Capillary Electrophoresis (CE) of Proteins

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Hand-out

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Further reading:

- https://www.agilent.com/cs/library/primers/public/5990_3777EN.pdf
  10 hardcopies are available here
- https://sciex.com/Documents/manuals/IntroductiontoCapillaryElectrophoresisVol-I.pdf

About the Author

- Undergraduate and graduate studies at University of Amsterdam, 1964-1976. Majors in Organic Chemistry and Chemical Engineering
- Post-doctoral research at State University of Ghent, Belgium, 1977 and post-doctoral training Analytical Chemistry, University of Amsterdam, 1978-1979
- R&D Chemist, group & project Leader, R&D section manager, HPLC column, HPLC system, CE capillaries and CE systenm development at Hewlett-Packard, Waldbronn, Germany, 1979-'99
- Since 2000, Agilent Technologies University Relations and External Scientific Collaborations Manager
- Retired September 1, 2012. Since then, working on freelance basis. Visit my website at http://www.rozing.com e-mail: gerard@rozing.com
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Content:

- Introduction of Electrophoresis
  - Basic principles and some theory of Capillary Electrophoresis
  - Methods of Capillary Electrophoresis
  - Instrumentation of Capillary Electrophoresis

- CE and CE-MS for Proteins
  - Interfacing with MS
  - CE and CE-MS of proteins

Introduction of Electrophoresis

Brief History of Electrophoresis of Proteins

Electrophoresis is a separation method based on the differential rate of migration of charged molecules in a buffer solution across which has been applied a **dc** electric field.

This separation technique called, *moving boundary electrophoresis,* was developed by the Swedish chemist Arne Tiselius in 1937 for the study of serum proteins*.

Placing protein mixtures in buffer solutions and applying an electric field, Tiselius found that sample components migrated into the direction of the electrode with opposite charge and at a rate determined by their charge and mobility.

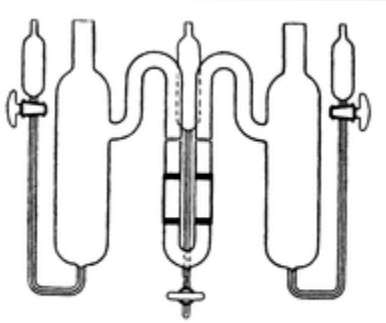


FIG. 1.—Electrophoresis apparatus for the moving boundary method (Tiselius, 1930).

In practice the high currents occurring caused convection and distorted the moving bands of protein which did not allow to keep the moving zones flat and sharp.

Therefore, later methods used gel matrices on a flat plat that inhibited convection and allowed to separate compounds into discrete and stable bands and therefore was called by Tiselius *zone electrophoresis*

*Tiselius, Arne (1937). "A new apparatus for electrophoretic analysis of colloidal mixtures". Transactions of the Faraday Society. 33: 524–531

Nowadays….

Electrophoresis is applied to a variety of difficult analytical separation problems, of organic and inorganic anions and cations and for amphoteric substances.

A particular strength of electrophoresis is its unique ability to separate charged, high-molecular weight (HMW) biomolecules like peptide, proteins and polynucleotides (DNA, RNA) in life science research and biotechnology and in the biopharmaceutical industry.

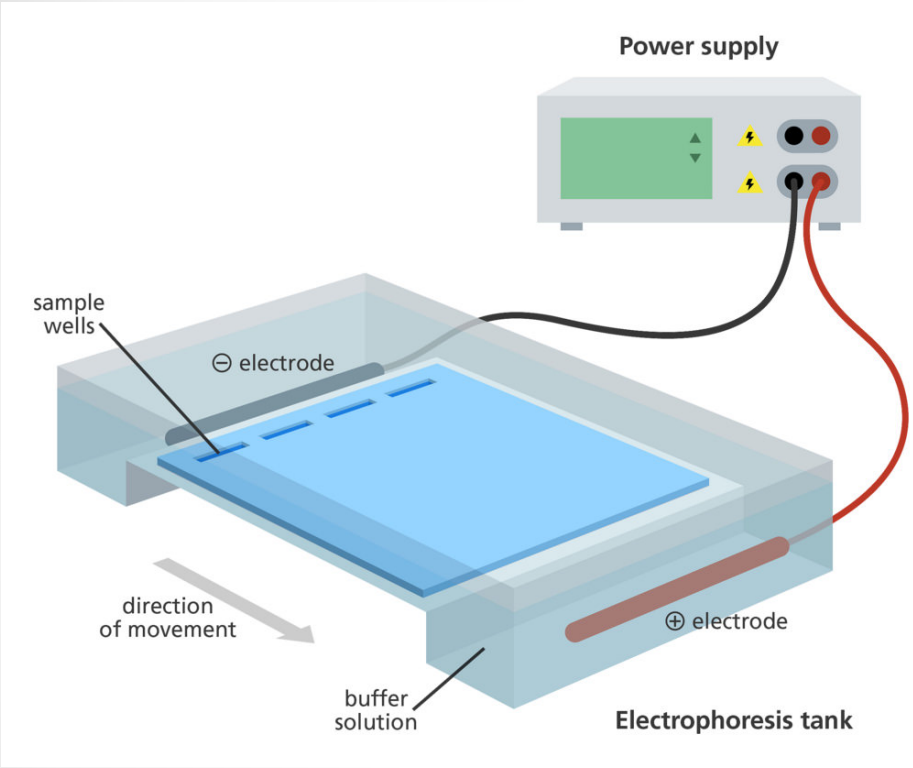
Nowadays….

Electrophoretic separations are currently performed in two quite different formats:

1. On a thin layer (slab) of an anticonvective medium (gel) deposited or immobilized on a flat, rectangular (glass or plastic) support
   Slab Gel Electrophoresis (SGE)

2. In a conduit like a fused silica capillary or a channel etched in a glass slide
   Capillary Electrophoresis (CE)

Schematic Representation of SGE of Polynucleotides*

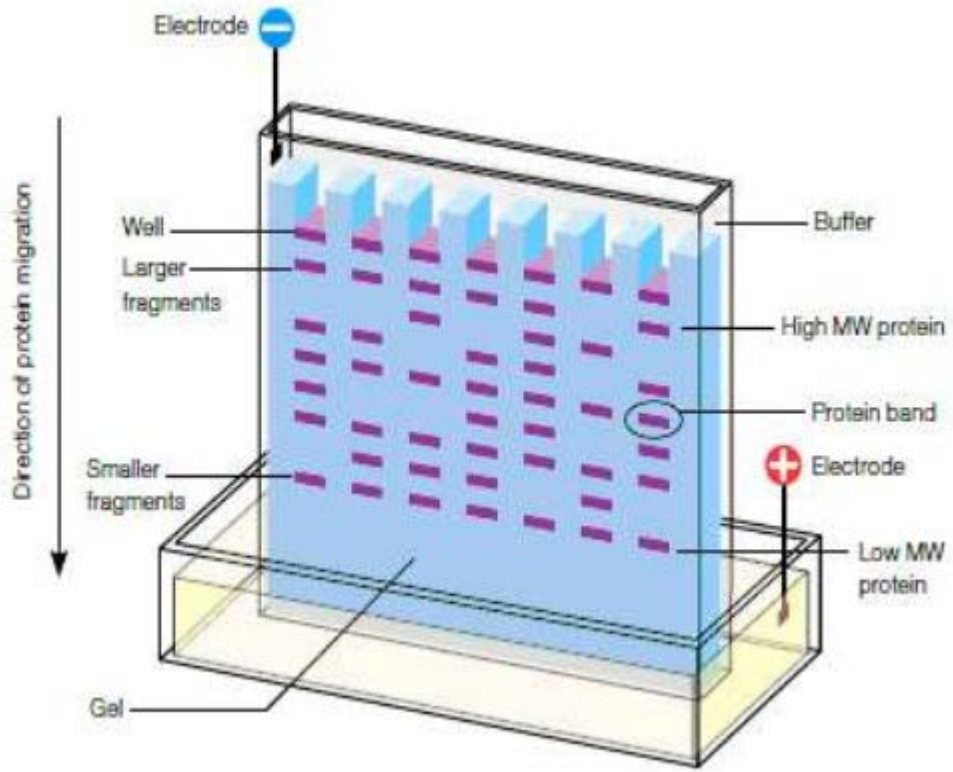


Most important methods of SGE for Polynucleotides, DNA, RNA

- Agarose Gel
- Size based separation
- Further work on separated bands
  - Southern blot (investigate ss DNA fragments from restriction enzymes)
  - Northern blot, RNA

*Picture taken from https://www.flickr.com/photos/yourgenome/26344970413

Schematic Representation of SGE of Proteins*



Most important methods of SGE for peptide and proteins

- Polyacrylamide gels (PAGE)
- Size based separation sodium dodecyl sulphate bound proteins (SDS-PAGE)
- pI based separation, iso electric focusing (IEF) on immobilized pH gradient gels (IPG)
- Multidimensional gel electrophoresis (MDGE), IEF on a strip followed by SDS-PAGE on a plate
- Further work on separated bands
  - Western and Eastern blotting (immuno-affinity methods)
  - Protein digestion followed by peptide mapping

*Picture taken from https://www.slideshare.net/brunobasil/electrophoresis-44229912

Capillary Electrophoresis

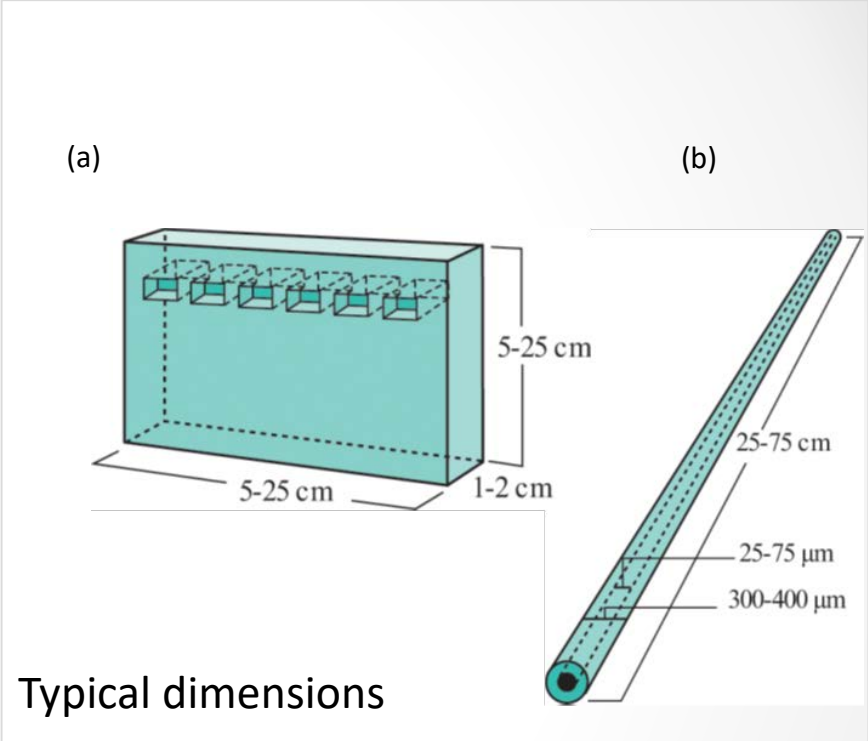
Basic principles and some theory

Capillary Electrophoresis - Principle

Separation efficiency in free solution, as performed by Tiselius, was limited by thermal diffusion and convection.

Since currents in narrow capillaries are low, they generate only small amounts of heat and are in principle anti-convective.

This allows the application of free-solution capillary zone electrophoresis, as well as the use of traditional gel media in the capillary.



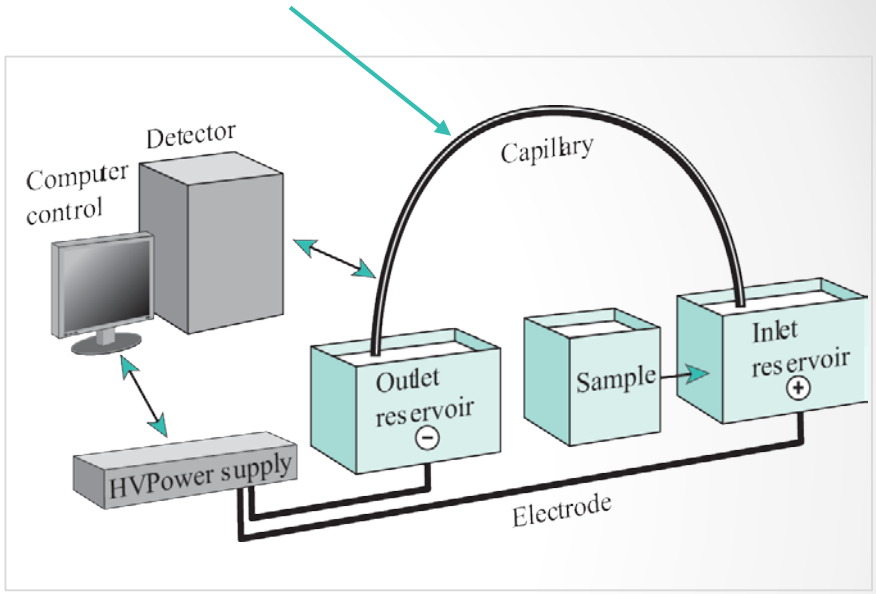
Comparison of gel used for slab gel electrophoresis, SGE (a) and capillary for capillary electrophoresis, CE (b).

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Capillary Electrophoresis - Principle

A schematic diagram of a generic capillary electrophoresis system is shown on the right.

- Ends of a narrow-bore, fused silica capillary are placed in buffer reservoirs.
- The reservoirs also contain platinum electrodes used to make electrical contact between high voltage power supply and capillary.
- Sample is loaded onto the capillary by replacing one of the reservoirs (usually at the anode) with a sample reservoir
- After replacing the buffer reservoir, the electric field is applied and the separation performed.
- Optical detection can be made at the opposite end, directly through the capillary wall.



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Capillary Electrophoresis – Capillary Dimensions

Diameter [a] I.D./O.D (µm)	Volume (µL)	Typical Injection Volume (nL) [b]	Injected Amount (fmol) [c]
75/365	2.2	22	440
50/365	1	10	200
25/180- 365	0.25	2.5	50

[a] capillary length L = 50 cm
[b] rule of thumb: < 1% of capillary volume (preconcentration methods)
[c] injection of 10 µg/ml of peptide (M = 500)

Theory of Capillary Electrophoresis - Mobility

Velocity of a charged molecule is proportional with the applied electrical field

$$v \propto E$$

$$v = \mu_{ep} E = \mu_{ep} \frac{V}{L}$$

where μ_{ep} is called electrophoretic mobility of the molecule which depends primarily on its charge and (hydrodynamic) size

In electrophoresis, separation is based on the differences in molecule velocity in an electric field.

Theory of Capillary Electrophoresis - Mobility

The velocity (mobility) is determined by the electric force that the ionized molecule experiences, balanced by the frictional drag force through the medium.
The electric force can be given by:

$$F_E = qE$$

and the frictional force (for a spherical ion) by Stoke's law:

$$F_F = -6\pi\eta r\nu$$

q	charge of the molecule
η	viscosity of the solvent
r	hydrodynamic radius of the molecule
υ	velocity

Theory of Capillary Electrophoresis - Mobility

At steady state conditions (equilibrium) these forces are equal but opposite and:

$$qE = 6\pi\eta r\upsilon$$

Solving for velocity (equation on slide 15) yields an equation that describes the mobility in terms of physical and molecular properties:

$$\mu_{ep} = \frac{q}{6\pi\eta r}$$

μ_{ep} electrophoretic mobility

From this equation it is evident that small, highly charged species have high mobilities whereas large, minimally charged species have low mobilities which forms the basis for separation.

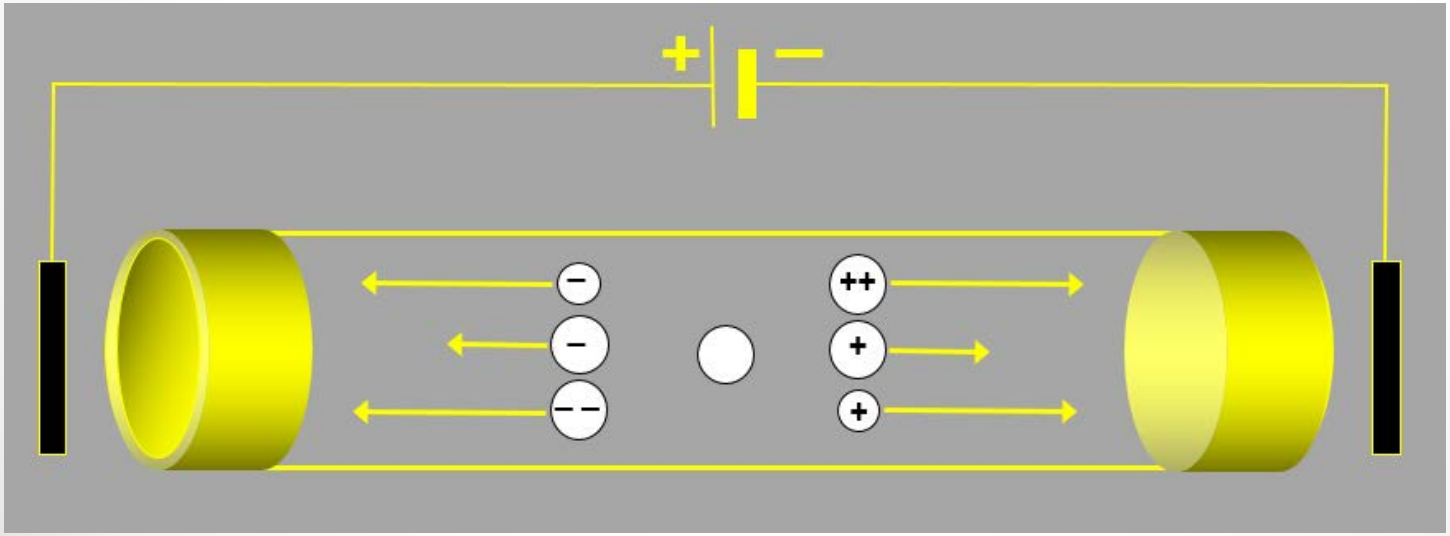
The differences in charge/size ratio are the decisive factor in separation

Theory of Capillary Electrophoresis - Mobility

- Magnitude of μ depends on ionization state of the molecule (pK_a) and therefore on the pH of the BGE

$\mu_{eff} = \alpha_i . \mu_p$ α_i degree of ionization (or dissociation)

- Direction of μ depends on molecule's charge sign

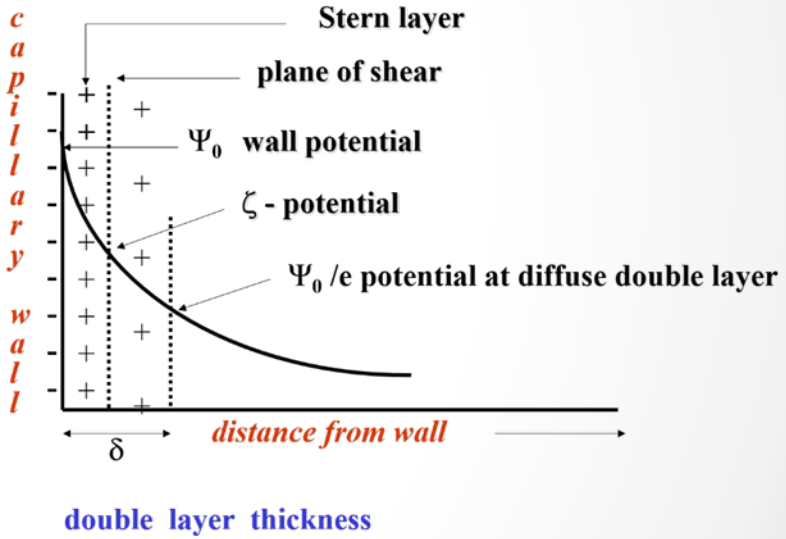


Theory of Capillary Electrophoresis – Electro-Osmotic Flow

Besides movement of the charged molecules by electrical force, a hydraulic flow is generated in the conduit used for separation:

➔ Electro-Osmotic Flow (EOF).

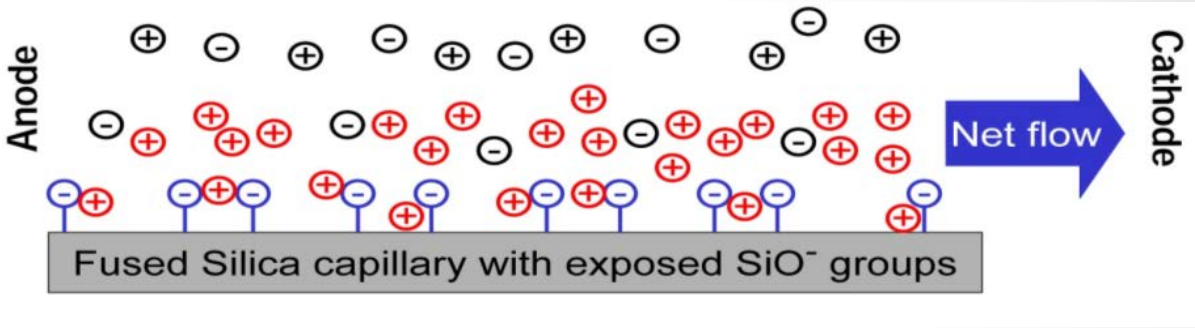
- The inside wall of a fused capillary is covered by silanol groups
- pK_a of silanol groups range 3-6 depending on structure, surface environment and solvent ion strength (background electrolyte)
- At *pH* > 3 the capillary surface becomes negatively charged
- SiO^- groups attract cations to the inside wall of the capillary
- The distribution of charge at the surface is described by the Stern electrical double-layer model and results in the zeta potential
- There is an excess of mobile cations in the diffuse layer



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Theory of Capillary Electrophoresis – Electro-Osmotic Flow

- Excess cations in the diffuse Stern double-layer flow are mobile and move towards the cathode
- Net hydraulic flow occurs as solvated cations drag the solvent along the capillary wall



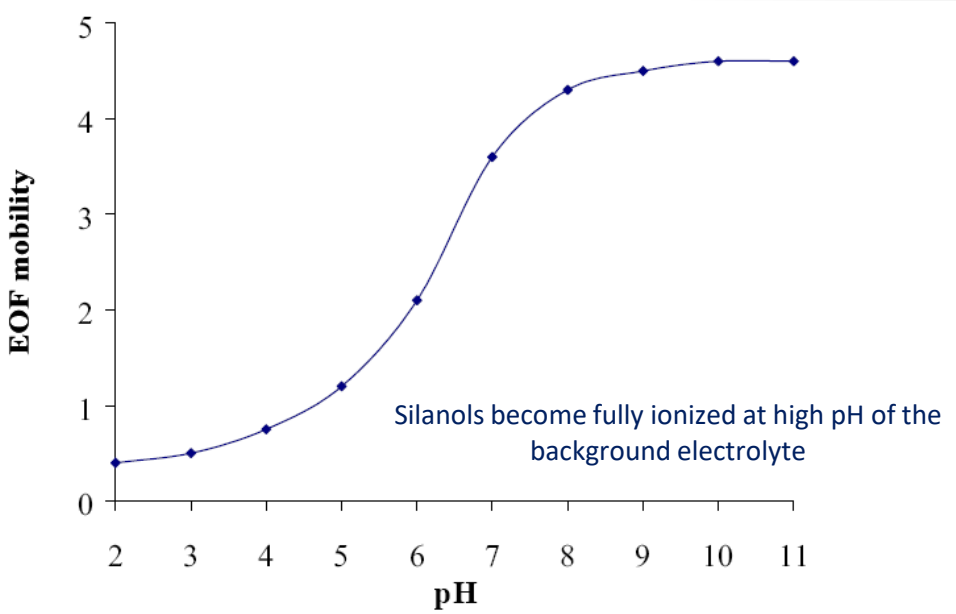


Figure 1.3 *The variation of EOF mobility with changing pH for a typical uncoated fused-silica capillary (simulated data)*

Theory of Capillary Electrophoresis – Electro-Osmotic Flow

Electroosmotic mobility is given by: (Smoluchowski equation)

$$\nu = \mu_{eo} E = \left(\frac{\epsilon_o \varepsilon \zeta}{4\pi\eta} \right) E$$

Where:

ν = electro-osmotic mobility

ε_o = dielectric constant of a vacuum

ε = dielectric constant of the buffer

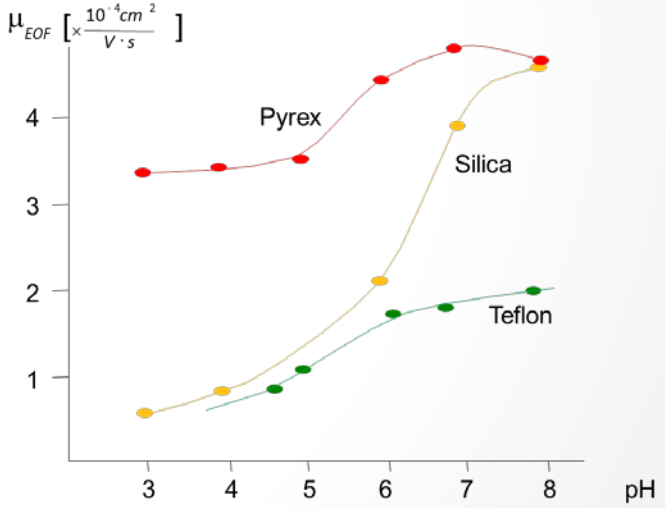
ζ = zeta potential

η = viscosity of the BGE

E = electric field

Experimental parameters that affect the EOF:

- Properties of the background electrolyte (BGE)
- surface charge properties of the capillary



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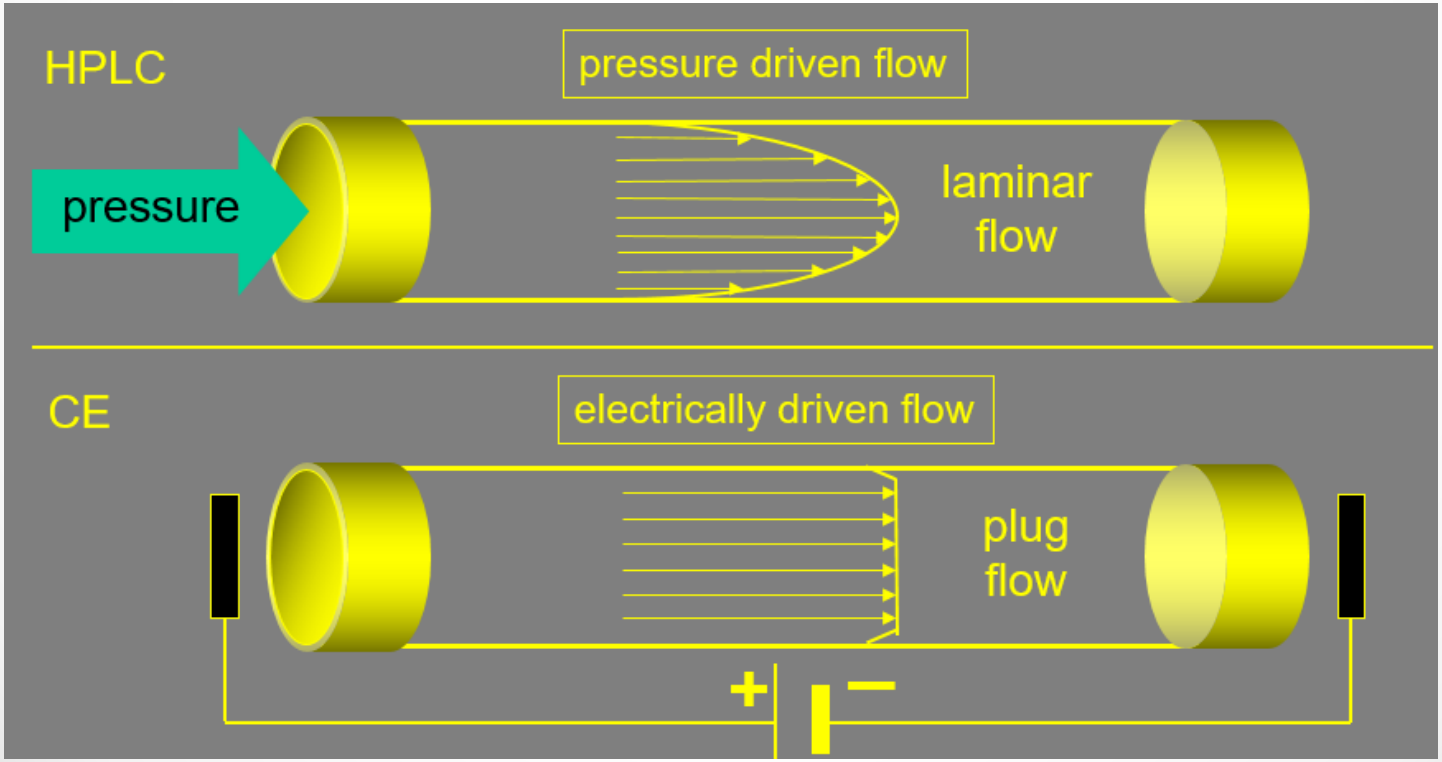
Theory of Capillary Electrophoresis – EOF Properties

- Magnitude
  - Typical 0 – 3 mm/s. In a 50 µm i.d. FS capillary; 0 – 0.35 µL/min,
- Direction
  - Depends on the surface charge of the capillary (coating of the capillary inner wall)
- Flow velocity profile

Theory of Capillary Electrophoresis – EOF Properties

Hydraulic flow
- pressure is the driving force
- velocity at the surface is zero
- solvent molecules do not start moving at the same time
- velocity in the middle is highest

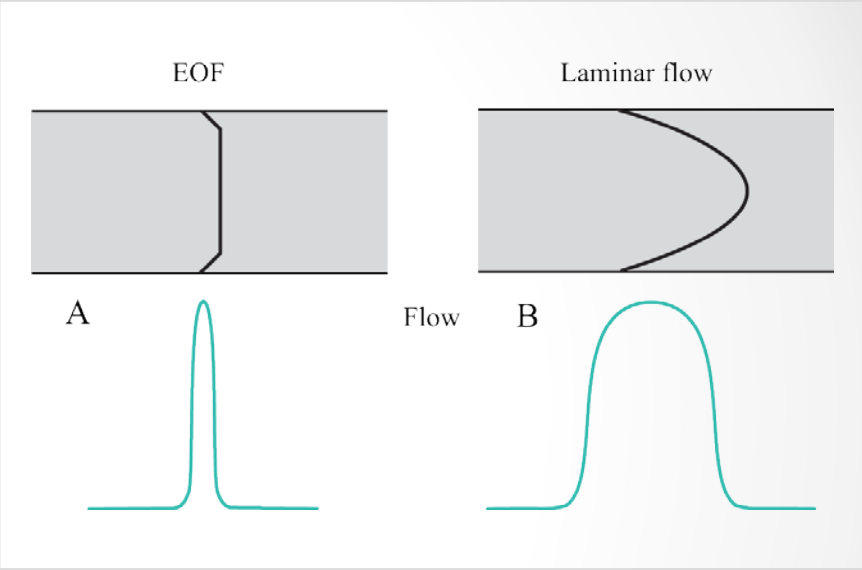
Electro-Osmotic Flow
- electrical field is the driving force
- solvent movement originates at surface
- all solvent molecules start to move at the same time with the same velocity
- no pressure drop is encountered
- flow velocity distribution is uniform across the capillary



Theory of Capillary Electrophoresis – EOF Properties

Electroosmotic flow does not contribute significantly to band broadening like pressure-driven flow in LC and related techniques

- Reduced broadening (dispersion) of solute zones
- Velocity profile is generally independent of capillary diameter
- Nearly all species, regardless of charge, move in the same direction
- But
  - it is not always desirable to have an EOF
  - it is sometimes unpredictable
  - It is difficult to control



Flow velocity profiles and corresponding solute zones in electro- and pressure driven flow.

