Department of Pharmaceutical Analysis and Nuclear Pharmacy, Faculty of Pharmacy Comenius University



Separation methods: Electromigration

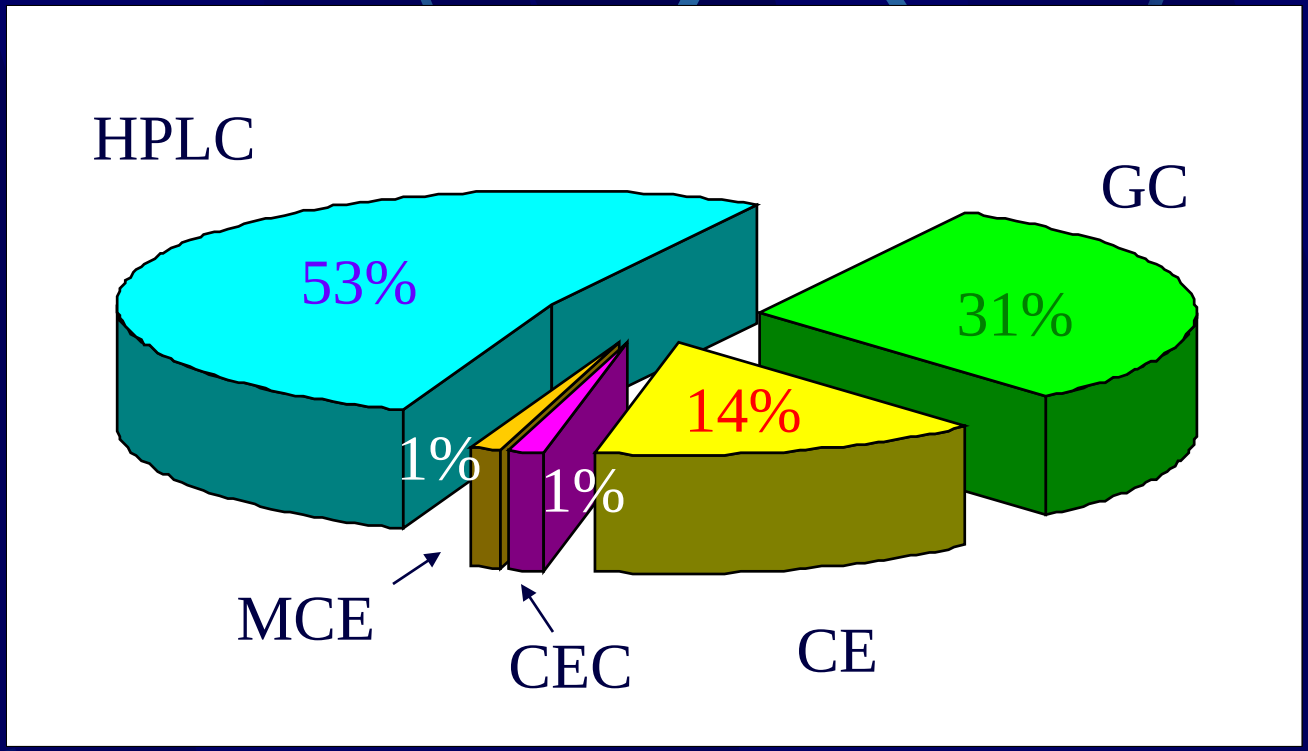
(lecture)

Bratislava, 2020

Peter Mikuš

The most important high-performance analytical separation methods

WOS – period of one decade (more than 100.000 papers)



Requirements for analytical method

- Selectivity ↑
- Sensitivity ↑
- Reliability ↑
- Sample preparation and handling ↓
- Sample throughput, analysis time ↓
- Economic parameters / cost ↓
- Automatization ↑

History

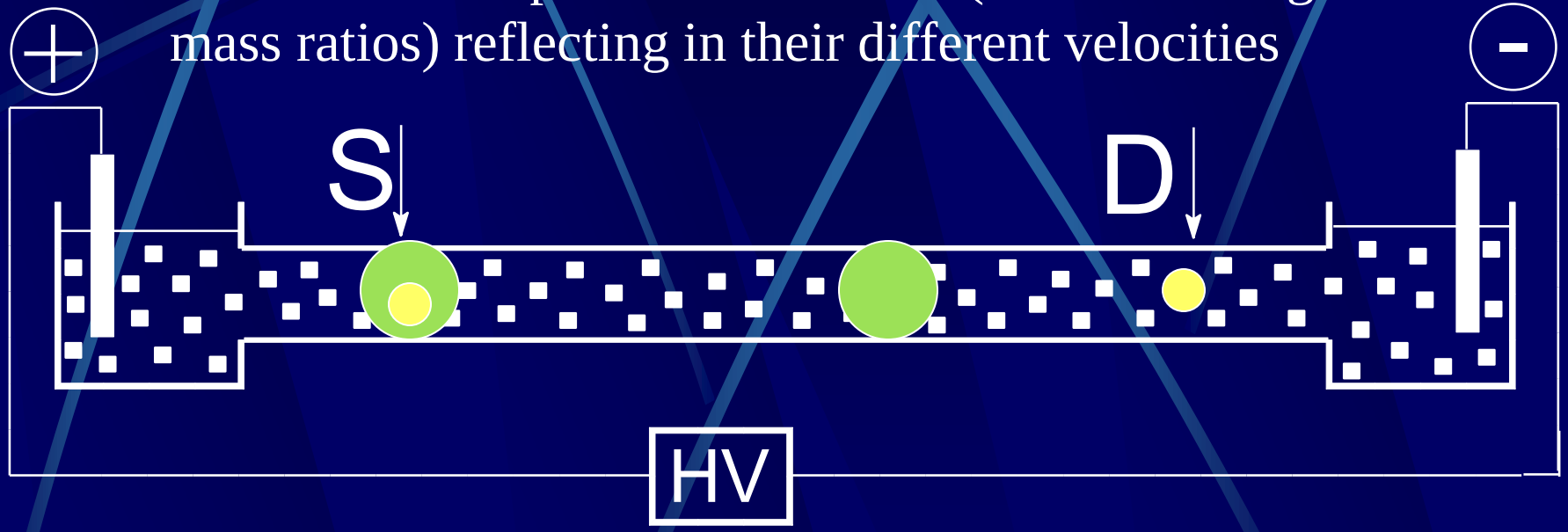
- Reuss 1905, electrophoretic principles
- Tiselius 1937, separation of protein mixture in free solution
- Electrophoresis in planar arrangement
- **Hjérten 1967, capillary electrophoresis**
- Capillary electrochromatography
- Microchips

What parameters are typical for CE?

- High separation efficiency ($N \sim 10^5$-10^6)
- Detection directly in separation capillary (on-capillary UV, CD, LIF, except from MS)
- Small volumes of samples and electrolytes (pl-nl-μl)
- Simple approaches for changing selectivity
- Flexibility in experimental arrangement
- Aqueous media are used the most frequently
- Wide scale of the analyzed compounds
- Short analysis times
- Low cost instrumentation and analyses
- Automatization
- Miniaturization

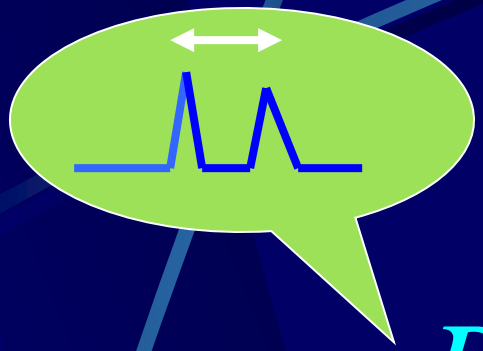
Electrophoretic principle and instrumental scheme

Compounds are separated in electric field due to their different electrophoretic mobilities (different charge-to-mass ratios) reflecting in their different velocities



$$v = m.E \qquad m \approx \frac{q}{M_{sol}}$$

Resolution of the separated compounds



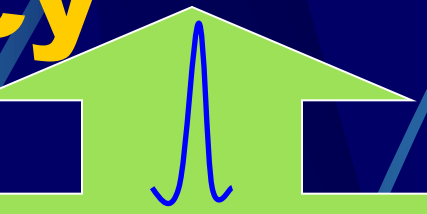
$$R = \frac{1}{4}\sqrt{N}\left(\frac{\Delta m}{m_{av}}\right)$$

efficiency

selectivity

Parameters influencing efficiency

Dispersion



Efficiency = ability of the separation system to provide narrow peaks

Diffusion

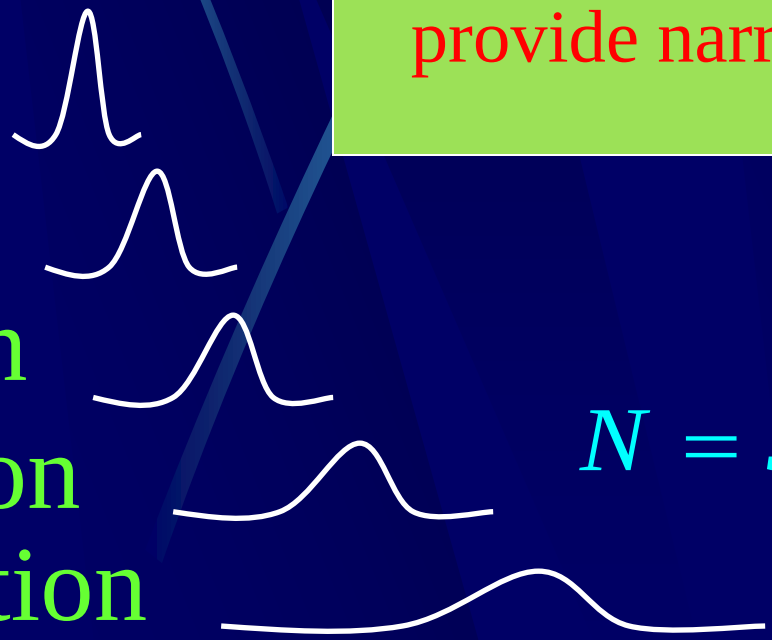
Thermal

Injection

Adsorption

Electromigration

Electroosmotic



$$N = 5.54\left(\frac{t}{w_{1/2}}\right)$$

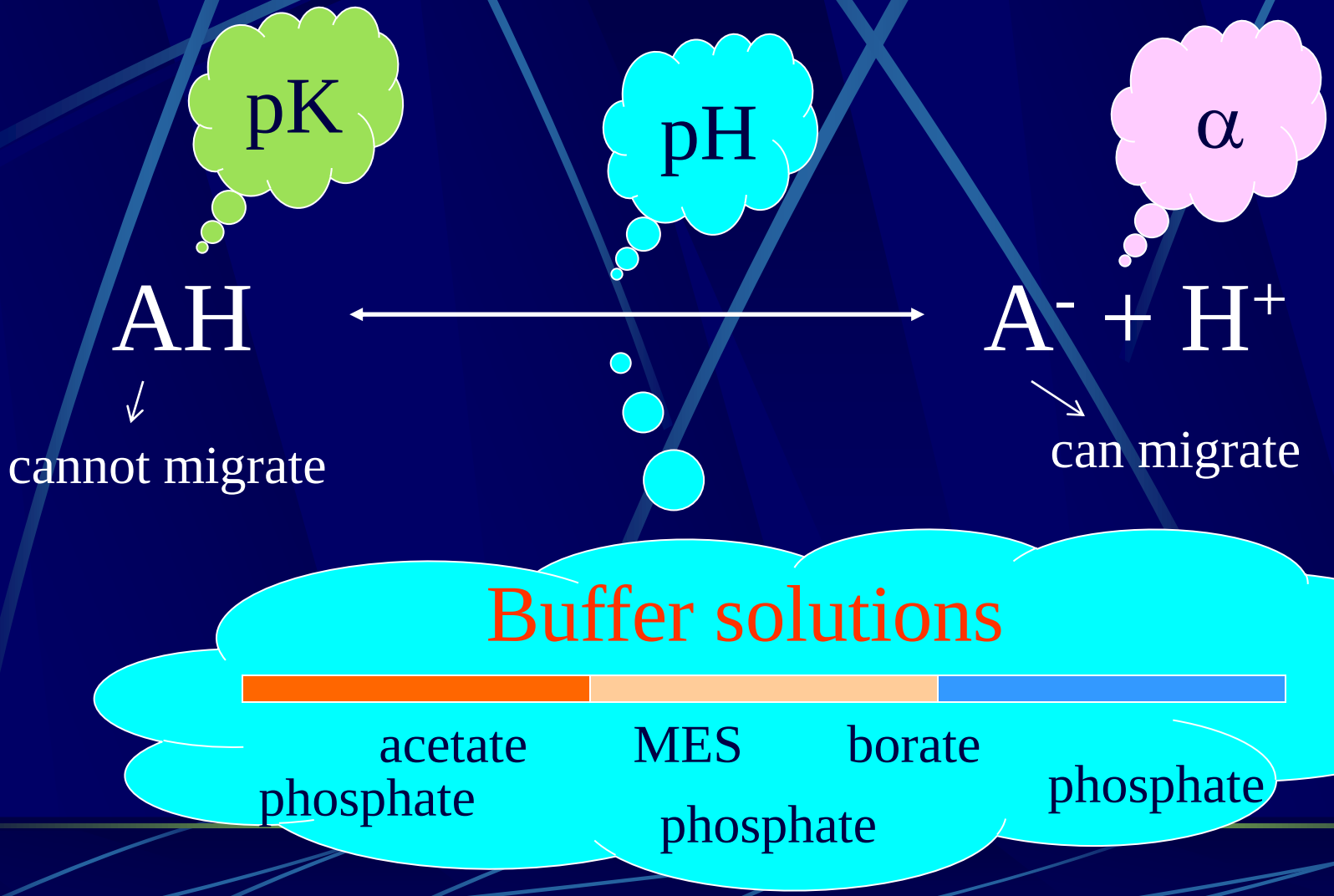
Electrophoretic mobility
how to influence it?

