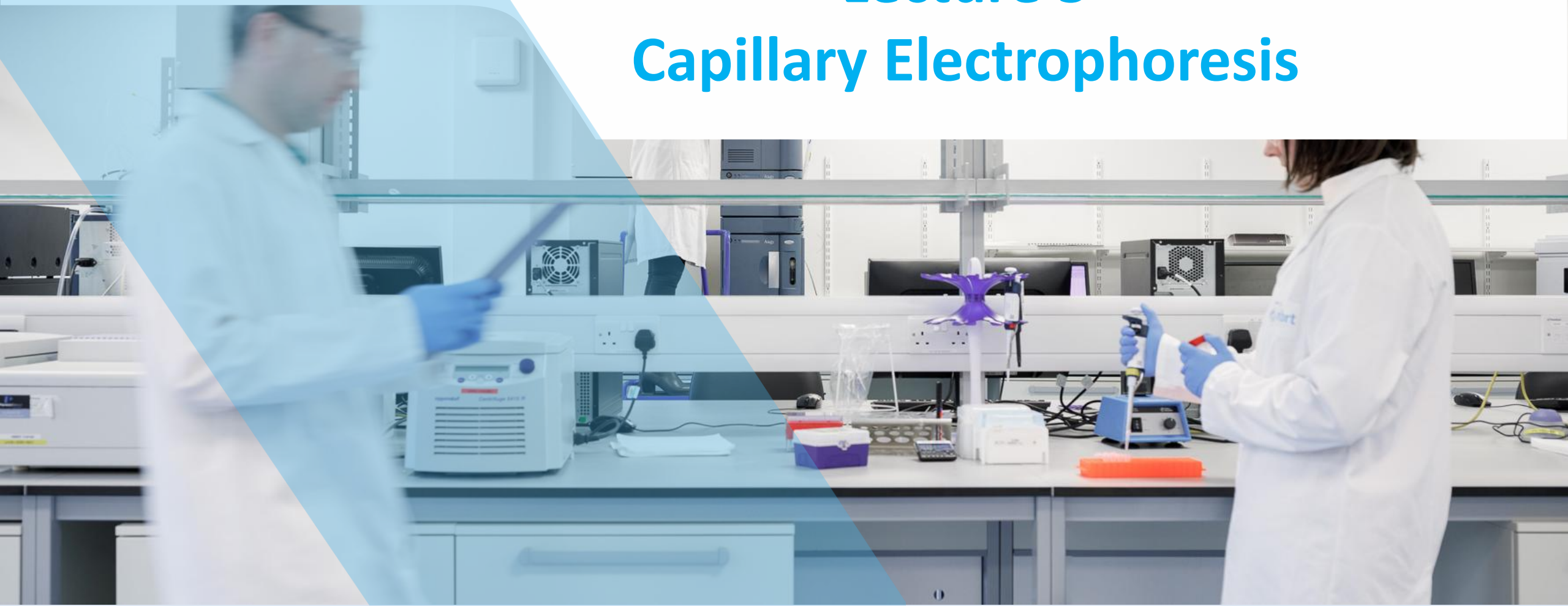
nibrt
National Institute for Bioprocessing Research and Training

Bioanalytical Techniques

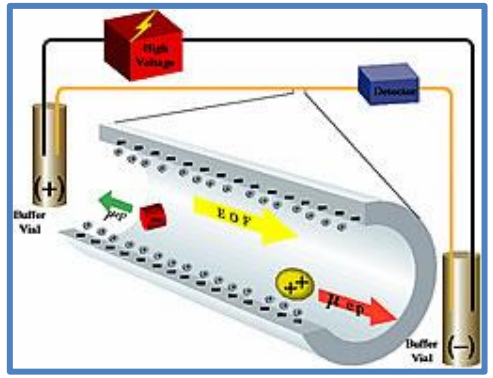
Lecture 5

Capillary Electrophoresis



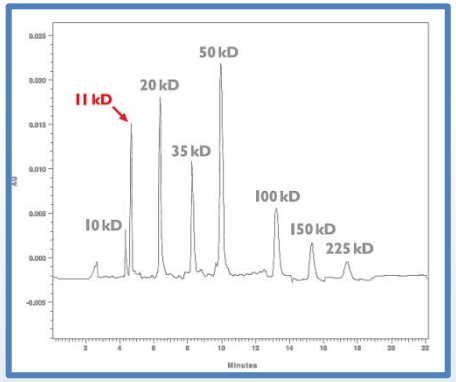
Learning Objectives

1. Describe the principles of capillary electrophoresis (CE)



2. Describe the main components of the CE instrument

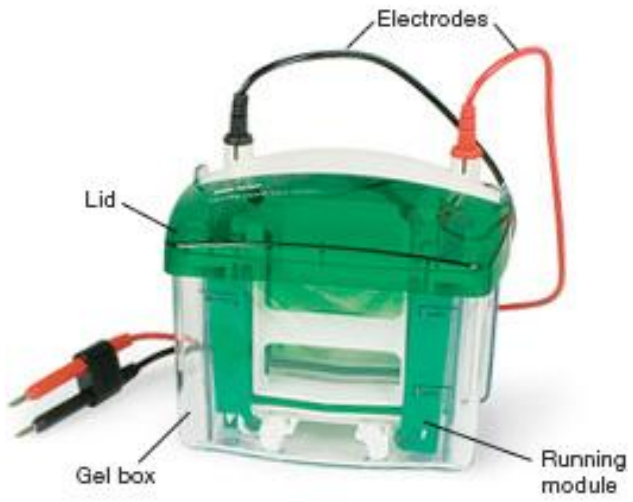
3. Discuss the advantages of CE over traditional slab gel

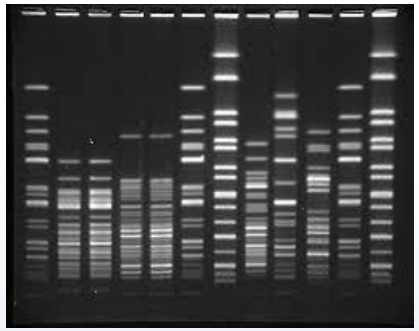


Topics

- Principles of Capillary Electrophoresis
- CE Instrumentation
- Separation Modes
- Applications

Classical Electrophoresis



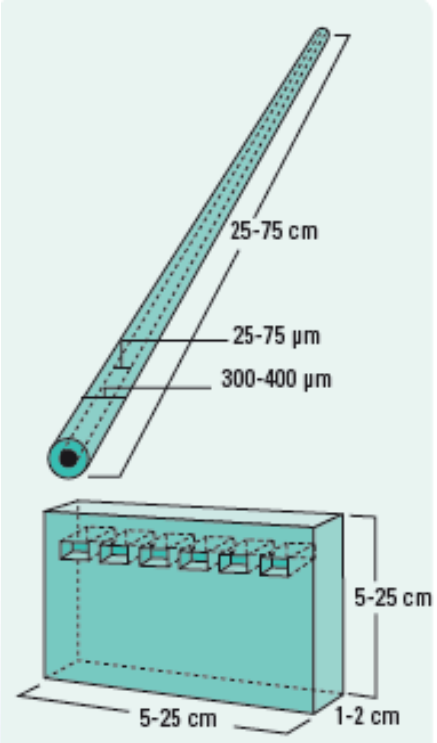


Classical electrophoresis

+ Inexpensive
+ Parallel operation
- Manual
- Time consuming
- Laborious
- Long analysis times
- Low efficiencies
- Difficulties in detection and automation

What is Capillary Electrophoresis?

- Similar to slab gel electrophoresis, but done in a capillary

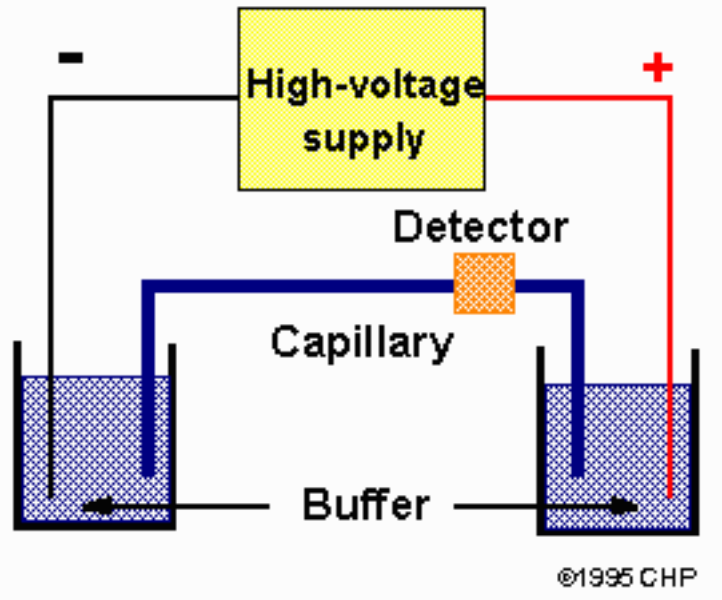


High Performance Capillary Electrophoresis, Agilent Technologies

- More **quantifiable and reliable**
- Provides information on the **size, charge, identity and purity**
- **Diverse application range**
  - Proteins
  - Peptides
  - Amino acids
  - Nucleic acids (RNA and DNA)
  - Inorganic ions
  - Organic bases
  - Organic acids
  - Whole cells

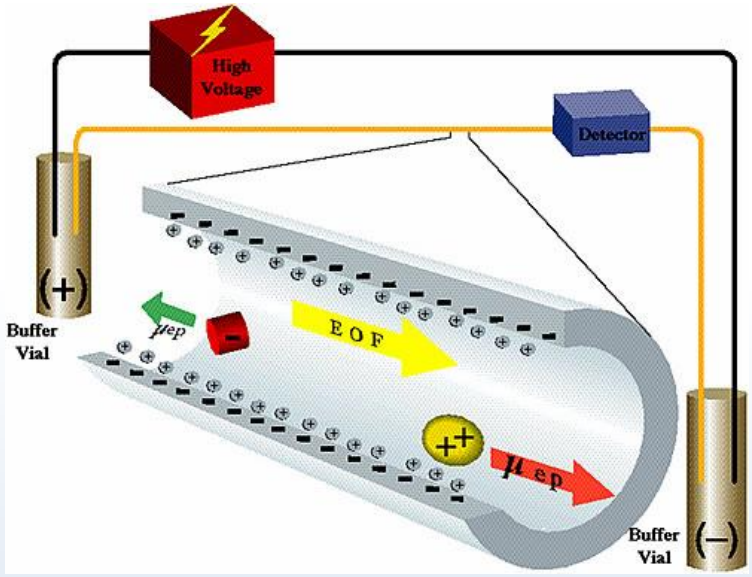
Principles of CE

- Same as gel electrophoresis, CE uses **electrophoresis** as the driving force of separation on a thin capillary