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Methods to Control the EOF in FS Capillaries - I

Variable	Result	Warning
Electric field	EOF changes proportionally with electrical field.	High field generates more heat in the capillary. Eventually short-circuits and sparking.
BGE pH	EOF decreases at low pH and increases at high pH.	Will change solute charge.
BGE ion strength or buffer conc.	Affects double layer thickness. EOF increases at low ion strength.	High ionic strength generates high current and Joule heating. Lower buffer capacity at low ion strength will cause possible solute adsorption Peak shape distorts if conductivity of the electrolyte differs from sample zone conductivity.
Temperature	EOF changes due to viscosity change (2 - 3%/°C).	Capillary temperature must be controlled.

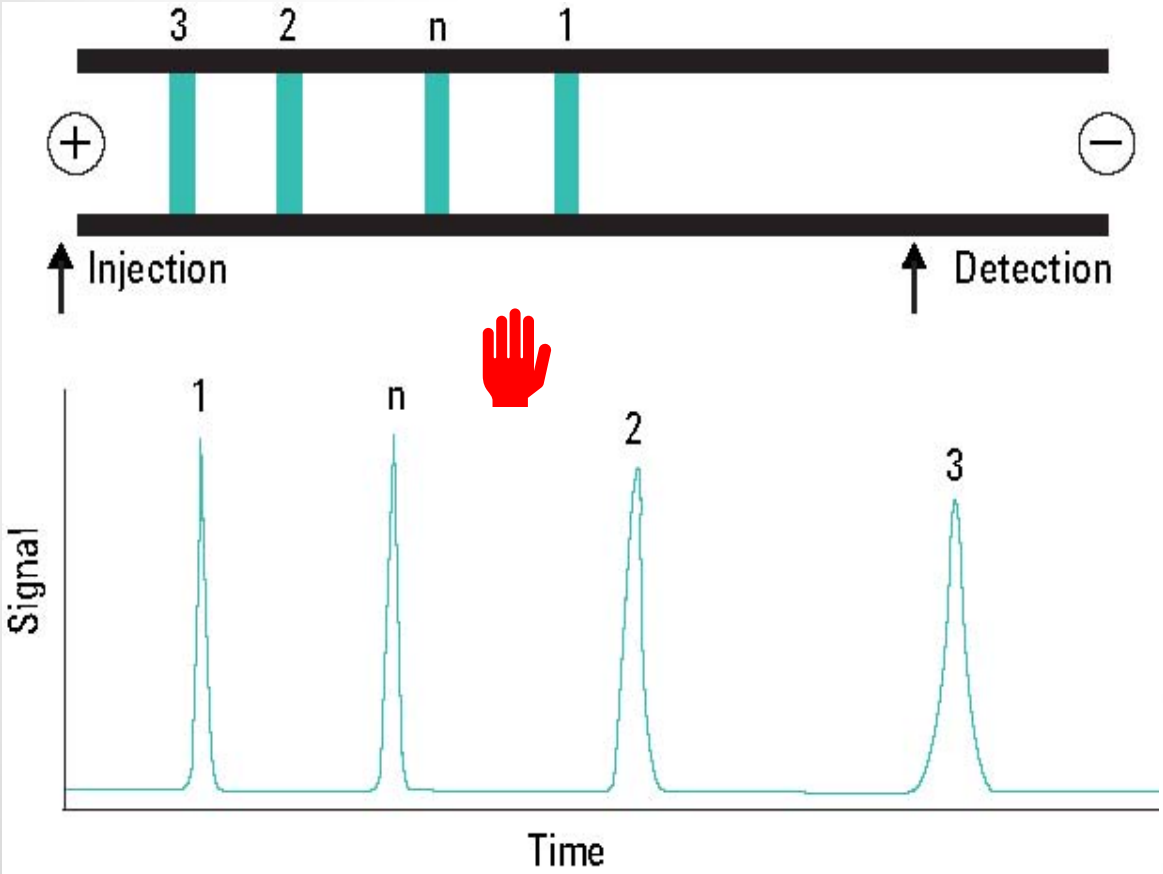
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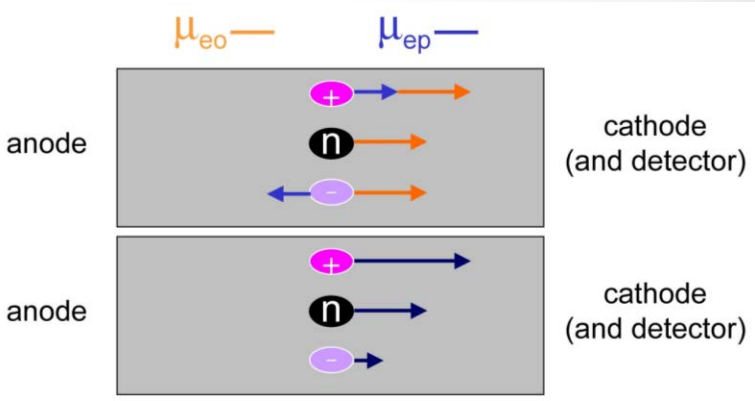
Methods to Control the EOF in FS Capillaries - II

Variable	Result	Comment
Organic modifier to the electrolyte	Changes zeta potential and electrolyte viscosity.	Complex changes of EOF; effect most easily determined experimentally. May alter selectivity of separation.
Additives to the electrolyte	Change magnitude and direction of EOFs. Anionic surfactants can increase EOF; Cationic surfactants can decrease EOF.	Dynamical adsorption to capillary wall via hydrophobic and or ionic interaction.
Neutral hydrophilic polymer	Decreases but controls EOF by shielding surface charge and increasing viscosity.	Adsorbs to capillary wall via hydrophilic interactions. In addition suppresses solute/wall interactions.
Covalent bonded surface coating	EOF depends on the charge and polarity of the coating or effectively suppress EOF	Many modifications possible (hydrophilicity or charge). Stability of coating is limited. Expensive

Capillary Zone Electrophoresis

$$v = (\mu_{ep} + \mu_{eo})E = (\mu_{ep} + \mu_{eo}).\frac{V}{L}$$





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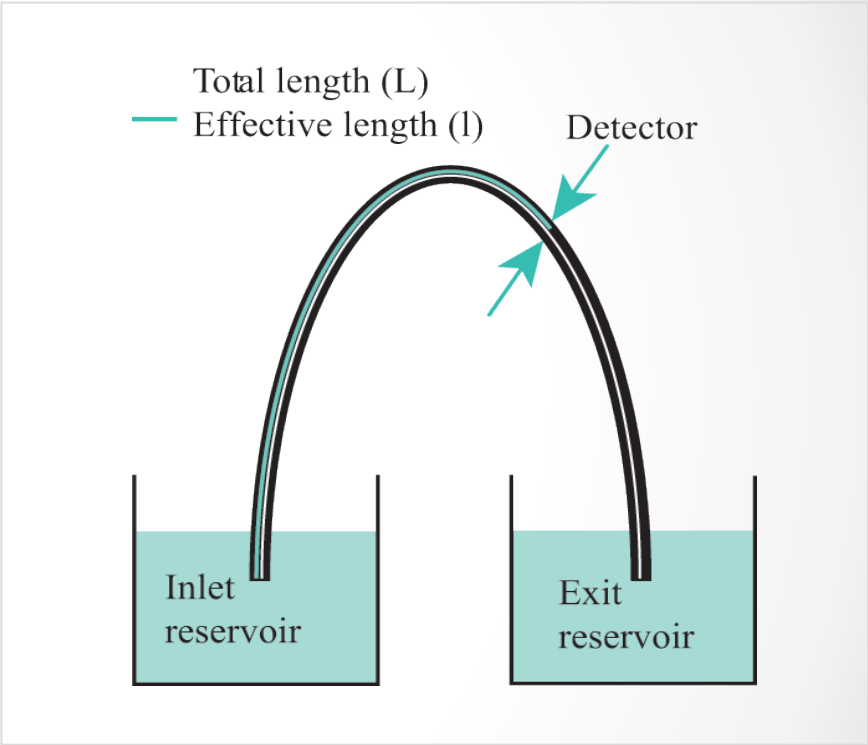
Mobility and Migration time

The time required for a solute to migrate to the point of detection is called the “migration time”.

$$\upsilon = \frac{l}{t_i} = \mu_i E = \mu_i \frac{V}{L}$$

$$\mu_i = \frac{l.L}{t_i.V}$$

μ_i	$\mu_{e,i} + \mu_{EOF}$
V	applied voltage
l	effective capillary length
L	total capillary length
t_i	migration time (analyte ion)
E	electric field



Effective capillary length is the distance from point of injection to point of detection.

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Theory of Capillary Electrophoresis – Zone Resolution

$$R = \frac{2\,(t_j - t_i)}{(w_i + w_j)} = \frac{t_j - t_i}{4\sigma}$$

t migration time

w width of the peak at base in length units (4σ for a gaussian peak)

With **constant cross sectional velocity** in the capillary and **absence of chromatographic interaction** with the capillary surface the solute zone only broadens by axial diffusion:

$$\sigma^2 = 2D_m t_i \quad \text{and} \quad N = \frac{l^2}{\sigma^2}$$

(D_m is the diffusion constant of the solute)

$$N = \frac{\mu_i l\, V}{2D_m L}$$

Plate number increases with applied voltage, and decreasing diffusion constant (i.e. HMW molecules!!)

$$R = \left(\frac{1}{4\sqrt{2}}\right)(\Delta\mu_a)\left(\frac{V}{D(\bar{\mu}_a + \mu_{EOF})}\right)^{1/2}$$

Resolution increases with square root of the applied voltage, and inverse square root of diffusion constant.

Selectivity $\Delta\mu_i$ works better

Capillary Electrophoresis – Other Sources of Zone Broadening

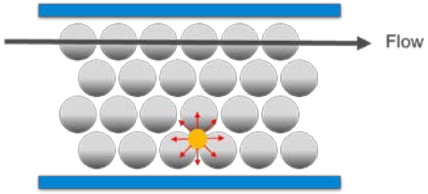
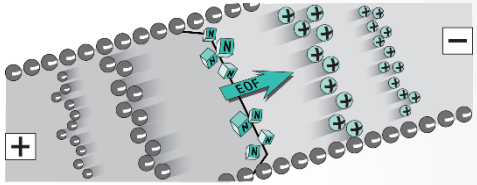
Source	Comment
Joule heating	Leads to radial temperature gradients differing flow velocity profiles across the capillary
Length of injection zone	Injection lengths should be less than the diffusion-controlled zone length. Detection limit difficulties often necessitate longer than ideal injection lengths
Sample adsorption	Interaction of solute with the capillary walls usually causes severe peak tailing.
Mismatched conductivities of sample and buffer (electrodispersion)	Solutes with higher conductivities than the running buffer result in fronting triangular peaks. Solutes with lower conductivities than the running buffer result in tailing triangular peaks.
Unleveled buffer reservoirs	Causes flow with parabolic flow velocity profile
Detector cell size	Volume should be small relative to volume of the zone

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Characteristics of Capillary Electrophoresis

- Electrophoresis is performed in narrow-bore (25- to 75-µm id), fused silica capillaries
- High voltages (10 to 30 kV) and high electric fields (100 to 500 V/cm) are applied; separation is based on compound mobility (mass/charge) in an electrical field
- High electrical resistance of the capillary limits current generation and internal heating
- High efficiency ($N>10^5$ to 10^6) and short analysis time (min)
- Detection performed on-capillary (DAD, CCD, LIF), with an external detection cell (DAD) or on-line MS
- Small sample volume required (1 to 50 nL injected)
- Operates mainly in aqueous media
- Orthogonal separation principle complementing HPLC
- Multiple principle modes of CE provided selectivity of separation and a wide application range

Compare HPLC and CE

	CHROMATOGRAPHY	ELECTROPHORESIS
Separation principle	Partitioning between mobile phase and stationary phase. All solutes move with the same velocity	Based on differences in solute mobility an electric field. Solutes move at different velocity
		
UV light path	Usually 1 cm to 6 cm (high sensitivity)	Usually 25 µm to 75 µm (HS-cell = 1.2 mm)
Columns	Packed with stationary phase particles	None, open tube (fused silica or polymers)
Flow rates	Constant depending on HPLC pump, usually in µL-mL/min range	pH dependent flow rate + compound specific migration, typically 30-300 nL/min
Peak width	Van Deemter equation	Depends on diffusion constant

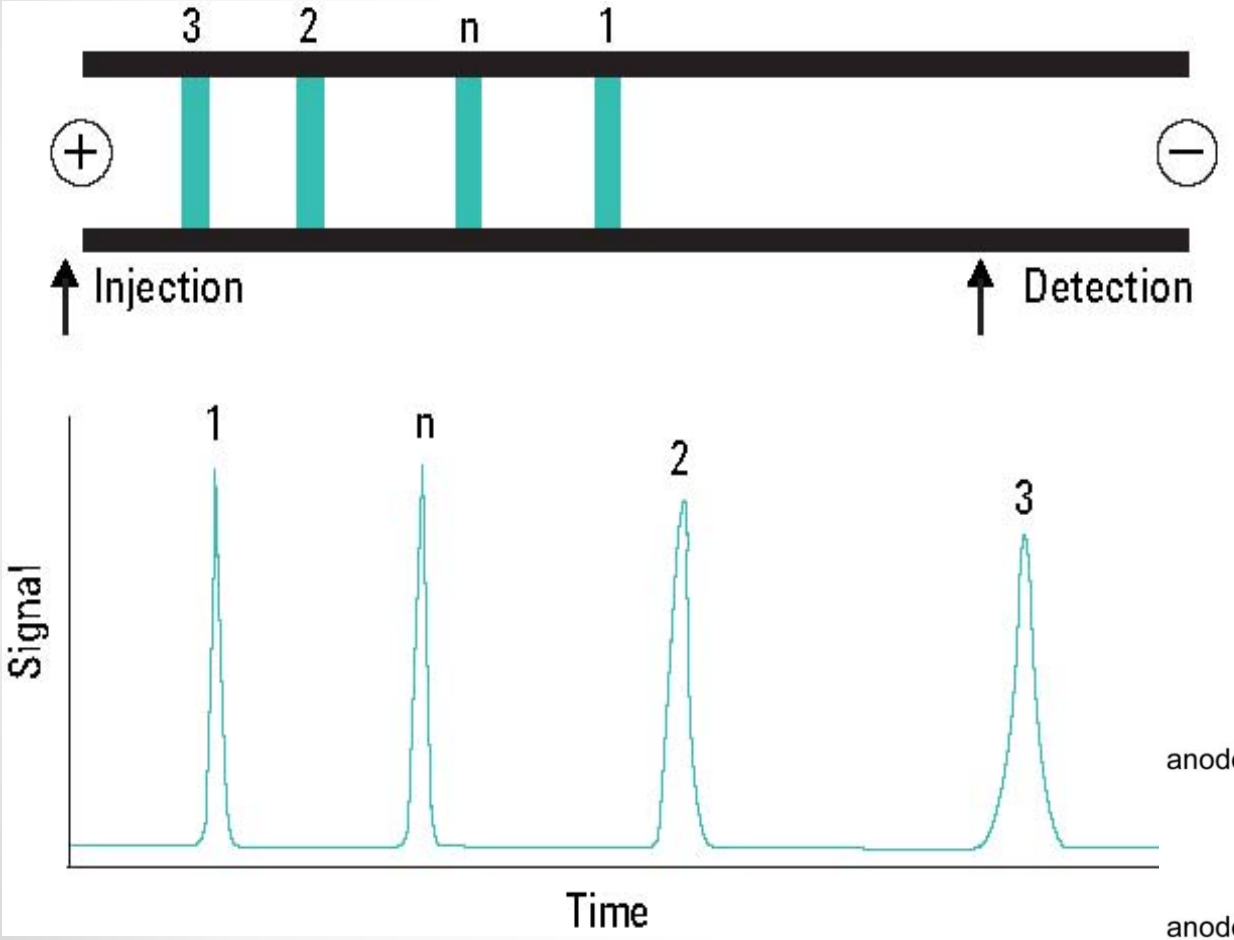
Methods of Capillary Electrophoresis

Modes of Operation

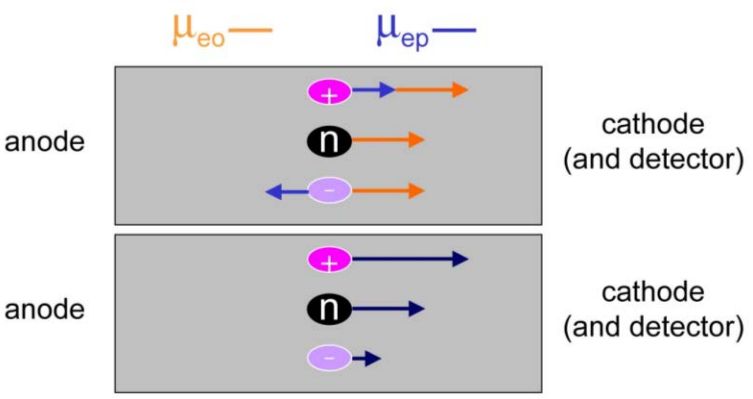
Modes of Operation of CE

Mode	Basis of Separation
Capillary Zone Electrophoresis	Difference in electrophoretic mobility of the solutes (all charged LMW and HMW molecules) in an open tube
Capillary Gel Electrophoresis	Difference in mobility by molecular size (SDS-proteins, DNA/RNA) in a gel sieving matrix
Capillary Isoelectric Focussing	Differences in isoelectric point (pI) of amphoteric solutes (peptides, proteins)
Capillary Isotachophoresis	Electrophoretic mobility (all charged molecules)
Micellar Electrokinetic Chromatography	Differences of molecules in hydrophobic interaction with micelles in the BGE (neutral or weakly ionized, LMW substances). Allows separation of neutral molecules
Capillary Electrochromatography	EOF driven HPLC (neutral and weakly ionized, LMW solutes)

Capillary Zone Electrophoresis



$$v = (\mu_{ep} + \mu_{eo})E$$



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Capillary Zone Electrophoresis - Buffers for BGE

A buffer used in CZE should possess the following properties:

- Good buffering capacity in the pH of choice
- Low absorbance at the wavelength of detection
- Low mobility to reduce the current (large, minimally charged ions)

Buffer	***pH Range***
Phosphate	1.14 - 3.14
Acetate	3.76 - 5.76
Phosphate	6.20 - 8.20
Borate	8.14 - 10.14
Zwitterionic Buffers (Good buffers*)	
MES	5.15 - 7.15
PIPES	5.80 - 7.80
HEPES	6.55 - 8.55
Tricine	7.15 - 9.15
Tris	7.30 - 9.30

*https://en.wikipedia.org/wiki/Good%27s_buffers

Capillary Zone Electrophoresis - Additives

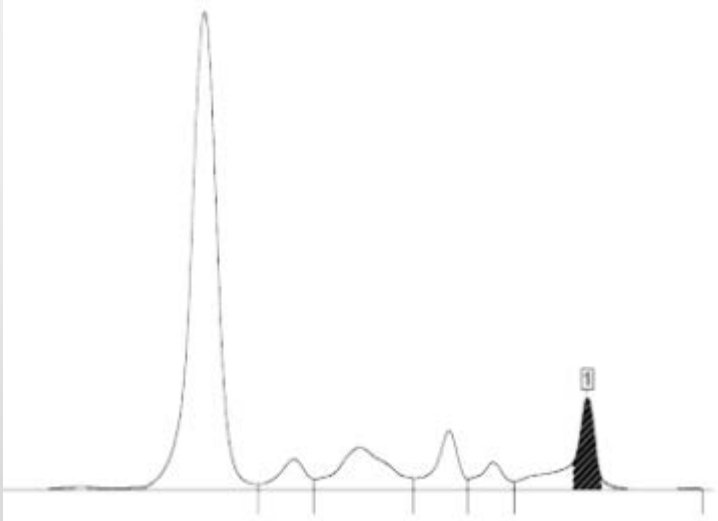
Change the chemical and physical properties of the FS capillary surface to influence the EOF and/or the mobility of solutes to improve selectivity of separation

Additive	Function
Organic solvents	Solubility sample compounds, modify electroosmotic flow
Organic acids or bases	Magnitude and direction of EOF, ionization state of solutes
Neutral, hydrophilic polymers	Reduce EOF, mask capillary surface e.g. hydroxymethyl cellulose
Surfactants	Below critical micelle concentration
Cyclodextrines	Direct separation of enantiomers!
Cationic or anionic polymers	Change the capillary surface charge
Urea etc.	Solubilizing agents

*Below the critical micelle concentration (CMC)

Main Application Areas of CZE

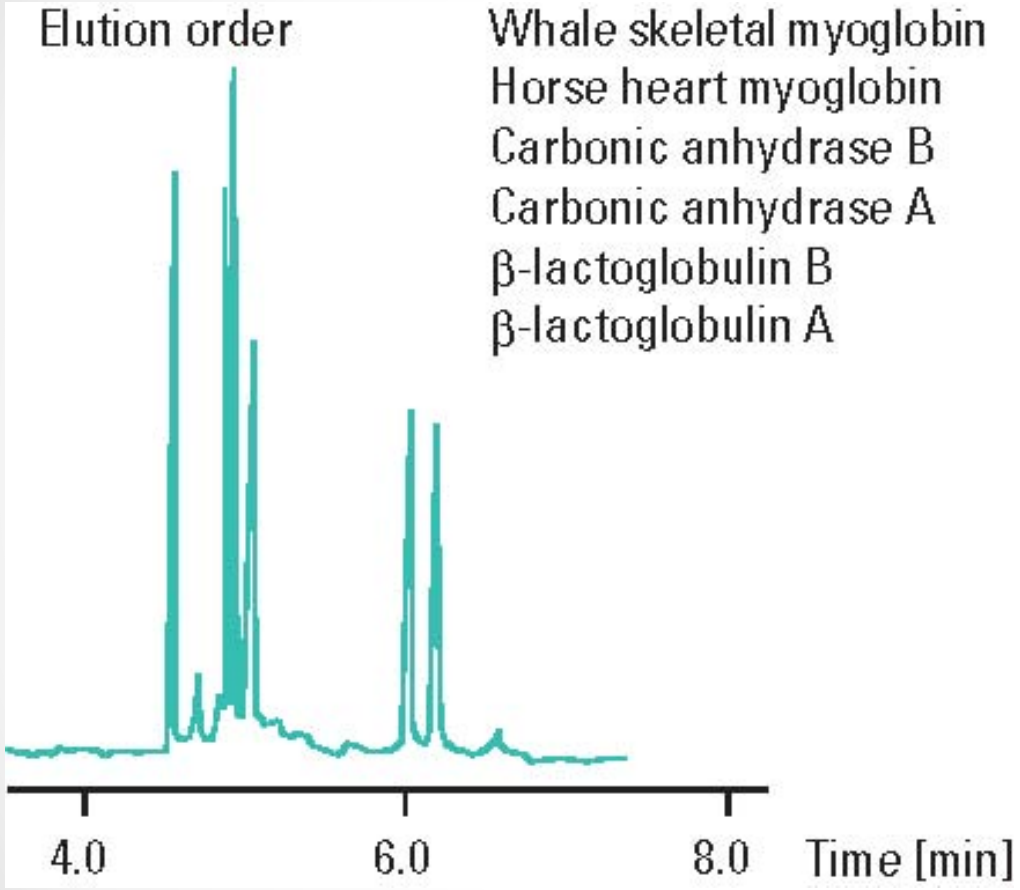
- LMW, ionizable molecules e.g. pharmaceuticals
- Chiral separations
- HMW molecules e.g. peptides and proteins (last section)
- Clinical analysis esp. serum protein profiling (CAPILLARYS



Protein electrophoresis is a well-established technique routinely used in clinical laboratories to screen samples to detect protein abnormalities (gammopathies, inflammatory pattern…).

Serum proteins into six major fractions:
Albumin, Alpha-1, Alpha-2,Beta-1, Beta-2, Gamma

CZE of Proteins using Buffer pH above Solute pI



Conditions:

20 mM borate pH 8.25

V = 30 kV

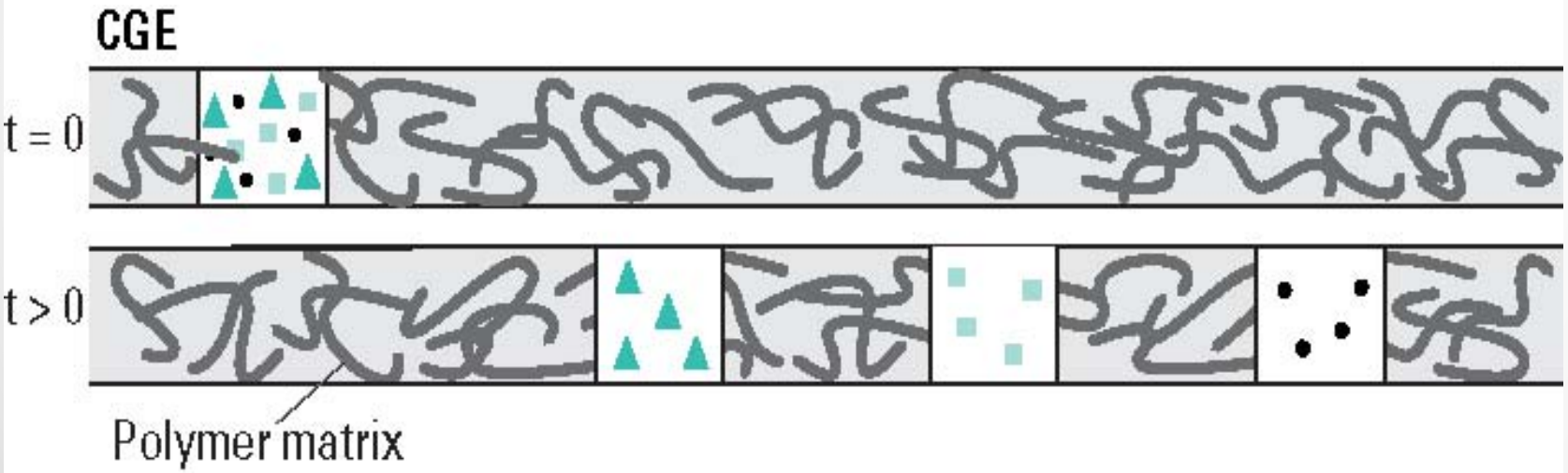
l = 55 cm, L = 101 cm, id = 52 µm

i = 38 µA

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Capillary Gel Electrophoresis (CGE)

- Separation of macromolecules by a “sieve” based on a physical or dynamic gel
- EOF is suppressed by the presence of gel



Polymer Matrices & Methods for CGE of Proteins[1]

Crosslinked polymers (physical gels):	**Poly-acrylamide/bis-acrylamide**
Linear polymers (chemically bonded):	**Linear poly-acrylamide**
Replaceable polymers[2]:	**LPA, dextran, polyethylene glycol, hydroxypropyl cellulose, polyvinyl alcohol, polyethylene oxide, pullanes**

Methods[2]

- Native protein CGE
- SDS protein CGE
- Oligonucleotides, DNA, RNA

1. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3227876/ , https://openwetware.org/wiki/Capillary_Gel_Electrophoresis
2. Anal Chim Acta. 2012 January 4; 709: 21–31. doi:10.1016/j.aca.2011.10.022.

Capillary Gel Electrophoresis of Proteins

- Sieving gel equivalent to SGE in a capillary
- Sodium dodecyl sulfate/protein complexes[1]
  - Approx. same mass/charge ratio ➔ in CZE all move with the same speed
  - Obstructed pathways in a gel ➔ speed proportional to hydrodynamic size of the complex
  - Replaceable polymers preferentially used nowadays
  - Capillary wall must be deactivated
  - MW calibration with protein ladder (commercial) or reference proteins

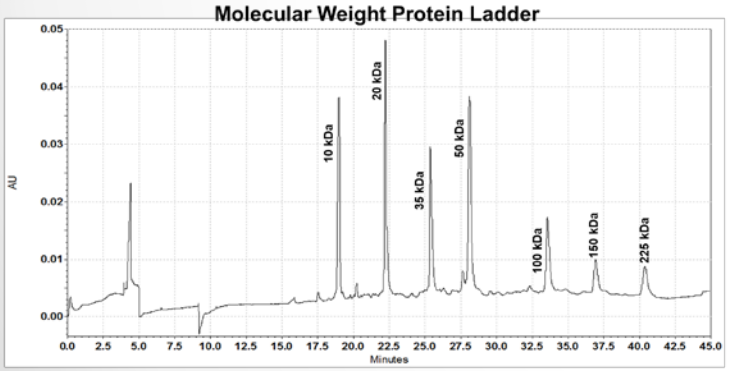


Figure 1. SDS-CGE electropherogram of commercial protein preparation for known molecular weights. The axes display time (Minutes) versus absorbance units (AU).