$$m_{A,ef} = \sum_i \alpha_{A,i} \cdot \beta_{A,i} \cdot \gamma_{A,i} \cdot m_{A,i,0}$$

By changing
pH

By creating
complexes

Acid-base equilibria



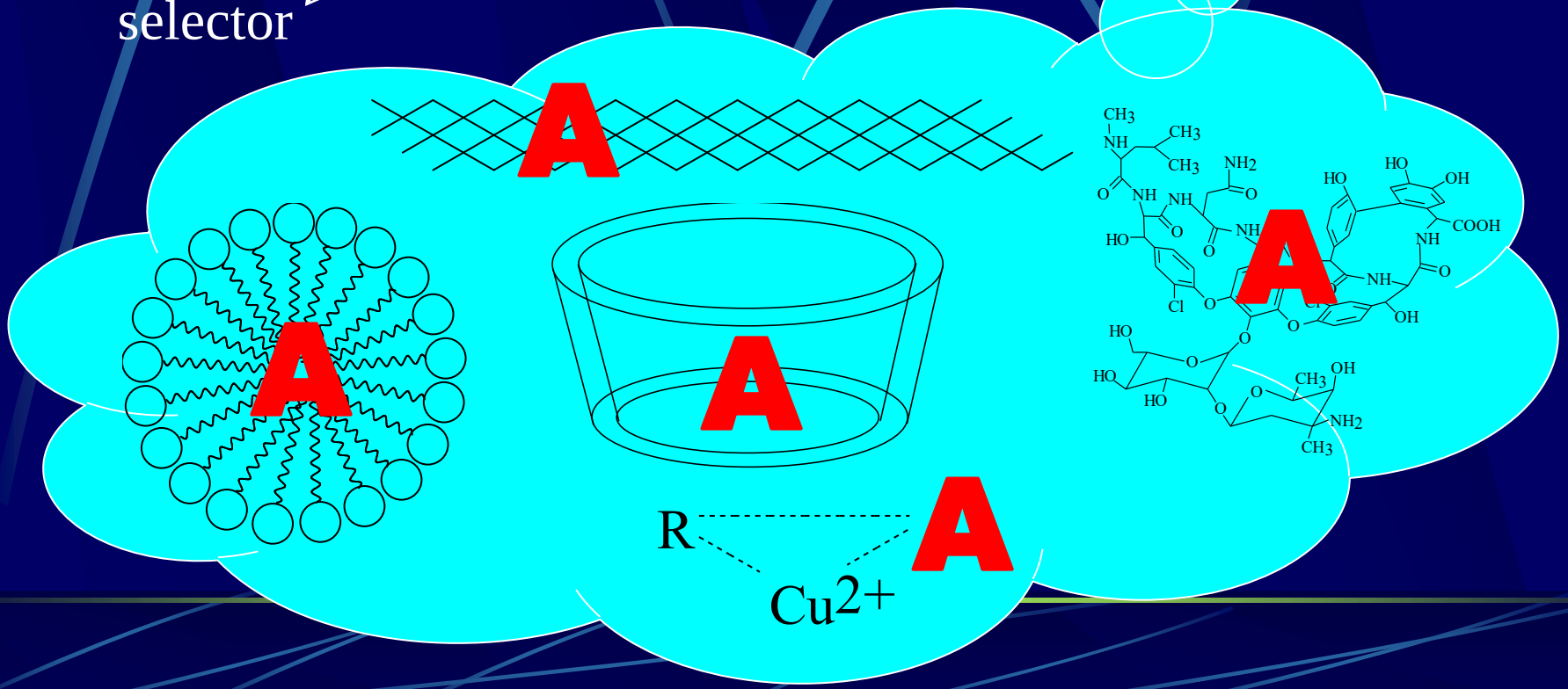
Complexing equilibria

β

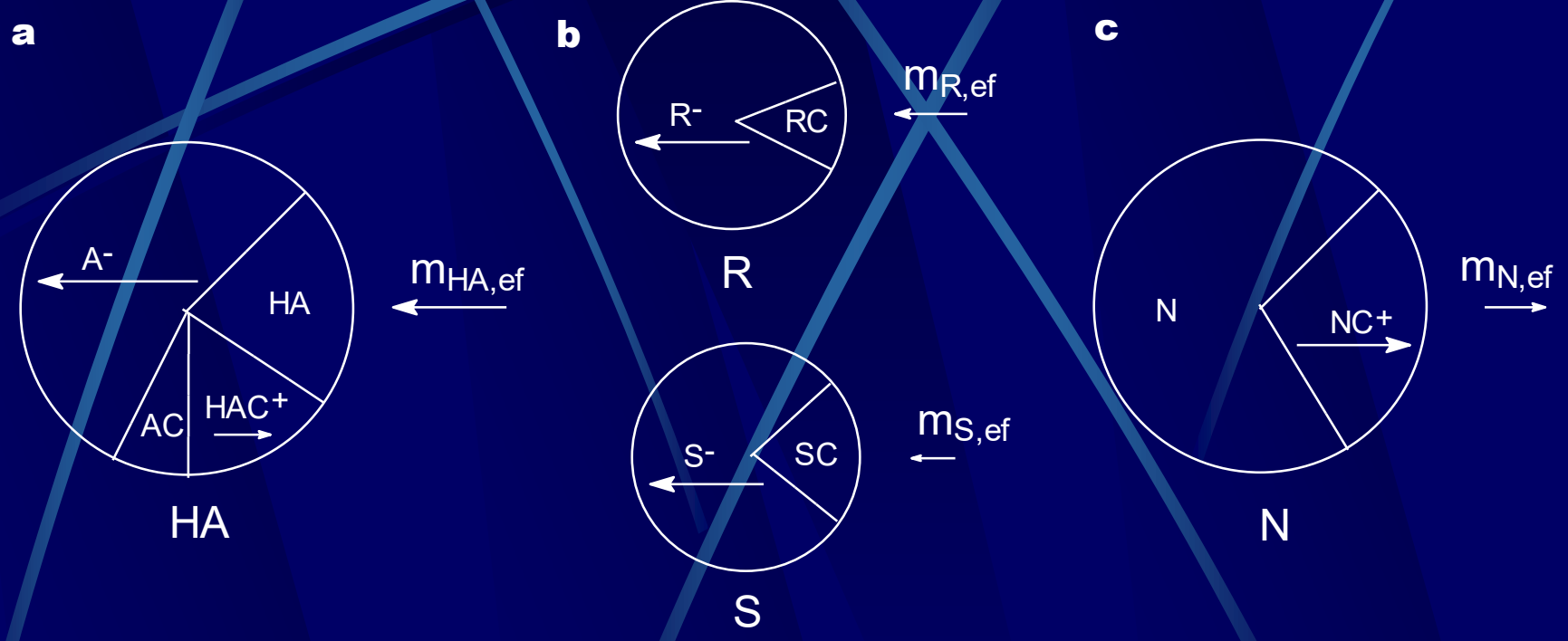
A + C ⟷ AC

pH, solvent

selector



Selectors, pH, and effective mobility



CZE

$$R = \frac{\sqrt{N}}{4}\frac{\Delta\mu}{\mu_{av}}$$

$$\Delta\mu = \frac{C(\mu_f - \mu_c)(K_1 - K_2)}{1 + C[K_1 + K_2] + C^2 K_1 K_2}$$

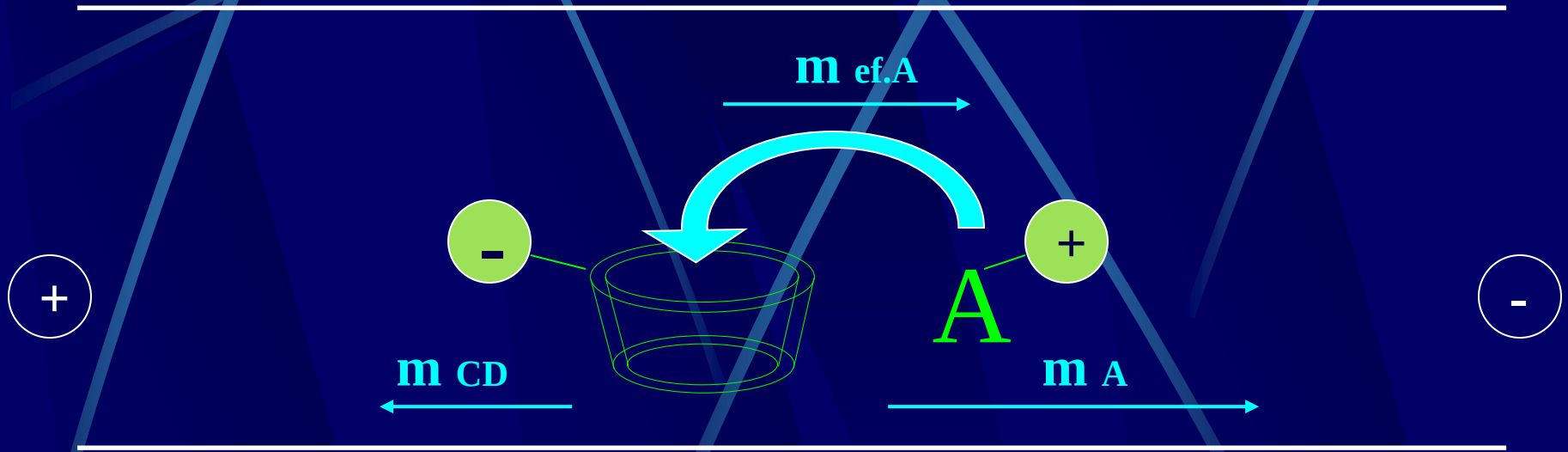
EKC

$$R = \frac{\sqrt{N}}{4}\left(\frac{\alpha - 1}{\alpha}\right)\left(\frac{k_2'}{1 + k_2'}\right)\left(\frac{1 - t_0/t_{CD}}{1 + (t_0/t_{CD})k_1'}\right)$$

$$k' = n_{CD}/n_{aq}$$

$$a = k_2'/k_1'$$

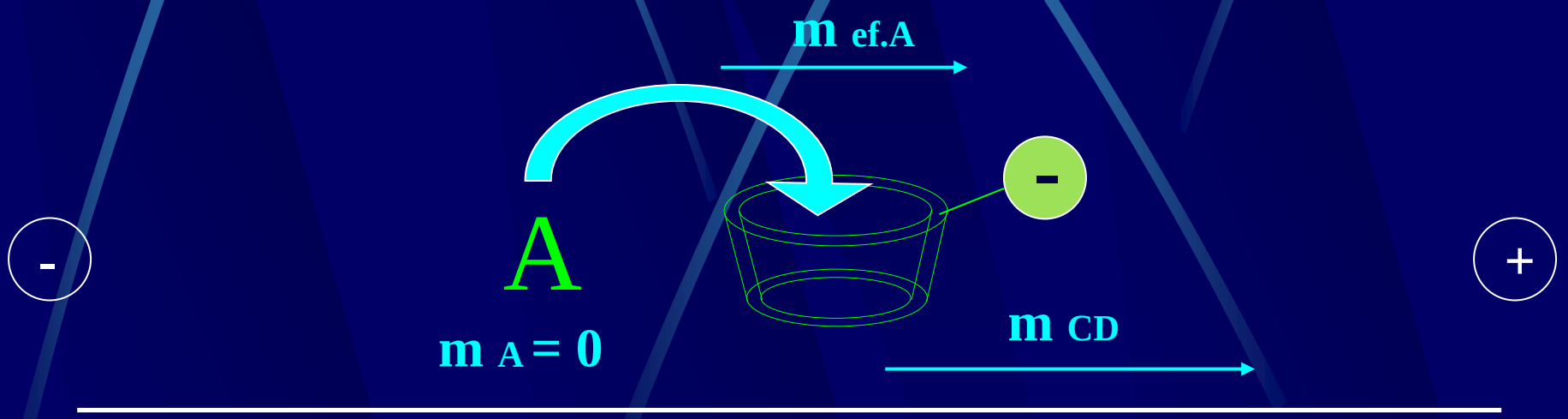
Enhancement of CE separations by oppositely charged CD



$$\Delta\mu = \mu_1 - \mu_2 = \frac{\mu_f + \mu_{c_1} K_1[C]}{1 + K_1[C]} - \frac{\mu_f + \mu_{c_2} K_2[C]}{1 + K_2[C]}$$

