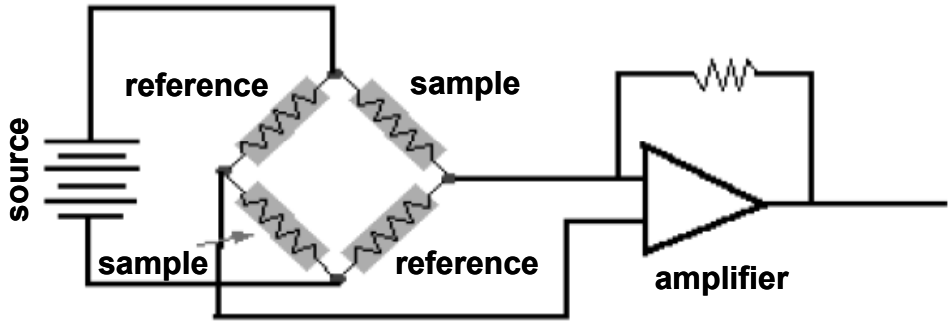


flame ionisation detector

FID

MDD
signal: current created by pyrolysis of carbon sample

: **noise** 10^{-13}
: **dyn. range** 10^{7}
: **sensitivity** 10^{-10} g/ml



thermal conductivity detector

TCD
catharometer

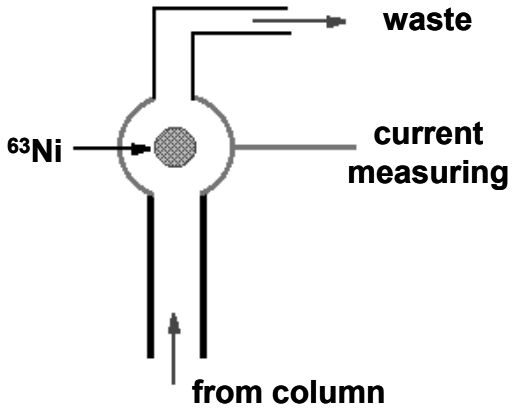
: noise 10^{-5}
: dyn. range 10^{6}
: sensitivity 10^{-9} g/ml

CDD
signal: sample molecules change (decrease) thermal conductivity of carrier gas
: carrier gas must have high thermal conductivity (He, H_2...)
: temperature dependent, universal

electron capture detector

ECD

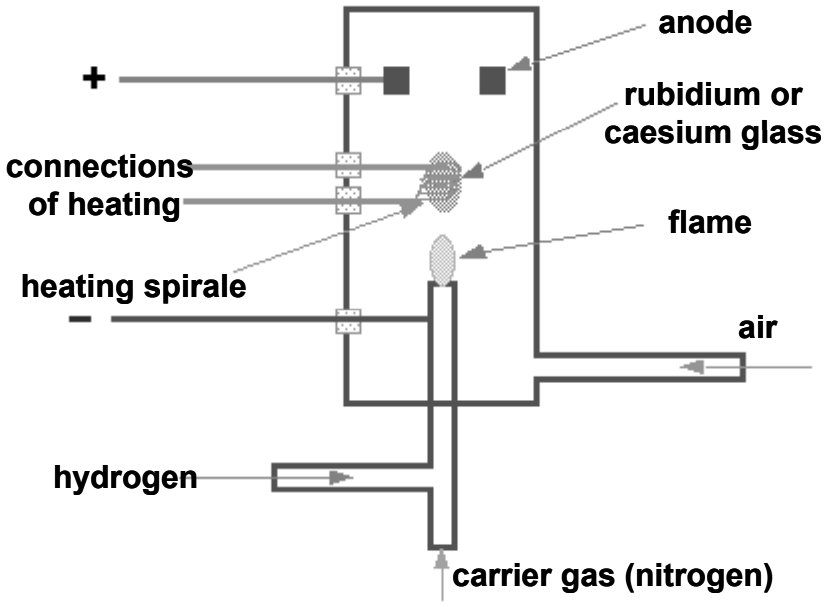
: noise 10^{-12}
: dyn. range 10^{5}
: sensitivity 10^{-14} g/ml



CDD
signal: analyte molecules decrease current generated by β-emitter
: halides, nitrites, cyano-compounds, peroxides, anhydrides, organometals

nitrogen phosphorus detector

NPD – thermoionisation detector

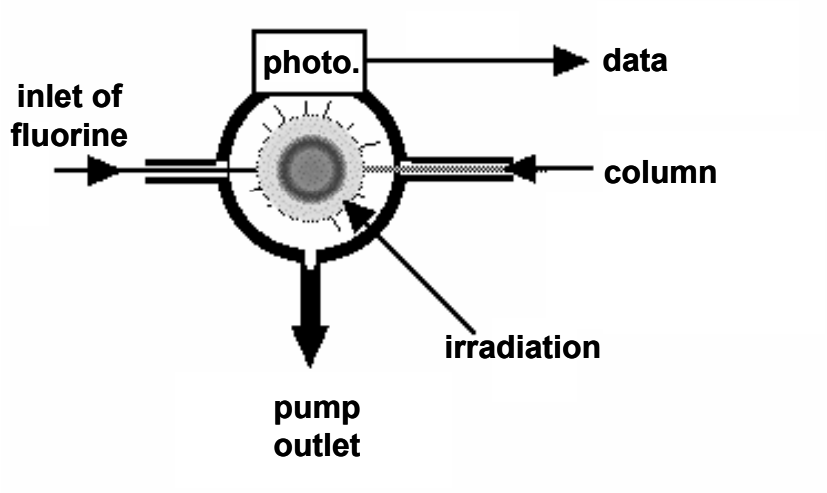


: noise 10^{-12}
: dyn. range 10^{6}
: sensitivity 10^{-11} g/ml

MDD

signal: Rb/Ce glass thermoionisation electron emission enhanced by N or P presence

chemoluminiscence detector



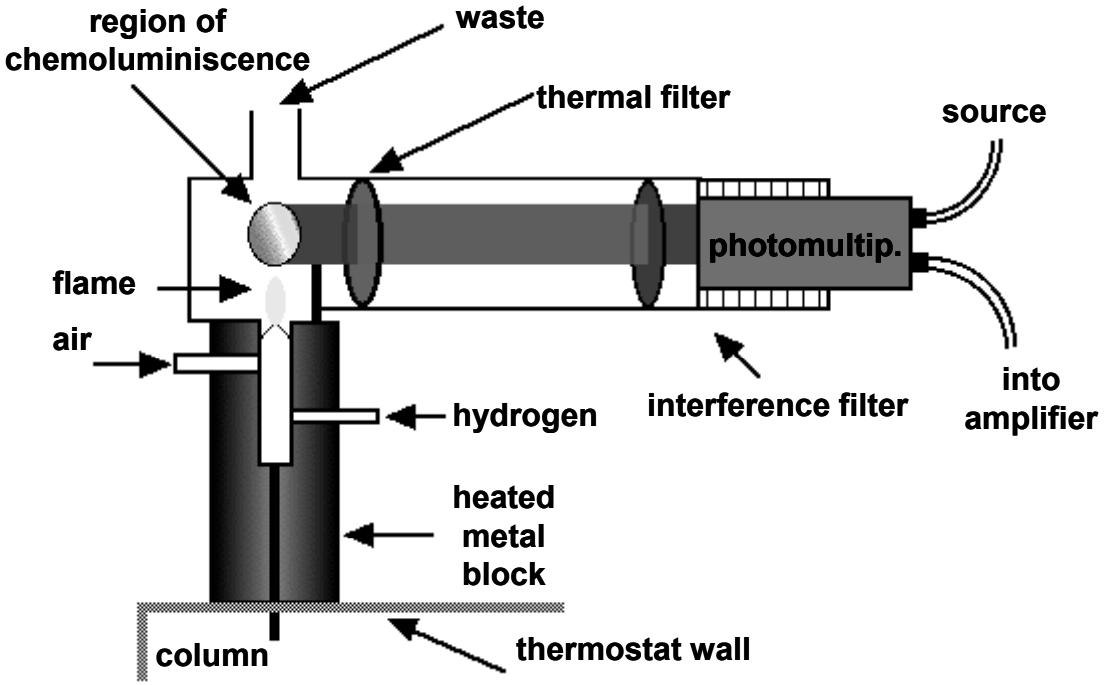
: noise 10^{-13}
: dyn. range 10^{4}
: sensitivity 10^{-12} g/ml

CDD

signal: reaction of F (strong oxidant) with analyte

flame photometric detector

FPD



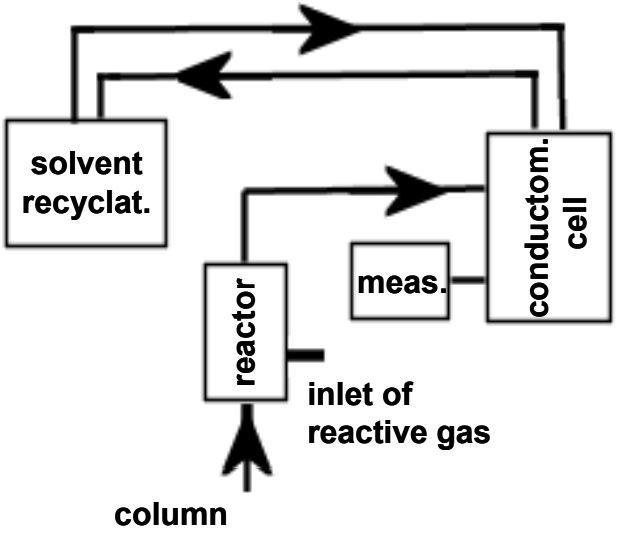
: **noise** 10^{-12}
: **dyn. range** 10^{7}
: **sensitivity** 10^{-11} g/ml

MDD
signal: chemoluminiscence
: selective S (394 nm), P (526 nm)

electrolytic conductivity detector

ELCD

: **noise** 10^{-13}
: **dyn. range** 10^{6}
: **sensitivity** 10^{-12} g/ml



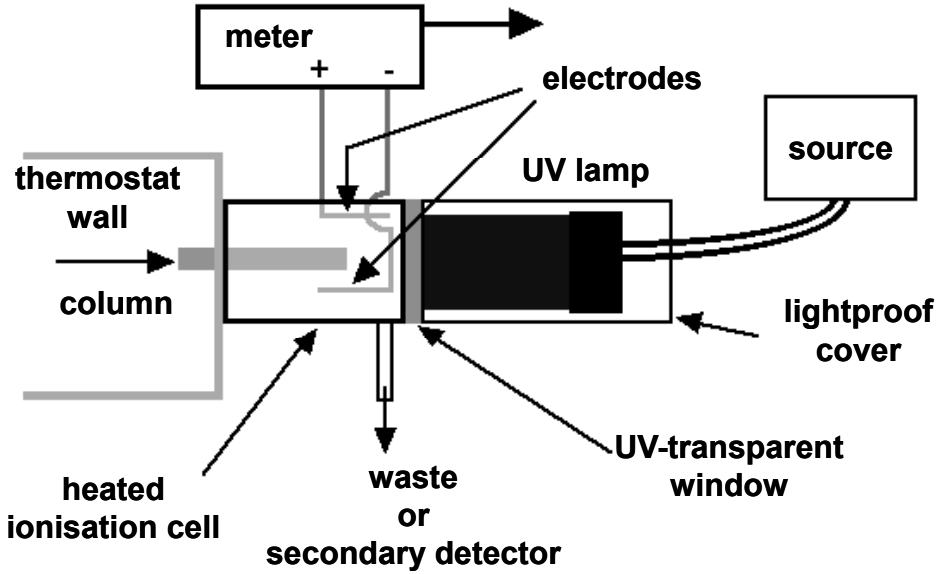
MDD
signal: appearance of special products
their conductivity measurement after mixing with solvent

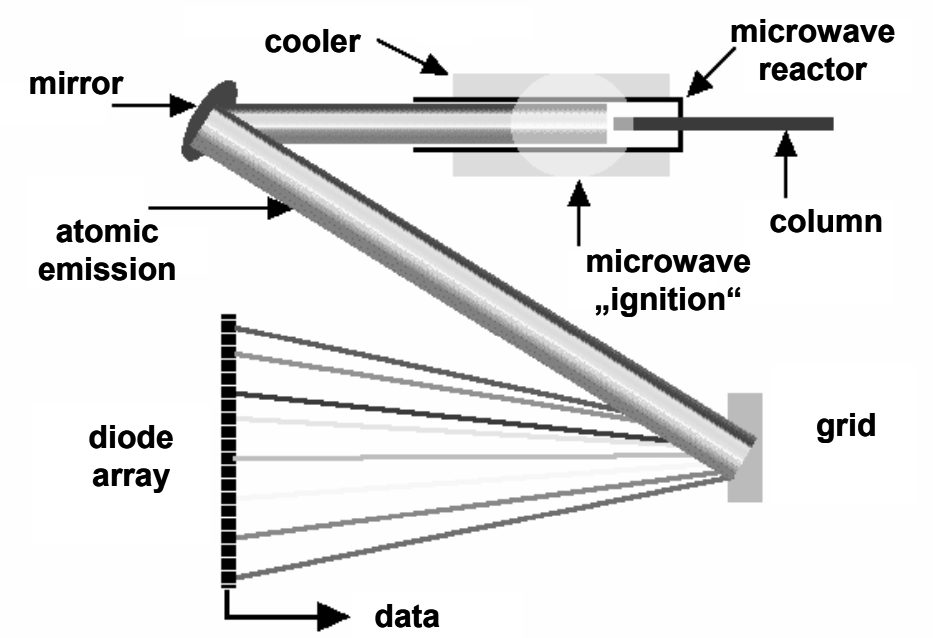
photoionisation detector

PID

: **noise** 10^{-13}
: **dyn. range** 10^{7}
: **sensitivity** 10^{-12} g/ml

CDD
signal: UV-irradiation ionisation



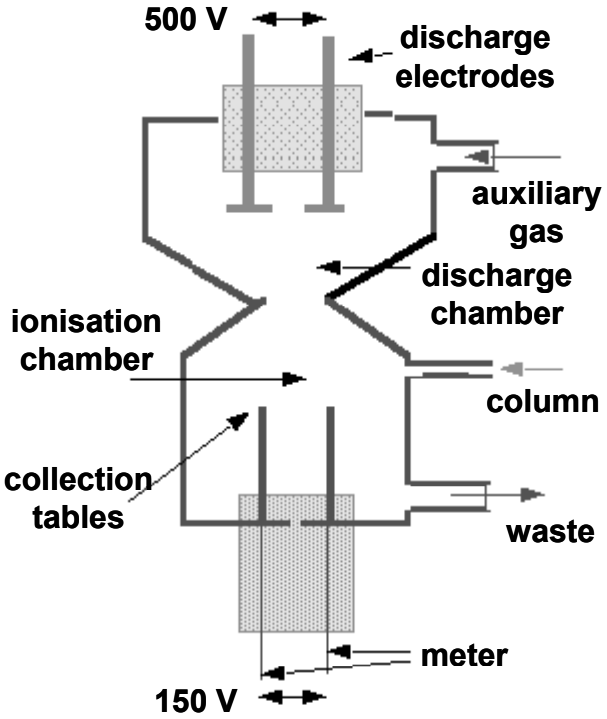


atomic emission detector

AED

: **noise** 10^{-14}
: **dyn. range** 10^{4}
: **sensitivity** 10^{-12} g/ml

MDD
signal: microwave induced plasma
: selective according to chosen emission wavelength
: very expensive



helium ionisation detector

HID

: noise 10^{-14}
: dyn. range 10^{6}
: sensitivity 10^{-13} g/ml

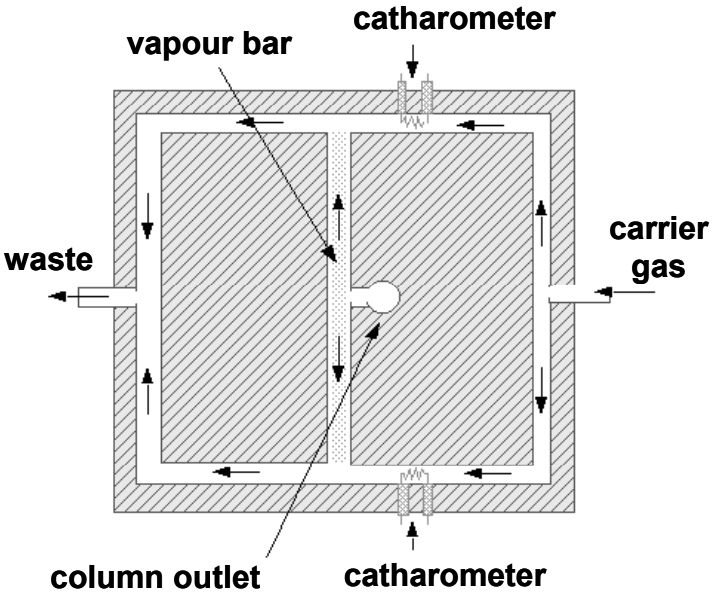
MDD
signal: auxiliary gas is ionised first (He, Ar),
its ions then secondary ionise sample molecules

gas density balance

GDB

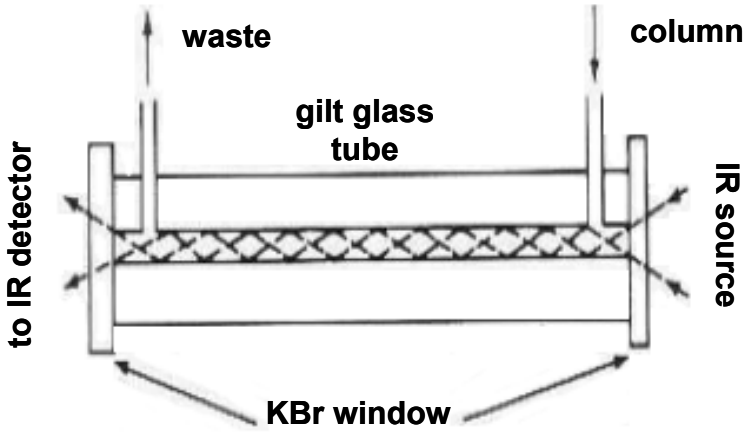
: noise 10^{-8}
: dyn. range 10^{3}
: sensitivity 10^{-7} g/ml

MDD
signal: pressure difference between upper and lower passage of gas in presence of eluent vapours



infrared detector

IRD



: noise 10^{-12}
: dyn. range 10^{5}
: sensitivity 10^{-11} g/ml

CDD
signal: IR absorbance

mass spectrometric detector

MS

: noise 10^{-14}
: dyn. range 10^{3}
: sensitivity 10^{-16} g/ml

CDD
signal: ion count
universal

ionisation:
: electron impact (EI)
: chemical i. (CI)

analysers:
: quadrupole (Q, Qq)
: ion trap (IT)
: magnetic sector
: time-of-flight (TOF)

definition of chromatographic system in GC

MF

carrier gas type

flow / pressure (ml.min^{-1} / kPa)

injection (*X* µl)
injection type (event. splitting rate)

SF

stationary phase type

length, inner diameter, manufacturer, SF type, film thickness
25m x 0.32 ID J&W DB-5 DF – 1.0

temperature gradient profile
initial temperature and its period, temperature increase; inlet temperature

(e.g. 130 °C 1 min, 130 – 250 °C at 5 °C/min, 250 °C 5 min; 250 °C)

detector

basic characteristic according to type

analytical information in chromatogram

qualitative information

retention time ≈ retention factor, distribution constant
: compound identification (*standard method*)

spectroscopic detectors: UV-Vis spectra
MS spectra (ESI / APCI; Qq / IT / o-TOF)
NMR spectra (^{1}H, ^{13}C)

retention time formulation

specific retention volume (V_p)

$$V_p = \frac{273.15 * V_N}{S * T_k}$$

relative retention time ($r_{A,B}$)
: comparison with internal standard

$$r_{A,B} = \frac{t'_R(A)}{t'_R(B)}$$

Kovats retention indices ($r_{A,B}$)
: linear dependence pf retention time logarithm of homologues on carbon number

quantitative information

peak area ≈ amount, concentration of compound

internal normalisation method

: all components are eluted (solvent does not count)
: all they have same/similar response factor

$$c_{\%} = A_{\%,j} = \frac{100 * A_j}{A_{tot}}$$

external standard method (*absolute calibration; calibration curve*)

: always same measurement conditions, same injection volumes
: indispensable matrix influence

$$c_{unknown} = \frac{A_{unknown}}{A_{known}} * c_{known}$$

internal standard method

$$c_{unknown} = \frac{A_{IS1}}{A_{IS2}} \frac{A_{unknown}}{A_{known}} * c_{known}$$

: need not to know injection volume
: standard must be chemically similar to analyte

standard addition method

: presumes calibration curve linearity

$$c_1 = \frac{V_S}{V_1} * \frac{c_S}{\frac{A_2}{A_1} * \frac{(V_1 + V_S)}{V_1} - 1}$$

A_1 – analyte peak area, unknown concentration **c_1**

A_2 – analyte peak area of unknown concentration **c_1**
after addition of standard of known concentration **c_S**

V_1 – sample volume, **V_S** – standard solution volume

electromigration methods

driving force – electric field

: charged particle motion in electric field
: extraction L-S

: electrolyte (liquid able to conduct current)
: separation channel wall (carries charge)
: stationary phase (SF, solid matter, micelles)

mobility of ions is influenced by charge, molecule size and surrounding ions

basic electromigration techniques

: column arrangement (in tube, in capillary)
: slab arrangement (in gel)

EMM history

1808-93

first experiments in U-tubes – F. von Reuss (1808), G. Wiedeman (1856), H. Buff (1858), O. Lodge (1886), W. Whetham (1893)