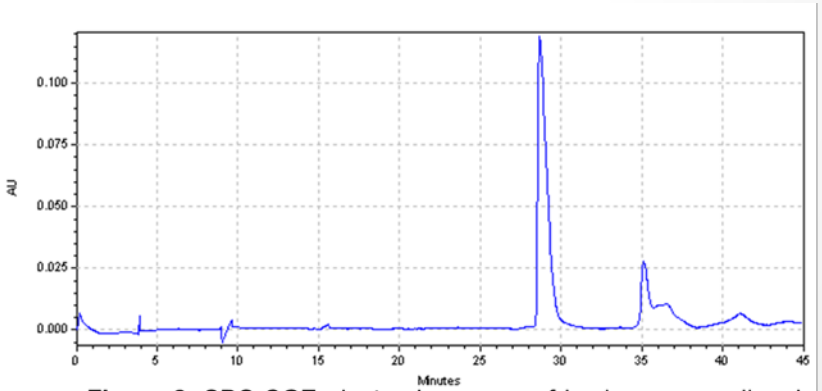
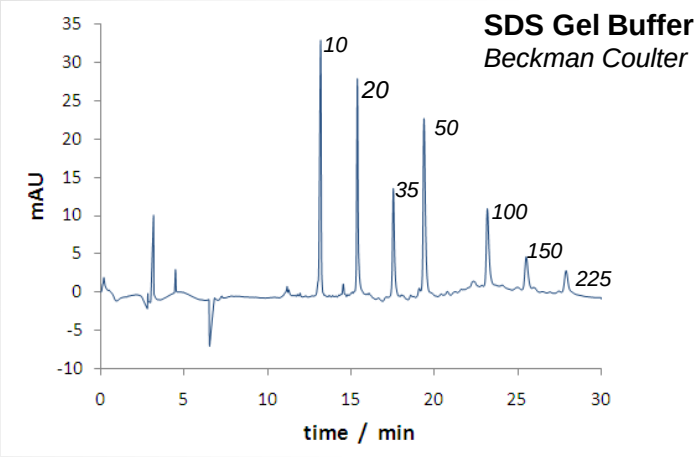


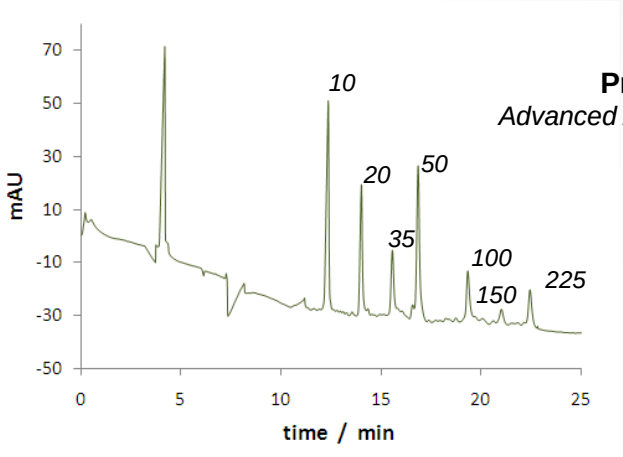
Figure 2. SDS-CGE electropherogram of bovine serum albumin. Albumin monomer, dimer, and trimer are shown.

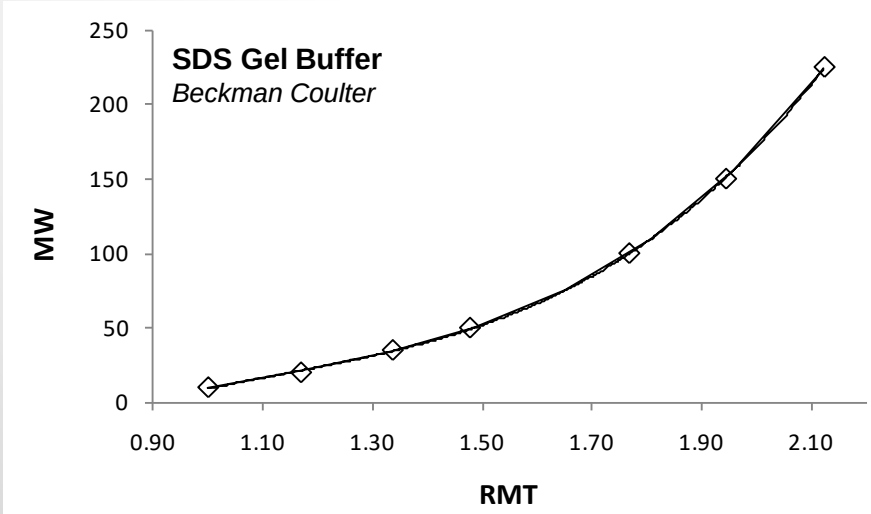
[1]A. Guttmann, Electrophoresis 1996, 17, 1333-1341

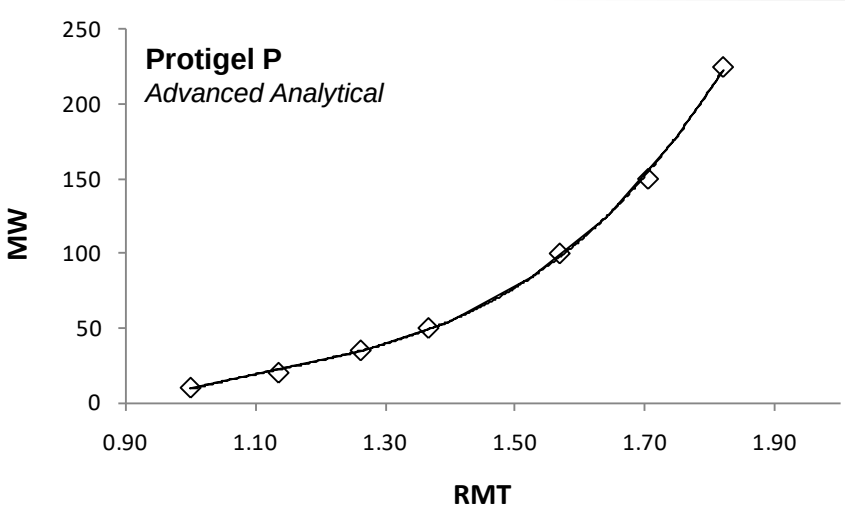
Capillary Gel Electrophoresis of Proteins

Representative electropherograms of molecular weight standards on commercial sieving gels*







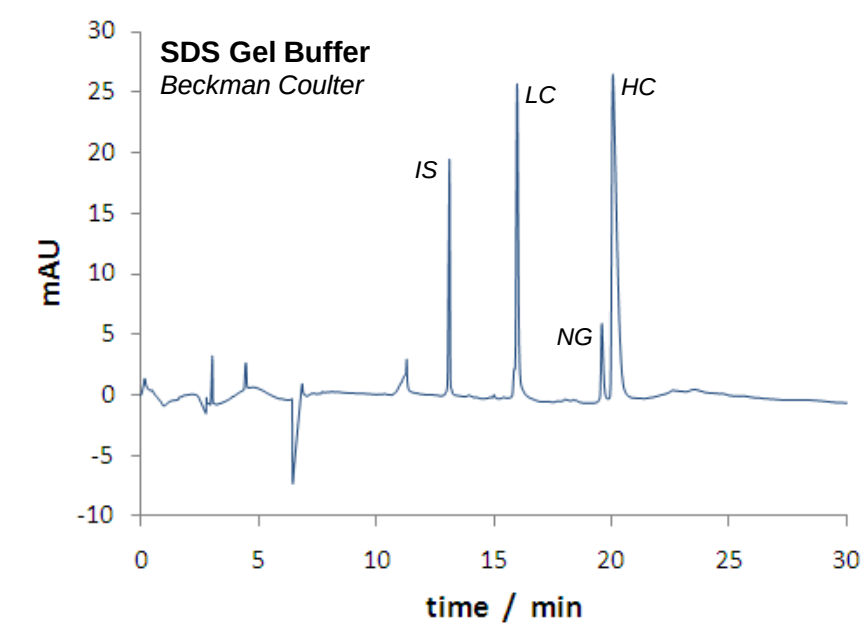


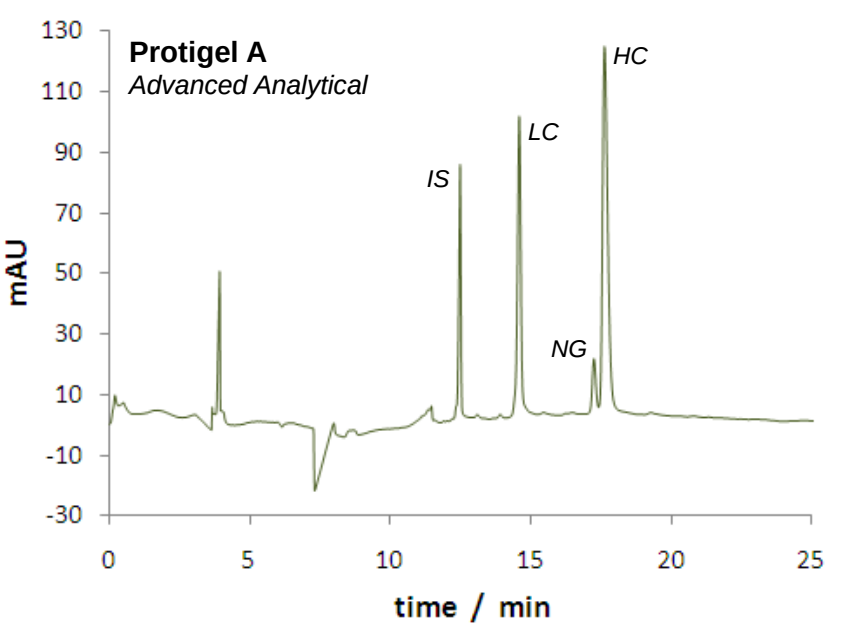
Slide courtesy of Agilent Technologies with permission

More information: https://www.agilent.com/search/?Ntt=5990-7976

Capillary Gel Electrophoresis of Proteins

IgG sample under reducing conditions. IS, internal standard; LC, light chain; NG, non-glycosylated heavy chain; HC, heavy chain





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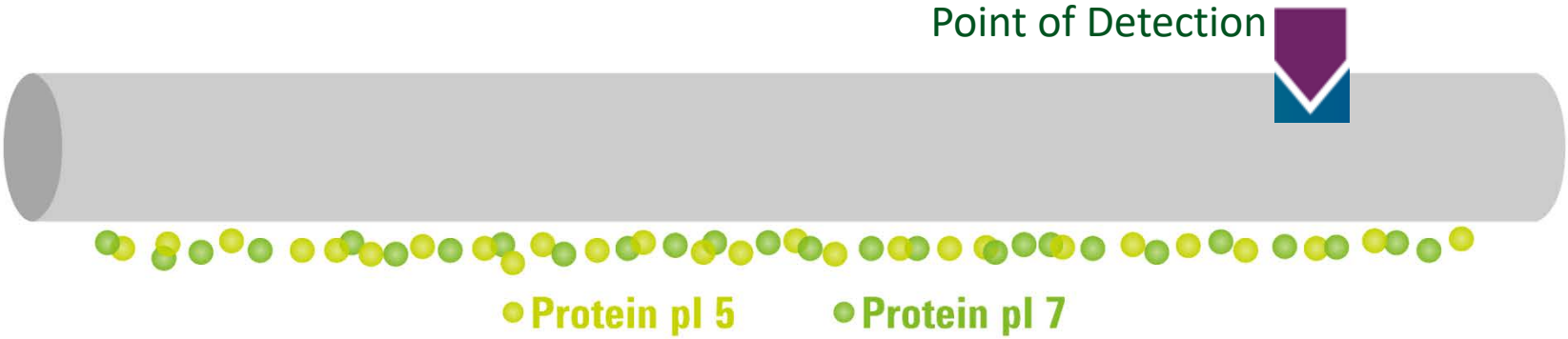
More information: https://www.agilent.com/search/?Ntt=5990-7976

Iso-Electric Focusing of Proteins*

- Proteins, like amino acids, are amphoteric molecules i.e. they can act as base by accepting a proton or as an acid by donating a proton
- For an amino acid *pI*= $\frac{(pK_a+pK_b)}{2}$, for a peptide/protein # of groups and environment
- A gradient is established from low to high pH by means of a special set of amphoteric molecules with pI 2-10.
- IEF separates in space domain
- Planar Format
  - Immobilized pH gradient
  - IEF strips for 2D GE
- Capillary Format

* https://www.agilent.com/cs/library/primers/public/5991-1660EN.pdf

Principles of Capillary IEF



Graphics courtesy of Agilent Technologies

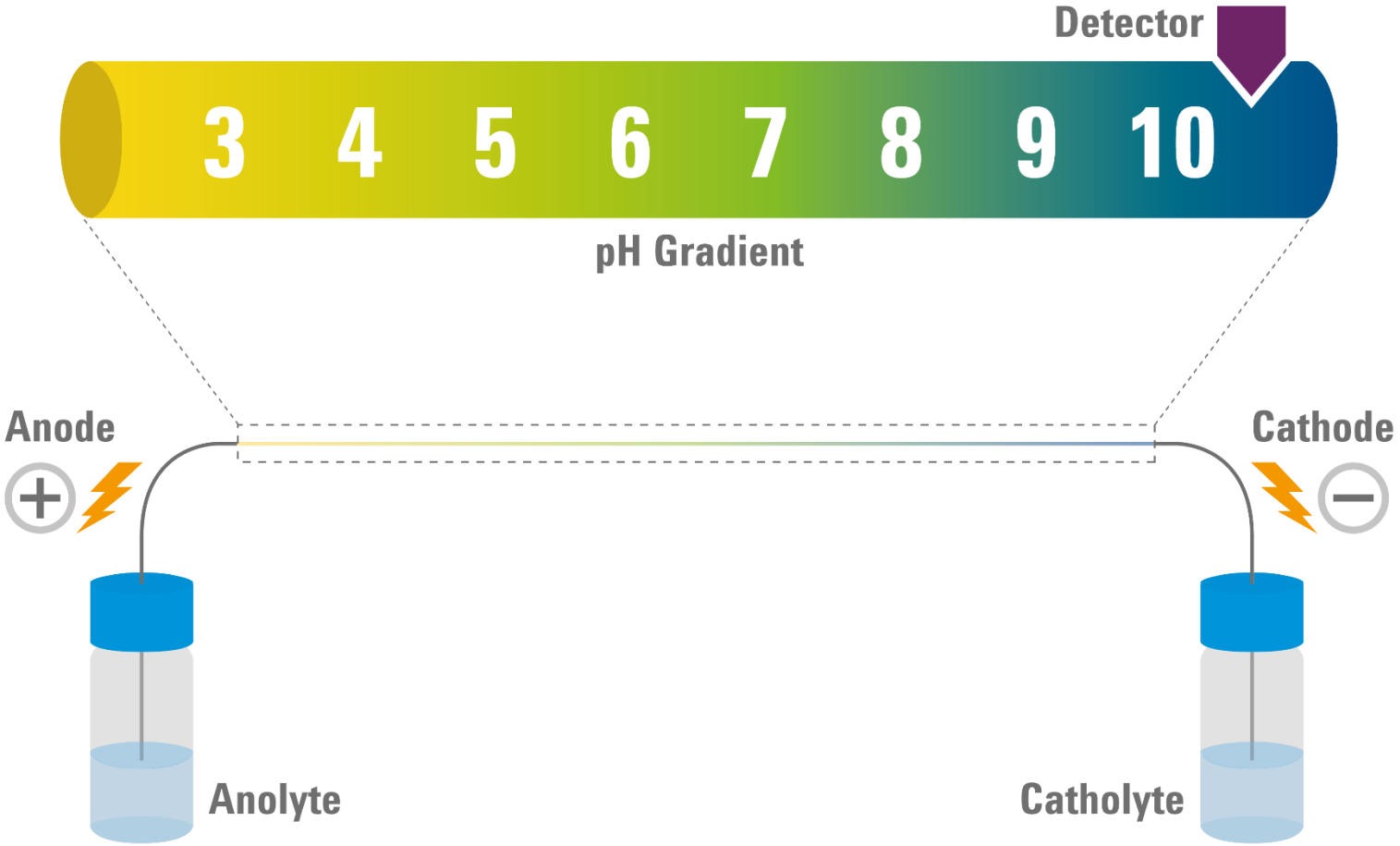
At the beginning of a CIEF run, the whole capillary (PFE coated fused silica) is filled with:

- Carrier Ampholytes (CA) (polyamino-polycarboxylic acid oligomers with pI's ranging from 3-10 and MW 500-800)*
- Protein or peptide sample to be analyzed (amphoteric molecules)
- pI markers (amphoteric molecules)
- pH of the solution average of all pKa's and pKb's; molecules with a low pI will be positively charged, molecules with high pH will be negative at start
- Sacrificing ampholytes to keep the focused zones within the capillary and before POD and additives that suppress residual EOF and improve solubility of focused protein zones
- The inlet side low pH (anolyte,H_3PO_4), high positive voltage, anode
- The outlet side high pH (catholyte, NaOH), grounded, cathode

*P. Righetti, Isoelectric Focusing: Theory, Methodology and Application, 1st Edition, 1983, Elsevier Science Publishers

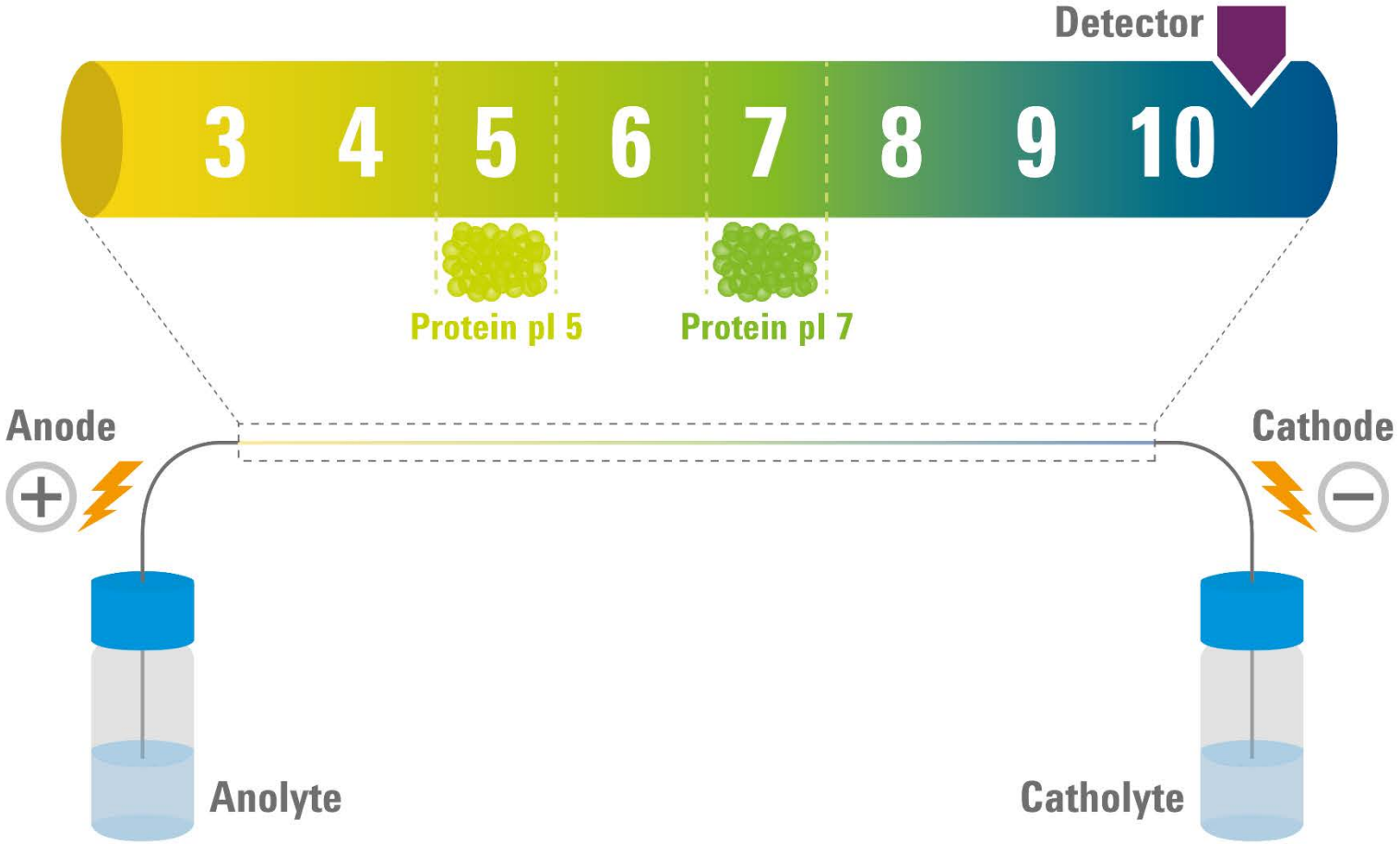
Slide courtesy of Agilent Technologies with permission

Principles of CIEF; pH Gradient



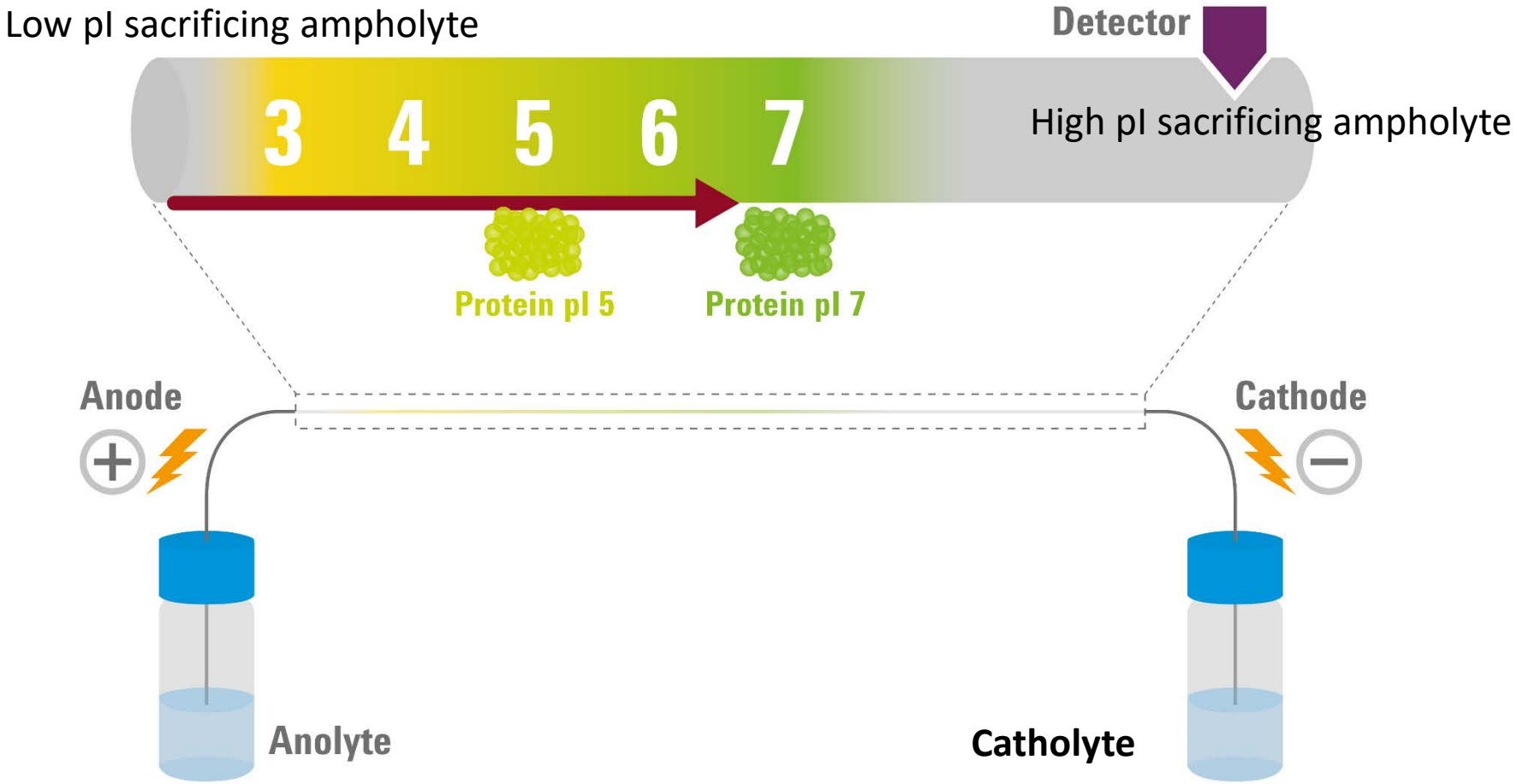
Graphics courtesy of Agilent Technologies

Principles of CIEF; Focusing



Slide courtesy of Agilent Technologies

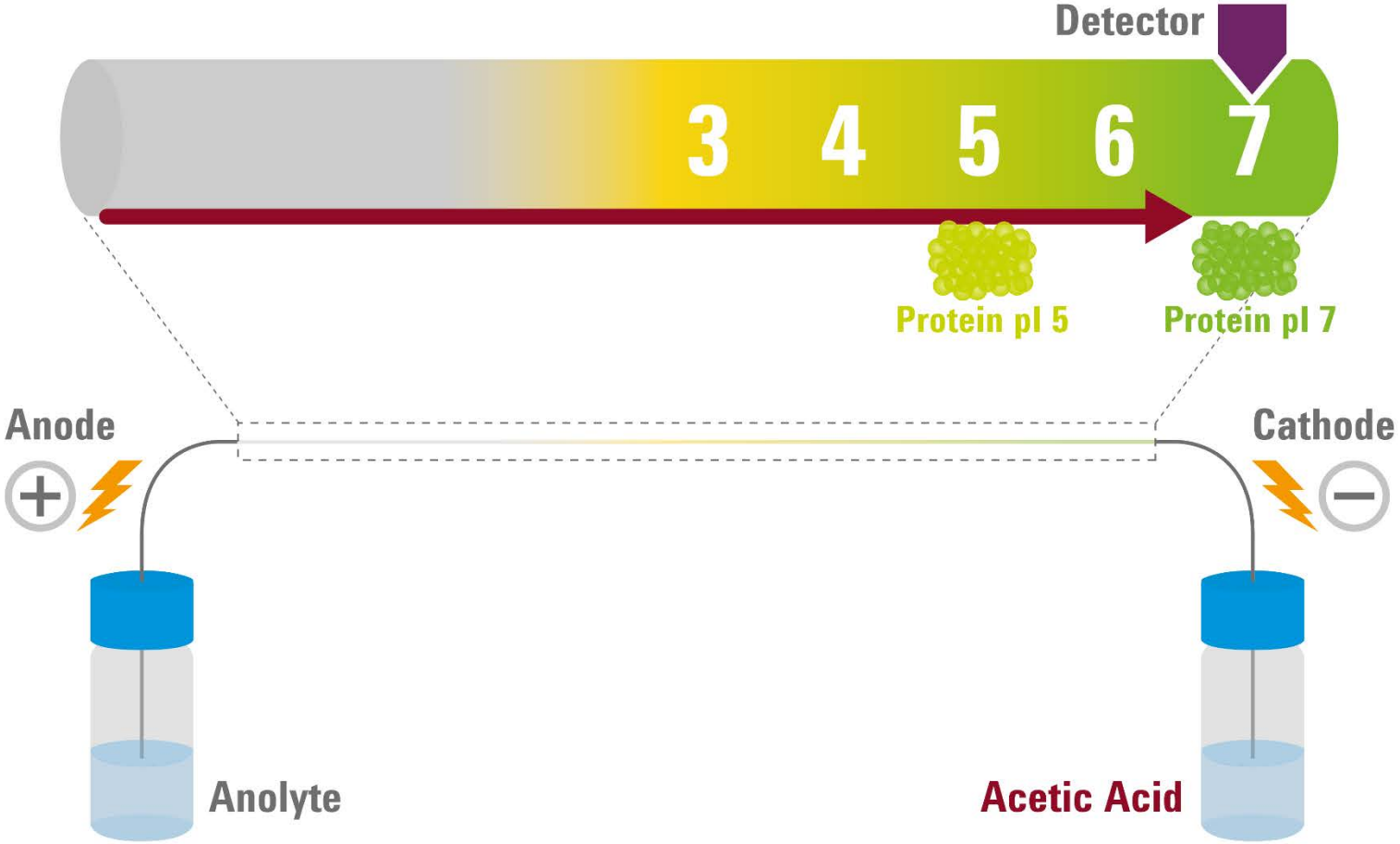
Principles of CIEF; Mobilization



Slide courtesy of Agilent Technologies

Principles of CIEF; Mobilization

Chemical Mobilization



Graphics courtesy of Agilent Technologies

Principles of CIEF



Pro's

- Available on commercial CE instruments (Sciex formerly Beckman-Coulter, Agilent Technologies
- Provides quantitative protein analysis in applicable concentration range (0.05 – 0.5 mg/mL)

Con's

- Long cycle time (focusing & mobilization takes about 1 run/hour)
- Time-consuming method development
- Non-reproducible peak patterns
- Loss of resolution during mobilization since focusing is absent

Whole Column Image Detection CIEF

- First described by Pawliszyn and Wu, Anal. Chem., 1992, 64, 224-227
- Commercialized by Convergent Bioscience in 2000, iCE280. Acquired by Cell Biosciences (now Protein Simple) in 2010.
- Established market leader Protein Simple, iCE280 and iCE3