Separation of electroneutral compounds (with ionic mobility close to zero)

- Neutral
- Weakly ionizable
- Zwitterionic

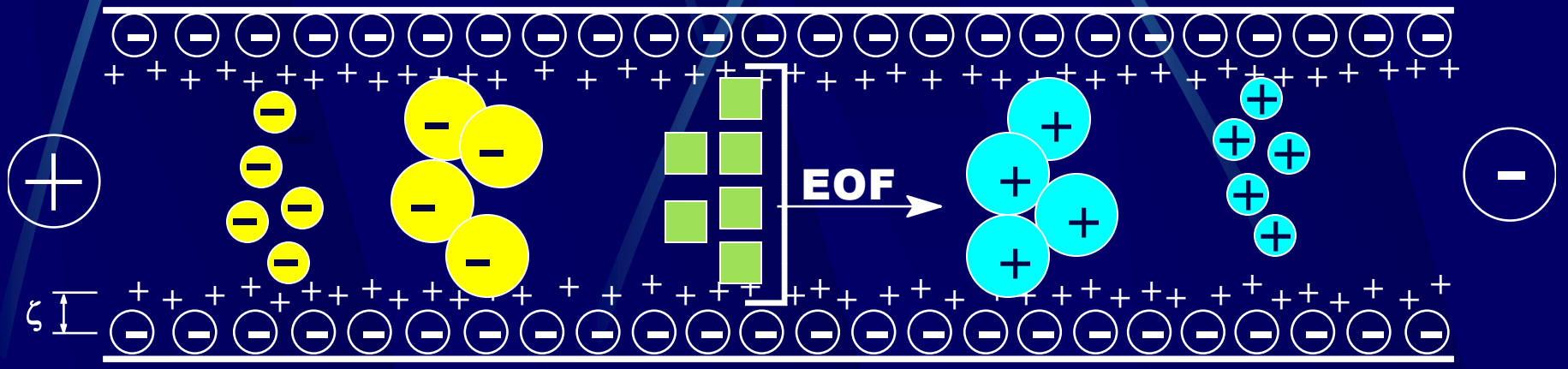


$k' = n_{CD}/n_{aq}$

$a = k_2'/k_1'$

Migration with electroosmotic flow

$$m_{A,ap} = m_{A,ef} + m_{EOF}$$

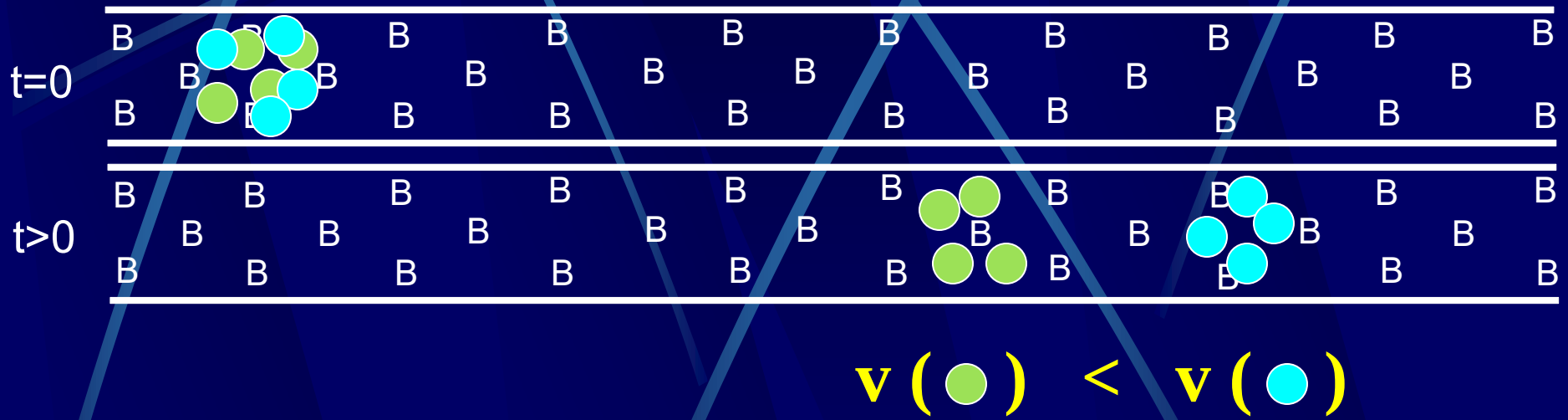


Capillary electrophoresis techniques

- Zone electrophoresis (ZE)
- Capillary zone electrophoresis (CZE) ⟶ CZE in free solution
- Electrokinetic chromatography (EKC) ⟶ CZE with selectors
- Capillary gel electrophoresis(CGE) ⟶ CZE with gel
- Isotachophoresis (ITP)
- Isoelectric focussing (IEF)
- Capillary electrochromatography

hybrid technique ⟶ CZE with chromatographic stationary phase

Zone electrophoresis

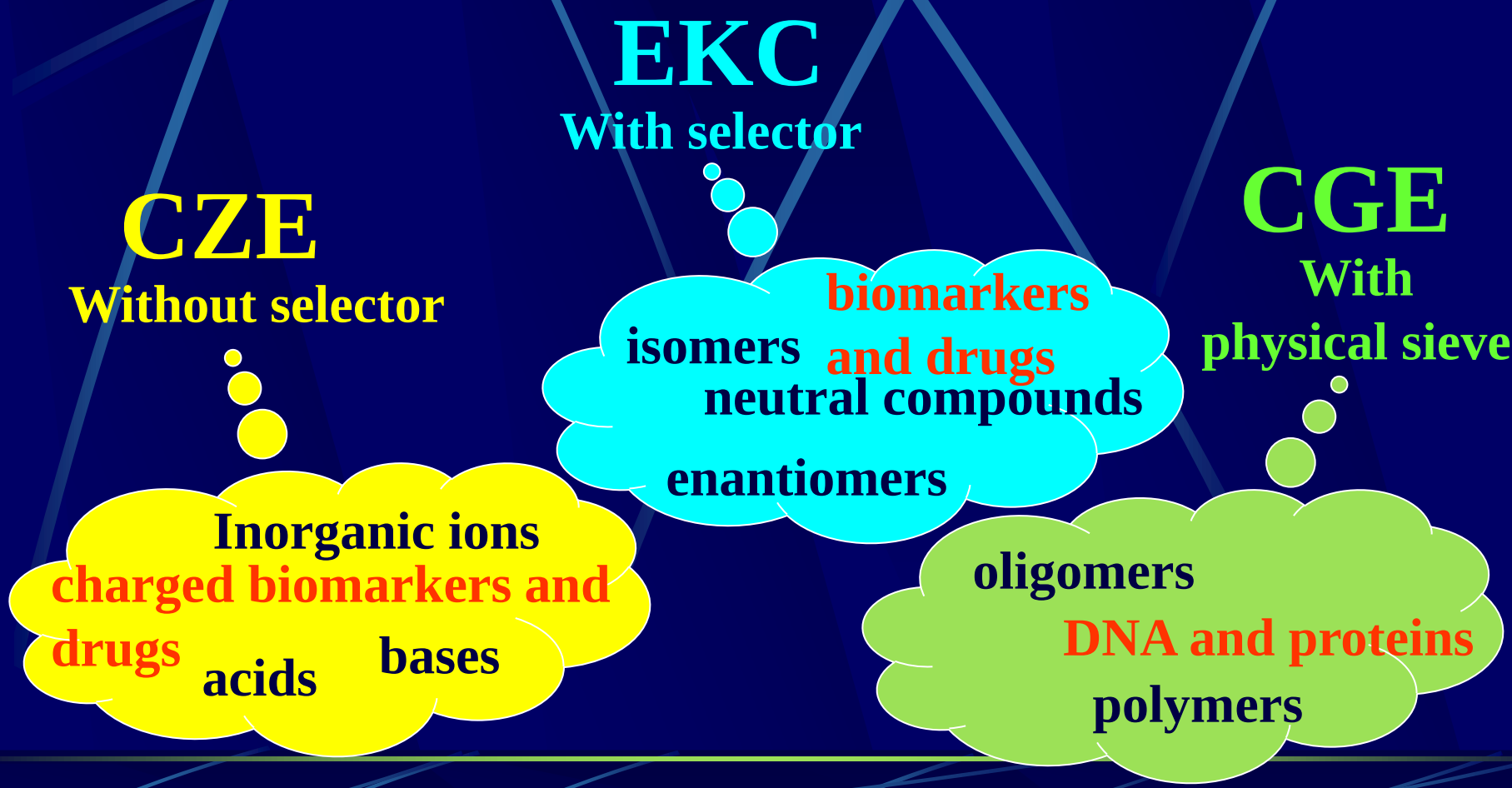


1 background electrolyte = constant separation conditions (pH, E) in whole capillary.

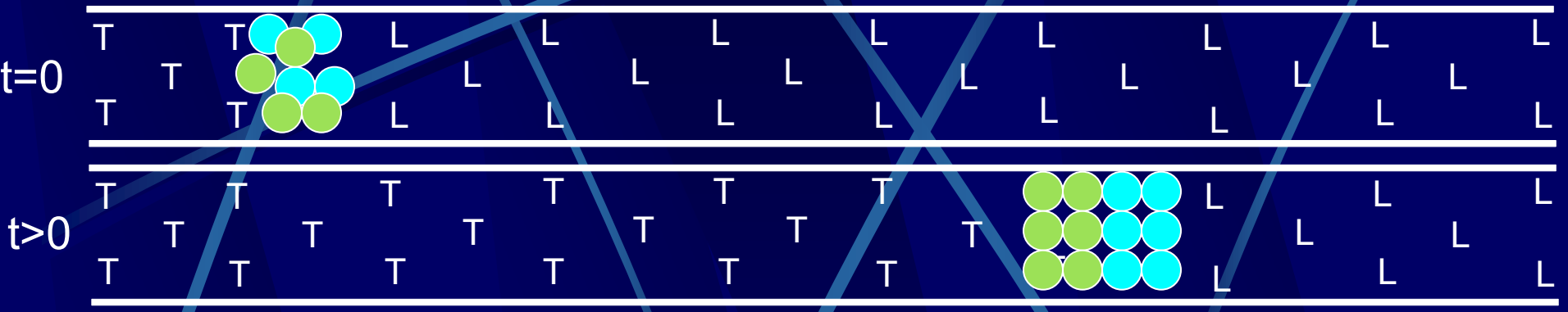
Zones are broadening due to diffusion.

Separation of cations, anions and neutral compounds in one experiment is possible.

Application of zone electrophoretic techniques



Isotachophoresis



$$v(\bullet) = v(\bullet)$$

$$m(T) < m(\bullet) < m(\bullet) < m(L)$$

2 electrolytes (Leading, Terminating) = separation conditions (e.g. E) in capillary are differing from zone to zone.

Zones are not broadened by diffusion.

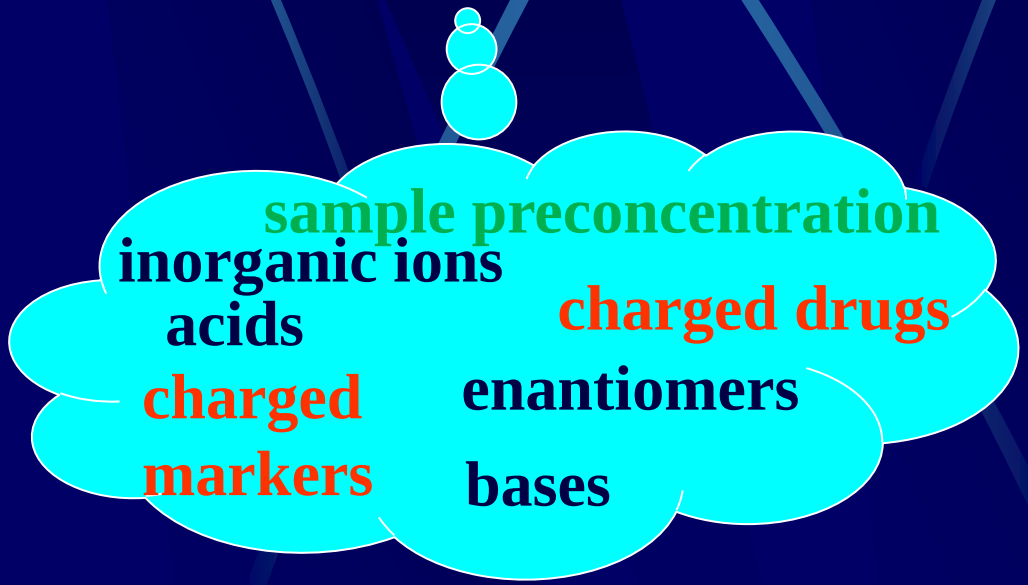
Zones are concentrated according to the concentration of L.

In one experiment just either cations or anions can be separated.

