SEPARATION METHODS **B**

jan havliš

: masaryk university

:: central european institute of technology

::: mendel centre for plant genomics and proteomics

:: faculty of science, national centre for biomolecular research

::: division of functional genomics and proteomics

separation methods B – syllabus

analytical separation

analytical separation method

: gas chromatography
:: GC

: separation of macromolecules
:: SEC, GPC, HCD a FFF

: separation in force field
:: CZE, MEKC, CIEF, ITP, CEC, ACE, NCE a CE-on-chip
:: MS (Q, QqQ, IT, TOF, FT-ICR, OT)

: membrane separation
:: dialysis, ultrafiltration

another aspects of analytical separations

: chiral separation
: separation method development and optimisation
: validation of analytical separation method

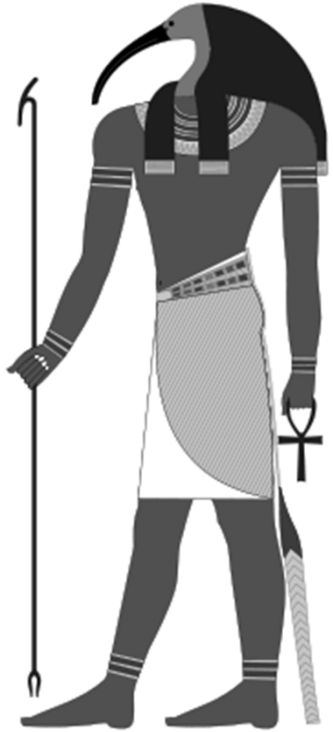
recommended reading

J. C. Giddings, **Unified separation science**, Wiley 1991

D. Hieger, **An introduction to high performance capillary electrophoresis**, Agilent Technologies 2000

C. F. Poole, **The essence of chromatography**, Elsevier 2003

R. L. Grob *et al*., **Modern practice of gas chromatography**, Wiley 2004



development of chromatographic method

choice of separation system – suitable SF type

knowing the sample, we choose SF

choice of separation conditions – suitable MF type

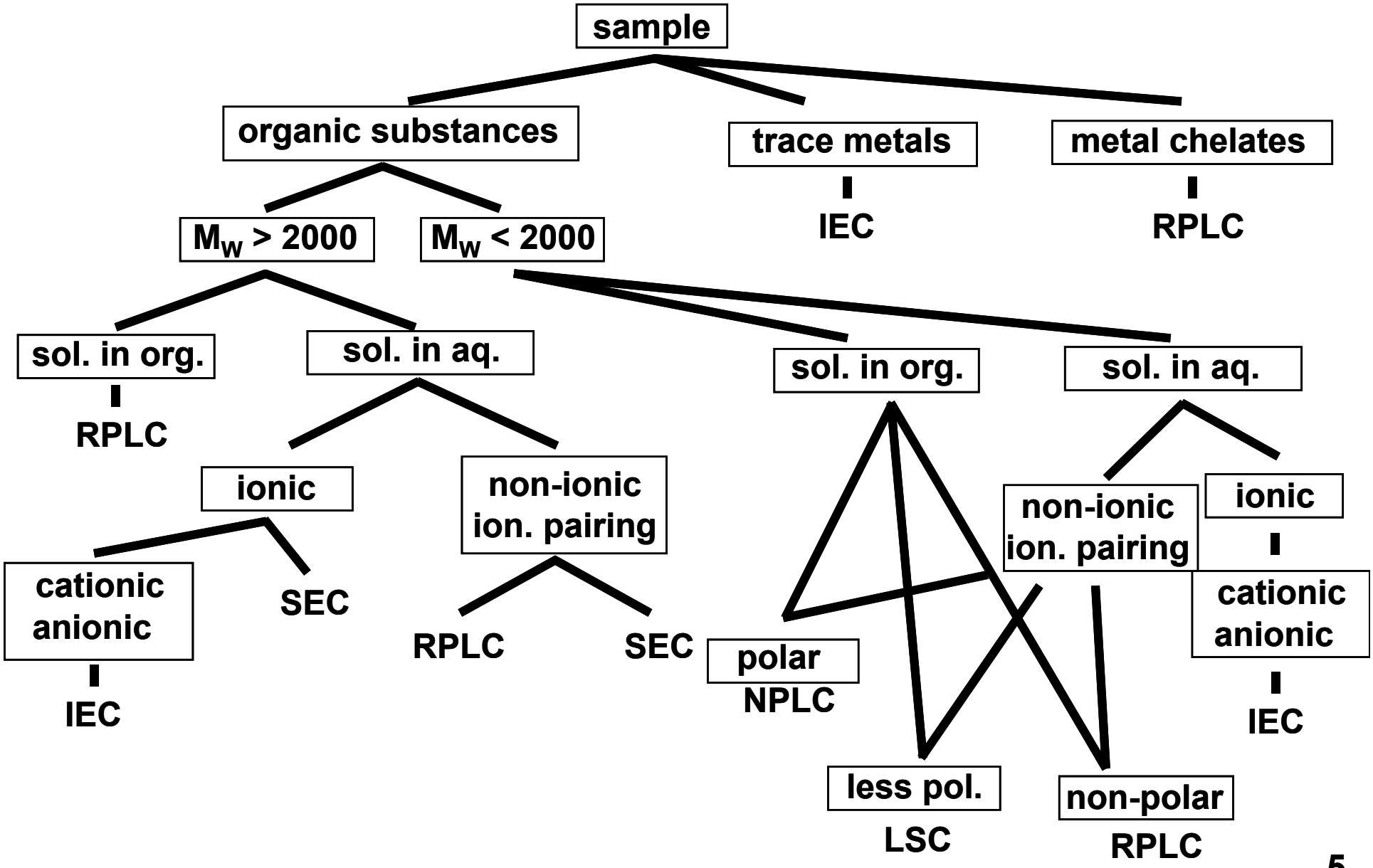
: MF composition may be derived from requested retention behaviour

: *practical*
: „unscientific“, we approach problem „from the end“
: for each case we need to do it again

: out of sample physico-chemical properties we derive retention properties

: *scientifically correct, but uneasy*

algorithm of separation system choice



choice of separation conditions

optimisation of separation conditions in dependence on demands

aim: $\mathbf{t_{R,i}}$ = min; $\mathbf{R_{ij}}$ = max; $\mathbf{n_j}$ = max; $\mathbf{dc_j / dt}$ = max

$$r_{(A,B)} = \frac{k_B}{k_A}$$

elution ratio

$$S_{(A,B)} = \frac{k_B - k_A}{k_A + k_B + 2}$$

separation ratio

means: separation conditions

$$Đ = f\left(T, u, c_{org}, pH, I, c_{pufr}, atp.\right)$$

dependent variables (Đ): retention times $\Rightarrow$ resolution, peak no., asymmetry

independent variables: buffer concentration, ion-pairing agent concentration, pH, % of organic component, temperature, gradient profile...

we study the dependence of dependent variables on independent

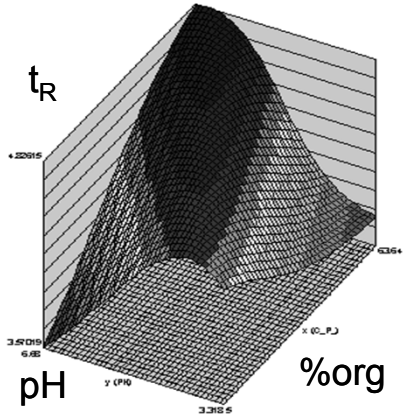
possibilities: modelling

hard m.: based on exact physico-chemical models

$$Đ = f(T, u, c_{org}, pH, I, c_{buffer}, etc.)$$

soft m.: based on approximation of real function
:: substitution to hard model

hyper-flat of approximated function
: relation between retention and separation conditions



tools and process of optimisation

single-criterial (semi-hard) evaluation of separation quality

criteria characterising by single value the level of separation of all sample components

chromatographic response function (CRF)

$$CRF = \prod_{i=1}^{n} \ln\left(\frac{\Delta t_{R,i}}{t_{R,min}} + \left(t_{R,min} - \Delta t_{R,i}\right)\right)$$

chromatographic optimisation function (COF)

$$COF = \sum_{i} \alpha \cdot \ln \frac{R_i}{R_{min}} + \beta\left(t_{R,max} - t_{R,posl}\right)$$

$$COF = \sum_{i} R_i + N^{\alpha} + \beta\left(t_{R,max} - t_{R,posl}\right) + \gamma \cdot \left(t_{R,prv} - t_{R,min}\right)$$

separation factor (S)

$$S = \frac{\prod_{i=1}^{n} \Delta t_{R,i}}{t_{R,max} - t_{R,min}}$$

resolution product (RP)

$$RP = \frac{\prod_{i} R_i}{\frac{\sum R_i^{(n-1)}}{n-1}}$$

normalised retention difference (NRD)

$$NRD = \prod_{i=1}^{n-1} \left(\frac{\Delta t_{R,i}}{\frac{1}{n-1} \cdot \sum_{i=1}^{n-1} \Delta t_{R,i}} \right)$$

multi-criterial evaluation of separation quality

single variable approach (**SVA**)

studies change of dependent variables while gradually changing one independent variable and keeping all other constant

method: relaxation method

!! *omits possible relations between independent variables*

multiple variable approach (**MVA**)

studies change of dependent variables while gradually changing more independent variable

method : partial least square (**PLS**)
: artificial neural network (**ANN**)

in combination with *experimental design* (**ED**)

experimental design

an experiment planning in a way, so that out of minimum of points we get maximum information and thus the best description of function of multi-variable function

factorial design

full factorial experimental design, FED

: contains all possible combinations of chosen factors

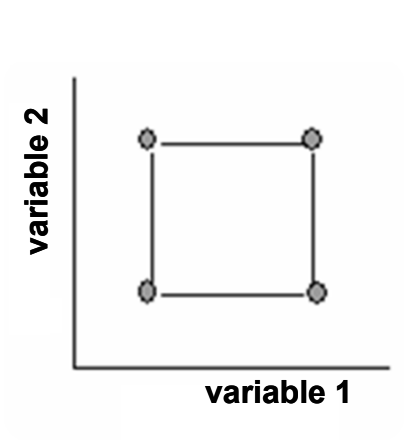
parameters: number of factors and levels for each factor

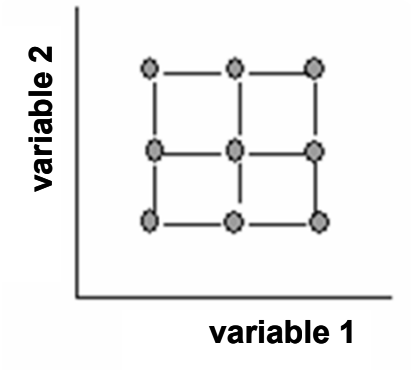
: *number of factors* (f) responds to number of input variables (***f*** components)
: *number of levels* (L) is number of values per each input variable (***L*** measured concentrations)

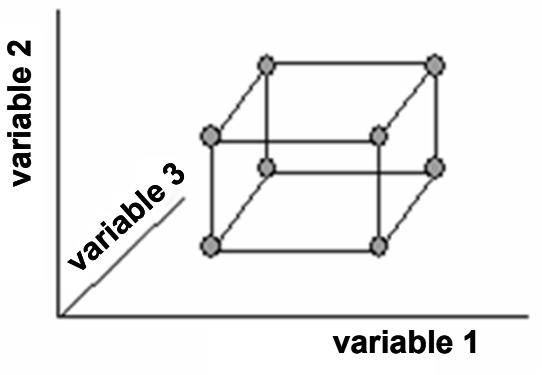
: *number of points of factorial design* (total number of experiments *n*)

$$n = L^f$$

three-level two-factorial design
(L = 3); 3^2 experiments







two-level two-factorial design
(L = 2) *simplest*; 2^2 experiments

two-level three-factorial design
(L = 3) 2^3 experiments

fractional factorial experimental design (FrED)

: **reduces** number of experiments of FED (sometimes to complex and laborious)

: still describes influence of each parameter and checks possible interactions

: proper in cases with expensive and time-consuming experiments

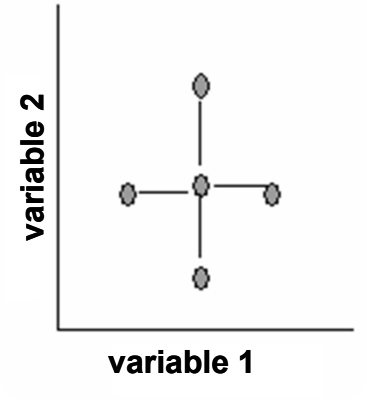
star design

other variant of experimental design
: it may be FrED variant of factorial design
:: three-level two-factorial design ⇒ two-factor star design

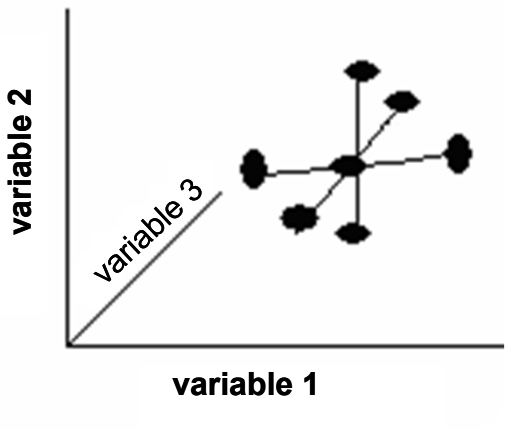
: contains (**2xf+1**) experiments, where **f** is number of factors (components)

: positioning of star design points is given by position of central point

: other points are located symmetrically around the centre



two-factorial star design
2xf+1 experiments



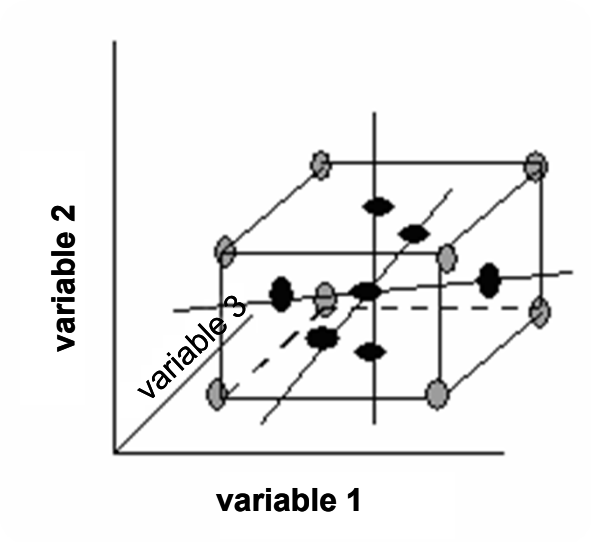
three-factorial star design
2xf+1 experiments

central and non-central composite designs

combination of factorial and star experimental design – *complex hyper-flat*

central composite designs – centres of both plans are equal

non-central composite designs – centres are not equal



five-level three-factorial central composite design
$2^f + 2xf+1$ experiments

approximative methods and algorithms

optimisation – effort to „uncover“ the numerical function of dependence of output on optimised parameters – ***approximation***

black box : algorithms do not describe the physico-chemical properties, but „only“ numerically describe the dependencies between variables

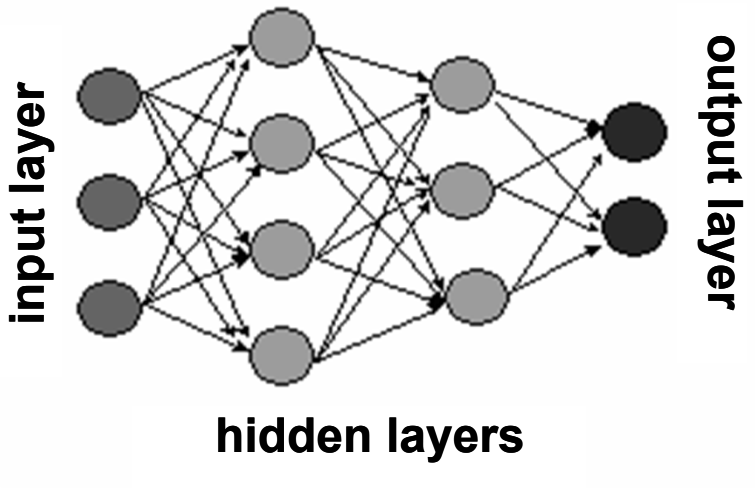
partial least squares (PLS)

MVA, values from all components of analysed mixture are calculated at once

canonical correlation (CC)

artificial neural networks (ANN)

: *mimics biologic system of mutually connected neurons*



processors – **neurons**
the way of connection – **network topology**

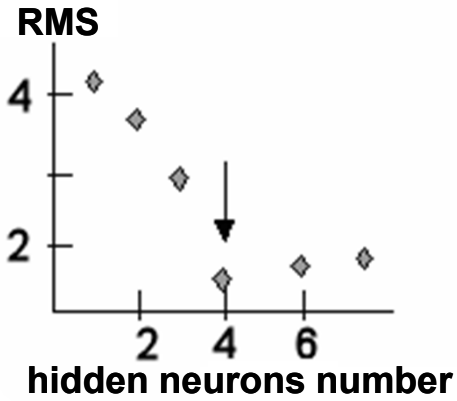
neurons are arranged in **layers**
outputs of n^{th} layer are directed into each neuron in **layer n + 1**

first, input layer
: inputs values for processing

last, output layer
: values are responses of whole ANN on changes of conditions of input parameters

numbers of neurons in input and outputs layers are given by numbers of input and output variables

inner, hidden layers
: number depends on approximated function complexity



connection between neurons

represented by rational number – *connection weight* (*w*)

learning of prediction of *output values* with *minimal deviation* of these predicted values by ANN *from values experimental* – by repeated setting of numerical inputs of transformation function and watching the outputs on real value

deviation – ***total sum of squares*** (*TSS*)
sum of squares of differences of predicted and input values

$$TSS = \sum_{i=1}^{n} (z_i - OUT_i)^2$$

$\mathbf{z_i}$ – value of output variable **z** for given triad (*x, y, z*), $OUT_\mathbf{i}$ (output) – its predicted value, **n** – number of elements of training set

each neuron (except input) sums values from preceding layer and multiplies them with connection weight ***w*** :

$$NET_j = \sum (INP_i \cdot w_i) + BIAS_i$$

$\boldsymbol{INP_i}$ – input value, $\boldsymbol{w_i}$ – weight value and $\boldsymbol{BIAS_i}$ – value of bias, which is so-called bias parameter and is essential for correct setup of neuron value NET_j and for whole performance of network

$\boldsymbol{NET_j}$ – neuron ***j*** in neural network

$\boldsymbol{OUT_i}$ – transformation of sum value NET_i (output)

$$OUT_i = 1/(1 + e^{-NET_j})$$

set training/learning – X parameter sets defined by experimental design
testing – at least 3 parameter sets inside boundaries given by ED
verification – at least 3 parameter sets inside boundaries given by ED (including boundaries)

gas chromatography

: extraction G-L
: extraction G-S

: **mobile phase** (**MF**, gas)
: **stacionary phase** (**SF**, liquid, solid, thin layer of liquid on carrier)

GC history

1941

Synge and **Martin** : theoretic principles of 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

principal differences between GC and LC

Raoult's law

$$p_A = p_A^0 \cdot x_A$$

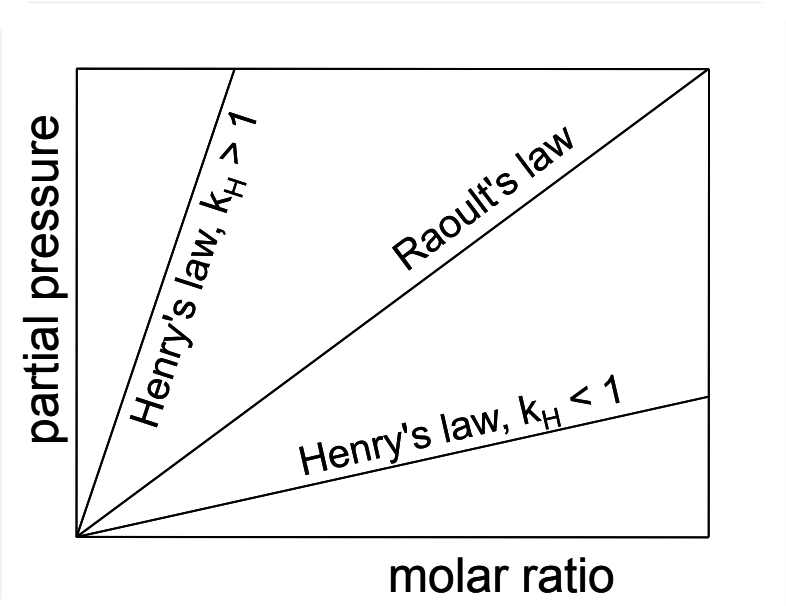
gas is **compressible** (liquid not)

x_A – molar ratio of **A** in mixture

p_A^0 – pressure of saturated vapours of **A**

Henry isotherm

$$c_A^S = k_H \cdot p_A$$



low concentrations of **A**, non-ideal solution

k_H – Henry's constant

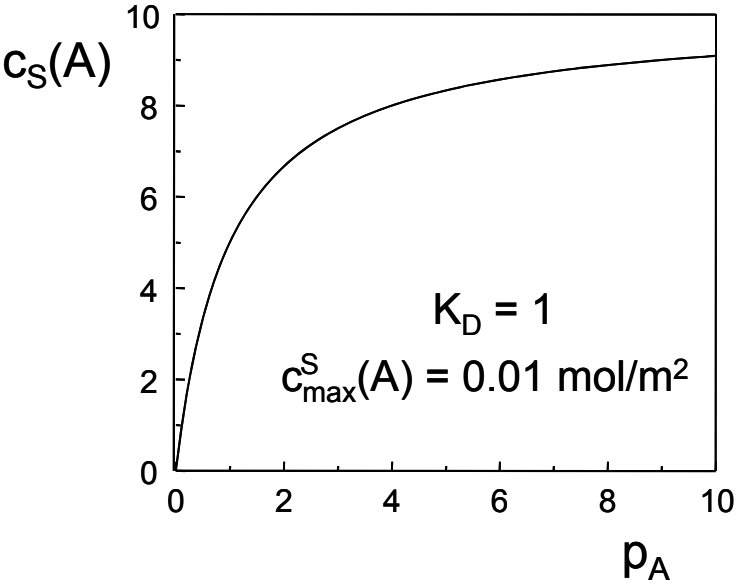
p_A – partial pressure of **A** over mixture

relation between
Raoult's and Henry's laws

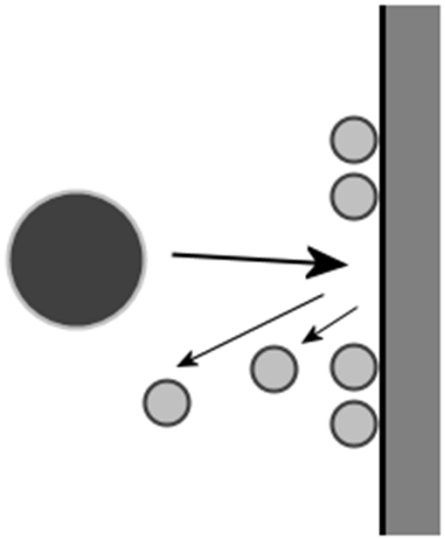
Langmuir isotherm

$$c_A^S = c_{A,max}^S \cdot \frac{K_D \cdot p_A}{1 + K_D \cdot p_A}$$

$\mathbf{c_{max}^S}$ – maximal bound concentration on SF

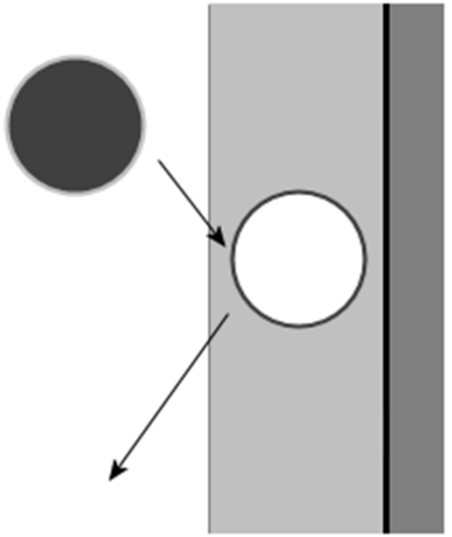


distribution constant is strongly dependent of **vapour púressure** and **volatility of analyte**



adsorption GC
GSC

distribution GC
GLC



adsorption (distribution) GC

distribution chromatography (GLC)

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

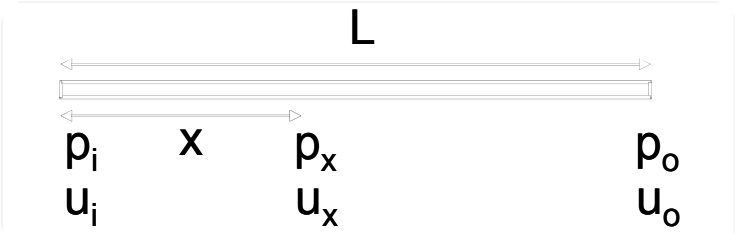
adsorption chromatography (GSC)

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

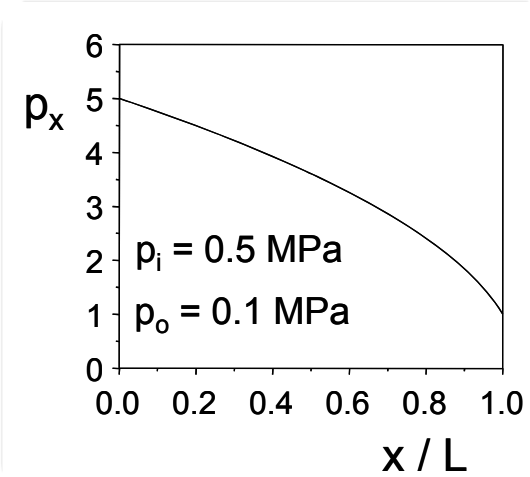
$$K_D = \frac{c_A^S}{c_A^M}$$

S – SF, ***M*** – MF

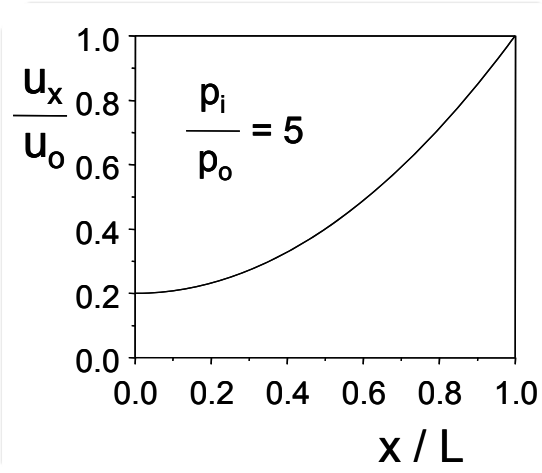
linear flow rate of carrier gas (MF)



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

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

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

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

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

$$\bar{u} = j \cdot u_o \cdot \left(\frac{T_{col}}{T_o}\right) \cdot \left(\frac{p_o - p_w}{p_o}\right)$$

$$\overline{F_m} = j \cdot F_o \cdot \left(\frac{T_{col}}{T_o}\right) \cdot \left(\frac{p_o - p_w}{p_o}\right)$$

T_o – temperature on outlet
T_{col} – column temperature
p_w – partial pressure of water at T_o

retention quantities

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

$$V_{R,i} = F_M \cdot t_{R,i}$$

void volume / time of column — V_m [ml], t_m [min]

$$V_m = F_M \cdot t_m = V_M$$

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

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

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

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

net retention volume — V_N [min]

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

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

specific volume — V_h [ml/g] or V_p [ml/m^2]

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

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

V_N related to 1 g or 1 m^2 SF and to 0 °C

temperature influence

$$T_{col} > T_{boil} \wedge T_{inj} \geq T_{col} \wedge T_{det} > T_{col}$$

T_{inj} – injection head temperature
T_{col} – column thermostat temperature
T_{det} – detector temperature

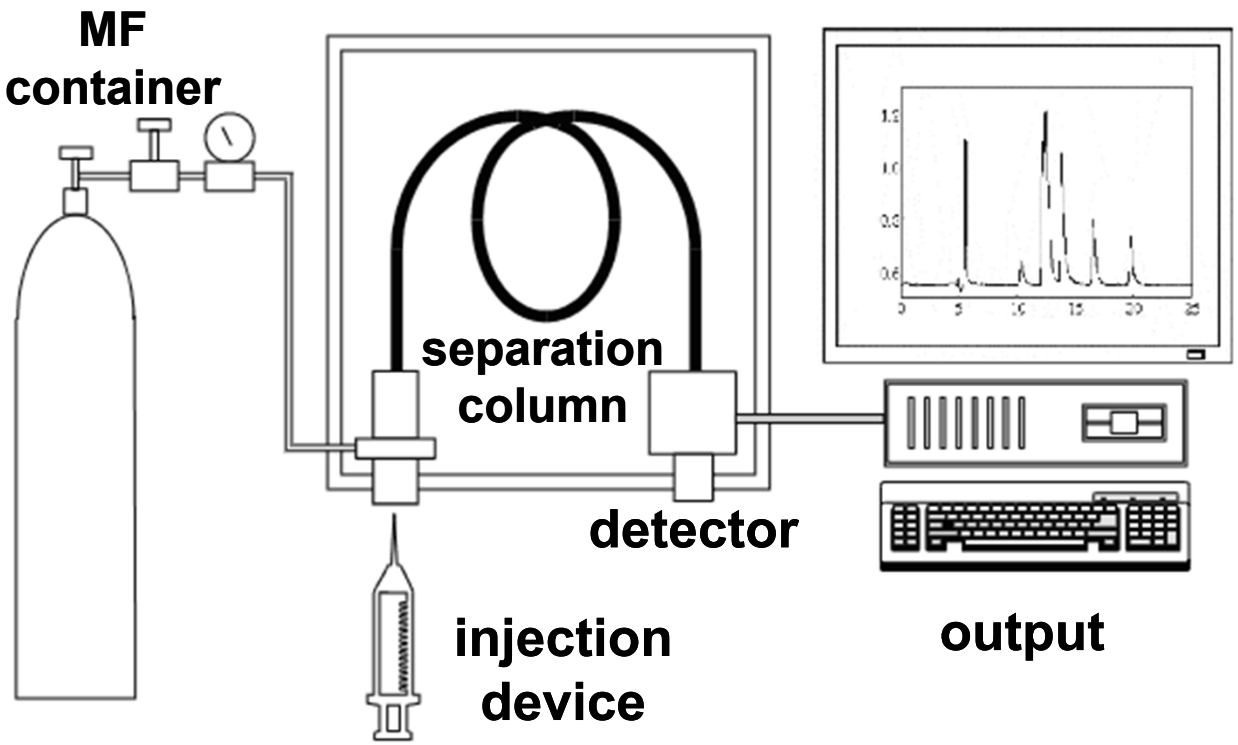
↑ T_{col} leads to faster analysis

↑ T_{col} demands ↑ MF pressure on column inlet for keeping **u** through column

isothermic analysis: $T_{col} = const.$

analysis with temperature gradient: $T_{col2} - T_{col1} > 0$

GC arrangement

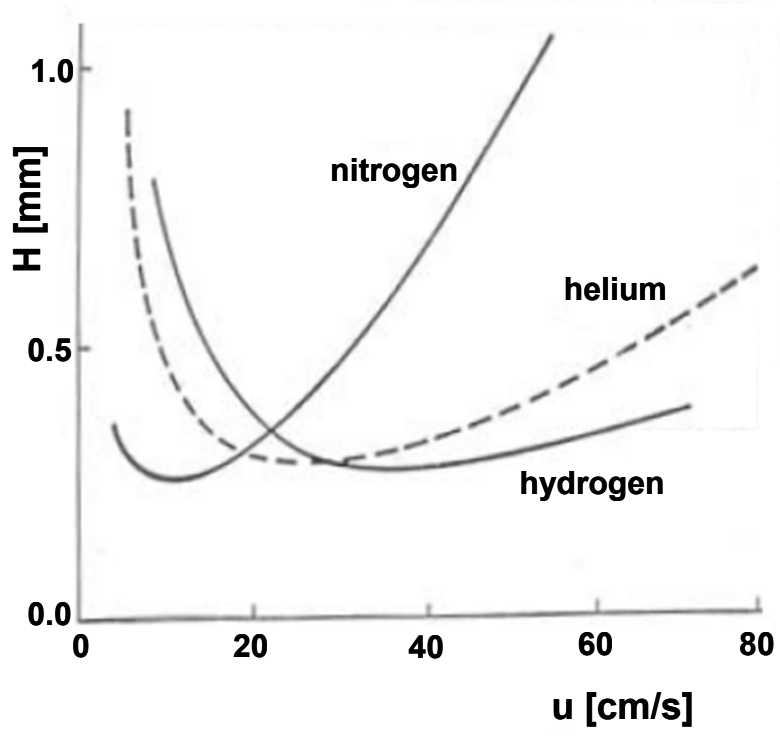


MF delivery

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

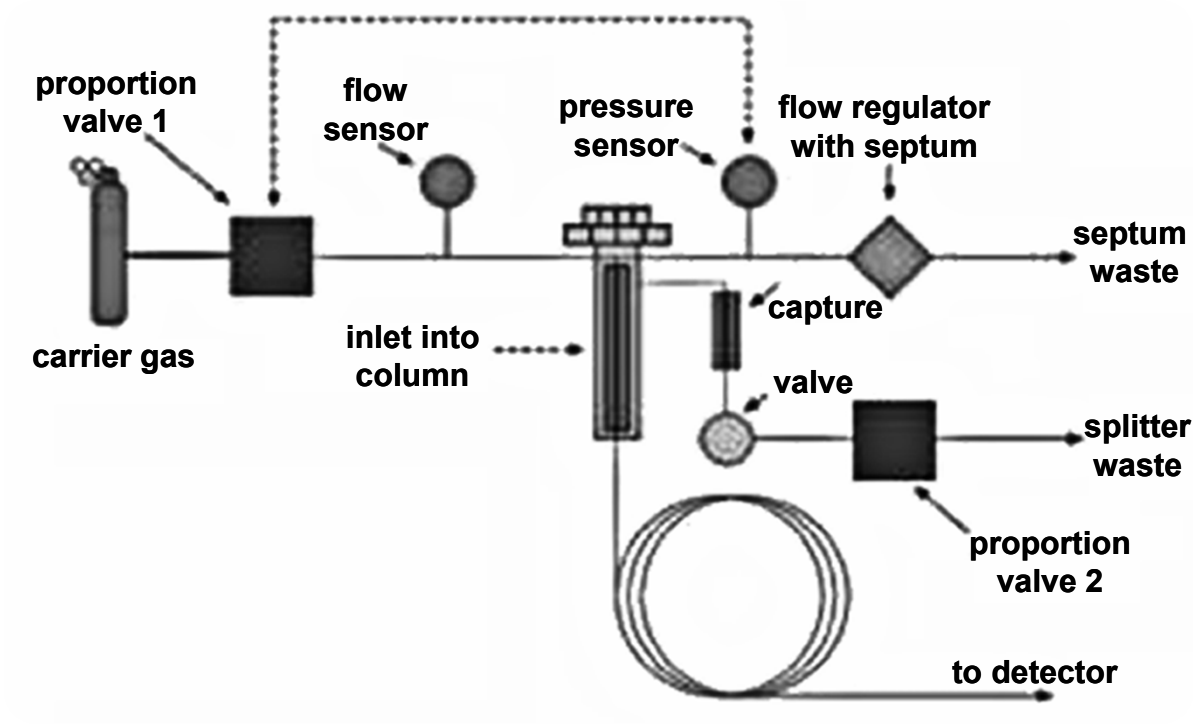
necessity to transform L and S samples into G state
: without changing the nature of sample

heated space on the beginning of the column

volatility increment

chemical derivatisation: *silylation* (N,O-bis(trimethylsilyl)acetamide)
silanisation (dimethylchlorsilane)
and *acetylation* (acetanhydride)

splitless injection



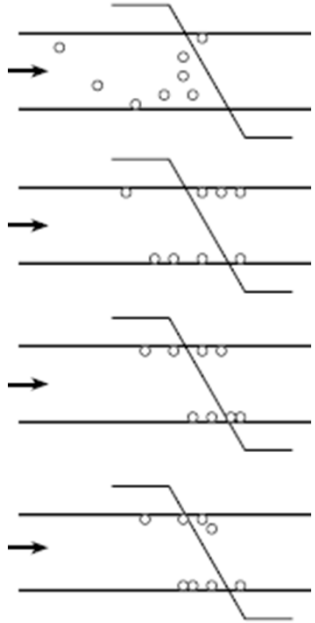
: with closed valve pressurise using proportion valve 1: flow sensor = 5 ml/min, pressure sensor = 70 kPa