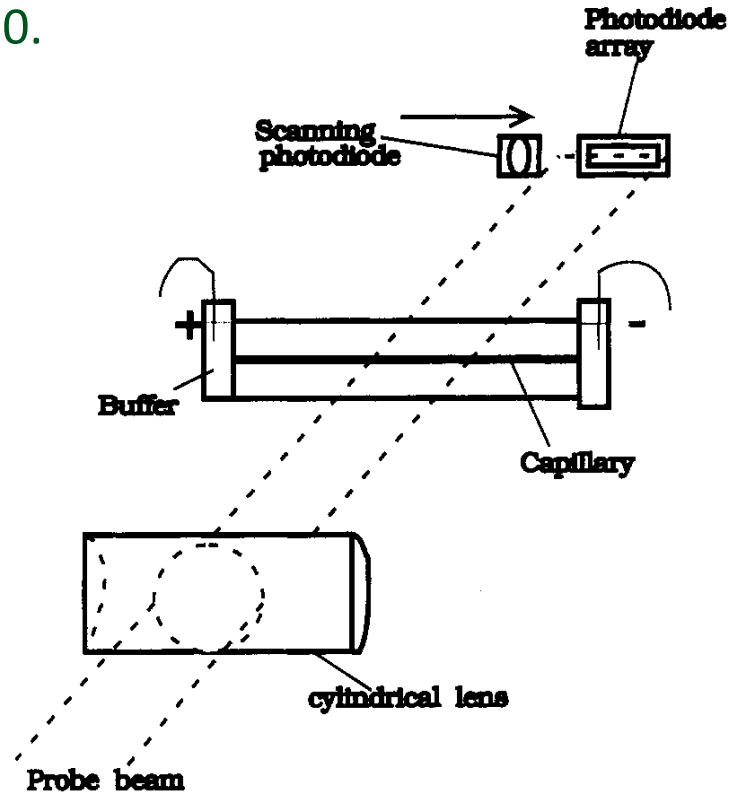
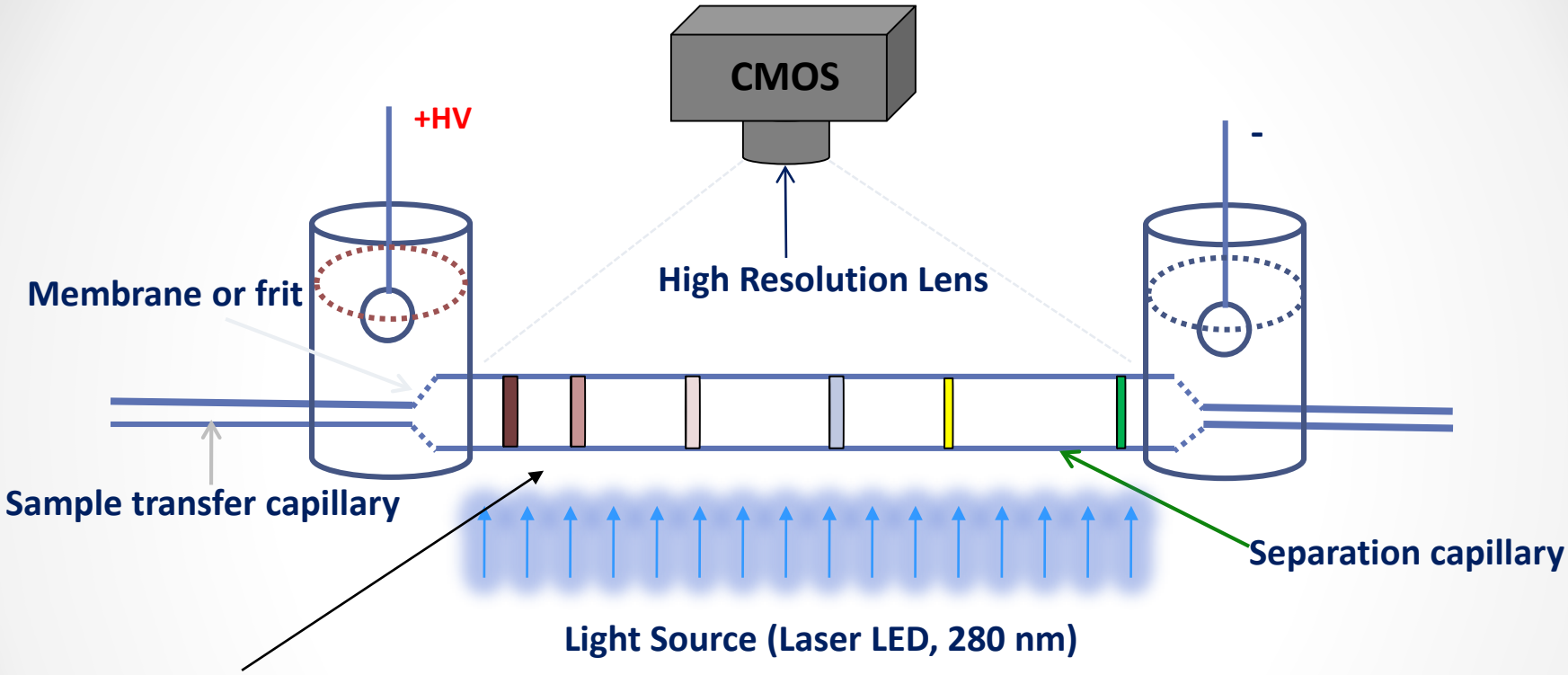


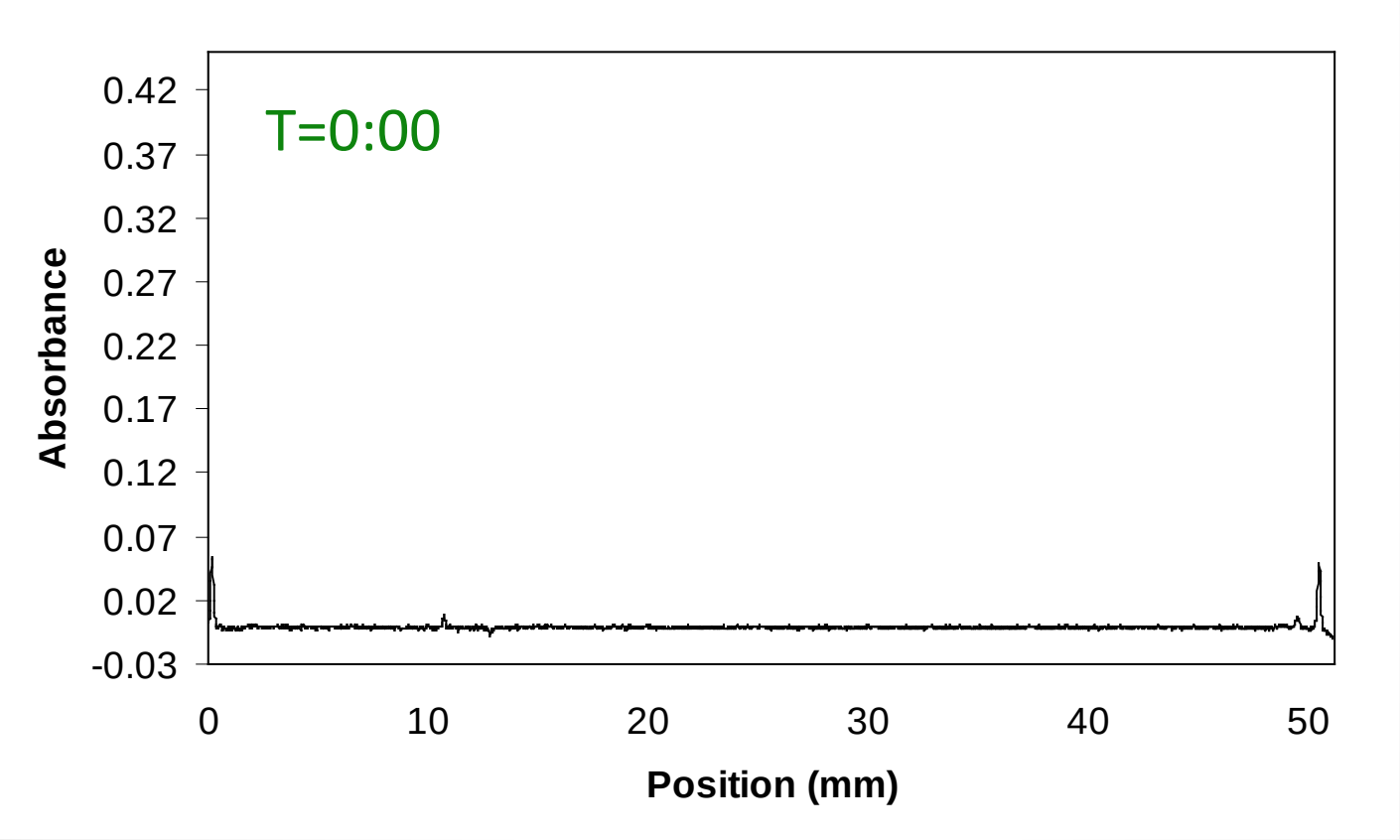
Figure 1. Diagram demonstrating the cartridge holding the capillary, and the probe beam alignment.

Whole Column Image Detection CIEF

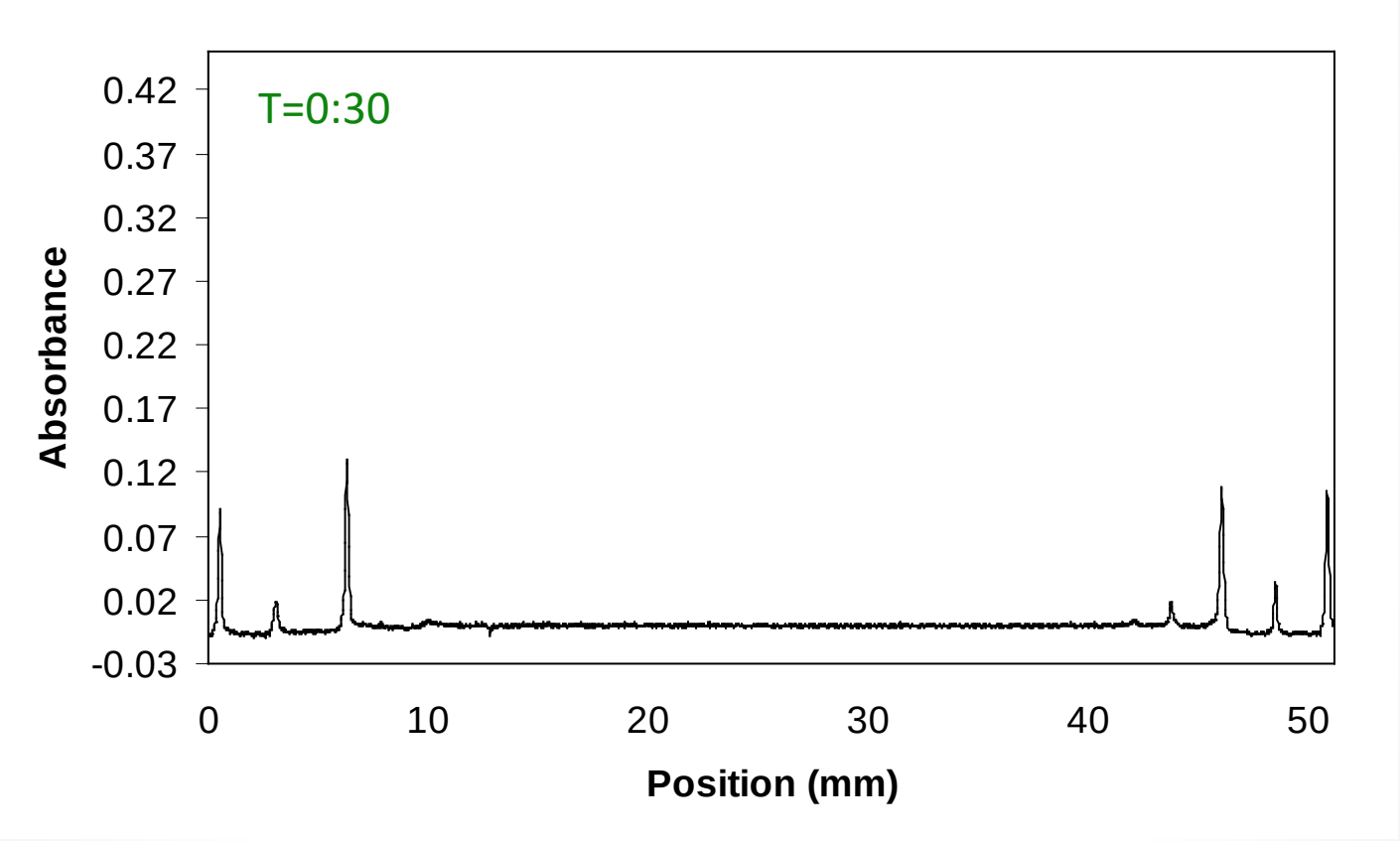


Separation capillary <u>50x0.1, 50x0.2 mm</u>

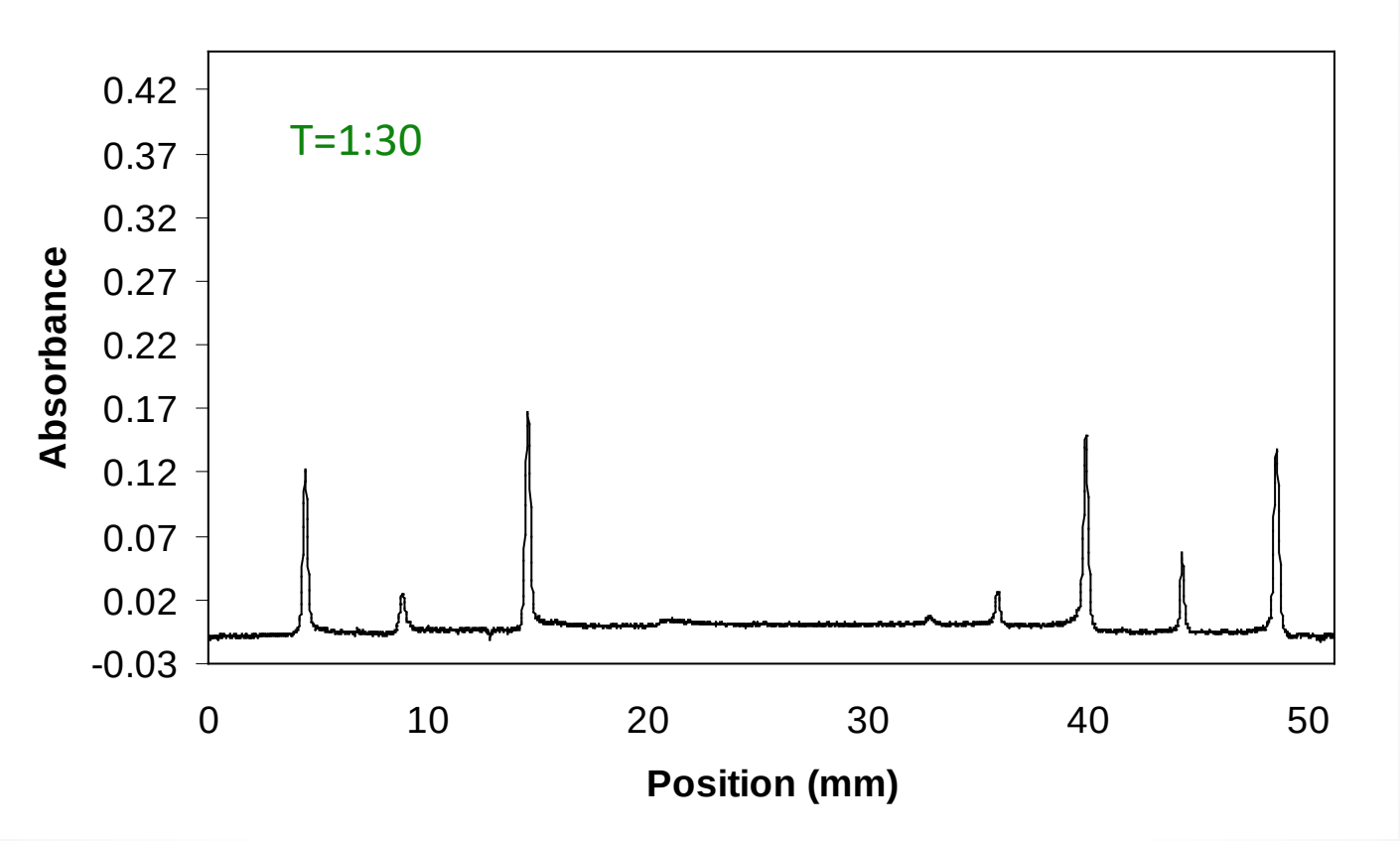
Development of Separation of five pI Markers



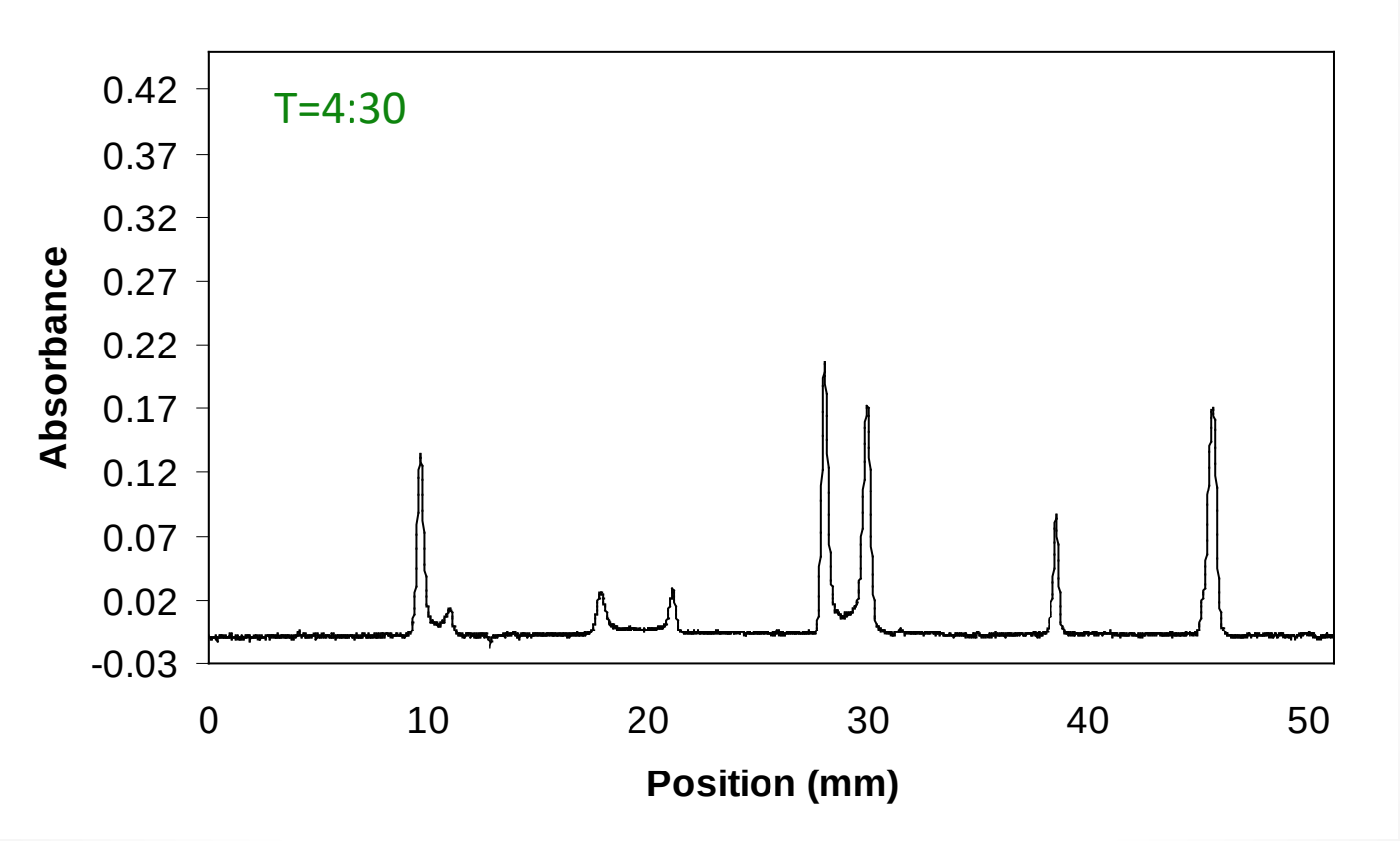
Development of Separation of five pI Markers



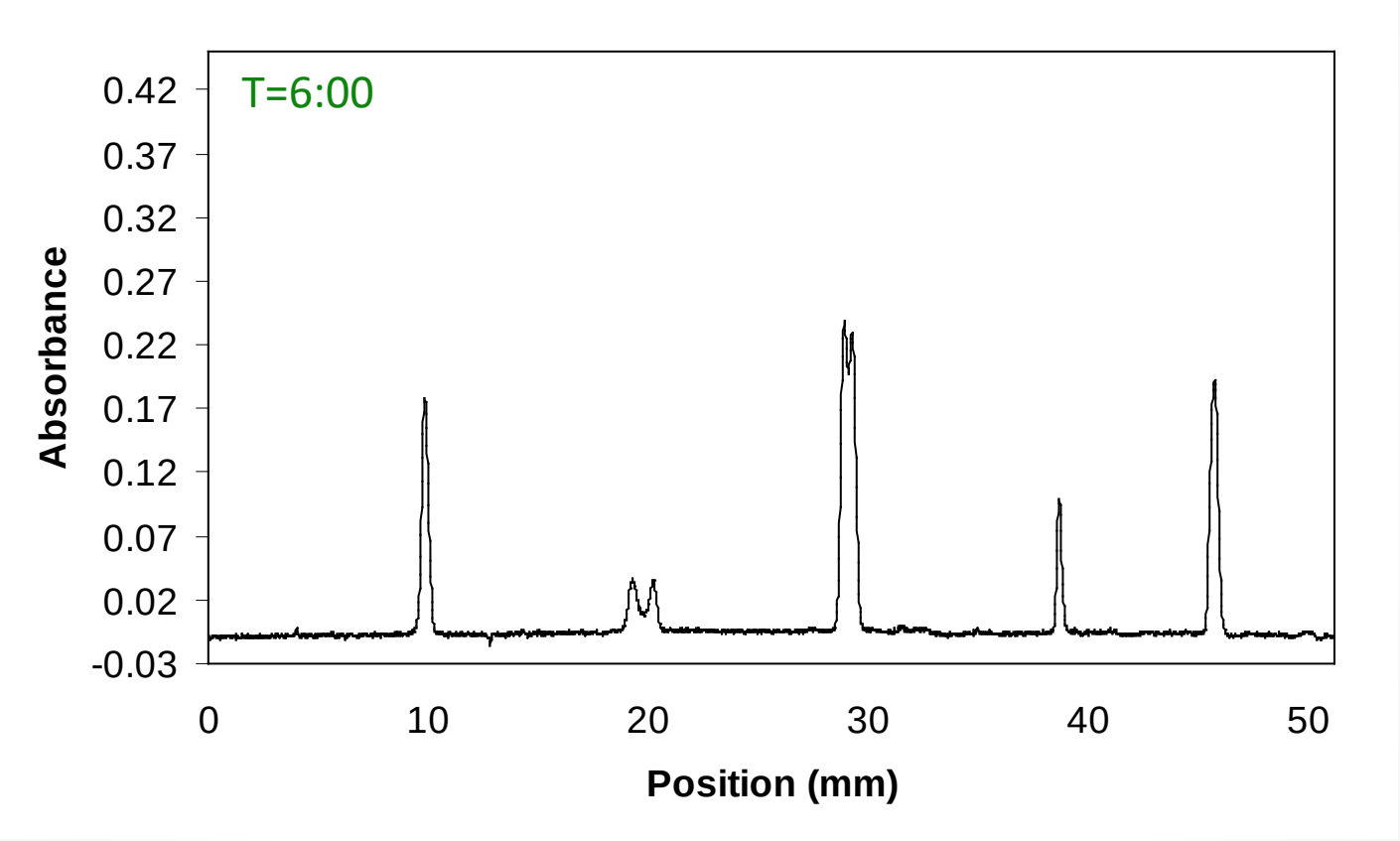
Development of Separation of five pI Markers



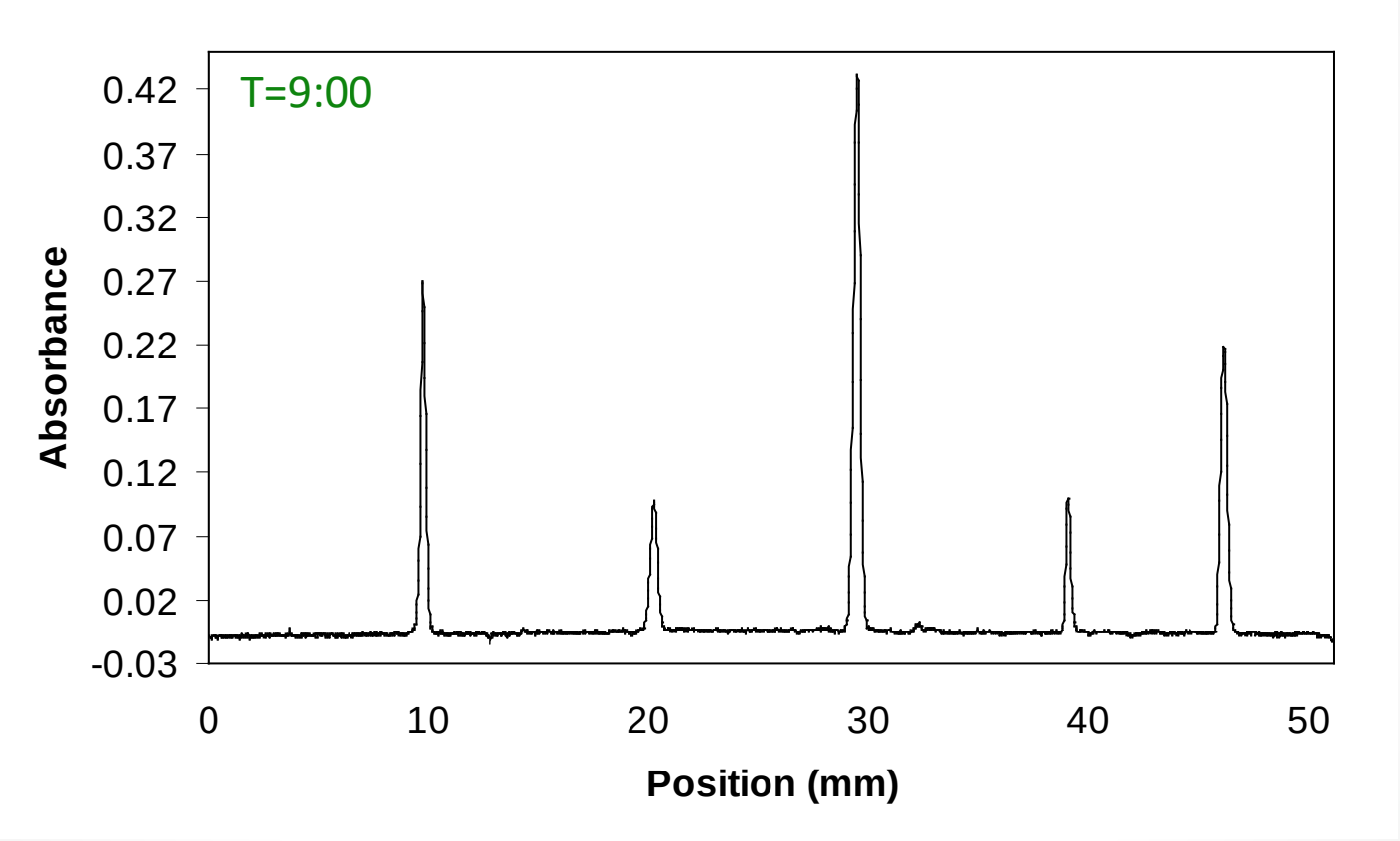
Development of Separation of five pI Markers



Development of Separation of five pI Markers

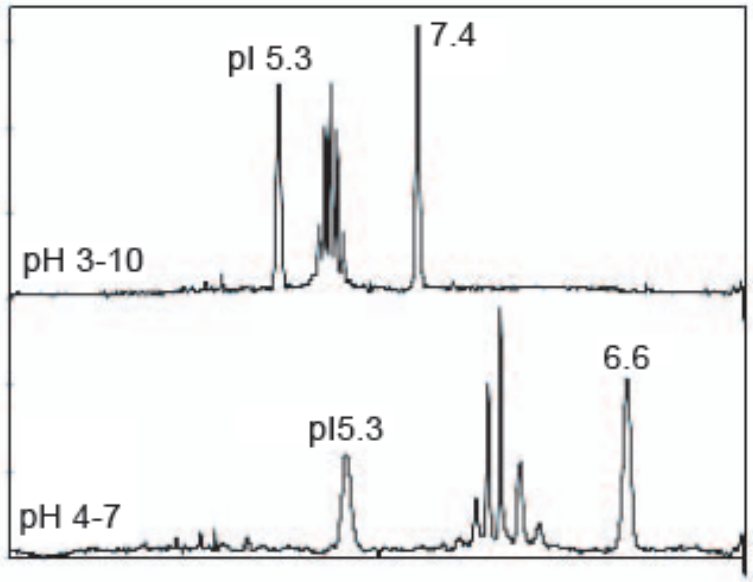


Development of Separation of five pI Markers



Narrow pH Range Carrier Ampholytes

Acidic Mab



Sample: Mab2

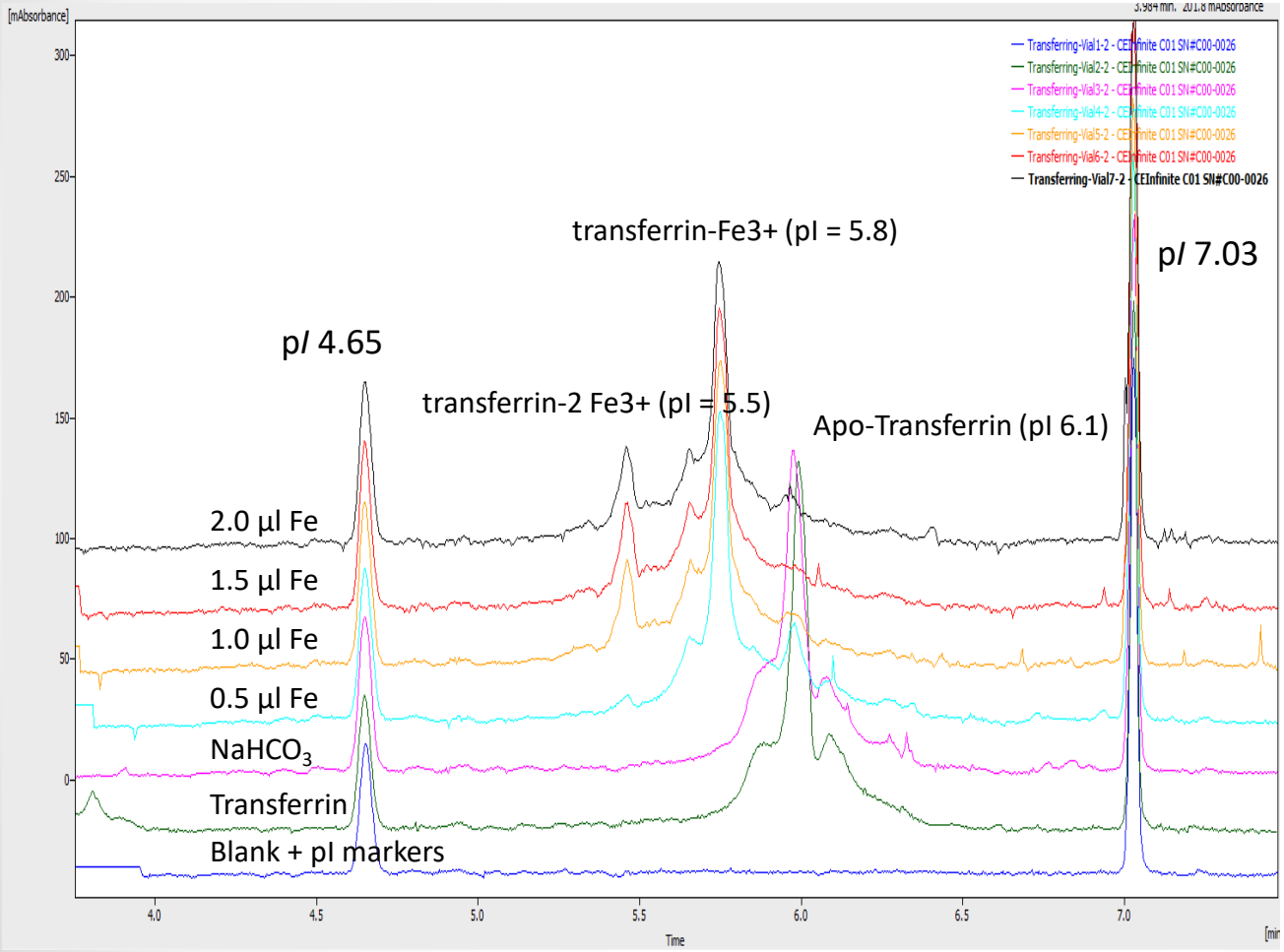
Trace 1: Pharmalyte 3-10
6 minutes focusing