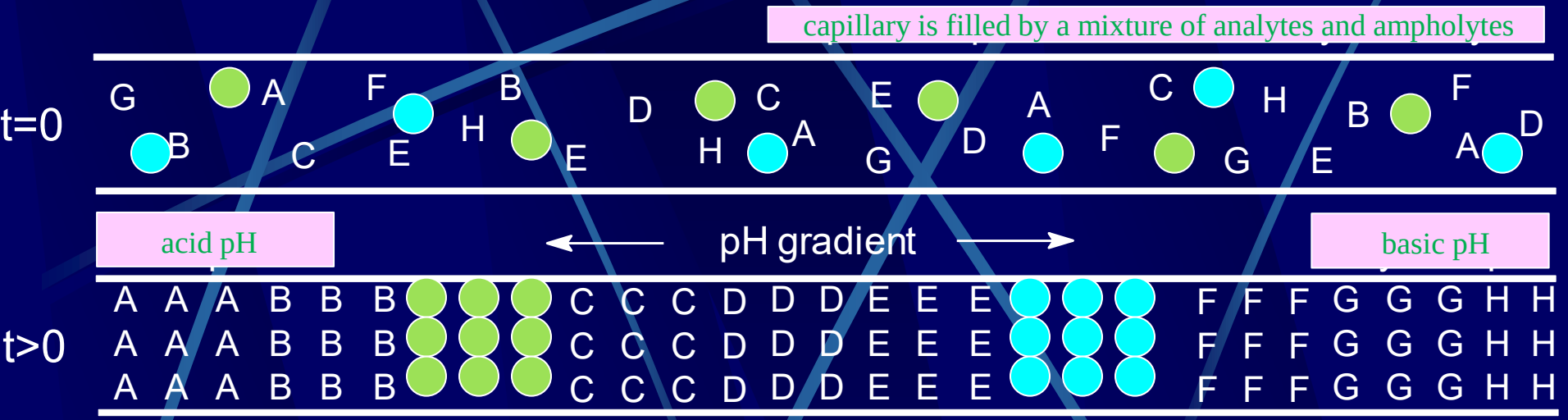
Application of isotachophoresis

ITP

with or without selector



Isoelectric focussing



v (●) = 0 **v (●) = 0**

pI (B) < pI (●) < pI (C) < pI (E) < pI (●) < pI (F)

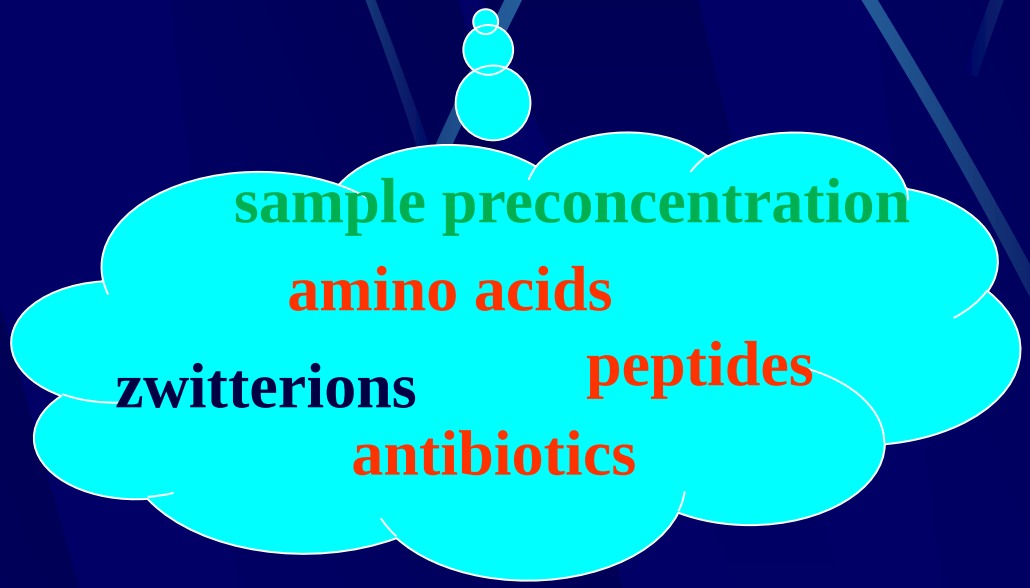
Many electrolytes (A-H) = separation conditions (e.g. pH, E) in capillary are differing from zone to zone (mixture of ampholytes= creates a pH gradient)

Zones are not broadened by diffusion.

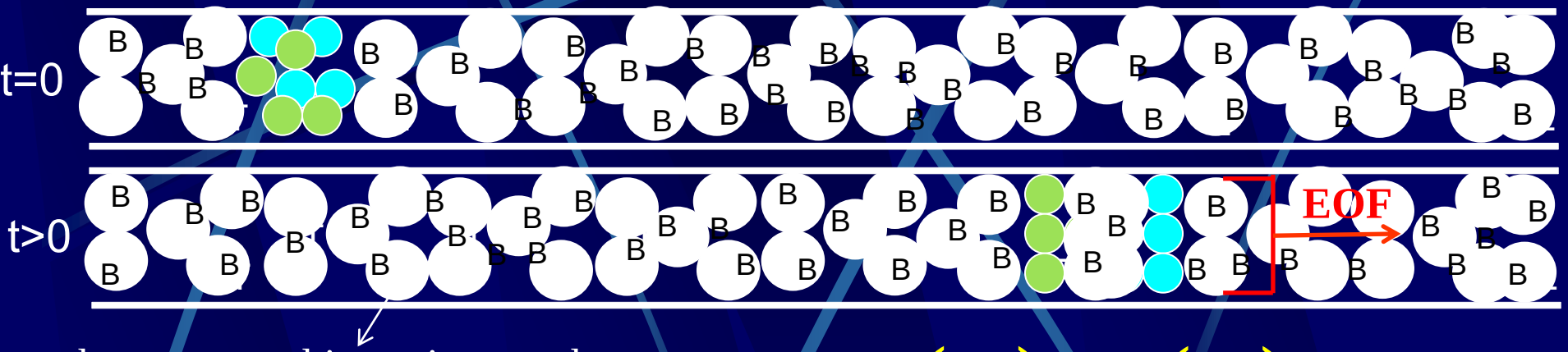
Separation of zwitterions.

Application of isoelectric focussing

IEF



Electrochromatography



v (●) < v (●)

m (●) < m (●)

1 background electrolyte = constant separation conditions (pH, E) in whole capillary. Chromatographic stationary phase in capillary.

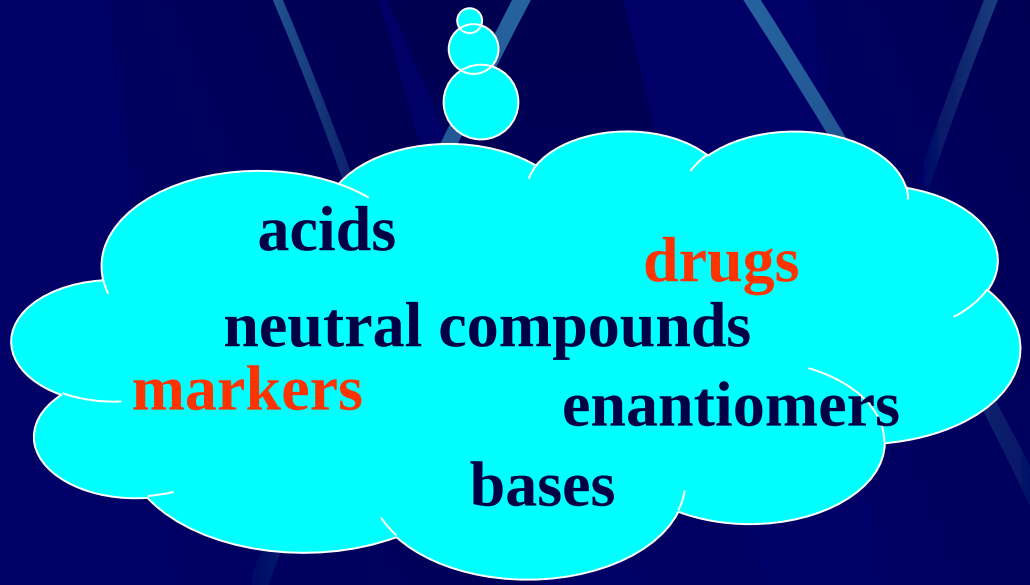
Zones are broadening due to diffusion.

Separation of cations, anions and neutral compounds in one experiment is possible (chromatographic selectivity, electrophoretic efficiency).

Application of electrochromatography

CEC

with or without selector



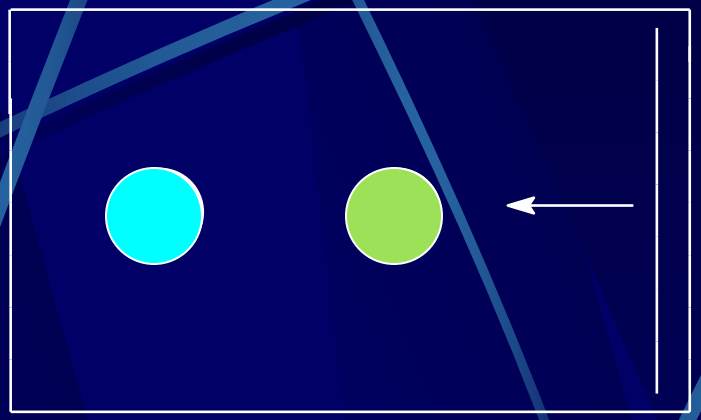
Detection in CE

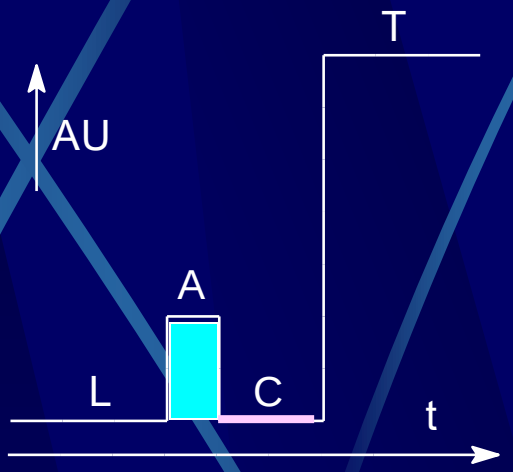
Detection method	Mass LOD (mol)	Concentr. LOD(mol/l)*	Benefits / limitations
UV-VIS absorption	10^{-13}-10^{-15}	10^{-5}-10^{-8}	•universal •DAD provides spectral information
Fluorescence	10^{-15}-10^{-17}	10^{-7}-10^{-9}	•sensitive •usually requires sample derivatization
Fluorescence - LIF	10^{-18}-10^{-20}	10^{-14}-10^{-16}	•extremely sensitive, selective •usually requires sample derivatization
Amperometric	10^{-18}-10^{-19}	10^{-10}-10^{-11}	•sensitive, selective •useful for electroactive compounds
Conductivity	10^{-15}-10^{-16}	10^{-7}-10^{-8}	•universal
Mass spectrometry	10^{-16}-10^{-17}	10^{-8}-10^{-9}	•MS/MS provides structural information •requires analyte ionization
Indirect UV, fluor, amper.	10-100-times higher than direct detection	-	•universal •lower sensitivity than direct methods

Other detection methods are based on radioactivity, refraction index, Raman spectra, etc.