1897

Kohlrausch – basic equation for ion migration in electrolyte solution

30. léta

Tiselius – gel elfo with glucose as medium

1937

Tiselius – first fully functional electrophoresis instrument, **1948** Nobel price

1955

Smithies – use of starch gels for elfo

1958

Hjertén – ZE in rotating tubes 1 – 3 mm

1959

Raymond and **Winstraub** – acrylamide gels, setting up gel porosity & stability

1965

Tiselius – ZE in 3 mm tubes

1967

Hjertén – elfo in tube, i.d. 1 – 3 mm, with inner coating against EOF

1969

Vesterberg and **Svensson** – IEF of proteins in ampholytes

1970

Laemmli – denaturing separation in gel, SDS and concentration gel use
Everaerts – ITP on own instrument

1974

Pretorius – EOF as a MF driving force through sorbent

1974 –79

Virtanen, and **Mikkers et al**. – glass and teflon capillaries, i.d. 200 µm

1975

O'Farrell – 2D GE, presetting IEF in gel to SDS elfo

1981

Jorgenson and **Lucas** – borosilicate glass capillary, i.d. 75 μm

1983

Hjertén – CGE for biological samples

1984

Terabe – micellar electrokinetic chromatography

1985

Hjertén – CIEF for biological sample

1987

Karger and **Cohen** – high efficiency CGE for NA
Knox and **Grant** – CEC in 50 μm capillaries with ODS

1988

Beckmann Instruments – first commercial instrument

motion of free charged particle in electric field
: charge and field orientation decided on direction and velocity

$$v = \mu * E = \mu * \frac{U}{l}$$

v – ion motion velocity
μ – electrophoretic mobility [$m^2 V^{-1} s^{-1}$]
E – electric field intensity
U – voltage
l – length of voltage gradient

influencing the motion by **ionic atmosphere** ⇒
⇒ decrease of velocity with increase of electrolyte concentration

μ_0 ionic (net) mobility – μ at zero ionic strength
10^{-9} $m^2 V^{-1} s^{-1}$ = 1 tiselius (Ti), sign implies ion polarity (anion has negative **μ**)

temperature influence: ↑**T** ⇒ ↑**μ_0**; with 1 °C about 2 %

$$\mu_T = \mu_{T_0} * \left[1 + 0.02 * \left(T - T_0\right)\right]$$

T – working temperature
T_0 – standard, tabulated temperature

ion mobility **estimation**

in a case, when value is not known (tabulated)

Stokes mobility; **a** – acceleration of spherical charged particle motion

$$a = 0$$

$$F_E = F_F$$

$$\frac{F_E}{F_F} = \frac{q * E}{6\pi * \eta * r * v} = \frac{q}{6\pi * \eta * r * \mu} \Rightarrow \mu = \frac{q}{6\pi * \eta * r}$$

q – charge
η – solution viscosity
r – ion radius
v – ion motion velocity

relation of ion mobility and **diffusion coefficient**

$$\mu = \frac{z * F}{R * T} * D$$

z – relative charge
F – Faraday constant
R – gas constant
T – temperature
D – diffusion coefficient

ion mobility estimation for small molecules

Jokl equation

$$|\mu_0| = |z| * \frac{a}{\sqrt{M}} - b$$

M – molecular mass

a, b – empiric constants

a ~ 485 x10^{-9} m^{-2} V^{-1} s^{-1}

b ~ 9.6 x10^{-9} m^{-2} V^{-1} s^{-1}

estimation error is *ca* 10 %

actual ion mobility

Onsager equation

$$|\mu| = |\mu_0| * (0.23 * |\mu_0 * z_+ * z_-| + 31.3 \cdot 10^{-9} * |z_{+/-}|) * \frac{\sqrt{I}}{1+\sqrt{I}}$$

z_+, z_- – relative ion and counter-ion charge

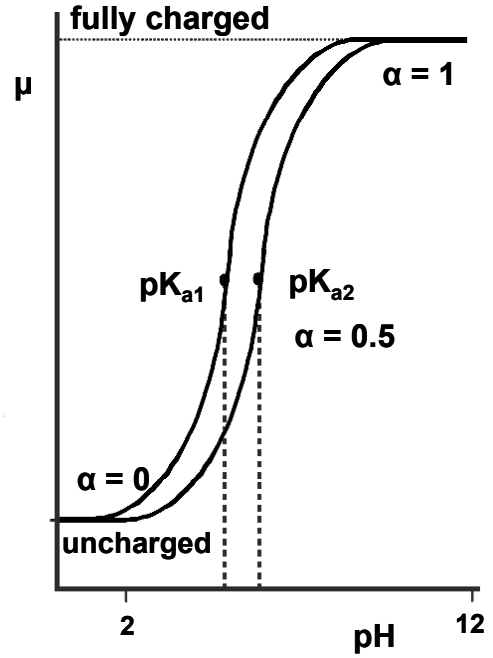
I – ionic strength

effective mobility

mobility of weak bases, acids or zwitterions
resulting mobility of all ion forms

$$\overline{\mu} = \sum_{i=1}^{n} \mu_i * x_i$$

μ_i – mobility of one ion form
x_i – its molar ratio



free mobility

mobility extrapolated to zero gel concentration

migration time

entry useful for mobility calculation

$$\mu = \frac{l_{tot} * l_{eff}}{U} * \left(\frac{1}{t_m} - \frac{1}{t_0}\right)$$

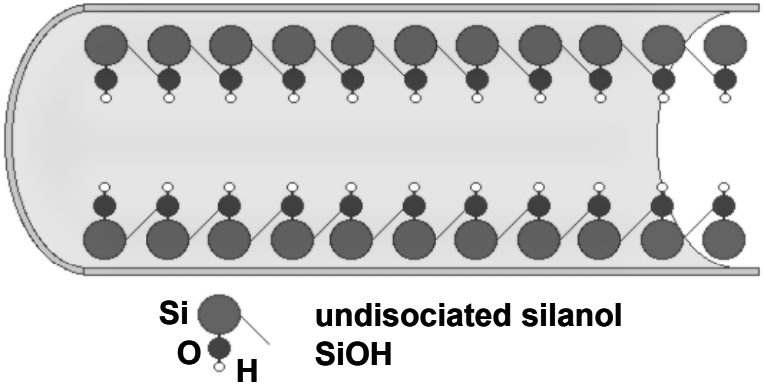
l_{tot} – separation channel total length
l_{eff} – separation channel effective length
t_m – migration time
t_0 – migration of neutral particle (EOF)

$$\mu_{tot} = \mu_{eff} + \mu_{EOF} = \frac{l_{eff}}{t_m * E} = \frac{l_{eff} * l_{tot}}{t_m * U}$$

electroosmotic flow

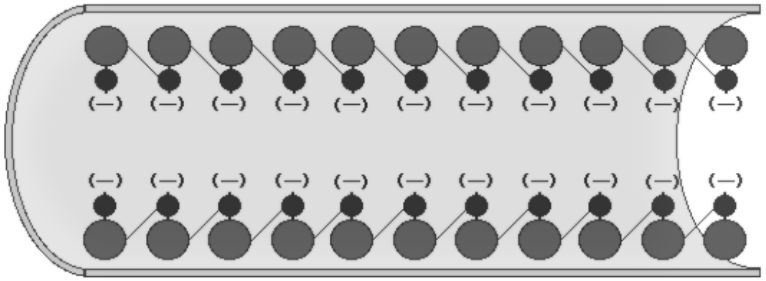
(EOF)

wall is charged **negatively** – until said others

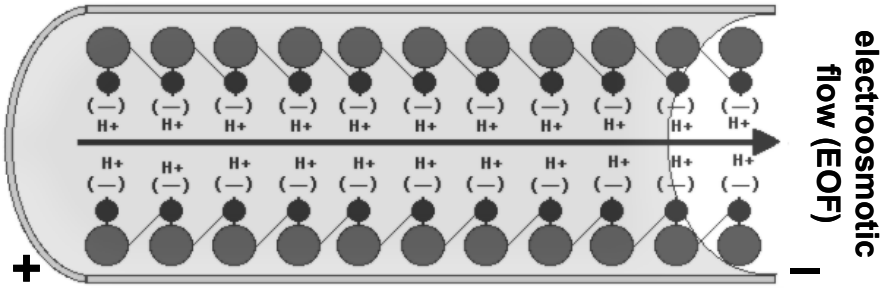


capillary = *endo-osmotic pump*

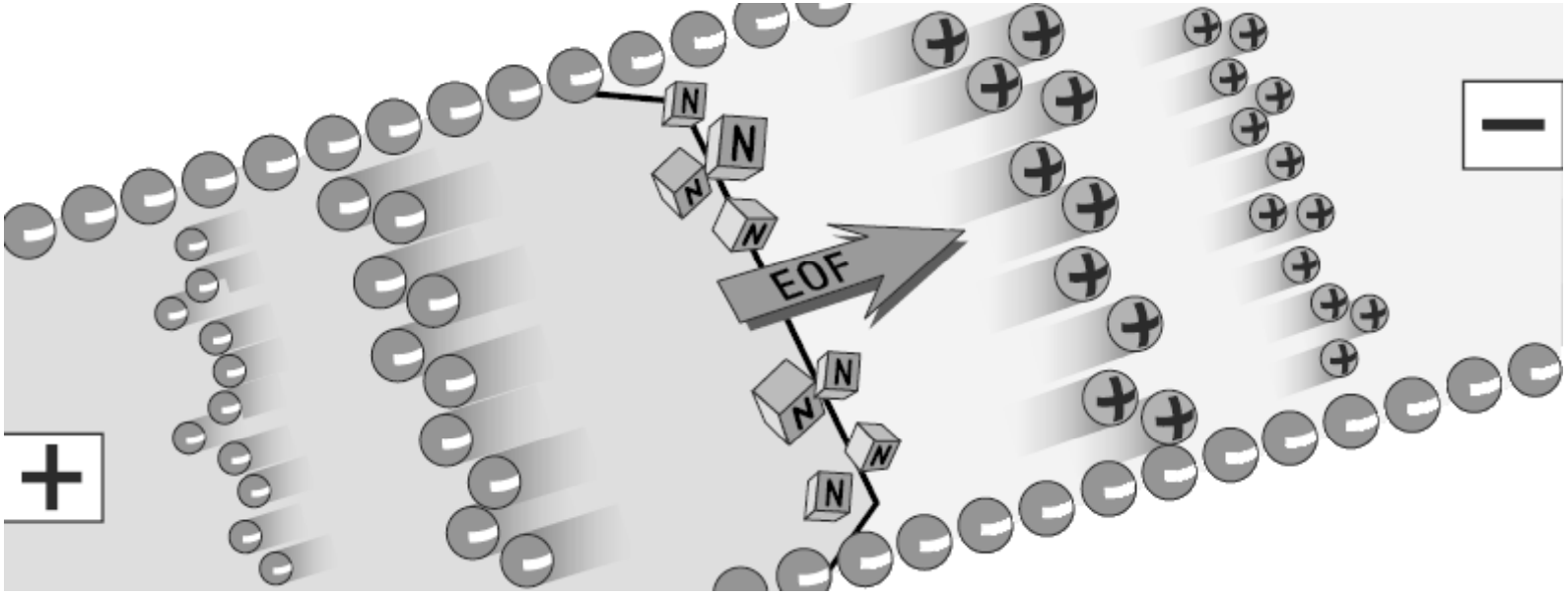
capillary made of fused silica
with exposed hydroxyl groups



dissociation of hydroxylgroups leaves
a negative charge on the inner wall



switching voltage on, liquid starts to move to cathode – it is mobilised by endoosmotic flow !



: **cations** migrate towards cathode and carry solvent molecules in the same direction – ***electroosmotic flow***

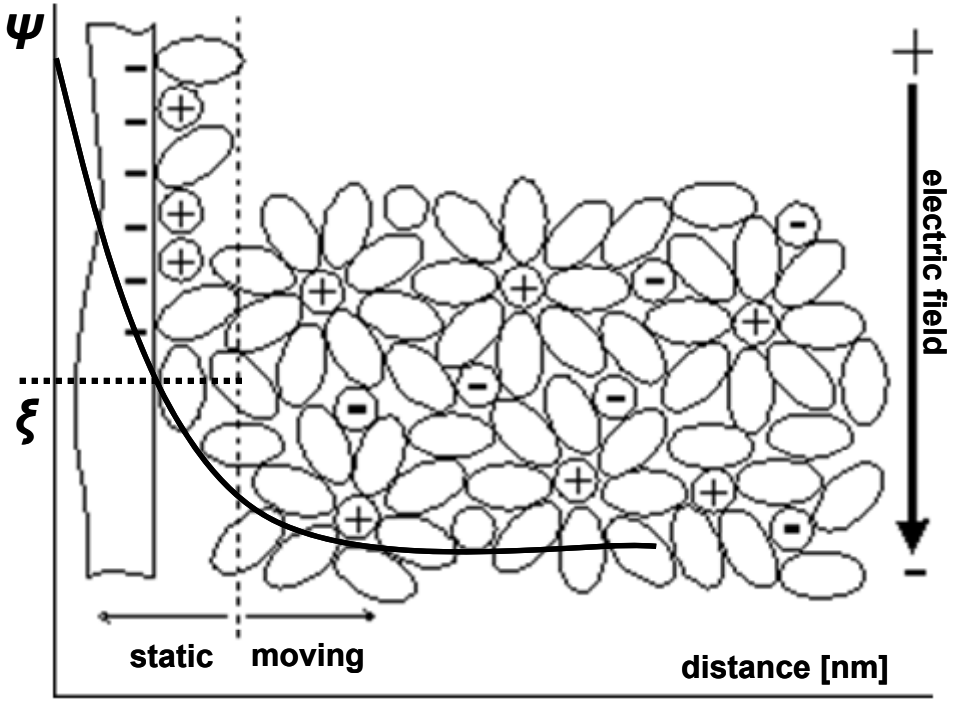
: **neutral molecules** are moving in the same direction as electroosmotic flow with negligible mutual separation

: **anions** are slowed on their way towards anode, electroosmotic flow is stronger than their electrophoretic mobility ⇒ **they proceed towards cathode too**

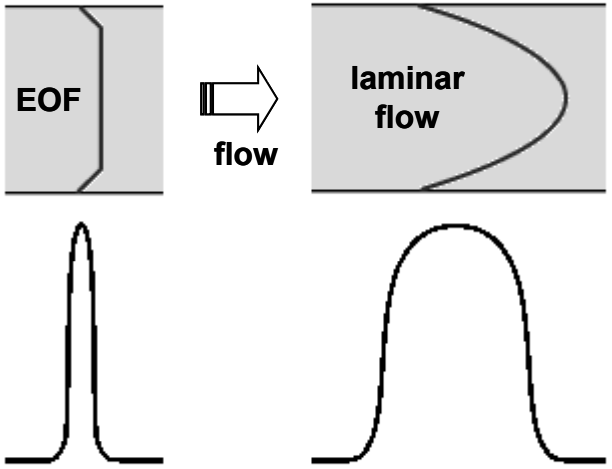
EOF = 0 ⇒ no mass flow, only ion exchange

$$v_{EOF} = \left(\frac{\varepsilon * \xi}{\eta} \right) * E \Rightarrow \mu_{EOF} = \frac{\varepsilon * \xi}{\eta}$$

ε – dielectric constant
ξ – zeta potential (electrostatic), appears as a consequence of charge on capillary wall
η – viscosity



EOF positively influences peak shape



influencing the EOF

high EOF – electrolyte carries cationic analytes out before reaching separation
low EOF – adsorption of cationic analytes

some EMM modes **demand EOF suppression** (IEF, ITF, GE)

what influences EOF?
: surface wall charge
: electrolyte viscosity
: electric field intensity

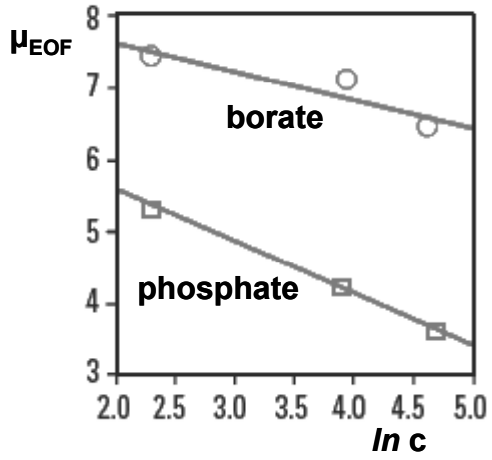
influence of voltage
: change of EOF is directly proportional

: low voltage ⇒ low efficiency of separation and resolution
: high voltage ⇒ high Joule heat

influence of ionic strength or background electrolyte concentration

: increasing value lowers ξ-potential and thus EOF

:: high values increase current and thus Joule heat
:: high values may cause analyte salting-out and adsorption to wall
:: low values supports adsorption to wall and limits sample concentration
:: changes peak shape, if electrolyte conductivity differs much from analyte



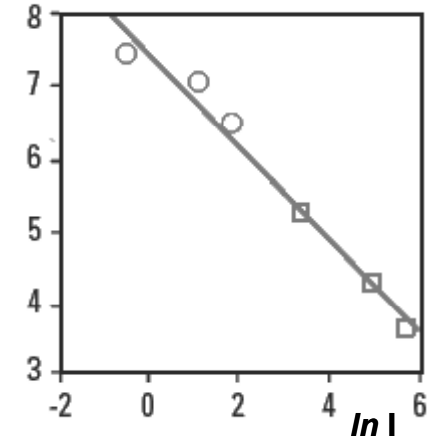
influence of organic solvent addition

: decreases ξ-potential and viscosity

:: may change selectivity, gathered only empirically

influence of tensides

: changes ξ-potential, may change wall polarity;
anionic tenside increases EOF, cationic decreases
(*if wall if negatively charged*)



influence of background electrolyte pH

: directly proportional EOF change; low pH ⇒ low EOF, high pH ⇒ high EOF

:: may change charge or structure of analyte

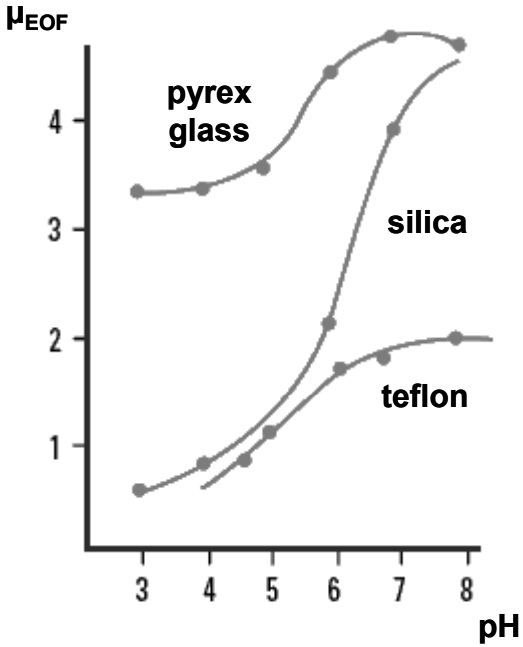
influence of temperature

: changes viscosity, higher temperature ⇒ higher EOF

:: thermolability of some samples

influence of covalent wall surface modification

: changes ξ-potential and wall charge polarity



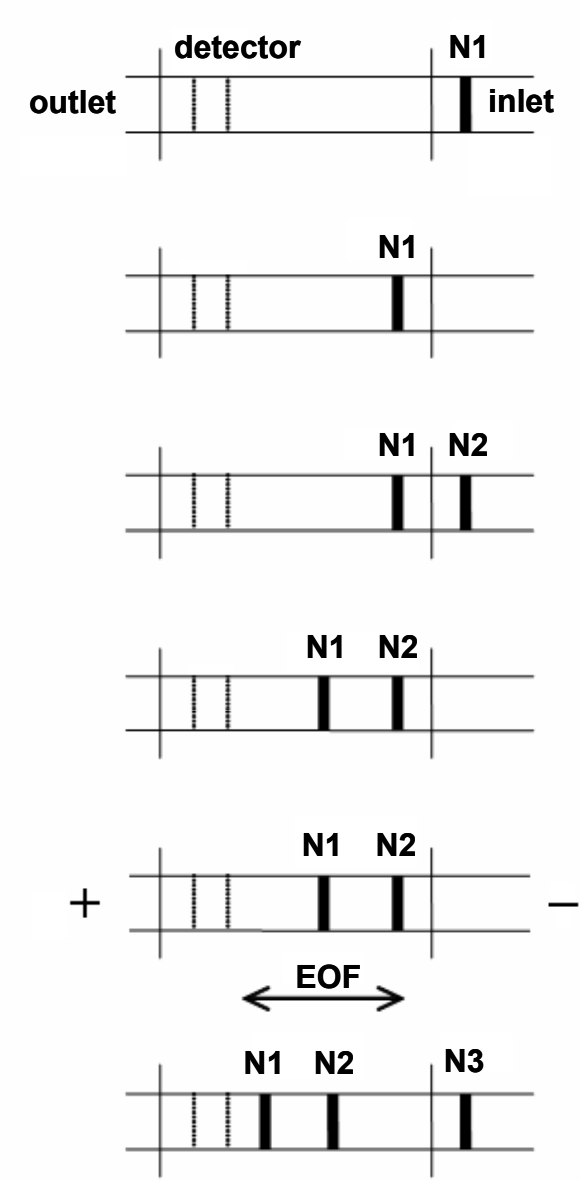
pH influence on EOF

influence of neutral hydrophilic polymers

: changes ξ-potential (decrease) and viscosity (increase), decrease EOF by charge shielding