Trace 2: Servalyt 4-7
12 minutes focusing

Anolyte: 80 mM H3PO4

Catholyte: 100 mM NaOH

iCIEF of Human Transferrin Standard

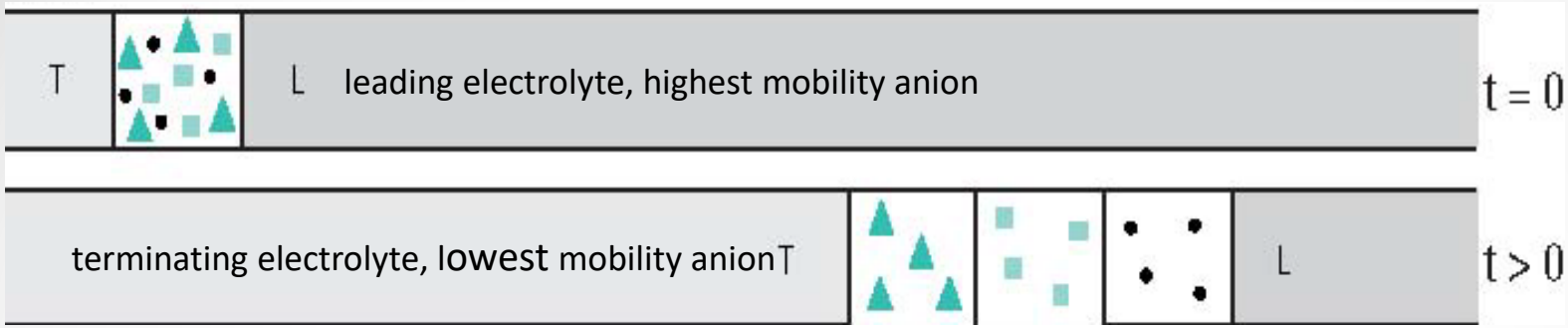


4% pH 4-7 Aeslyte HR

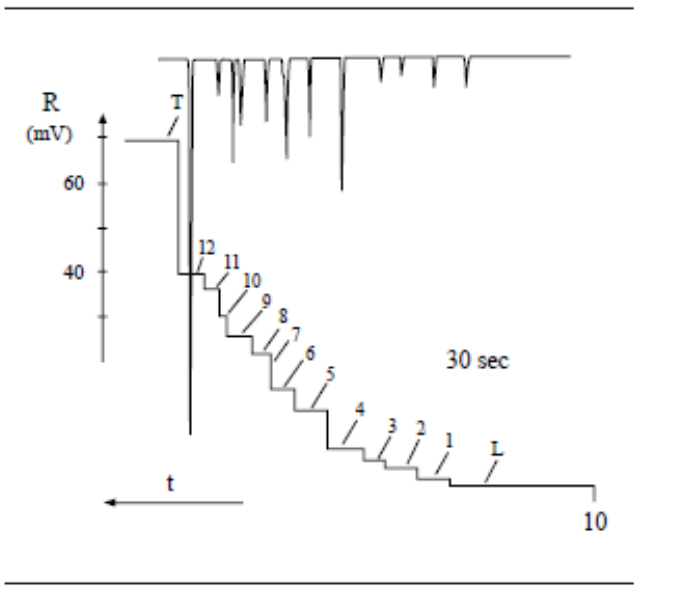
Remaining Modes of Operation of CE

Mode	Basis of Separation
Capillary Zone Electrophoresis	Difference in electrophoretic mobility of the solutes (all charged molecules)
Capillary Gel Electrophoresis	Difference in molecular size (proteins, DNA/RNA)
Capillary Isoelectric Focussing	Differences in isoelectric point (pI) of amphoteric solutes (peptides, proteins)
Capillary Isotachophoresis	Electrophoretic mobility (all charged molecules)
Micellar Electrokinetic Chromatography	Differences in hydrophobic interaction with micelles in the BGE (neutral or weakly ionized, LMW substances)
Capillary Electrochromatography	EOF driven HPLC (neutral and weakly ionized, LMW solutes)

Capillary Isotachophoresis (CITP)

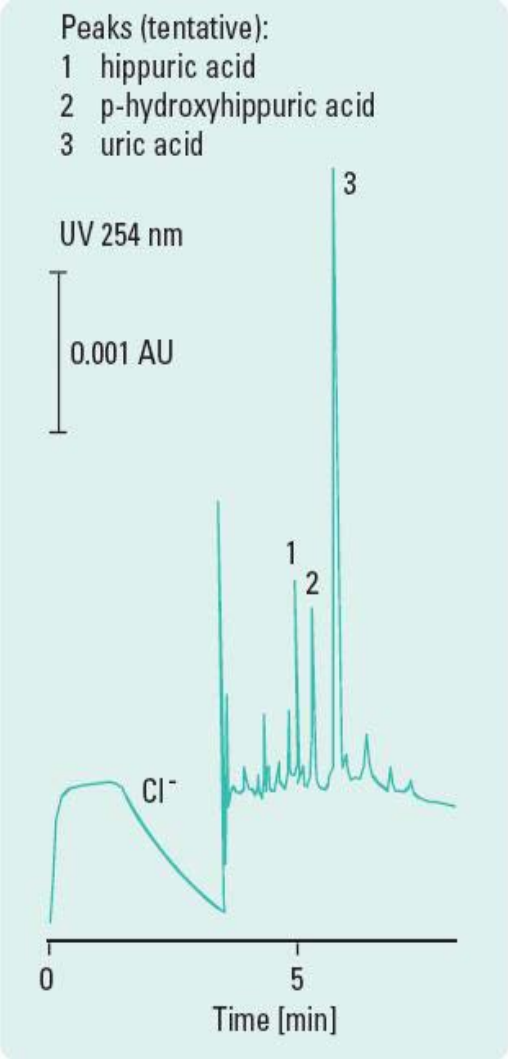


- Moving boundary technique
- Only anion or cations in one run
- Zones stack after each other
- Length of the zone proportional to concentration



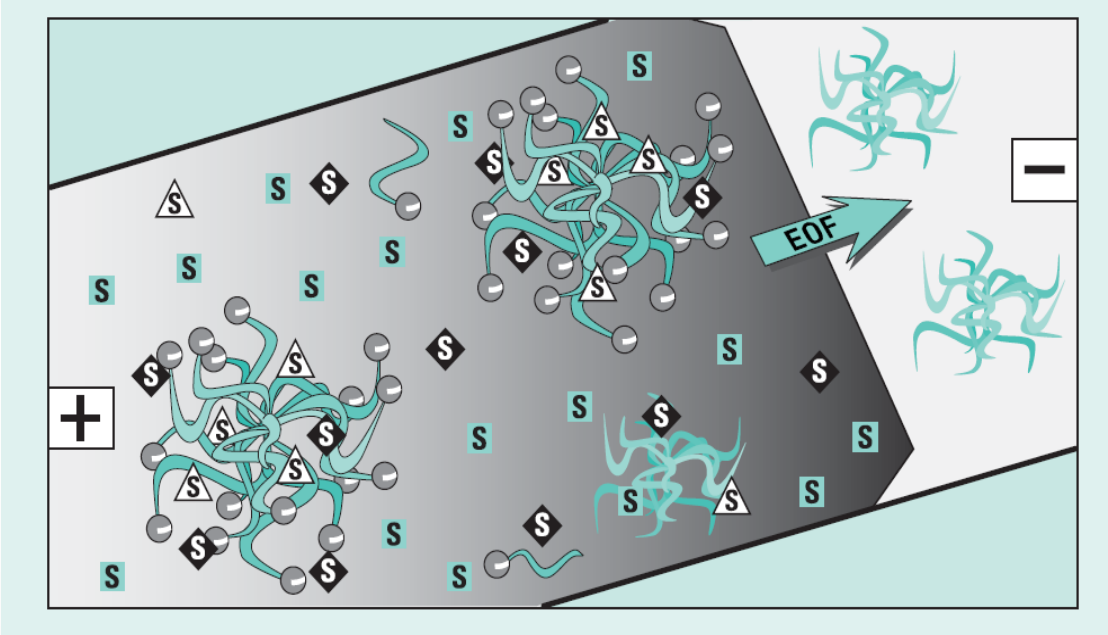
Further reading, J. Sep. Sci. 2006, 29, 2705 – 2715

CITP-stacked Uraemic Sera, Separation by CZE

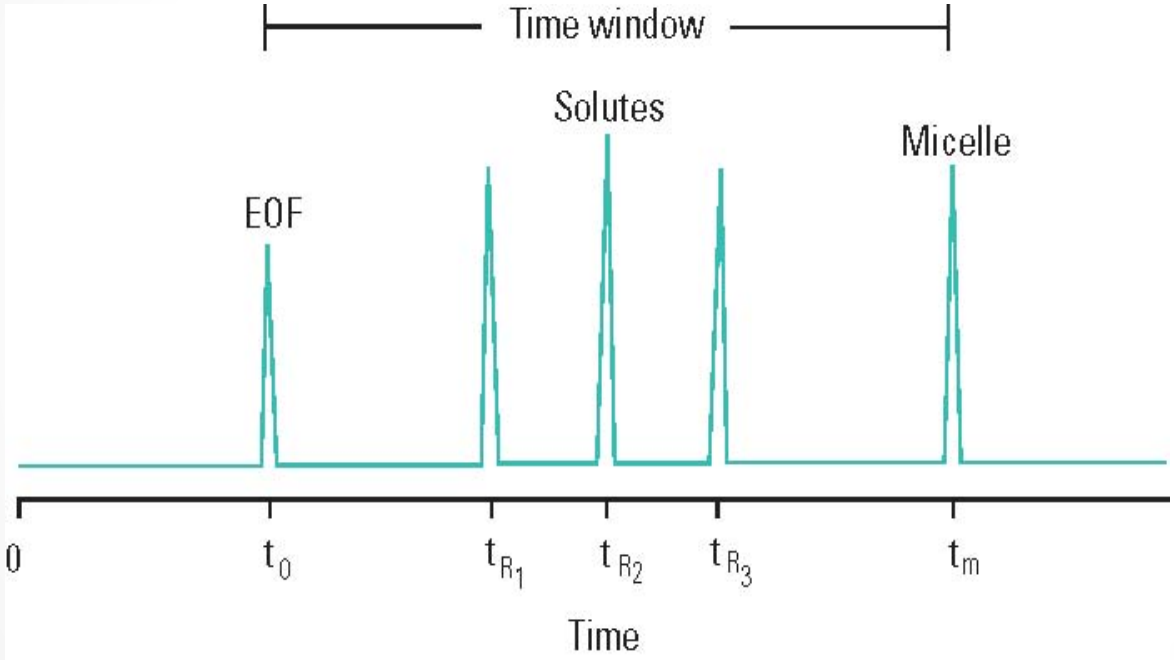


Conditions:
10 mM MES, histidine, pH 6.05,
0.05 % methyl hydroxyethyl cellulose,
Constant current = 35 mA
l = 25 cm, id = 200 µm, PTFE capillary

Micellar Electrokinetic Chromatography (MEKC)



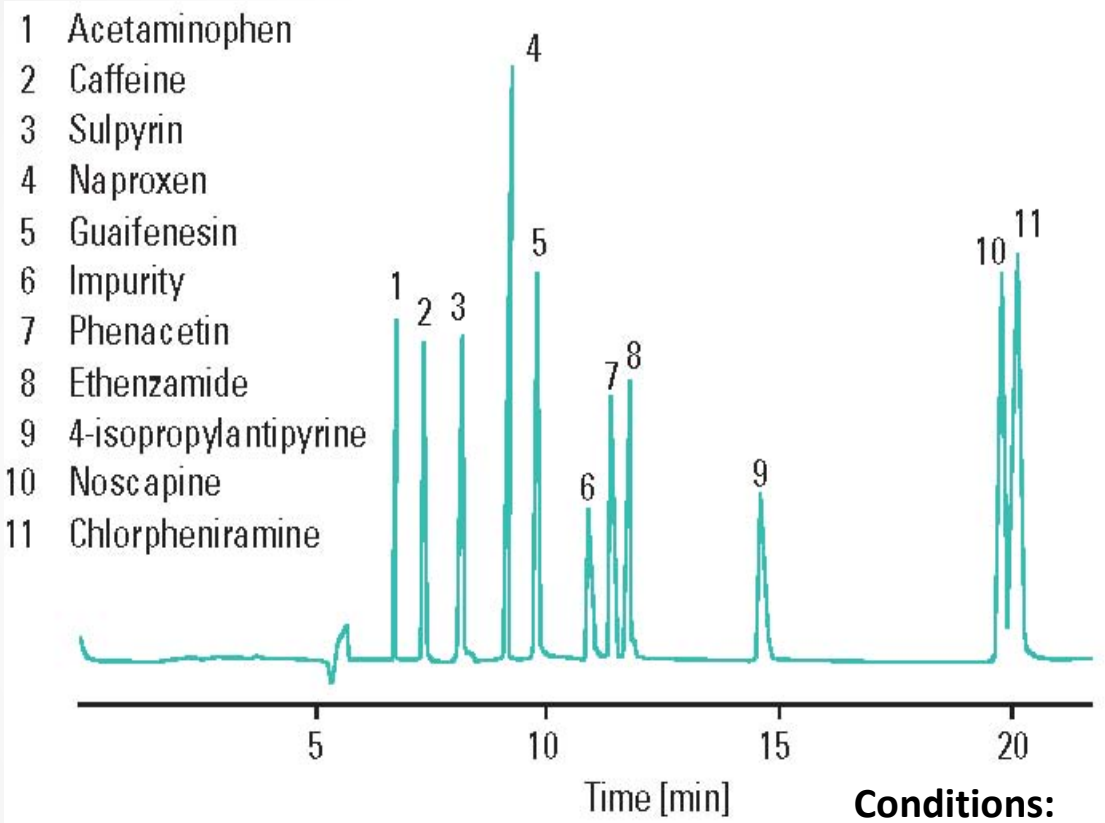
Micellar Electrokinetic Chromatography (MEKC)



Micellar Electrokinetic Chromatography - Surfactants

	Biological detergents	Critical micelle conc. [mM]	Aggregation number
Anionic	SDS	8.2	62
Cationic	DTAB	14	50
	CTAB	1.3	78
Non Ionic	Octylglucoside	---	---
	n-Dodecyl-β-D-maltoside	0.16	---
	Triton X-100	0.24	140
Zwitterionic	CHAPS	8	10
	CHAPSO	8	11
Bile Salt	Cholic acid	14	2-4
	Deoxycholic acid	5	4-10
	Taurocholic acid	10-15	4

MEKC Separation Of Cold-relief Medicine Constituents



Conditions:

20 mM phosphate-borate,100 mM SDS, pH 9

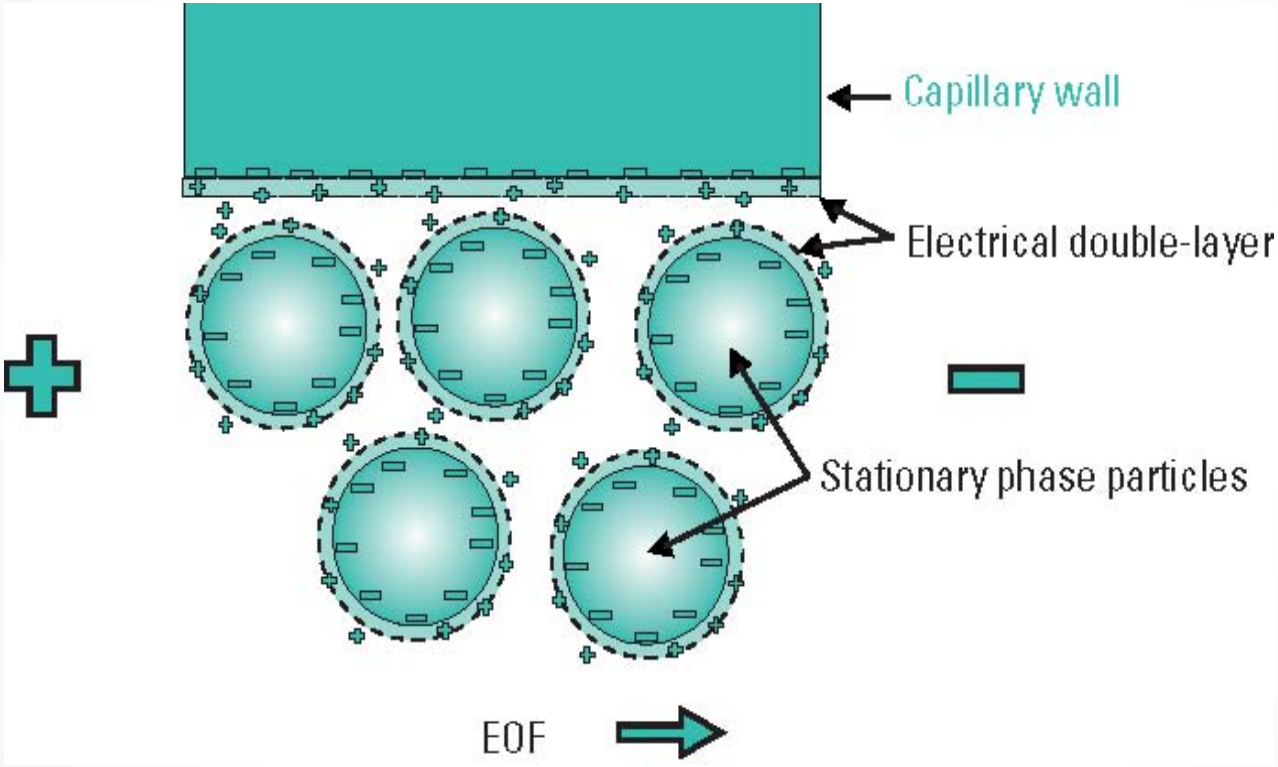
V = 20 kV

L = 65 cm

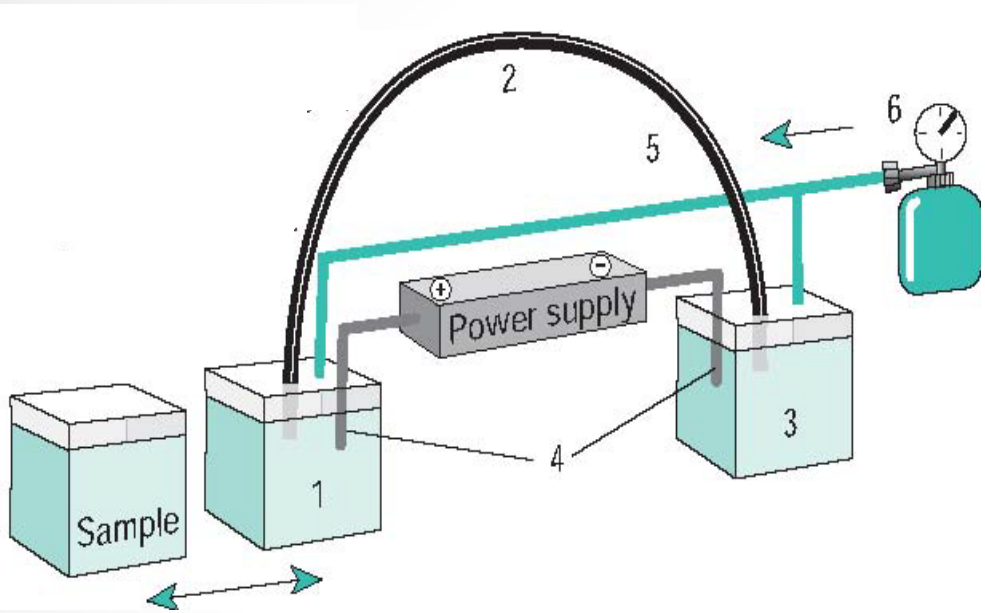
id = 50 µm

λ = 210 nm

Capillary Electrochromatography - Generation of EOF

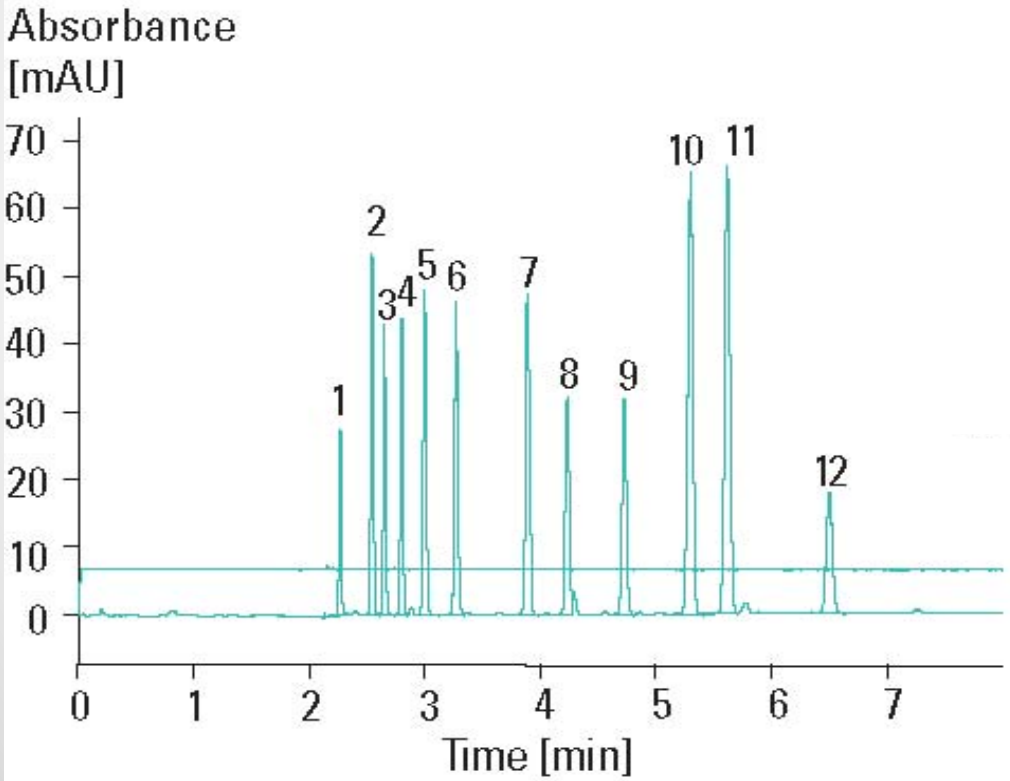


Capillary Electrochromatography (CEC)



1, 3 Buffer vials
2 Packed fused silica capillary
50-200 µm id, 200-500 mm length,
4 Electrodes
5 Point of detection
6 External pressure 2-12 bar

CEC Separation after Electrokinetic Injection



1 Thiourea
2 Methylparabene
3 Ethylparabene
4 Propylparabene
5 Butylparabene
6 Pentylparabene
7 Hexylparabene
8 Naphthalene
9 Biphenyl
10 Fluorene
11 Athrancene
12 Fluoranthene

Conditions:
Column: CEC-Hypersil C18, 3 µm, 250(335) x 0.1 mm
Mobile phase: ACN/MES 25 mM pH 6 80/20,
Temperature: 20 °C,
Voltage: 25 kV
Electrokinetic sample injection

Instrumentation of Capillary Electrophoresis

Systems and Components

Capillary Electrophoresis – Instrumentation Suppliers

Main Suppliers

- Sciex Separations (formerly Beckman-Coulter)
- Agilent Technologies
- Prince Technologies
- Wynsep
- Lumex









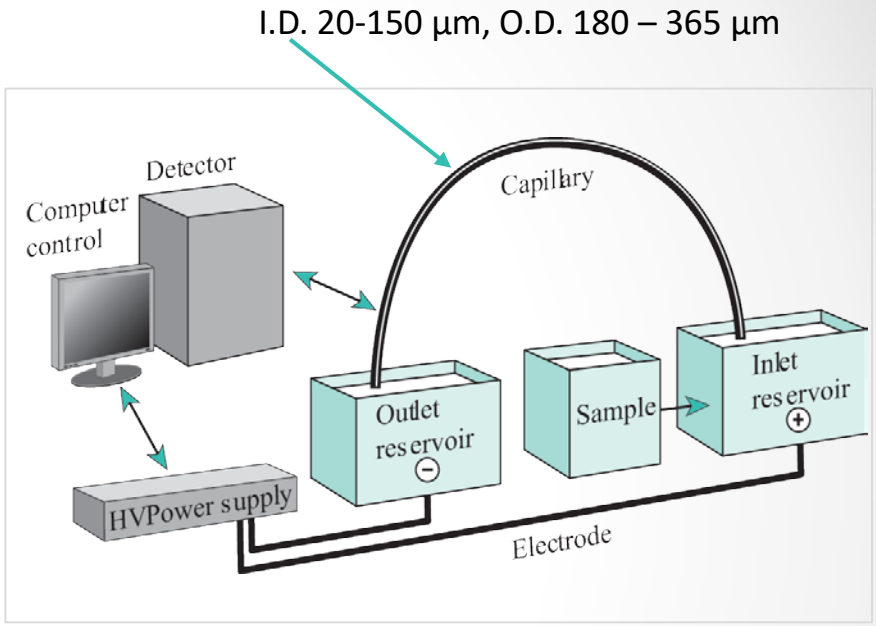


Capillary Electrophoresis – High Voltage Power Supply

A schematic diagram of a generic capillary electrophoresis system is shown on the right.

High voltage power supply

- 30 kV, dual polarity
- Maximum current 300 µA
- Stability 0.1%
- Constant voltage, current, and power mode
- Programmable voltage, current and power



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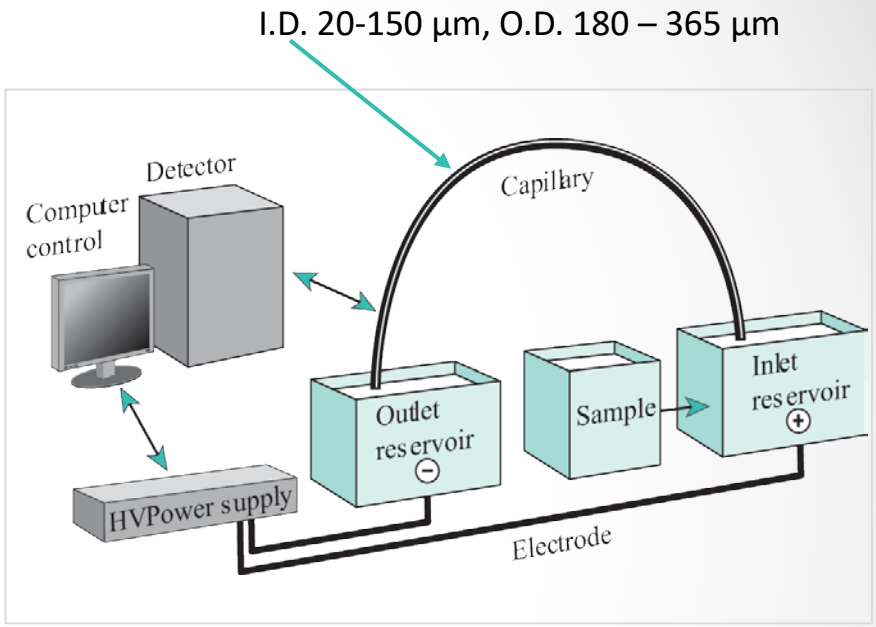
Capillary Electrophoresis – Sample injection

A schematic diagram of a generic capillary electrophoresis system is shown on the right.