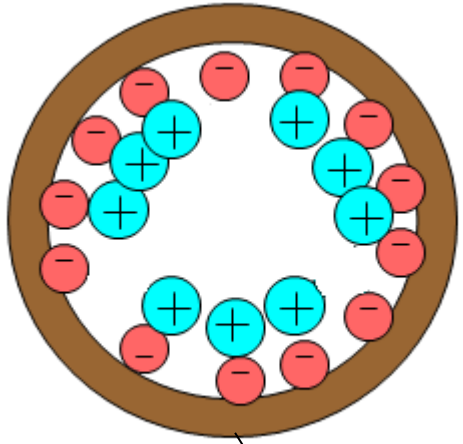
Principles of CE

- The small diameter of the capillary contributes to another aspect of the separation process
  - The phenomenon known as **electroosmosis or electrooosmotic flow (EOF)**

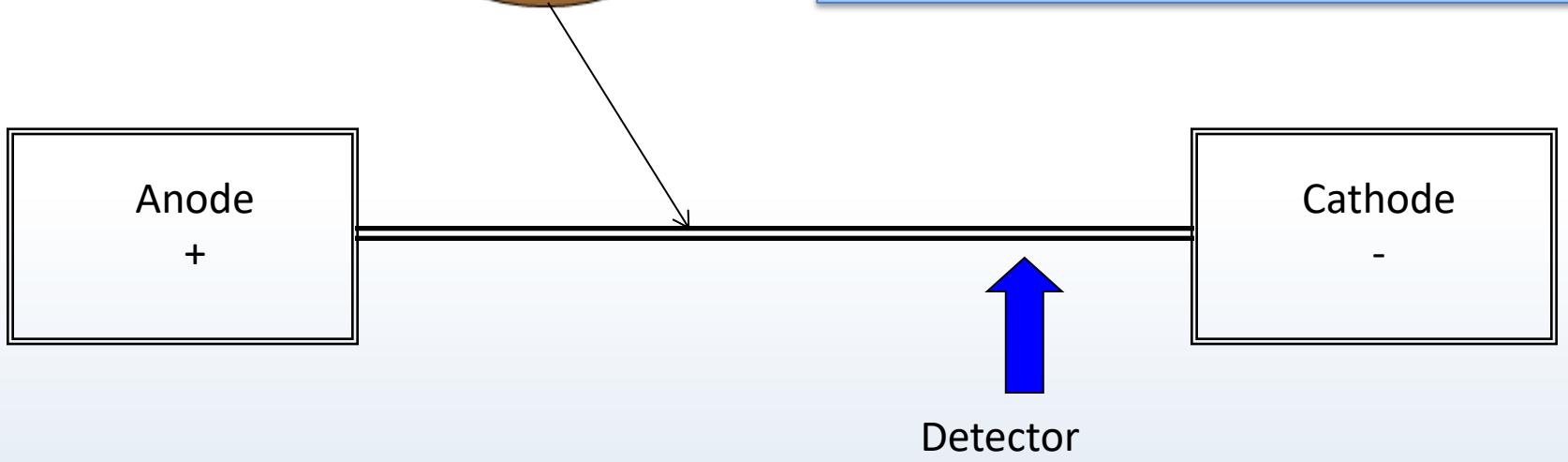


Electroosmotic Flow

Inner wall of the fused silica capillary is negatively charged at pH>3 (due to numerous silanol groups, SiO^-)

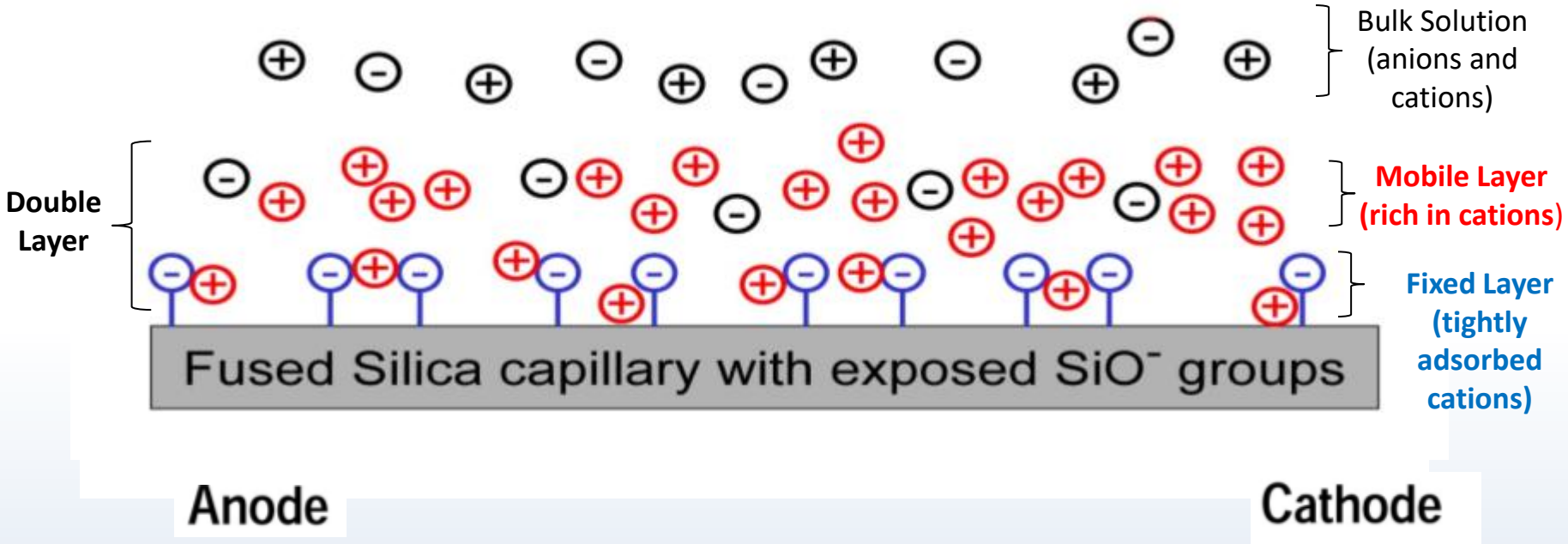


The negatively-charged wall attracts positively-charged ions (cations) from the buffer, creating an electrical layer.



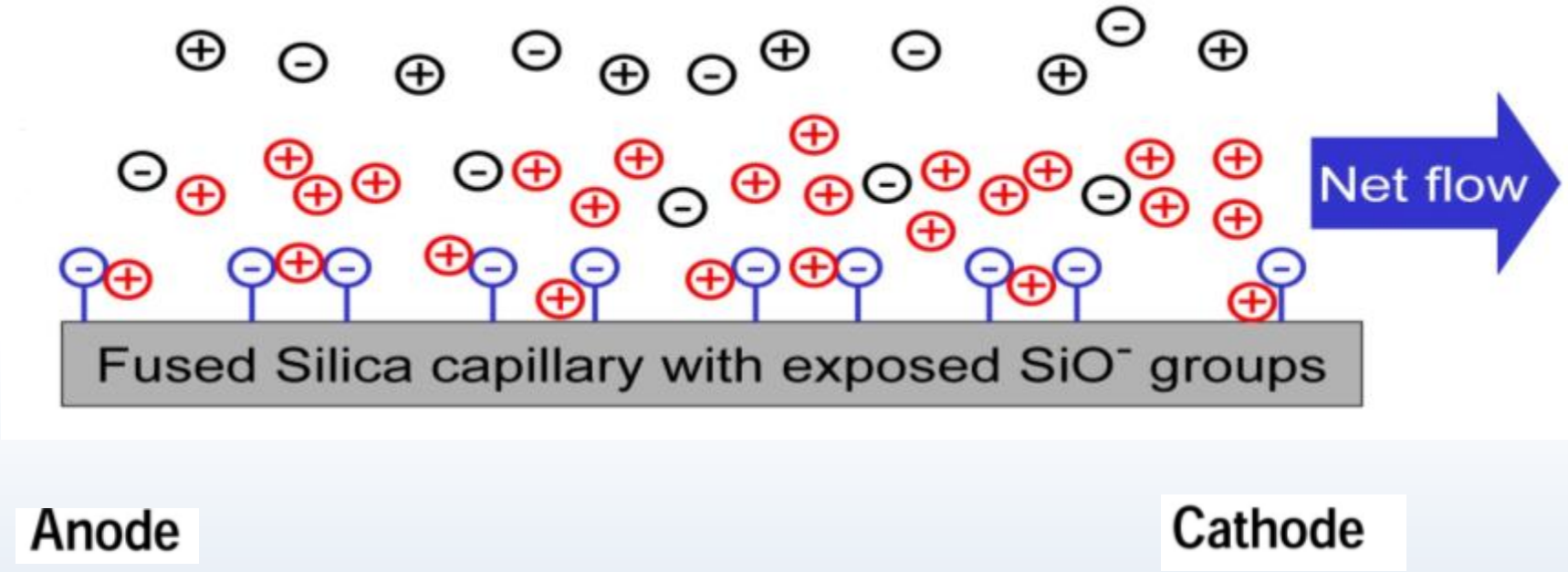
EOF

This first layer of cations (**fixed layer**) is not of sufficient density to totally neutralise the negative charges of the silanoate, so a second outer layer of cations forms (mobile layer)



EOF

EOF leads to *en masse* **movement of all charged species towards the cathode**



Controlling EOF

- The EOF is usually beneficial, but it needs to be controlled
- For example:

At high pH the EOF may be too rapid, resulting in elution of solutes before separation has occurred.