: 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

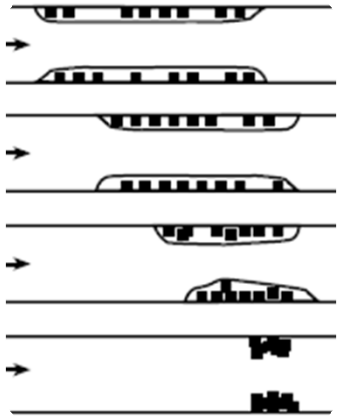


cold trapping

: 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 re-concentration

solvent effect

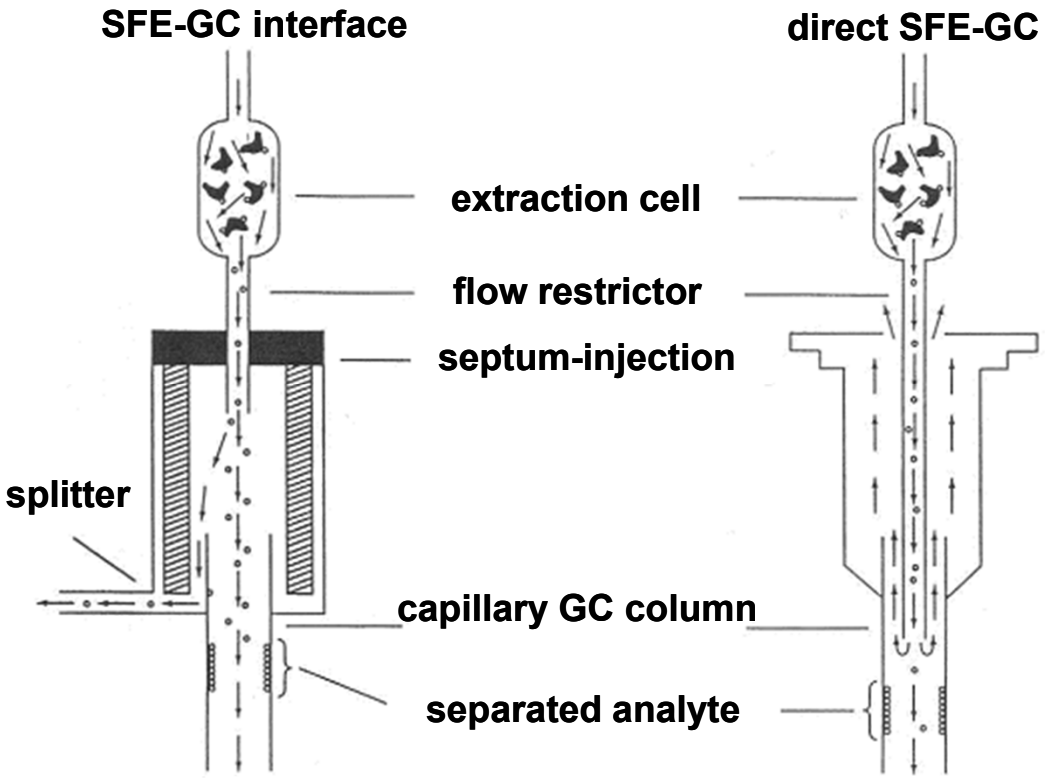


: first few centimetres of column has negative temperature gradient
(~ 250 °C /injection/ >> capture region is *ca* 20 °C bellow solvent T_{boil})

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

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

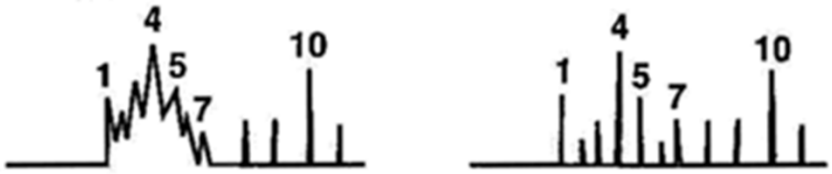
hyphenation of SFE with GC
(cold-trapping)



separation of supercritical fluid from sample increases quality GC analysis

separation by means of cold-trapping

1. T_{col} in time (t = 0) ≤ 25 °C
2. d_f ≥ 2 µm SF



a) w/o utilisation

b) w/ utilisation

split injection

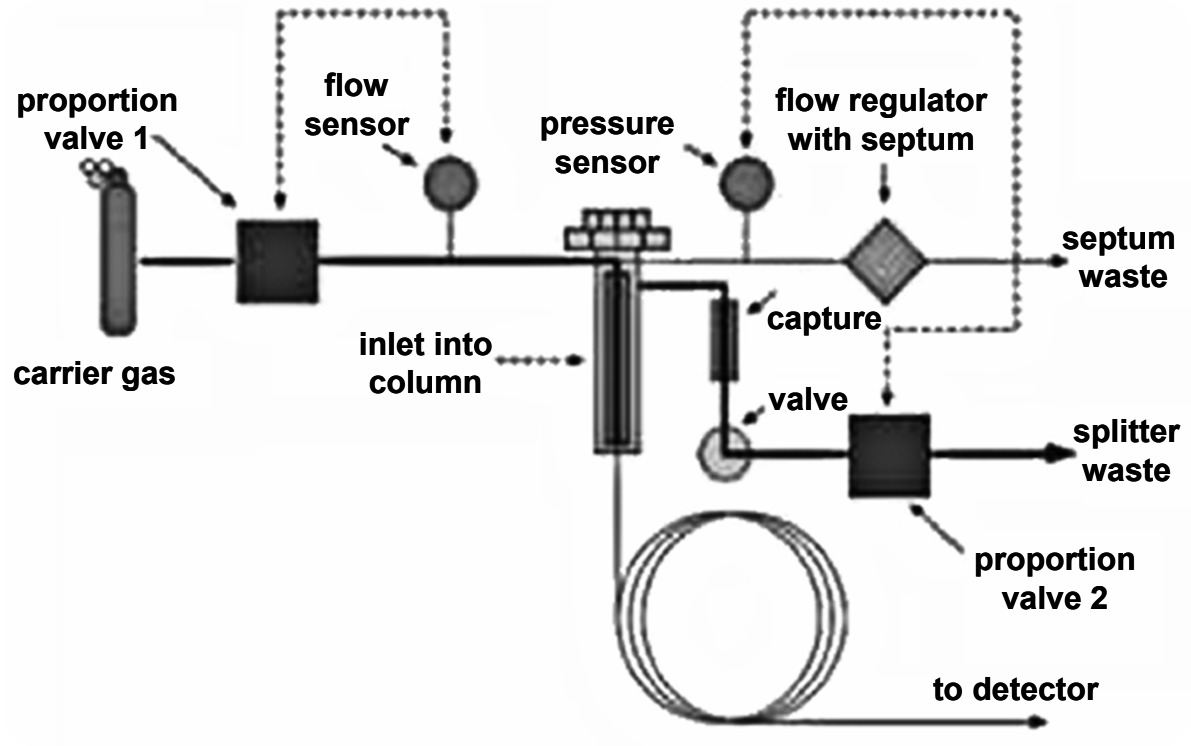
splitter allows: easy injection of *small volume*
: is related to sharp zone entering onto column and column capacity

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

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

gas injection

step 1
equilibration

step 2
HSE syphoning

step 3
injection

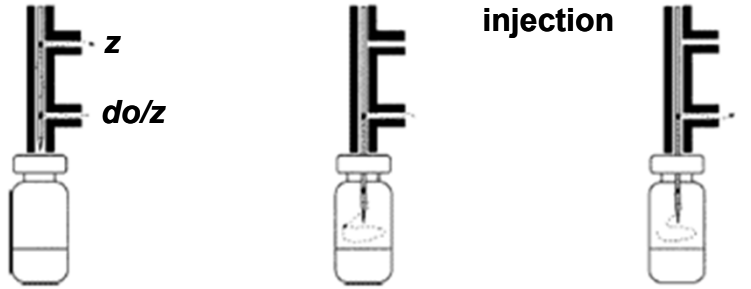
hyphenation HSE-GC

injection in system with pressure equilibrium

step 1
equilibration

step 2
pressurising

step 3
syphoning and
injection

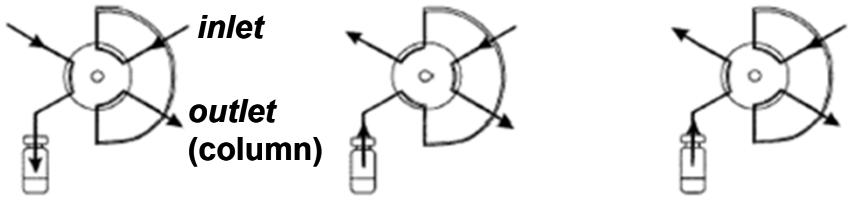


pressure valve

step 1
pressurising

step 2
HSE syphoning

step 3
injection



separation column

tubular
: analytical
: preparative

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

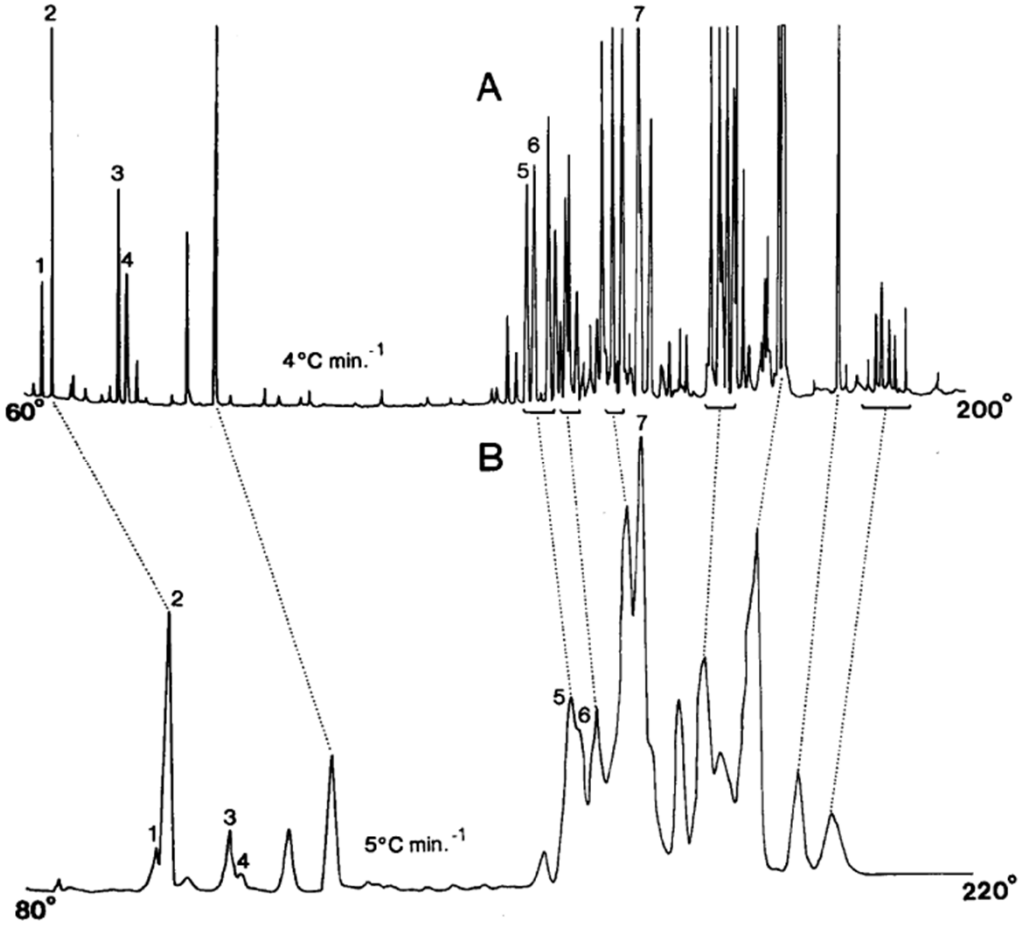
length: 2 – 6 m
diameter: > 6 mm

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

tubular columns

cover: glass, steel, copper, polymers

carriers

modified infusorial earth

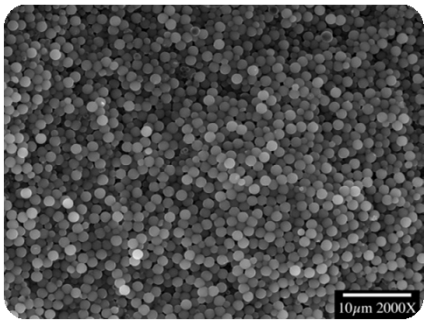
active centres (silanols and siloxanes) ⇒ tailing of more polar components

suppression – *silylation*

adsorbents

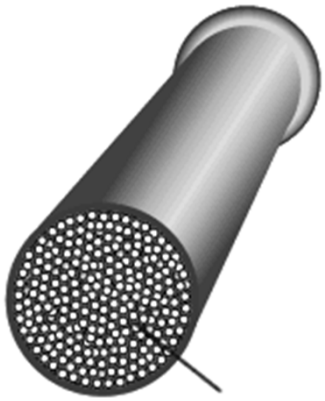
: *unspecific* (activated carbon)

: *specific* (silicagel, alumina, molecular sieves etc.)



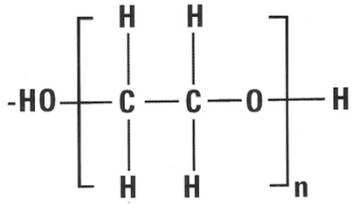
carrier – fine, solid and inert material (spherical silicagel)

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



solid SF

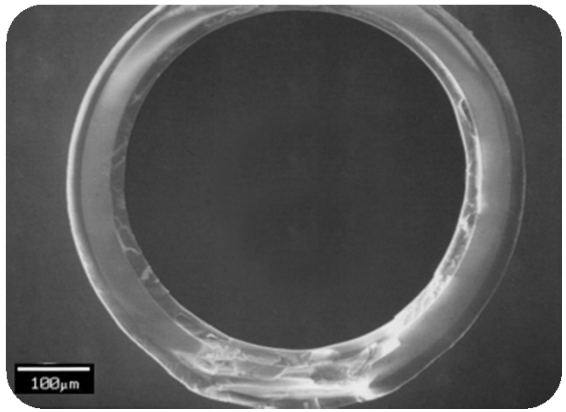
non-polar
: methylated polysiloxane, squalene, apolane C-87



mildly polar
: phenylated polysiloxane

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

capillary columns



silica

surface enlargement by etching
polyimide cover ⇒ increase of mechanical stability

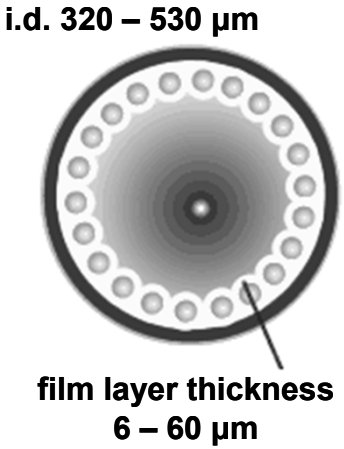
SF universal non-polar silicon phases or immobilised Carbowax

wall-coated open tubular columns

(WCOT)
liquid SF anchored directly on the capillary wall
: GLC

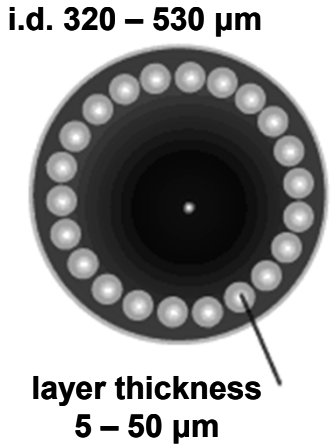
fused silica open tubular

(FSOT)
thin wall with outer polyimide cover (mechanical stability)
: GSC



support-coated open tubular columns

(SCOT)
carrier is on capillary wall, SF is on it
: GLC



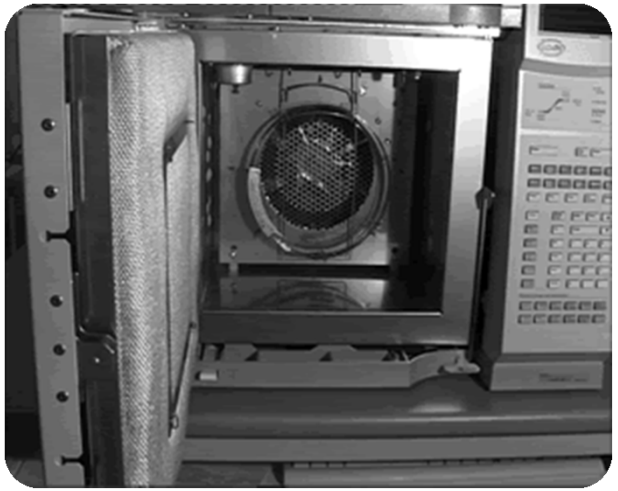
porous-layer open tubular columns

(PLOT)
layer of solid active sorbent on an inner capillary wall
: GSC

column thermostat

importance of temperature of GC

: 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

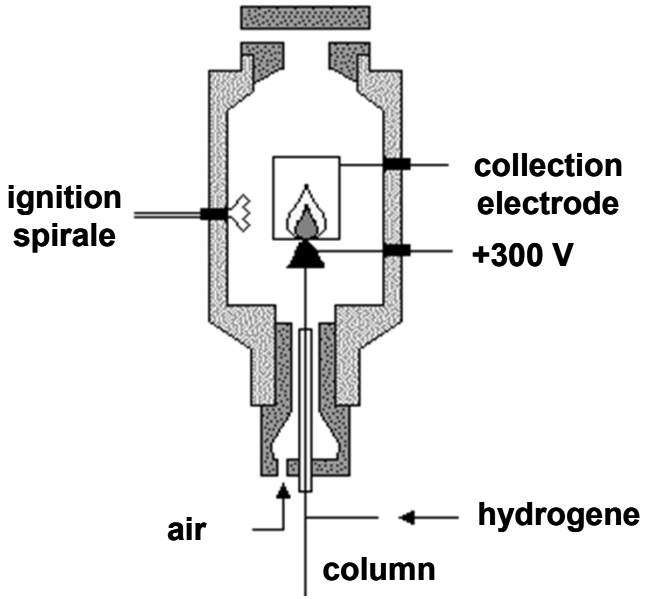
wide range of T_{boil} of separated components ⇒
⇒ *temperature programme / column gradient* (Δ temperature during experiment)
temperature may be increased gradually or in steps

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

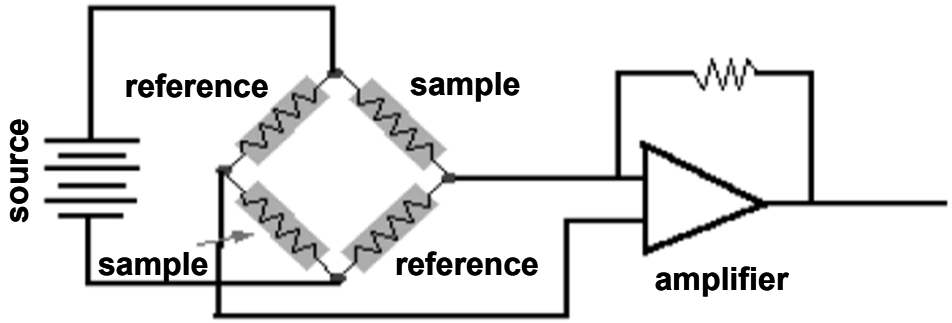


flame ionisation detector

FID

MDD
signal: current created by pyrolysis of carbon sample

: **noise** 10^{-13}
: **dyn. range** 10^{7}
: **sensitivity** 10^{-9} M



thermal conductivity detector

TCD
catharometer

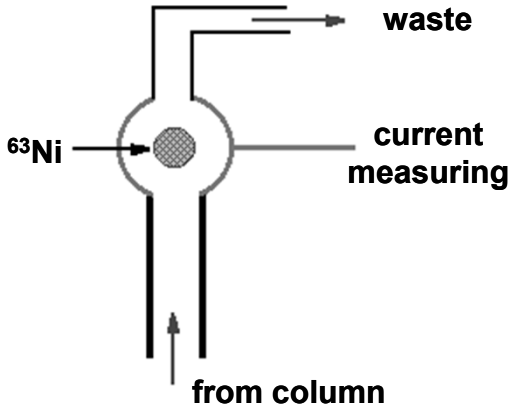
: noise 10^{-5}
: dyn. range 10^{6}
: sensitivity 10^{-8} M

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

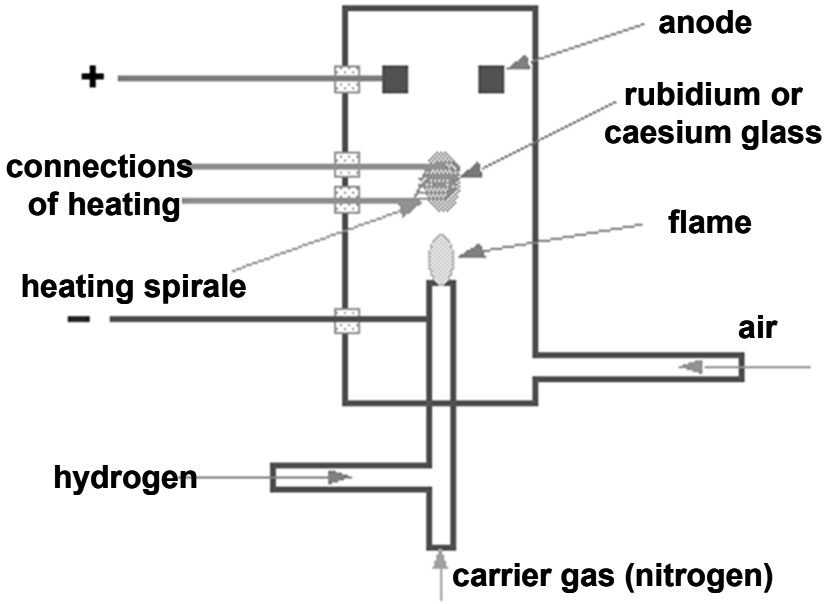
: noise 10^{-12}
: dyn. range 10^{5}
: sensitivity 10^{-13} M



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

nitrogen phosphorus detector

NPD – thermoionisation detector

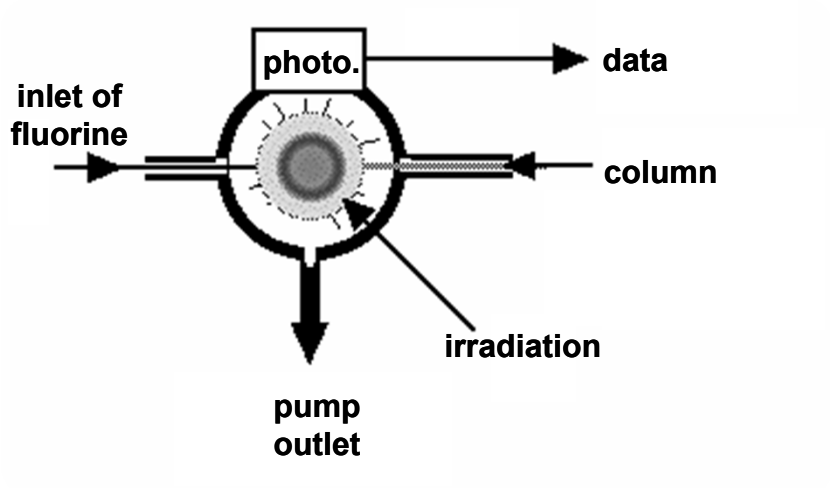


: noise 10^{-12}
: dyn. range 10^{6}
: sensitivity 10^{-10} M

MDD

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

chemoluminiscence detector



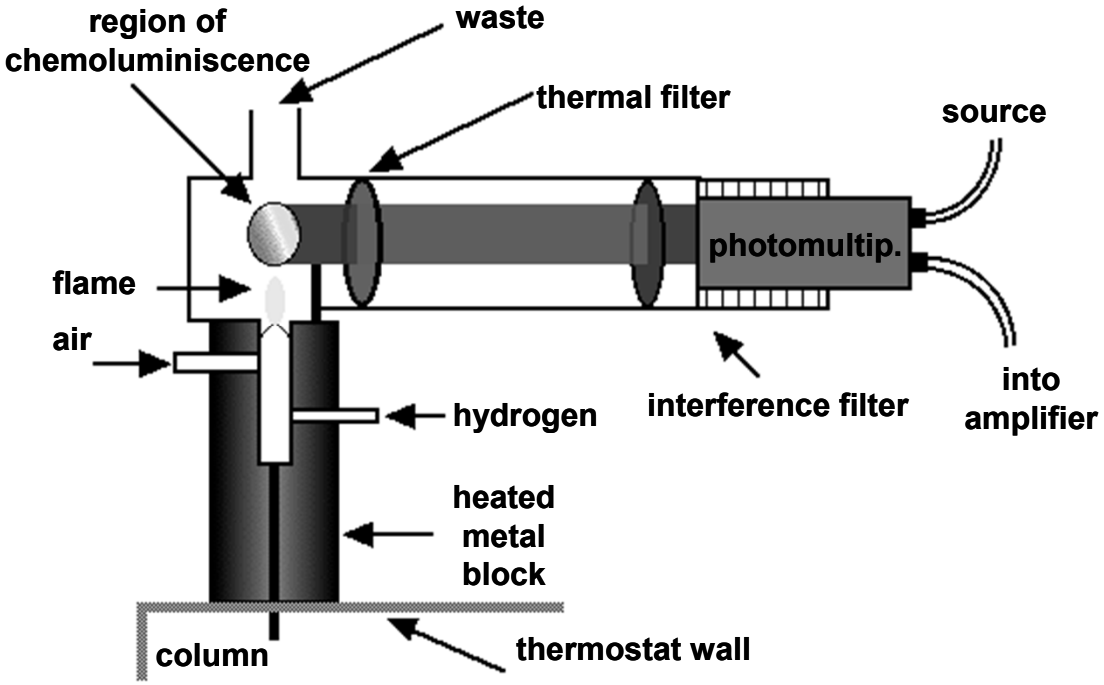
: noise 10^{-13}
: dyn. range 10^{4}
: sensitivity 10^{-11} M

CDD

signal: reaction of F (strong oxidant) with analyte

flame photometric detector

FPD



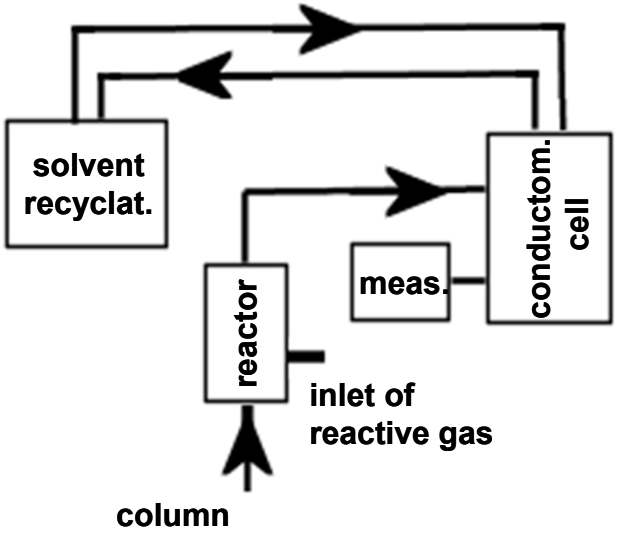
: **noise** 10^{-12}
: **dyn. range** 10^{7}
: **sensitivity** 10^{-10} M

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

electrolytic conductivity detector

ELCD

: **noise** 10^{-13}
: **dyn. range** 10^{6}
: **sensitivity** 10^{-11} M



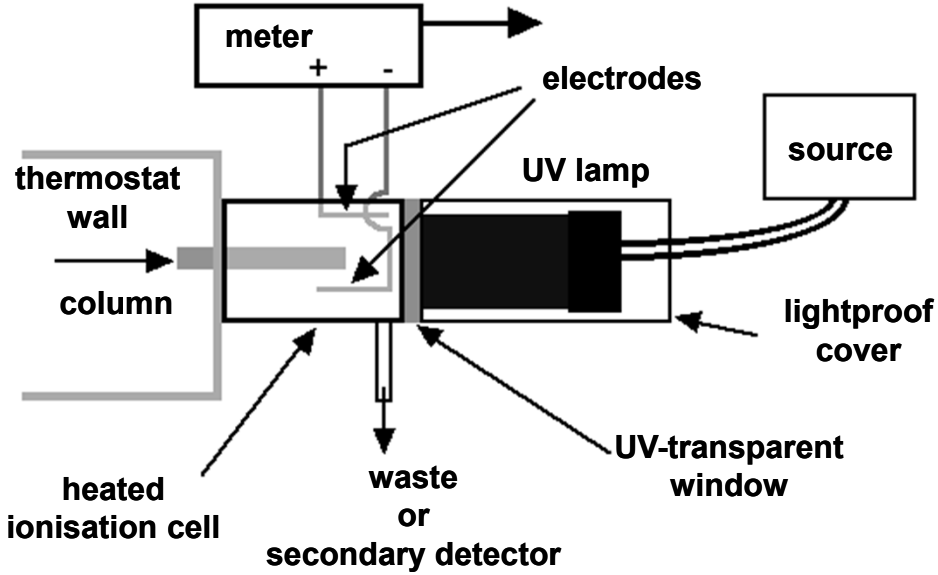
MDD
signal: appearance of special products
their conductivity measurement after mixing with solvent