EOF measuring

B.A. Williams, G. Vigh, *Anal. Chem.*, 68, (1996) 1174-1180



: first EOF marker injection

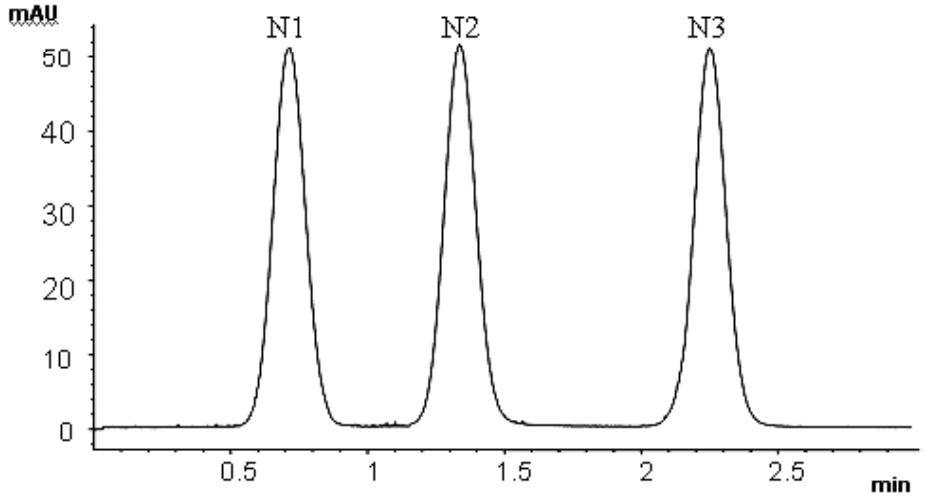
: shifting the marker zone to detector by pressure

: second EOF marker injection

: shifting both marker zones to detector

: voltage application – electrophoretic mobilisation

: third EOF marker injection and consequent application of pressure – shifting all marker zones to detector



$$l_{EOF} = (t_3 - 2*t_2 + t_1)* \frac{l_{eff}}{t_3 + t_{inj}/2}$$

$$\mu = \frac{l_{EOF} * l_{tot}}{U*(t_m - t_{ru}/2 - t_{rd}/2)}$$

l_{EOF} – length, which marker travels during electrophoresis
t_1, t_2, t_3 – migration times of zone N_1, N_2, N_3
t_{inj} – time period of marker injection by pressure
l_{eff} – effective capillary length
l_{tot} – total capillary length
U – applied voltage
t_m – time period of electrophoretic shifting
t_{ru} and t_{rd} – time periods, for which the voltage (inc-/dec-)reases linearly to given value

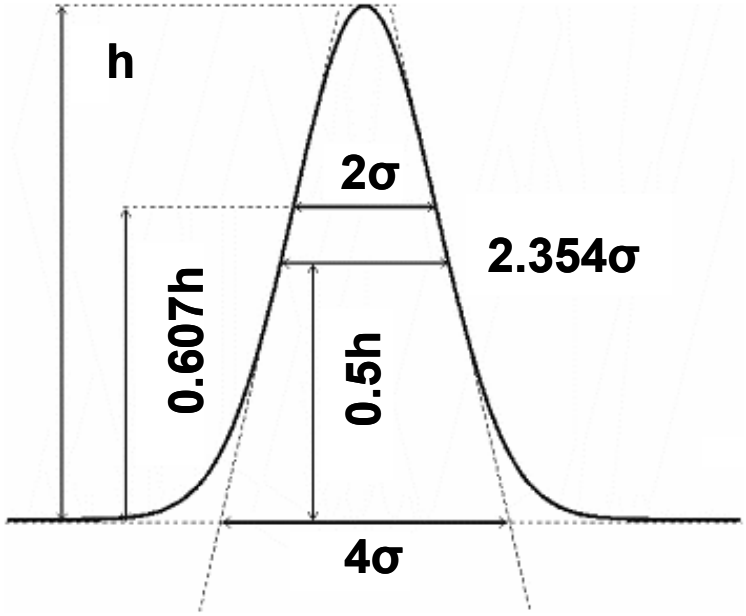
common EOF calculation

$$\mu_{tot} = \mu_{eff} + \mu_{EOF} = \frac{l_{eff}}{t_m * E} = \frac{l_{eff} * l_{tot}}{t_m * U}$$

description of separation

maximum function $I_{sign} = f(t)$

electrophoretic peak (*Gaussian peak*)



<u>width of zone</u> **A** in separation channel

a) peak width at baseline $w = 4\sigma$

b) peak width in half of peak height $w_{1/2} = 2{,}354\sigma$

c) peak width between inflex points $w_i = 2\sigma$

σ^2 – dispersion; defines zones broadening

peak width is given in **temporal** units

<u>peak area</u> $A = 1{,}064 * h * w_{1/2}$

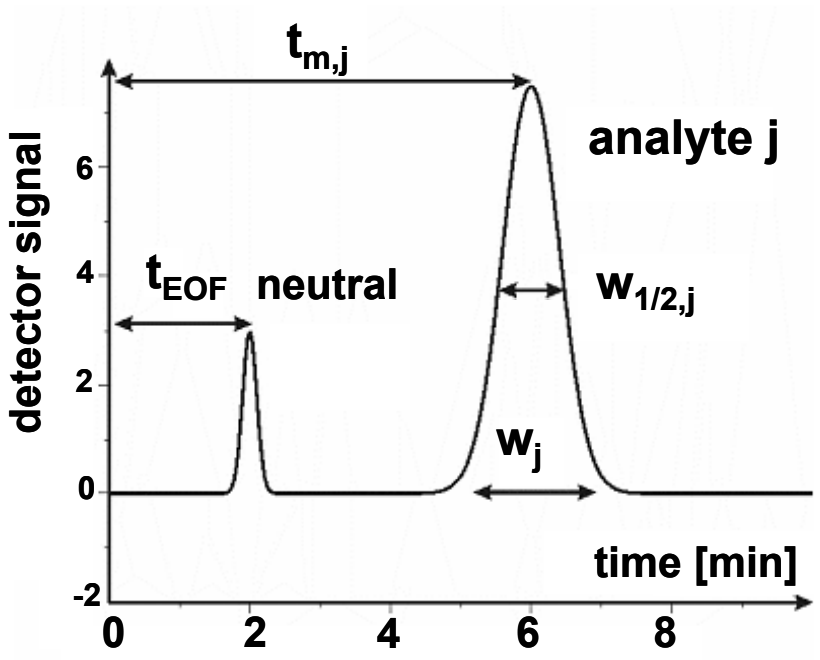
could be neglected and rectangle may be used $A = (h * w)/2$

separation efficiency

zones of **A broaden** during separation

and **become asymmetric**

(*electrodispersion*)



number of theoretical plates

$$n = \left(\frac{t_{m,j}}{\sigma}\right)^2 = 16 \cdot \left(\frac{t_{m,j}}{w_j}\right)^2 = 5{,}545 \cdot \left(\frac{t_{m,j}}{w_{1/2,j}}\right)^2$$

height equivalent of theoretical plate

(*comparison of separation channels of different length*)

$$H = \frac{\sigma^2}{L} = \frac{L}{n}$$

number of theoretical plates

$$n = \left(\frac{l_{eff}}{\sigma} \right)^2$$

under ideal conditions (short injection length, no sorption, ...)
the only influencing is ***diffusion*** (zone broadening)

$$\sigma^2 = 2D * t = \frac{2D * l_{eff} * l_{tot}}{\mu_{eff} * U} \quad \Rightarrow \quad n = \frac{\mu_{eff} * U * l_{eff}}{2D * l_{tot}} = \frac{\mu_{eff} * E * l_{eff}}{2D}$$

principal difference from **n** in LC

factors influencing efficiency

$$\sigma^2 = \sigma^2_{dif} + \sigma^2_{el.disp} + \sigma^2_{inj} + \sigma^2_{heat} + \sigma^2_{sorp} + \sigma^2_{det} + \ldots$$

diffusion influence

$$\sigma^2_{dif} = 2 * D * t$$

D – diffusion coefficient
t – time

basic factor
analytes with low D create sharp zones

detection cell length influence

should be smaller than length / width of analyte zone ⇒ better peak depicture

sorption influence

sorption causes peak tailing

$$\sigma_{ads}^2 = \frac{k' * v_{EOF} * l_{eff}}{(1+k')^2} * \left(\frac{r^2 * k'}{4D} + \frac{2}{K_d} \right)$$

$$k' = \frac{t_{m,ret} - t_{m,unret}}{t_{m,unret}}$$

k‘ – capacity factor
$\mathbf{K_d}$ – first order dissociation constant

$\mathbf{t_{m,ret}}$ – retained analyte migration time
$\mathbf{t_{m,unret}}$ – unretained analyte migration time

sorption could be prevented by capillary **inner coating**
: serves to change also other system properties (reverts EOF...)

injection length influence

: injection length must be shorter than diffusion controlled zone width
: low sensitivity demands often longer injections

$$\sigma_{inj}^2 = \frac{t_{inj}^2}{12}$$

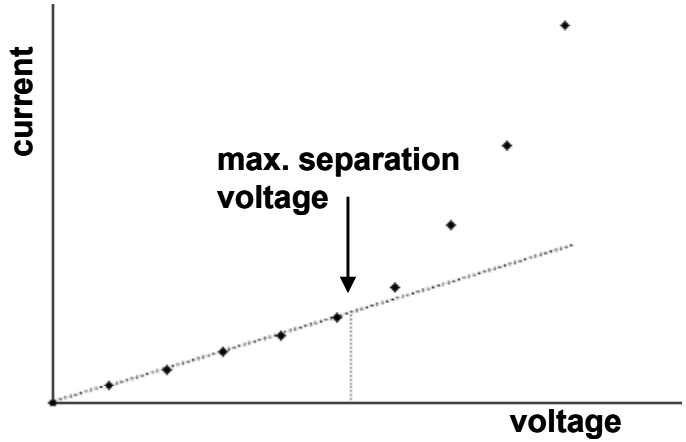
$\mathbf{t_{inj}}$ – injection pulse length

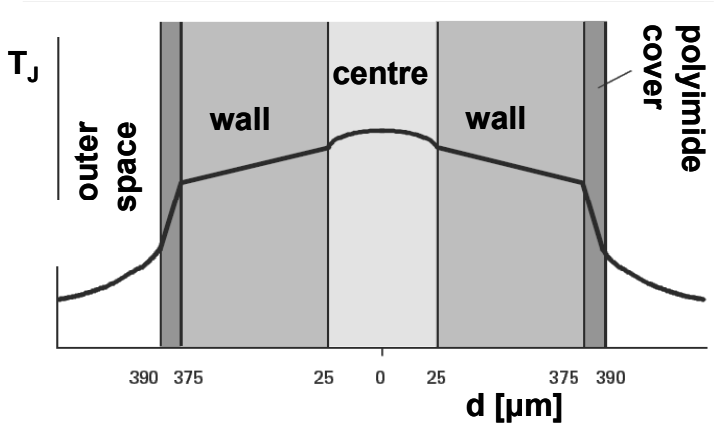
Joule heat influence leads to temperature gradient and laminar flow

$$\Delta T_J = \frac{Q * r_1^2}{2}\left[\frac{1}{\kappa_{sil}} * \ln\left(\frac{r_{o.d.sil}}{r_{i.d.sil}}\right) + \frac{1}{\kappa_{polyim}} * \ln\left(\frac{r_{o.d.polyim}}{r_{o.d.sil}}\right) + \frac{1}{r_{o.d.polyim}} * \frac{1}{h}\right]$$

Q – output
r – radius

κ – thermal conductivity
h – heat transfer rate off capillary



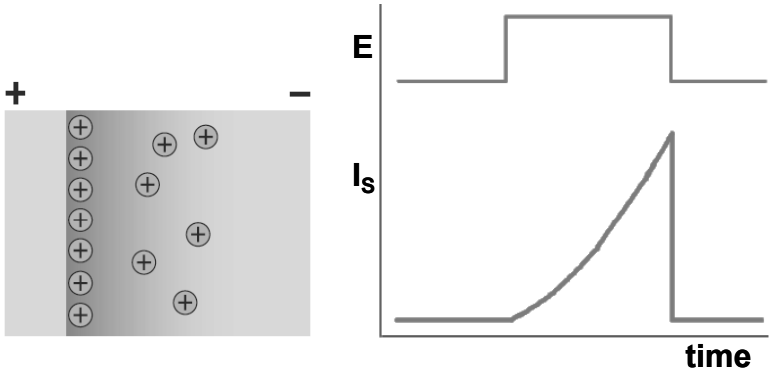


decreasing voltage : decreasing generated heat, low sensitivity and resolution
lowering capillary i. d. : current decrease with i. d. square, low sensitivity, adsorption!
decreasing BGE concentration : decreasing current, increasing adsorption
thermostating : draining heat

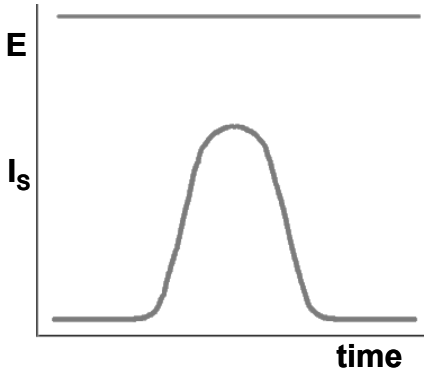
electromigration dispersion influence

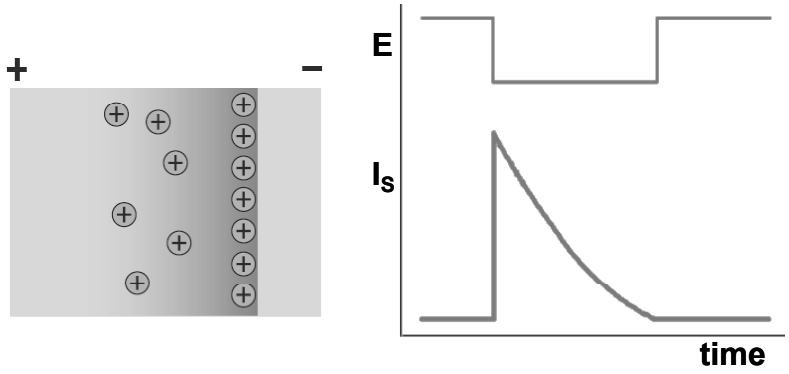
influences peak shape

difference between conductivity of sample and electrolyte leads to
1) peak tailing
2) focusation (low sample conductivity), broadening (high sample conductivity)
3) ITF effect (peak fronting) because of certain ion surplus (e.g. Cl^-)

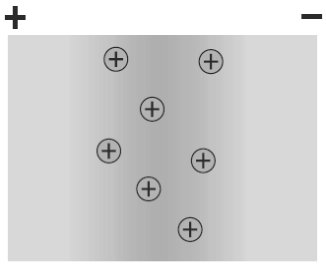


$\mu_S > \mu_{BGE} \Rightarrow$ front gets broad and tail focuses





$\mu_S < \mu_{BGE} \Rightarrow$ front focuses and tail gets broad



$\mu_S = \mu_{BGE} \Rightarrow$ sharp zone

resolution

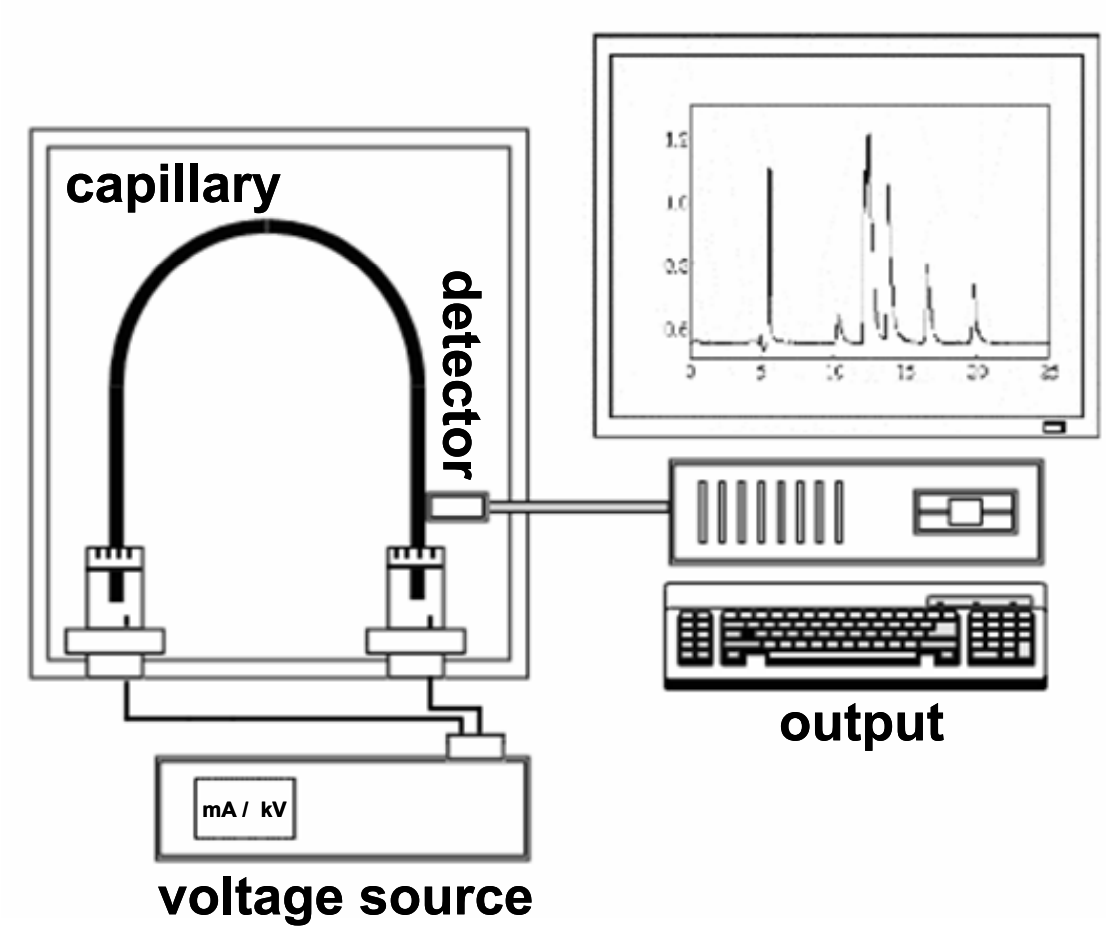
$$R_{i,j} = \frac{2 \cdot (t_{m,i} - t_{m,j})}{w_i + w_j} = \frac{2 \cdot \Delta t_m}{w_i + w_j}$$

$$R_{i,j} = \frac{\sqrt{n}}{4} * \frac{\Delta\mu}{\overline{\mu}}$$

Δμ – difference, **(μ_2 - μ_1)**
$\overline{\mu}$ – median, **(μ_2 + μ_1) / 2**

$$R_{i,j} = \frac{1}{\sqrt{32}} * \Delta\mu * \sqrt{\frac{U}{D * (\overline{\mu} + \mu_{EOF})}}$$

EMM arrangement

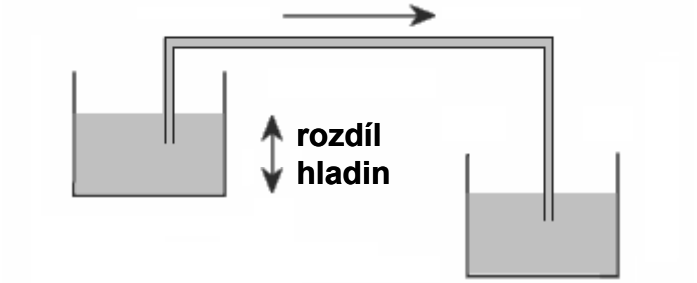


instrumentation

injection device

hydrostatic

siphon effect



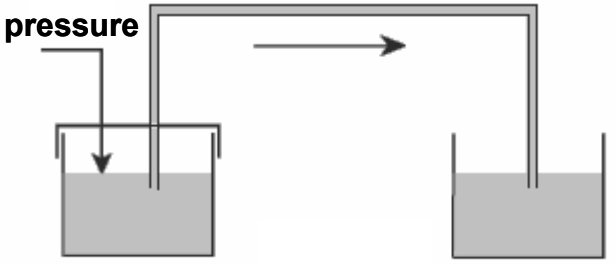
typical volumes: 10 – 100 nl (capillary ~ 1 – 2 µl)

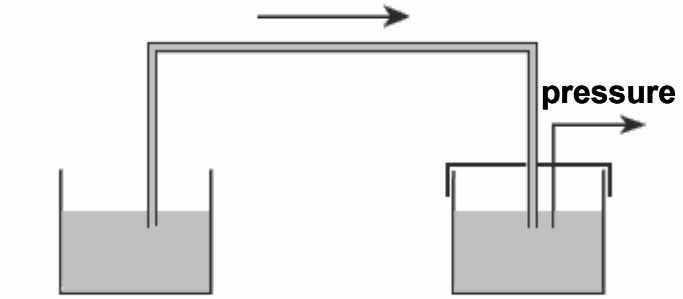
normal – longer part before detector
reverse (*short-end*) – the other end

hydrodynamic

$$V_{inj} = \frac{\Delta P * d^4 * \pi * t_{inj}}{128 * \eta * l_{tot}}$$

injected volume $\mathbf{V_{inj}}$

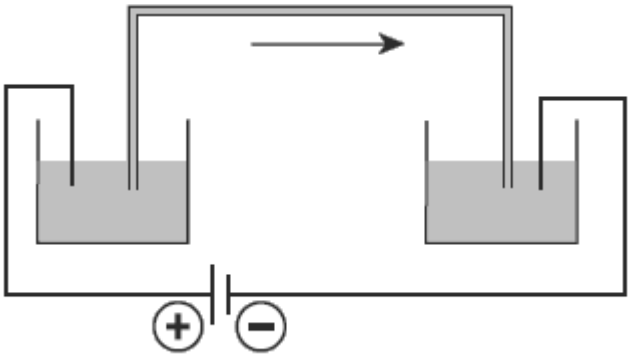




ΔP – pressure difference
d – capillary i. d.
$\mathbf{t_{inj}}$ – time length of injection
$\mathbf{l_{tot}}$ – total capillary length
η – background electrolyte viscosity