At low or moderate pH, the low negative charge of the inner capillary surface can cause adsorption of cationic solutes

Some electrophoretic separation modes (isoelectric focusing, capillary gel electrophoresis require reduction or absence of EOF)

Controlling EOF

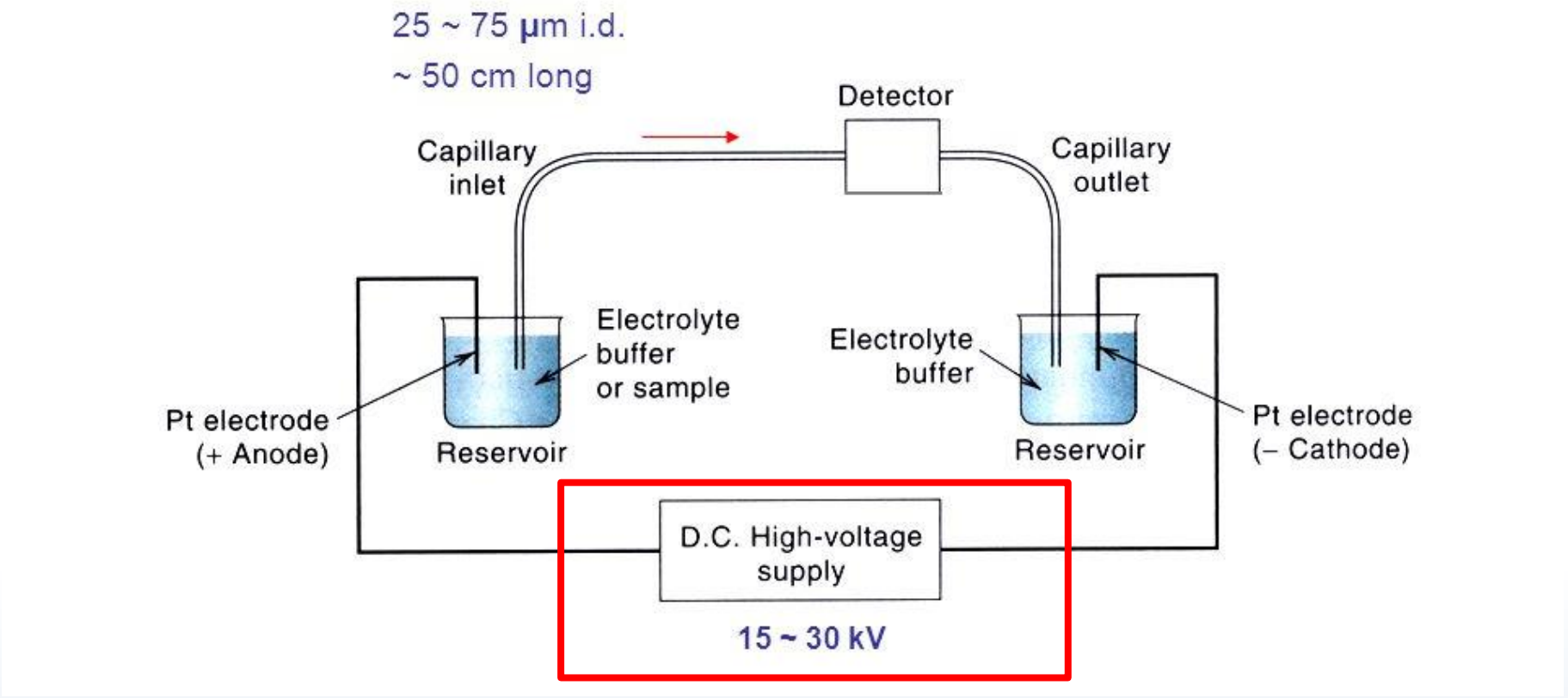
Variable	Result	Notes
Electric Field	Proportional change in EOF	Joule heating may result
Buffer pH	EOF decreased at low pH, increased at high pH	Best method to control EOF, but may change charge of analytes
Ionic Strength	Decreases ζ and EOF with increasing buffer concentration	High ionic strength means high current and Joule heating
Organic Modifiers	Decreases ζ and EOF with increasing modifier	Complex effects
Surfactant	Adsorbs to capillary wall through hydrophobic or ionic interactions	Anionic surfactants increase EOF Cationic surfactants decrease EOF
Neutral hydrophilic polymer	Adsorbs to capillary wall via hydrophobic interactions	Decreases EOF by shielding surface charge, also increases viscosity
Covalent coating	Chemically bonded to capillary wall	Many possibilities
Temperature	Changes viscosity	Easy to control

ζ = Zeta potential, the electrokinetic potential at any given point in an ion double layer

Topics

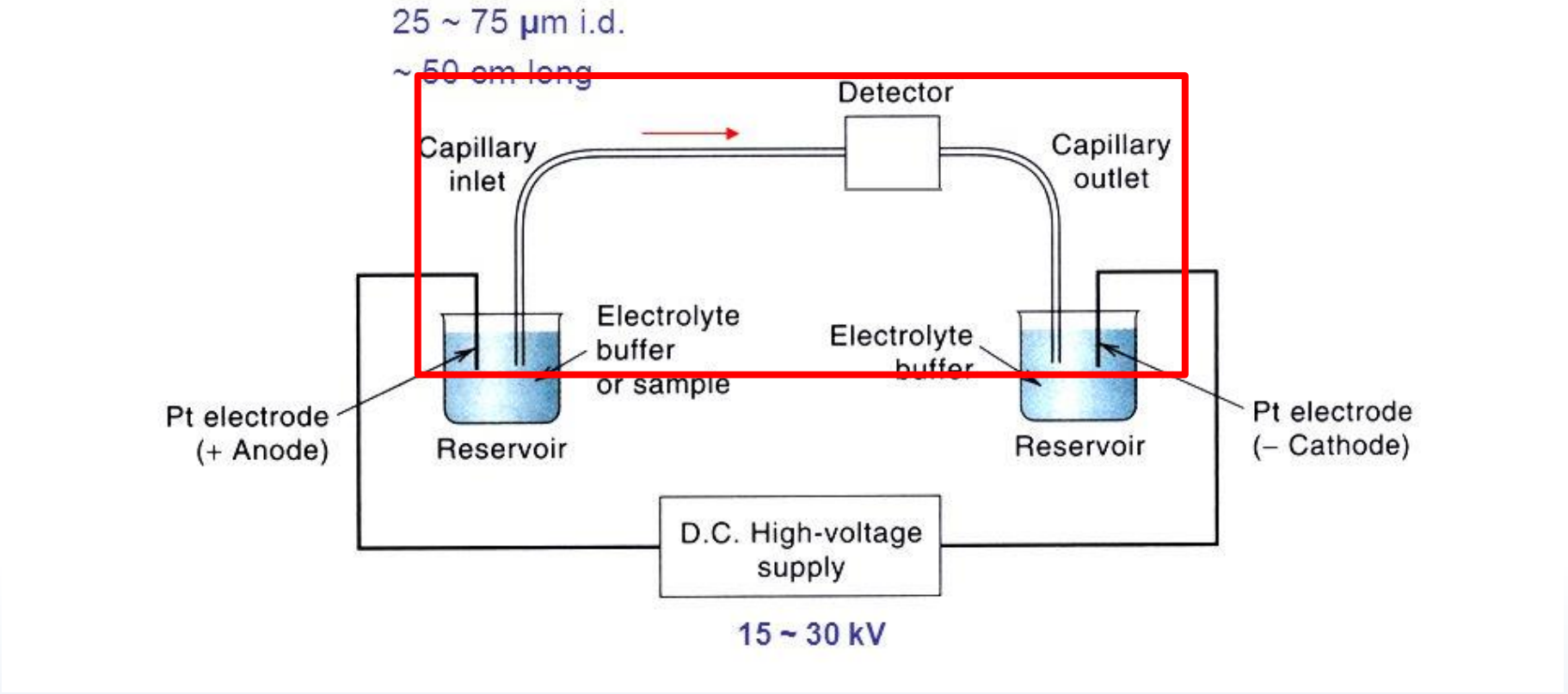
- Principles of Capillary Electrophoresis
- CE Instrumentation
- Separation Modes
- Applications

High-voltage Supply



G. D. Christian, Analytical Chemistry, 6th ed., John Wiley, 2004, p. 632

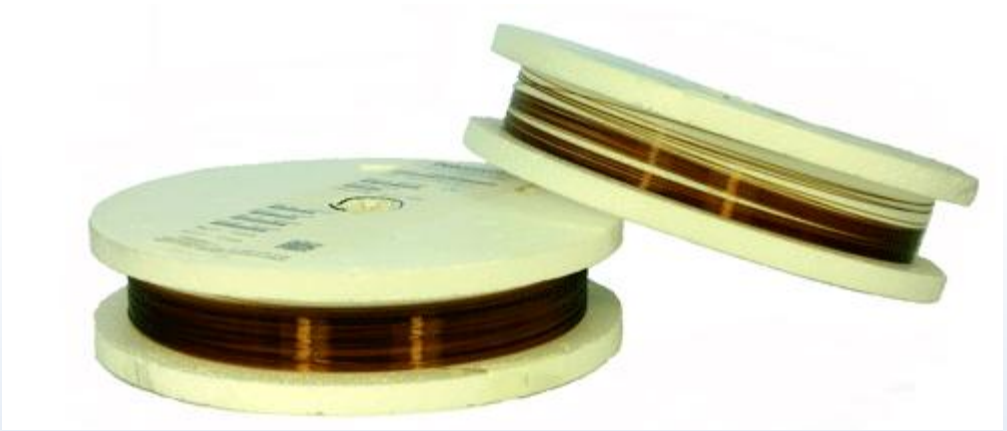
The Capillary



G. D. Christian, Analytical Chemistry, 6th ed., John Wiley, 2004, p. 632

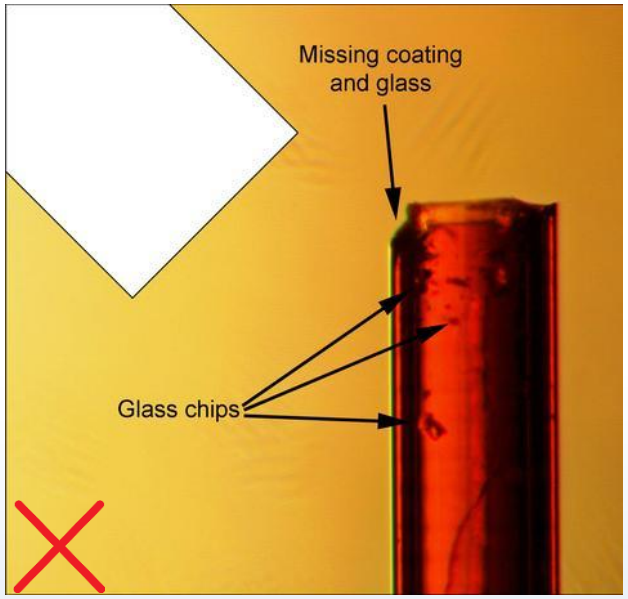
The capillary

- Diameter of 25-100μm, up to 1m long (coiled)
- Large surface area to volume ratio which enhances efficiency of **heat dissipation** from the system
- This allows operation at higher current density, which speeds up the rate of migration through the capillary
- A window (no polyimide coating) allows the detector to read a signal from the capillary