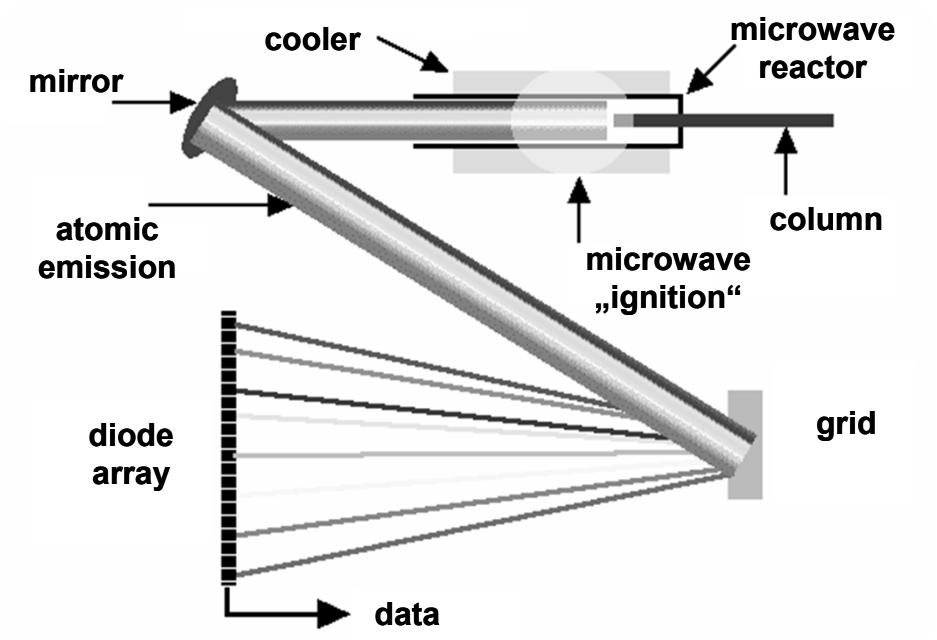
photoionisation detector

PID

: **noise** 10^{-13}
: **dyn. range** 10^{7}
: **sensitivity** 10^{-11} M

CDD
signal: UV-irradiation ionisation



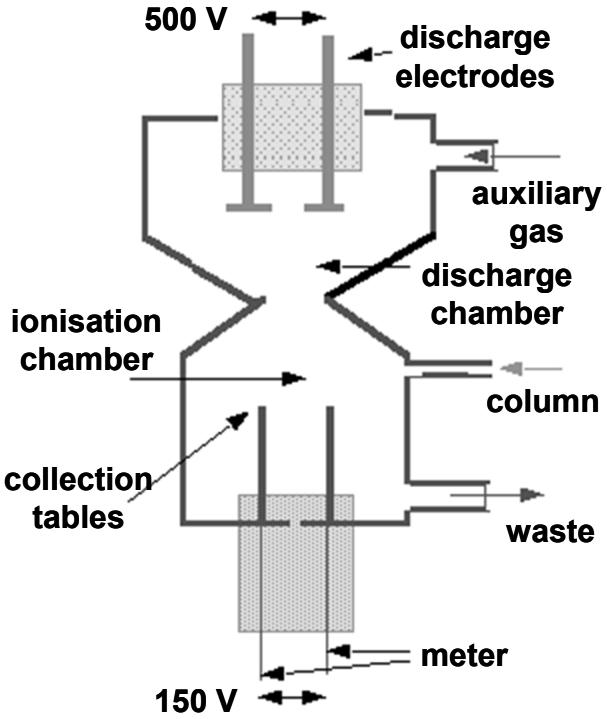


atomic emission detector

AED

: **noise** 10^{-14}
: **dyn. range** 10^{4}
: **sensitivity** 10^{-11} M

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^{-12} M

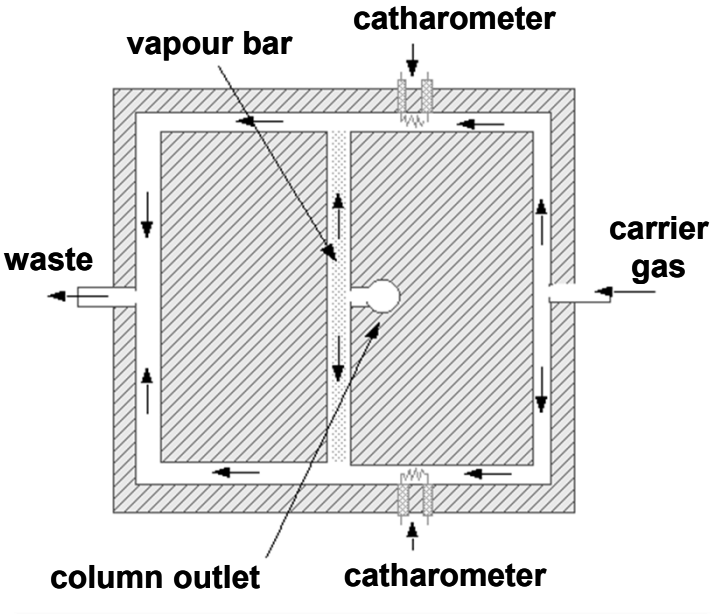
MDD
signal: auxiliary gas is ionised first (He, Ar),
its ions then secondary ionise sample molecules

gas density balance

GDB

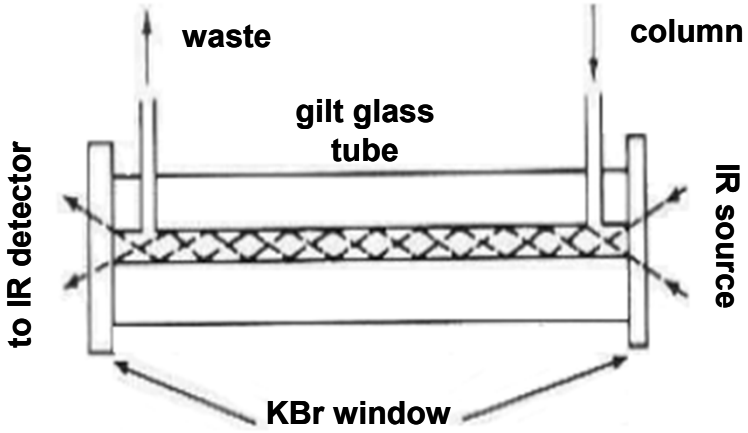
: noise 10^{-8}
: dyn. range 10^{3}
: sensitivity 10^{-8} M

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^{-10} M

CDD
signal: IR absorbance

mass spectrometric detector

MS

: **noise** 10^{-14}
: **dyn. range** 10^{3}
: **sensitivity** 10^{-15} M

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
: 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)

$$V_p = \frac{273.15 \cdot V_N}{S \cdot T_{col}}$$

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
: because of narrow peaks frequently only height

internal normalisation method

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

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

external standard method
(absolute calibration; calibration curve)

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

$$c_{unknown} = \frac{A_{uknown}}{A_{known}} \cdot c_{known}$$

internal standard method

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

: need not to know injection volume

: standard must be chemically similar to analyte

standard addition method

: presumes calibration curve linearity

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

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

test measurements in GC

column testing

in dependence on time (at const. flow rate) ***we observe***

efficiency

: normalised retention times of components
: height of peaks
: symmetry of peaks

testing mixture for uncoated carriers

n-decane, 1-aminoacetate, 3,5-dimethylpyrimidine, n-dodecane, 1-aminodecane, 2,6-dimethyl-aniline, N,N-dicyclohexylamine, 1-aminododecane and n-heptadecane

MF – H_2, $\mathbf{T_{initial}}$ = 40 °C, $\mathbf{T_{terminal}}$ = 180 °C

testing mixture for coated carriers **(Grob test)**

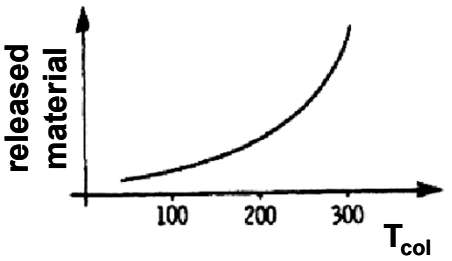
methyl decanoate, methyl undecanoate, methyl dodecanoate, n-decane, n-undecane, n-dodecane, 1-octanol, nonanal, 2,3-butanediol, 2,6-dimethylaniline, 2,6-dimethyl-phenol, dicyclohexylamine, 2-ethylhexanoic acid

MF – H_2 or He, $\mathbf{T_{initial}}$ = 40 °C, $\mathbf{T_{terminal}}$= 100 °C, resp. 175 °C

thermostability ***column bleeding***

n-C_{22}

MF – He, $\mathbf{T_{initial}}$ = 40 °C, $\mathbf{T_{terminal}}$ = 300 °C



separation of macromolecules

separation of macromolecules 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*)

theoretical fundaments of separation of macromolecules

what is that macromolecule?

molecule of M_W >10 000

synthetic polymers

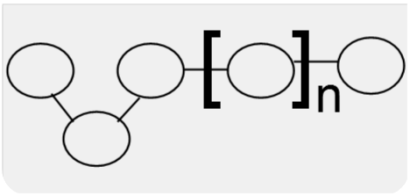
monomer, oligomer (10 – 100), polymer

homopolymers (PE, PP, PS, PTFE...)

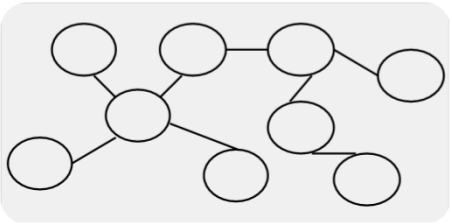
$$nM \rightarrow [M]_n$$

: one repeated unit (monomer)

linear



branched



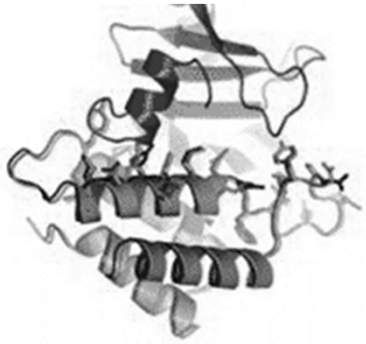
heteropolymers

: more of different units

$$nX + mY \rightarrow X_n Y_m$$

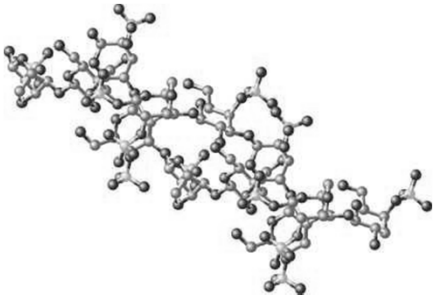
biological polymers

$M_W \approx 10\,000 – 1\,000\,000$



proteins

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



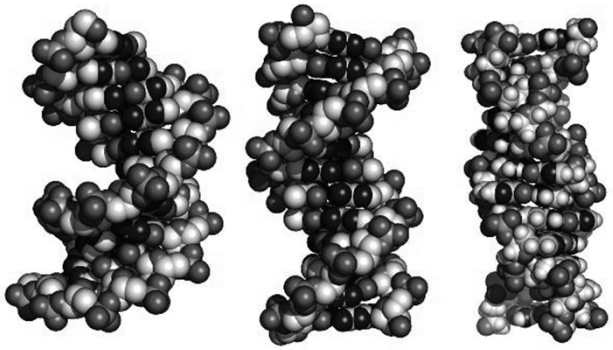
glycans (polysaccharides, oligosaccharides)

(starch, glycogen, chitin, cellulose, dextrans, pullulans)

nucleic acids (polynucleotides, oligonucleotides)

nucleotide = phosphate + nucleoside
nucleoside = saccharide + base

DNA – saccharide – deoxyribose
RNA – saccharide – ribose



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

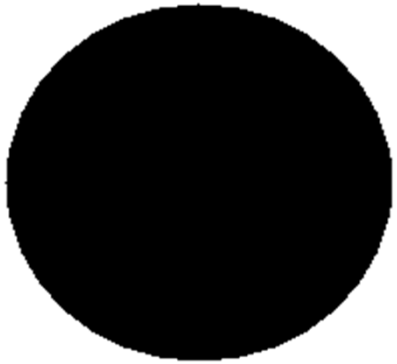
primary ⇒ *secondary, tertiary, ternary structure – native form*

description of macromolecule

macroscopic forms

random coil

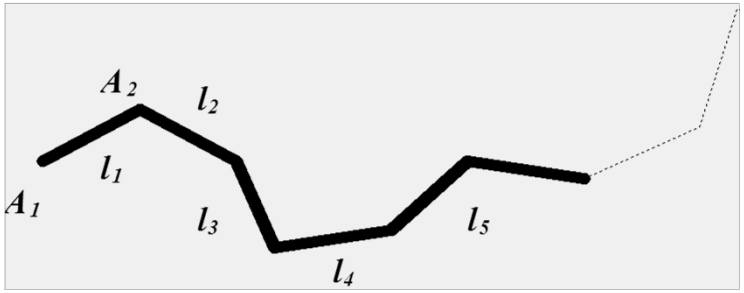
rod



sphere

size of macromolecule

flexible molecule



contour length (**L**)

$$L = n \cdot l$$

n – number of bonds
l – monomer length

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

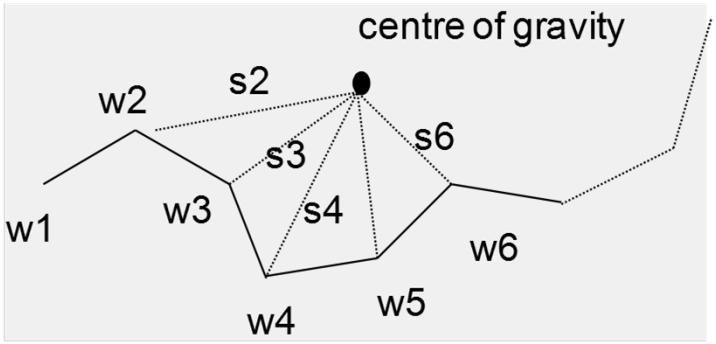
$$\vec{r} = \sum_i \vec{l}$$

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*)

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

for macromolecules

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

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

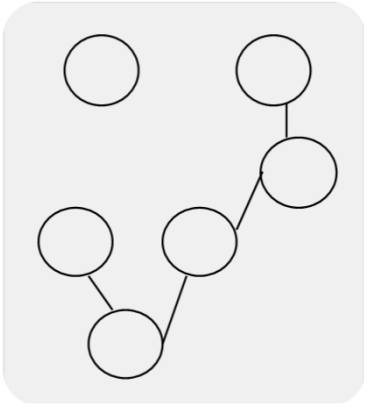
$$\Rightarrow P = \frac{M_w}{M_n} \geq 1$$ polydispersity
~ distribution

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

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

example 8

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



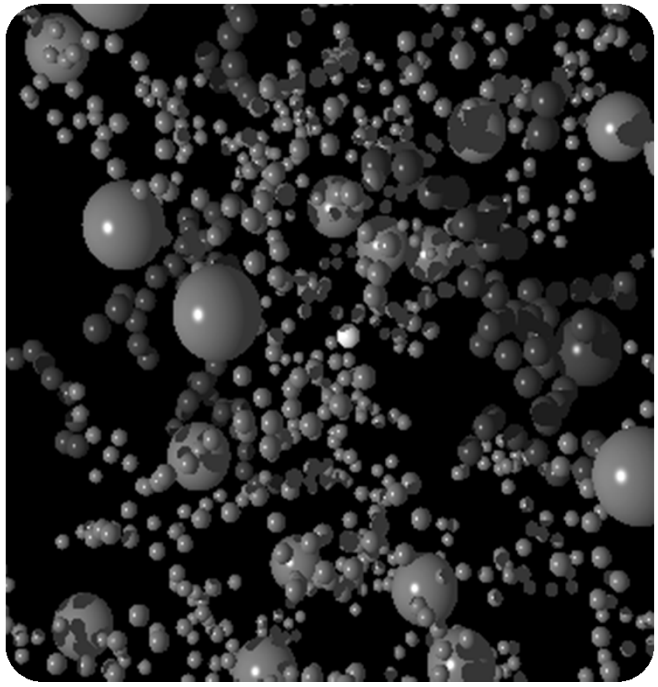
???

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)



SEC, size exclusion chromatography

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

principle

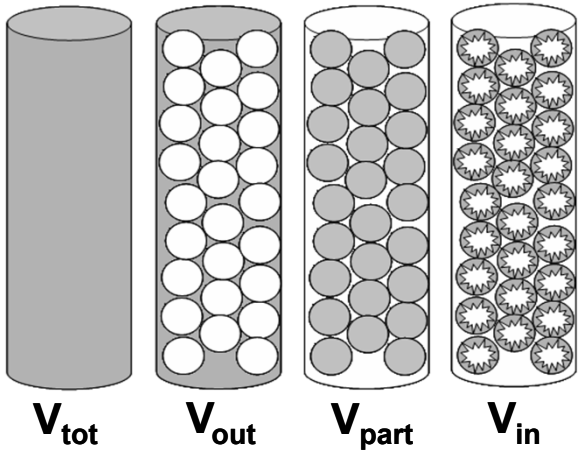
: analyte is distributed between MF outside of particles and inside of particles
:: sieving effect, steric exclusion
:: diffusion
:: pressure of carrier liquid – motion of liquid and its flow profile

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

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

V_R – retention volume
K'_D – distribution constant

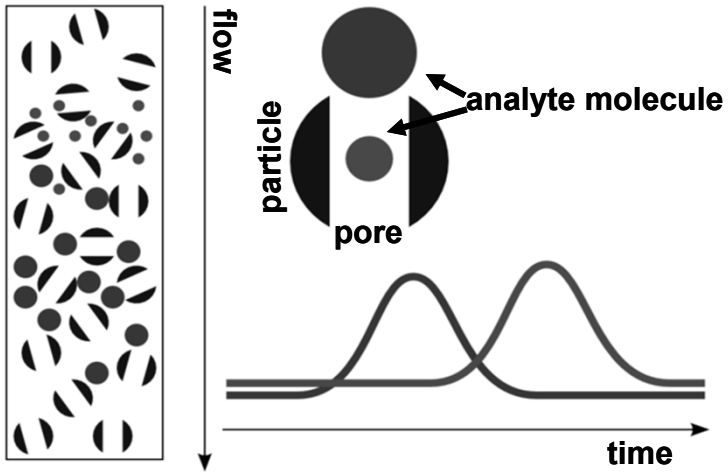
tot – total volume
out – MF outside of particles
in – MF inside of particles
part – volume of particle material

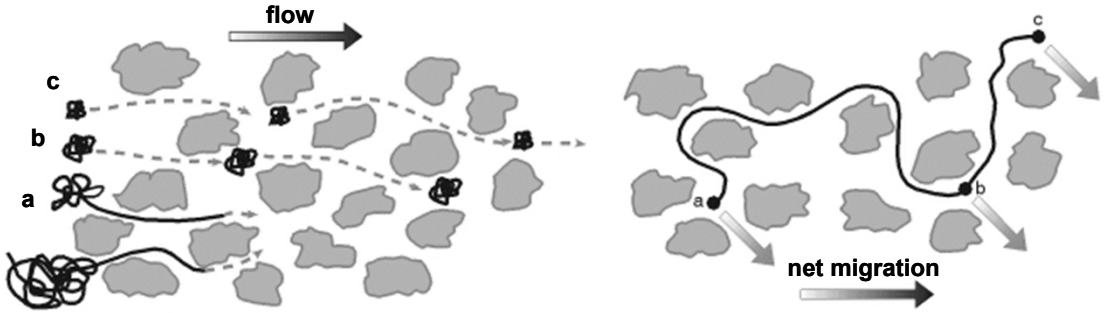


$$V_R = V_{out} + K'_{AV} \cdot (V_{tot} - V_{out})$$ where $$(V_{tot} - V_{out}) = V_{in} + V_{part}$$

K'$_{AV}$ – elution constant

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





molecular sieve effect
: uniform pore diameter (determines cut-off)
: distribution of pores with different diameter

thermodynamic interpretation

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

$\Delta H \sim 0 \Rightarrow$ process is **entropically controlled**

$$K'_D = \frac{c_A^{in}}{c_A^{out}}$$

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

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

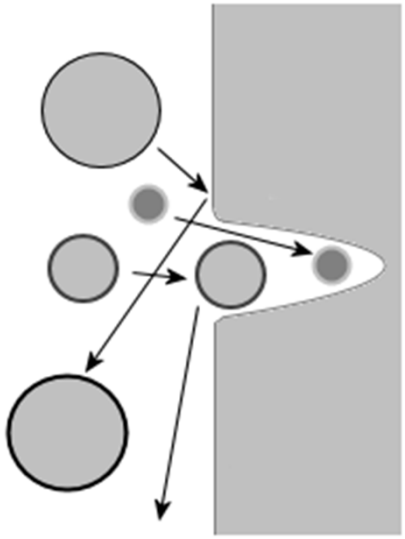
$\mathbf{k_1}$, $\mathbf{k_2}$ – numeric constants

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

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

$$K_D = \frac{a_A^{qS}}{a_A^{M}}$$

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

not classic LC, **no** chemical affinity

qS – quazi SF, ***M*** – MF

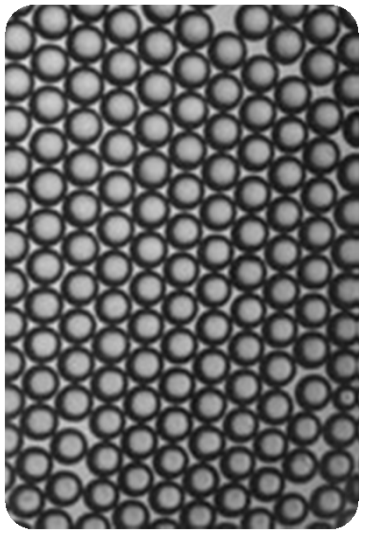
use of SEC

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

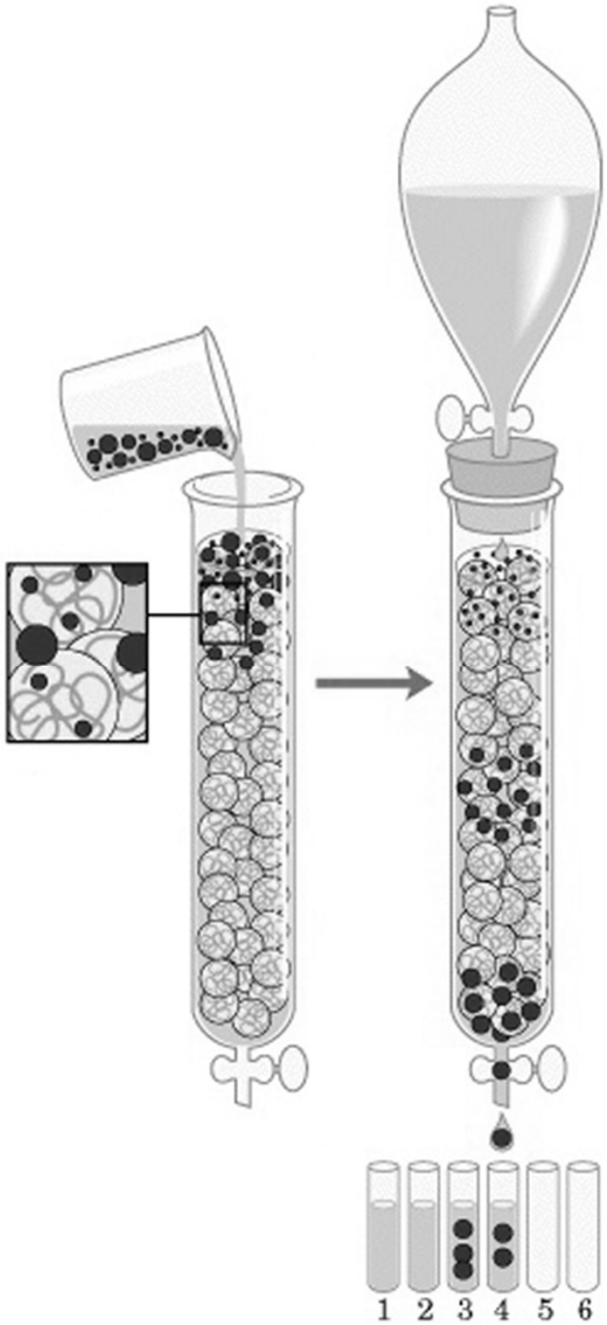
: comparison with standards (in line increasing M_W)
: polymer polydispersity and distribution

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

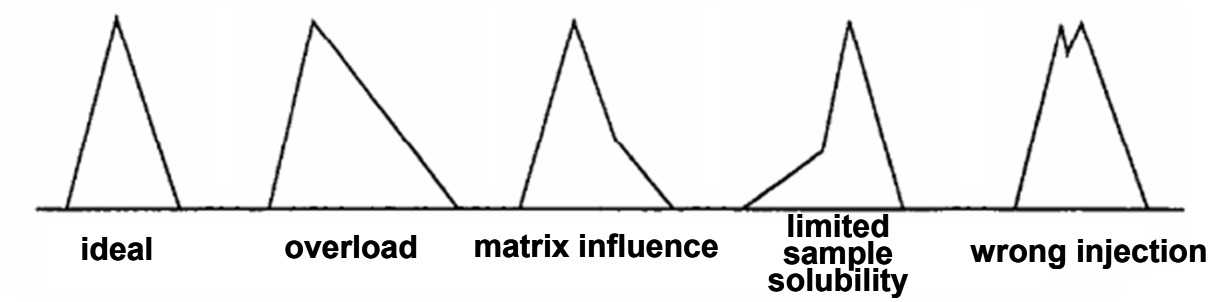
elution

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

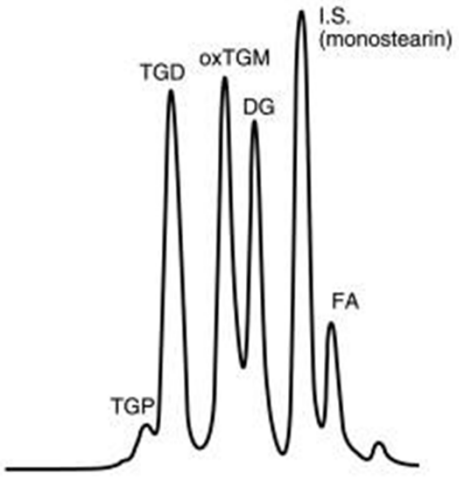
0.02 % sodium azide
0.05 % trichlorobutanol (Chloreton)
0.005 % ethylmercurythiosalicylate (Mertiolate)
0.002 % chlorhexidine

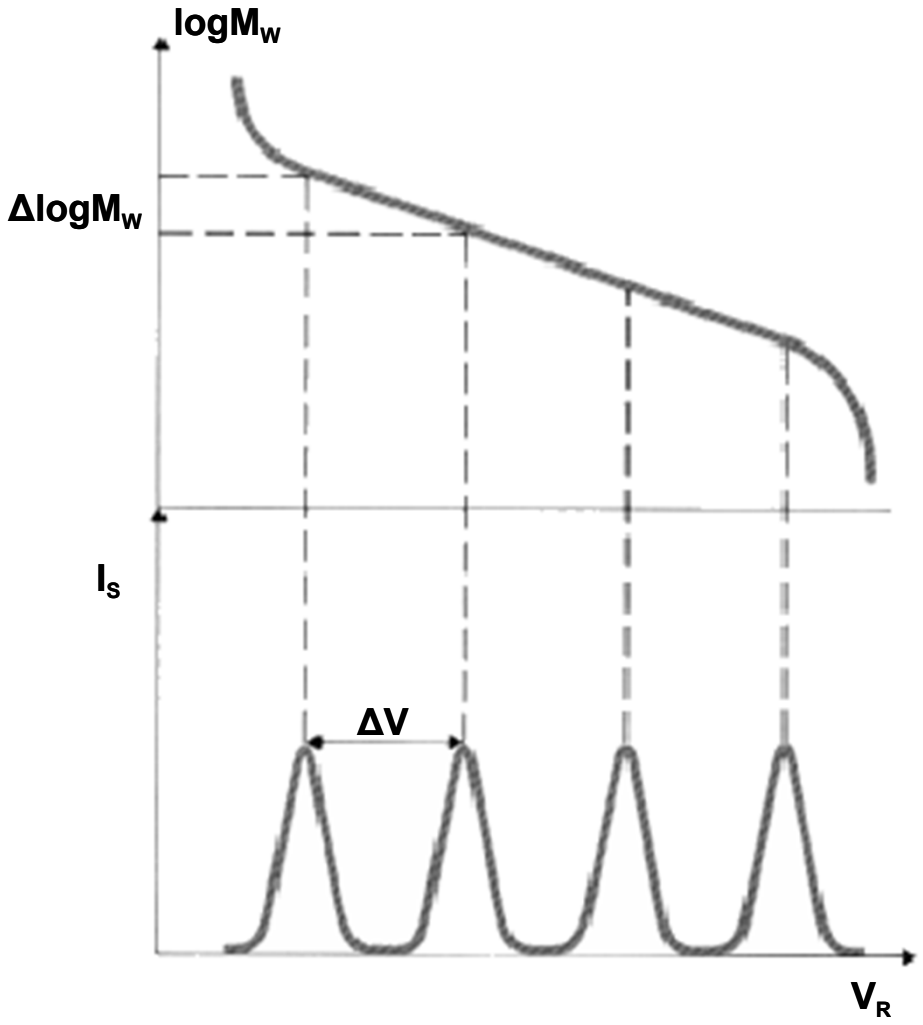


calibration

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

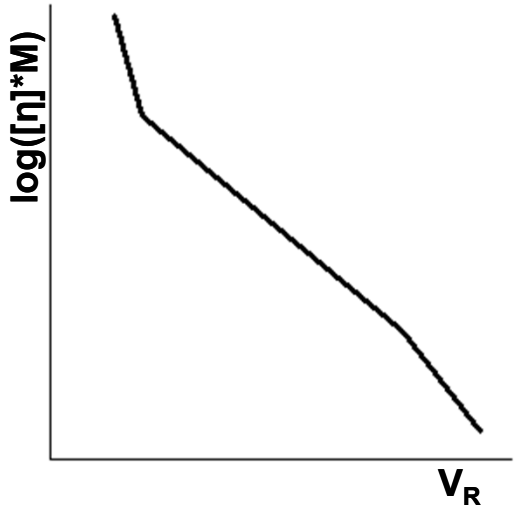
$$[\eta] = \lim_{\rho \to 0} \frac{(\eta / \eta_{solv}) - 1}{\rho} = \frac{k \cdot V_R}{M} \Rightarrow [\eta] \cdot M = k \cdot V_R$$

independent on macromolecule structure

$$[\eta] = K \cdot M^{\alpha} \qquad \Rightarrow \qquad [\eta]_A \cdot M_A = [\eta]_S \cdot M_S = f(V_R)$$