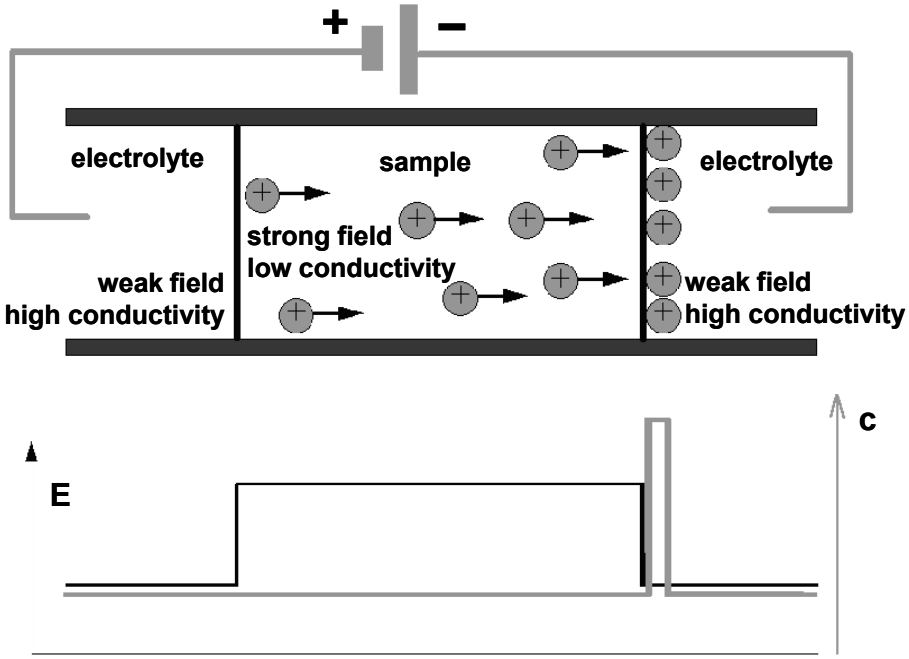
electrokinetic

for CGE the only possible
: non-quantitative – more mobile ions go easier



stacking effect

sample conductivity < electrolyte conductivity
⇒ sample ions carry the current
⇒ stacking/concentration on inter-phase sample-electrolyte



$$V_{inj} = \pi * r^2 * l_{eff} * \frac{t_{inj} * U_{inj}}{t_{EOF} * U_{sep}}$$

injected volume $\mathbf{V_{inj}}$

$\mathbf{U_{inj}}$ – injection voltage
$\mathbf{U_{sep}}$ – separation voltage
r – capillary i. d.
$\mathbf{l_{eff}}$ – capillary effective length
$\mathbf{t_{inj}}$ – injection time length
$\mathbf{t_{EOF}}$ – EOF marker migration time

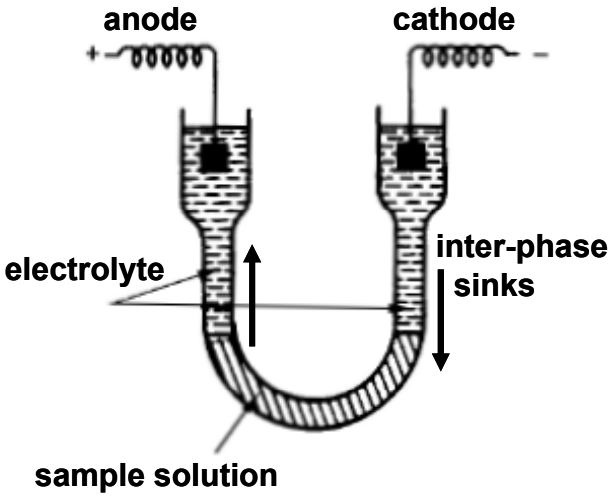
voltage source

typical range: 0 – 30 kV; recommended gradient 400 V/cm
0 – 300 mA

too high voltage decreases analysis time, lead to discharges (*ca* 20 – 25 kV)

ZE – constant voltage, ITF – constant current
one electrode always grounded – that one closer to detector

separation channel



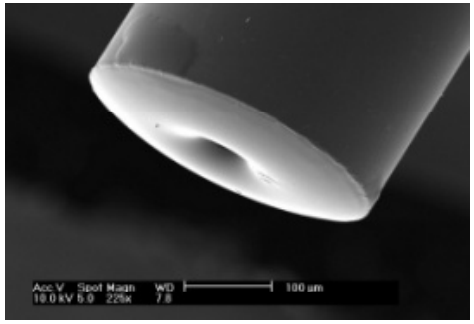
tube

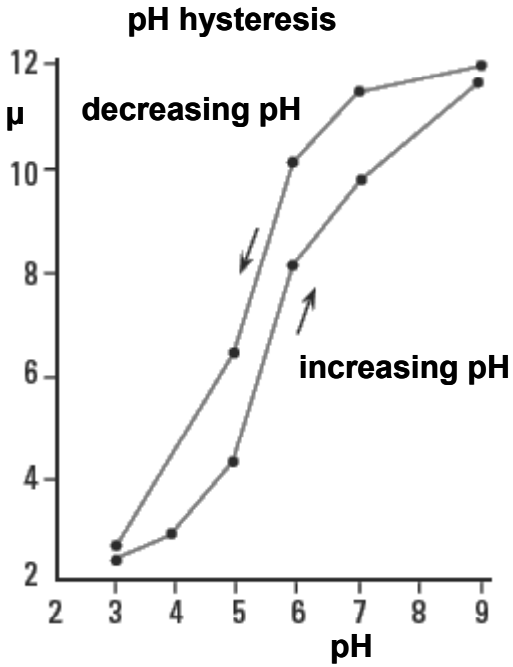
the oldest (proposed 1892, done 1930)

glass U-tube

electrophoresis in free solution
: separation detection by moving inter-phase observation
: coloured solution and clean electrolyte solution

capillary





fused silica

i. d. 10 – 200 μm
o. d. 350 – 400 μm
length 10 (CGE) – 100 cm; 50 – 75 cm most common

outer coating – polyimide (mechanical properties)

conditioning:
establishing the properties of capillary inner surface

surface cleaning: 1 M NaOH, 0.1 M HCl, BGE
other: strong acids, organics (DMSO), detergents

teflon

reproducible EOF
worse heat conductivity

other materials based on SiO_2 – glass (Pyrex)

covalent coating

suppressing EOF, in range pH 4 – 5 relatively low (~ 0), pH 6 – 7 slowly increases
at high pH is almost about 4/5 lower than in un-coated silica capillary

Si-O-Si-R

polyacrylamide-, arylpentafluoro-, 3-glycidoxypropyltrimethoxy-siloxan
protein or amino acid, sulphonic acids, maltose, PEG, polyvinylpyrrolidon

: relatively easy preparation
: limited long-term stability

Si-C

polyacrylamide using Grignard reaction

: stabile between pH 2 – 10
: difficult to prepare

SF from GC and LC

C2-18, PEG, phenylmethylsilicon

: easy to hydrolyse
: increased adsorption

adsorbates

cellulose, polyethylene glycol, polyvinyl alcohol, polyethylene imine

: only short-term stability in acidic range pH 2 – 4 (PEG, PVA)
: stabile in neutral pH (PEI)
: relatively hydrophobic
: reverts EOF (PEI)

dynamic coating

part of BGE, stems in the praxis of adsorbates use

pH extremes

reduction of coulombic interactions

: pH range 2 – 12
: EOF elimination at low pH, EOF high at high pH
: unsuitable for proteins – denaturation
: decreasing the charge differences decreases separation efficiency

high BGE concentration (ionic strength)

reduction of coulombic interactions

: decrease of EOF often limited by Joule heat

hydrophilic polymers

alkylcellulose, polyvinyl alcohol, dextrans, polyacrylamide

shield wall charge of capillary and decreases EOF

: increases viscosity
: in high concentration = entangled gel electrophoresis (CEGE)

tensides

anionic: sodium dodecylsulphate (SDS),
cationic: cetyltrimethylammonium bromide (CTAB)
non-ionic: Brij-35, BRIS
zwitterionic: 3-[(-cholamidopropyl)dimethylammonio]-1-propansulphate (CHAPS)

deactivate capillary surface by hydrophobic or ionic interactions

: wide possibility of compounds, easy use
: decrease or revert EOF
: may irreversibly denaturise protein
: suitable in combination with RP-LC surfaces

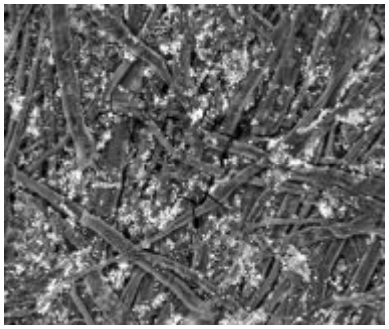
quaternary amines

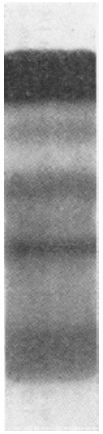
decrease or revert EOF

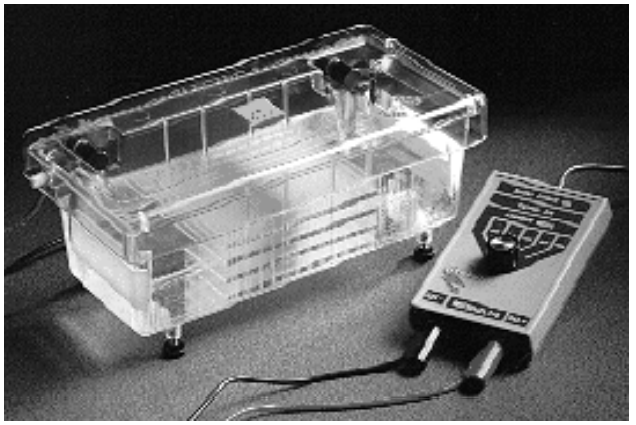
: work also as ion pairing agents (MEKC)

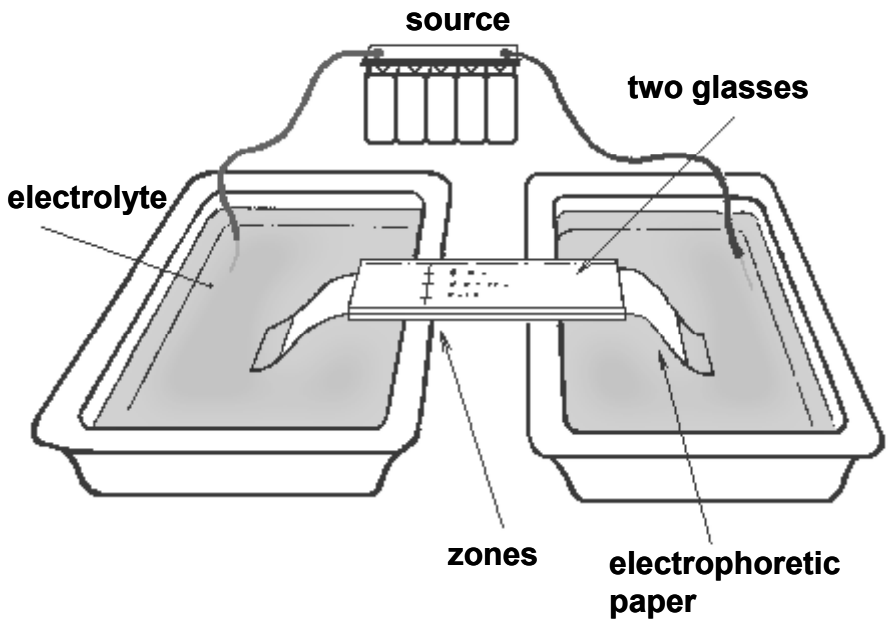
paper / membrane

100 % cotton / **cellulose**
0.17 – 0.30 mm thick
pore size 2.5 µm

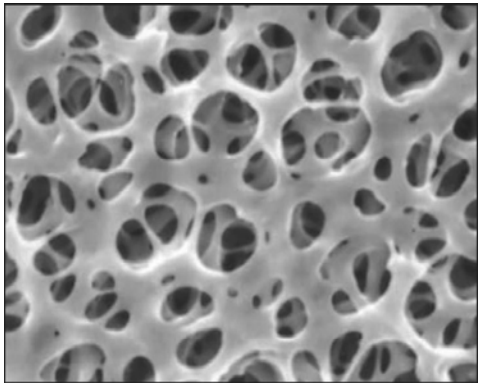


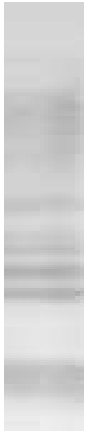




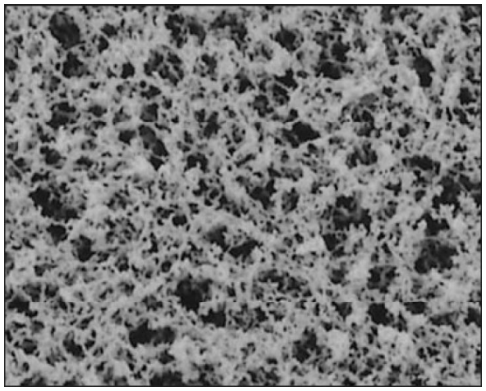


acetate cellulose
pore size 0.2 µm

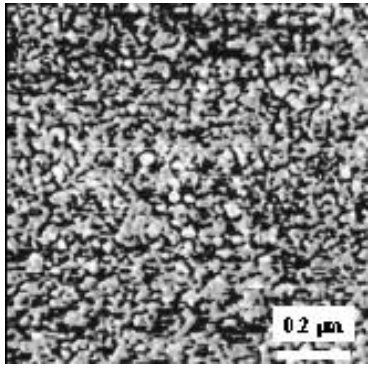




nitrocellulose
pore size 0.2 µm

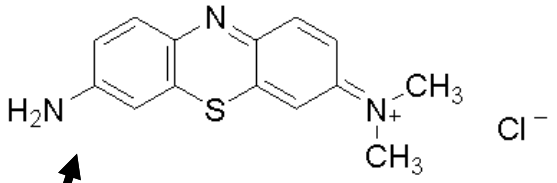


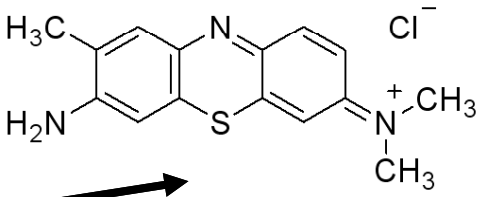
nafion (PTFE, sulphonated tetrafluoroethylene)
1 – 2 nm and 5 – 6 nm

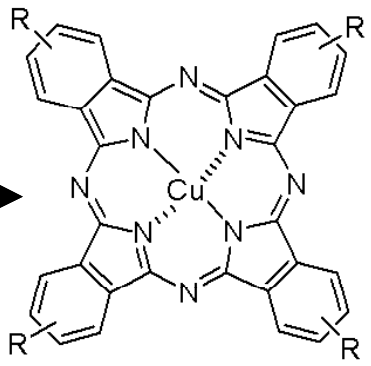


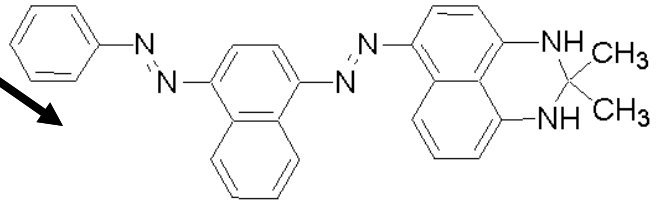
visualisation

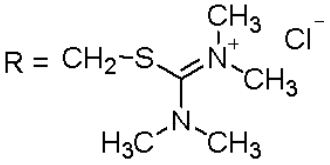
bromophenol blue
dimethylthionine (azure A)
toluidine blue
alcian blue
sudan black
naphthalene black









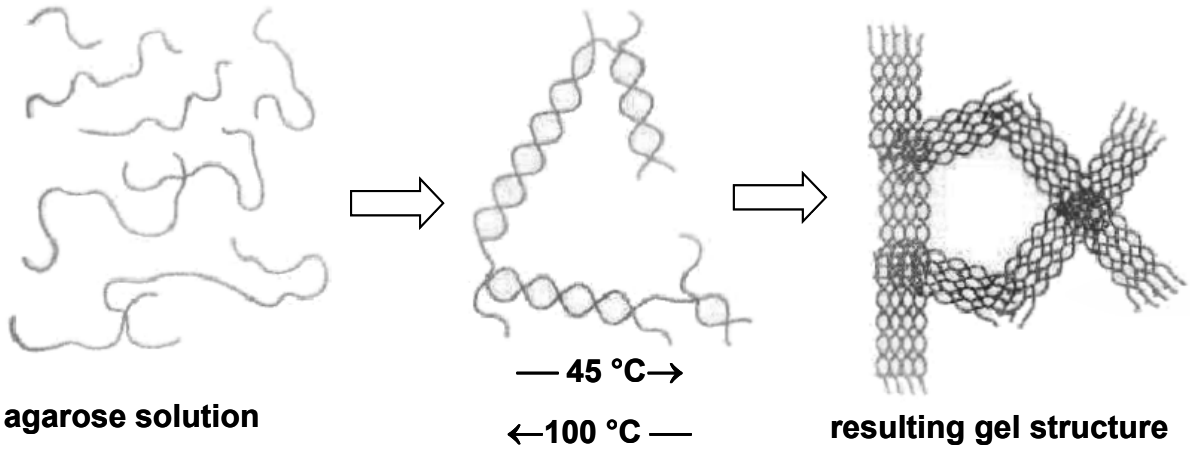


agarose gel

gel

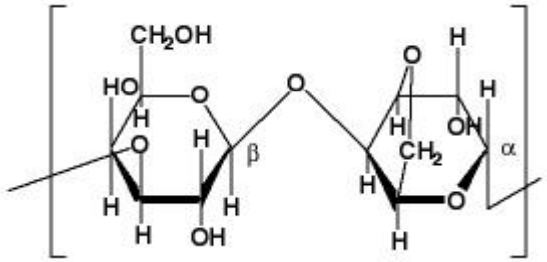
: non-toxic, cheap, no additional components for polymerisation
: fragile

0.8% large molecules
1 – 2% common separation
4% small molecules
% w/v

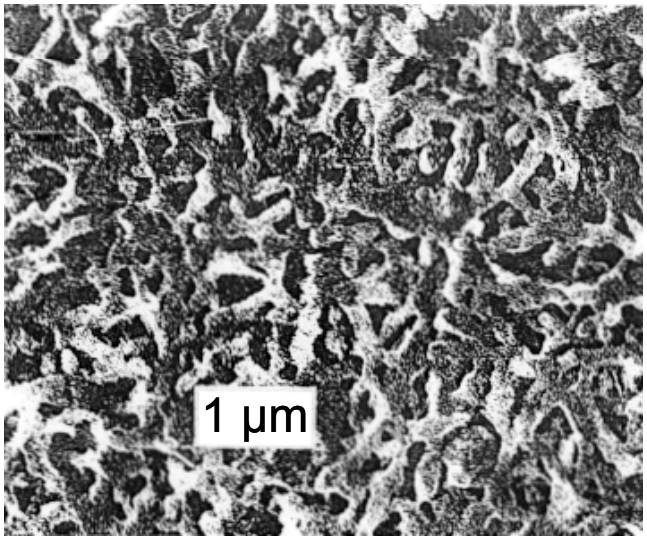


agarose solution **resulting gel structure**

D-galactose



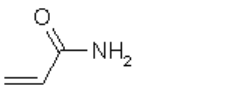
3,6-anhydro-L-galactose



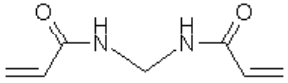
polyacrylamide gel

: toxic (bis-acrylamide), inert

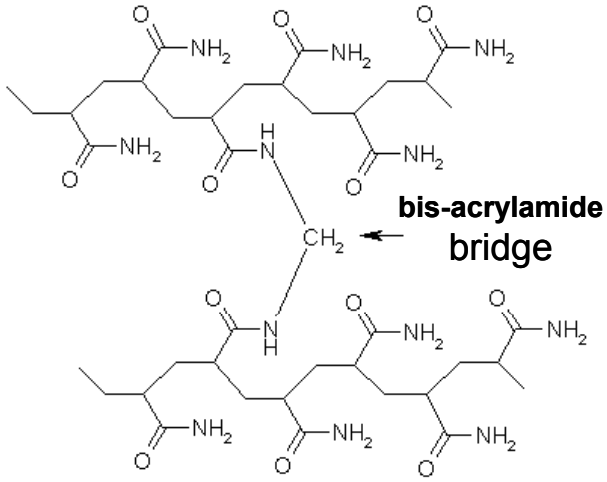
: fragile, reinforcement by RhinoHide™ or DurAcryl™