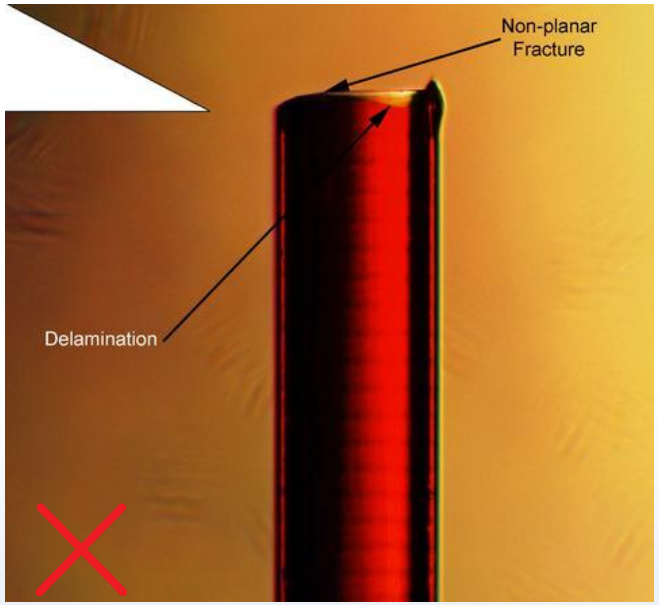


The capillary

- Capillary sometimes needs to be cut and fitted into a cartridge
- Cutting is done using a cleaving stone
- Capillary needs to be examined under a microscope to ensure it was not damaged during cutting and fitting
- Many QC labs prefer to use pre-cut capillaries



Result of dragging a cleaving stone across capillary

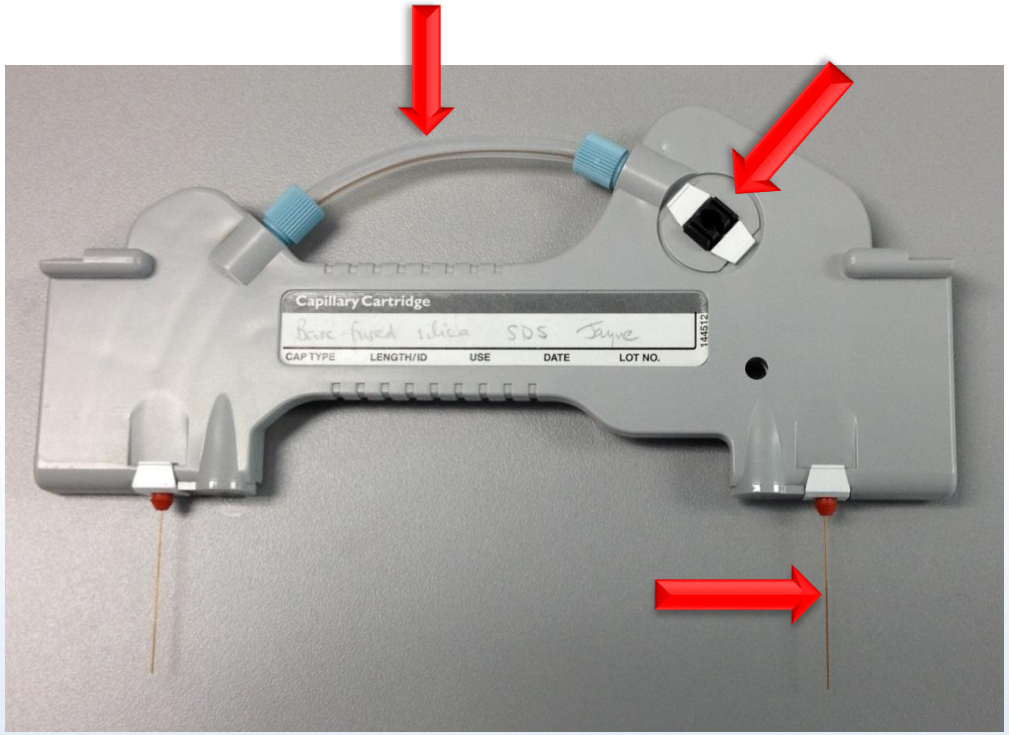


Result of folding the column to snap it

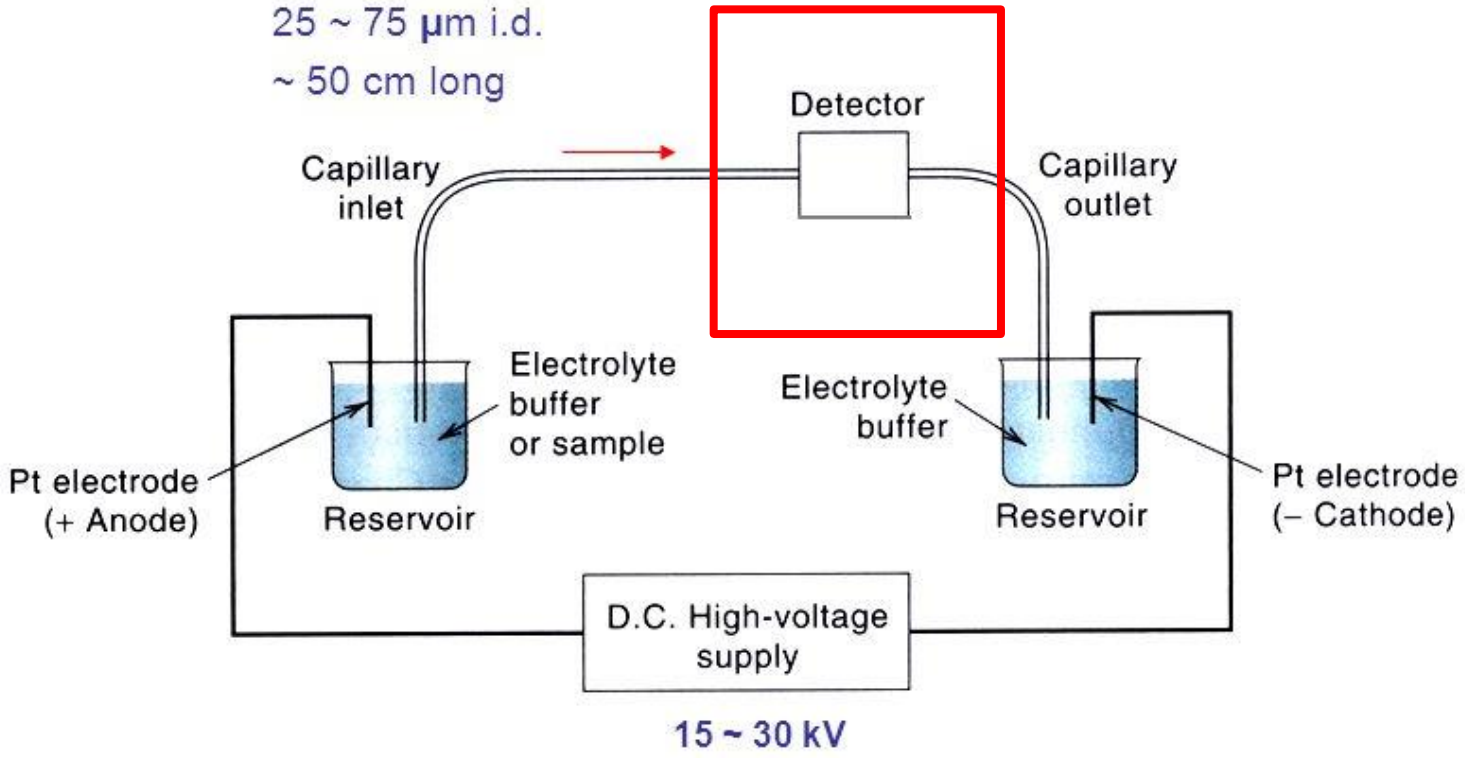
https://innovaquartz.com/blogs/news/14905873-how-to-properly-cut-capillary-columns

The cartridge

- The capillary is pushed through a cartridge to prepare it for use in the CE
- It can have different length loops attached to allow different lengths of capillaries to be used
- It has a window to give the detector access to the capillary window



Detector



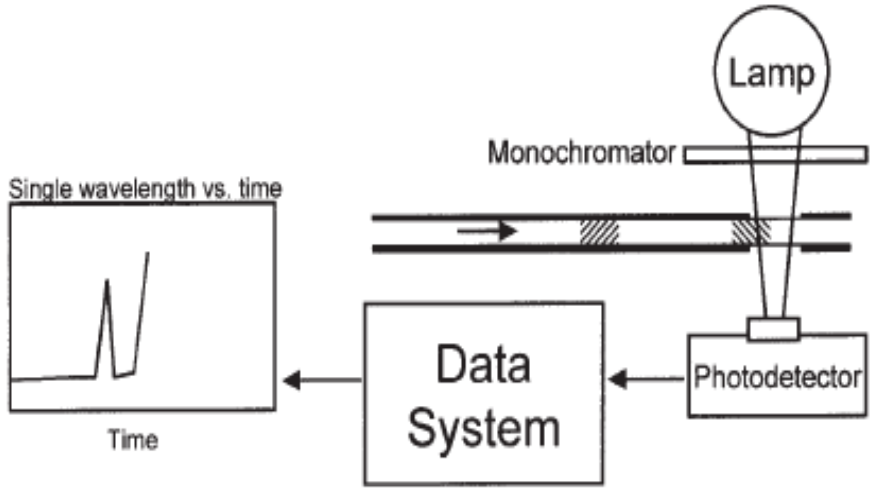
G. D. Christian, Analytical Chemistry, 6th ed., John Wiley, 2004, p. 632