Mark-Houwink's equation

A – analyte, **S** – standard



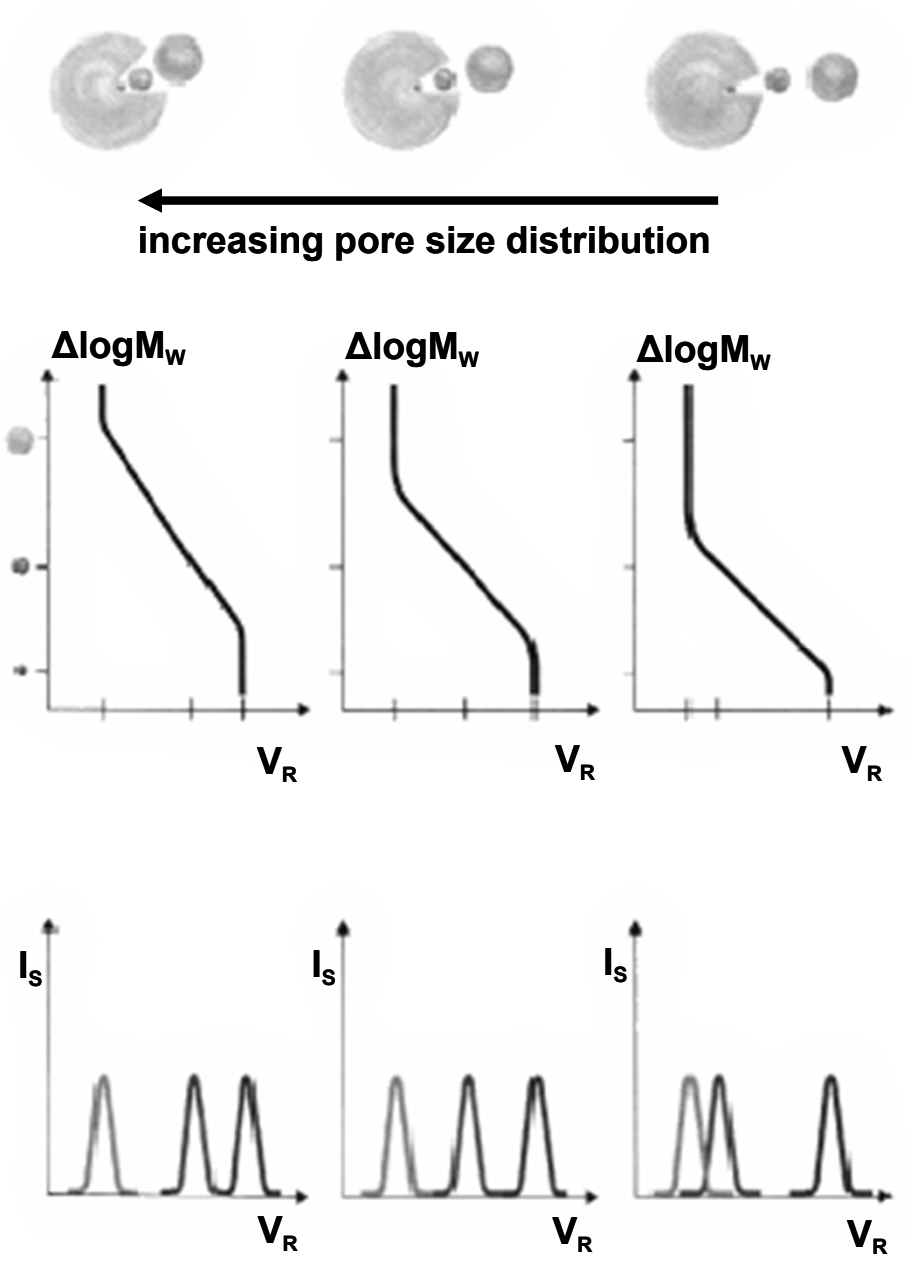
$$K_A \cdot M_A^{\alpha_A + 1} = K_S \cdot M_S^{\alpha_S + 1}$$

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

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

[η] – by viscosimetry

selectivity in relation to 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

hydroxymethyl metacrylate + ethylendimethyl methacrylate
elution: polar and non-polar solvents

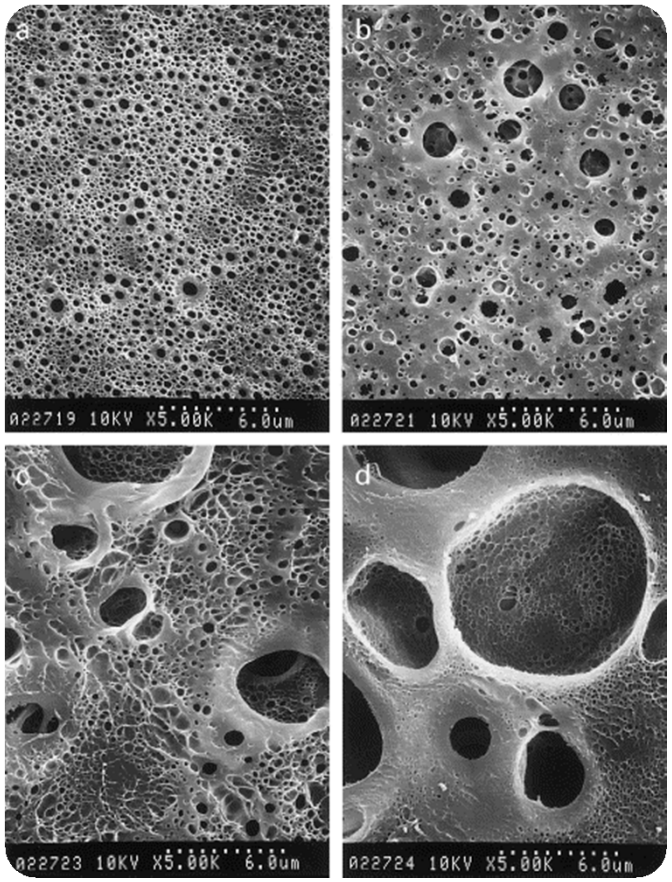
Spheron

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 ⇒ indirect detection

refractometric detector

: universal

fluorimetric (fluorescence) detector

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

viscosimetric detector

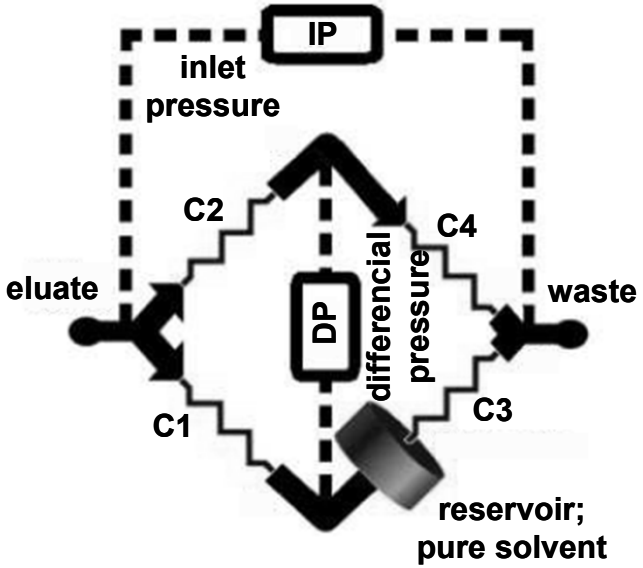
MDD

signal: pressure unbalance in bridge

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

$$[\eta] = KM^{\alpha} = \lim_{\rho \to 0} \frac{(\eta / \eta_{solv}) - 1}{\rho}$$

Mark-Houwink's equation



different **η** of solutions in **C1**,**C2**,**C4** & **C3** ⇒ **ΔP**

$$[\eta] = \frac{4 \cdot \Delta P}{P_{IP} - 2 \cdot \Delta P}$$

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

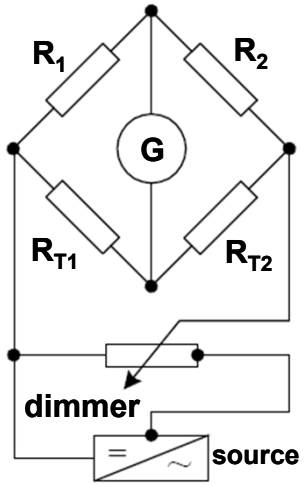
osmometric detector

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

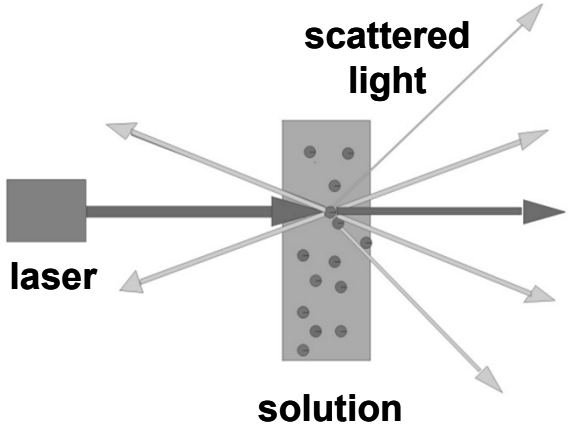
scattering of light beam on particles of suspension or colloid solution

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)

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

c – concentration
N – number of particles; scattering centres
$\bar{n}_0$ – refractive index of solvent
$(\partial n/\partial c)_{\mu}$ – particle refractive index changes at constant **μ**

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

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

intensity ratio of scattered (i_s) and original light I_0 (non-polarised)

V – unit volume
λ_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)

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

N_A – Avogadro's number
M – molecular mass

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

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

Rayleigh radius

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

summing constants into one, K

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

in polydisperse sample, **M** is substituted

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

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

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

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)

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

use of P(θ) parameter to express scattering

$$\frac{K \cdot c}{R_\theta} = \left[\frac{1}{P(\theta)}\right] \cdot \left[\frac{1}{M} + 2A_2 \cdot c\right] \quad \Rightarrow \quad \text{if } \mathbf{(1-x)^{-1} \approx (1+x)}$$

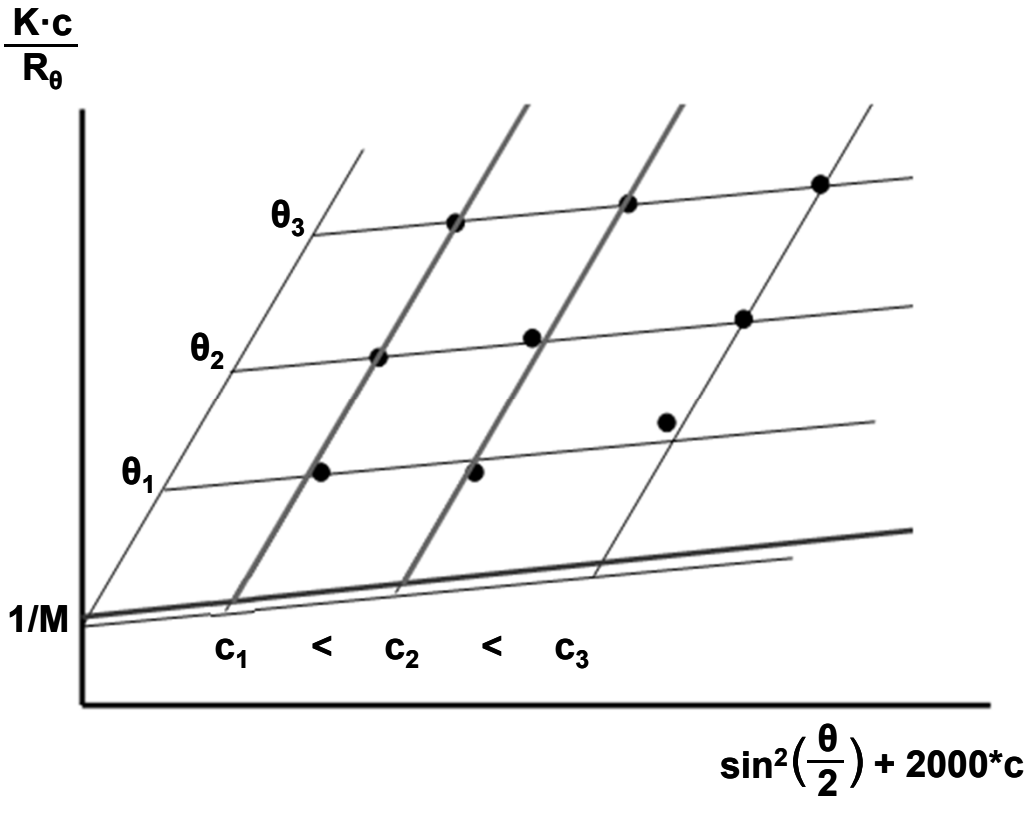
$$\Rightarrow \quad \frac{K \cdot c}{R_\theta} = \left[1 + \frac{16\pi^2 \langle s^2 \rangle}{3\lambda_0^{\,2}} \cdot \sin^2\left(\frac{\theta}{2}\right)\right] \cdot \left[\frac{1}{M} + 2A_2 \cdot c\right]$$

experimental bases for calculation of **gyration radius**

multiple angle laser light scattering

(MALLS)

Zimm's graph



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

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

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)

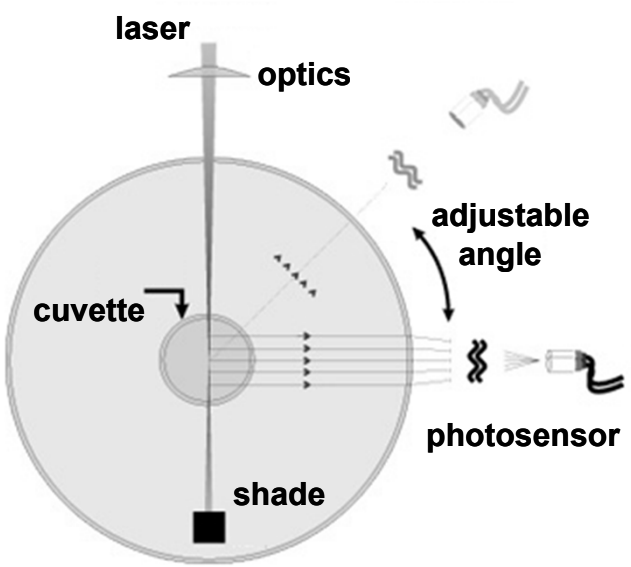
at small angles θ (< 7 °) $\sin^2(\theta/2) \sim 0 \Rightarrow P(\theta) \rightarrow 1$

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

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)

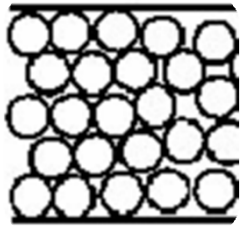
disadvantages:
: *dust* – demanding high solution purity

HC, hydrodynamic chromatography

principle

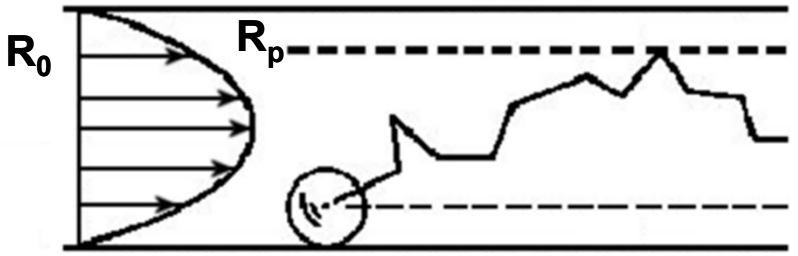
: combination of *steric exclusion* with *surface* (colloid) *interaction* sample-filling, eventually *solute retardation behind streamlines of laminar flow with profile* (**wall effect**)

sample moves with MF flow →



non-porous material

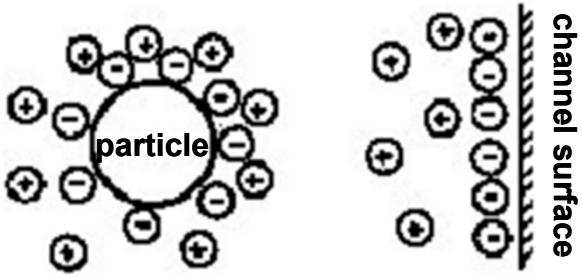
⇒



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

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

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



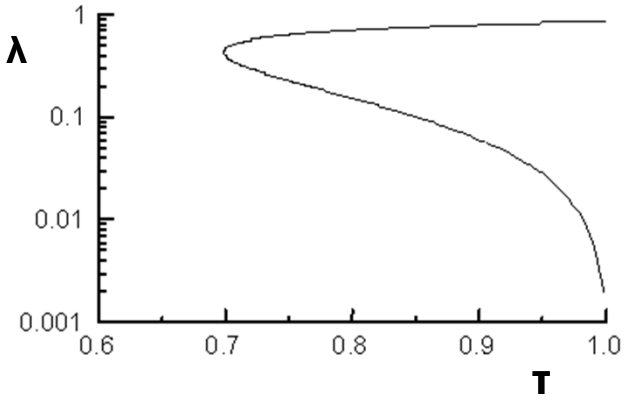
⇒ sample moves in channel *hydrodynamically* or *electrically*

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

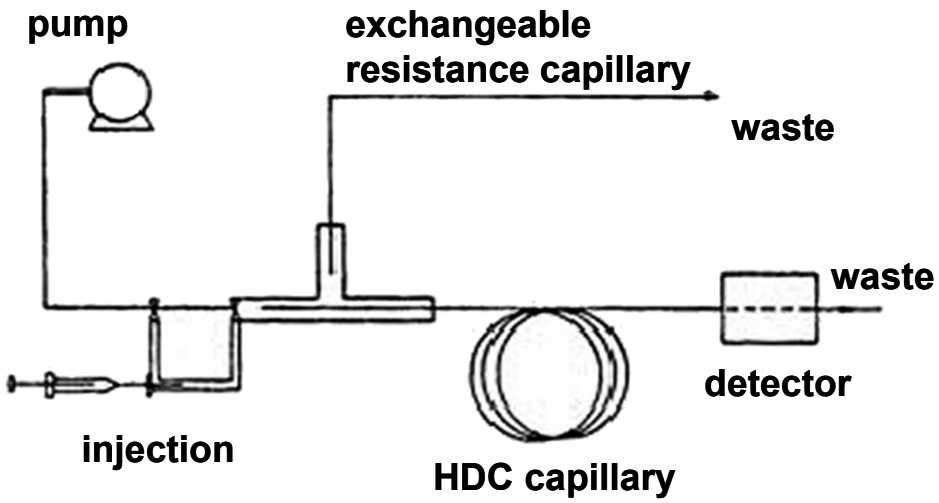
porous material

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*)

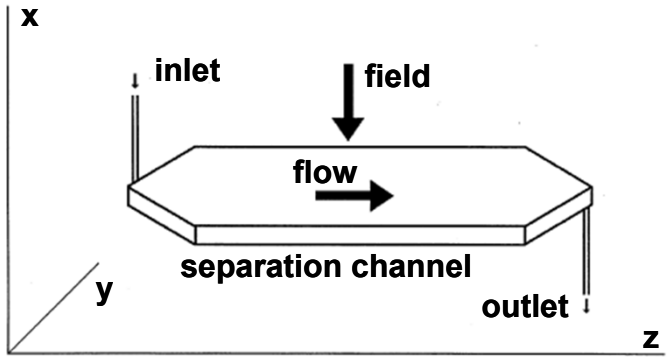


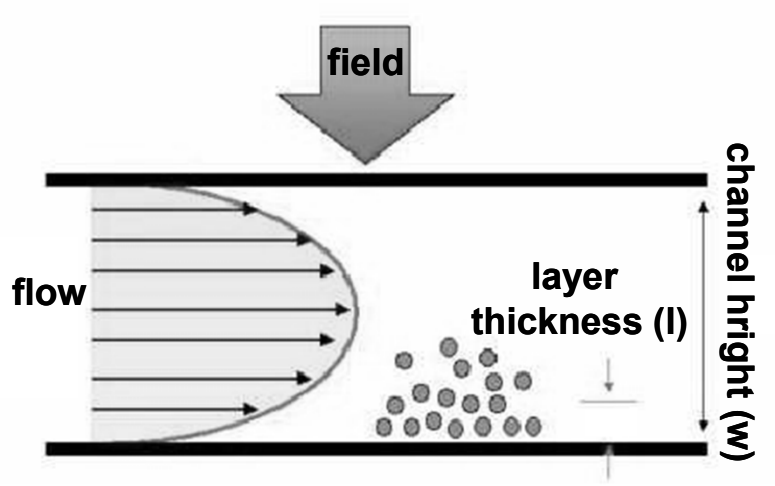
FFF, flow-field fractionation

1966 – J. C. Giddings

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





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

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)

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

$$\lambda = l / w$$

use of FFF

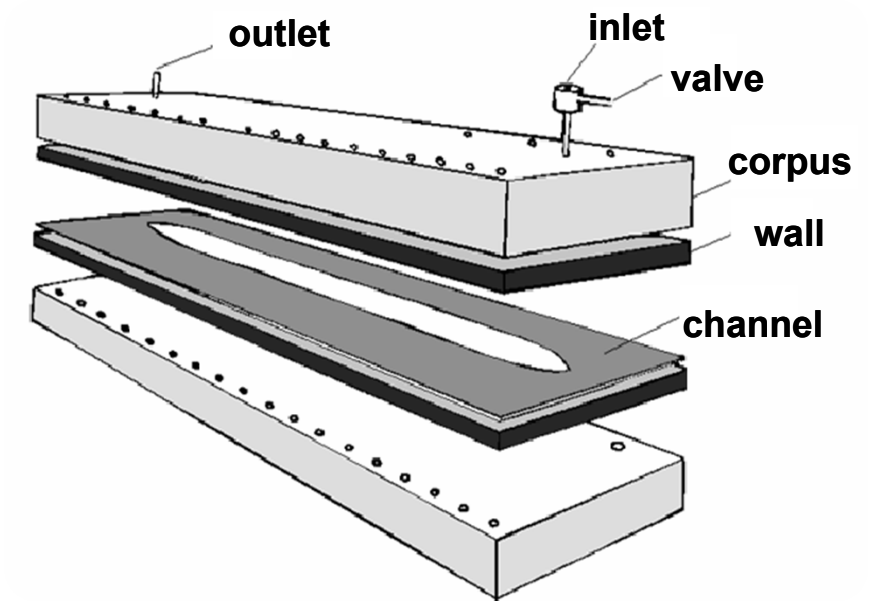
: no SF (one-phase chromatography) ⇒ no interactions with active surface

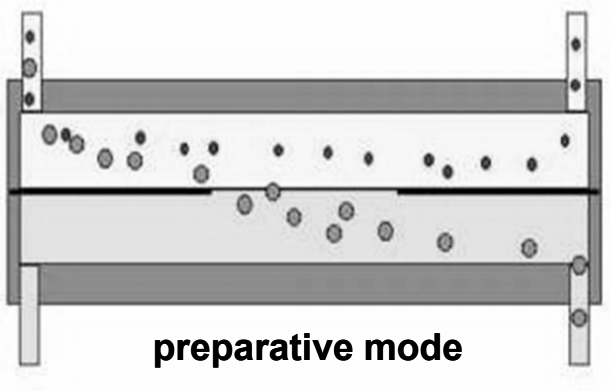
: MF is carrier liquid, influences separation indirectly only

: variables influencing separation may be changed continuously in wide range

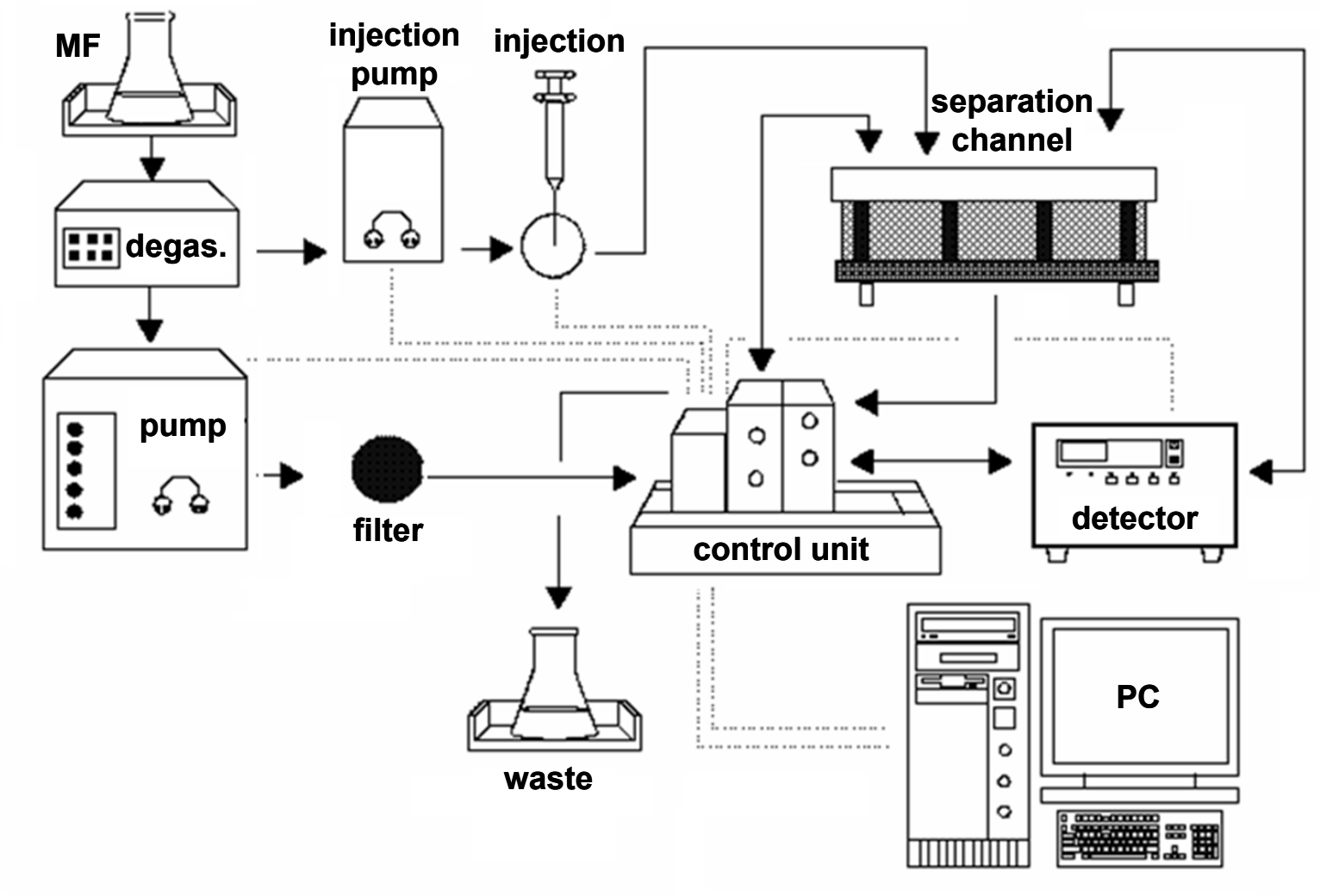
separation of macromolecules and particles **10^3 – 10^{15} Da**

FFF proceeding





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

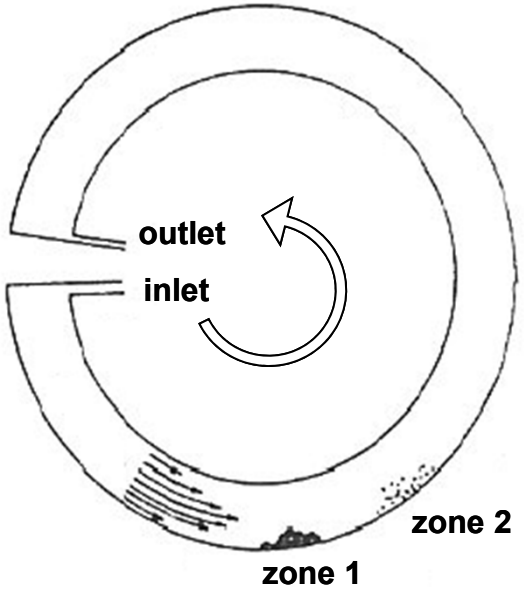
: refractometer
: photometer – absorption, fluorescence, optical rotation, scattering
: other – viscosimeter, densitometer, osmometer...

SdFFF, sedimentation flow-field fractionation

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

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

G – gravity (g) or centrifugal acceleration
Δq – density difference between particles and solvent
$\mathbf{d_p}$ – 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)

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

$\mathbf{D_T}$ = thermal diffusion coefficient

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

TFFF : to describe thermal diffusion

EFFF, electric flow-field fractionation

walls – semipermeable cellulose membranes

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

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

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

$$\lambda = R \cdot T \cdot V_0 / 3\pi \cdot N \cdot \eta \cdot V_c \cdot w^2 \cdot d$$

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

FFFF : > 1 nm

electromigration methods

basic principles of 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 arrangement

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

electromigration methods 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

theoretical fundaments of electromigration methods

separation **in external** field

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

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

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**)

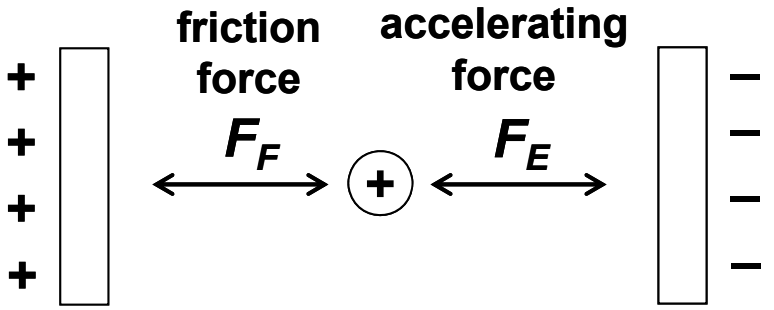
temperature influence: ↑**T** ⇒ ↑μ_0; with 1 °C about 2 %

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

T – working temperature
$\mathbf{T_0}$ – standard, tabulated temperature

ion mobility **estimation**

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



Stokes mobility

$$a = 0$$

$$F_E = F_F$$

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

a – acceleration of spherical charged particle motion
q – charge
η – solution viscosity
r – ion radius
v – ion motion velocity

relation of ion mobility and **diffusion coefficient**

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

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

ion mobility estimation for small molecules

Jokl equation

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

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

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

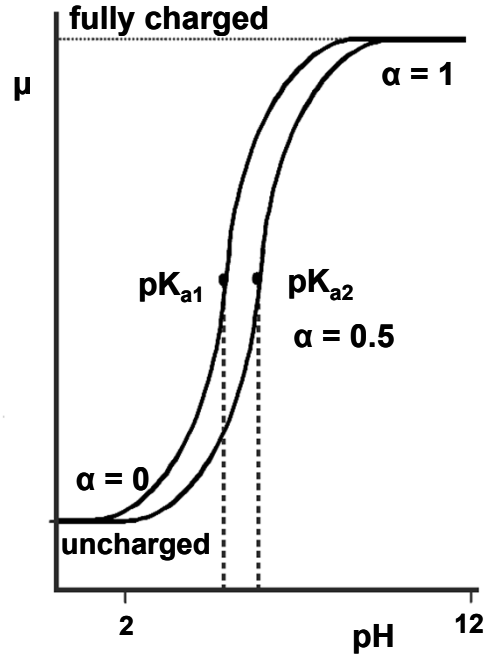
z_+, z_- – relative ion and counter-ion charge
I – ionic strength

effective mobility

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

$$\bar{\mu} = \sum_{i=1}^{n} \mu_i \cdot x_i$$

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

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

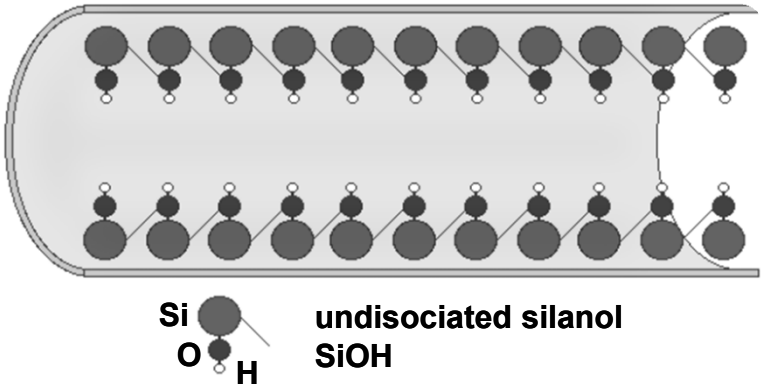
l_{tot} – separation channel total length
l_{eff} – separation channel effective length
t_m – migration time
t_0 – migration of neutral particle (EOF)

$$\mu_{tot} = \mu_{eff} + \mu_{EOF} = \frac{l_{eff}}{t_M \cdot E} = \frac{l_{eff} \cdot l_{tot}}{t_M \cdot U}$$

electroosmotic flow

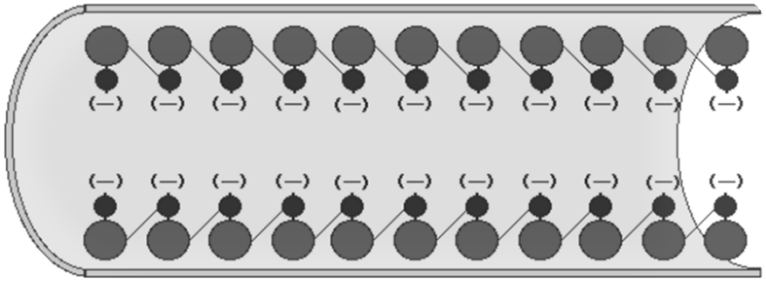
(EOF)