*given data correspond to a 10 nl volume of the injected sample

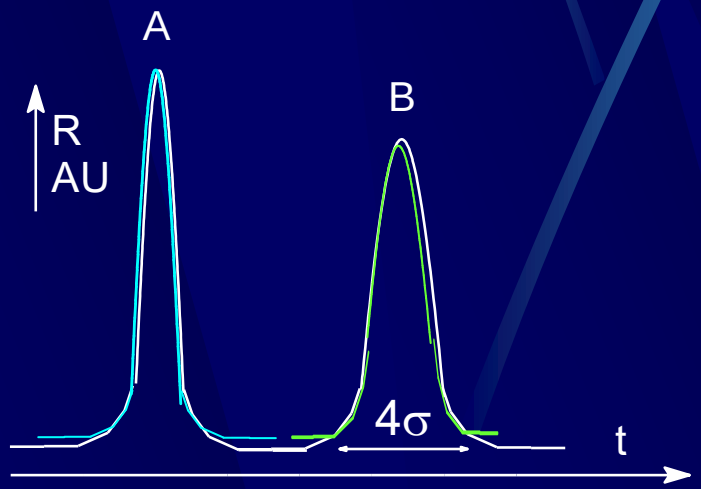
Detection signal

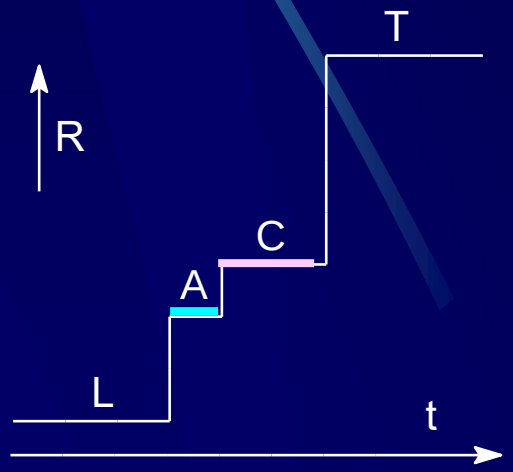




ITP

CZE,
EKC,
CEC



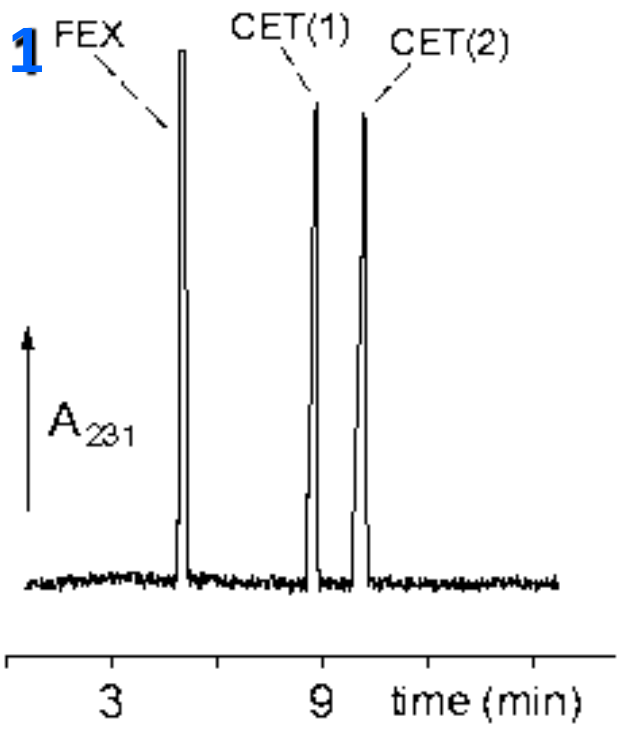


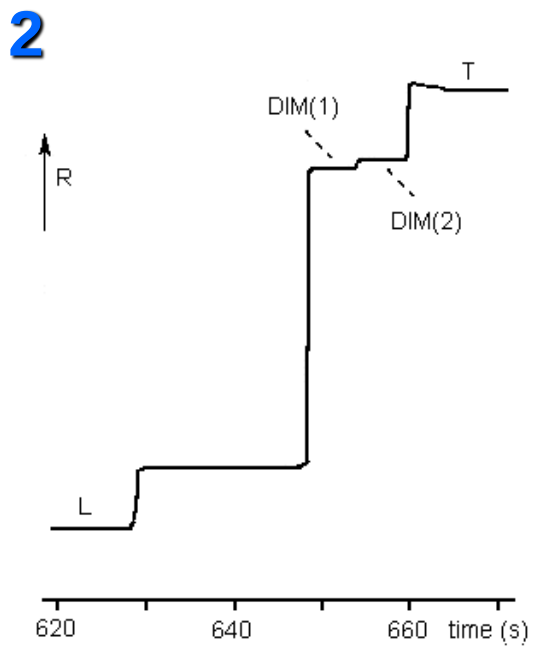
Qualitative parameter: migration time
Quantitative parameter: height or area of the peak

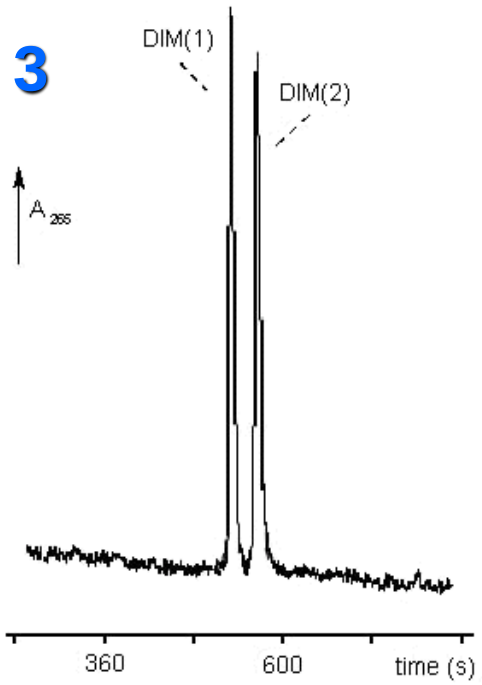
Qualitative parameter: height of the zone
Quantitative parameter: length of the zone

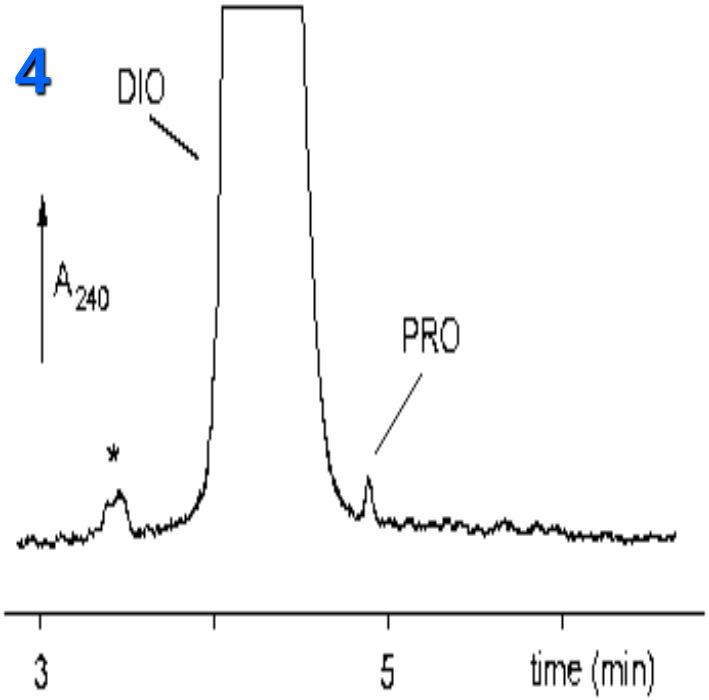
Examples: enantiomeric purity control of drugs

1. **S-β-CD/EKC, tablets**
2. **CE-β-CD/ITP, gel**
3. **CE-β-CD/EKC, syrup**
4. **CE-β-CD/EKC, instilation**