Detectors

- Detectors:
  - UV
  - Photodiode array detector (PDA)
  - Laser-induced fluorescence (LIF)

UV Detector

- Most widely used detection
- Modular, selectable wavelengths (below 200nm up to the visible spectrum)

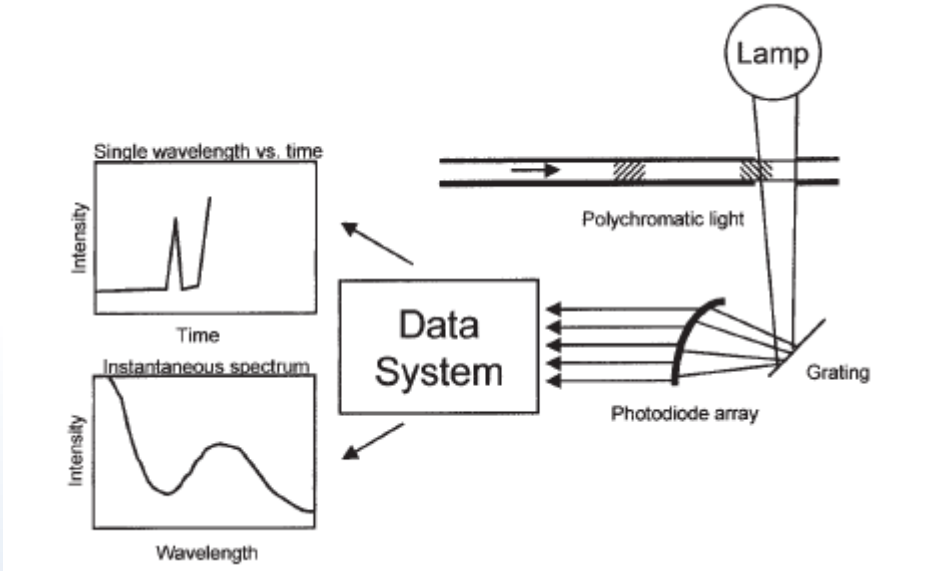


Basic Principles and Modes of Capillary Electrophoresis, *Clinical and Forensic Applications of Capillary Electrophoresis,* 2001.

Diode Array Detector

- Allows spectral data to be collected so **scans across all wavelengths at the same time**, rather than only reading at one absorbance as with UV detector
- Any wavelength in the scan range may be viewed during a run and selected for extraction and analysis after the run
- Useful for method development (finding optimal wavelength) and for confirmatory analysis (identity, purity)

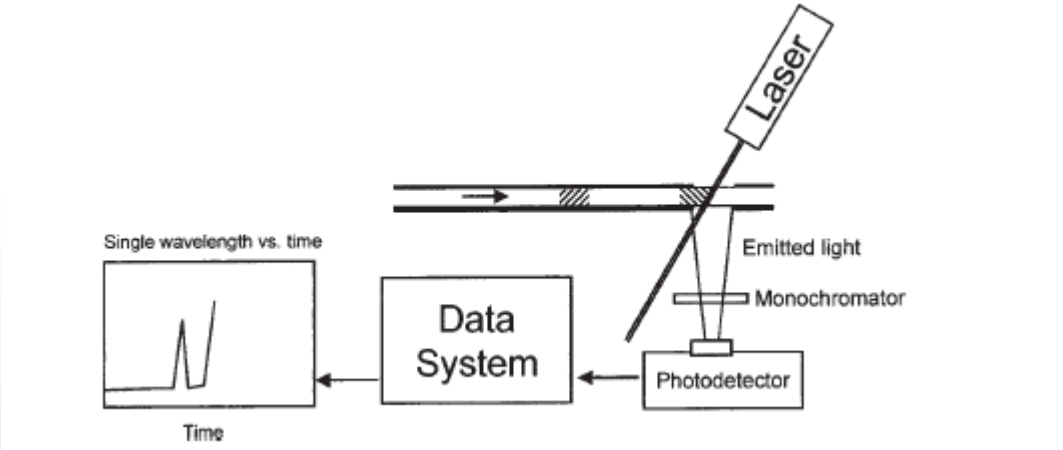
All the energy of the source lamp focused onto a very small region of the capillary - Some capillary coatings and buffers will decompose under this conditions!



Basic Principles and Modes of Capillary Electrophoresis, *Clinical and Forensic Applications of Capillary Electrophoresis,* 2001.

LIF Detector

- Laser-induced fluorescence
- Used for analysis of carbohydrates, nucleic acids & other compounds which **either naturally fluoresce or that can be fluorescently labelled**
- LIF is **selective and highly sensitive** and typically yields 10 - 1000 times better sensitivity than UV absorbance
- Range of commercially available reagents have been developed which allow for the addition of a fluorescence molecule to a specific functional group on the analyte



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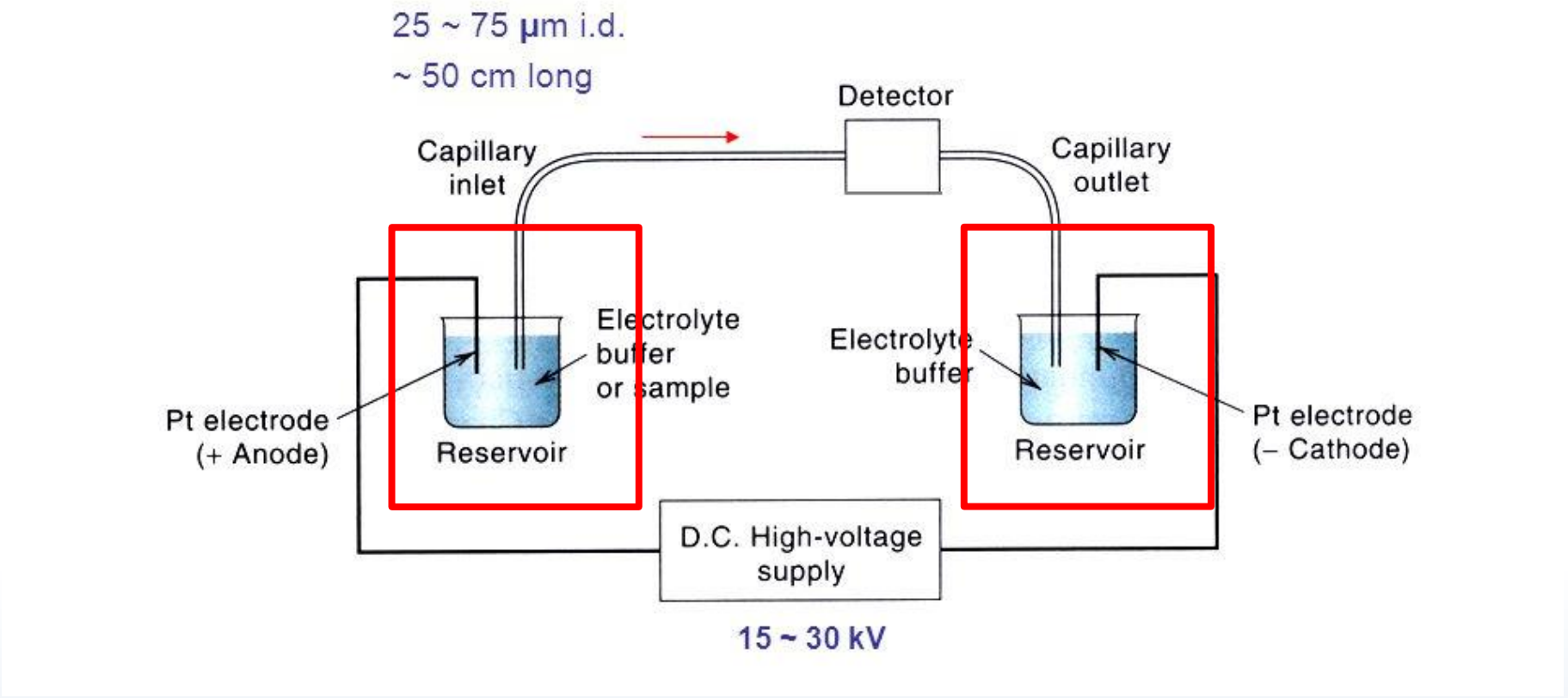
Buffer and Sample Reservoirs



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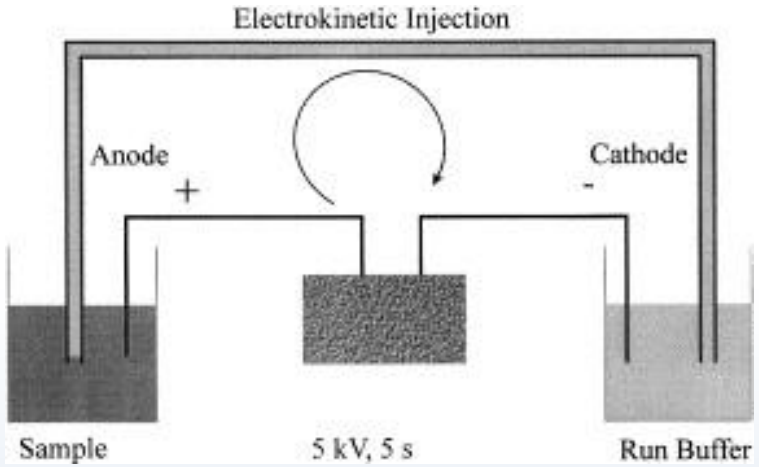
Methods of Sample Injection

Electrokinetic

- The capillary inlet is inserted into the sample and the outlet into a buffer vial
- Voltage is briefly applied – sample is drawn into the capillary through a combination of electrophoresis and electroosmotic flow



Components that migrate more rapidly in the electrical field will
be over-represented in the sample compared to slower moving components!