High voltage, power supply

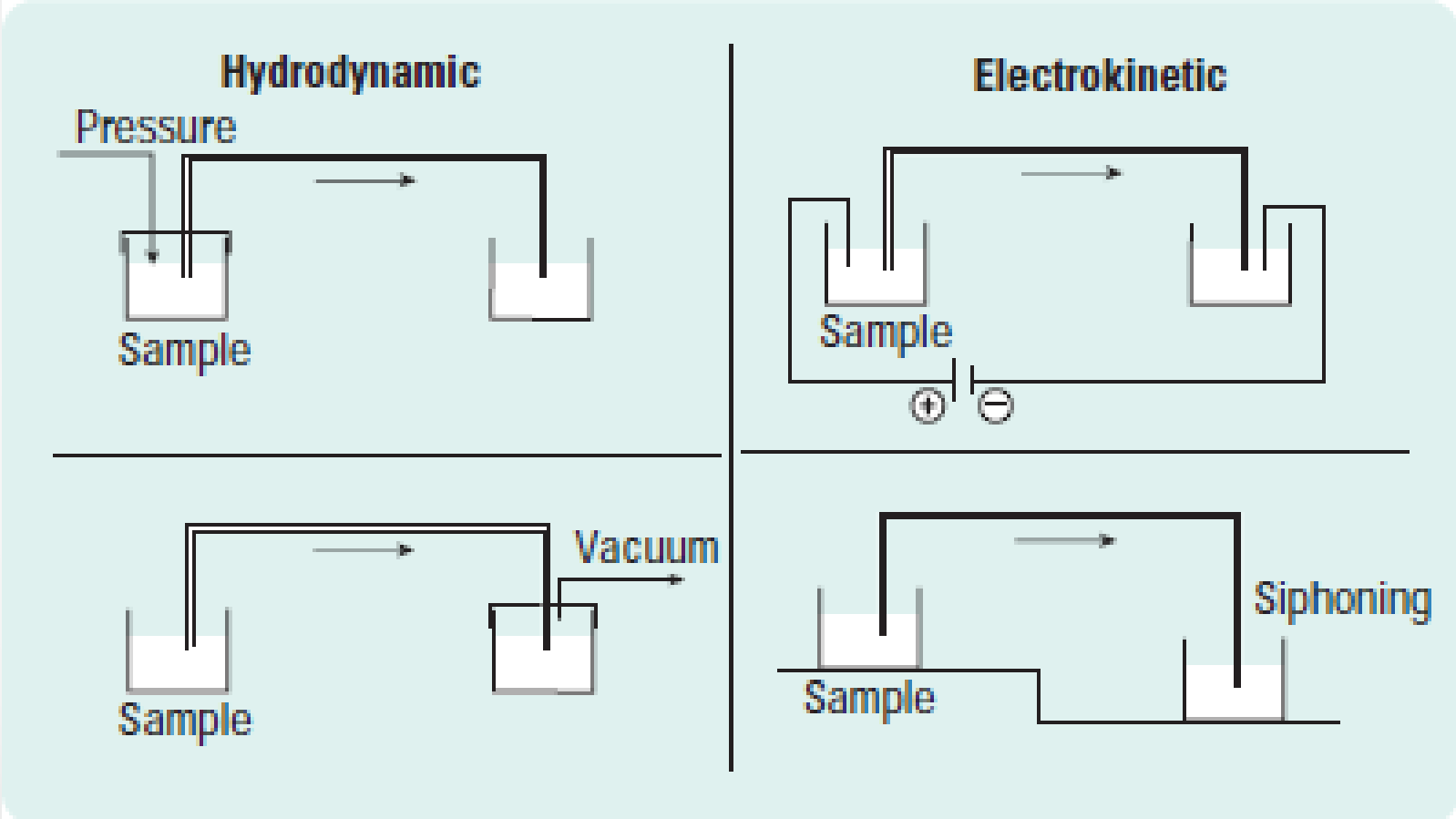
Sample and BGE handling

- Exchange inlet vial with sample vial
- Inject by pressure – hydrodynamic injection
- Inject by E-field – electrokinetic injection
- Injection volume 1-2% of capillary volume, 1-50 nl
- Many preconcentration methods employ differences in conductivity, pH between sample zone and BGE
- Optional automatic replenishment of BGE



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Methods of Sample Injection



Hydrodynamic Injection

$$V_{inj} = \frac{\Delta P \, d^4 \, \pi t_{inj}}{128 \eta L}$$

where ΔP = pressure differences across capillary [Pa]
d = capillary inside diameter [m]
t_{inj} = Injection time [s]
η = buffer viscosity [Pa·s]
L = total capillary length [m]

- Injection plug length should be <1-2 % of total capillary length
- Injection volume typically 1-50 nL depending on capillary dimensions
- Injection should be defined by quantity of pressure x time [mbar s]
- Determine optimal injection empirically (inject largest volume without loss of resolution)

Electrokinetic Injection

$$Q = \frac{(\mu_e + \mu_{EOF})\, V\, \pi\, r^2\, C\, t_{inj}}{L}$$

where μ_e = electrophoretic mobility
μ_{EOF} = EOF mobility
V = voltage
r = capillary radius
C = analyte concentration
t_{inj} = injection time
L = capillary total length

- The local electric field (E=V/L) depends on sample conductivity
- Used primarily for samples with non-conductive matrices or viscous buffer systems (e.g. gels)
- Employ internal standard calibration if sample conductivity is variable
- Only one electrokinetic injection per sample vial
- Injection parameter is voltage x time

Capillary Electrophoresis – Capillary Environment

I.D. 20-150 µm, O.D. 180 – 365 µm

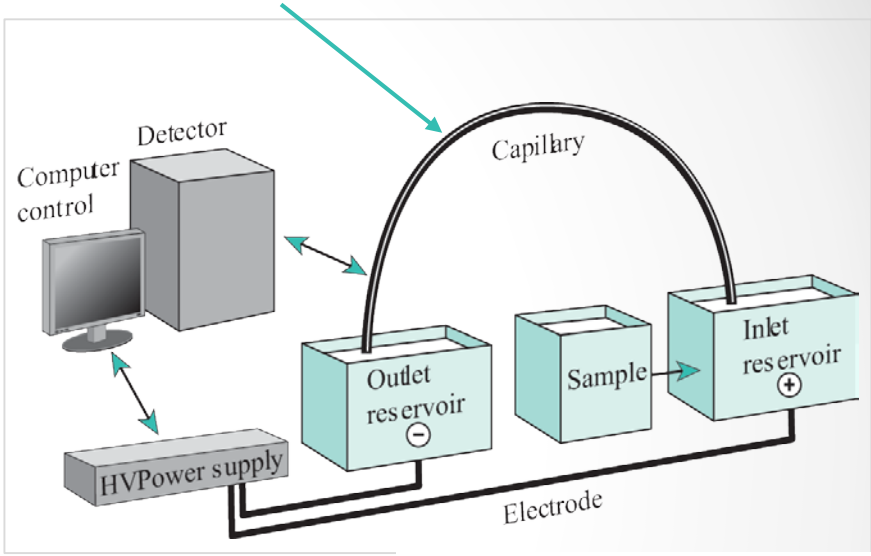
A schematic diagram of a generic capillary electrophoresis system is shown on the right.

High voltage, power supply

Sample and BGE handling

Capillary environment

- Thermostat to control Joule heating
  P=V.I (typical 0.1 – 3 W)
- Temperature stability < 0.1%
- Temperature range 4 – 40 °C
- Environment rejection
- Cassette holder for the separation capillary
- Interface with built in detector



Alignment interface with selfaligning detection window



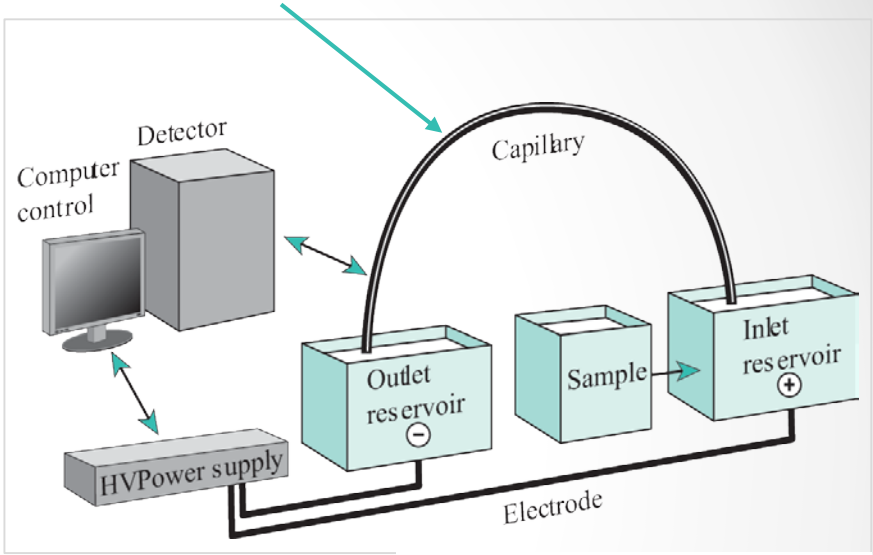
Capillary ends go into the vials

Slide courtesy of Agilent Technologies with permission

Capillary Electrophoresis – Detection

I.D. 20-150 µm, O.D. 180 – 365 µm

A schematic diagram of a generic capillary electrophoresis system is shown on the right.



High voltage, power supply

Sample and BGE handling

Capillary environment

Detection

- Spectrophotometric detection
  UV-Vis (diode array), 10^{-6} M;
  Extended light path (bubble, Z-shaped cell) 10^{-7} M
  Indirect mode for non-adsorbing species
- Fluorescence, direct 10^{-7} – 10^{-8} M or laser induced 10^{-9} – 10^{-12}M
- Conductivity,10^{-5} - 10^{-6} M
- Mass spectrometry (next section)

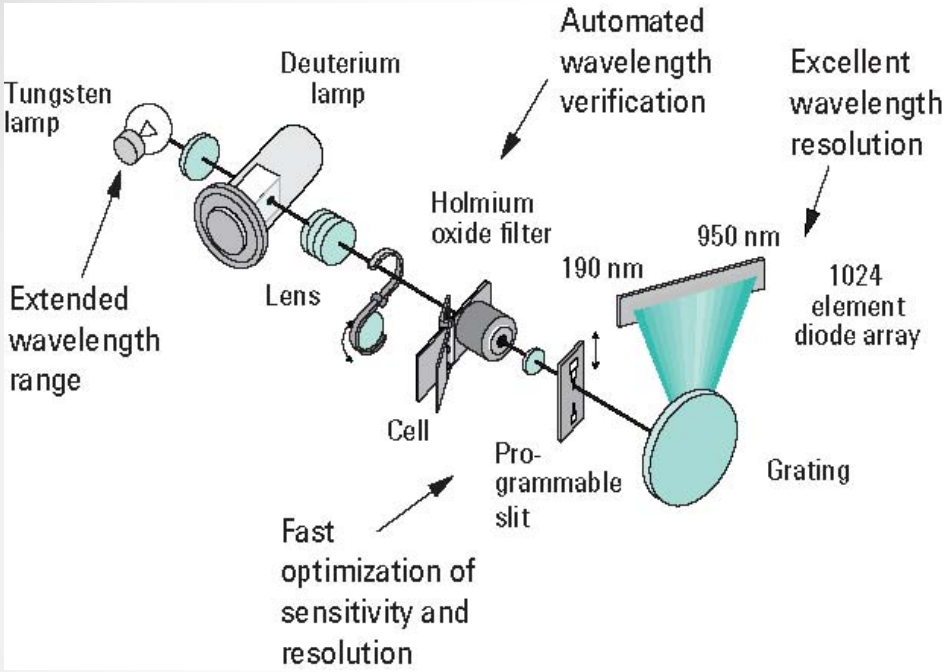


Alignment interface with selfaligning detection window

Capillary ends go into the vials

Slide courtesy of Agilent Technologies with permission

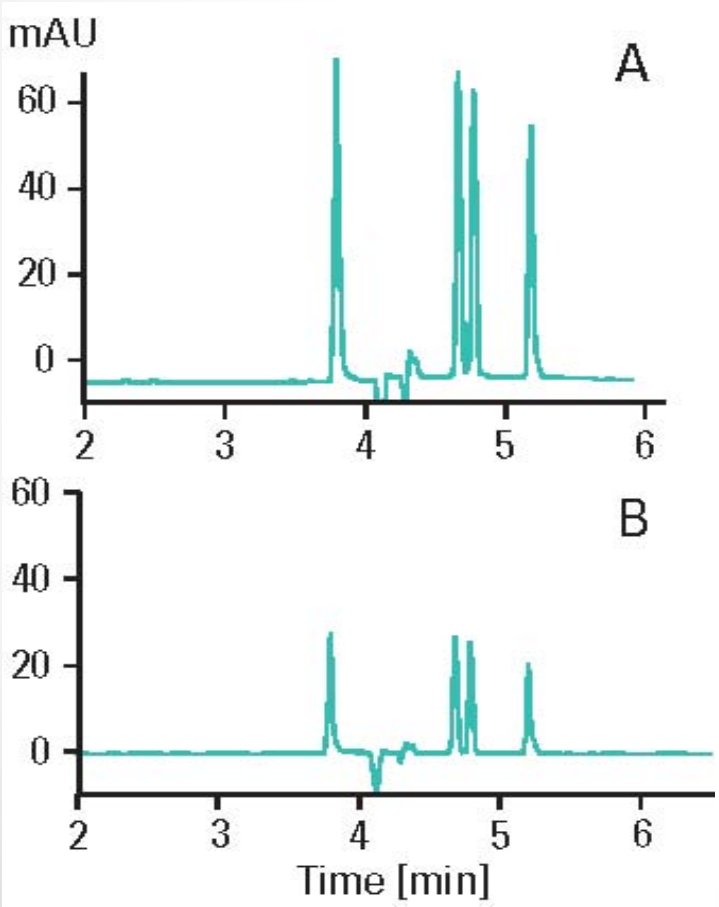
CE Spectrophotometric Diode Array Detection



- Most widely used detection method;
- High efficiency due to on-capillary detection, no zone broadening
- Wavelength range (190-600 nm)
- Optical slit allows to achieve short axial path length for high resolution

CE Spectrophotometric Diode Array Detection

Effect of the Extended Light Path Capillary

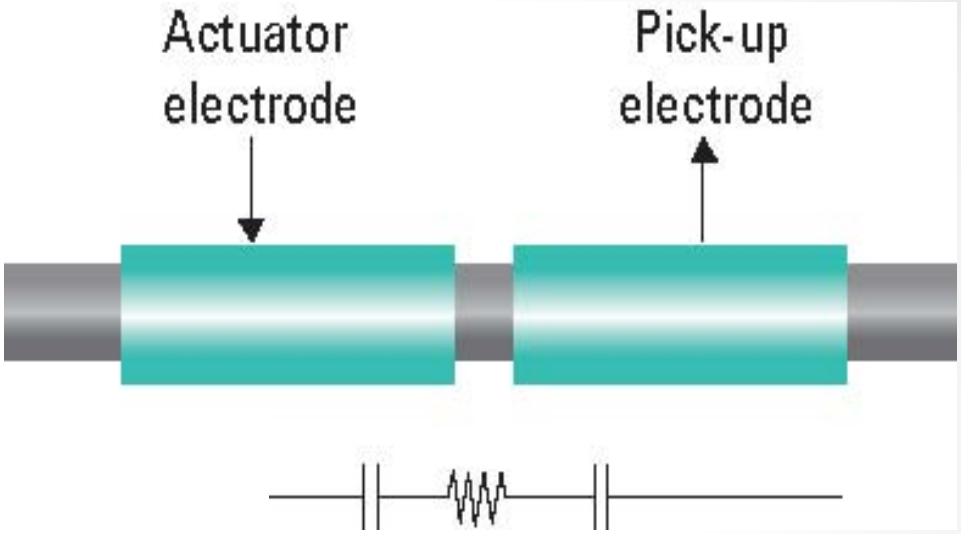


Conditions:
Extended light path capillary:
56 cm x 50 µm id
with 150 µm detection cell

Conditions:
Normal straight capillary:
56 cm x 50 µm id

Buffer: 89 mM Tris-borate, pH 8.2
Injection: 100 mbar s
Voltage: 25 kV (i = 13 µA)

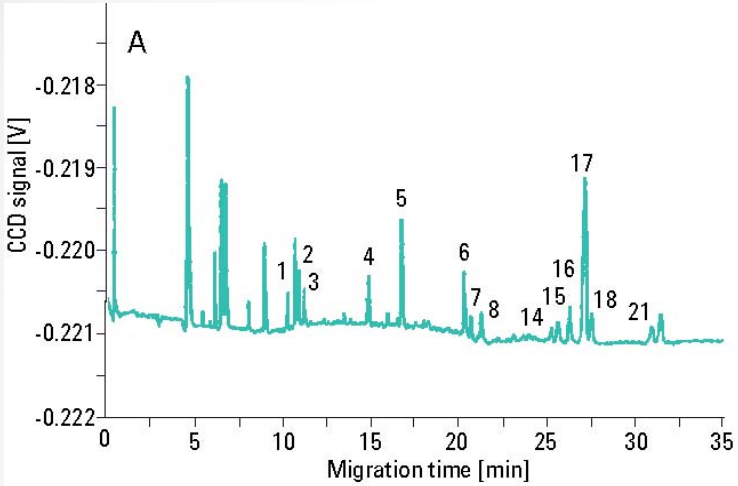
CE Contactless Conductivity Detector Design



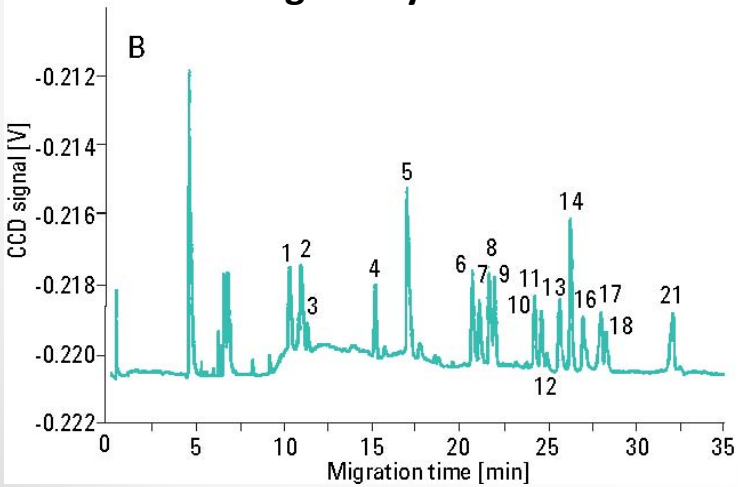
- On-capillary detection
- No electrode fouling
- No necessary connections
- Hyphenation with other detection systems (UV, LIF, MS)
- Ability to work with packed capillaries (CEC)
- Besides fused silica, other materials (Peek, Teflon) can be used
- Limits of detection for inorganic and small organic ions in the ppm →ppb region

Profiles of Amino Acids Measured by CZE-CCD

Budvar beer



Pangamin yeast



Conditions:

2.3 M acetic acid pH 2.1 + 0.1 % HEC

L_{eff} = 66.5 cm; L_{tot} = 80 cm; id = 50 µm

Injection: 300 mbar s

Voltage: + 30 kV

Current: 15.4 mA

Temperature: 25 °C

Detection: CCD

Peaks: 1, Lys; 2, Arg; 3, His; 4, Gly; 5, Ala; 6, Val; 7, Ile; 8, Leu; 9, Ser; 10, Thr; 11, Asn; 12, Met; 13, Gln; 14, Trp; 15, Glu; 16, Phe; 17, Pro; 18, Tyr; 21, Asp

Capillary Electrophoresis – Capillaries

I.D. 20-150 µm, O.D. 180 – 365 µm

A schematic diagram of a generic capillary electrophoresis system is shown on the right.

High voltage, power supply

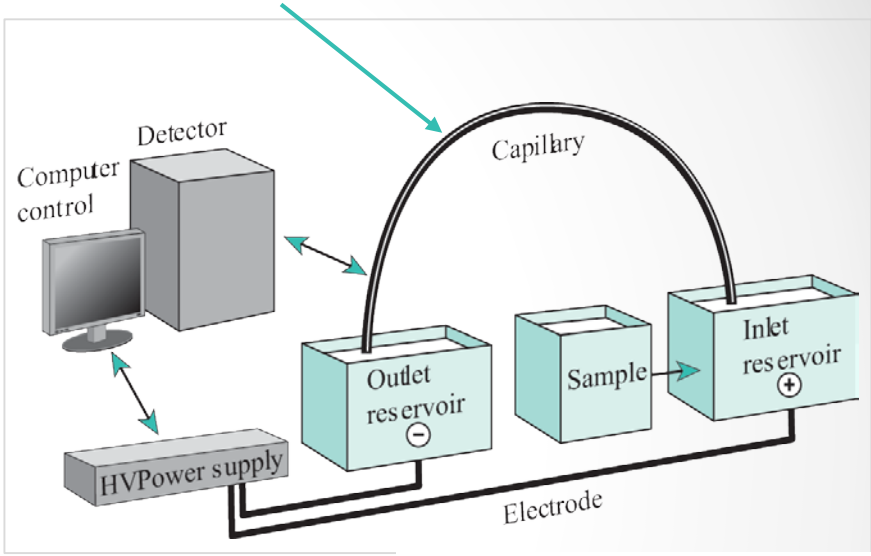
Sample and BGE handling

Capillary environment

Detection

Capillary and capillary coatings

- Fused silica
- Permanently coated capillaries polyvinyl alcohol, polyethylene glycol, poly acrylamide, Teflon (tradenames)
- Dynamic coatings, polyethylene imine, polybrene, dextran sulfate, polyvinyl sulfate



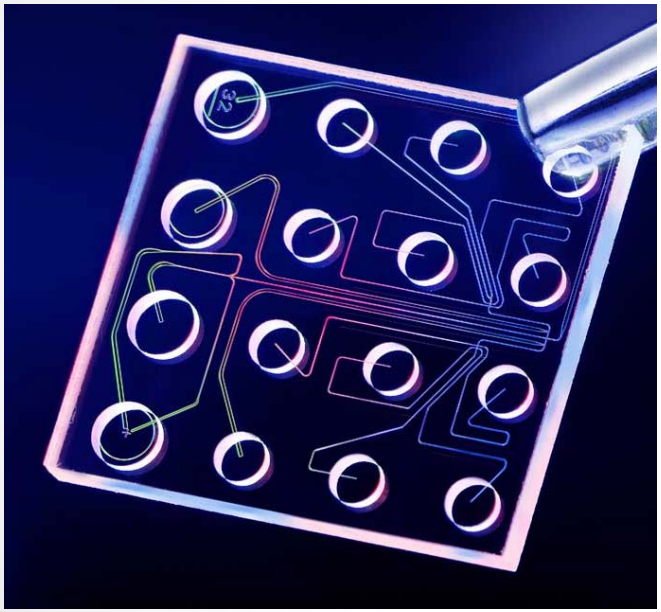


Alignment interface with selfaligning detection window

Capillary ends go into the vials

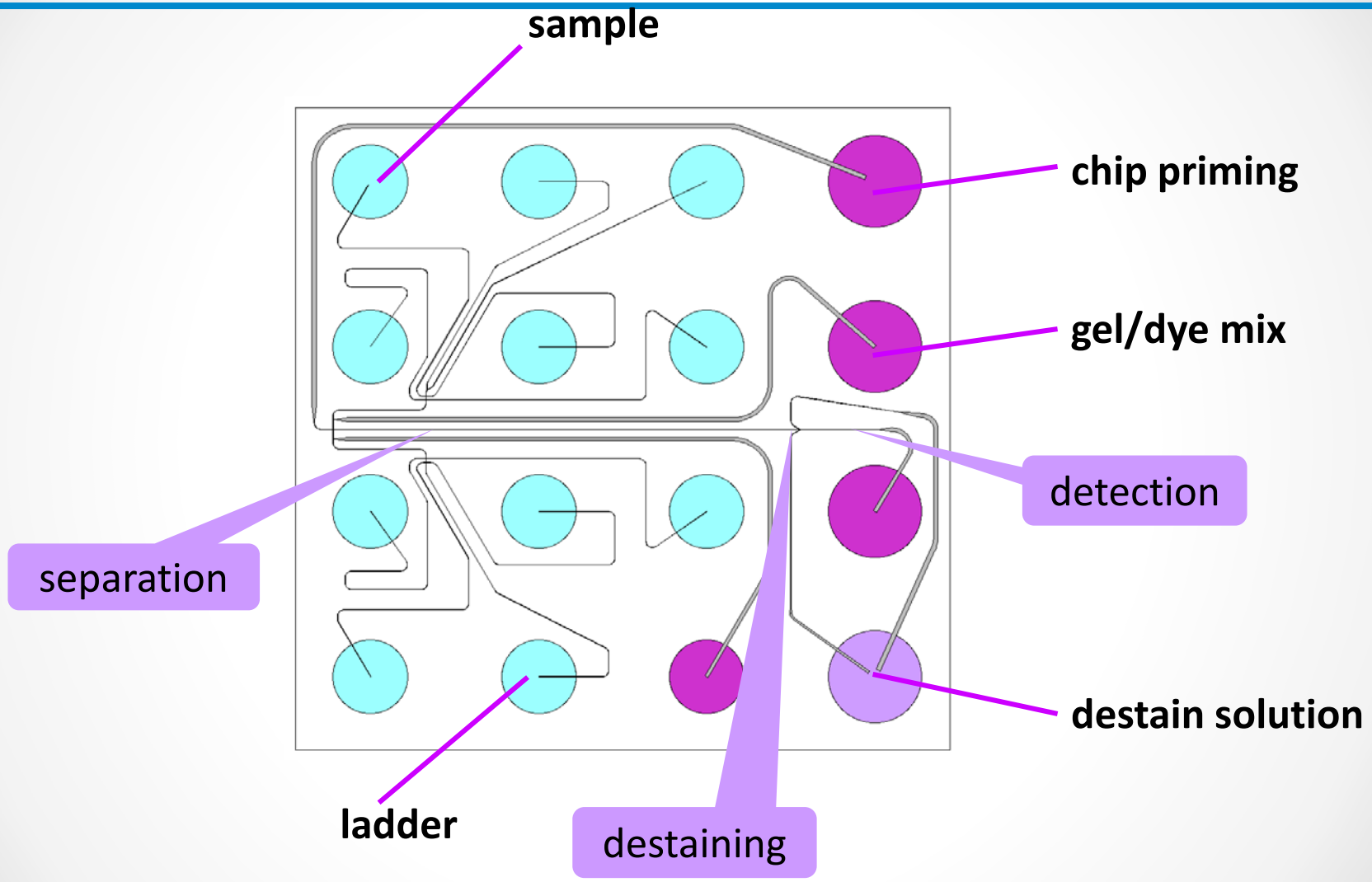
Slide courtesy of Agilent Technologies with permission