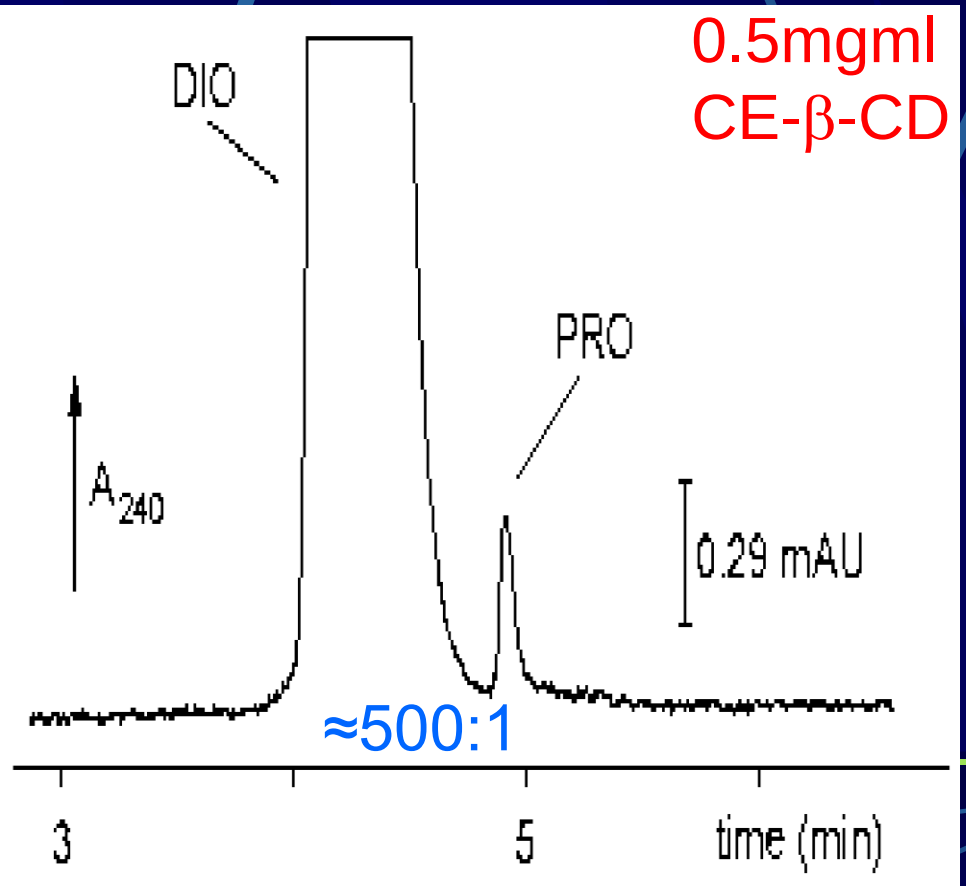


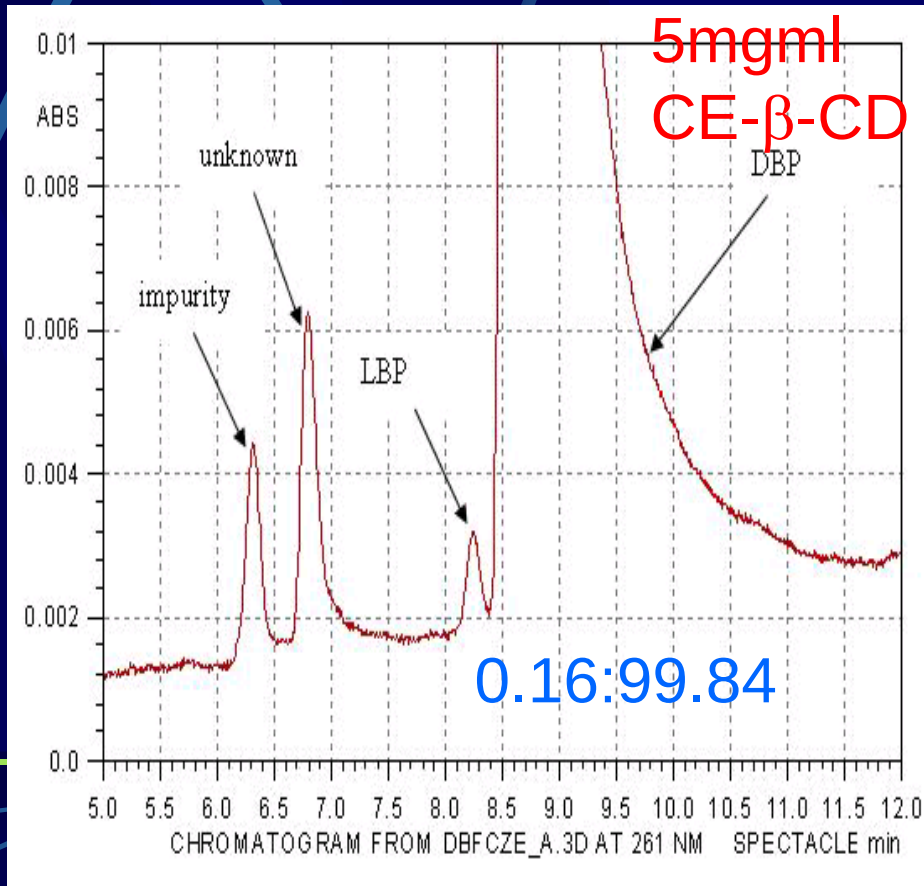


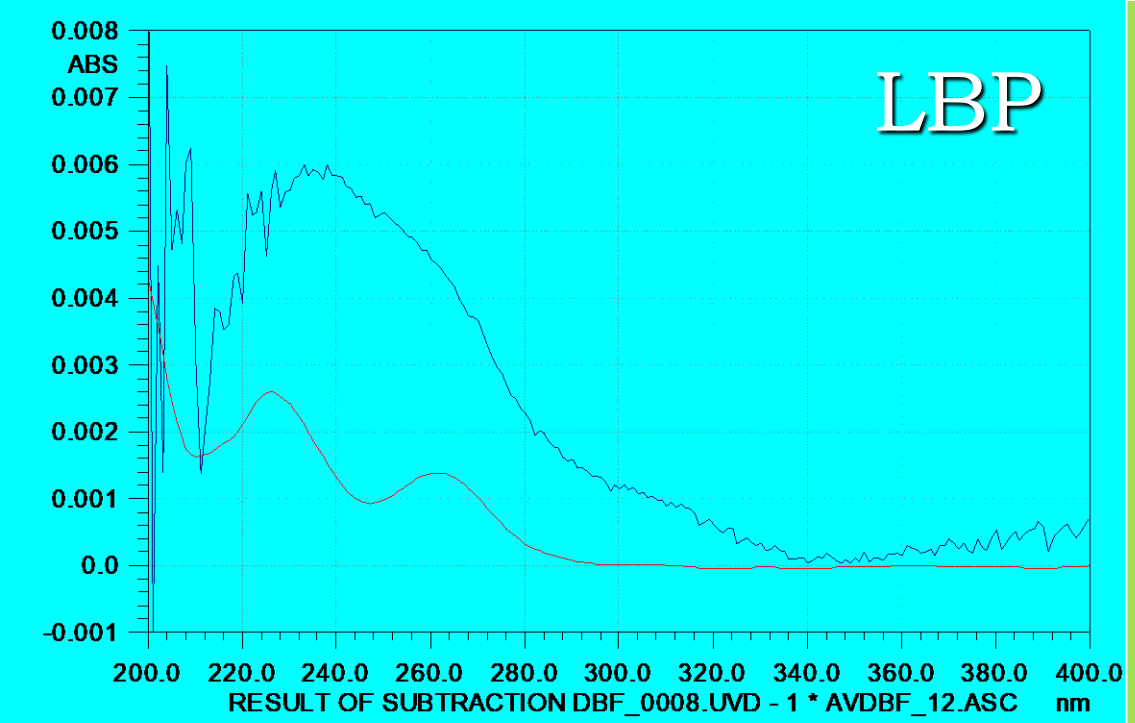


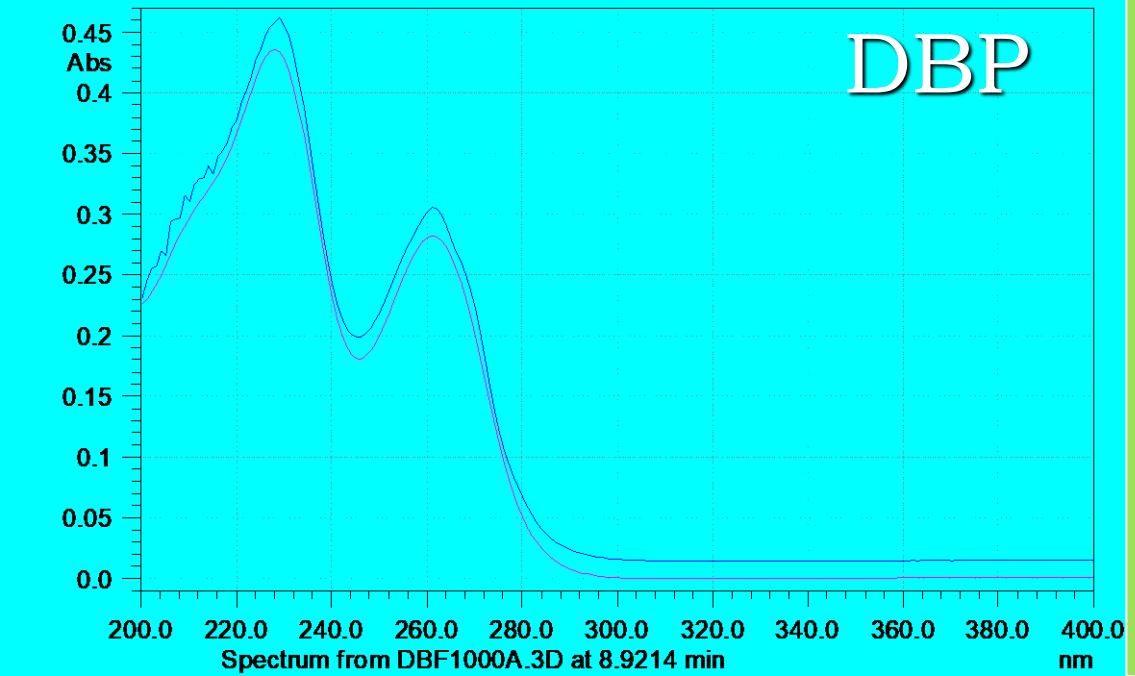
Examples: Separation of compounds with different concentration ratio

- **Structurally related compounds (e.g. drug and impurity)**
- **High concentration ratio (>10^2)**









DAD spectra

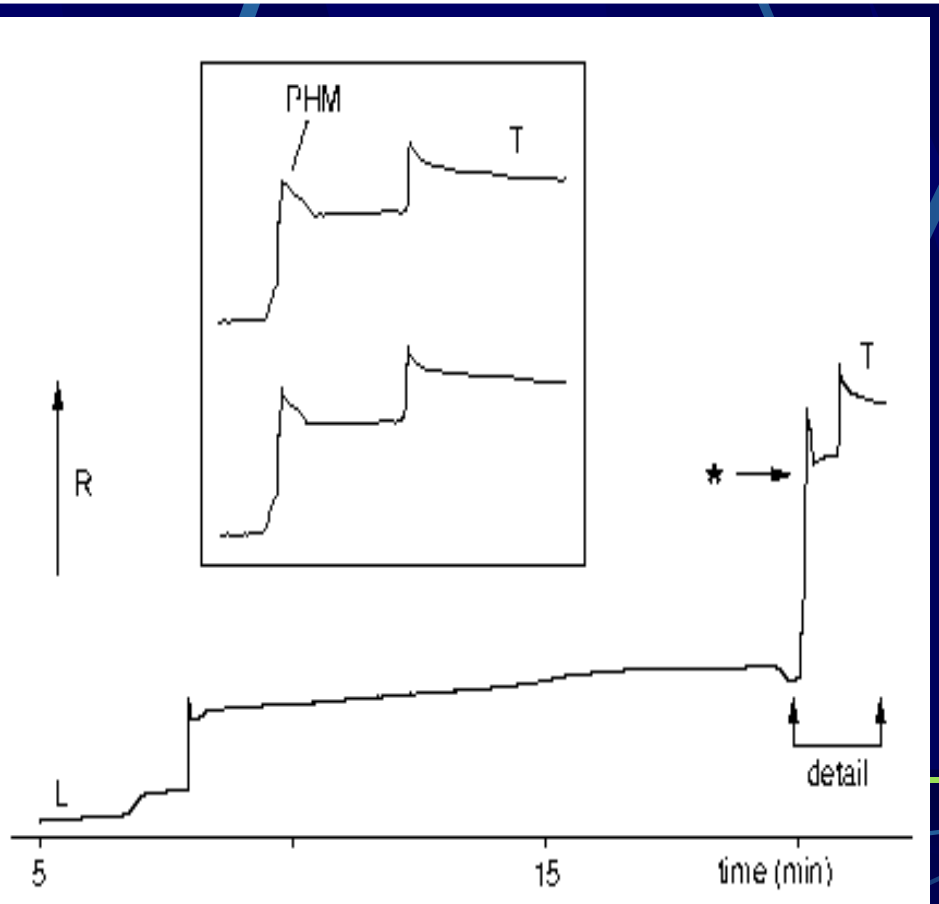
- Confirmation of identity
  - Pearson correlation coefficient
  - Smoothing of crude spectrum

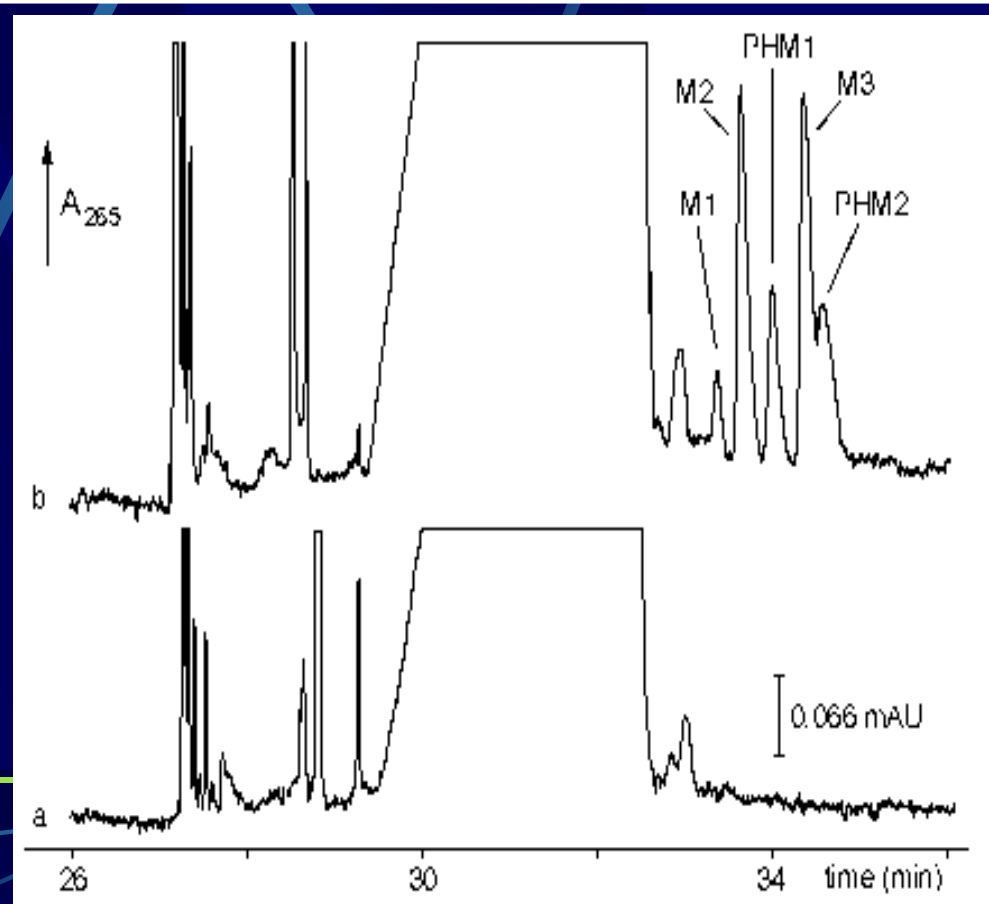


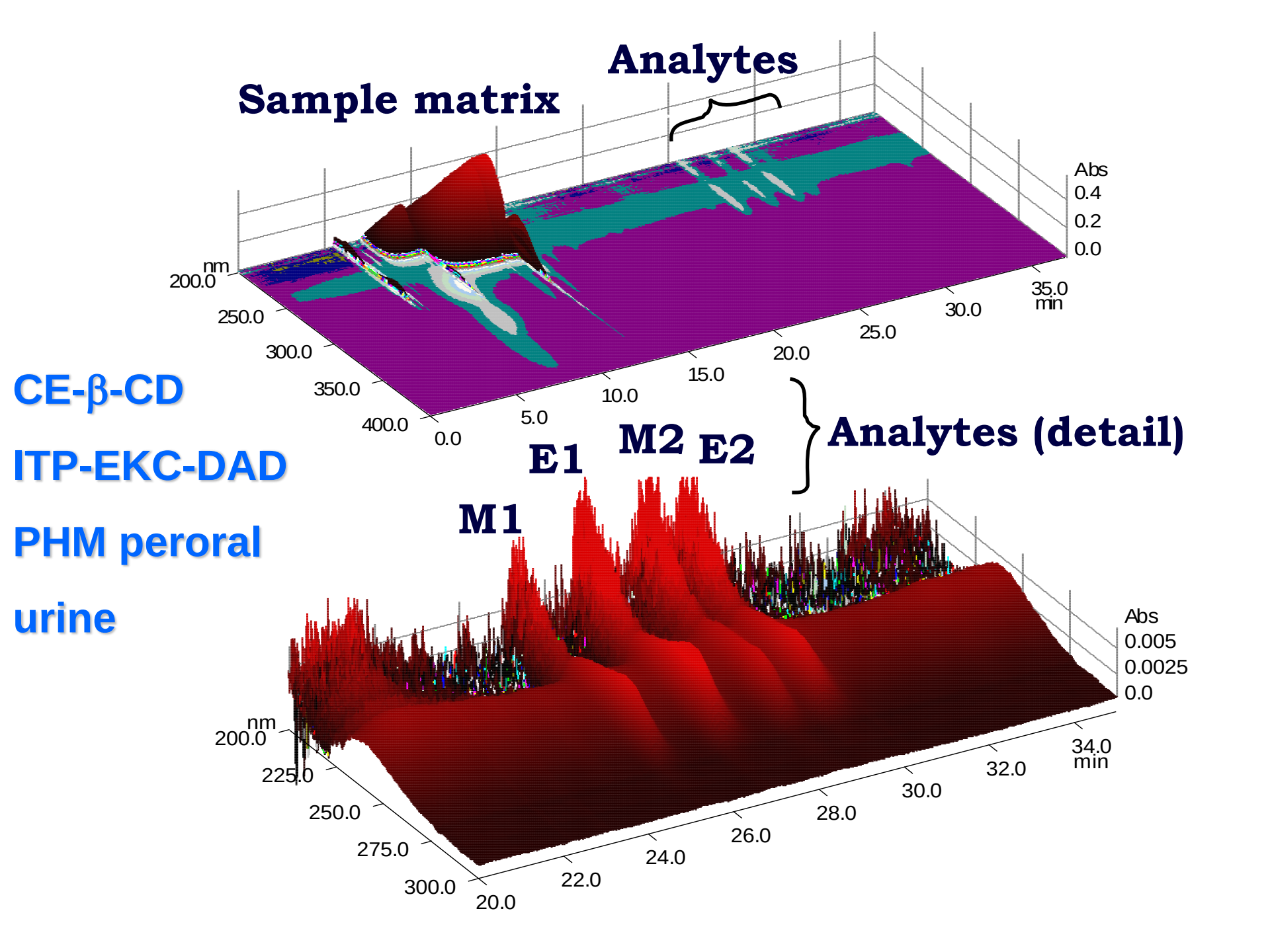
Reliability

Examples: Clinical monitoring of enantiomeric drugs and their metabolites

CE-β-CD/ITP-EKC-UV, urine analysis, PHM peroral administration






Analytes
Sample matrix
Abs
0.4
0.2
0.0
nm
200.0
250.0
300.0
350.0
400.0
0.0
5.0
10.0
15.0
20.0
25.0
30.0
35.0
min
CE-β-CD
ITP-EKC-DAD
PHM peroral
urine
E1
M2
E2
Analytes (detail)
M1
Abs
0.005
0.0025
0.0
nm
200.0
225.0
250.0
275.0
300.0
20.0
22.0
24.0
26.0
28.0
30.0
32.0
34.0
min