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

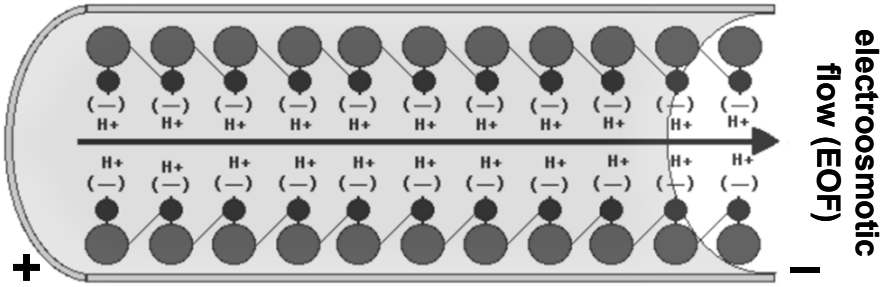


capillary = *endo-osmotic pump*

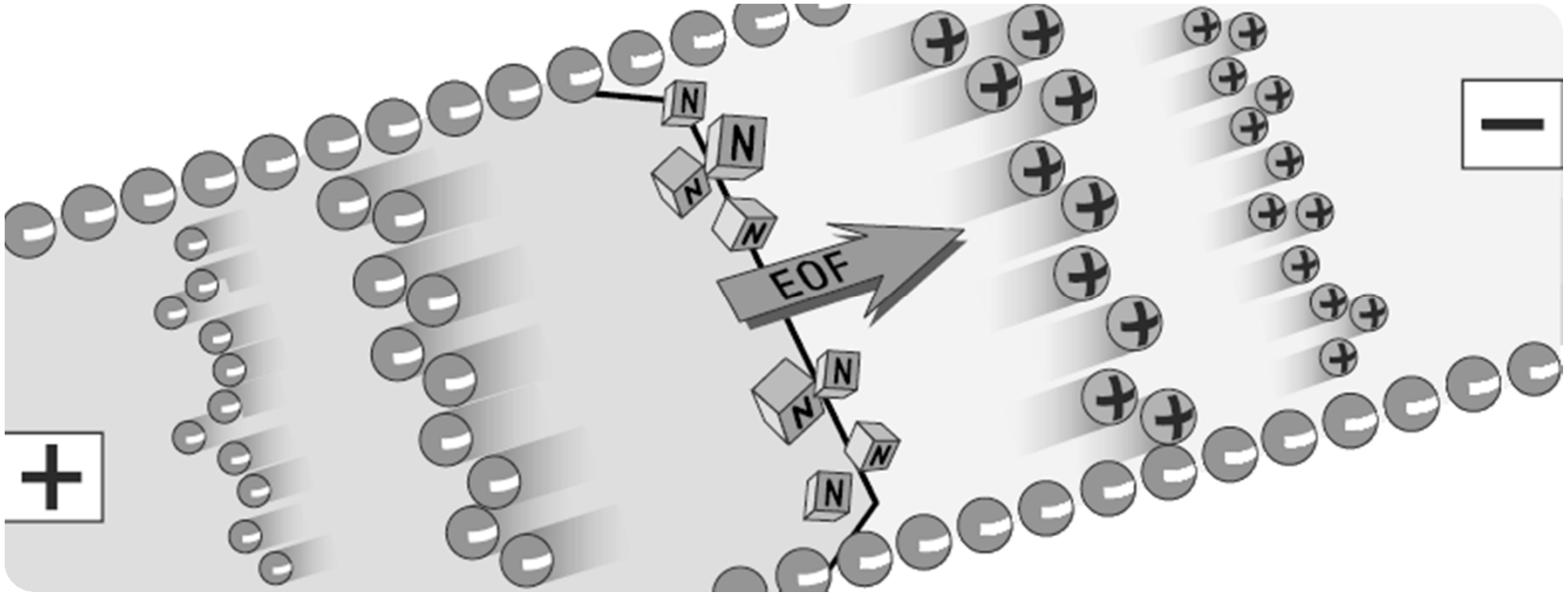
capillary made of fused silica
with exposed hydroxyl groups



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***

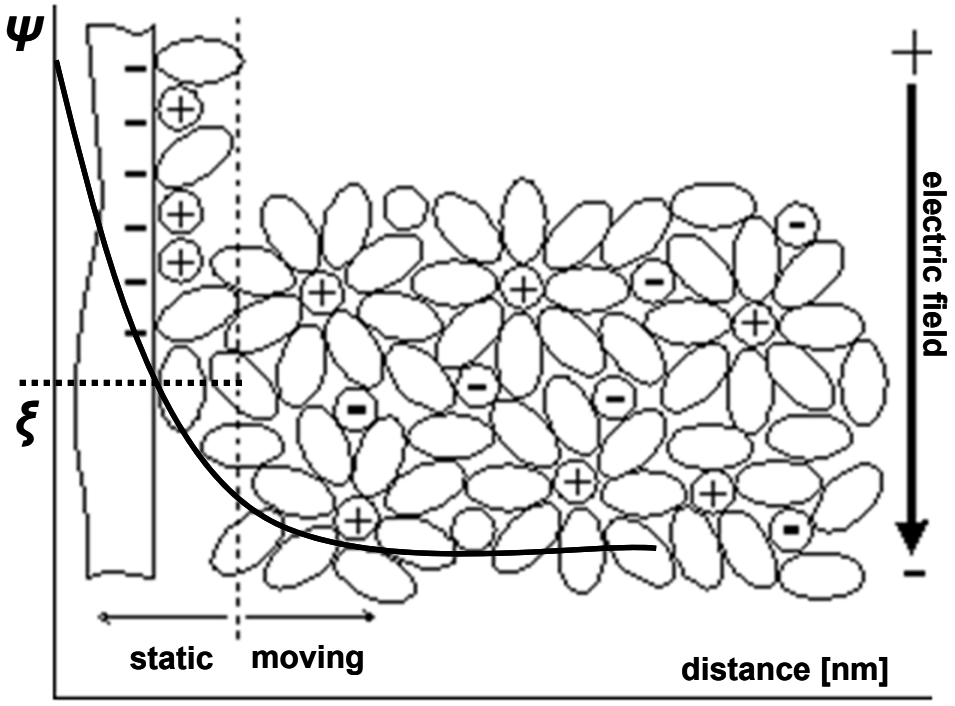
: **neutral molecules** are moving in the same direction as electroosmotic flow with negligible mutual separation

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

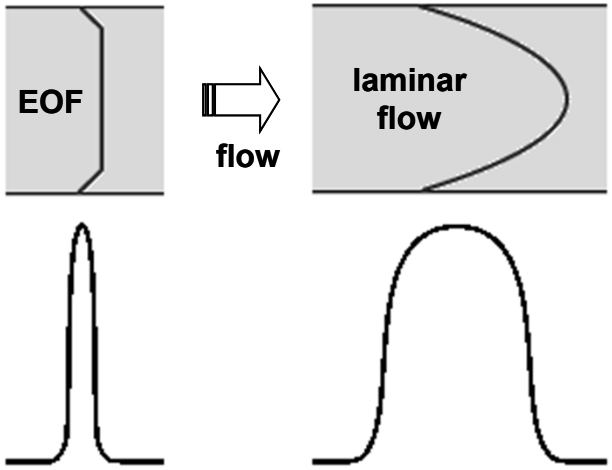
EOF = 0 ⇒ no mass flow, only ion exchange

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

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

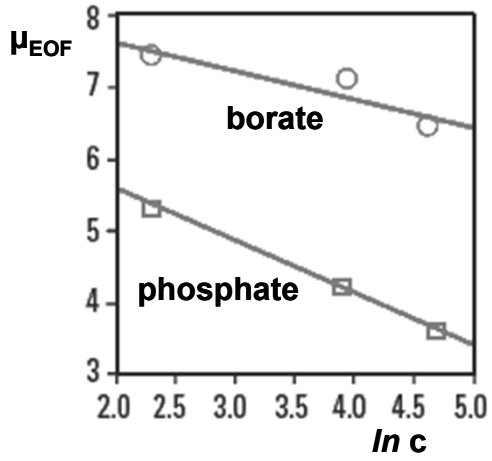
: change of EOF is directly proportional

: 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

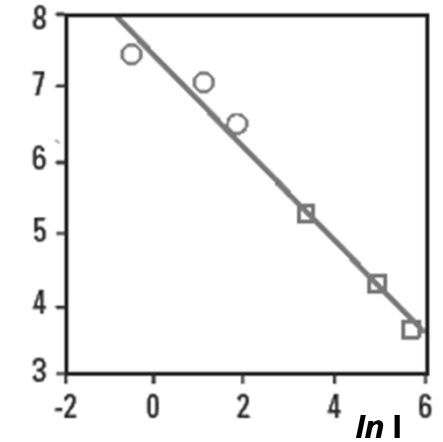
:: high values increase current and thus Joule heat
:: high values may cause analyte salting-out and adsorption to wall
:: low values supports adsorption to wall and limits sample concentration
:: changes peak shape, if electrolyte conductivity differs much from analyte



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

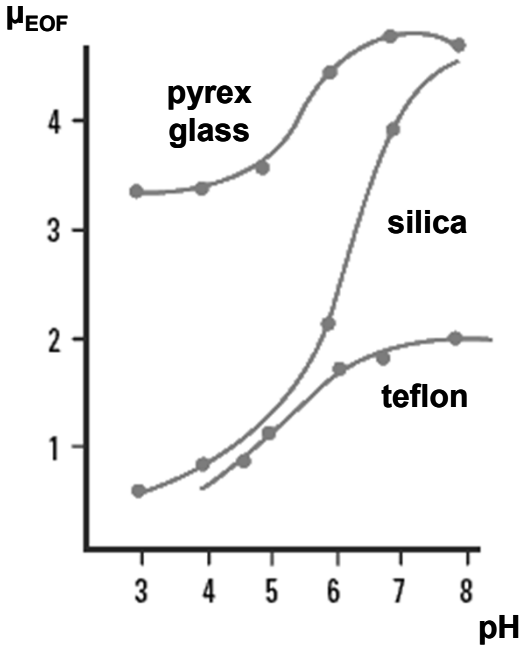
influence of temperature

: changes viscosity, higher temperature ⇒ higher EOF

:: thermolability of some samples

influence of covalent wall surface modification

: changes **ξ**-potential and wall charge polarity



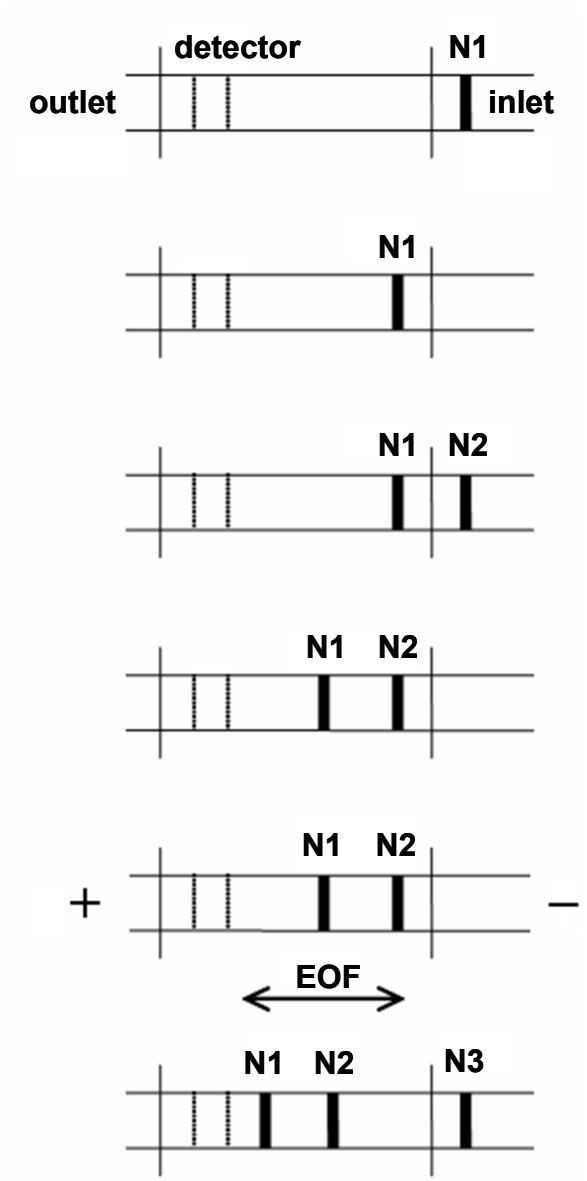
pH influence on EOF

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

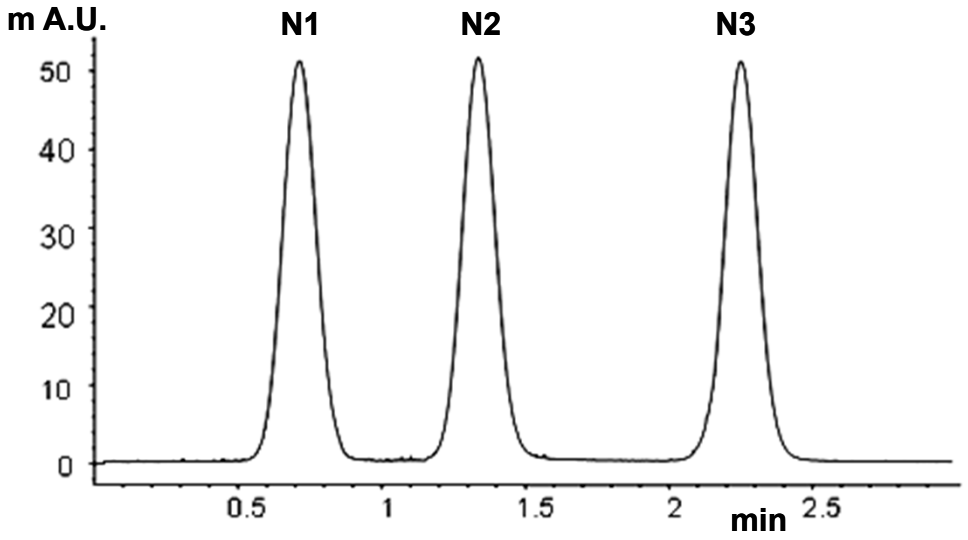
: shifting the marker zone to detector by pressure

: 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



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

$$\mu = \frac{l_{EOF} \cdot l_{tot}}{U \cdot (t_M - \frac{t_{ru}}{2} - \frac{t_{rd}}{2})}$$

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

common EOF calculation

$$\mu_{tot} = \mu_{eff} + \mu_{EOF} = \frac{l_{eff}}{t_M \cdot E} = \frac{l_{eff} \cdot l_{tot}}{t_M \cdot U}$$

graphical illustration of separation

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

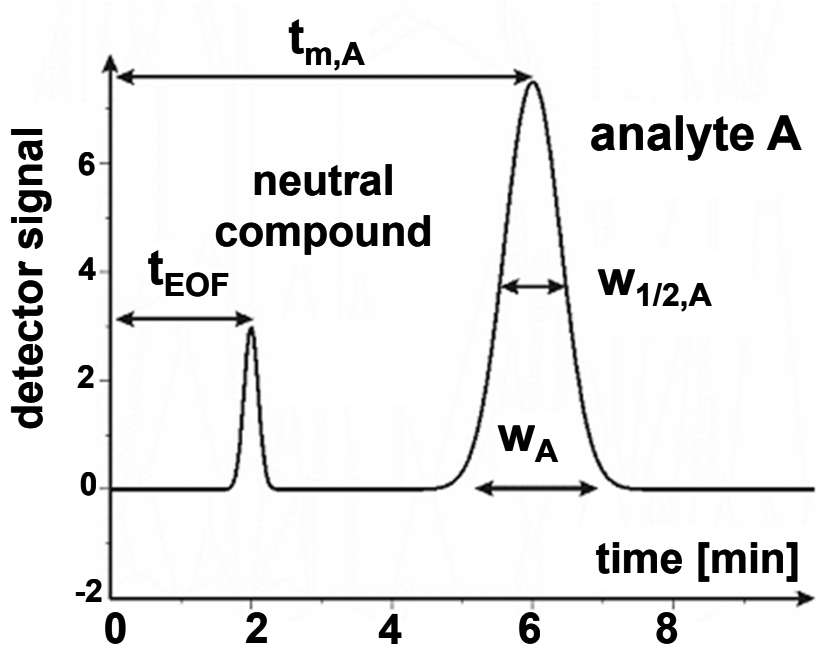
electrophoretic peak

: **also** *Gaussian shape as in chromatography*

electrophoretogram

: *electropherogram*, *electrophoregram*, *electrophoreogram*

migration time of ***i***-th analyte $\mathbf{t_M}$ [min]



separation efficiency

zones of **A broaden** during separation

and **become asymmetric**

reasons behind zone broadening

: lateral diffusion

: electrodispersion

number of theoretical plates (N)

$$N = \left(\frac{t_M}{\sigma}\right)^2 = 16 \cdot \left(\frac{t_M}{w}\right)^2 = 5.545 \cdot \left(\frac{t_M}{w_{1/2}}\right)^2$$

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

analogically as in chromatography

height equivalent of theoretical plate (H)

(*comparison of separation channels of different length*)

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

$$H = A + \frac{B}{u} + C \cdot u$$

A = 0 : in absence of particles

C = 0 : is there is no SF

$$\Rightarrow \quad H = \frac{B}{u}$$

increasing voltage causes **increasing of flow rate**, but it also releases heat and it **increases rate of lateral diffusion**

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

$$H = \sigma^2 = 2D \cdot t = \frac{2D \cdot l_{eff} \cdot l_{tot}}{\mu_{eff} \cdot U}$$

$$H = \frac{B}{u} = \frac{2D}{u} = \frac{2D}{\mu \cdot E} = \frac{2D \cdot L}{\mu \cdot U}$$

$$\Rightarrow \quad N = \frac{\mu_{eff} \cdot U \cdot l_{eff}}{2D \cdot l_{tot}} = \frac{\mu_{eff} \cdot E \cdot l_{eff}}{2D}$$

principal difference from **N**
in chromatography

contributions to zone broadening in electromigration methods

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

diffusion influence

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

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

electromigration dispersion influence

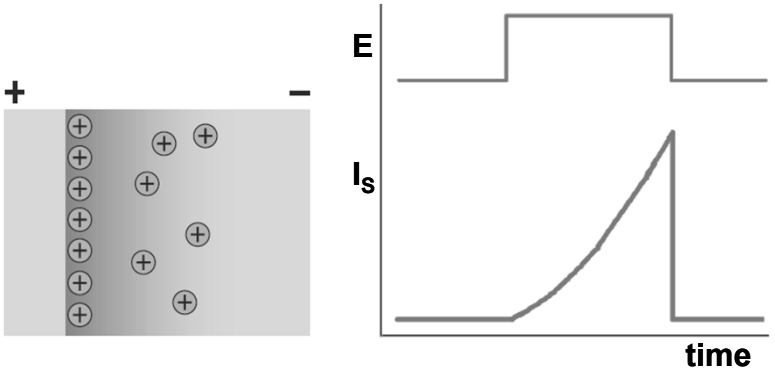
influences peak shape

difference between **conductivity of sample** and **electrolyte** leads to

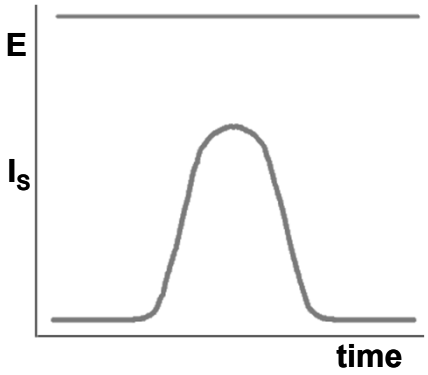
: peak **tailing**

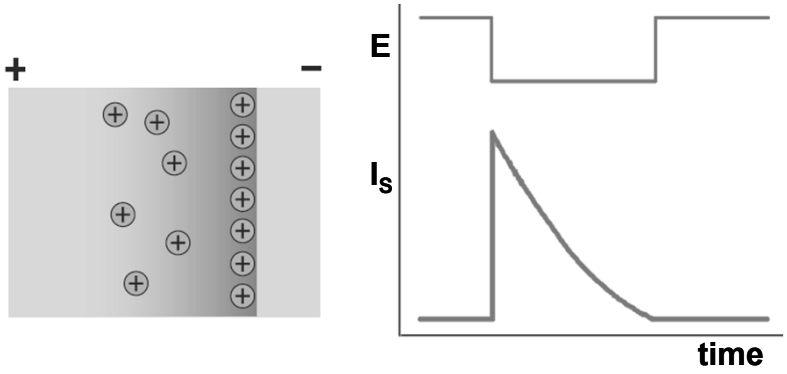
: **focusing** (low sample conductivity), **broadening** (high sample conductivity)

: **ITF effect** (peak fronting) because of certain ion surplus (e.g. Cl^-)

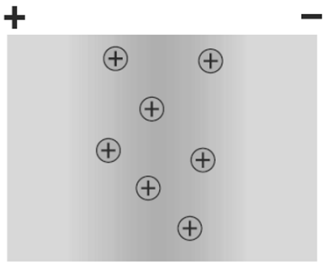


$\mu_S > \mu_{BGE} \Rightarrow$ front gets broad and tail focuses





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



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

sorption influence

sorption causes peak tailing

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

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

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

$$\sigma^2_{inj} = \frac{t^2_{inj}}{12}$$

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

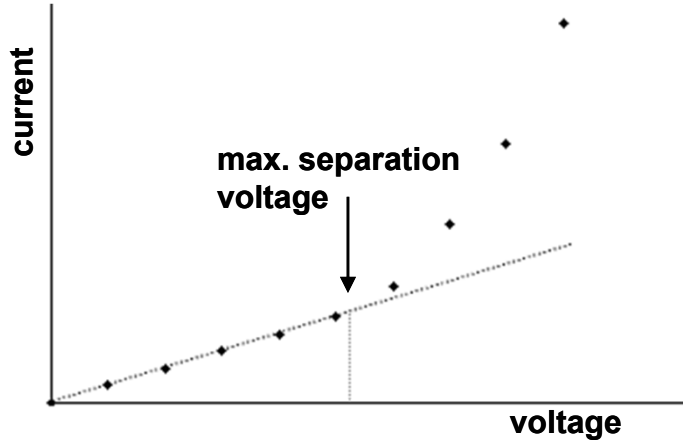
$\mathbf{t_{inj}}$ – injection pulse length

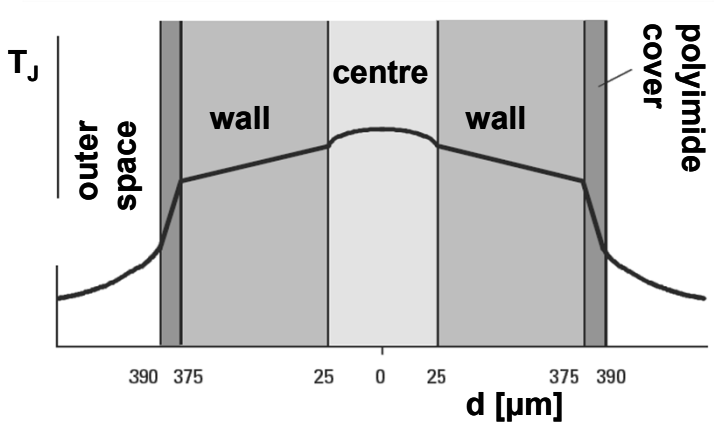
Joule heat influence leads to temperature gradient and laminar flow

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

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

resolution

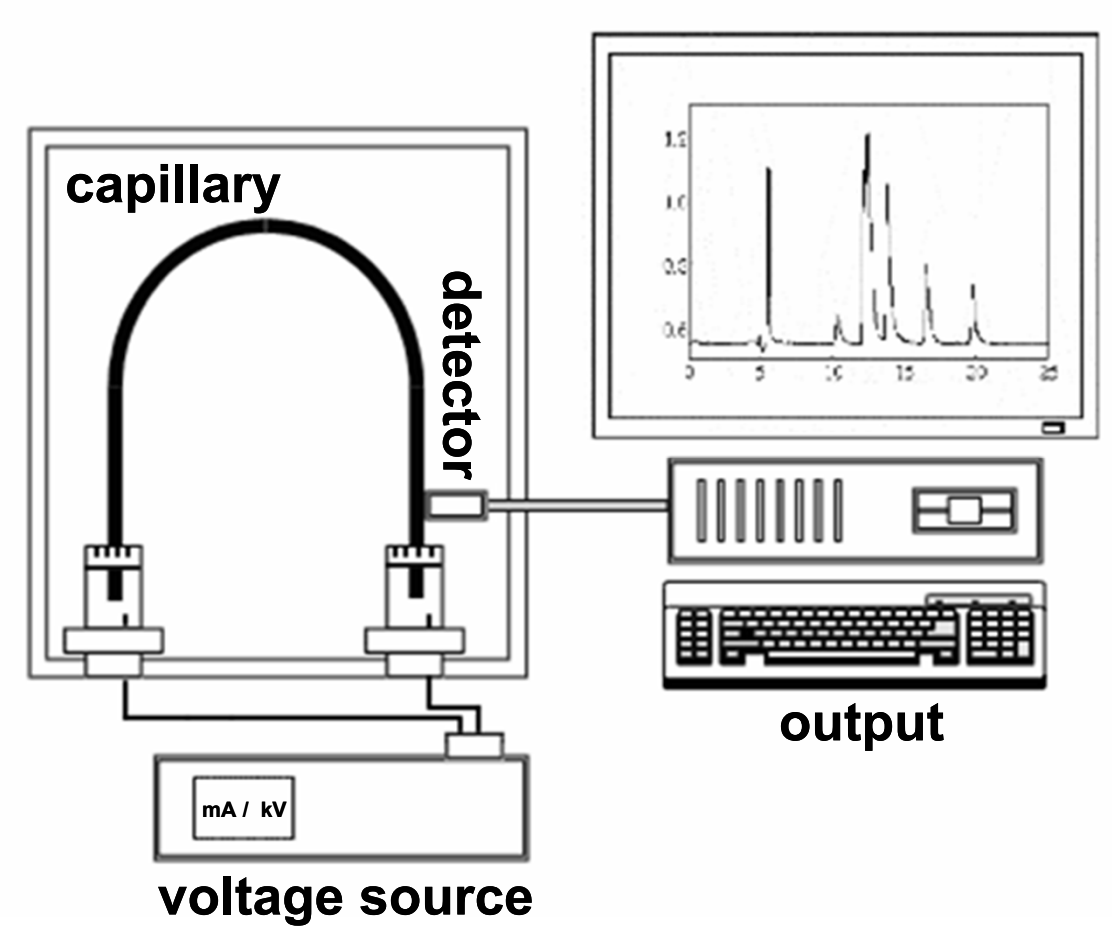
$$R_{(A,B)} = \frac{2 \cdot (t_{M,A} - t_{M,B})}{w_A + w_B} = \frac{2 \cdot \Delta t_M}{w_A + w_B}$$

$$R_{(A,B)} = \frac{\sqrt{n}}{4} \cdot \frac{\Delta\mu}{\bar{\mu}}$$

$\Delta\mu$ – difference, **$(\mu_2 - \mu_1)$**
$\bar{\mu}$ – median, **$(\mu_2 + \mu_1) / 2$**

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

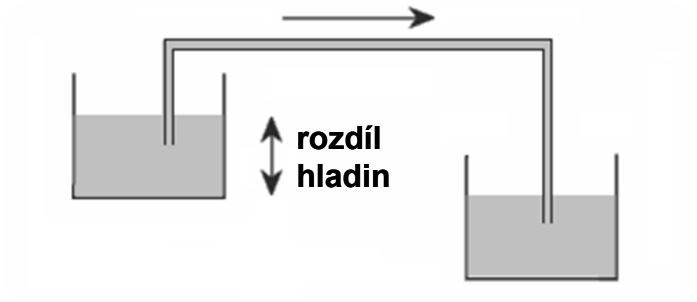
electromigration methods arrangement



injection device

hydrostatic

siphon effect



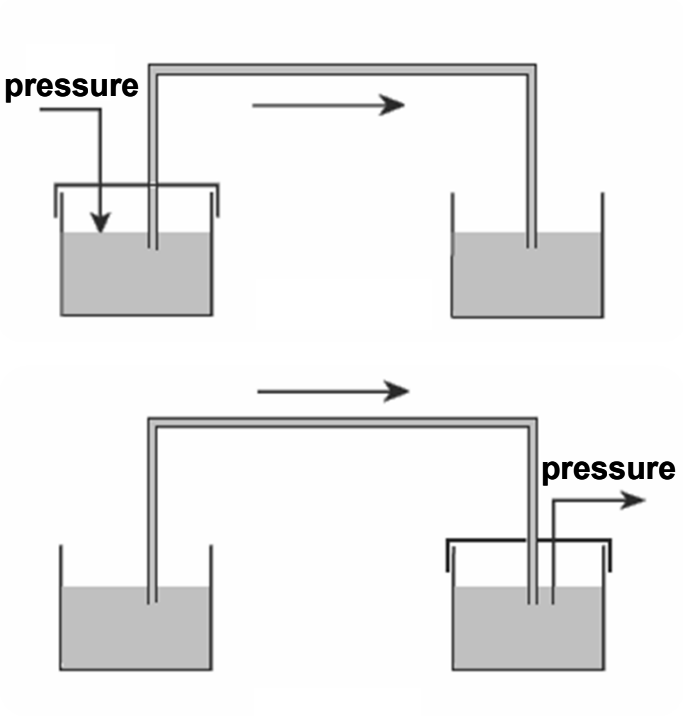
typical volumes: 10 – 100 nl (capillary ~ 1 – 2 µl)

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

hydrodynamic

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

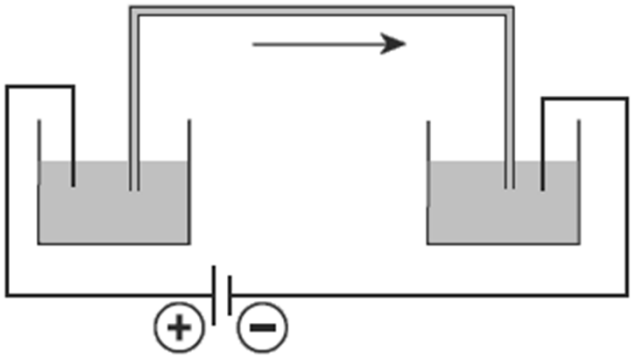
injected volume $\mathbf{V_{inj}}$



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

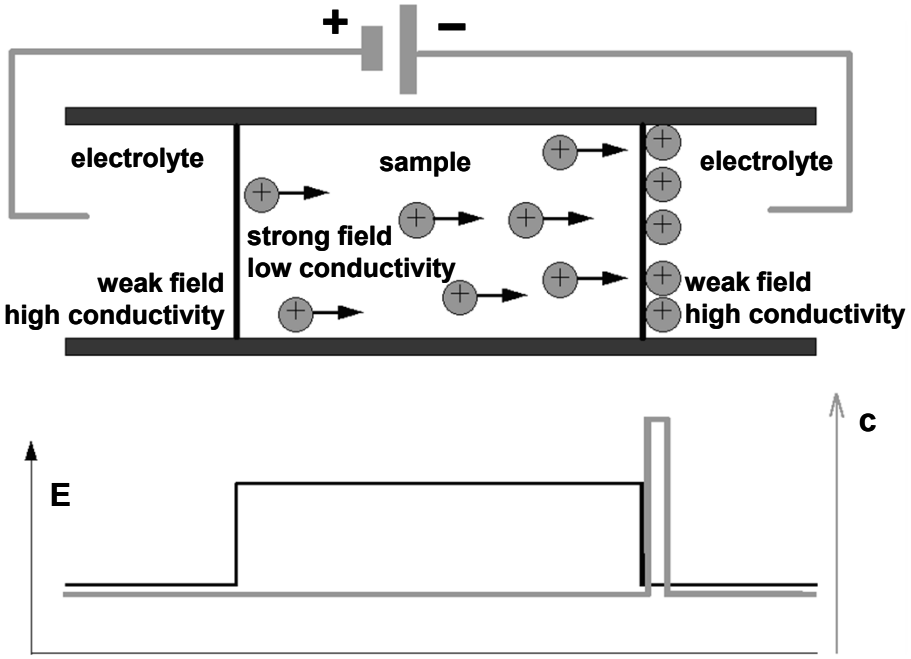
electrokinetic

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



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

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

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

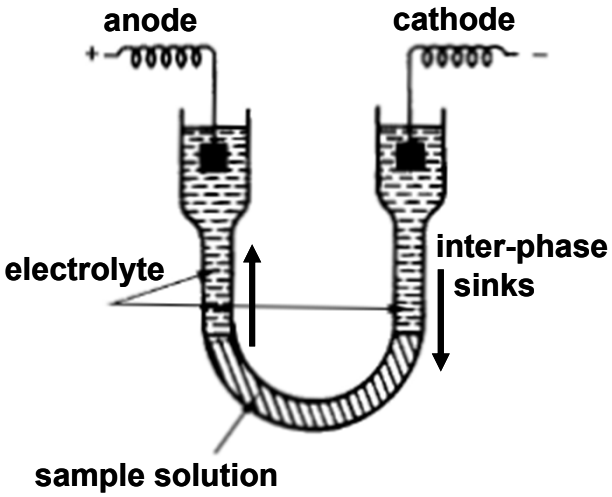
voltage source

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



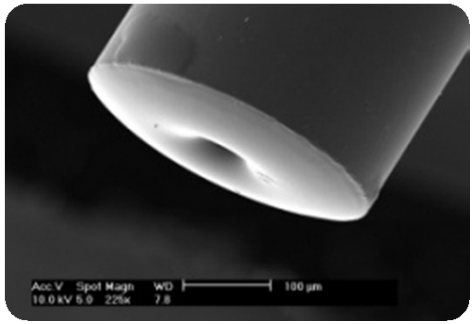
tube

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



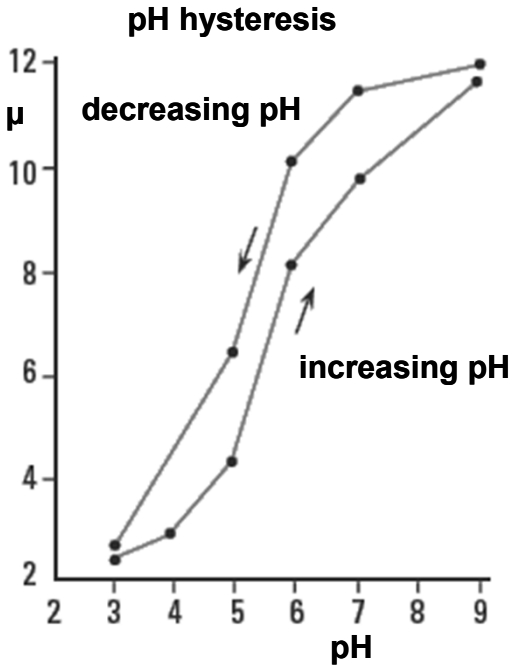
fused silica

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)