Microchip CE

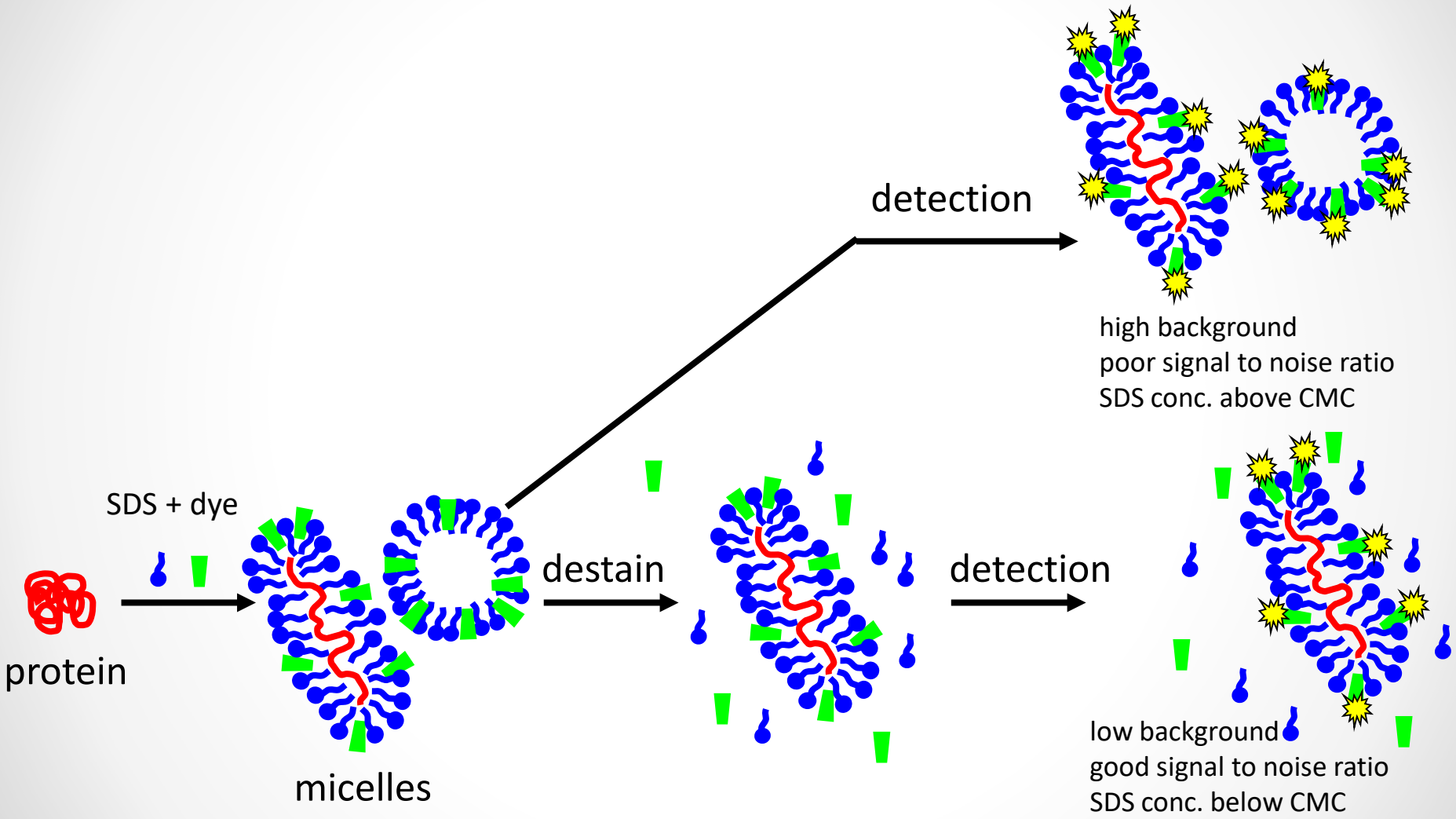




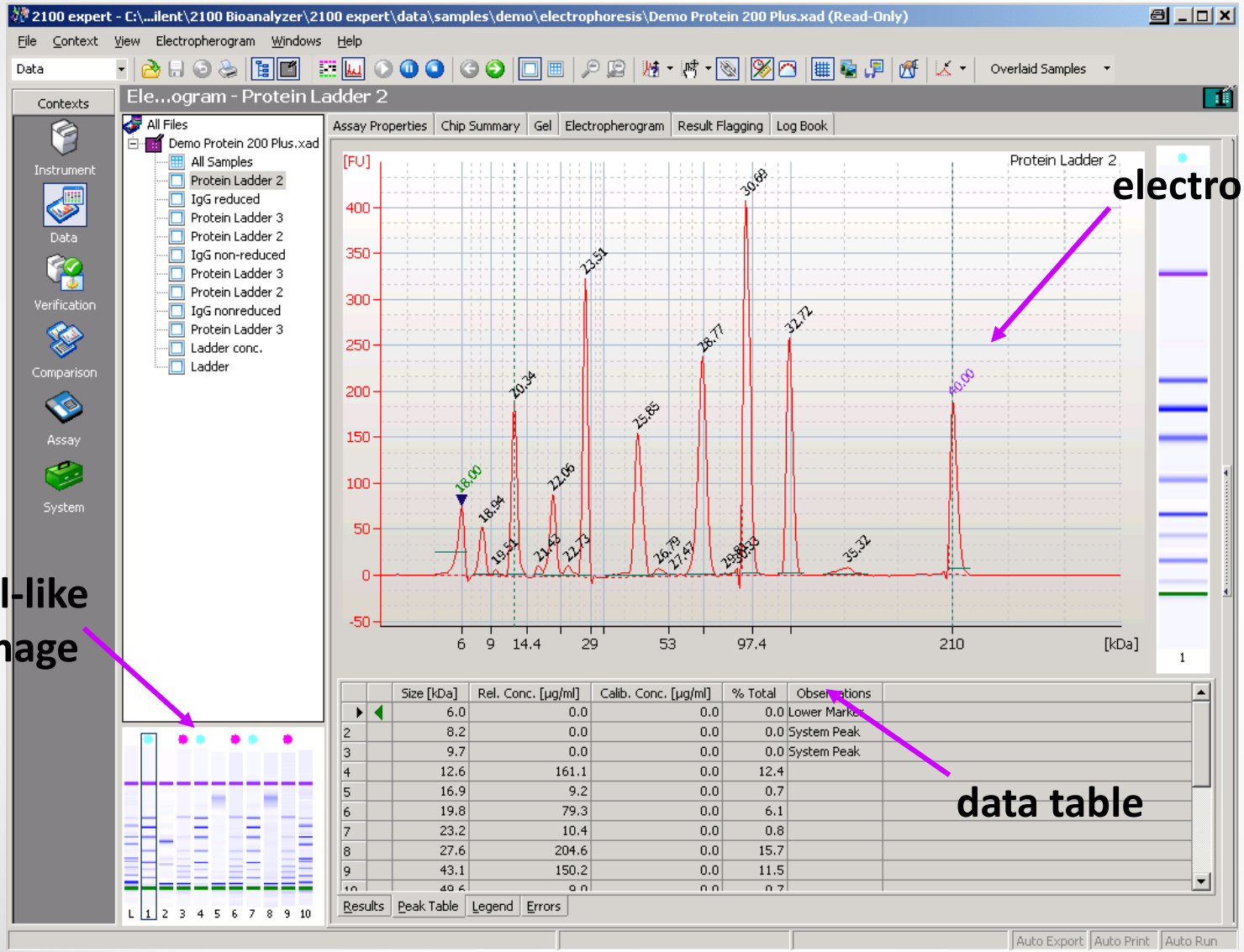
Microchip CE Protein Analysis - Chip



Microchip CE Protein Analysis - Destaining



Microchip CE Protein Analysis - Separation



electropherogram

gel-like image

data table

CE and CE-MS for Proteins

Interfacing of CE with MS

Principle Methods for Coupling CE with MS

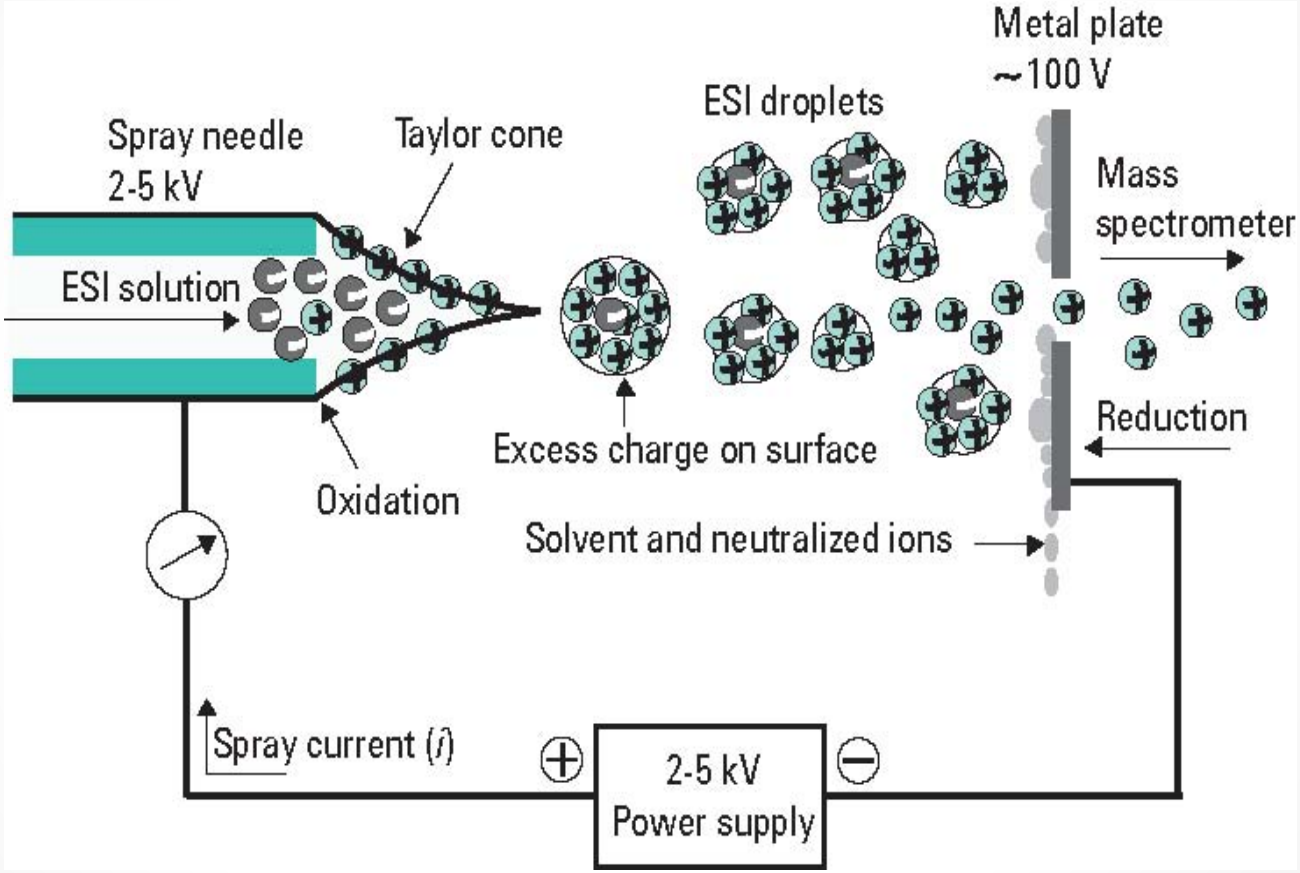
Ionization

- Nano-electrospray ionization (ESI-MS)
- MALDI MS

Challenges for CE-MS Interfacing

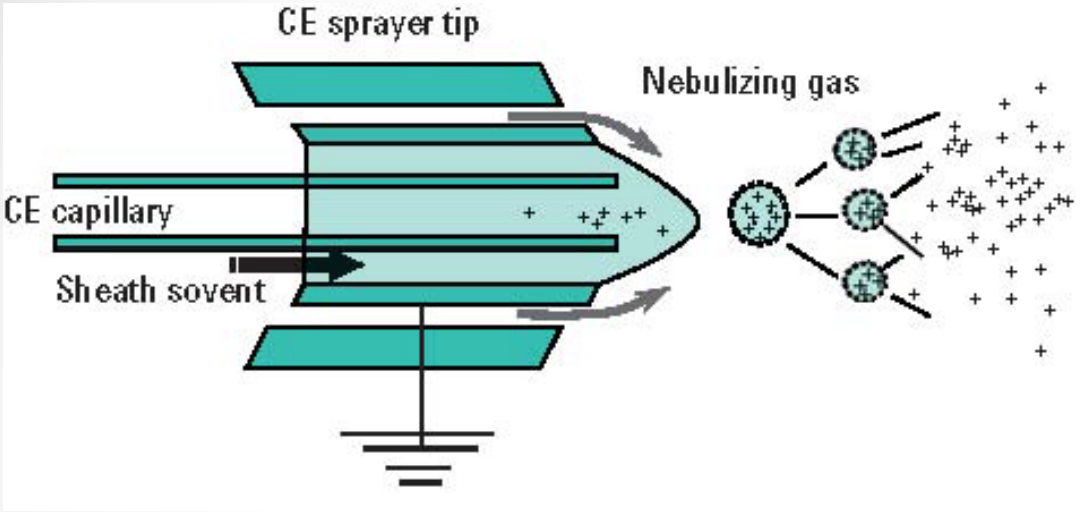
- Electrical - 2 high voltage connections on one liquid conductor
  - 30 kV to drive the solutes toward the outlet by EOF and electrophoretic mobility
  - 3 kV to generate the electrospray at the outlet
  - Voltages may be of opposite sign
  - Return path for the current is needed (ground connection!)
- Hydraulic – outlet must be open to allow spray formation
  - Flow too low to generate stable spray, use sheath solvent flow and pneumatic assistance
  - Since MS is a vacuum detection technique, the BGE may not contain non-volatile components (salts, non-volatile additives)
- Compromised CE separation (EOF, efficiency)

Electrospray Ionization Process



ElectroSpray Ionization (ESI)

CE MS Interfacing- Triple Tube Sprayer



*R.D. Smith et al, Anal. Chem., 60, 1948 (1988)

Sheath Liquid Interface for CE-MS
– Triple Tube Design*

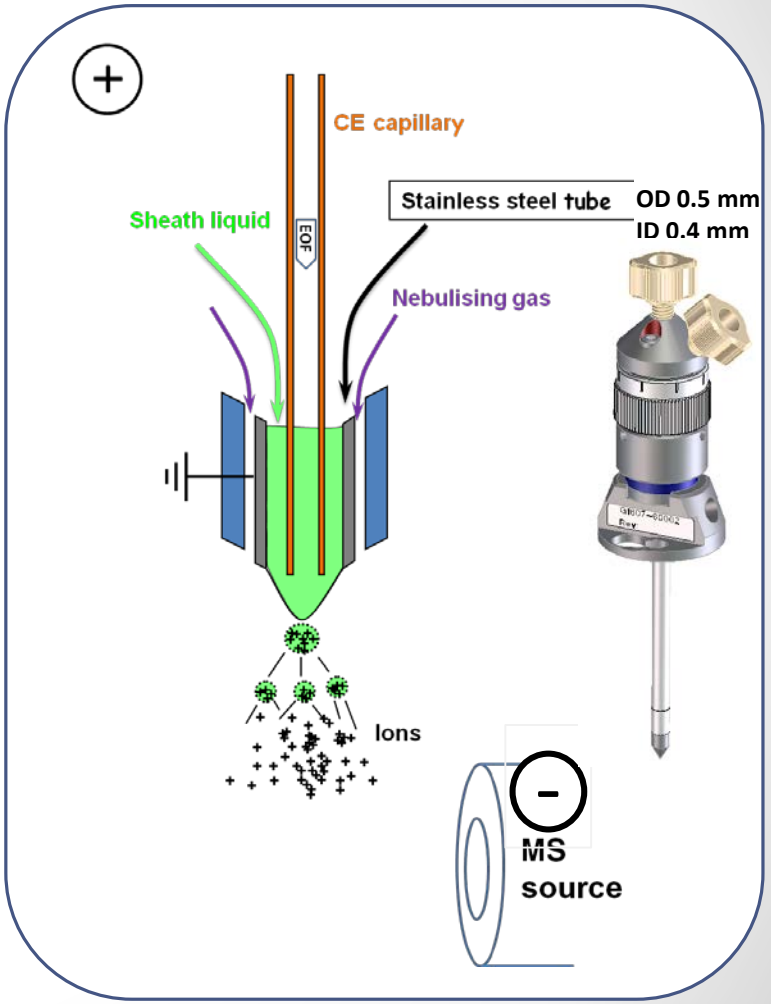
Sheath solvent is added to the CE effluent at a rate of typically 1 - 5 µL/min. Spray becomes independent of EOF.

Spray needle (gray) is grounded. Common ground for CE and ESI. Bubbles are transported out

Spray formation pneumatically assisted spray. Nitrogen gas at 5-20 p.s.i.

Sheath solvent composition dominates electrospray ionization chemistry. Typical sheath solvents contain formic acid, methanol, water → low pH, positive ion detection or ammonia → high pH for anion detection

Orthogonal configuration let neutrals & big droplets pass



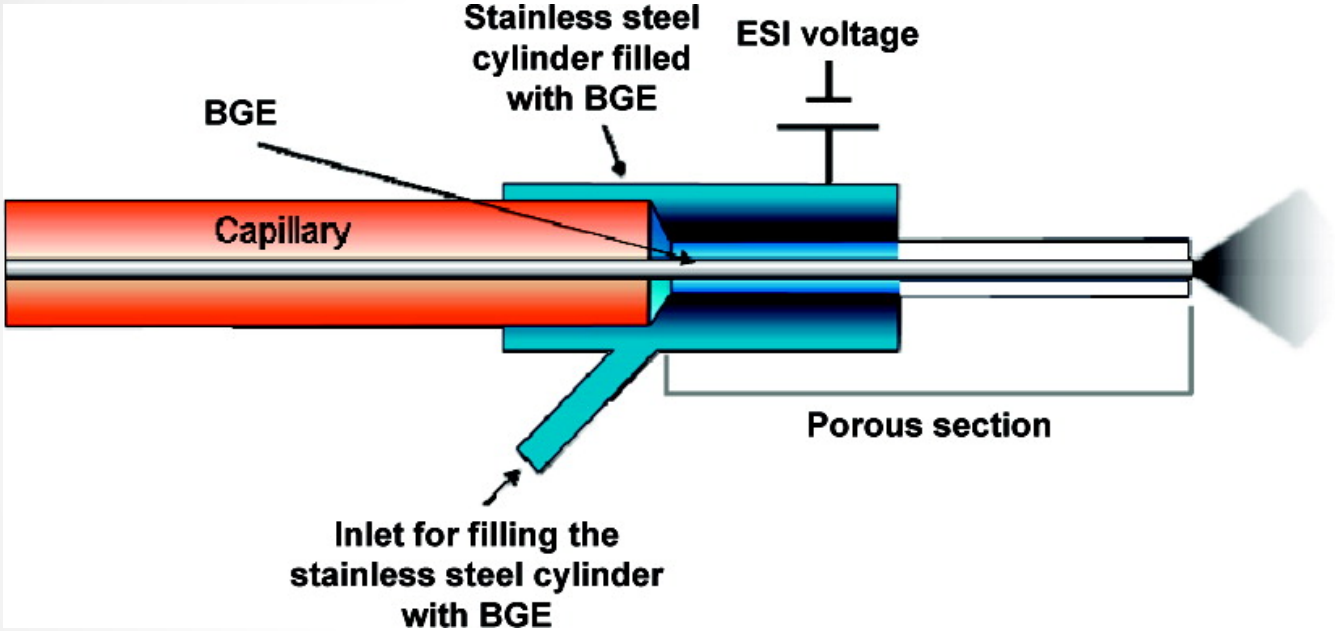
Sensitivity is compromised!!
- ➔ ESI-MS is concentration sensitive detection!
- ➔ Dilution 5 - 50x with the sheath solvent
- ➔ Higher flow rate compromises nano-electrospray

Pneumatic assistance required to establish the spray
- ➔Hydraulic flow observed ("venturi" effect)

*R.D. Smith et al, Anal. Chem., 60, 1948 (1988)

New Developments in CE-MS “Sheathless” Interfacing

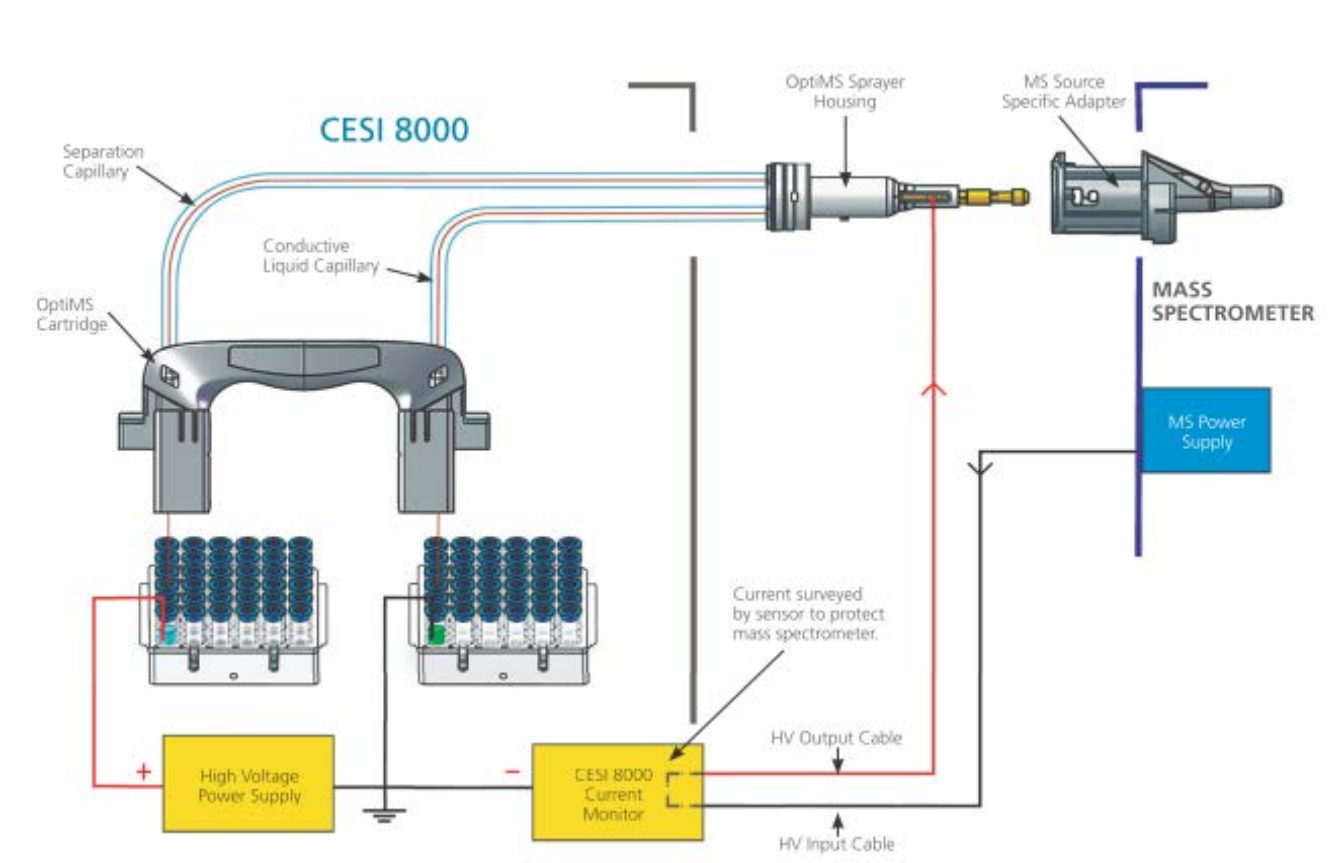
Porous tip approach, Mehdi Moini Anal. Chem., 2007, 79 (11), pp 4241–4246



Sensitivity sub-µM – nM
In absence of EOF, pressure assistance required to obtain a stable spray
Commercialized by Beckman-Coulter CESI 8000
Capillary OD/ID 220/30 µm

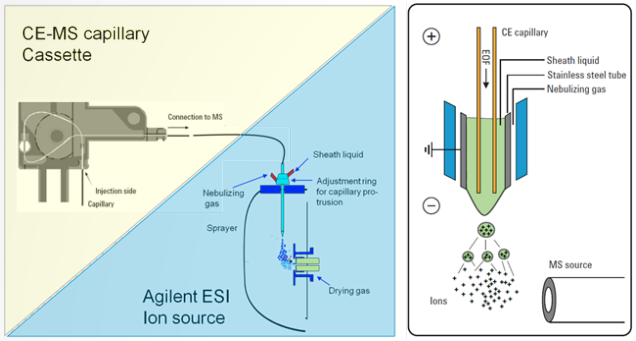
Figure published in: Jean-Marc Busnel; Bart Schoenmaker; Rawi Ramautar; Alegria Carrasco-Pancorbo; Chitra Ratnayake; Jerald S. Feitelson; Jeff D. Chapman; André M. Deelder; Oleg A. Mayboroda; *Anal. Chem.* **2010,** 82, 9476-9483. Copyright © 2010 American Chemical Society

New Developments in CE-MS "Sheathless" Interfacing

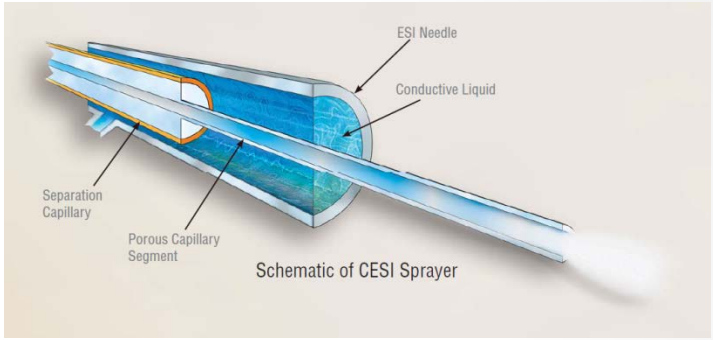


Sheath vs. sheathless CE-MS interfaces

Agilent CE-MS



Sciex Separation CESI-MS



Triple tube sheath liquid interface

- for Agilent and Bruker standard ESI
- uses standard FS capillaries
- flow rate controlled by makeup flow (low µL/min range)
- for all standard capillaries with 360 µm OD (cheap)
- Capillary and electrospray share ground connection
- Pneumatic assistance may cause suction on the outlet
- For all modes of polarities on CE and MS
- Decoupling of the chemistry for separation and for ionization
- Non-aqueous buffers can be used
- Best robustness and ease of use available

Most robust interface for routine and unattended operation. Dilution by make-up flow limits sensitivity (10^{-6}-10^{-7} M) but addition of sheath chemistry can recover sensitivity partially due to improved MS ionization efficiency

Porous glass sheathless interface

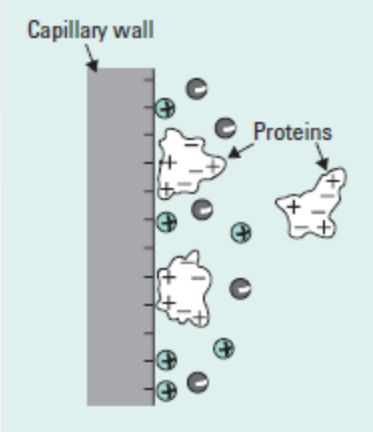
- real NanoESI MS, high sensitivity (20-50x better)
- exclusively Beckman-Coulter CE-MS capillaries (150 µm OD, ID 30 µm, expensive!)
- EOF is required. Polarity must allow EOF towards MS ➔ wall coated capillaries
- capillary end electrically on high voltage. CE current drain required!
- neutral coated capillaries require pressurized flow
- Electrolyte must match the CE separation and MS ionization requirements
- non-aqueous buffers can not be sprayed (ionized) efficiently
- limited robustness, need expert handling

Very high sensitivity for HMW substances like proteins / peptides analysis due to absence of dilution and very low flow rate enhancing electrospray efficiency. Limited in application range and robustness

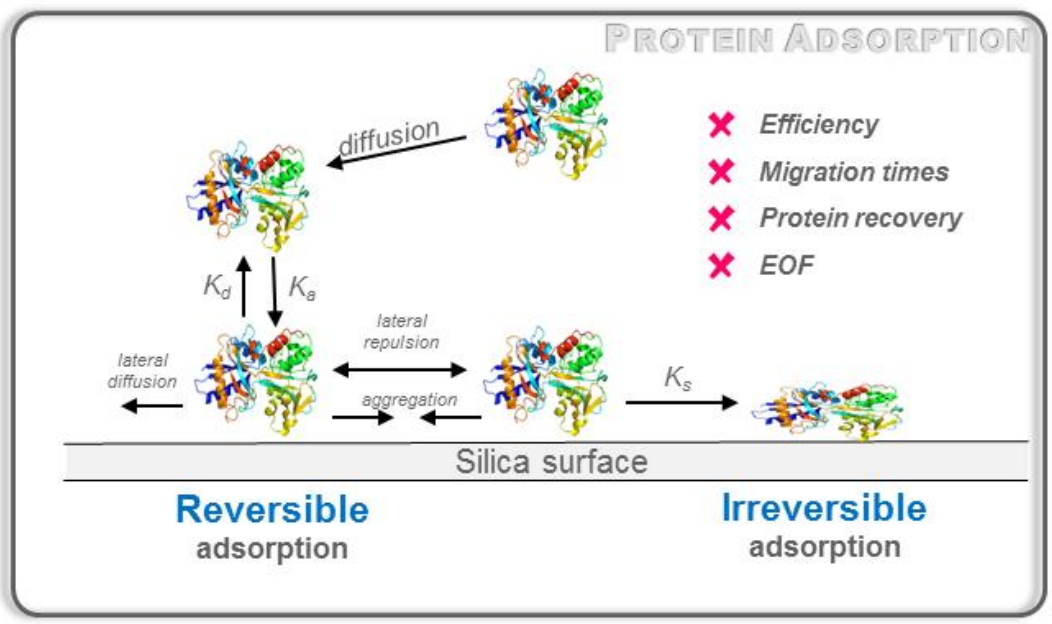
CE and CE-MS of Proteins

Protein Separation by CE – Surface Interactions

Bare fused silica



- Active silanol groups (Si-OH)
- Different structure, pKa 2-5
- Hydrophobic siloxane regions
- Amorphous structure subject to change



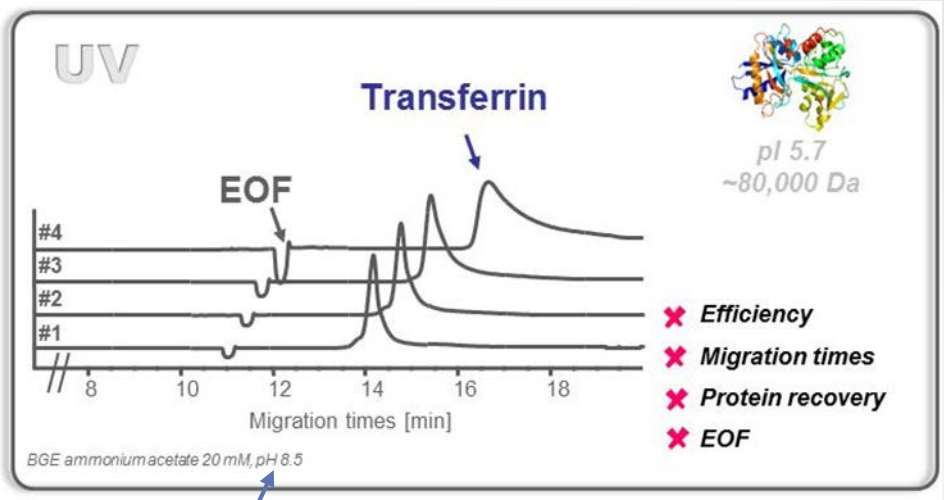
Adsorption/desorption slow process, causing retention (like HPLC),peak broadening and tailing, conformation change

Causes surface property changes affecting precision and repeatability. Low recovery

Slide courtesy of Dr. Julie Schappler, Geneva University

Protein Separation by CE – Surface Interactions

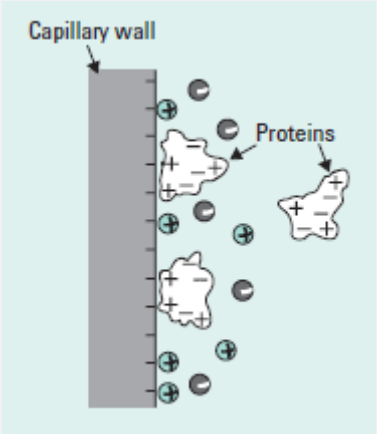
- ✓ Irrepeatable mobilities of protein and EOF
- ✓ Peak tailing
- ✓ Loss in efficiency
- ✓ Poor recovery



Slide courtesy of Dr. Julie Schappler, Geneva University

Minimize Protein-Surface Interaction in CZE

Bare fused silica



- Active silanol groups (Si-OH)
- Different structure, pKa 2-5
- Hydrophobic siloxane regions
- Amorphous structure subject to change

Approaches to minimize protein-surface interactions

- pH of BGE clearly above or below pI of the proteins to reduce ionic interactions*
- Add low amount of organic solvent to the BGE
- Use buffers with high ionic strengths to reduce coulombic interactions
- Apply a rigorous protocol for capillary pre-treatment and storage
- Capillary surface modification

*Staub et al., Electrophoresis 2010, 31, 3326–3333

Minimize Protein-Surface Interaction by Capillary Surface Deactivation

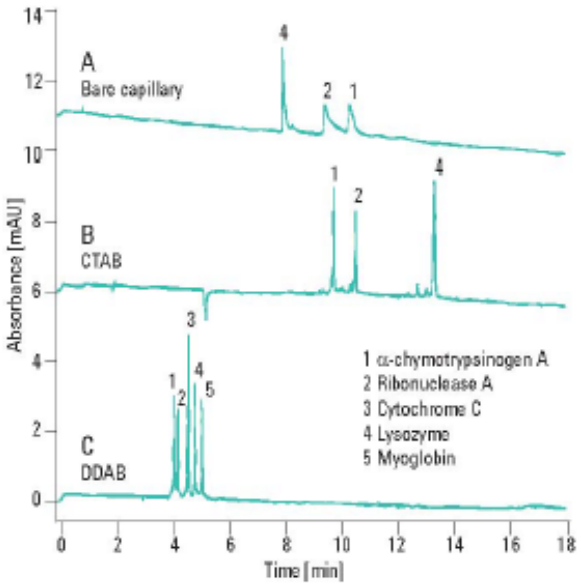
Type	Effect	Comment
Neutral hydrophilic polymers e.g. hydroxy methylcellulose	Mask surface charge and reduces EOF	Low concentration used (see slide 37)
Cationic polymers e.g. polyethylene amine, polybrene	Bind to silanol surface groups and reverse EOF	Form a semi permanent layer
SMIL, e.g. dextran sulphate layer upon cationic polymer layer	Returns a stable negative surface layer	
Cationic Surfactants like CTAB or DDAB	Deactivate surface	Below CMC
Covalent coatings, PVA, CEP, PA, FEP	Suppress EOF	Expensiv

Polymers dissolved in BGE and become replenished.
SMIL are stable and donot need replenishment

SMIL, successive multiple ionic layer, CTAB, cetyltrimethylammonium bromide, DDAB, didodecyldimethylammonium bromide, PVA, polyvinyl alcohol, CEP, crosslinked ethylene glycol polymer, PA, PDMA poly (dimethyl) acrylamide, fluoroethylene polymer

Minimize Protein-Surface Interaction by Capillary Surface Deactivation

Dynamic Coating with Single and Double Chain Surfactant



Conditions:
50 cm x 50 µm id capillary (40 cm to detector)
UV detection at 214 nm