New trends in CE

- Implementation of modern detection techniques (LIF, MS, voltamperometry)
- Implementation of new electrolytes and selectors
- Capillary electrochromatography with new stationary phases
- Capillary electrophoresis with on-line coupled columns (automatization)
- Microchip capillary electrophoresis (miniaturization)

New trends in CE

two-dimensional CE systems

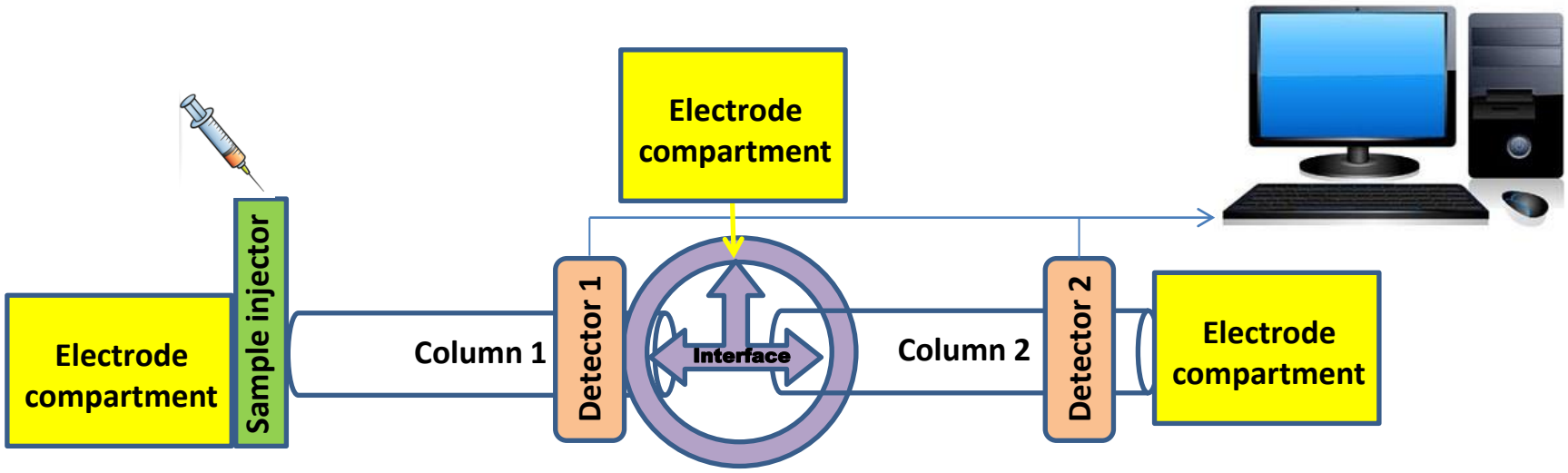
2D CE (two-dimensional capillary electrophoresis):

- CE-CE (fraction or „heart-cut“ two-dimensional capillary electrophoresis)
- mCE-CE (multiple „heart-cut“ two-dimensional capillary electrophoresis)
- Various CE combinations: CZE-CZE, ITP-CZE, IEF-CZE, ITP-CEC, etc.

2D-CE enables:

to enhance separation capacity and selectivity – prerequisite to achieve sufficient resolution of analytes present in multicomponent matrices, and to enhance sensitivity of determination, e.g.:

- Endogenous and exogenous compounds in various matrices
- Drugs, metabolites and biomarkers in biological samples
- Biopolymers (proteins, DNA, RNA) in biological samples

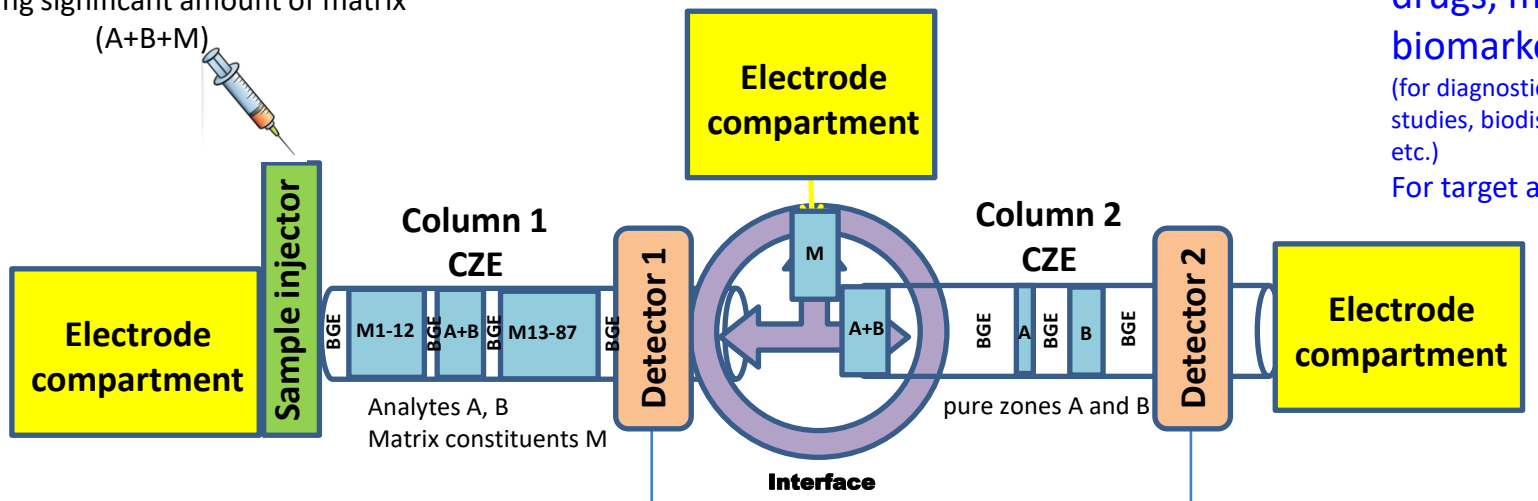


2D-CE instrumental arrangement

New trends in CE

two-dimensional CE systems – (m)CZE-CZE

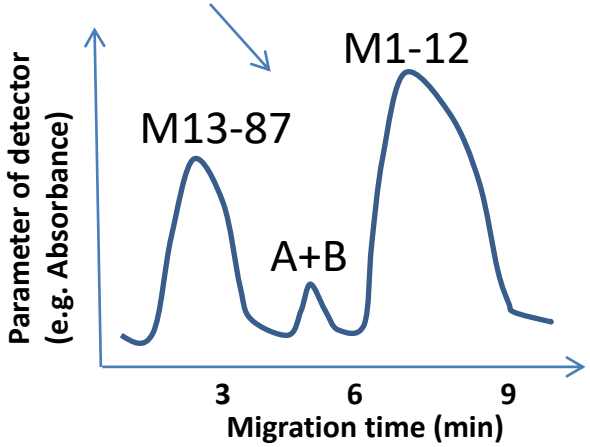
Practical use
Selective determination of: drugs, metabolites, biomarkers
(for diagnostics, pharmacokinetic studies, biodistribution studies, etc.)
For target analysis

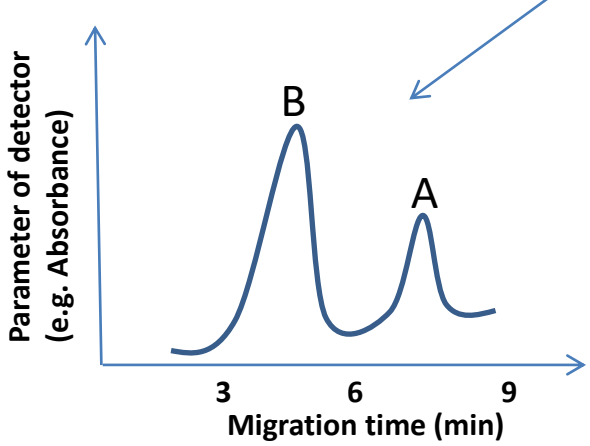


Separation of the analytes from the sample matrix

peaks of pure compounds
(suitable for indentification and quantification)

Sample fraction containing analytes (A+B) separated from the sample matrix constituents (M1-87) in the 1st column is cut, transferred and separated to its individual constituents (A and B) under different separating conditions in the 2nd column; M is not entering the 2nd column





(m)CE-CE: selected sample fraction(s) is(are) analyzed

Notice: CE-CE = one analyte fraction = one cut; mCE-CE = more than one analyte fraction = more than one cut

New trends in CE

two-dimensional CE systems – (m)ITP-CZE

Practical use

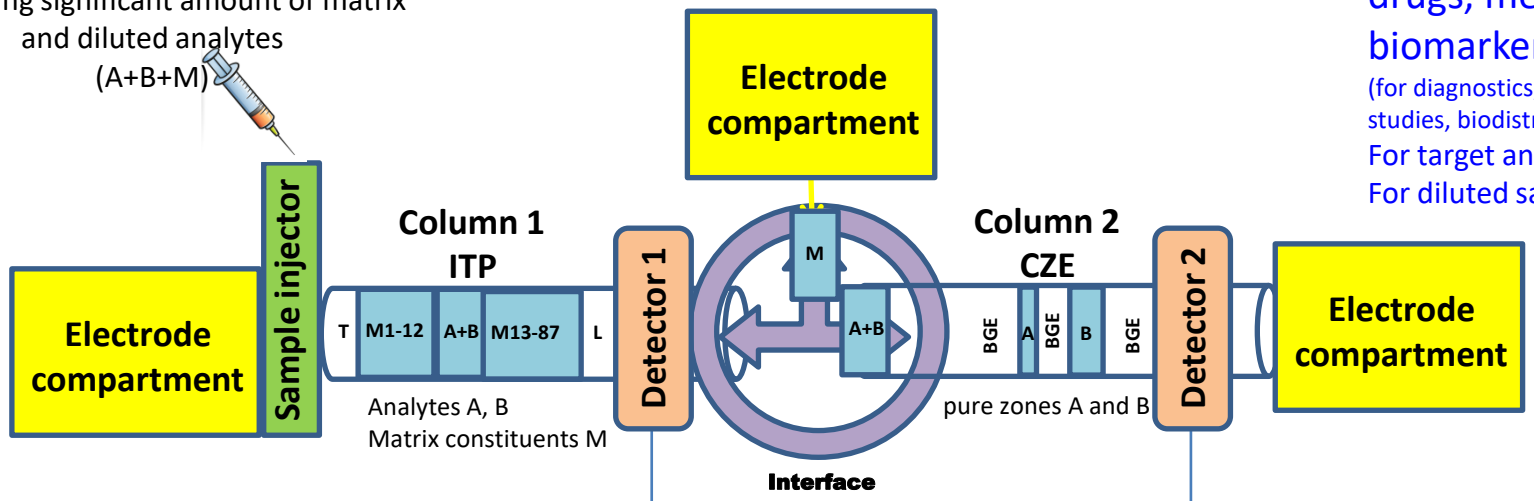
Selective and sensitive determination of: drugs, metabolites, biomarkers

(for diagnostics, pharmacokinetic studies, biodistribution studies, etc.)