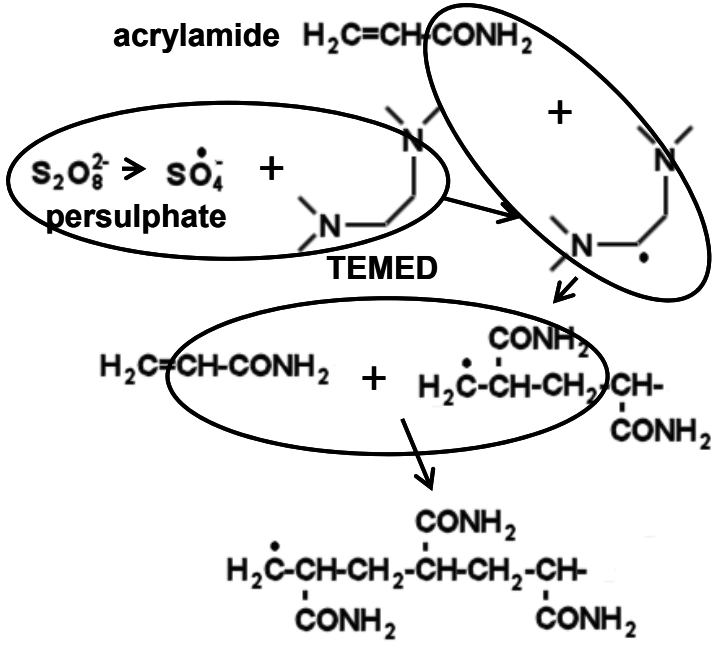
acrylamide



methylene-
bis-acrylamide

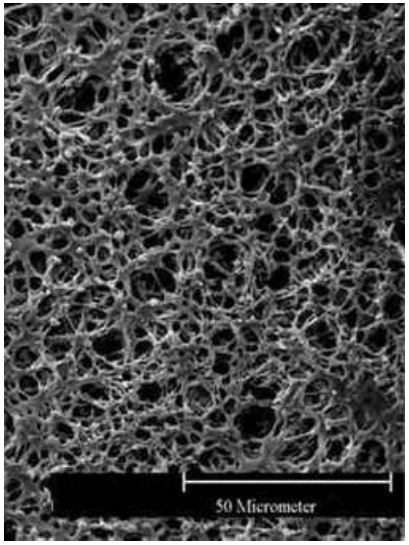


bis-acrylamide bridge



persulphate /ammonium/ – initiator

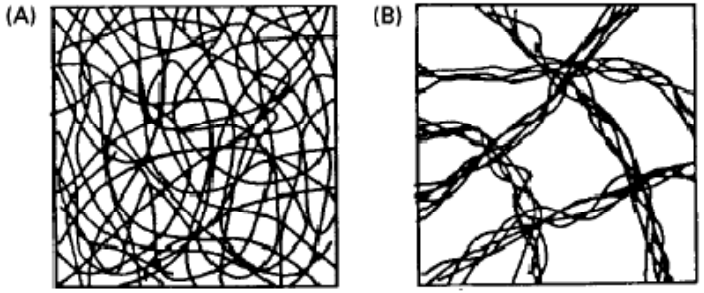
tetramethylene ethylenediamine (TEMED)
– catalyser



gel density
(cross-linking percentage;
acrylamide and *bis-acrylamide* ratio)

↓ % cross-linking
⇒ easier motion of very large molecules

12% – common for 15 kDa – 60 kDa
8% – molecules 30 kDa – 120 kDa
25% – < 15 kDa;
special protocol according to Schägger-von Jagow



12%-gel

viscosity	~100 $m^2 s^{-1}$
cavity diameter (12%)	~ 4.4 nm

: isocratic (continuous) (8 – 15 %)
: discontinuous gel (4% concentration and 12 % separation)
: gradient gel (Schäger-von Jagow)

visualisation

ethidium bromide (EtBr)

Kongo red

Coomassie blue R-250, G-250

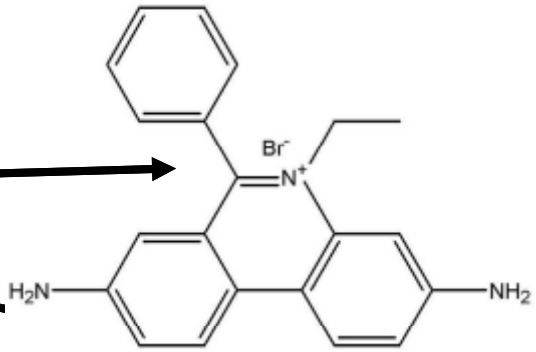
SYPRO ruby

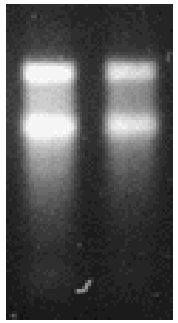
SYBR II green

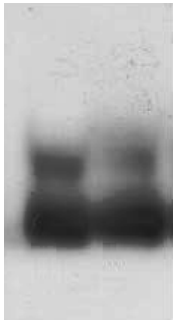
silver

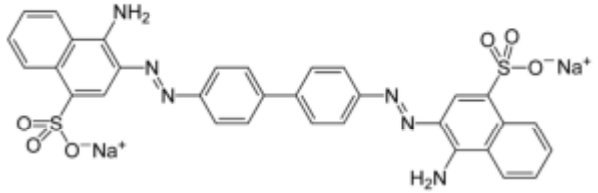
zinc

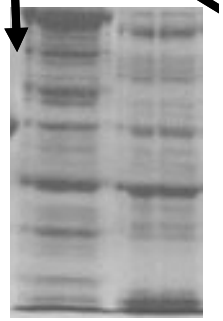
copper



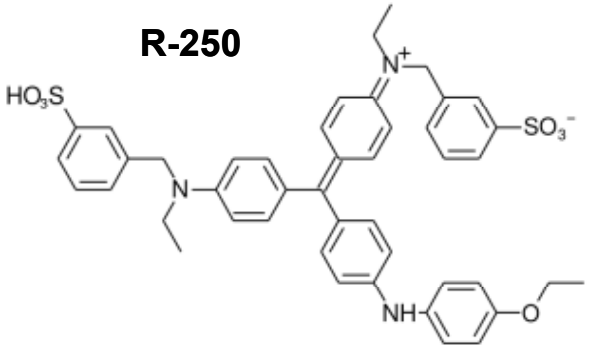


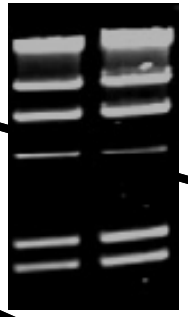


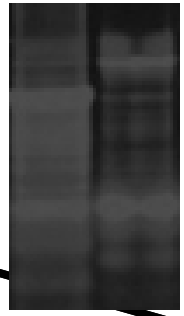


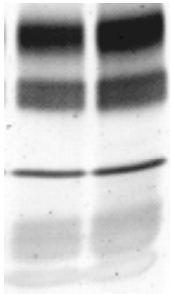


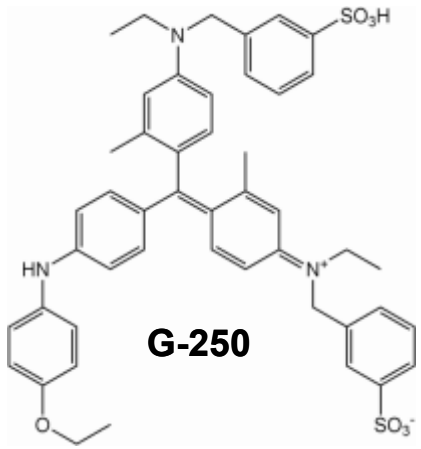
R-250



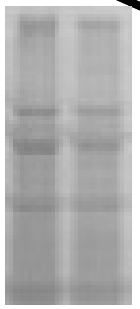




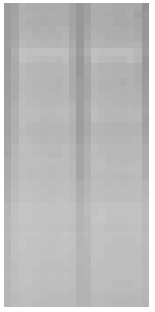




G-250



0.3 M $CuCl_2$



0.2 M $ZnSO_4$
0.2 M imidazole

0.02% $Na_2S_2O_3$

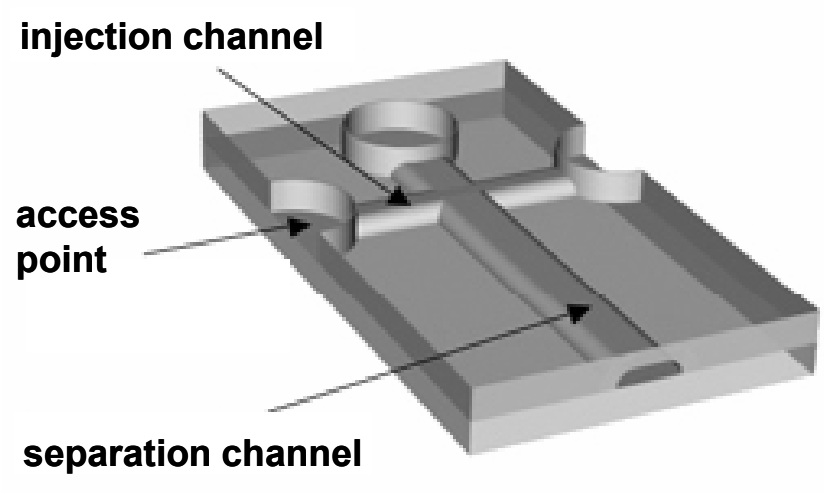
0.1% $AgNO_3$

37% HCOH

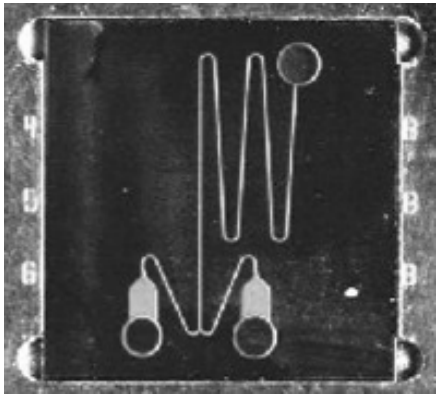
1% CH_3COOH

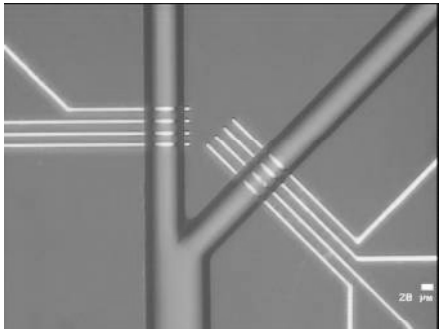
chip (CE-on-chip)

simpler arrangement than LC-on-chip
: easy application of driving force
: simple separation channel
: suitable detection

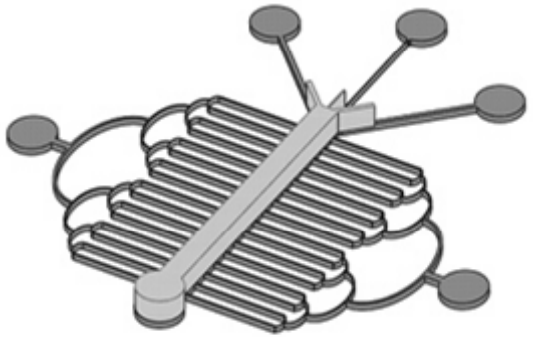


ZE, ITF, IEF...





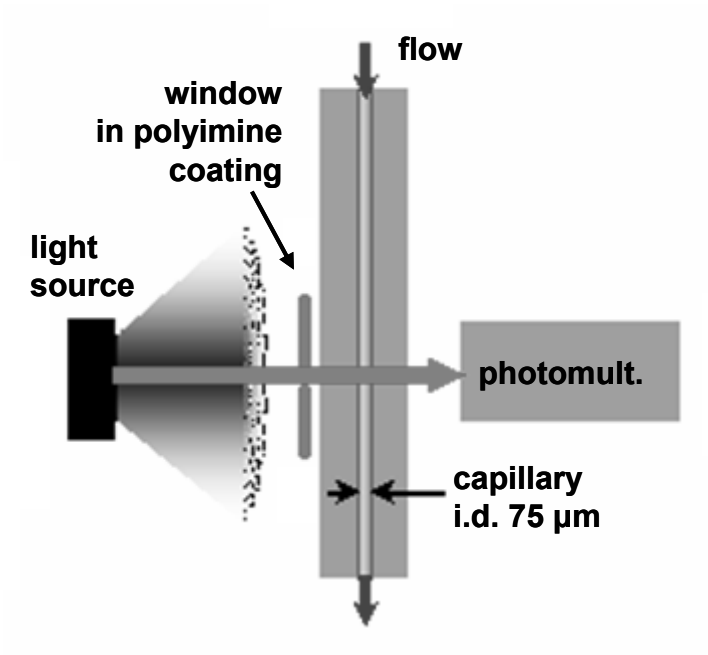
electrochemical detection



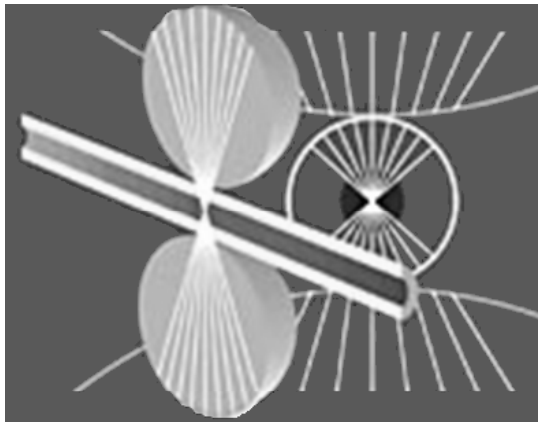
lab-on-chip
LC + CE

absorption photometric detector

diode array detector



problems : **beam focusation**
: **optical path length**



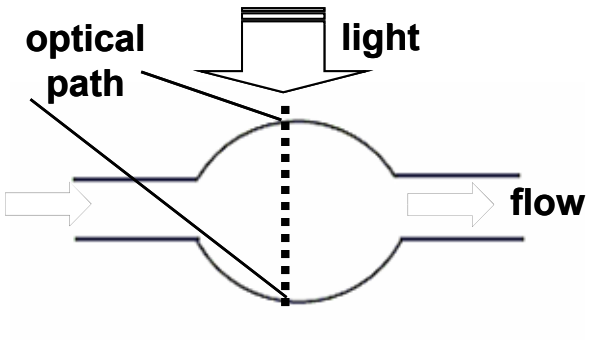
focusing optics – two spherical lenses

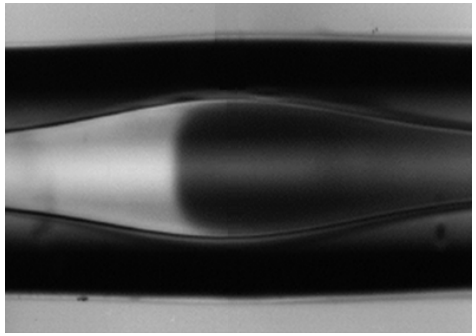
absorbance
: **sensitivity** 10^{-7} g/ml

indirect detection
: **sensitivity** 10^{-5} g/ml

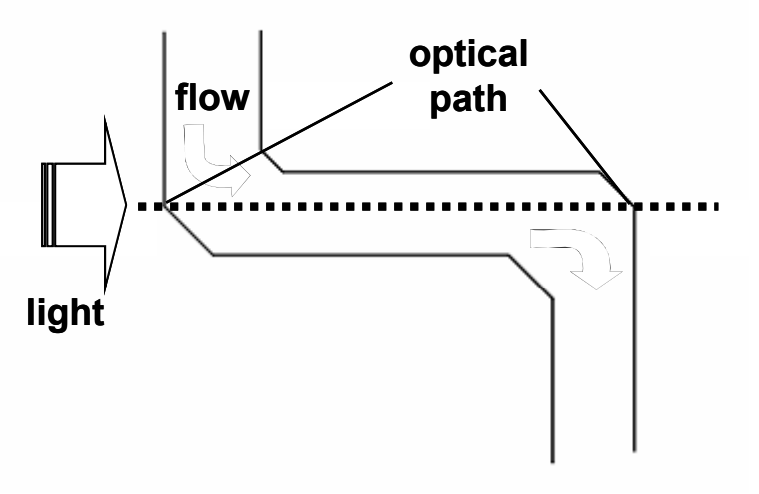
prolongation of optical path

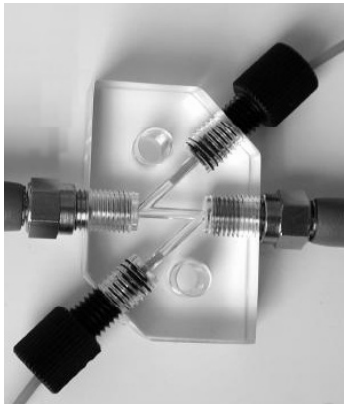
bubble cell



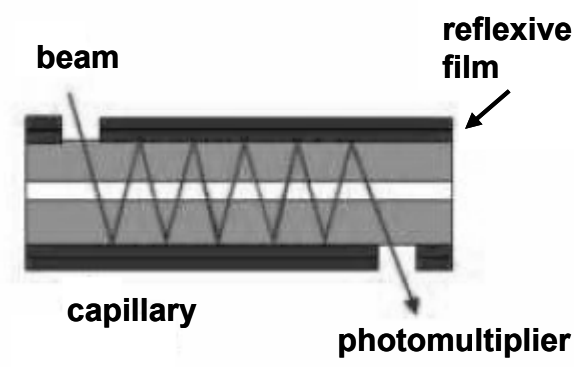


Z-cell

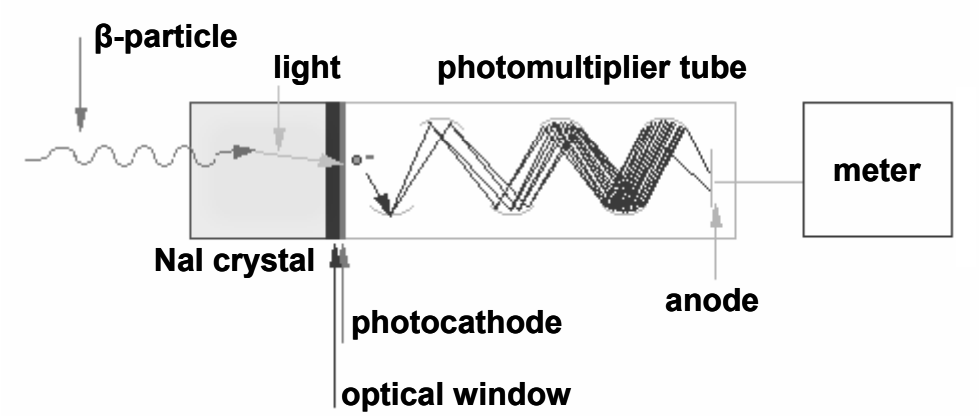




reflexive inner coating



radioactive (scintillation) detector



scintillation
: sensitivity 10^{-11} g/ml

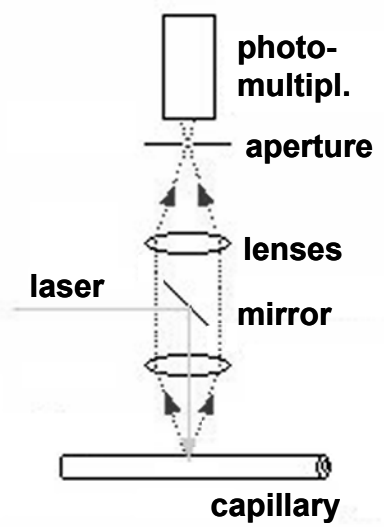
fluorescence detector

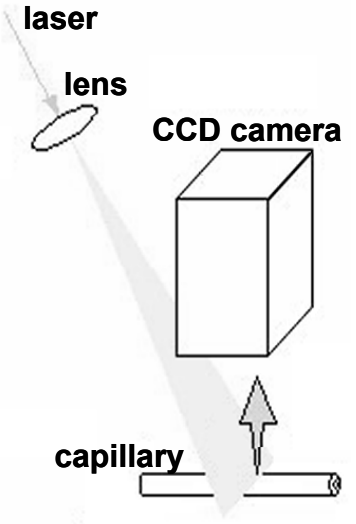
laser induced fluorescence

(LIF)

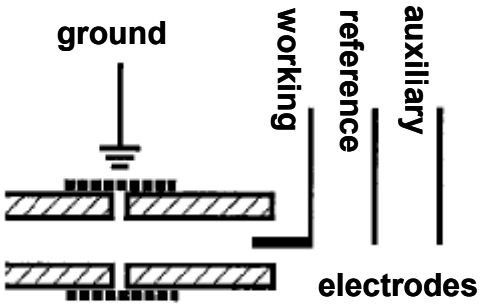
fluorescence
: sensitivity 10^{-9} g/ml

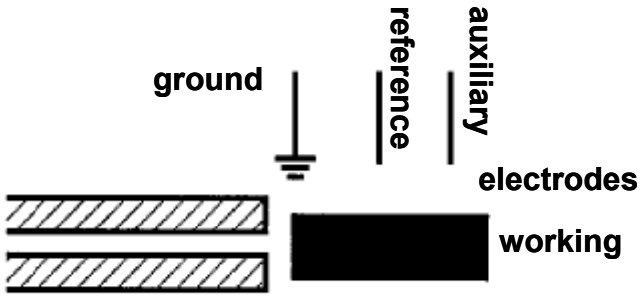
LIF
: sensitivity 10^{-11} g/ml





amperometric detector

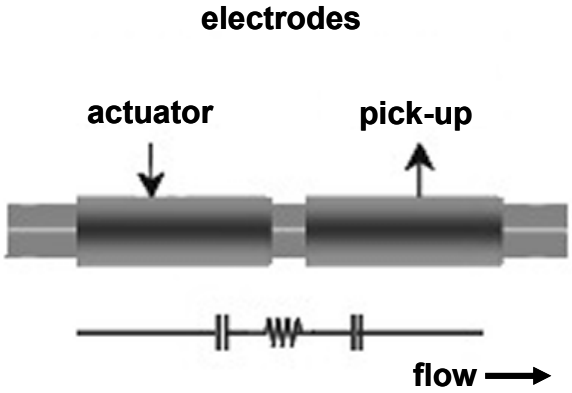




amperometry
: sensitivity 10^{-8} g/ml

conductivity detector

conductivity
: sensitivity 10^{-6} g/ml



: two metallic electrodes around capillary

: when applying AC voltage on an actuator, the current flows through wall, in-between electrodes towards the pick-up electrode