(A) applied voltage:+15 kV
BGE: 25 mM phosphate at pH 3.0
without surfactant

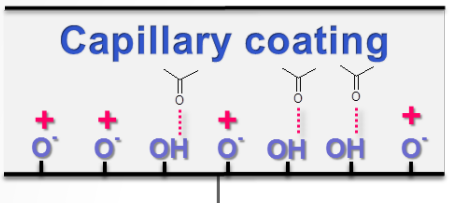
(B) applied voltage:-1 5 kV
BGE: 25 mM phosphate at pH 3.0
+ 0.5 mM CTAB

(C) applied voltage: -1 5 kV
BGE: 25 mM phosphate at pH 3.0
+ 0.1 mM DDAB

Agilent Technologies

8

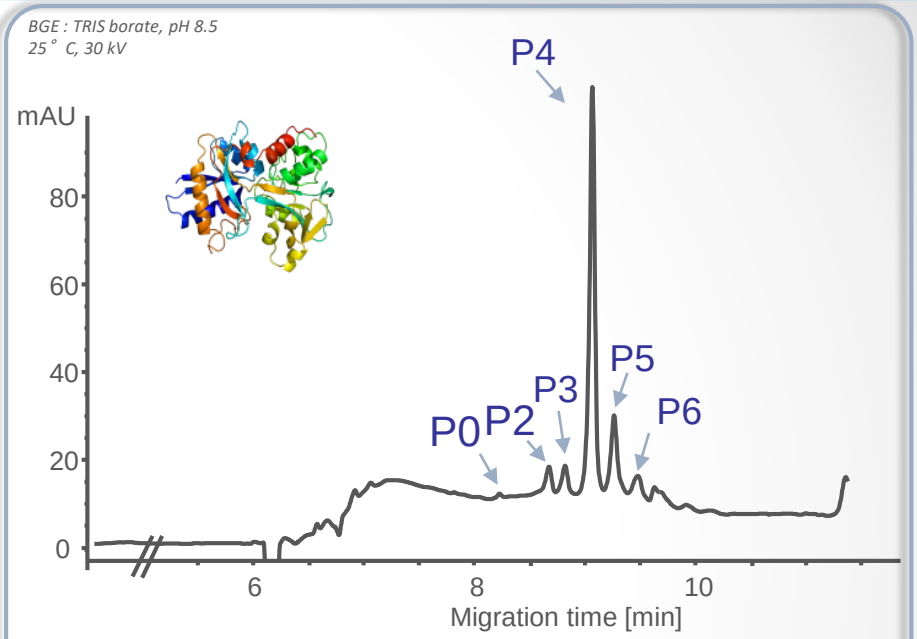
Minimize Protein-Surface Interaction by Capillary Surface Deactivation



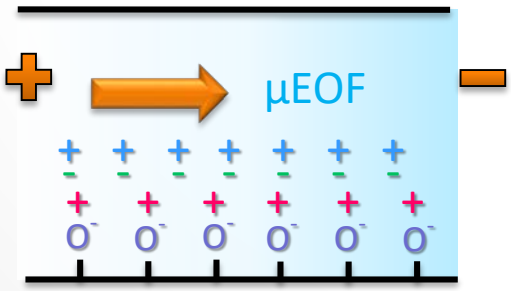
Dynamic coating

Water-soluble polymers added to BGE

CEofix™ CDT kit (Analis, Belgium)



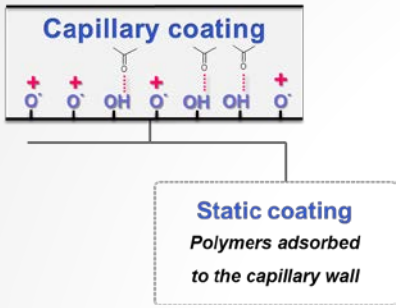
Transferrin, good R_s between glycoforms



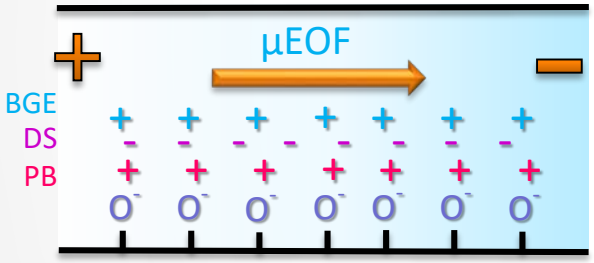
BGE counterions
BGE with polyanions
Initiator (polycations)
Capillary silica wall

Slide courtesy of Dr. Julie Schappler, Geneva University

Minimize Protein-Surface Interaction by Capillary Surface Deactivation



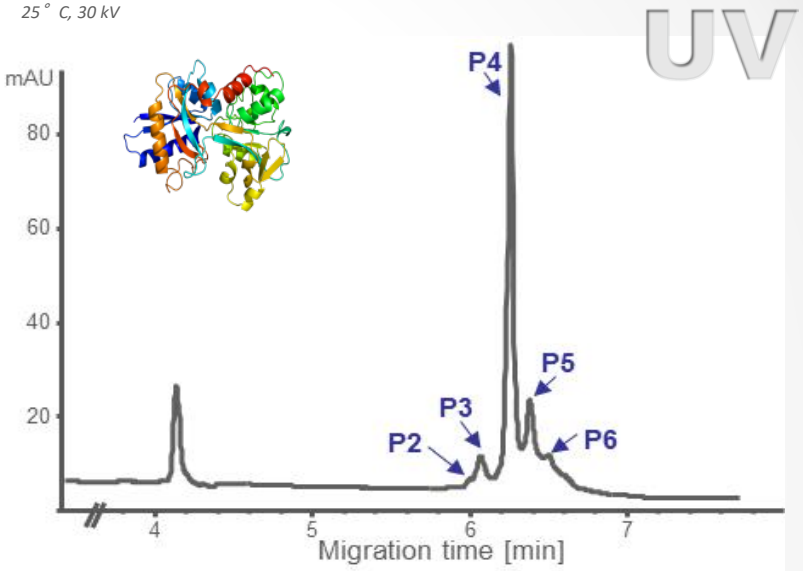
PB = *Polybrene (10%)*
DS = *Dextran sulfate (10%)*





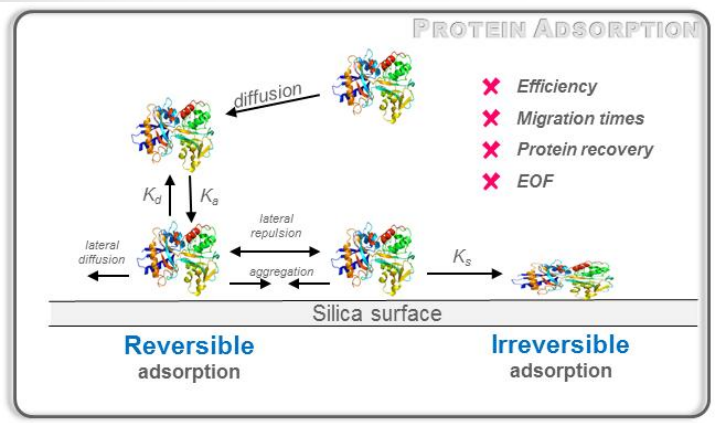
Slide courtesy of Dr. Julie Schappler, Geneva University

BGE : ammonium acetate 20 mM, pH 8.5
25 ° C, 30 kV



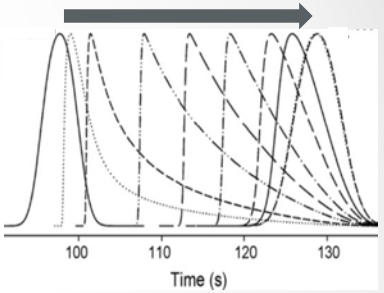
- ✓ Sufficient resolution between glycoforms (R_s 1.0 – 2.5)
- ✓ Acceptable efficiencies (N = 130'000 – 800'000)
- ✓ Low protein adsorption
- ✓ Acceptable coating stability (RSD = 2.0%, *n*=8)

Minimize Protein-Surface Interaction by Capillary Surface Deactivation

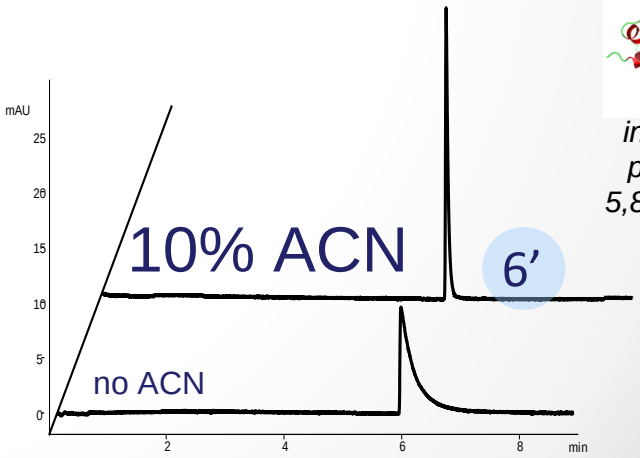


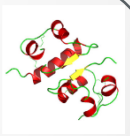
Protein Absorption

- non reproducible mobilities of protein and EOF
- Peak tailing
- Loss in efficiency
- Loss in protein recovery



Alternative method for direct MS-coupling
Addition of organic solvent to the BGE





insulin
pI 5.2
5,808 Da

Staub *et al., Electrophoresis* (2010)

Slide courtesy of Dr. Julie Schappler, Geneva University

CE-ESI-TOF/MS | human growth hormone (hGH)

Staub *et al., Electrophoresis* (2010)



hGH
pI 5.1
22,125 Da

μ+

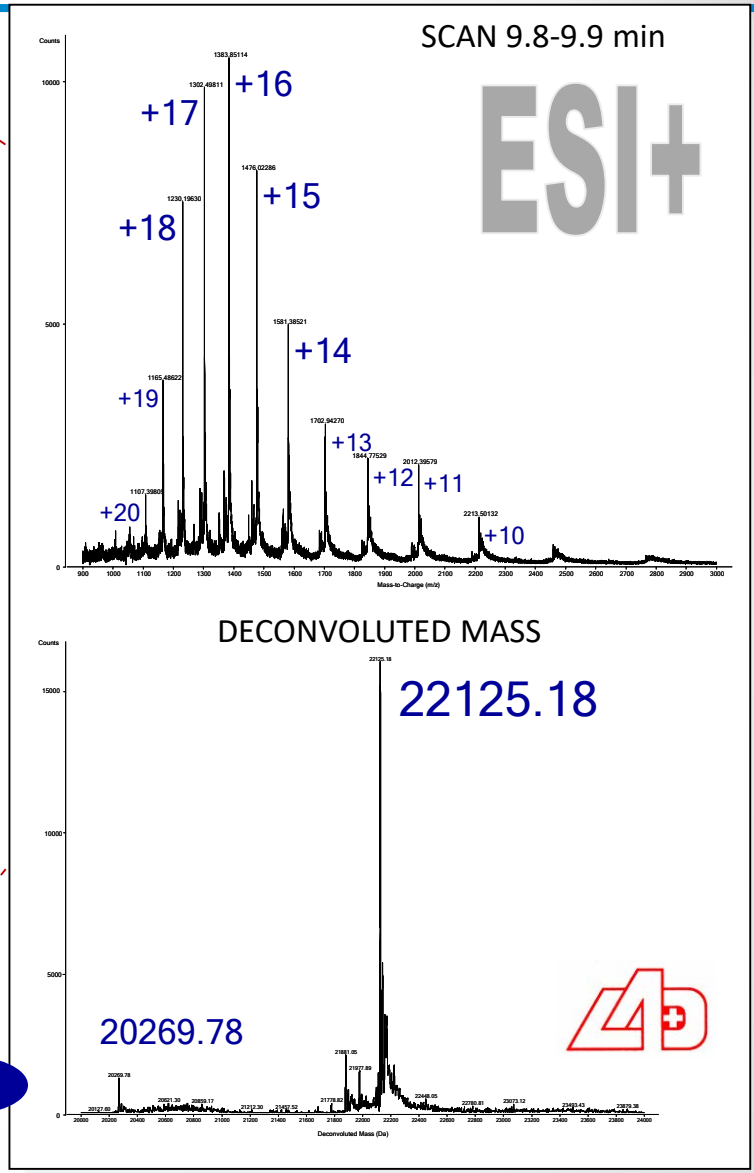
Acidic buffer + ACN

- ✓ very limited adsorption
- ✓ no degradation
- ✓ high efficiency, N = 750000

CE-ESI-TOF/MS

- ✓ unambiguous identification
- ✓ 22 kDa / 20 kDa ratio | *ca.* 5%

→ LOD (TIC/MS) | 50 ppm | 2.3 µM
36 fmol (788 pg) injected

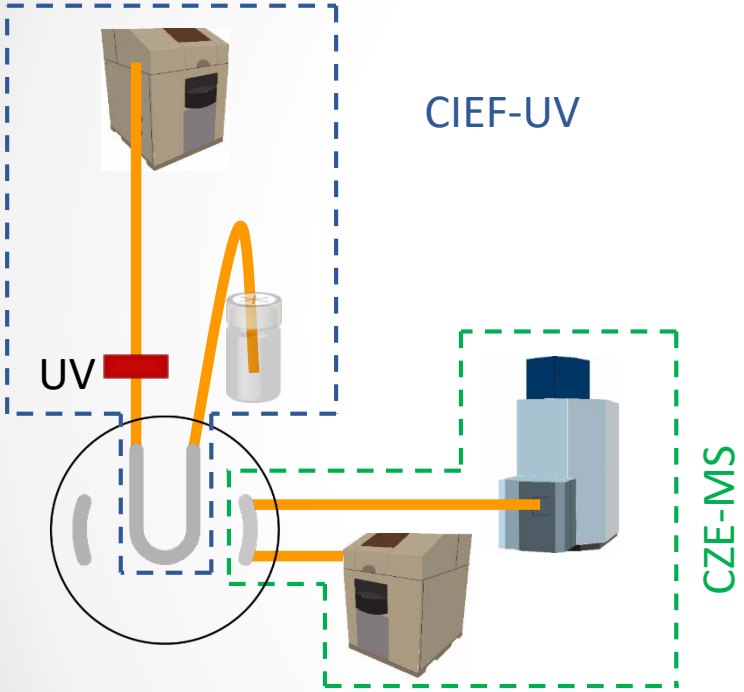


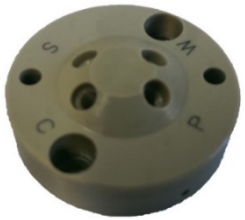
Slide courtesy of Dr. Julie Schappler, Geneva University

Special Challenges in Coupling CIEF with ESI-MS

- Transport a separated/discrete small zone to the ESI interface
  - Chemical Mobilization ??
  - Pressure Mobilization → zone dispersion?
- Interference/ion suppression of the ESI process by ampholytes, catholyte (high pH) and other additives?
- Presence of non-volatile neutral additives may cause contamination of the MS inlet
- Deal with two high voltage sources on one liquid conductor

Neusüß et al. – CIEF coupling with MS[1,2]







Stator: PEEK

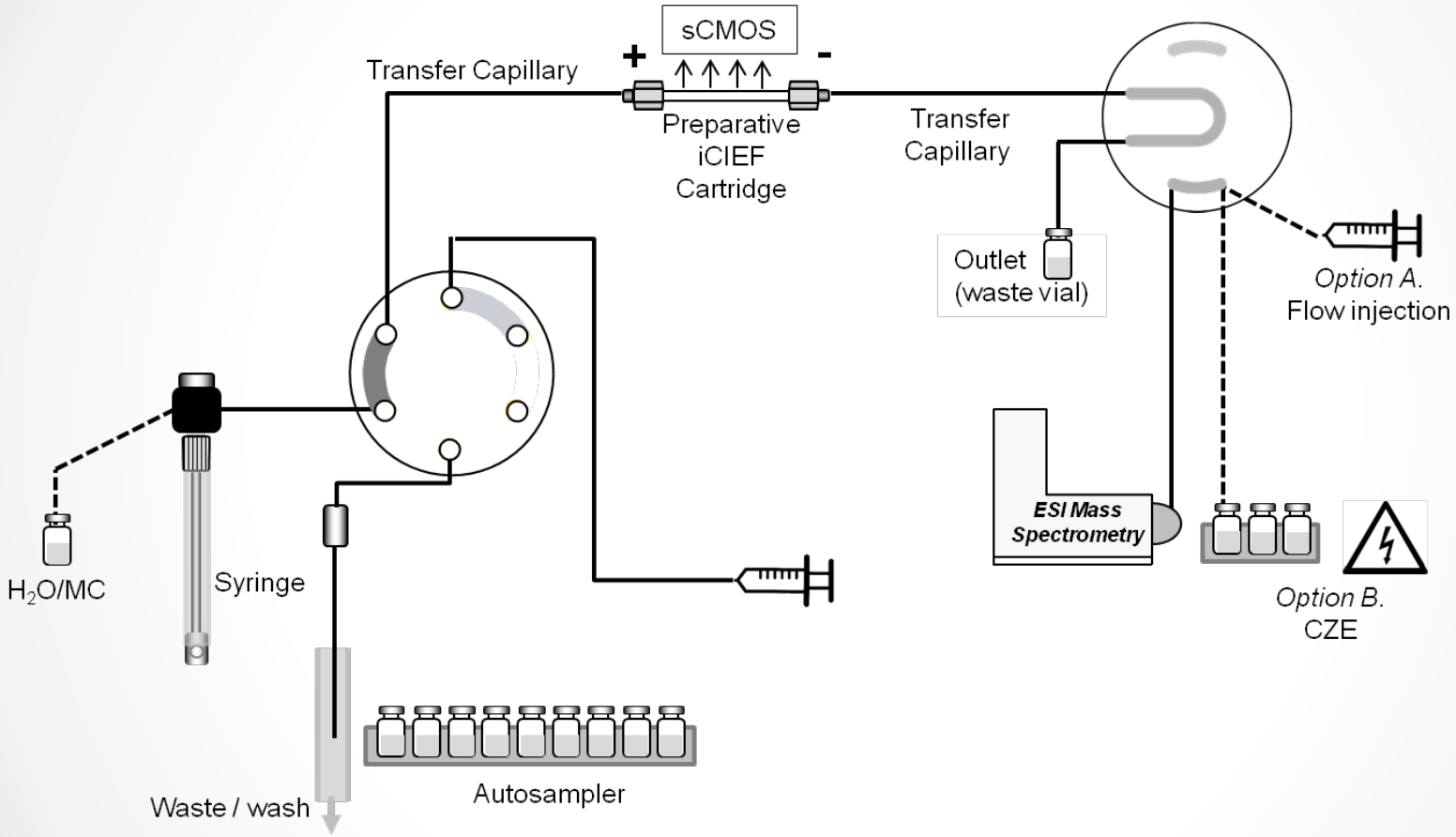
Rotor: PAEK/PTFE
loop: 4, 10, or 20 nl



4-port-nl-valve

[1]Hühner, Neusüß, Anal. Bioanal. Chem. 2016, 08, 15, 4055-4061
[2]Kohl, Montealegre, Neusüß, Electrophoresis 2016, *37,* 954–958

Coupling iCIEF with MS by the 4-port nanoliter valve

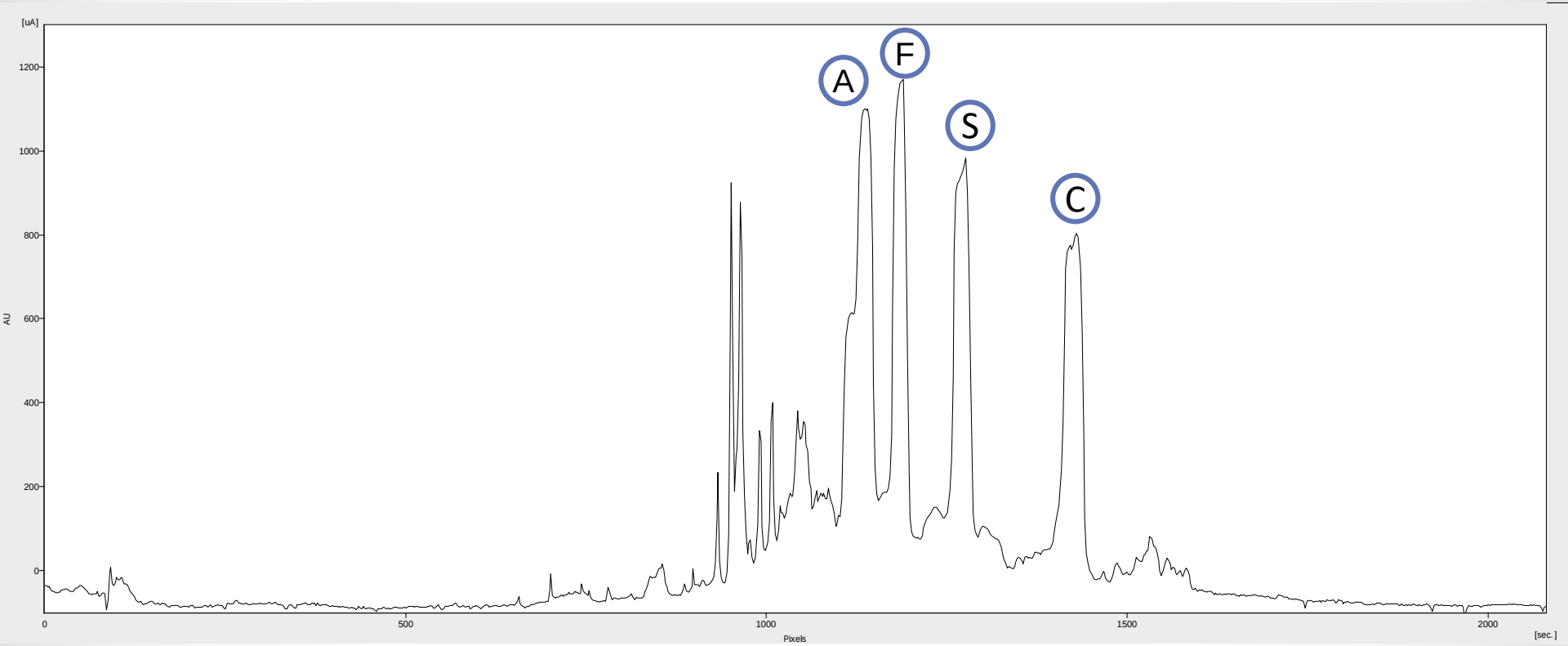


- **Easy hydraulic coupling of iCIEF with nanoliter valve, and MS**
- **CE and ES voltages are decoupled ➔ no dedicated CE-MS interface required, µHPLC ESI interface suffices**

Neusüß et al. Electrophoresis 2018, 39, 1151–1154

iCIEF-flow injection-MS – Hemoglobin

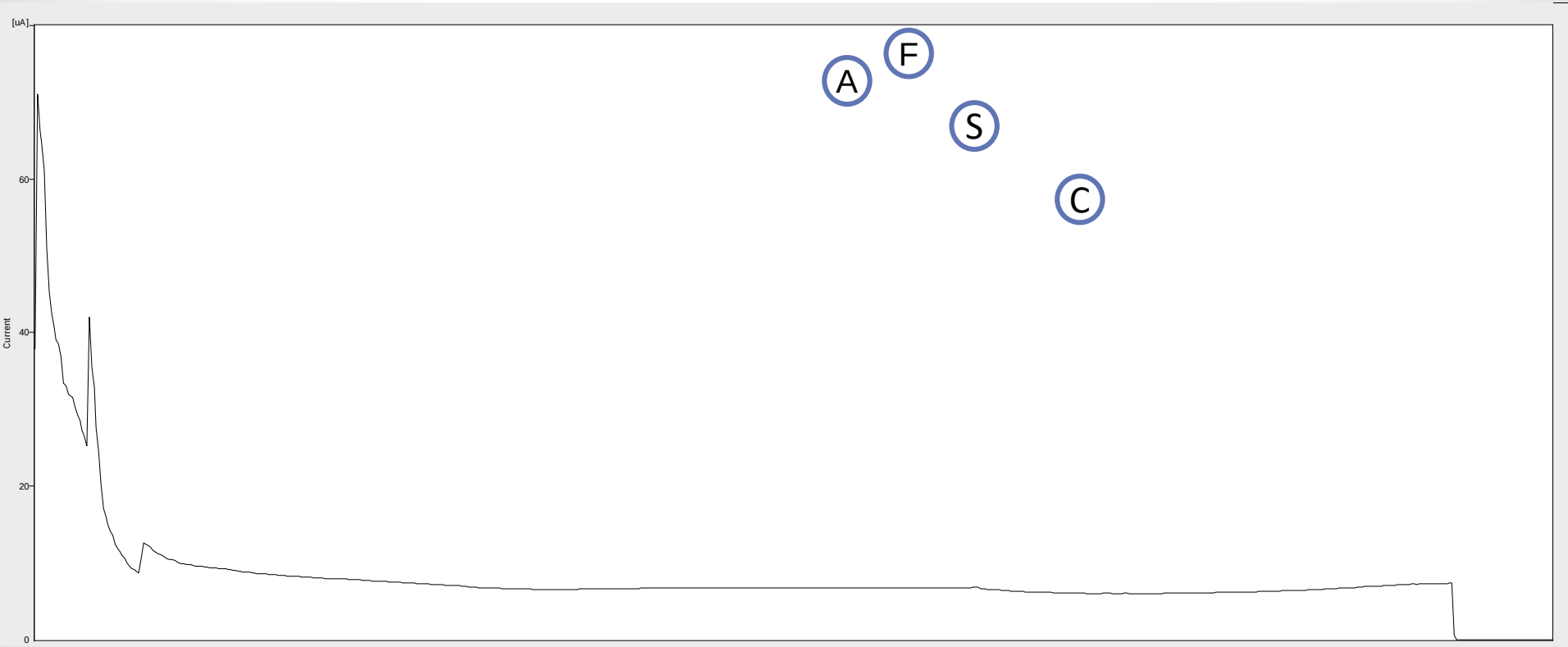
Focusing



Sample: 2% ASFC Hb, 2% AESlyte 6-9, 70% MC 0.5 %
Focusing: 1000 V (1 min), 2000 V (1 min), 3000 V (6 min), 15 s interval time
Separation capillary 50x0.2 mm

iCIEF-flow injection-MS – Hemoglobin

Transfer



Sample: 2% ASFC Hb, 2% AESlyte 6-9, 70% MC 0.5 %
Mobilization: 0.120 µL/min + 3000 V (25 min), 15 s interval time

Including CZE as second dimension

Triple tube CE-MS sprayer



1st D: iCIEF



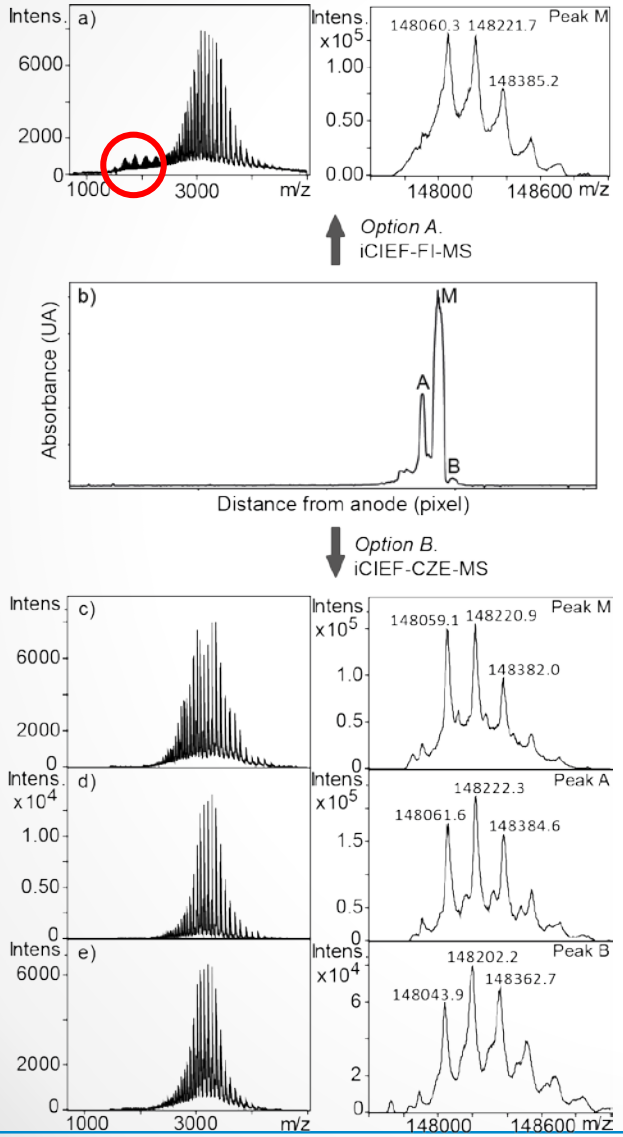
Valve

2nd D: CZE-MS



To CE

iCIEF of mAB coupled to CE-MS



a) Option A (direct coupling) for the main variant M (1.25 mg/mL).
b) Example of image after focusing for the mAb in 1% AESlyte pH 16 3-10, 2% AESlyte pH 8 - 10.5 HR, and 70% methylcellulose 0.5
c) Option B (CE-MS coupling) for the main variant M (1.25 mg/mL),
d) the main acidic variant A (2.0 mg/mL) and
e) the main basic variant B (6.0 19 mg/mL)

Summary CE of Proteins

- CZE is very well suited for separation of proteins provided that interaction of proteins with capillary surface is minimized and under control
- CIEF is a ultra high resolution separation method requiring a dedicated instrument
  - Uses standard commercial CE instruments
  - High resolution (ΔpI > 0.02 units) quantitative protein analysis in applicable concentration range (0.05 – 0.5 mg/mL).
  - Compromised method (focusing in front of detector, separate mobilization
- Imaged CIEF
  - Requires a dedicated CE instrument
  - High resolution - ΔpI > 0.02 units quantitative protein analysis in concentration range 0.1 – 1.0 mg/mL
  - Fast method development and short analysis time (up to 6 runs/hour)
  - Wide pI range (pH 2-11)

Summary CE-MS of Proteins

- CE-MS of proteins rivals LC-MS (resolution, fast)
- Porous tip interface generic nano electrospray ionization → highest sensitivity
- Triple tube interface most robust, rugged, easy to use interface → compromised sensitivity*
- CZE-MS straightforward technique. Commercial available
- CIEF-MS** and iCIEF-MS high potential but require further development

*https://www.agilent.com/search/?Ntt=5991-9073EN
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