<u>conditioning</u>:
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

inner 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

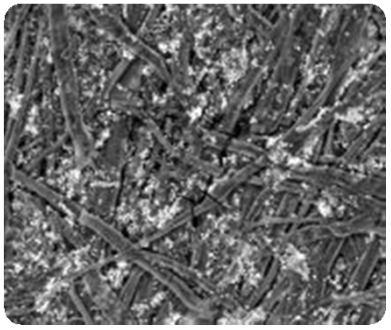
decrease or revert EOF

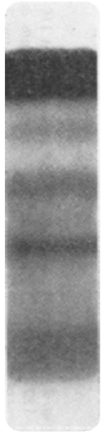
: work also as ion pairing agents (MEKC)

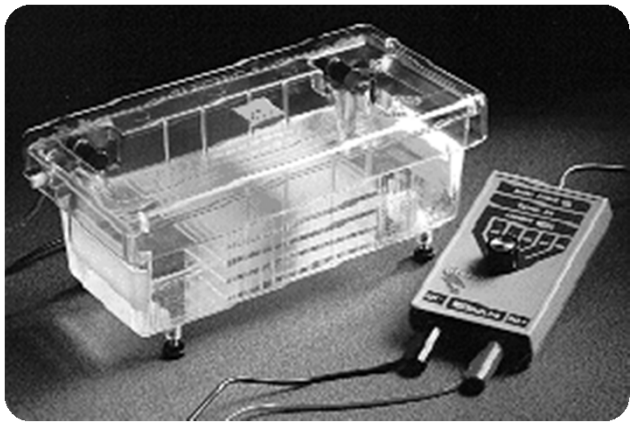
paper / membrane

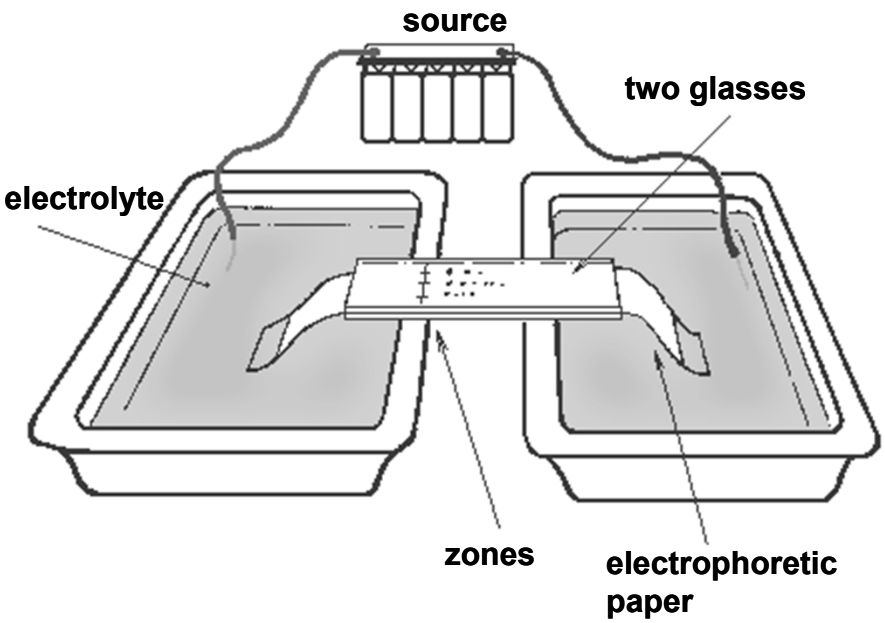
100 % cotton / **cellulose**

0.17 – 0.30 mm thick
pore size 2.5 µm

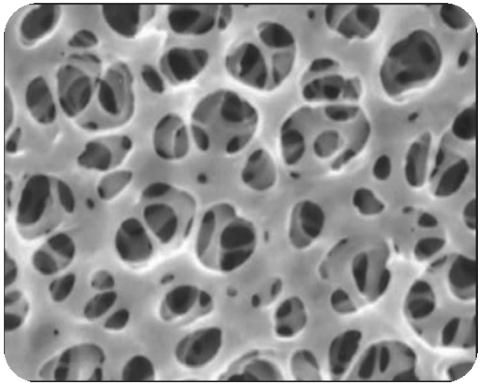






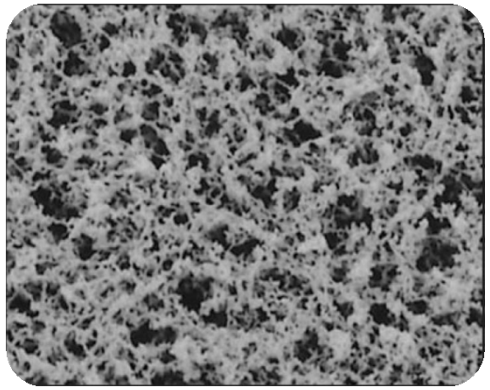


acetate cellulose
pore size 0.2 µm



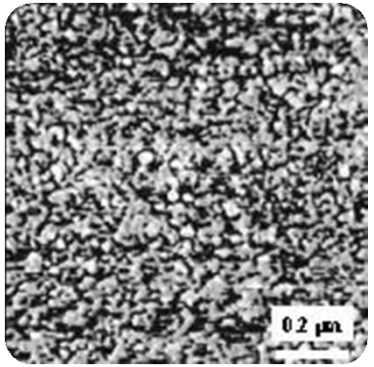
nitrocellulose

pore size 0.2 µm



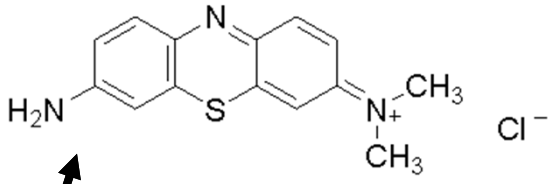
nafion (PTFE, sulphonated tetrafluoroethylene)

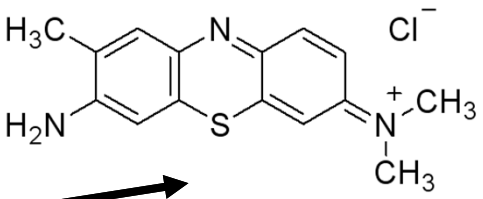
1 – 2 nm and 5 – 6 nm

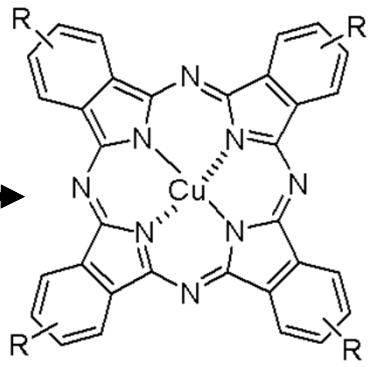


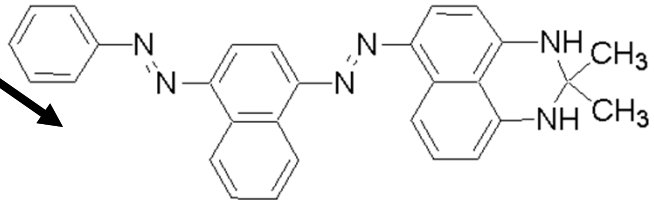
visualisation

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







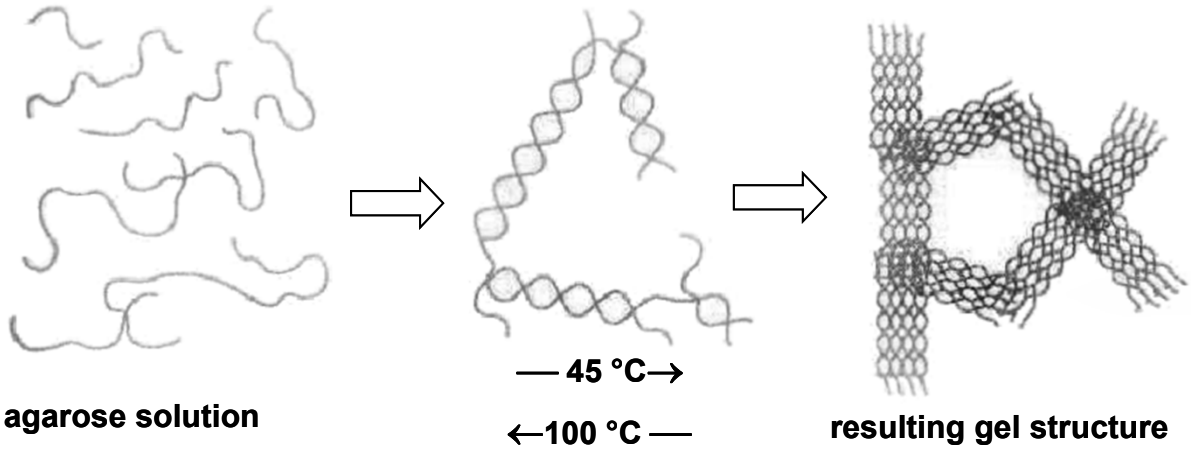


agarose gel

gel

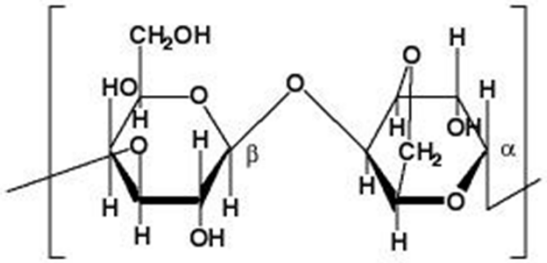
: non-toxic, cheap, no additional components for polymerisation
: fragile

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

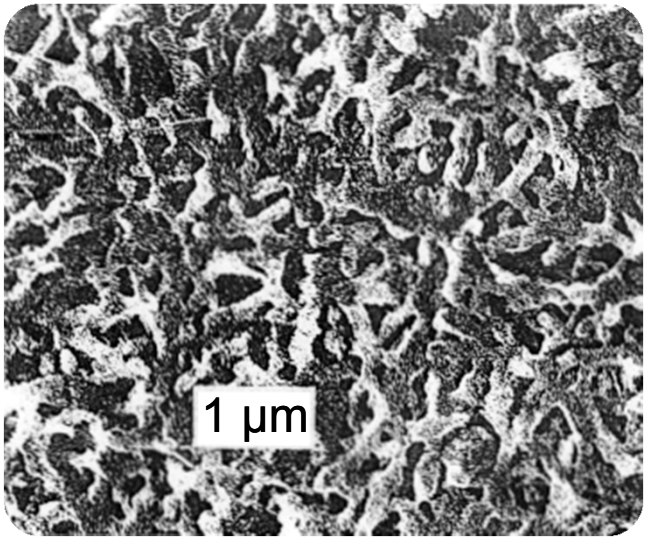


agarose solution **resulting gel structure**

D-galactose

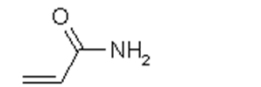


3,6-anhydro-L-galactose

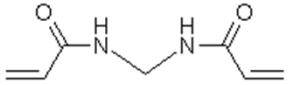


polyacrylamide gel

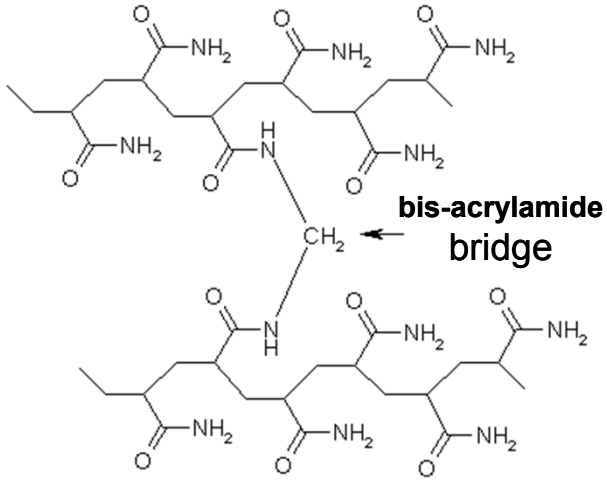
: toxic (bis-acrylamide), inert
: fragile, reinforcement by RhinoHide™ or DurAcryl™



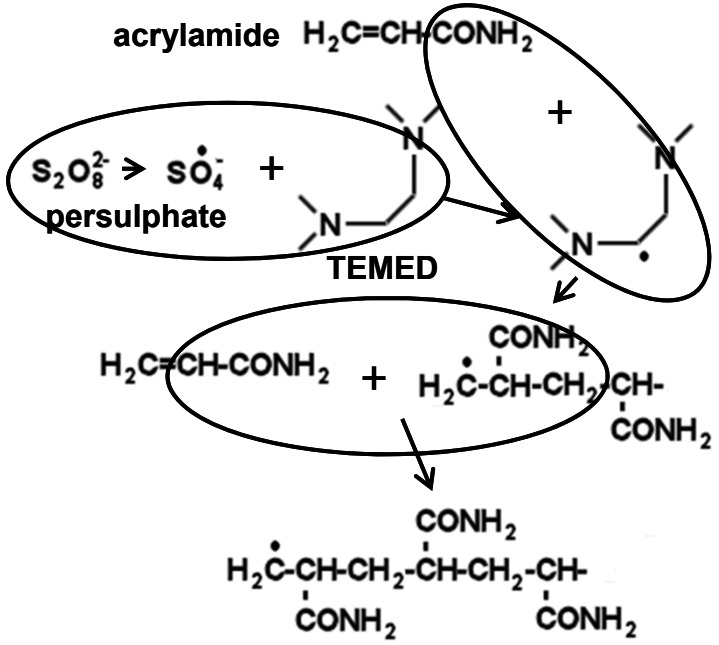
acrylamide



methylene-
bis-acrylamide

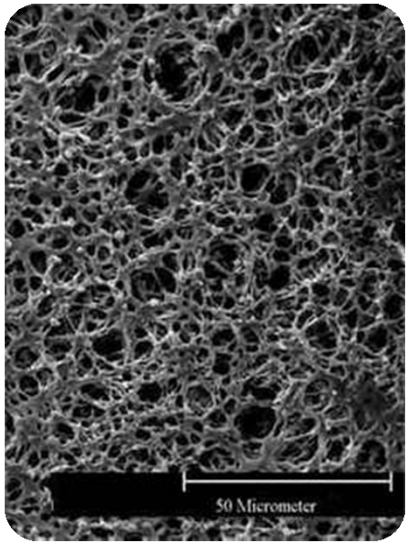


bis-acrylamide bridge



persulphate /ammonium/ – initiator

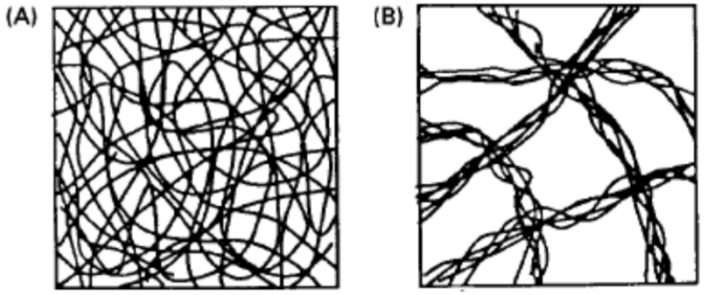
tetramethylene ethylenediamine (TEMED)
– catalyser



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



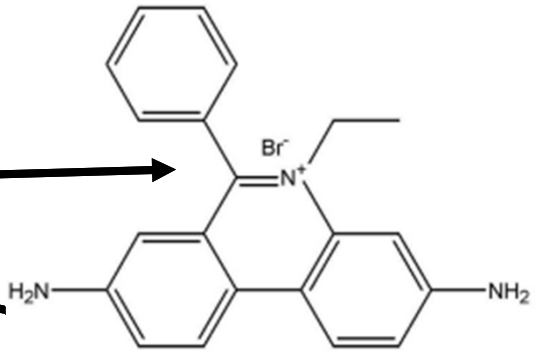
12%-gel

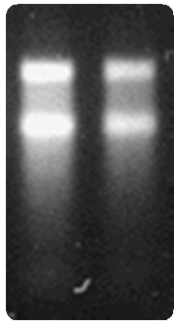
viscosity	~100 $m^2 s^{-1}$
cavity diameter (12%)	~ 4.4 nm

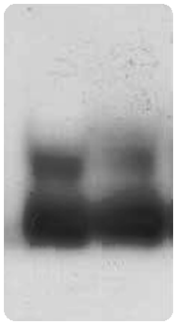
: isocratic (continuous) (8 – 15 %)
: discontinuous gel (4% concentration and 12 % separation)
: gradient gel (Schäger-von Jagow)

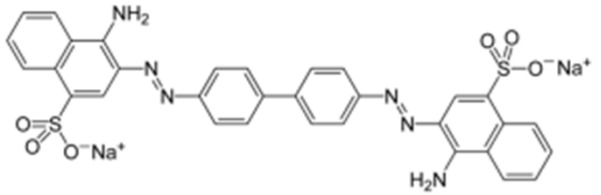
visualisation

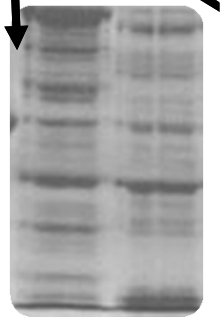
ethidium bromide (EtBr)
Kongo red
Coomassie blue R-250, G-250
SYPRO ruby
SYBR II green
silver
zinc
copper



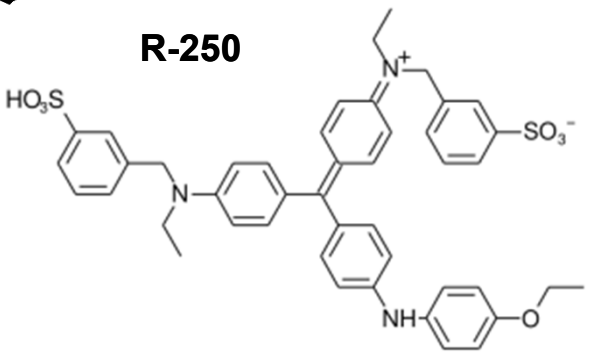


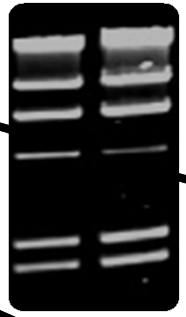


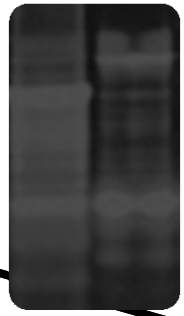


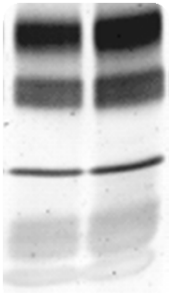


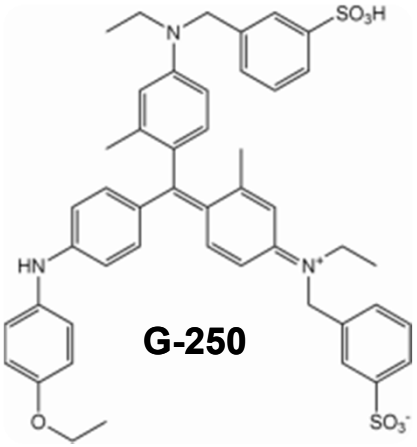
R-250





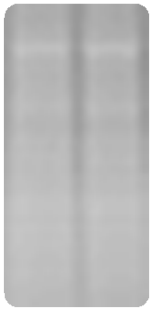






G-250

0.3 M $CuCl_2$



0.2 M $ZnSO_4$
0.2 M imidazole

0.02% $Na_2S_2O_3$

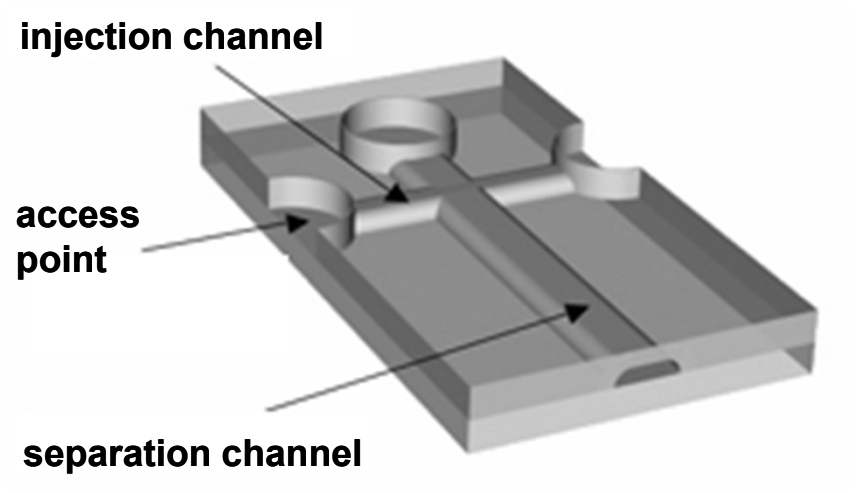
0.1% $AgNO_3$

37% HCOH

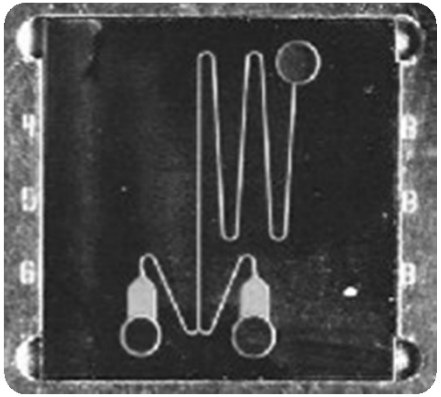
1% CH_3COOH

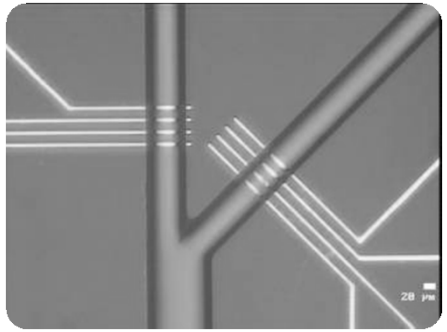
chip (CE-on-chip)

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

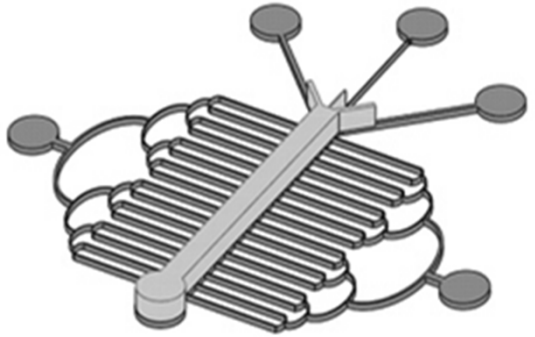


ZE, ITF, IEF...





electrochemical detection

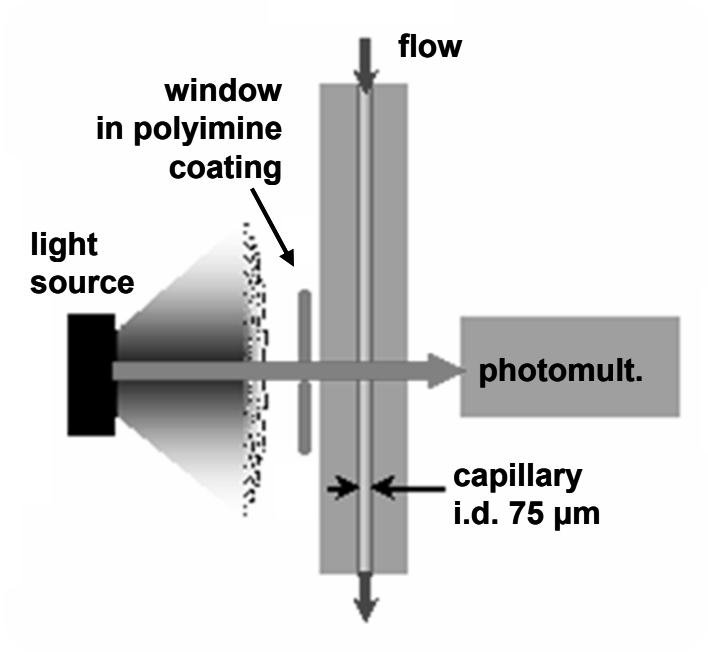


lab-on-chip
LC + CE

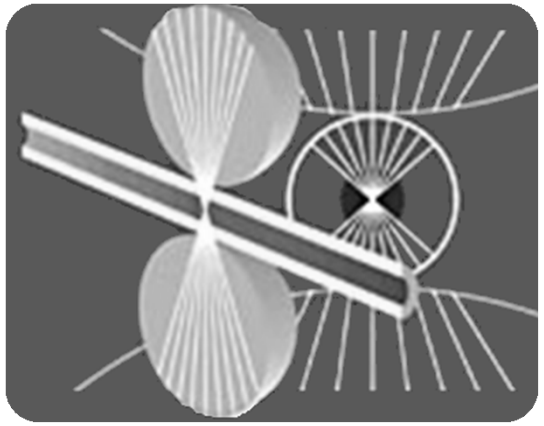
absorption photometric detector

diode array detector

detectors



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



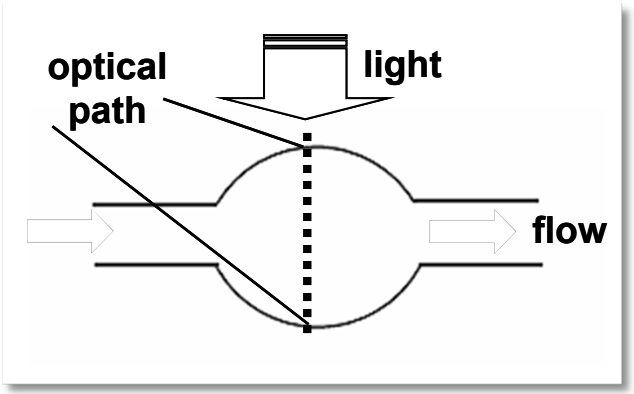
focusing optics – two spherical lenses

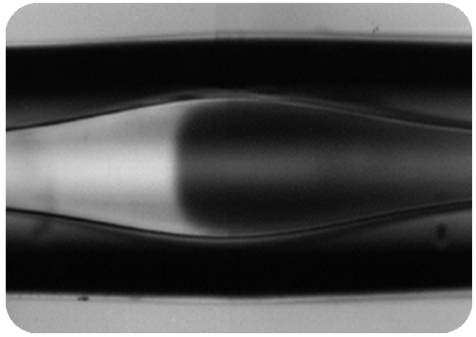
absorbance
: sensitivity 10^{-6} M

indirect detection
: sensitivity 10^{-4} M

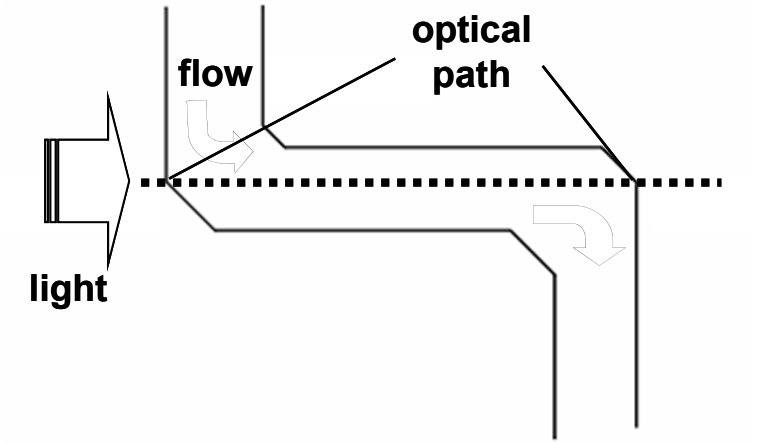
prolongation of optical path

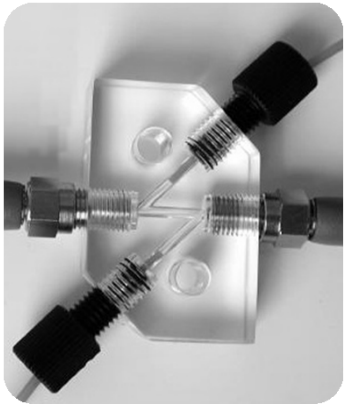
bubble cell



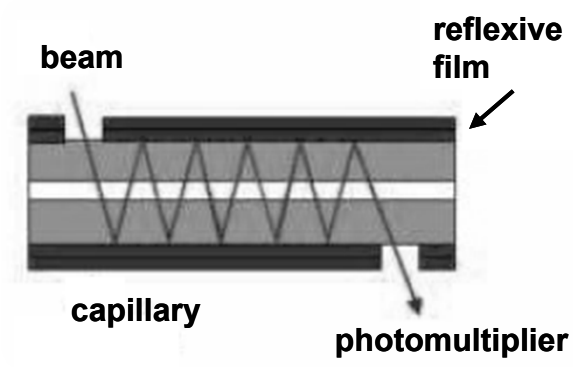


Z-cell

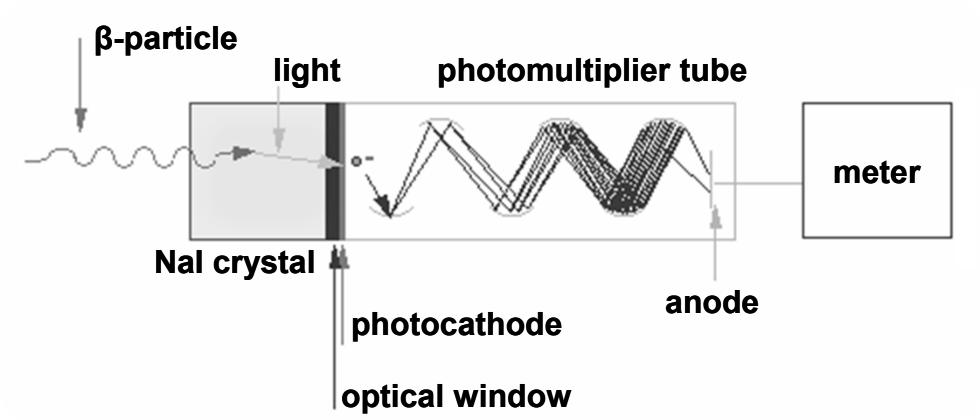




reflexive inner coating



radioactive (scintillation) detector



scintillation
: sensitivity 10^{-10} M

MDD
signal: beam of β-particles (e^-)

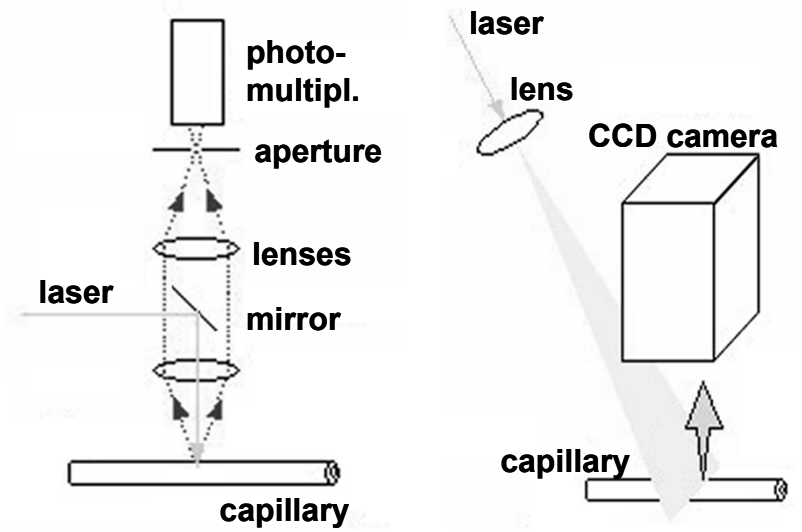
fluorescence detector

laser induced fluorescence

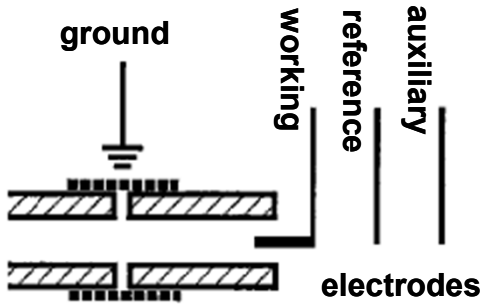
(LIF)