Image from Majidi, V., (2000) Microchem J 66, 3-16

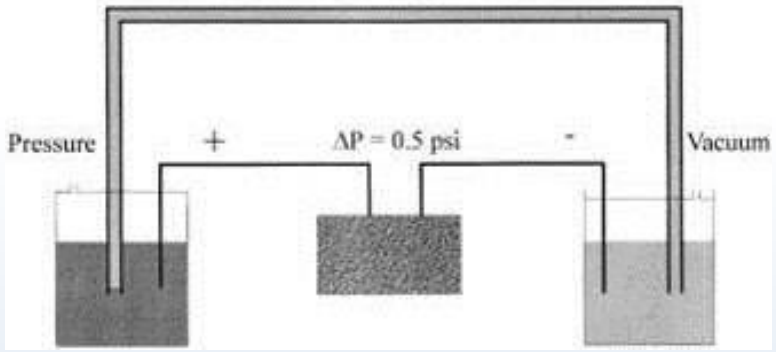
Methods of Sample Injection

Hydrodynamic

- By **pressurising** the sample to 'push' it into the capillary
- By creating a **vacuum** at the receiving reservoir, 'pulling' the sample into the capillary

- Injection volume can be controlled by adjusting delivery pressure and delivery time (lower pressure injection usually gives better performance)

Injection by pressure or vacuum:



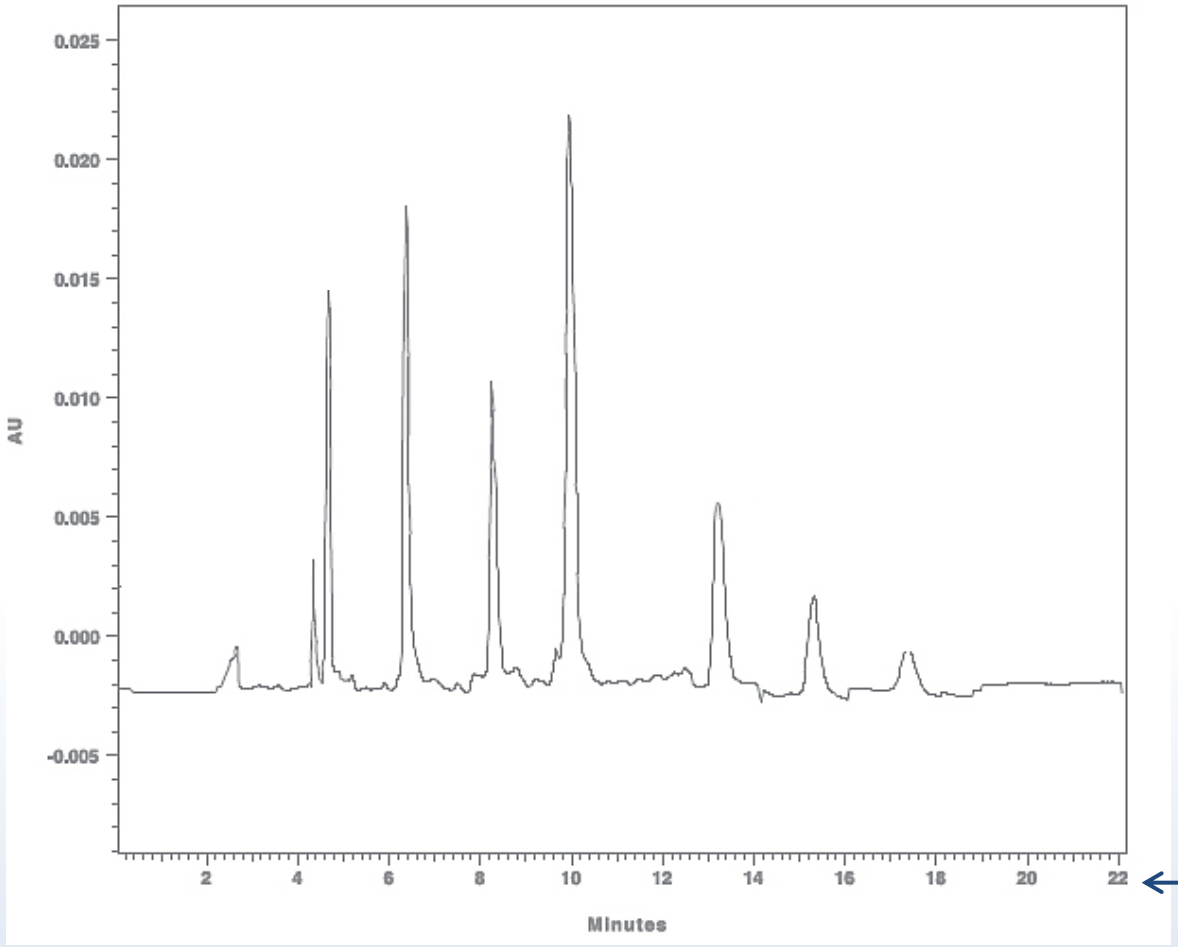
Temperature (and thus viscosity) affects reproducibility of injection volumes in both cases!

Image from Majidi, V., (2000) Microchem J 66, 3-16

CE Results

- Results are shown as an **electropherogram** (similar to a chromatogram in HPLC): time v absorbance/fluorescence units

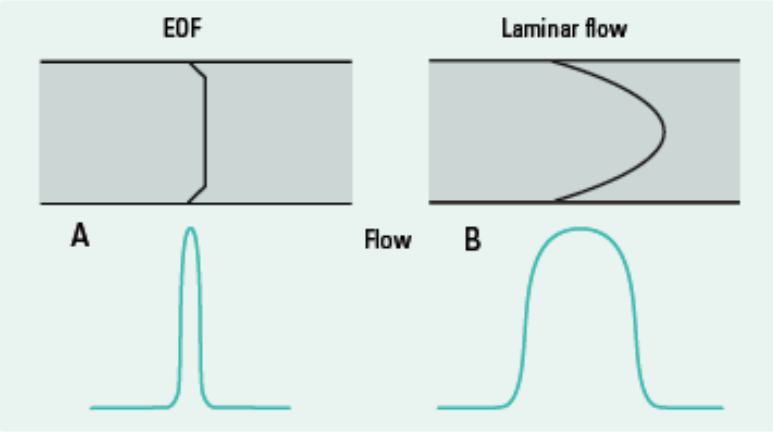
Detector response



Migration Time

Flat Flow Velocity Profile

- A unique feature of EOF in the capillary is the **flat flow velocity profile**
- The flat flow velocity profile is beneficial since it does not contribute to the broadening of sample zones.
- This is in contrast to the flow velocity profile generated by pressure (e.g. HPLC), which yields a laminar flow velocity profile due to the shear forces at the wall



The flat movement of sample through capillary leads to very high resolution

Topics

- Principles of Capillary Electrophoresis
- CE Instrumentation
- Separation Modes
- Applications

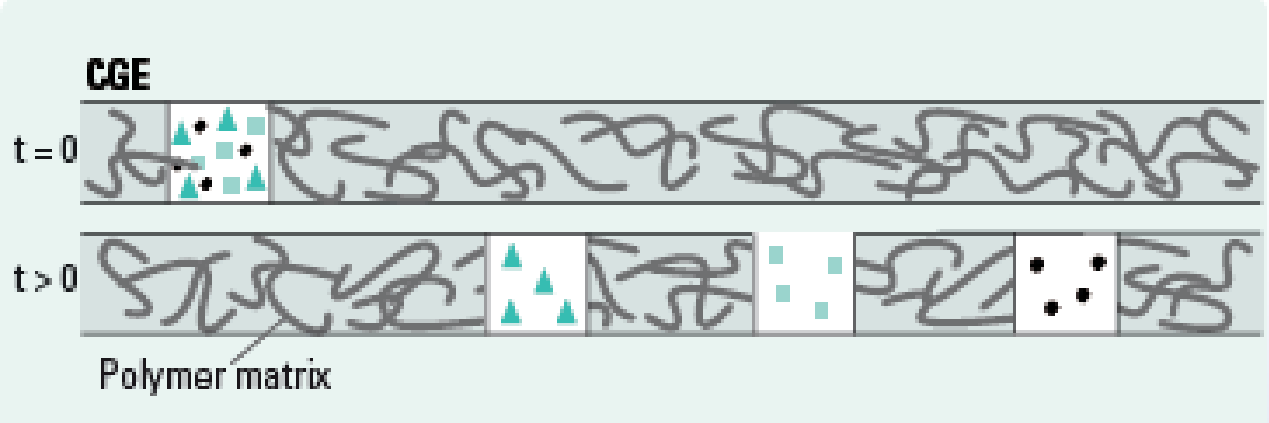
CE separation techniques

- Several separation techniques can be used depending on the capillary and the electrolytes used

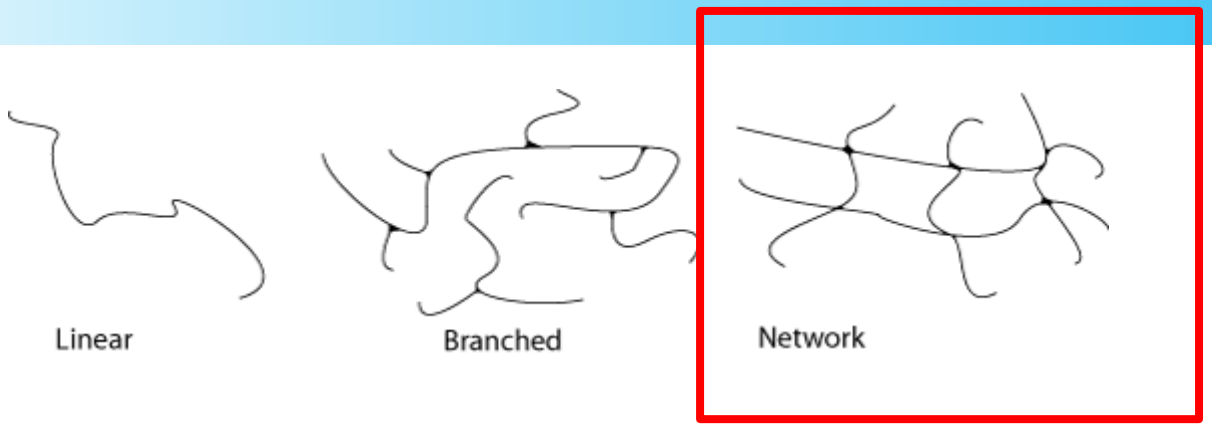
  - **Capillary Zone Electrophoresis (CZE):** simplest form of CE, separation is by charge-to-mass ratio
  - **Capillary Gel Electrophoresis (CGE):** separation occurs in a gel in the capillary e.g. polyacrylamide gel. SDS can be used to coat protein in negative charge so separation is by size
  - **Capillary iso-electric focusing (CIEF):** separation is by charge & occurs along a pH gradient so proteins migrate depending on their isoelectric point

Capillary Gel Electrophoresis (CGE)