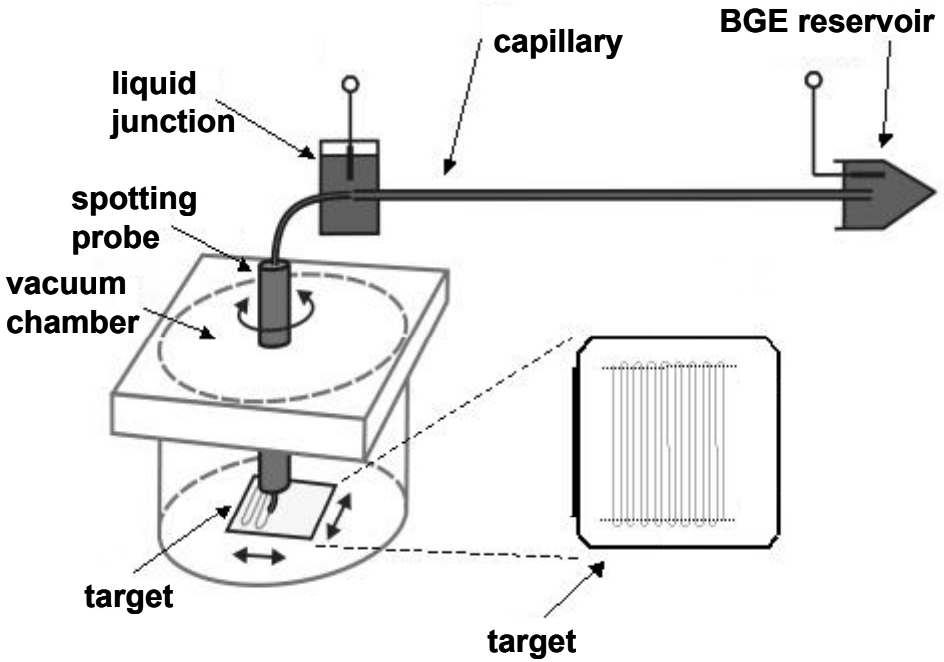
: signal is then amplified

matrix assisted laser desorption / ionisation

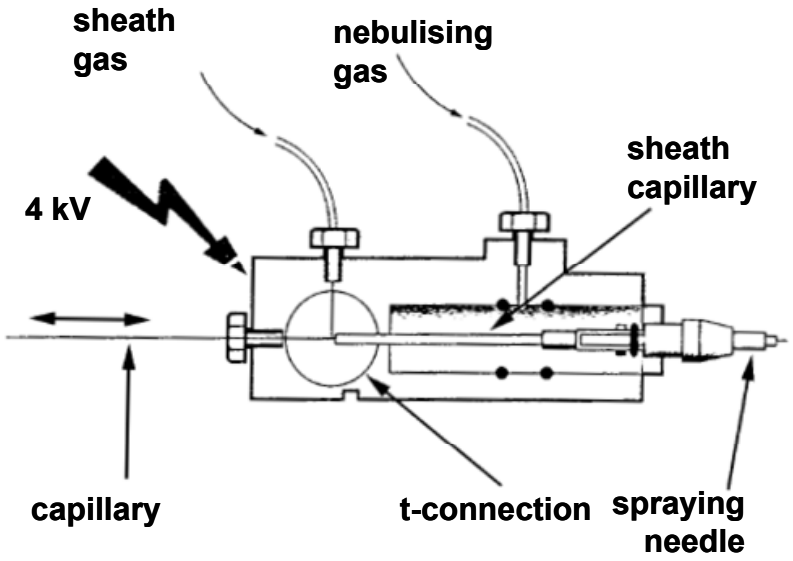
MALDI

discrete points (fractions)
mixing with matrix
: before outlet
: after outlet

continuous trace
mixing with matrix
: in liquid junction
: pre-spotted matrix trace



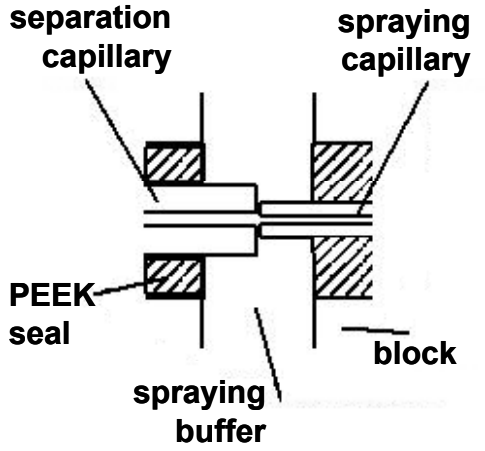
ion count
: sensitivity 10^{-8} g/ml



electrospray ionisation

ESI

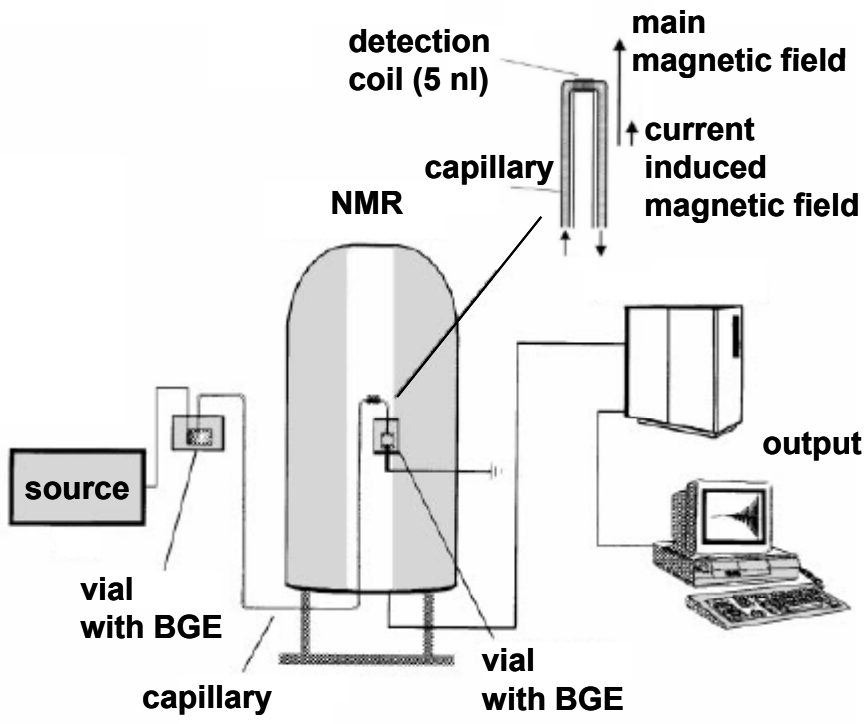
key point
liquid junction



nuclear magnetic resonance

may use **bubble cell**

^{1}**H** and ^{13}**C** – NMR



NMR
: sensitivity 10^{-6} g/ml

preparation small volumes (nl) ⇒ elution into **collection vials** (10 – 15 µl)

peak detection ⇒ volume **calculation** / distance from capillary end

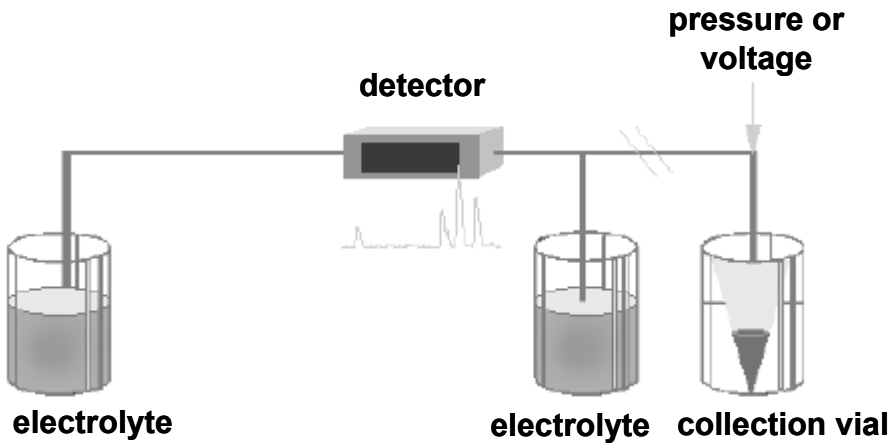
pressure elution: (**CZE**, **ITP;** MEKC, IEF**;** CGE – ***no***)
: pressure application (5 kPa) during pre-calculated time period

electrokinetic elution: (CZE, ITP, CGE, MEKC**;** IEF – ***no***)
: voltage application during pre-calculated time period
: collection vial must contain BGE or other electrolyte

elution in IEF mode:
: it is necessary to consider that µ = 0

collection electrolytes:

CZE	2% acetic acid
ITP	2% acetic acid
CGE	BGE
MEKC	BGE
IEF	ampholyte



definition of electrophoretic system

BGE

composition: buffer concentration, pH, additives

injection: type, its characteristics (time, pressure, voltage)

mode

separation channel type

capillary
length, i. d., material, manufacturer
30 cm x 50 μm i. d., fused silica, J&W Scientific

conditioning – coating, rinsing

applied voltage,
current or output

application time period

slab
size (height x length x thickness), material
6.5 x 10 cm x 1 mm, polyacrylamide

continuous, discontinuous, gradient; leading colour

detector

basic characteristic according to type

analytical information from electrophoretogram

electropherogram, *electrophoregram*, *electrophoreogram*

migration time normalisation:
wrong reproducibility; *adsorption* or *EOF changes*

: on one marker (either *EOF* or *very fast*)

: on two markers inclosing separated components
first: carries no charge, moves with EOF
second: highest mobility

peak area normalisation:
peak area is function of migration velocity (migration time)

$$A_N = A^*\left(l_{eff} / t_m\right) \Rightarrow A / t_m$$

only within *EOF changes*;
within *ionic strength* or *injection length changes* – no correction effect

$$A_{N2} = A_N / A_{N,IS}$$

correction of *injection length* change
within pressure injection
IS – internal standard; might be a peak in mixture

basic modes of electromigration methods

electrophoresis (ZE)

isoelectric focusation (IEF)

isotachophoresis (ITF)

electrochromatography (EC)

micellar electrokinetic chromatography (MEKC)

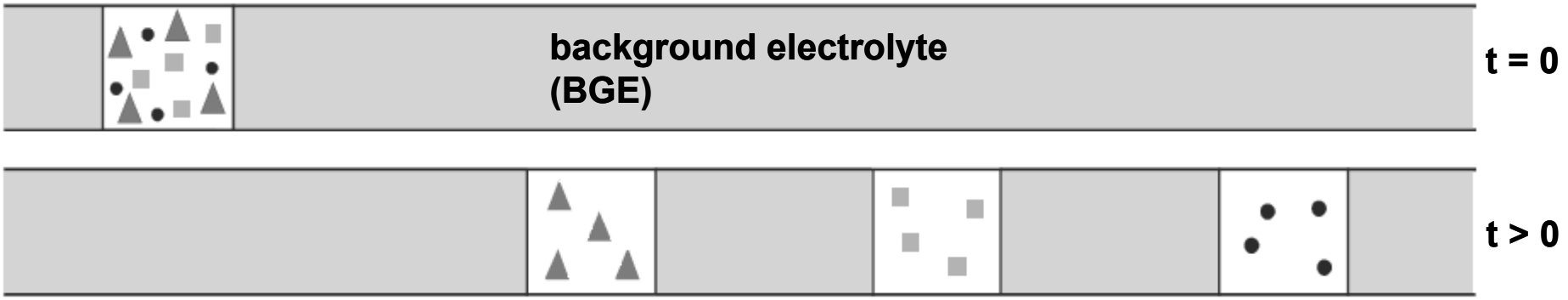
affinity electrophoresis (ACE)

non-aqueous electrophoresis (NCE)

CZE, capillary zone electrophoresis

electrophoresis – greek **ήλεκτρον** (amber) and **φορέω** (I carry)

one **background electrolyte** (BGE)
⇒ constant electric field intensity in whole separation channel



$$\alpha = \frac{\overline{\mu}_A - \overline{\mu}_B}{\overline{\mu}_B}$$

selectivity of separation, analytes **A** and **B**

choice of background electrolyte

: sufficient buffering capacity in chosen pH range
: low background signal in detector
: low mobility (large, low charged molecules) ⇒ low Joule heat

additives

tensides

all types

changes EOF; give charge to non-polar molecules
changes CZE into **MEKC** (if the critical micellar concentration is exceeded)

zwitterions

CHAPS (3-[(-cholamidopropyl)dimethylammonio]-1-propansulphate)

increases ionic strength without increase in conductivity (heat)
influences selectivity

chiral selectors

cyclodextrins, crown-ethers ...

similar to chiral additives in MF within **LC**

metal ions

K^+, Na^+, Cu^{2+}, Li^+ ...

influence selectivity in MEKC and **GE**

chaotropic agents

urea ...

solubilise NA and proteins; influence selectivity in MEKC

linear hydrophilic polymers

methylcellulose, polyacrylamide, polyethylene glycol, polyvinyl alcohol ...

decrease EOF; decrease analyte adsorption in low concentrations, ZE ⇒ GE

organic agents

methanol, acetonitrile ...

generally decrease EOF; influence selectivity in MEKC and **chiral separations**

complexing buffers

borate ...

allow separation of saccharides and **catechols**

CGE, capillary gel electrophoresis

classical – cross-linked gel in capillary

relatively fast, reproducible and quantitative

compared to *slab gel electrophoresis* : on-line detection in UV-VIS without visualisation
disadvantages: capillary filling (homogeneous polymerisation, bubbles...)
commercially filled capillaries – high price

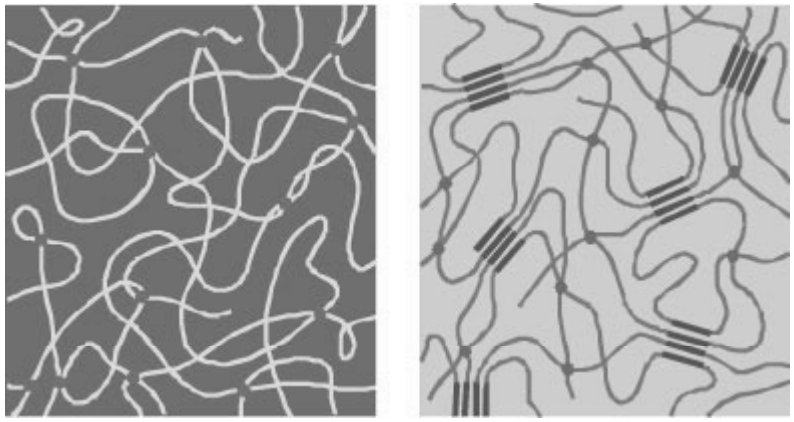
chemical gels: polyacrylamides – porous structure with strong covalent bonds

physical gels: agarose – weak intermolecular bonds of different molecule parts

entangled gel – linear gel as part of BGE

entangling medium (e.g. polymerous net) is present in background electrolyte

similar to physical gels – characteristic intermolecular interactions
rapid increase in viscosity (= $f(M_W)$) at liminal concentration values



mostly used polymers

: linear polyacrylamide

: N-substituted acrylamides

N-acryloyl aminopropanol (AAP)

N-acryloyl aminobutanol (AAB)

N-acryloyl aminoethoxyetanol (AAEE)

: polyethylene glycol (PEG)

: polyethylene oxide (PEO)

: polyethylene alcohol (PEA)

: polyvinyl alcohol (PVA)

: cellulose derivatives

methylcellulose (MC)

hydroxyethylcellulose (HEC)

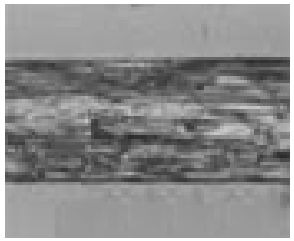
hydroxypropylcellulose (HPC)

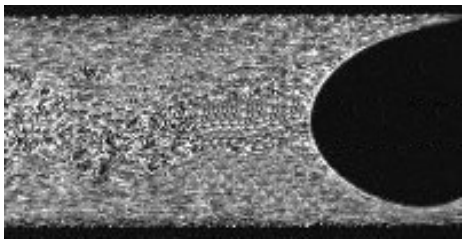
hydroxypropylmethylcellulose (HPMC)

: galactomannan (GalMan)

: glucomannan (GluMan)

capillary filling





bubbles : monomer solution looses volume when polymerising

⇒ *isotachophoretic polymerisation*

capillary and anodic space: acrylamide, bisacrylamide, triethanol amine (catalyser)
cathodic space: ammonium persulphate (initiator)

when the source is switched on, the initiator enters the system
ITF interface chloride / persulphate keeps initiator zone sharp
⇒ supervised polymerisation

such a voltage that initiator flow ~ rate of polymerisation (*ca* 2 – 4 V/m)

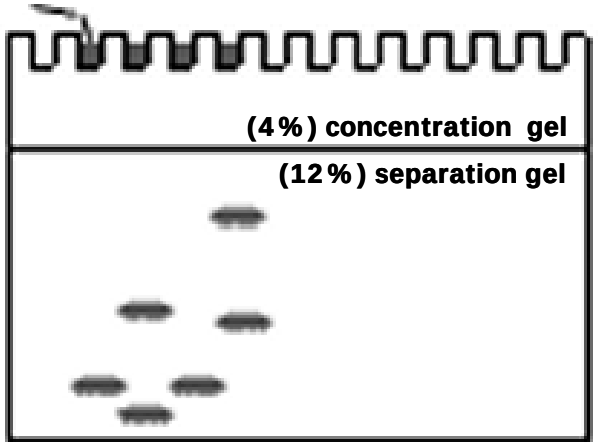
GE, slab-gel electrophoresis

denaturing (SDS, *Lämmli*) – separation according to $\mathbf{M_w}$

non-denaturing (native) – separation according to **pI**, **shape** and $\mathbf{M_W}$

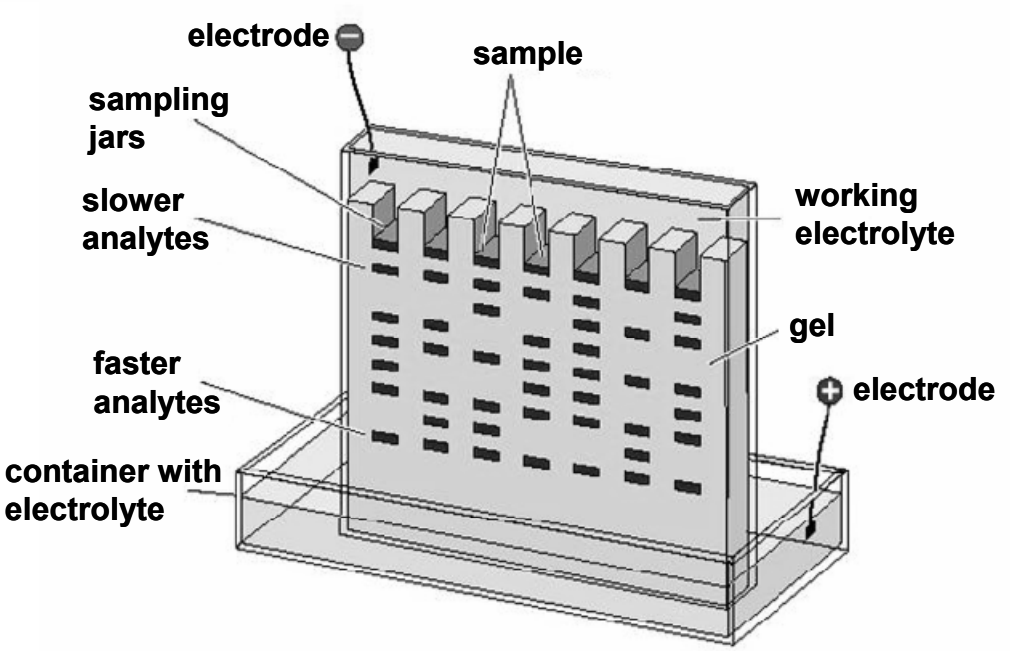
one dimensional gel electrophoresis (1D-GE)

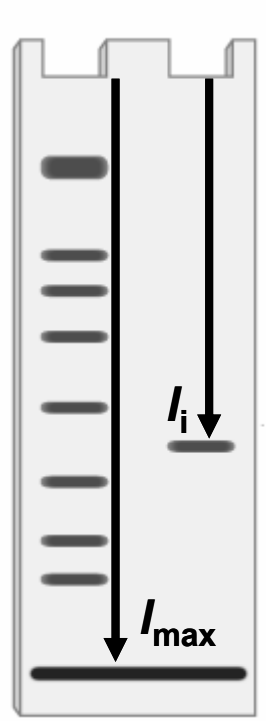
: slab gel polymerises between glass plates, separated by spacers
: loading jars are created by special spacer – *comb*



basic procedure

1. sampling buffer is added to sample
2. sample is loaded into jars
3. gel is put in-between buffers and voltage is applied
4. gel is washed and stained