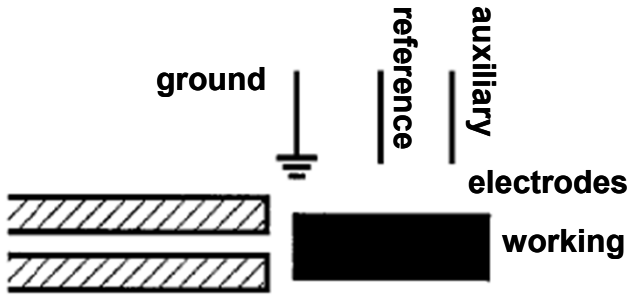
fluorescence
: sensitivity 10^{-11} M

LIF
: sensitivity 10^{-13} M



amperometric detector

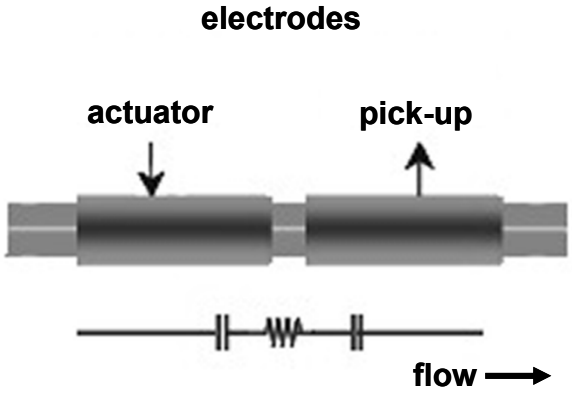




amperometry
: sensitivity 10^{-7} M

conductivity detector

conductivity
: sensitivity 10^{-5} M



: two metallic electrodes around capillary

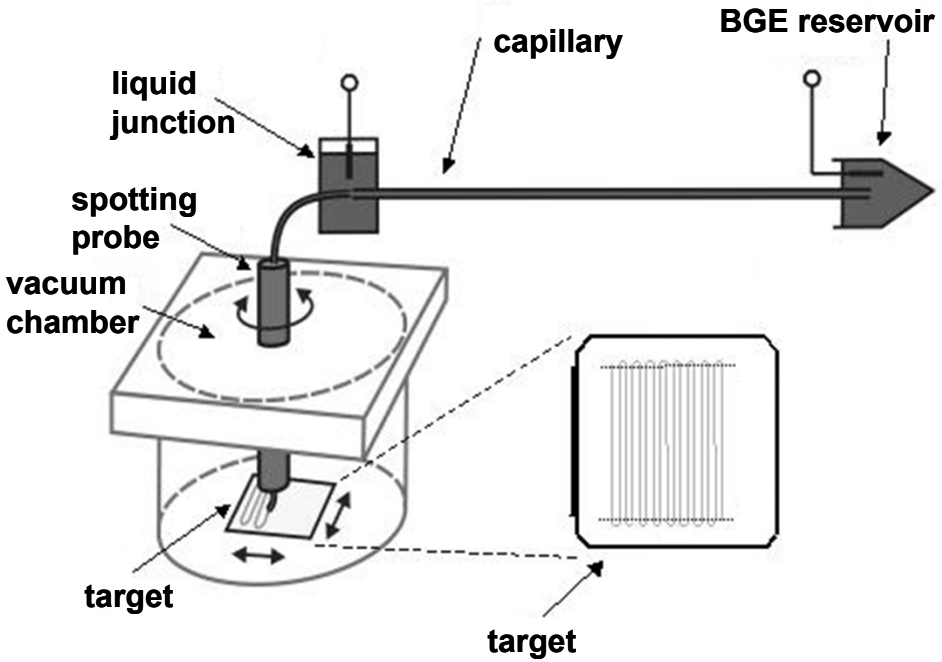
: when applying AC voltage on an actuator, the current flows through wall, in-between electrodes towards the pick-up electrode

: signal is then amplified

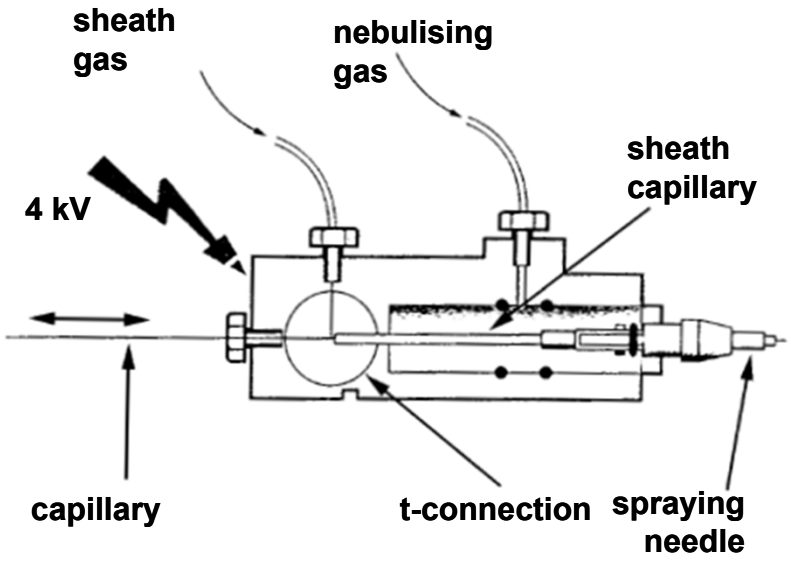
matrix assisted laser desorption / ionisation

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

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

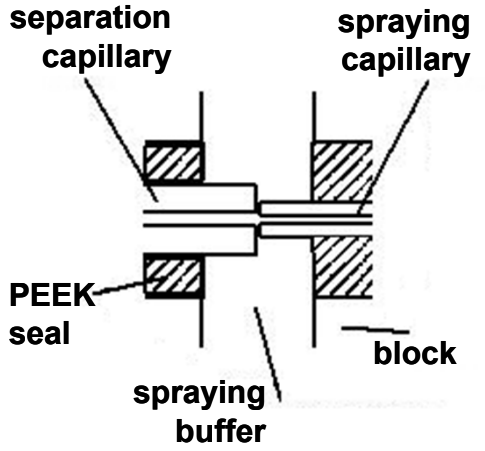


ion count
: sensitivity 10^{-7} M



electrospray ionisation

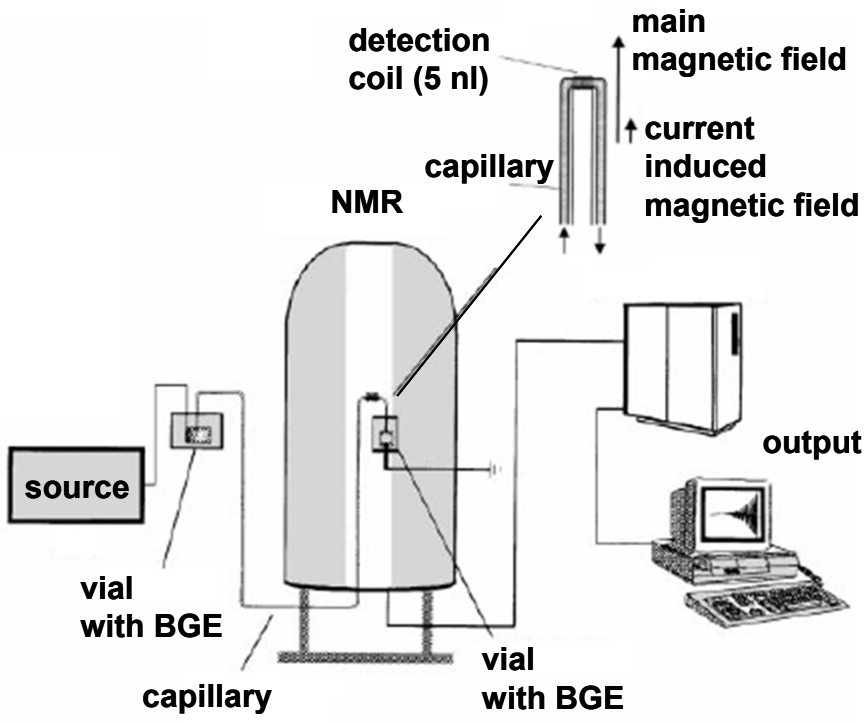
key point
liquid junction



nuclear magnetic resonance

may use **bubble cell**

^{1}**H** and ^{13}**C** – NMR



NMR
: sensitivity 10^{-5} M

preparative electromigration methods

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

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

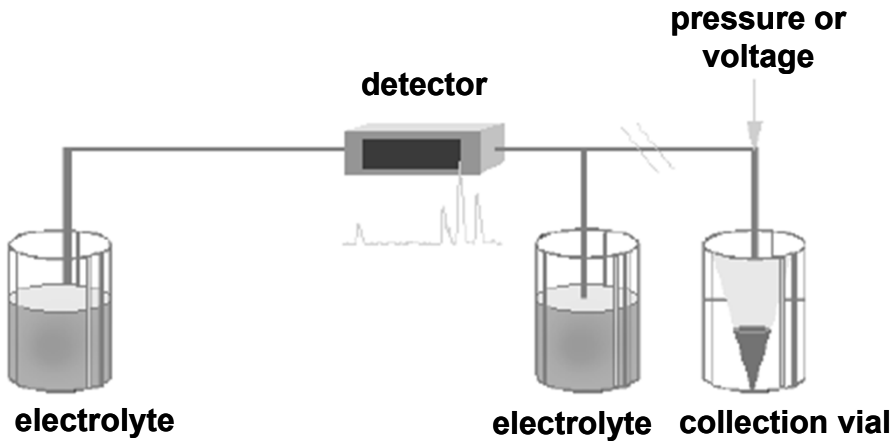
pressure elution: (**CZE**, **ITP;** MEKC, IEF**;** CGE – ***no***)
: pressure application (5 kPa) during pre-calculated time period

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)

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

planar

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

qualitative information

migration time normalisation

bad 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)

$$A_N = A \cdot \left(l_{eff} / t_M \right) \Rightarrow A / t_M$$

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)

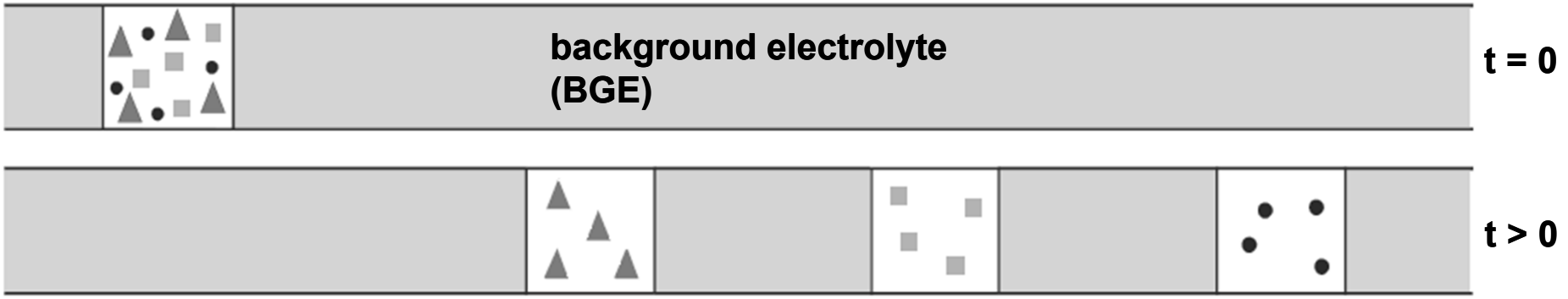
affinity electrophoresis (ACE)

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



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

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

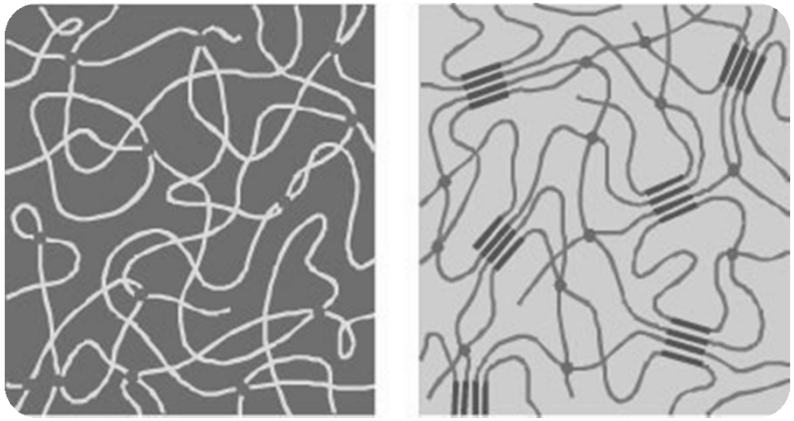
physical gels: agarose – weak intermolecular bonds of different molecule parts

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)

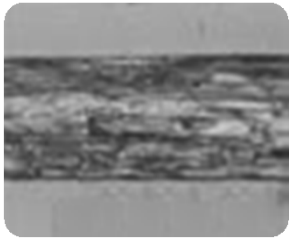
hydroxypropylcellulose (HPC)

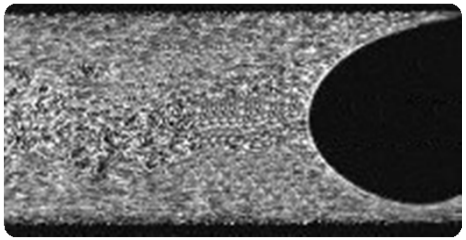
hydroxypropylmethylcellulose (HPMC)

: 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)

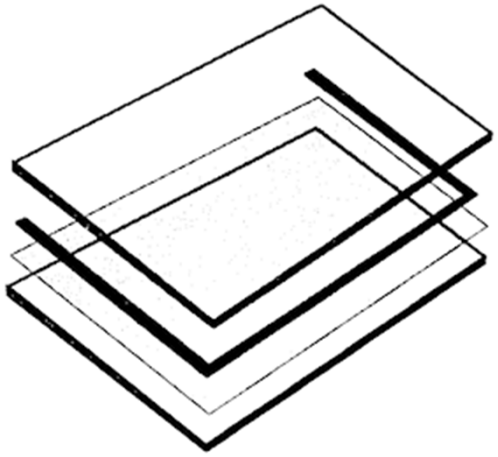
GE, slab-gel electrophoresis

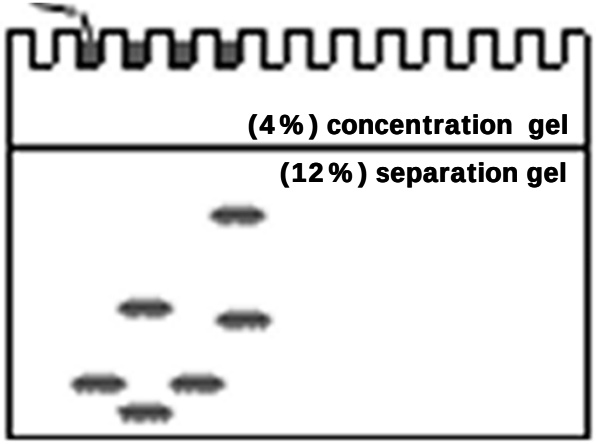
denaturing **(SDS, *Lämmli*)** – separation according to $\mathbf{M_w}$

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

one dimensional gel electrophoresis (1D-GE)

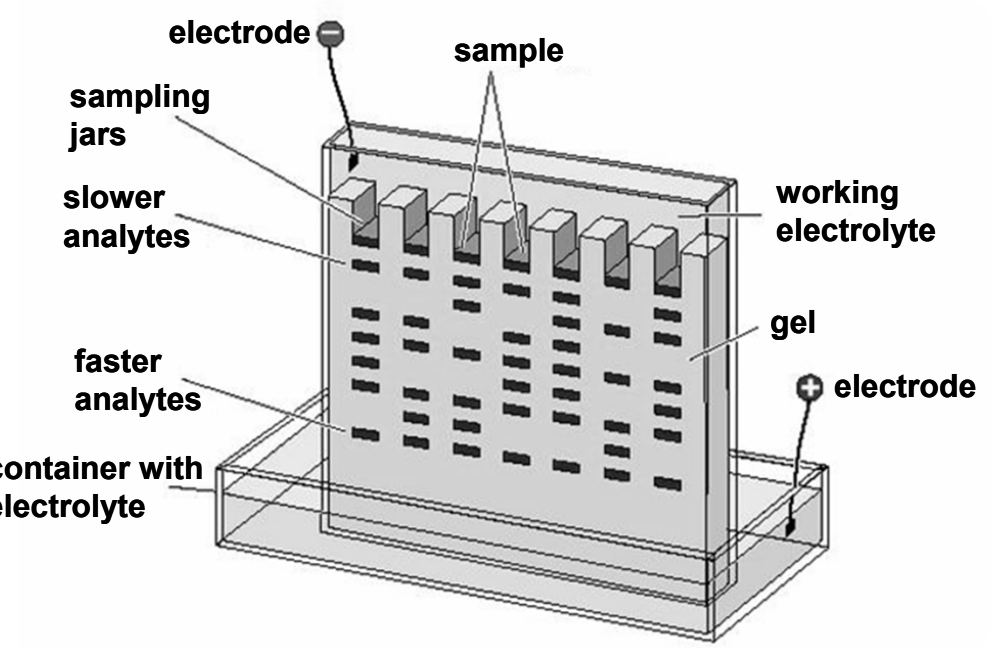
: slab gel polymerises between glass plates, separated by spacers
: loading jars are created by special spacer – *comb*

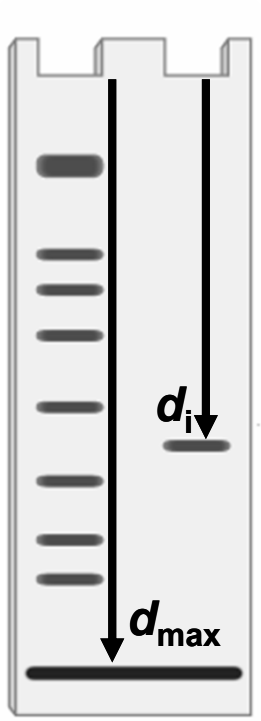




basic procedure

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



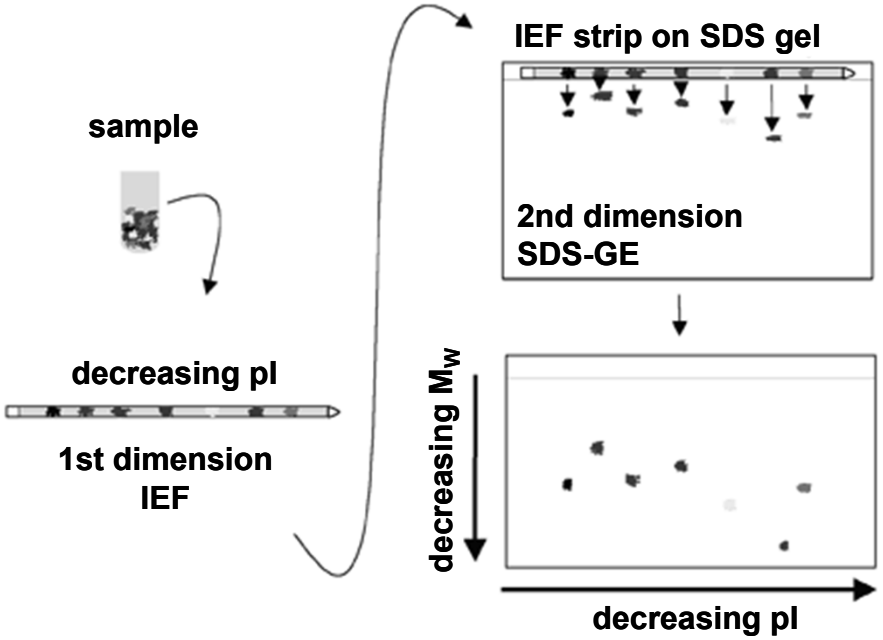


$$R_f = \frac{d_i}{d_{max}}$$

retention factor

two dimensional gel electrophoresis (2D-GE)

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



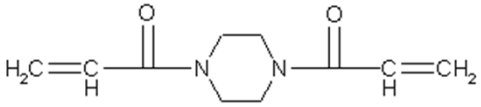
1. isoelectric focusation (IEF)

immobilised pH-gradient in gel strip

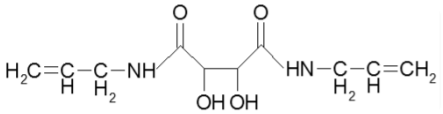
2. denaturing gel elfo (SDS-GE)

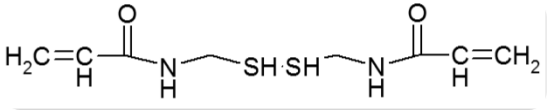
SDS is not in gel since polymerisation (as with 1D)
: micelles would be created

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



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

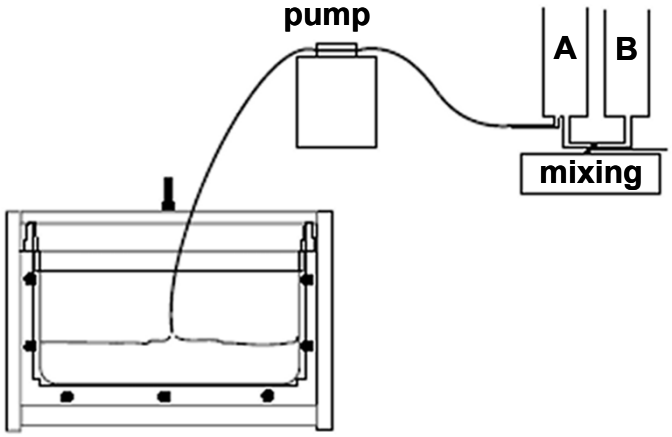




sodium thiosulphate in gel – low background with Ag-staining

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

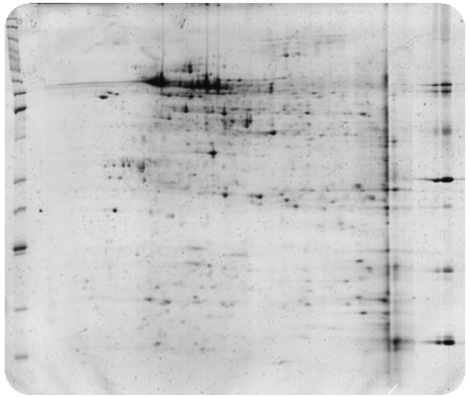


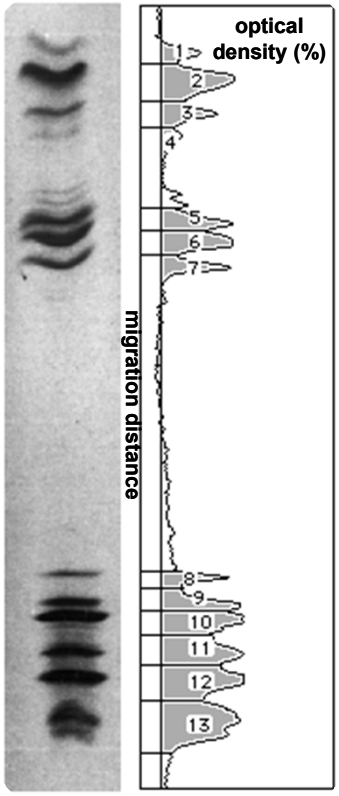
gradient profile is given by the shape of containers

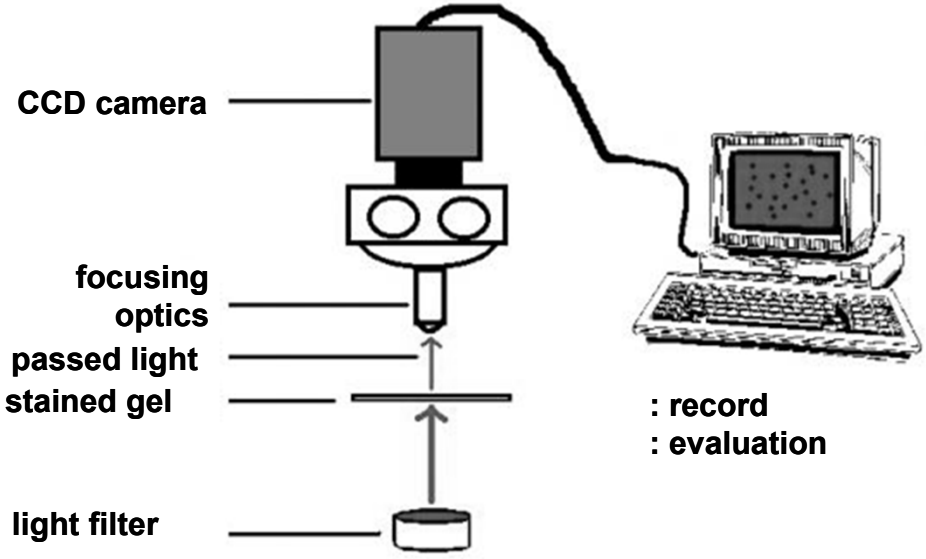
new – non-linear pH gradients in IEF

after staining

: densitometry
:: UV-Vis
:: fluorimetry



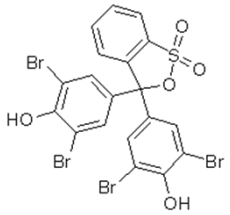


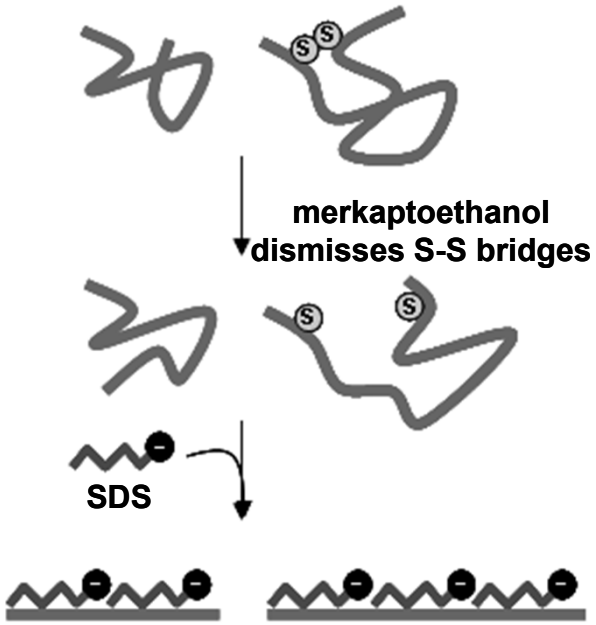


denaturing GE

: 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

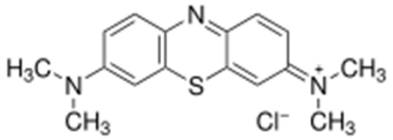




non-denaturing (native) GE

: 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)

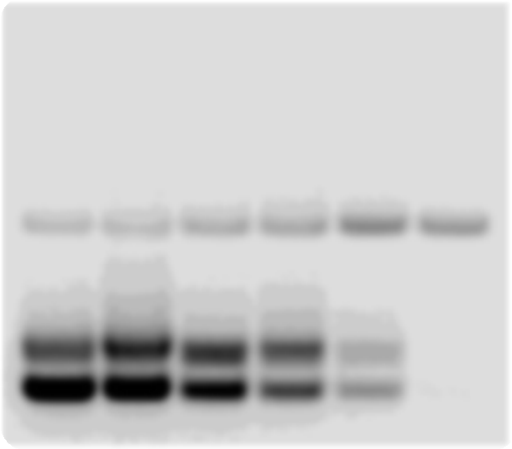
clean native PAGE (CN-PAGE) – n-dodecyl-β-maltoside and digitonin

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



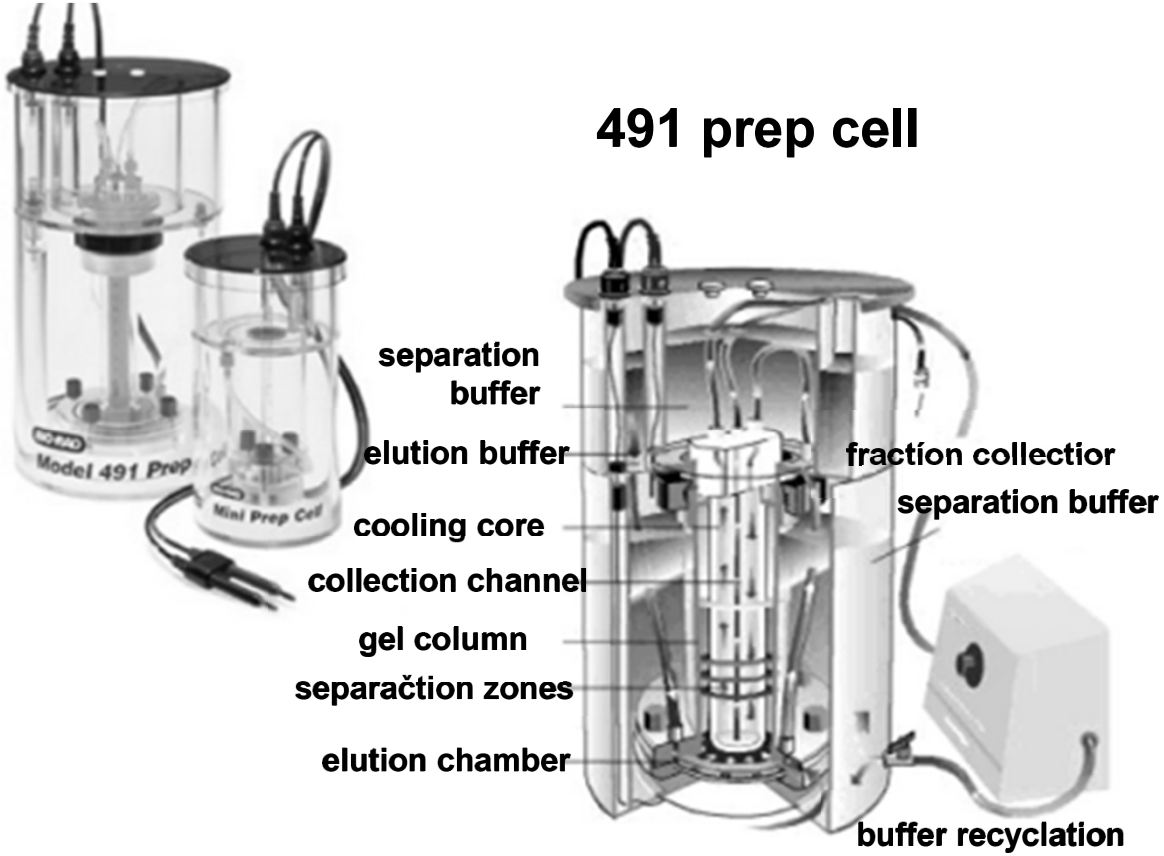
leading colours
: xylene and bromophenol blue, cresol red, orange G

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

CEGE, column continuative elution gel electrophoresis

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

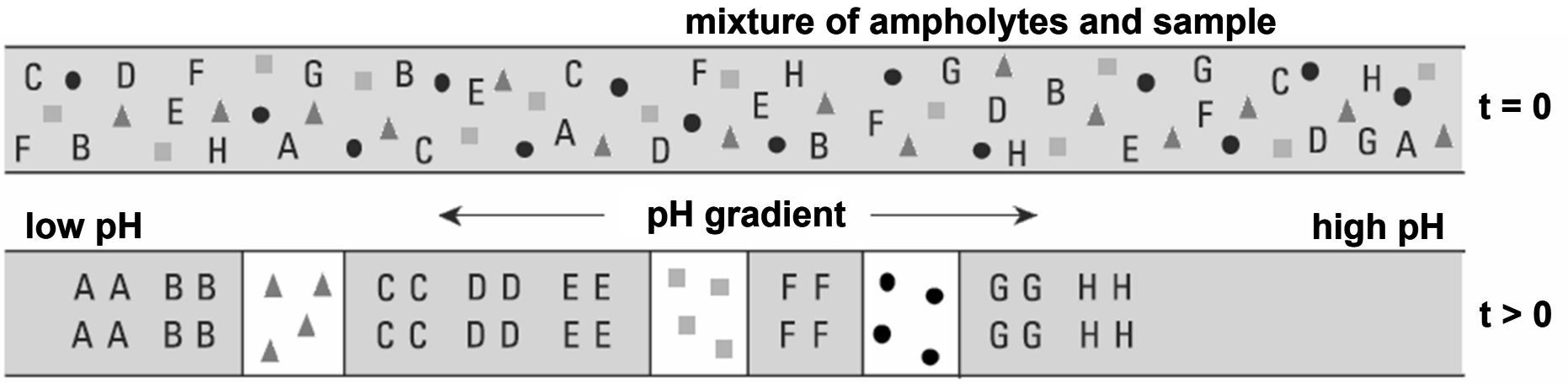


CIEF, capillary isoelectrofocusing

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, movement towards detector only due to EOF (or pressure)