For target analysis

For diluted samples

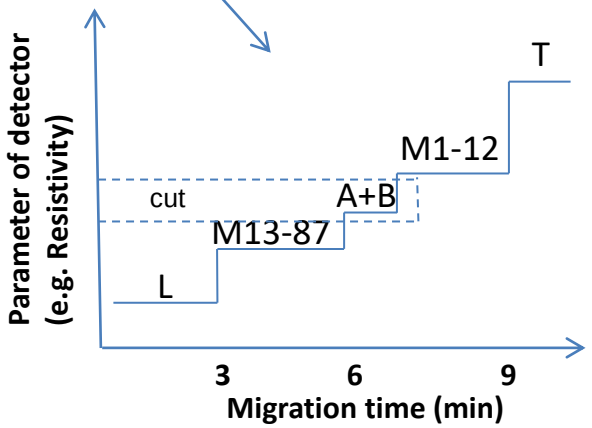
Sample mixture containing significant amount of matrix and diluted analytes (A+B+M)

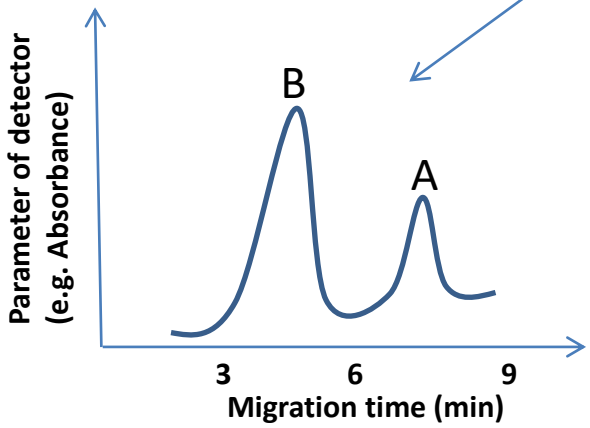


Separation of the analytes from the sample matrix plus preconcentration

peaks of pure compounds (suitable for indentification and quantification)

Sample fraction containing analytes (A+B) separated from the sample matrix constituents (M1-87) and also preconcentrated in the 1st column is cut, transferred and separated to its individual constituents (A and B) under different separating conditions in the 2nd column; M is not entering the 2nd column





(m)CE-CE: selected sample fraction(s) is(are) analyzed

Notice: CE-CE = one analyte fraction = one cut; mCE-CE = more than one analyte fraction = more than one cut

New trends in CE

two-dimensional systems: non electrophoretic technique-CE

nonCE-CE, e.g.:

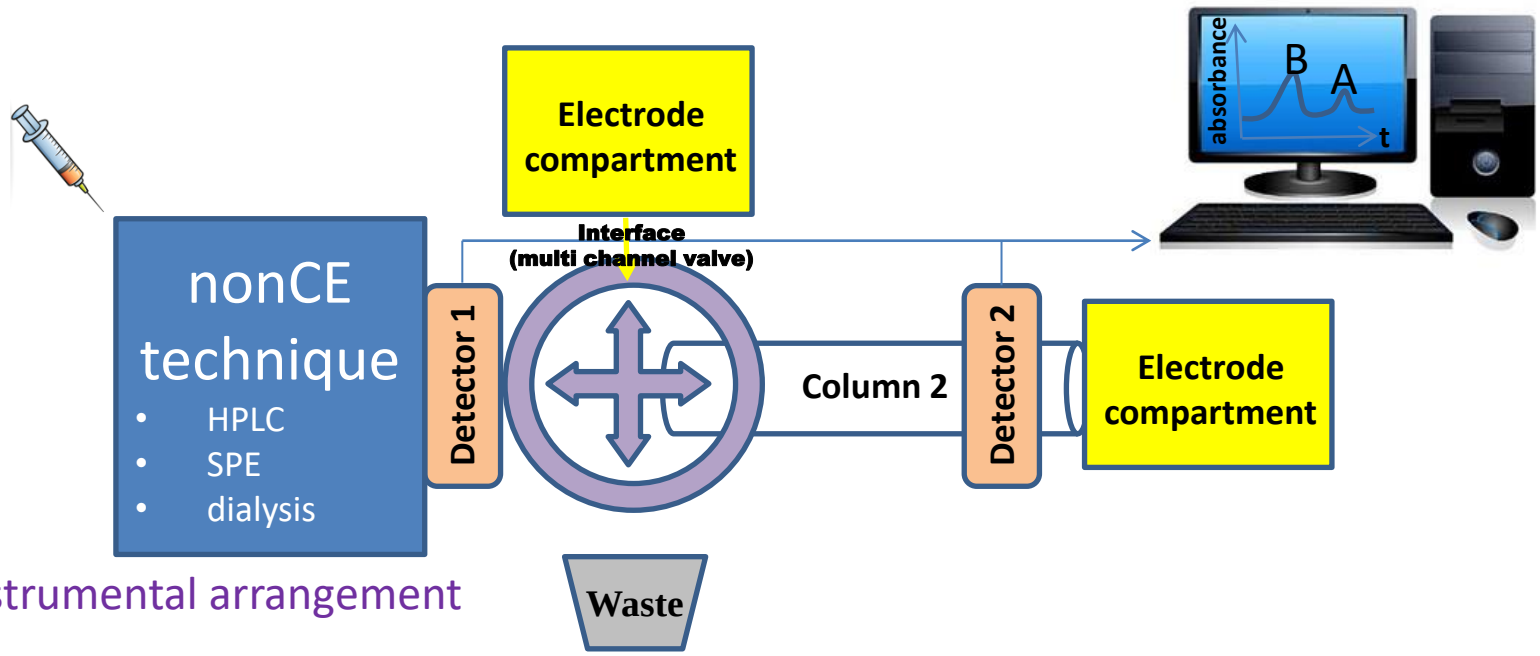
- HPLC-CE
- SPE-CE
- Dialysis-CE

In comparison with CE-CE various other separation mechanisms can be utilized

nonCE-CE enables:

to enhance separation capacity and selectivity – prerequisite to achieve sufficient resolution of analytes present in multicomponent matrices, and/or to enhance sensitivity of determination, e.g.:

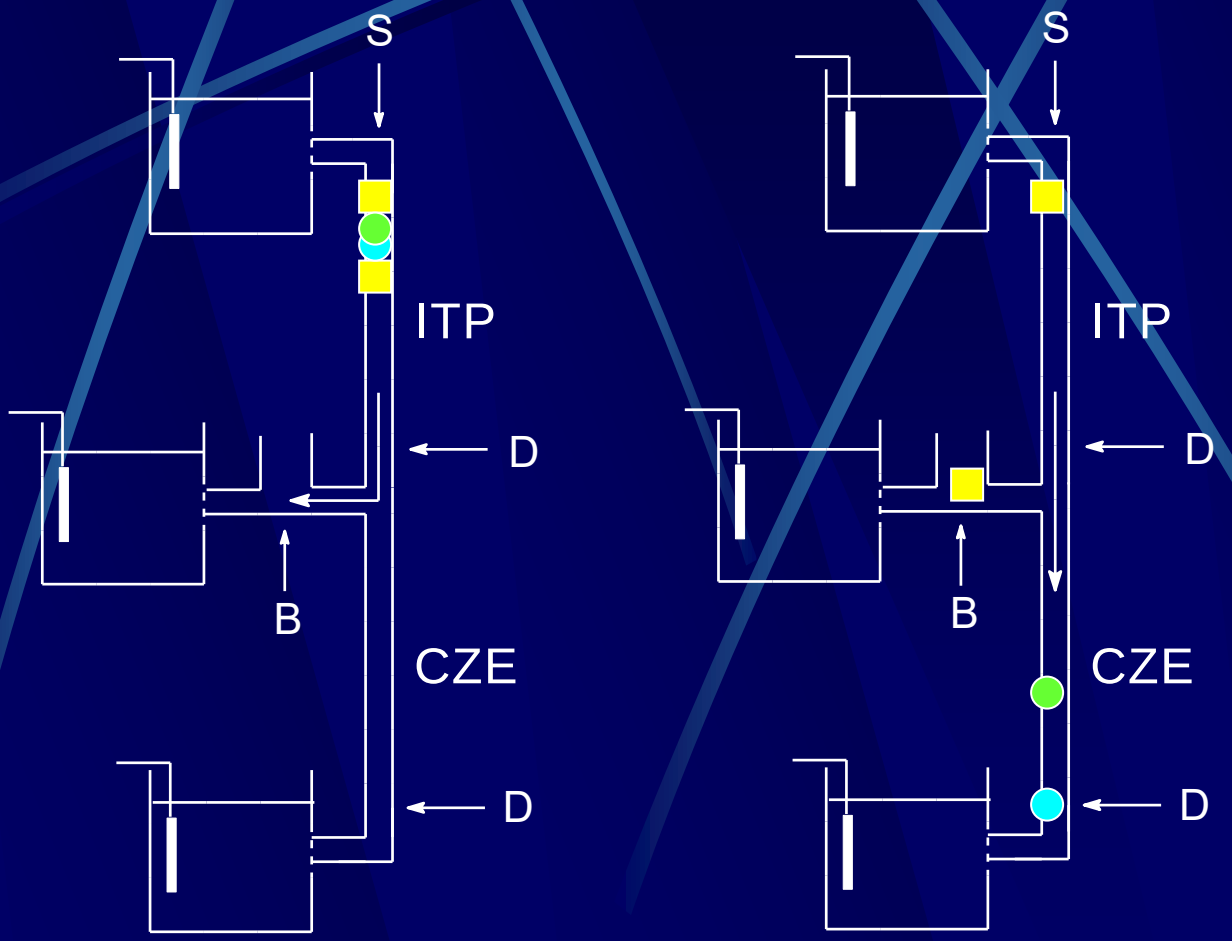
- Endogenous and exogenous compounds in various matrices
- Drugs, metabolites and biomarkers in biological samples
- Biopolymers (proteins, DNA, RNA) in biological samples



nonCE-CE instrumental arrangement

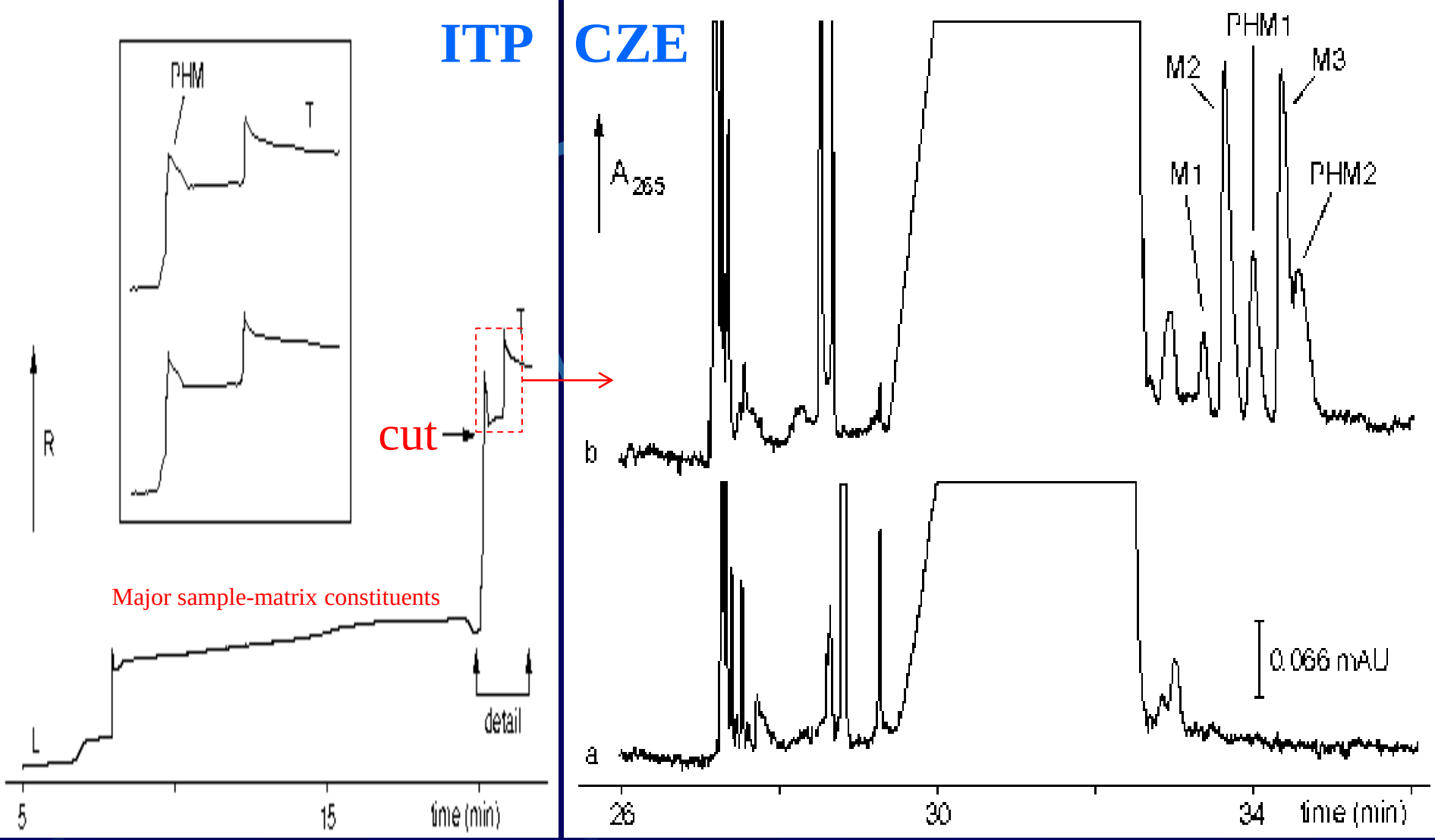
CE-CE

(an illustrative example)





(ITP-CZE, ITP-ITP, CZE-CZE, ITP-EKC, ...)



ITP-EKC integrating ITP sample purification and preconcentration and EKC analytical separation of on-line pretreated sample.

Analysis of human urine 48 h after peroral administration of granuled powder Fervex® with pheniramine drug (PHM). M1-M3 = metabolites.





Lab-on-a Chip

http://www.dfki.uni-kl.de/~aschulen/projects/labonachip.html

Recommended literature

- Heiger, D.: High performance capillary electrophoresis, Agilent Technologies, Publication No. 5968-9963E, Germany 2000, 134 pp.
- Mikuš, Peter; Maráková, Katarína: HYPHENATED ELECTROPHORETIC TECHNIQUES IN ADVANCED ANALYSIS, KARTPRINT, Bratislava, 2012