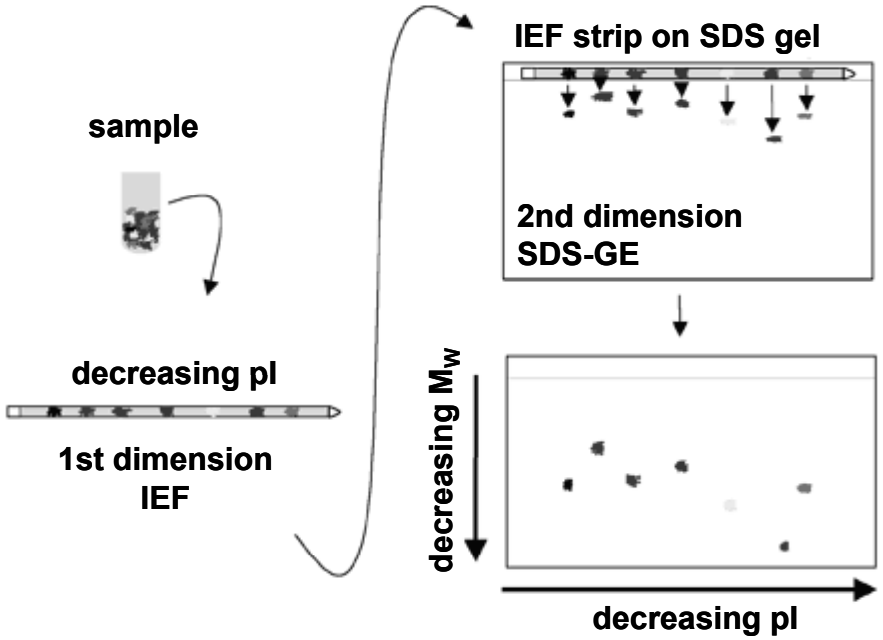


$$R_f = \frac{l_i}{l_{max}}$$

retention factor

two dimensional gel electrophoresis (2D-GE)

two dimensions:
1. **IEF**
2. **SDS-GE**



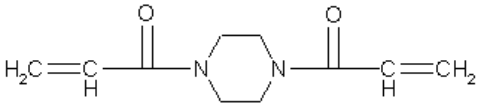
1. isoelectric focusation (IEF)

immobilised pH-gradient in gel strip

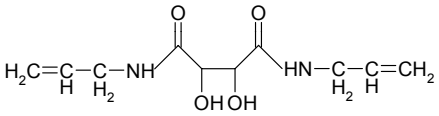
2. denaturing gel elfo (SDS-GE)

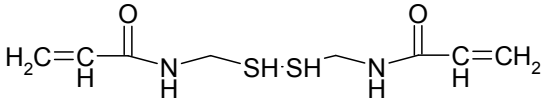
SDS is not in gel since polymerisation (as with 1D) – micelles would be created

necessary **to cool** more than 1D (5 – 12 °C)



as cross-linking agent **piperazine diacrylyl** (PDA), **diallyltartarate diamide** (DATD), **bisacrylyl cystamine** (BAC)

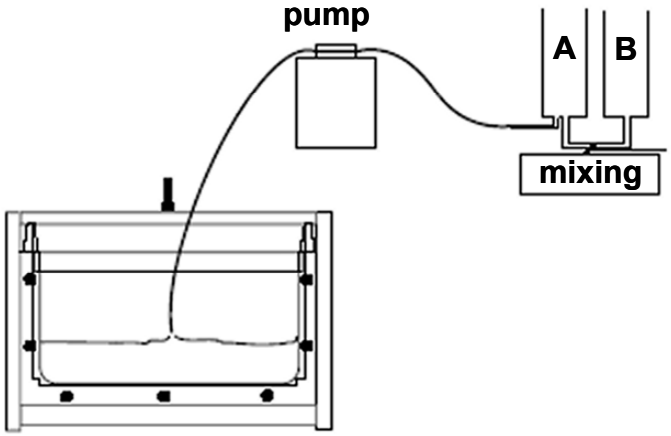




sodium thiosulphate in gel – low background with Ag-staining

in 2D density gradient (9 – 16 %) is used

in connected containers are mixed
A) solution **without cross-linker**
B) solution **with *max* cross-linker concentration**
: at outflow, increasing cross-linker gradient is formed

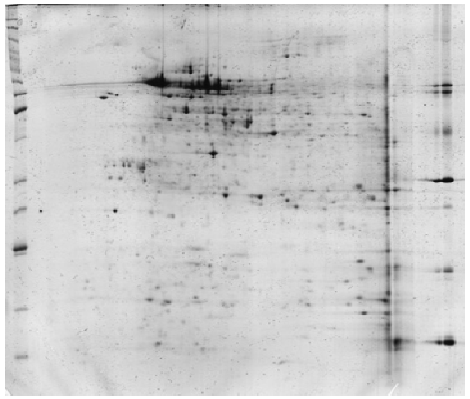


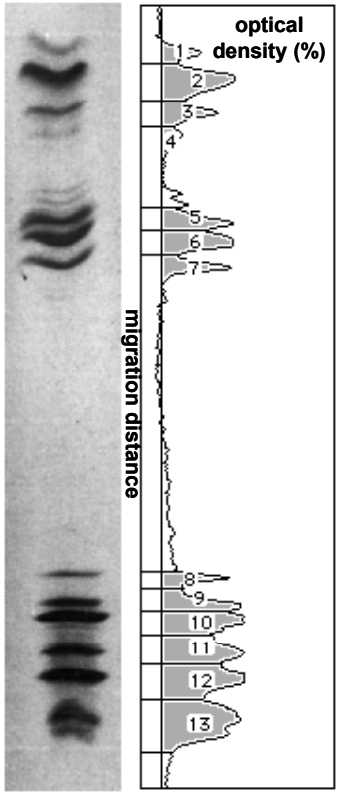
gradient profile is given by the shape of containers

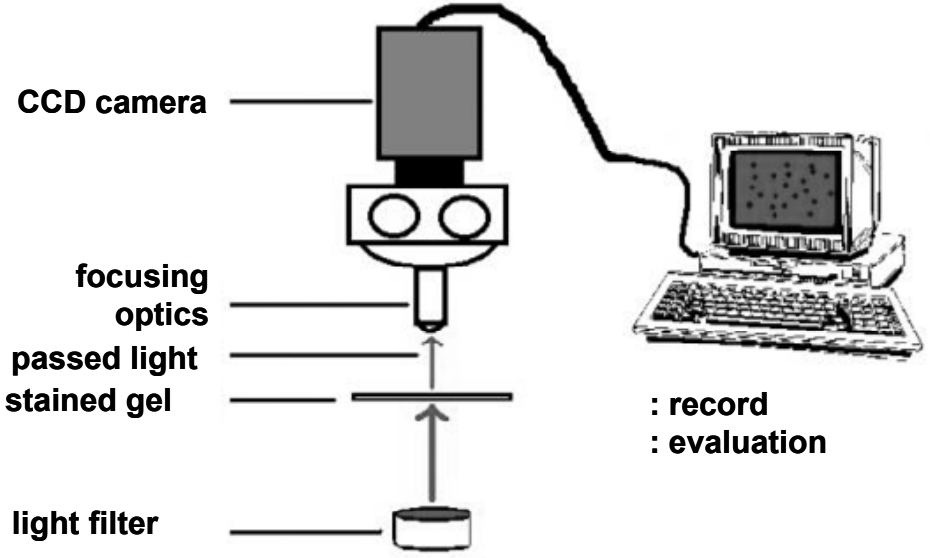
new – non-linear pH gradients in IEF

<u>after staining</u>

: densitometry
:: UV-Vis
:: fluorimetry

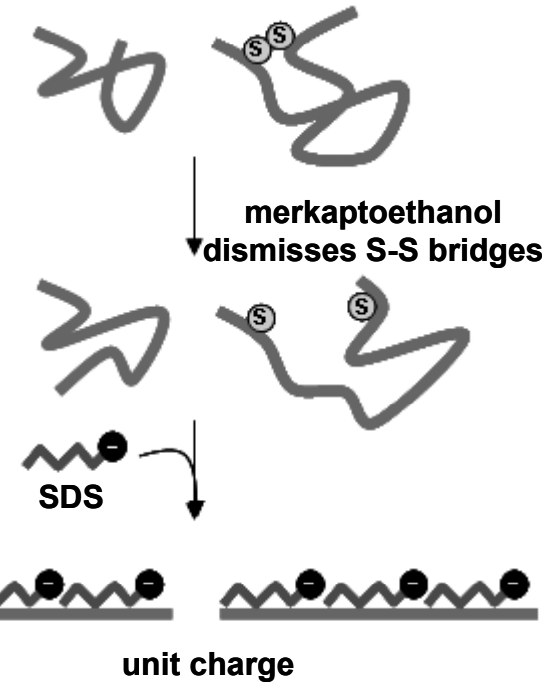




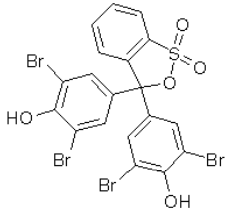


denaturing GE

: prior to analysis, sample is denatured
(+ EtSH, 95 °C, 5 min)
:: breaking of di-sulphidic bonds
:: turn into random coil conformation



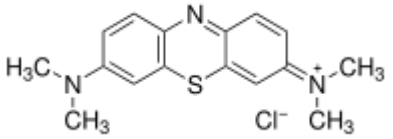
: leading colour: bromphenole blue



non-denaturing (native) GE

: separation of **acidic and basic** proteins **– separately:**

: leading colour: bromphenole blue for acidic
methylene blue for basic



: separation of **acidic** and **basic** proteins **– together:**
giving them a unit charge without denaturation

blue native PAGE (BN-PAGE) – CBB R-250 (~ 1 g to 1 g of protein)

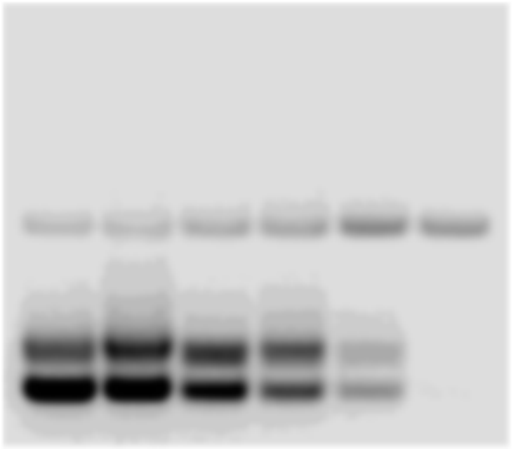
clean native PAGE (CN-PAGE) – n-dodecyl-β-maltoside and digitonin

polyacrylamidove gel electrophoresis – PAGE

: for separation of proteins
in native and denaturing mode; 1D and 2D

agarose gel electrophoresis – AGE

: for nucleic acids separation	0.8%	50 – x1000 kbp
only one mode (1D)	1 – 2%	20 – 50 kbp
NAs already have unit charge	4%	< 20 kbp



leading colours:
xylene and bromophenol blue, cresol red, orange G

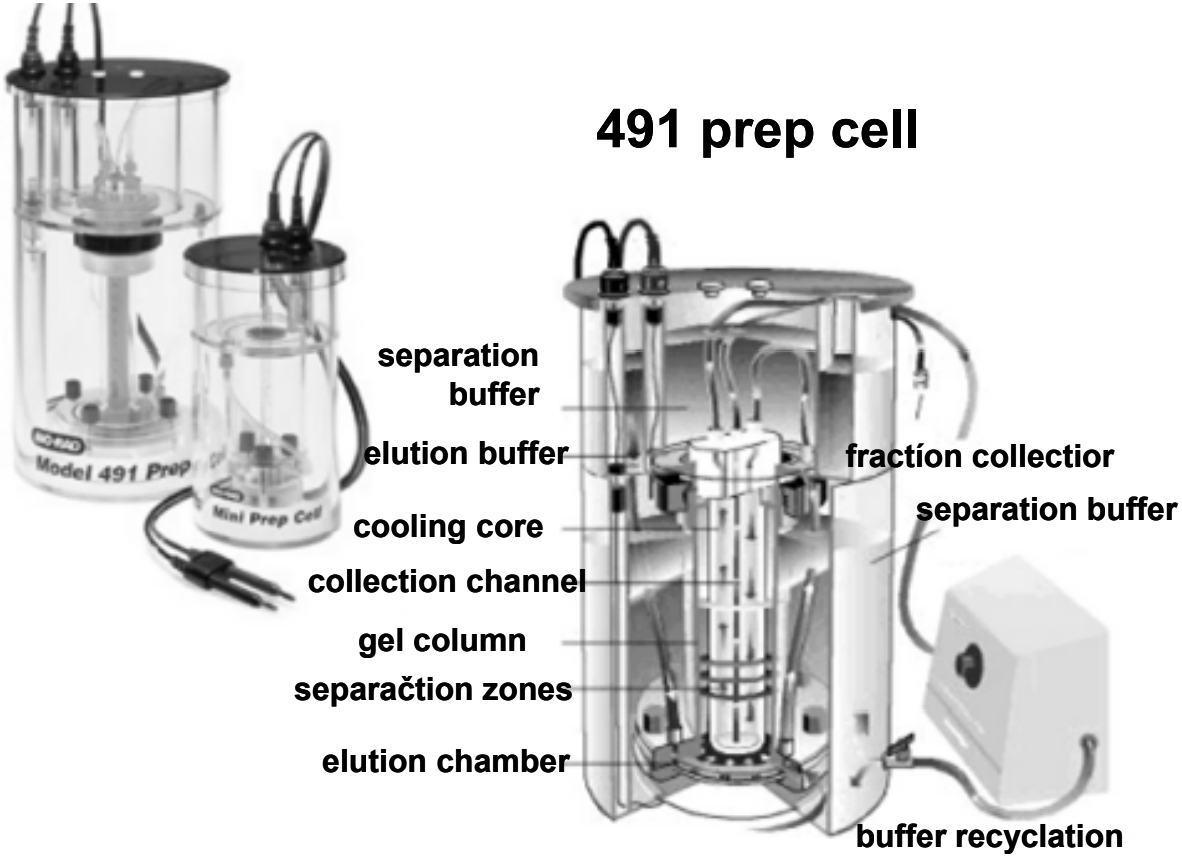
separation conditions:

TRIS-acetate EDTA (TAE) **:** low voltage, large molecules (50 – x000 kbp)
TRIS-borate EDTA (TBE) **:** 20 – 50 kbp
sodium borate (SB) **:** high voltage (35 V/cm), small molecules < 5 kbp

column continuative elution gel electrophoresis

(**CEGE**)

: new technique similar to ***slab GE*** – *primarily preparative*
:: mostly SDS-PAGE
:: native isoelectrofocusation QPNC-PAGE
(*quantitative preparative native continuous*)
: suitable for on-line connection with detection techniques (MS)

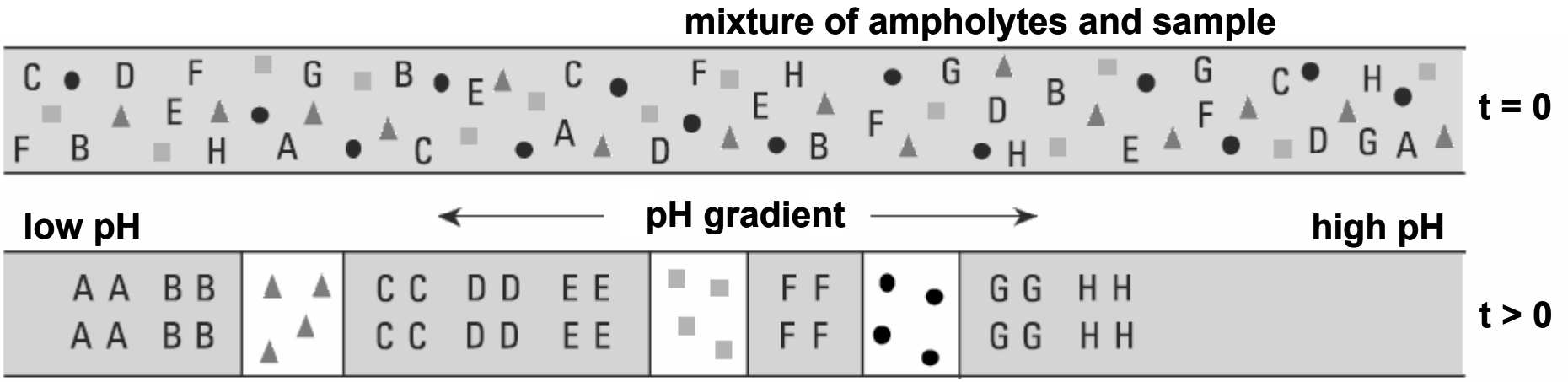


CIEF, capillary isoelectrofocusation

isoelectrofocusation – greek **ίσος** (same), **ήλεκτρον** (amber) and latin **focus**

solution contains **ampholytes**
during separation, the **pH gradient** is established

pH = pI, analyte is not moving



zones are sharp, **self-focusation effect**

$$w_A = \sqrt{D \Big/ \left(\left(\frac{\partial \mu}{\partial pH} \right) * \left(\frac{\partial pH}{\partial x} \right) \right)}$$

w_A – zone width
x – length coordinate

resolution in IEF

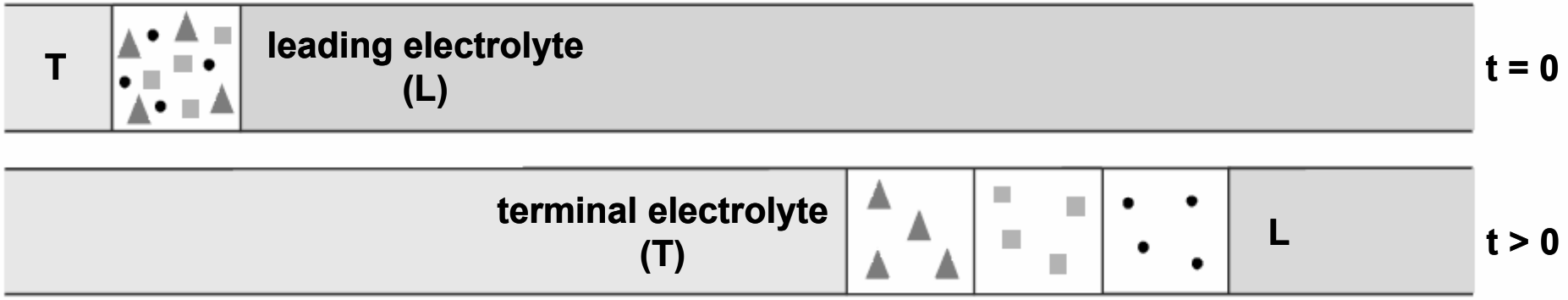
$$\Delta pI = \sqrt{\left(\frac{\partial pH}{\partial x} \right) \Big/ E * \left(- \frac{\partial \mu}{\partial pH} \right)}$$

E – electric field intensity [V/cm]
∂pH / ∂x – pH gradient
∂μ / ∂pH – mobility slope at given pI

CITF, capillary isotachoforesis

isotachophoresis – greek **ίσος** (same), **ταχύς** (speed) and **φορέω** (I carry)

two **electrolytes**
: **leading** – leading ion has absolutely highest mobility in system
: **terminal** (*trailing*) – terminal ion has absolutely lowest mobility in system
⇒ electric field intensity increases from leading to terminal ion



component concentration in zone is according to Kohlrausch **ω**-function
analytical concentration of compound A, $\mathbf{c_A}$:

$$c_A = c_L * \frac{\mu_A}{\mu_A - \mu_{CI}} * \frac{\mu_L - \mu_{CI}}{\mu_L}$$

for strong univalent electrolytes

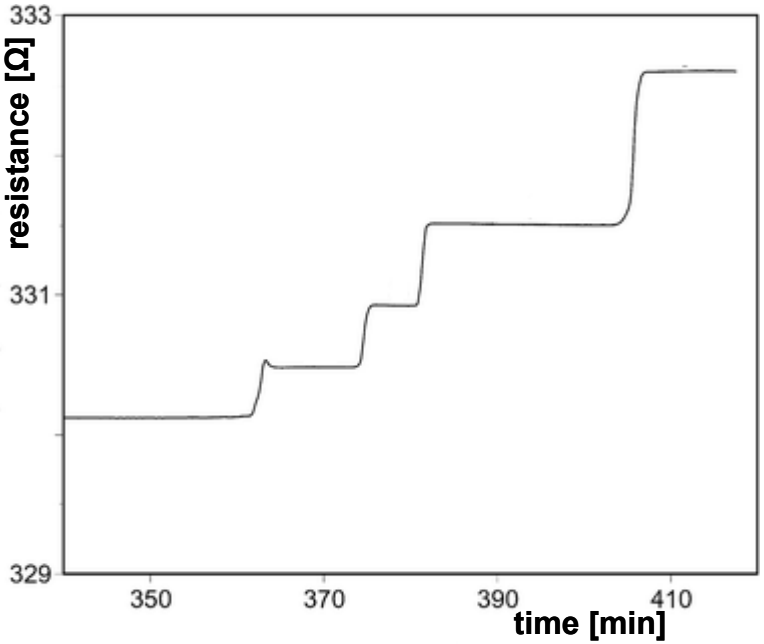
CI – analyte counter-ion

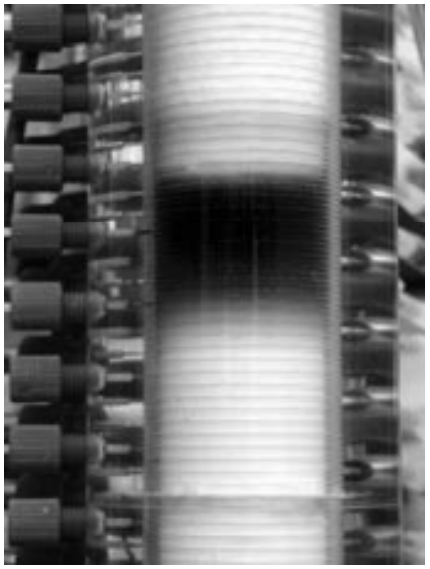
self-focusing effect

zones are **sharp** and **do not broaden** ⇒ concentrating minor components in few orders

if ion **L** because of diffusion goes to **zone X**, because of ↑ **E**
also **increases its migration velocity** and it **goes back to zone L**

if ion **X** because of diffusion goes to **zone L**, because of ↓ **E**
also **decreases its migration velocity** and it **goes back to zone X**