- CGE is the adaptation of traditional gel electrophoresis into the capillary using polymers in solution to create a molecular sieve
- SDS negatively charges all proteins so **separation is by size: CE-SDS**
- Larger molecules tend to be retarded more by the viscous separation medium than smaller molecules

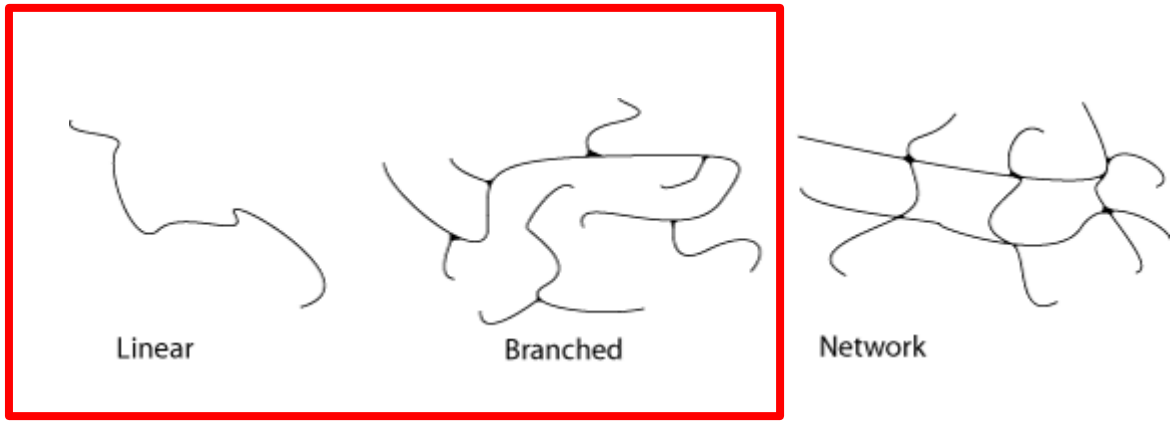


CGE Separation Medium



- **Agarose and cross-linked polyacrylamide** (CPA) were used as sieving matrices, and these matrices were **prepared directly inside the capillary**
- **Issues:**
  - CPA sometimes shrinks and breaks during polymerisation leading to bubble formation inside the capillary
  - Over-time large molecules and particulates accumulate at the end of the capillary and clog the capillary
  - Run-to-run reproducibility poor

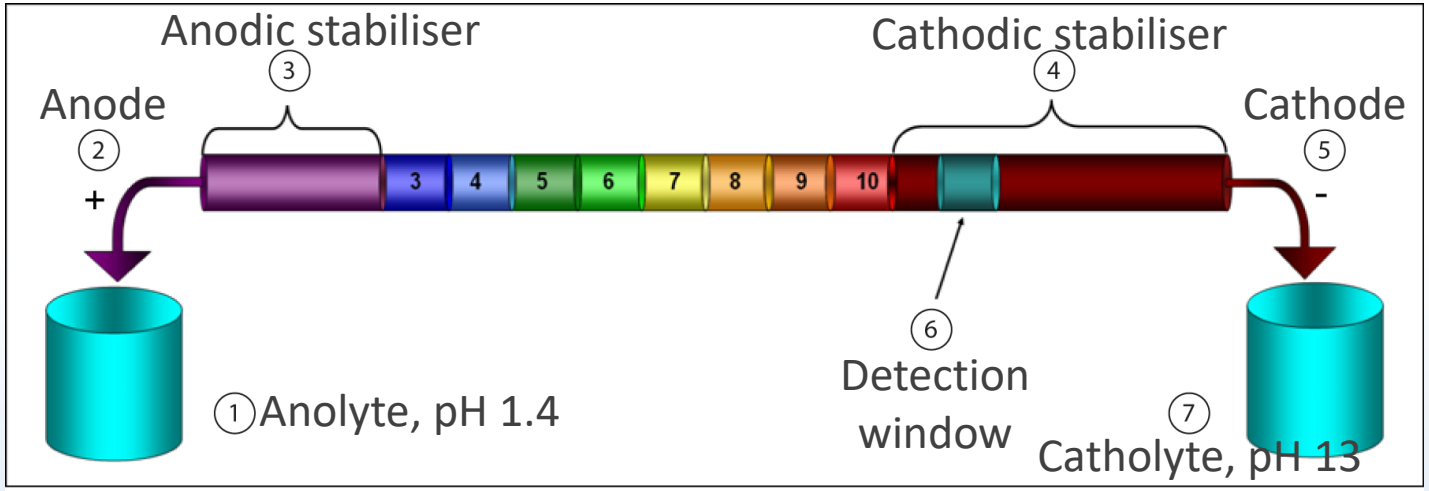
CGE Separation Medium



- Currently, **water-soluble, replaceable linear or slightly branched polymers**, are used (linear polyacrylamide polyethylene glycol, polyethylene oxide, dextran, pullulan)
- Can be dissolved in buffer and hydrodynamically loaded into the capillary
- **The capillary wall needs to be coated to eliminate EOF**

Capillary iso-electric focusing (CIEF)

- CIEF can be used for **charge heterogeneity determination**
- Useful for determining the **identity, purity, post-translational modifications** (e.g. sialylation, phosphorylation, deamidation) and **stability** of therapeutic proteins
- Separation of samples **by their iso-electric point** in a pH gradient
- EOF needs to be minimised!



pH gradient in a capillary

E9108255.png

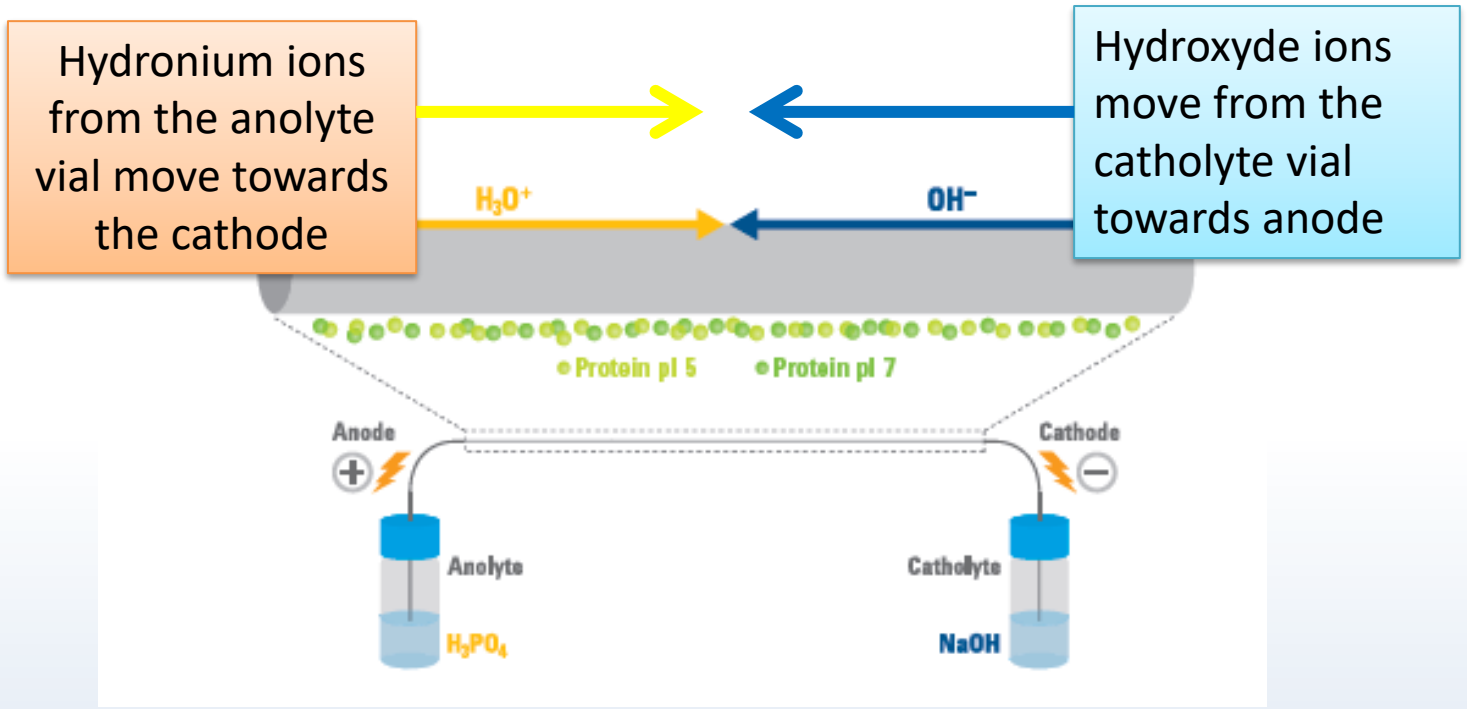
CIEF

Different solutions are premixed and filled into the capillary:

- Carrier ampholytes,
- Sample proteins,
- Standards (pI markers),
- Optional additives

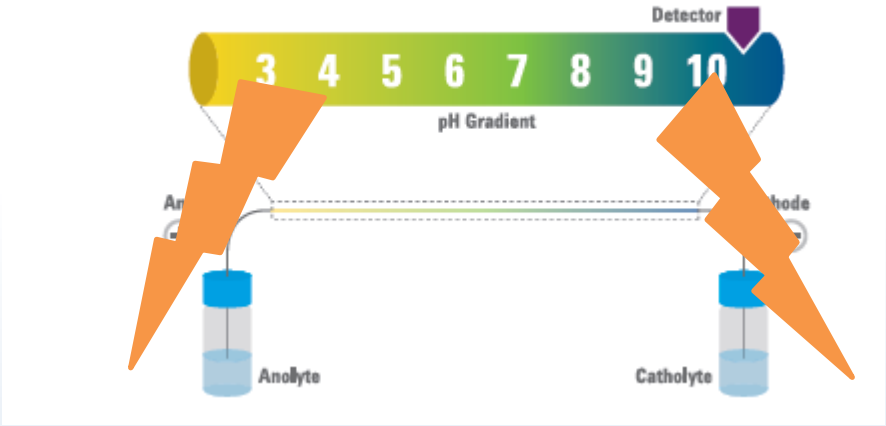
CIEF

- One end of the filled capillary is connected with the low pH electrolyte (**anolyte**) and the other end with the high pH electrolyte (**catholyte**) and **electric filed is applied**