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

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

$\mathbf{w_A}$ – zone width
x – length coordinate

resolution in IEF

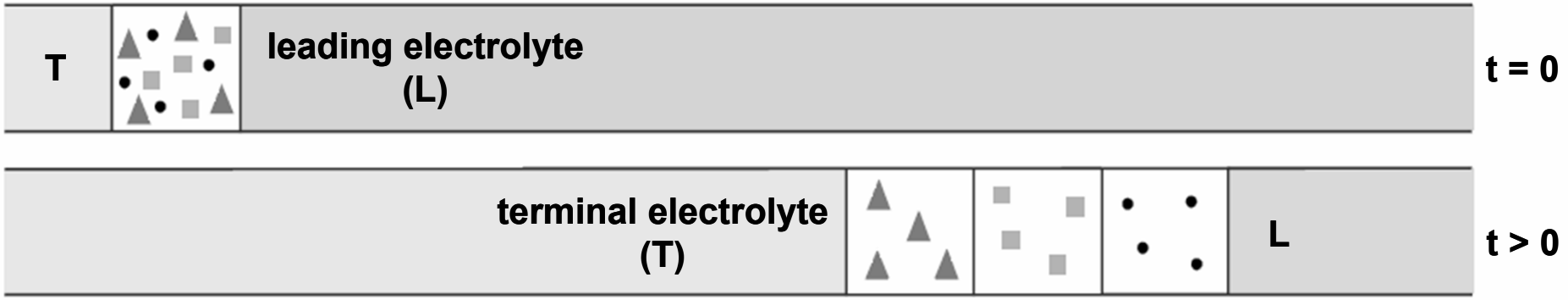
$$\Delta pI = \sqrt{\left(\frac{\partial pH}{\partial x} \right) \Big/ E \cdot \left(- \frac{\partial \mu}{\partial pH} \right)}$$

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, c_A:

$$c_A = c_L \cdot \frac{\mu_A}{\mu_A - \mu_{Cl}} \cdot \frac{\mu_L - \mu_{Cl}}{\mu_L}$$

for strong univalent electrolytes

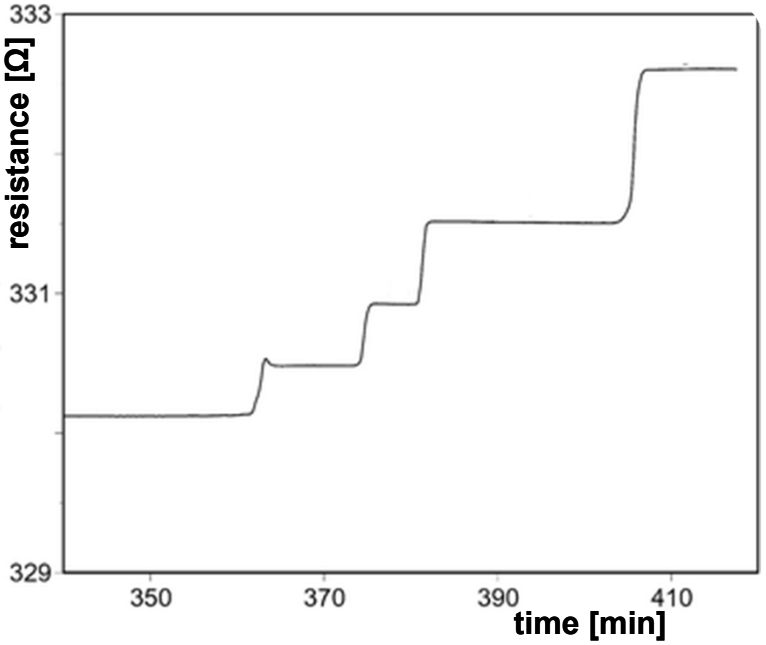
Cl – analyte counter-ion

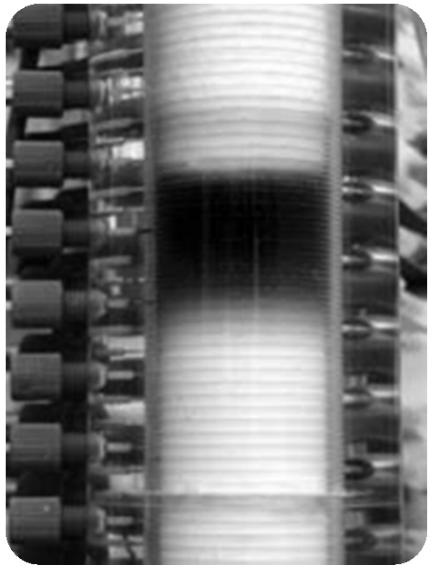
self-focusing effect

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

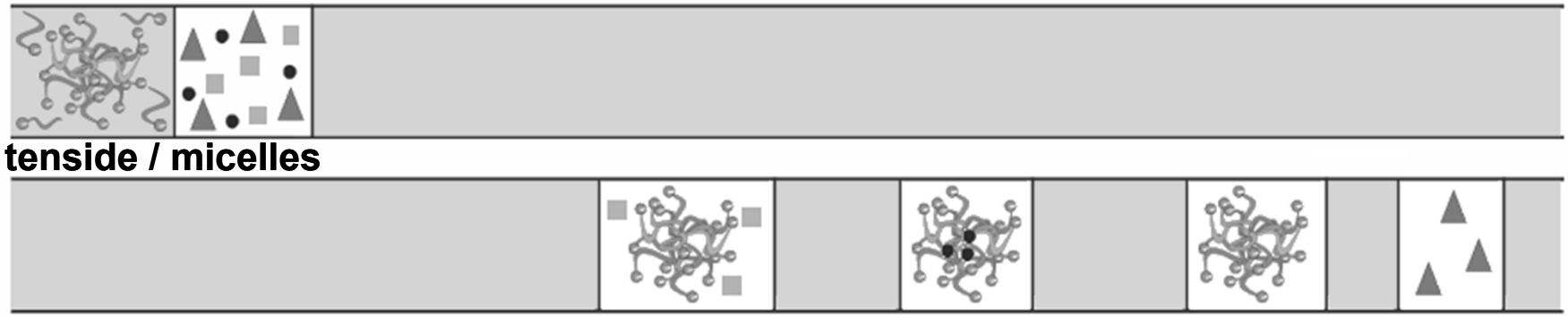
typical **detection** – resistance; others methods – conductivity, thermometry, UV-Vis

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**

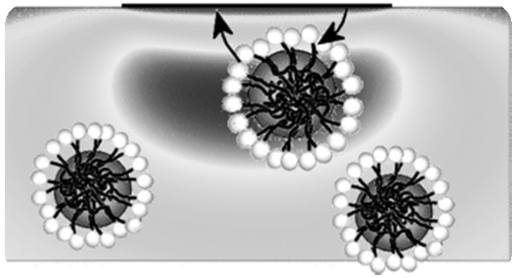
analyte **does not enters** micelles ⇒ **K = 0,** analyte **enters completely** ⇒ **K =** ∝



$$k' = \frac{t_M - t_m}{t_m\left(1 - \left(t_M / t_{M,mic}\right)\right)} = K \cdot \left(V^S / V^M\right)$$

k' – capacity factor
t_m – void retention time
t_M – retention time
$t_{M,mic}$ – 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

$$R = \left(\frac{\sqrt{N}}{4}\right) \cdot \left(\frac{\alpha - 1}{\alpha}\right) \cdot \left(\frac{k'_2}{k'_2 + 1}\right) \cdot \left(\frac{1 - (t_m / t_M)}{(1 - (t_m / t_M)) \cdot k'_1}\right)$$

efficiency selectivity retardation

α – selectivity
N – number of theor. plates

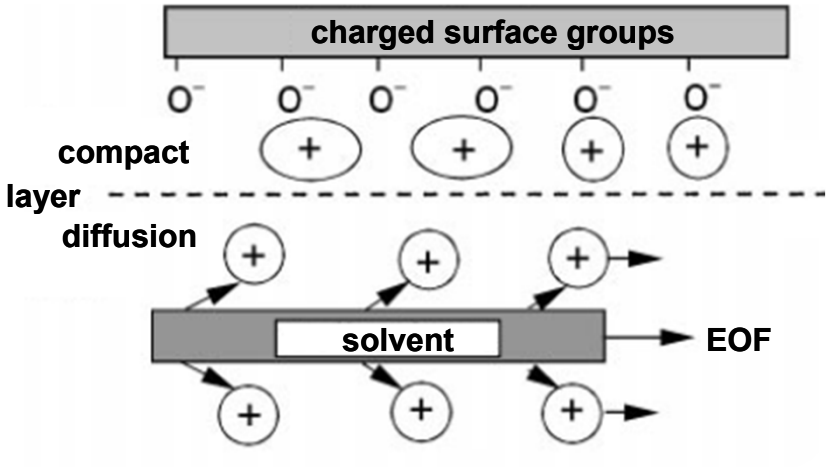
disadvantage: difficult *reproducibility*

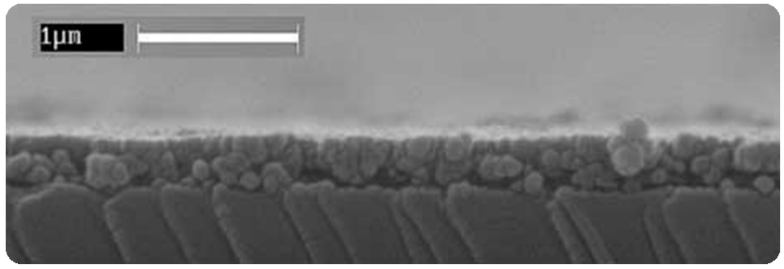
TLE, thin layer electrochromatography

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)





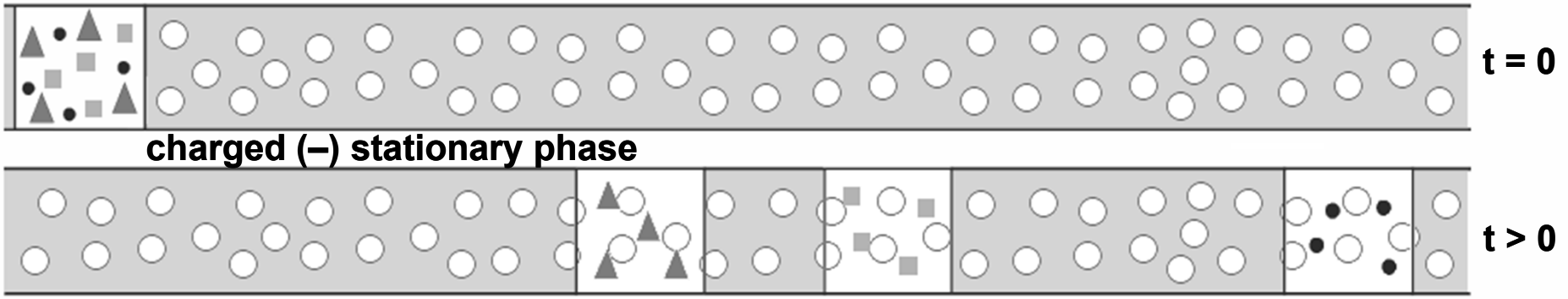
fast : applied voltage is driving force; comparing to TLC where it is capillary elevation
: fast also comparing to capillary variant (up to three orders of magnitude)
: voltage 160 V/cm ⇒ migration velocity 100 µm.s^{-1}

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

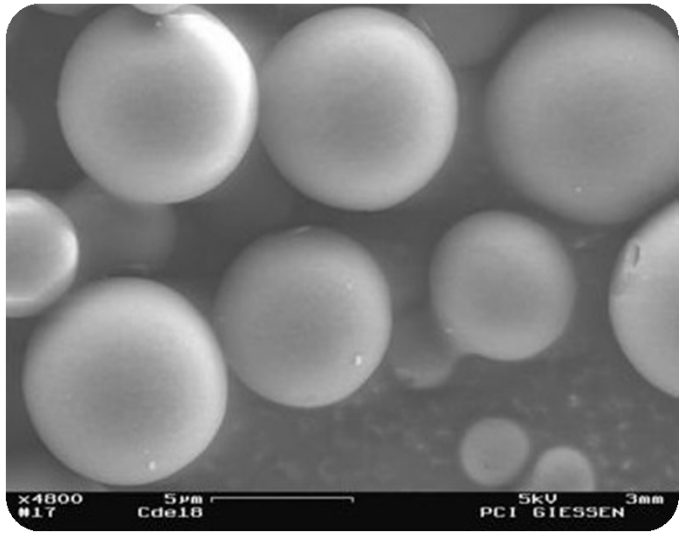
low currents: max 10 μA
Joule heat 0.1 W.cm^{-2} (1500x more heat than within pressure heating by HPLC)



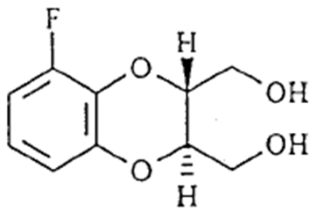
SF

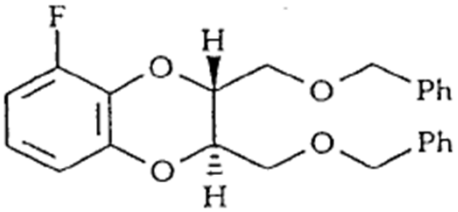
: C18 bound on silicagel (reverse CEC)
: β-CD bound on silicagel (chiral CEC)
: SCX cation exchanger (-$CH_2CH_2CH_2SO_3H$)

90 % SF for separation equilibrium
10 % SF (pure silica) for EOF stabilisation

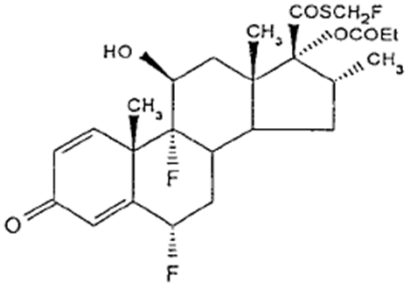


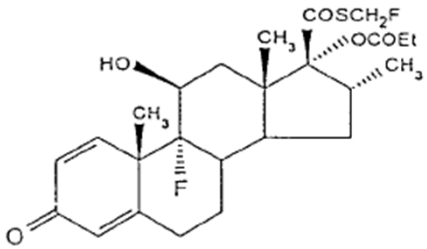
testing mixture



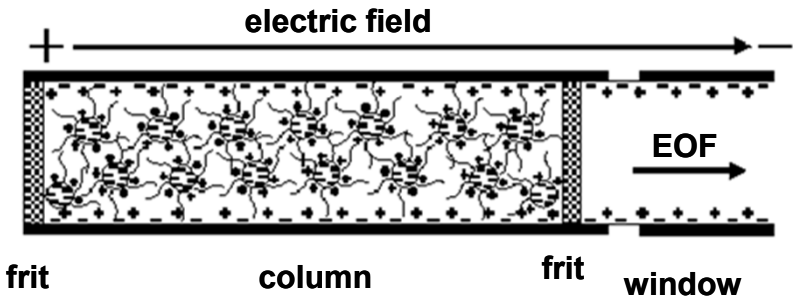


thiourea
GR 57888X, GR 57994X
fluticason proprionate, des-6-α-fluoro-fluticason proprionate





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

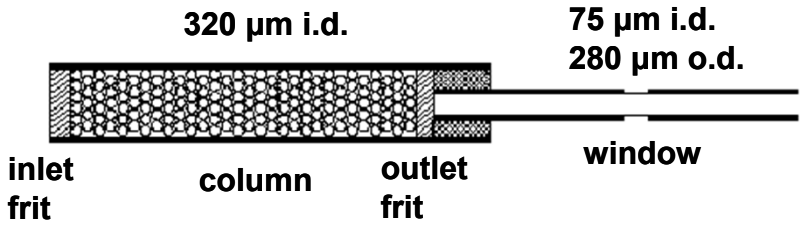


advantages

: 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

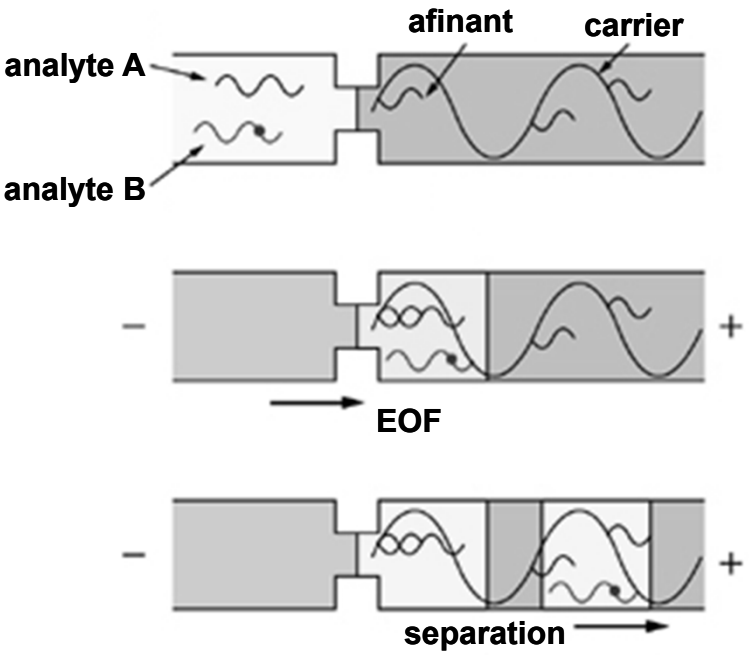
: bubbles (EOF differences, Joule heat)
: electrokinetic injection (internal standard)
: lower sensitivity

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



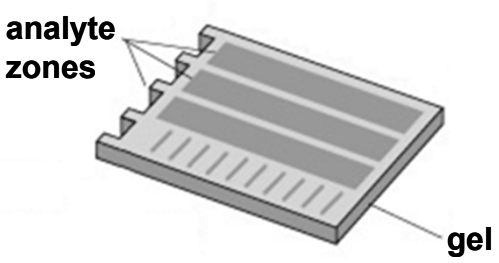
in **capillary** and in **gel**

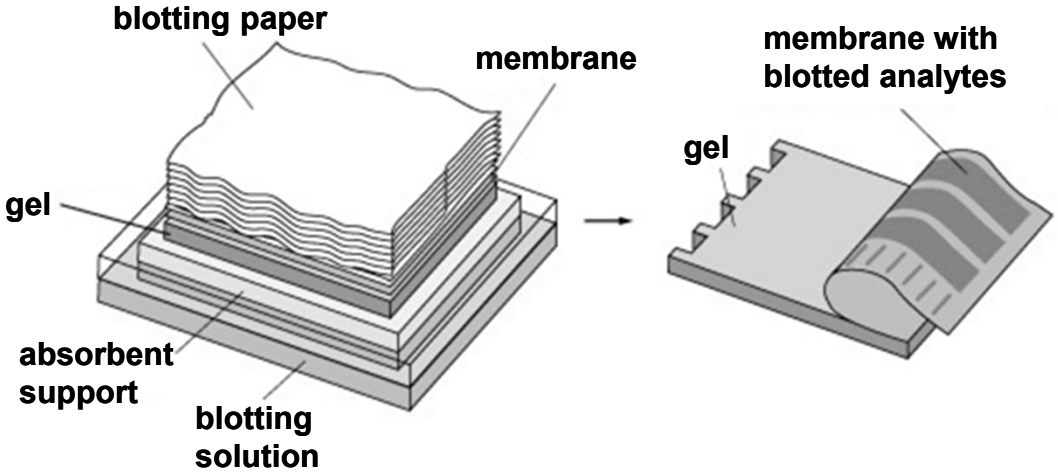
: separation
highly selective

: purification
shot-gun

: interaction study
compatibility
association constants

blotting

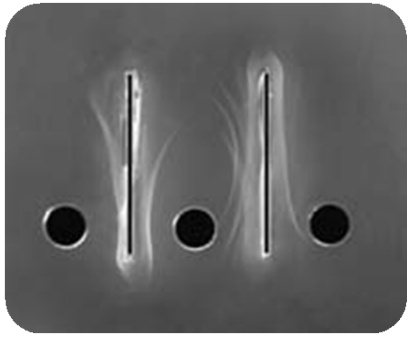




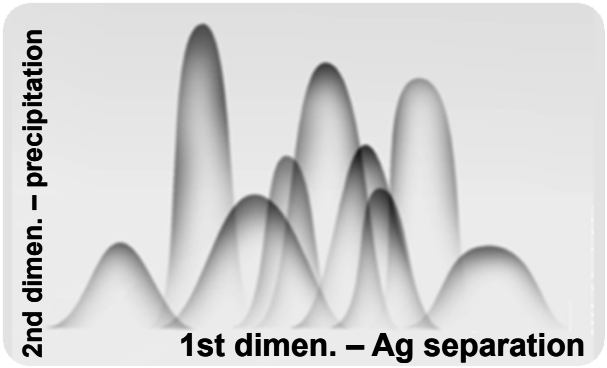
Southern blot – DNA
Northern blot – RNA
Western blot – proteins

immunoelectrophoresis

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



1D gel immunophoresis



2D gelová immunophoresis

NAE, non-aqueous electrophoresis

separation in **non-aqueous solvents**

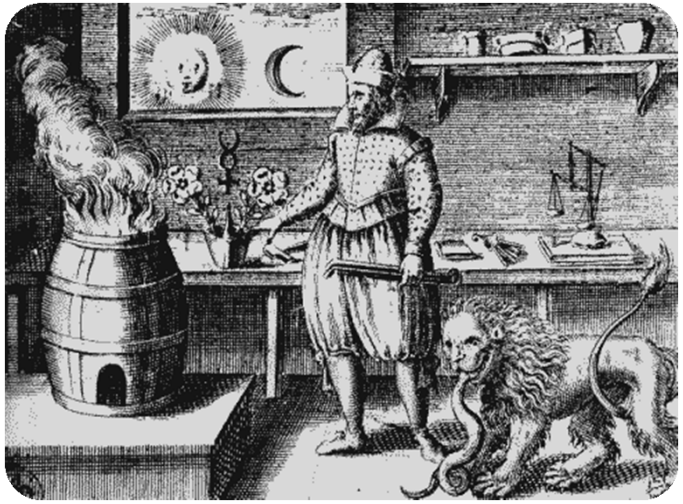
1978 – non-aqueous **TLE**
1984 – non-aqueous **CE (NACE)**

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**

validation of analytical separation method

procedure *– demonstration and documentation of quality of analytical separation method by means of establishment of defined criteria and by estimation of values of these criteria*

statistical proof of reliability of separation method
: including whole manipulation/analytical background

validated property – subject of validation

: identity and concentration of principal substance
: impurity concentration
: physico-chemical parameter

when to validate?

: when ***introducing new*** method
: when ***transferring validated*** methods
(e.g. out of development into target laboratory; published validated methods)
: when ***checking competence*** of system
: when ***revalidating*** method; revalidation conditions should be strictly given

kinds of validation

internal validation

in a frame of one laboratory

pilot validation

: limited number of samples
: piloting the suitability of chosen analytical method for full scale validation
: *validation parameters*: selectivity, robustness, reproducibility

full validation

: demonstration of method suitability for intended use
: all required validation parameters

validation by method transfer

: introduction of published validated analytical method
: *validation parameters*: laboratory accuracy and reproducibility

retrospective validation

: checking the validity of previously fully validated method
: checking the calibration line (linearity and sensitivity)
: *validation parameters*: reproducibility

external validation

inter-laboratory comparison tests

: internal validation + comparative method validation from more laboratories
: *validation parameters*: repeatability

validation programme

summarises **basic rules**:

: for planning and organisation of analytical data validation
: for introduction and use of such defined parameters in praxis

items of validation programme

a) operating sequence
b) validation parameters
c) system revalidation conditions
d) validation protocol
e) literature (critical research and consultations)

operating sequence

: complete analytical formula serving to reproduce whole analytical method

: contains all needful instructions: *precise*, *detailed* and *complete*

: must be optimised and as such used with statistical check of measurements

characteristics of operating sequence

scope of method use, sequence principle, chemical reactions and interactions of determined component, analyte and matrix, range of content of determined component, measurement principle and units

chemicals

chemical purity of chemicals used, their processing and purification, preparation of solvents, agents and support chemicals, stability and concentration

standard operation procedure

mechanical sample procedure, chemical sample procedure, calibration, measurement, calculations and evaluation

method identity

validation parameters

measure of agreement tightness between independent results under defined conditions

independent result – result obtained uninfluenced by any previous result on the same or similar sample

expression: standard deviation of results ($\mathbf{s_X}$)

absolute value of $\boldsymbol{s_X}$ – if not dependent on content (X)
relative value of $\boldsymbol{s_X}$ (%) – if dependent on content

relative standard deviation – if standard deviation is constant in whole range of measured values; related to the highest value of set x_{max}

standard deviation

characterises deviation of individual values x_i around average $\bar{x}$

coincidental quantity – it is not valid characteristics of given analytical method

must be specified

: must content all sources of variability (also those of operation sequence – sample decomposition, dilutions, extractions, dissolutions, final instrumental measurement)

: changing the operation sequence – revalidate the standard deviation value

for obtaining must be *sufficiently high number of samples of the same material* not from one series, but from long-term measurements

reproducibility

consistency of method under conditions of reproducibility
: depends only on coincidental error distribution; has no relation to accuracy

tightness of identity between mutually independent results of tests obtained under conditions of reproducibility

conditions
mutually independent results of tests by repeated use of the same test method on identical material, in the same laboratory, by the same operator using the same instruments, during short time range

$$R_{max} = q \cdot s_x$$

s_x is standard deviation, q is tabulated value of studentised distribution

conditions of s_X and reproducibility determination

at least 5 levels (H), sample number **m** ≥ 20, parallel measurement number $\mathbf{n_A}$=2

validation protocol: all measured quantities, calculated $\boldsymbol{s_X}$

if we presume $\mathbf{R_{max} = f\,(H)}$, we need to test

: linear dependence $R_{max} = a + b \cdot H$

: exponential dependence $\log R_{max} = c + d \cdot \log H$

for *a* = 0 and *d* = 1 are these equation equivalent
in case *b* = 0 (for majority of cases $b \leq 0{,}1$) is R_{max} (resp. s_X) constant in a whole range of X values

if $\boldsymbol{s_X}$ is not dependent on content, the relative standard deviation is calculated in regard to the highest set value $\mathbf{x_{max}}$

method precision/accuracy

tightness of identity of obtained value with real one

source of real value

: standard
: reference material
: validated independent methods
: reference laboratory (same method)

yield of method

$R = n_{ex} / n_{ref}$ must be 0.95 – 1.05 for each concentration level

yield test I.

$$t = \frac{|x_{ref} - \overline{x_{ex}}|}{s_x} \cdot \sqrt{m}$$

m – number of parallel determination of reference
$\mathbf{s_x}$ – determined out of min. 7 values on one concentration level; RSD_{max} = 3 %

if $\mathbf{t \geq t_\alpha}$; method is subjected to systematic error

yield test II.

test to if systematic error is
: constant **≠ *f* (c)**
: proportional **= *f* (c)**

$$t_a = \frac{|1-a|}{s_a} \qquad t_b = \frac{|b|}{s_b}$$

if $\mathbf{t_b \geq t_\alpha}$; method is subjected to **constant systematic error**

eliminable – new blank experiment

if $\mathbf{t_a \geq t_\alpha}$; method is subjected to **proportional systematic error**

calibration choice

linear or **non-linear**?

linearise? and if non-linear, so which and why such?

aspect of linearity: $\mathbf{r_{xy} \geq 0.98}$
min: 5 points in concentration scale, 3 points per each point of scale

evaluation of importance of segment **b**

$$t = \frac{|b' - b|}{s_b} \qquad t = \frac{|a' - a|}{s_a}$$

recalibration

new adjustment of parameters **a** and **b**
: difference test of new and old values by F-test
:: if not similar, it is necessary to calibrate again

sensitivity

$$\frac{dy}{dx} = \frac{df(x)}{dx}$$

first derivation of calibration function

limit of detection

analyte concentration at which the signal is statistically different from noise

uses blank experiment
max deviation of baseline (h_{max}) in range of 20-fold of $w_{1/2}$ of signal peak

$y_{LOD} = 3 \cdot h_{max} \Rightarrow x_{LOD} = y_{LOD} / a_h$; $\mathbf{a_h}$ is calibration on peak height $\mathbf{y = a_h \cdot x}$

limit of quantification

analyte concentration at which the relative standard deviation predicted from calibration is small (~ 0.1)

uses blank experiment
max deviation of baseline (h_{max}) in range of 20-fold of $w_{1/2}$ of signal peak

$y_{LOQ} = 10 \cdot h_{max} \Rightarrow x_{LOQ} = y_{LOQ} / a_h$; $\mathbf{a_h}$ is calibration on peak height $\mathbf{y = a_h \cdot x}$

selectivity

ability of *precise* and *accurate* determination of analyte in *matrix* presence

determination
comparison of analyte signal in *standard sample* and in *sample with matrix*

all minimally **3x** and at concentration **close to LOQ**

: determine quantity and deviation of background signal
: determine the difference importance of background signal to substance concentration at LOQ

interferent < 1% of response close to LOQ

robustness

extent of influence of individual parameter deviation on resulting determination

robustness optimisation
: choose purpose quantity/function; has an extreme in optimum; **Z**
: consider and choose factors, which may influence result; $\mathbf{Q_i}$
: for each Q_i choose extreme of purpose quantity/function – *min* or *max*

reduces multifactorial analysis by Plackett and Burman

: use of 2-level reduced experimental design
: minimal number of runs **m** (= 4), minimal number of factors **n** (= m – 1 = 3)
: to each factor assign two extreme values *higher* (+) and *lower* (–)
: *in the first line,* **m/2** factors is **+** and **(m/2)-1** is **–**
: *each next line* has same representation, but different composition
: *last line* has all **–**

if factor number < than possible (m-2) $\Rightarrow$ use of **dummy factors** (+1 or -1)

((m/2)-1) dummy factors tests errors by prediction of main effect

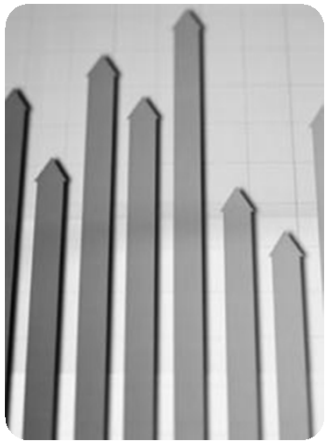
run	factors							purp. funct.
	Q_1	Q_2	Q_3	Q_4	Q_5	Q_6	Q_7	
1	+	+	+	–	+	–	–	Z_1
2	+	+	–	+	–	–	+	Z_2
3	–	–	+	–	–	+	+	Z_3
4	–	+	–	–	+	+	+	Z_4
5	+	–	–	+	+	+	–	Z_5
6	–	–	+	+	+	–	+	Z_6
7	–	+	+	+	–	+	–	Z_7
8	–	–	–	–	–	–	–	Z_8
weight	W_1	W_2	W_3	W_4	W_5	W_6	W_7	

$$W_i = \frac{\sum Z_i^+ - \sum Z_i^-}{m/2}$$

$$s_W = \sqrt{\frac{\sum W_{i,a}^2}{m-n-1}}$$

if $W_i \geq W = s_W \cdot t_\alpha$; influence of factor Q_i is **statistically important**

statistical testing



deviation agreement	F-test
agreement of mean values	Student t-test
outlying values	Grubbs T-test Q-test according to Dean-Dixon Cochran test C-test

instrumental validation

validation by manufacturer – norms ISO 9000 – 9004

other: individual validation program of instrumentation

revalidation

conditions cannot be generally defined

each change in the analytical system must lead to *its revalidation*
influence on final outcome should be considered individually

revalidation *should not be complex*, only as a partial step of validation program (e.g. calibration, sensitivity); standard deviation must be retrospectively determined (resp. R_{max}), i.e. influence of revalidation on value of R_{max} resp. s_x

validation protocol

in regard to *particular validation program*

: records all measurements, calculations
: results and conclusions are clearly defined

mention the date of individual tests, name of responsible operator and names of all collaborators, which worked on validation program

scheme of validation procedure