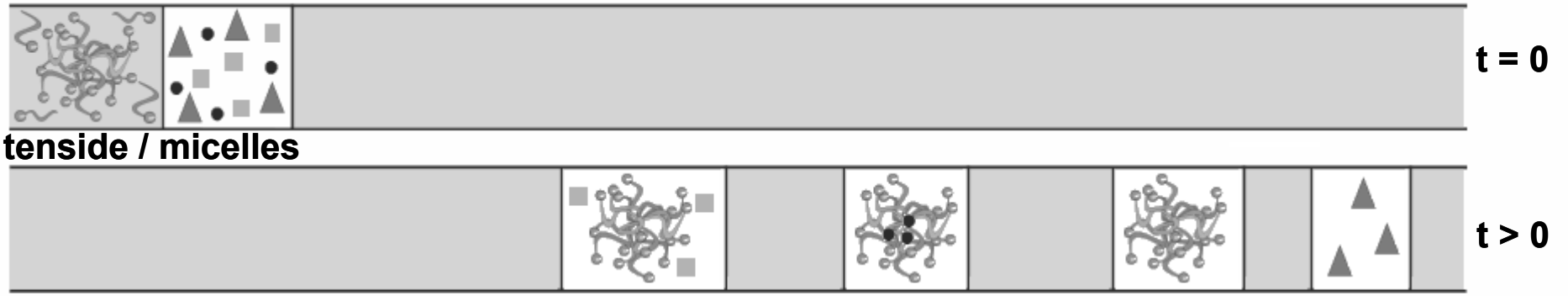
isotachophoretogram
typical detection – resistance; others methods – conductivity, thermometry, UV-Vis

MEKC, micellar electrokinetic chromatography

one **electrolyte** containing **ionogenic tenside** over critical micellar concentration
⇒ **micelles** are created

analyte **is separated between micelles** and **electrolyte** acc. **distribution coefficient** (K)
MEKC may be seen as ZE of two entities – **analyte** and **micelles with it**

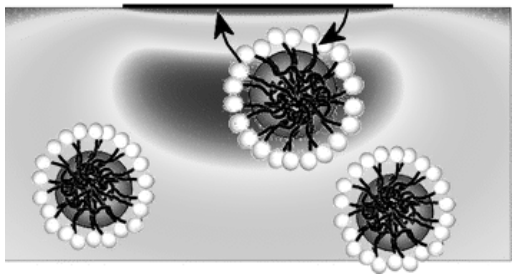
analyte **does not enters** micelles ⇒ **K = 0,** analyte **enters completely** ⇒ **K =** ∝



$$k' = \frac{t_m - t_M}{t_M\left(1-\left(t_m / t_{mc}\right)\right)} = K * \left(V_{SF} / V_{MF}\right)$$

k' – capacity factor
$\mathbf{t_M}$ – void retention time
$\mathbf{t_m}$ – retention time
$\mathbf{t_{mc}}$ – retention time of micelles

commonly used tensides



anionogenic : sodium dodecylsulphate ...
cationogenic : cetyltrimethylammonium bromide, septonex ...

to *decrease migration velocity* of micelles **non-ionogenic tenside** (Triton X-100) is added

micelles may be substituted with *microemulsion* or *polyions*

addition of organic phase: solvatation changes, micellar structures,
smoother setting – mixture of tensides

resolution in MEKC

$$R = \left(\frac{\sqrt{N}}{4}\right) * \left(\frac{\alpha - 1}{\alpha}\right) * \left(\frac{k_2'}{k_2' + 1}\right) * \left(\frac{1 - (t_M / t_m)}{(1 - (t_M / t_m)) * k_1'}\right)$$

efficiency selectivity retardation

α – selectivity
N – number of theor. plates

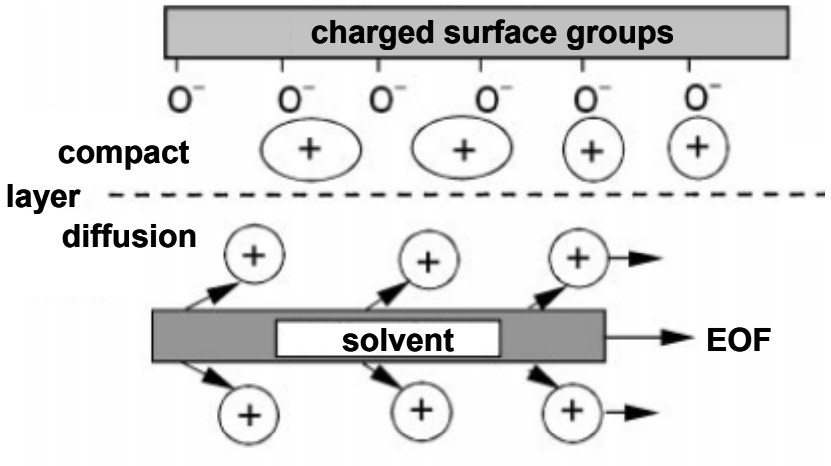
disadvantage: difficult *reproducibility*

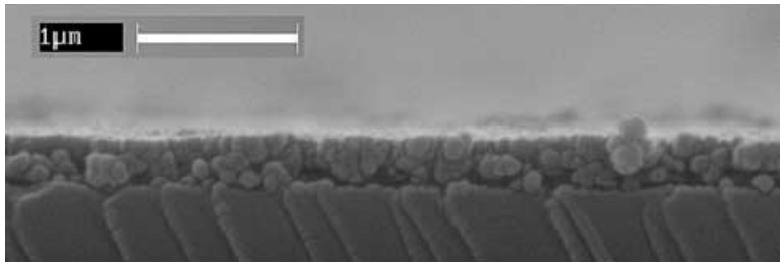
TLE, thin layer electrochromatography

paper electrophoresis, slab electrochromatography

charged (mostly negative) **SF**; often silicagel, cellulose and its derivatives

analyte **is separated between SF** and **electrolyte** acc. **distribution coefficient** (K)





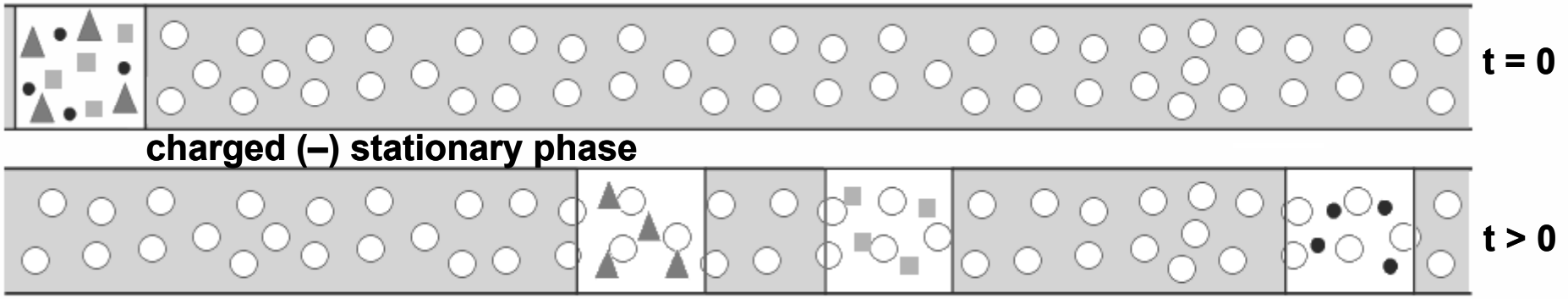
fast : applied voltage is driving force; comparing to TLC where it is capillary elevation
: fast also comparing to capillary variant (up to three orders of magnitude)
: voltage 160 V/cm ⇒ migration velocity 100 µm.s^{-1}

CEC, capillary electrochromatography

charged (mostly negative) **SF**; porous particles of o.d. 1.5 – 5.0 μm
column: either *broader* (320 μm) or *narrower* capillary (50, 75 or 100 μm)

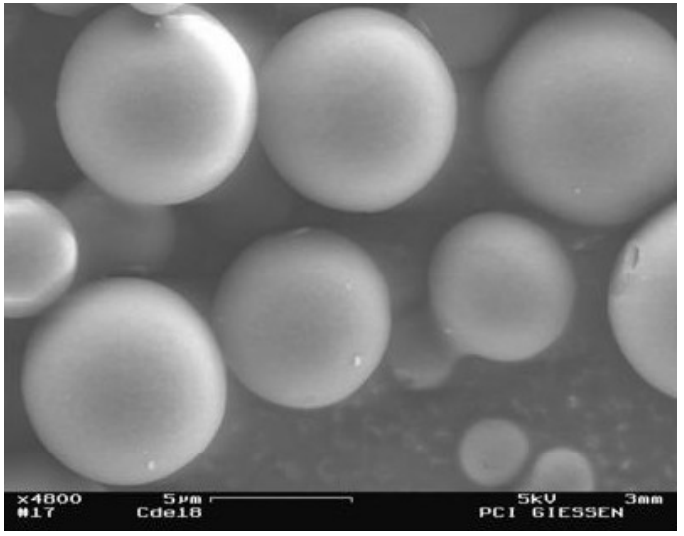
analyte **is separated between SF** and **electrolyte** acc. **distribution coefficient** (K)
: applied voltage is separation driving force ⇒ flow of the liquid is not laminar
: EOF is created on the surface of SF rather than on a wall of separation channel

low currents: max 10 μA
Joule heat 0.1 W.cm^{-2} (1500x more heat than within pressure heating by HPLC)

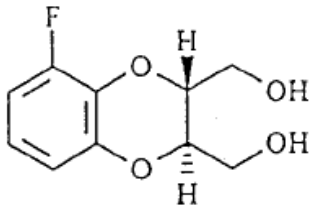


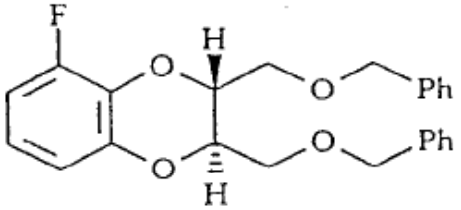
SF

: C18 bound on silicagel (reverse CEC)
: β-CD bound on silicagel (chiral CEC)
: SCX cation exchanger (-$CH_2CH_2CH_2SO_3H$)

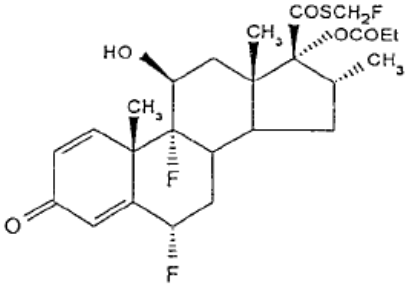


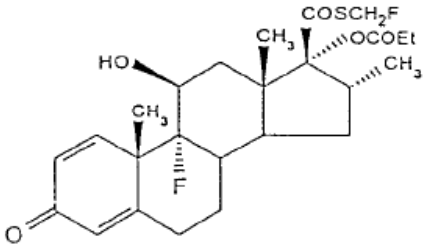
testing mixture



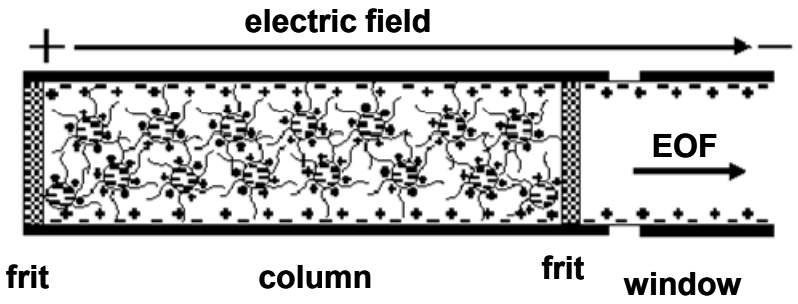


thiourea
GR 57888X, GR 57994X
fluticason proprionate, des-6-α-fluoro-fluticason proprionate





: thiourea indicates EOF
: components 2 and 3 determine hydrophobicity
: components 4 and 5 determine resolution

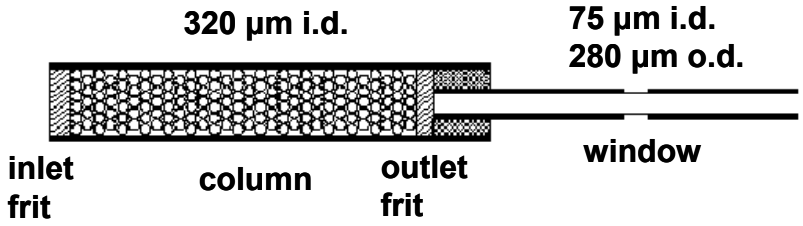


advantages

: higher efficiency than HPLC
up to 300 000 plates / m (i.e. 3 – 4x)

: may use very small particles
no high back pressure

: separation of neutral, lipophilic and water-insoluble analytes
: low sample and MF consumption
: isocratic and gradient elution
: may use MS detection
: same instrumentation as for CZE, CEC or CLC



disadvantages

: column
filled capillaries with frits; fragility

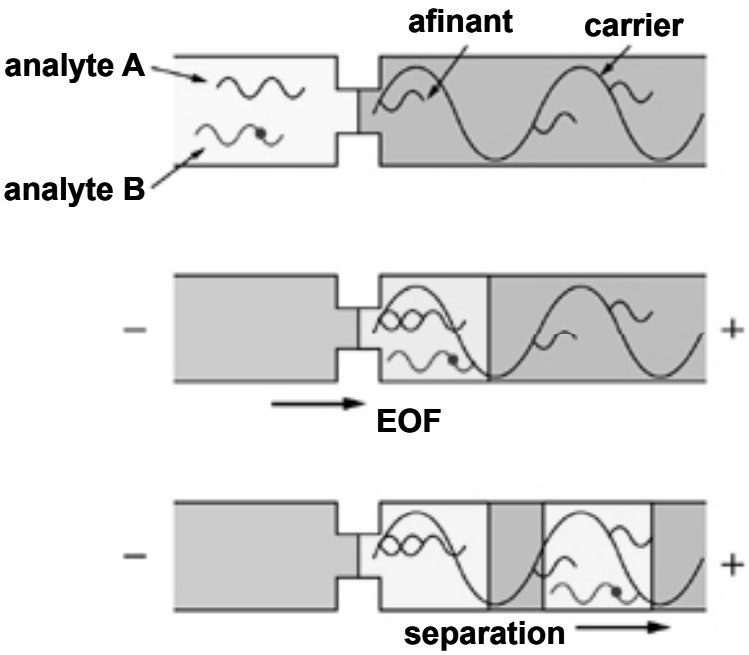
: bubbles (EOF differences, Joule heat)
: electrokinetic injection (internal standard)
: lower sensitivity

AE, affinity electrophoresis

uses combination of **separation in filed** and **affinity separation**

affinity separation – specific interaction of analyte and ligand

enzyme	:	coenzyme, substrate, inhibitor
nucleic acid	:	complementary chain, histone
antigen	:	antibody
receptor	:	signal molecule



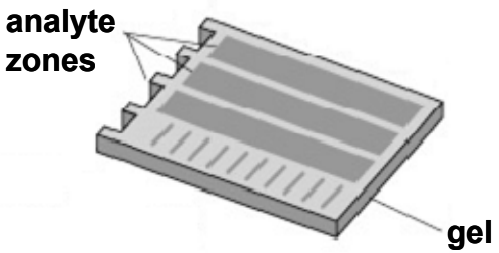
in **capillary** and in **gel**

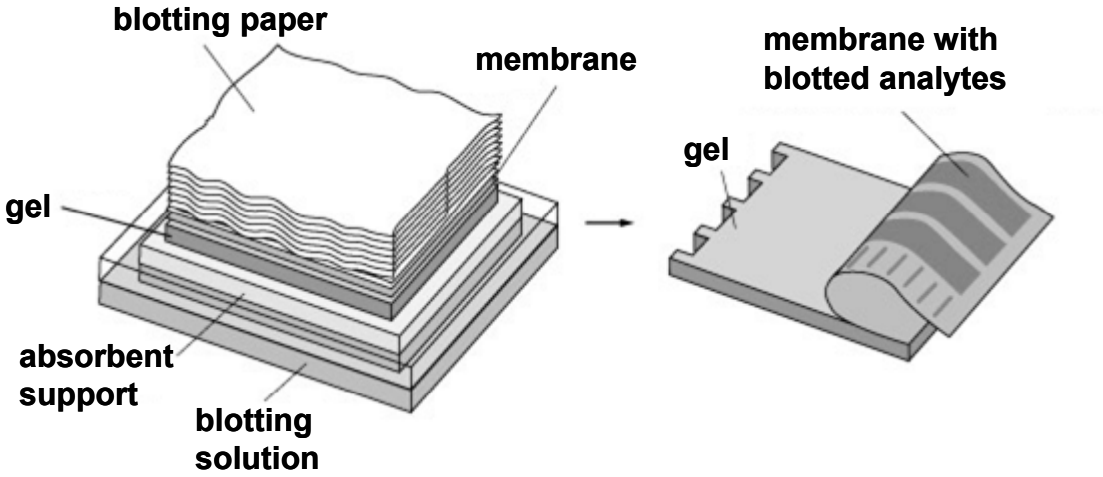
: <u>separation</u>
highly selective

: <u>purification</u>
shot-gun

: <u>interaction study</u>
compatibility
association constants

blotting

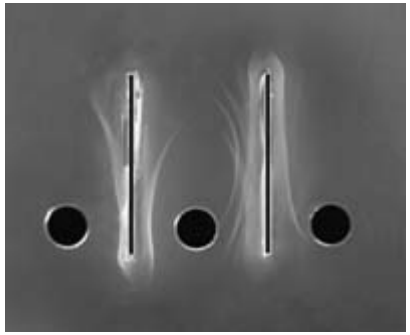




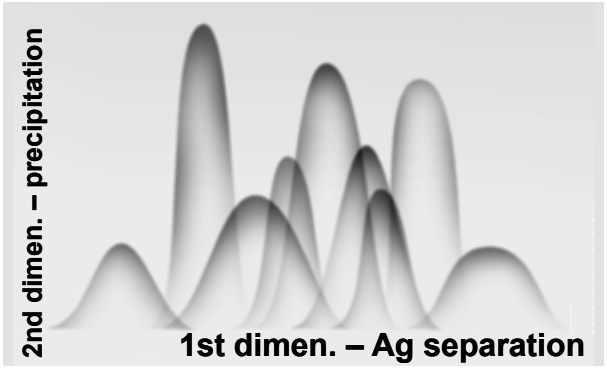
Southern blot – DNA
Northern blot – RNA
Western blot – proteins

immunoelectrophoresis

interaction **antigen** (Ag) + **antibody** (Ab)



1D gel immunophoresis



2D gelová immunophoresis

NAE, non-aqueous electrophoresis

separation in **non-aqueous solvents**

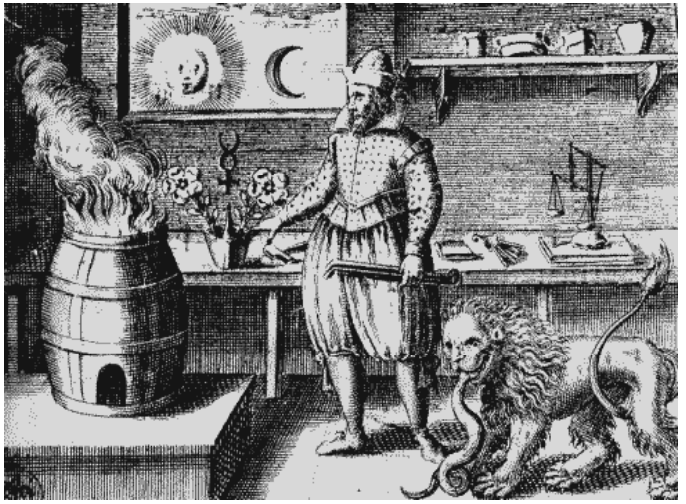
1978 – non-aqueous **TLE**
1984 – non-aqueous **CE (NACE)**

advantages:

: elimination of *levelling effect of solvent* ⇒ higher selectivity of separation
: low current
: separation of hydrophobic (water-insoluble) analytes

solvent choice:

: volatility
: ability to solve BGE and analyte
: viscosity
: dielectric constant
: transparency in UV



solvents:

water content *max* 1 %

amphiprotic

: neutral	(+;+) : MeOH, glycerol, phenol, *tert*-butylalcohol
: protogenic	(+;–) : sulphonic a., formic a., acetic a.
: protophilic	(–;+) : liquid ammonium, formamide, N-methylformamide
: dipol. protophilic	(–;+) : DMSO, dimethylformamide, THF, 1,4-dioxan, pyridine

aprotic

: dipol. protophilic	(–;–) : AcN, acetone, nitrobenzene, sulpholane, PC
: inert	(–;–) : alif. hydrocarb., benzene, 1,2-dichloret., tetrachlorom.

relatively basic or acidic (*;*)

background electrolytes:

: ammonium acetate, sometimes with addition of acetic a. or sodium acetate

: quaternary ammonium salts

: Tris, magnesium acetate, citric a., formic a., trifluoroacetic a. ...

additives: polyalcohols and surfactants ⇒ **decreasing EOF**