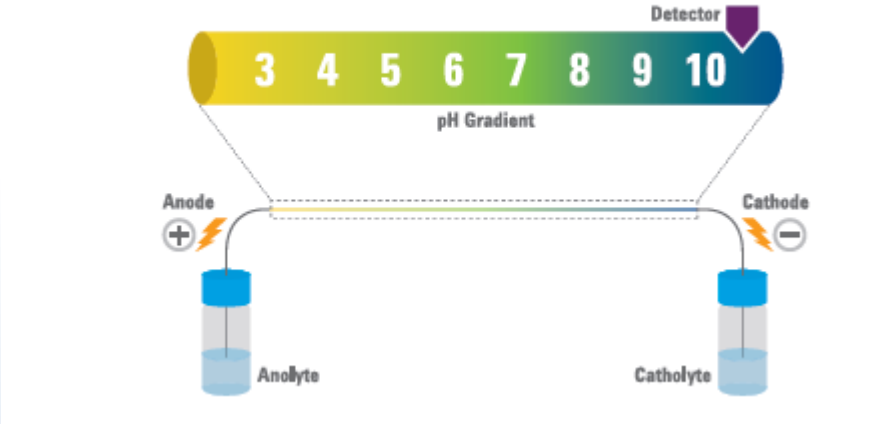
Focusing

- Proteins and the ampholyte become sorted in the capillary from low to high pI – **FOCUSING**
- At the beginning of the focusing process a high current is observed, which continuously decreases until it reaches a minimum (usually 10% of the initial value)



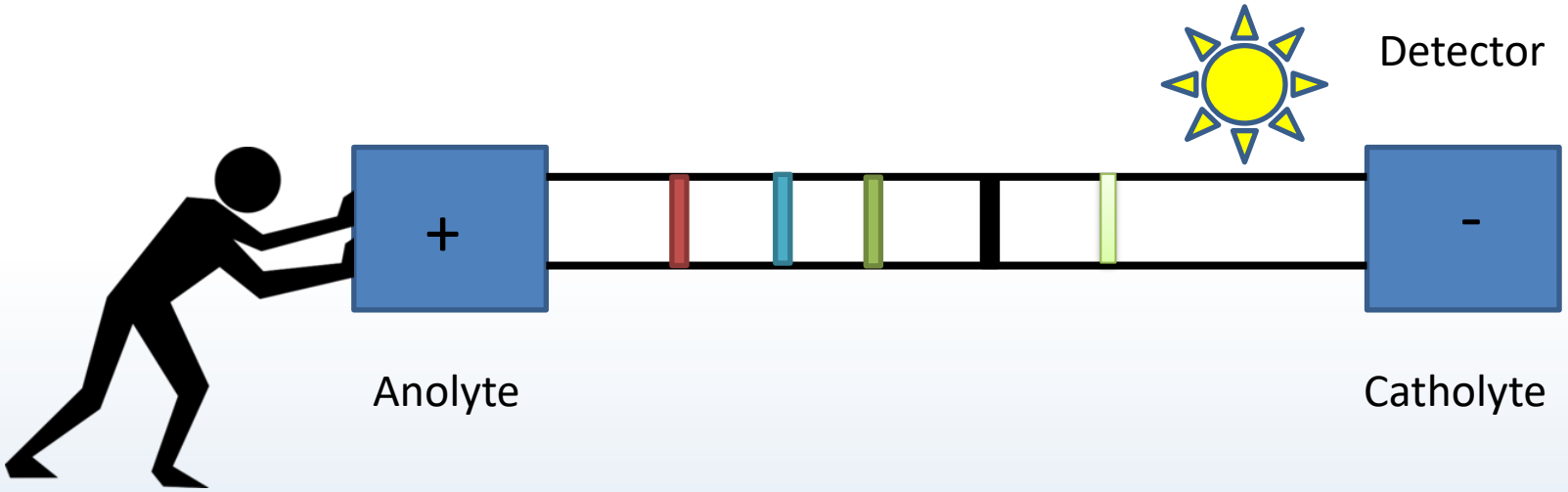
Mobilisation

- To be detected the analytes must be moved past the point of detection – **MOBILISATION**
- Two approaches:
  - **Hydrodynamic or pressure mobilisation**
  - **Chemical mobilisation**



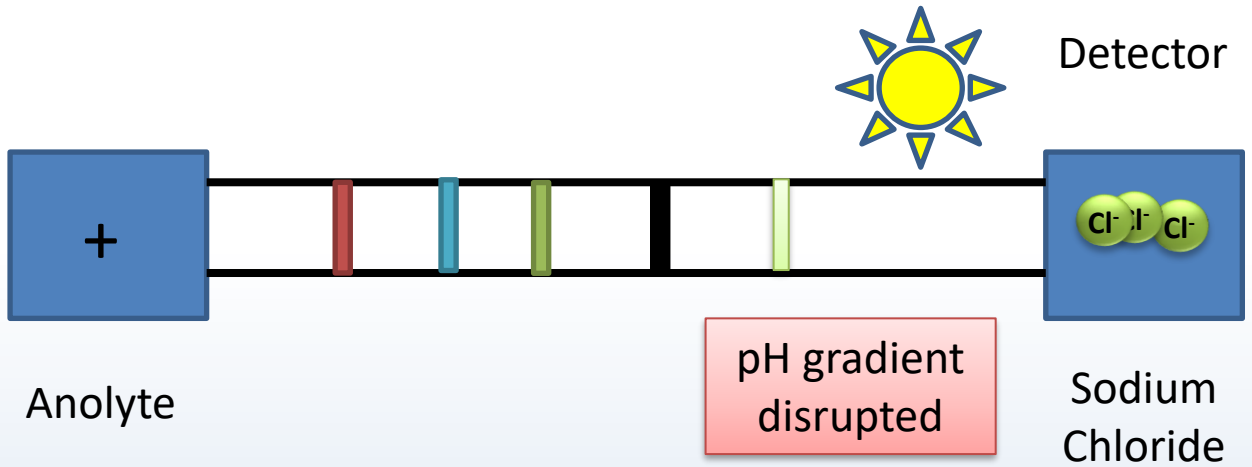
Hydrodynamic mobilisation

- A slight **pressure is applied** to the anolyte vial
- The content is pushed forward and every zone is driven to the point of detection
- Some broadening of the focused analyte zones might occur



Chemical mobilisation

- The anolyte or catholyte is replaced by another **electrolyte** solution **with different pH or ionic strength** (e.g sodium chloride, acetic acid)
- This **disrupts the pH gradient** causing analytes to acquire the net charge and start moving towards the outlet



Capillary Electrophoresis

Capillary electrophoresis

+ Automatic
+ High resolution
+ On-line detection
+ Quantitative
+ Minimal sample volume requirements
- Parallel operation

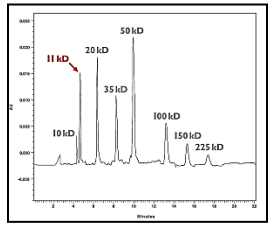
Topics

- Principles of Capillary Electrophoresis
- CE Instrumentation
- Separation Modes
- Applications

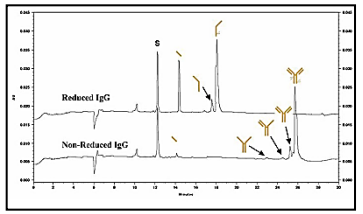
Applications

Examples

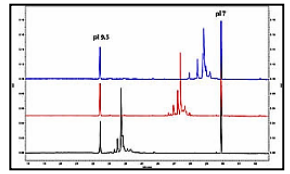
- Determination of SDS molecular weight of protein



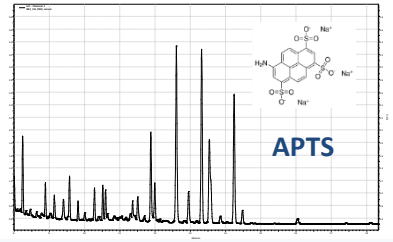
- IgG purity and heterogeneity determination



- Capillary Iso-electric focusing (CIEF): determination of charge heterogeneity



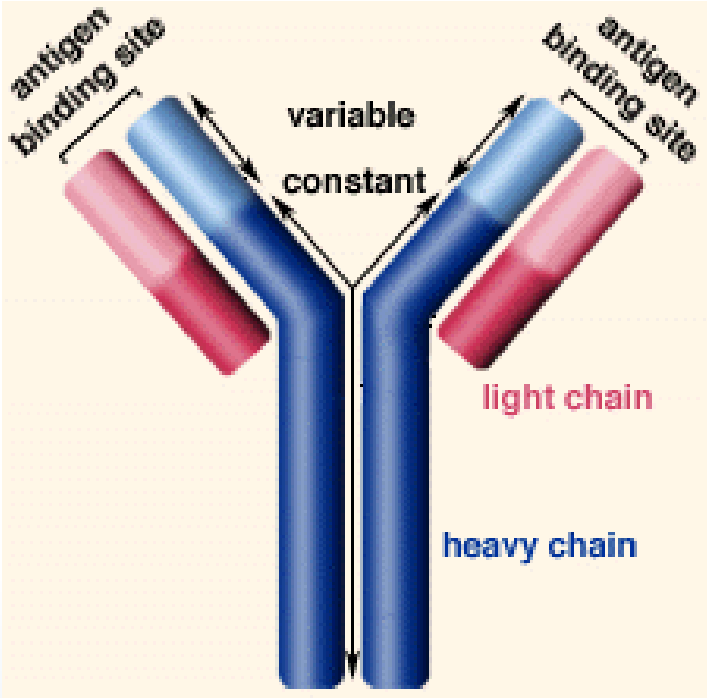
- Carbohydrate analysis with CE-LIF



Images from Beckman Coulter

1. IgG purity and heterogeneity determination

- Separation by CGE: **by size**
- Allows resolution of IgG isoforms and impurities
- Can analyse IgG in non-reduced or reduced forms:



Non-reduced IgG:

- Intact non-glycosylated tetramer (**2HC**, **2LC**)
- Intact glycosylated tetramer (**2HC**, **2LC**)

Reduced IgG:

- **Light chain** (~25kDa)
- **Non-glycosylated heavy chain** (<50kDa)
- **Glycosylated heavy chain** (~50kDa)

1. IgG purity and heterogeneity determination

Example:

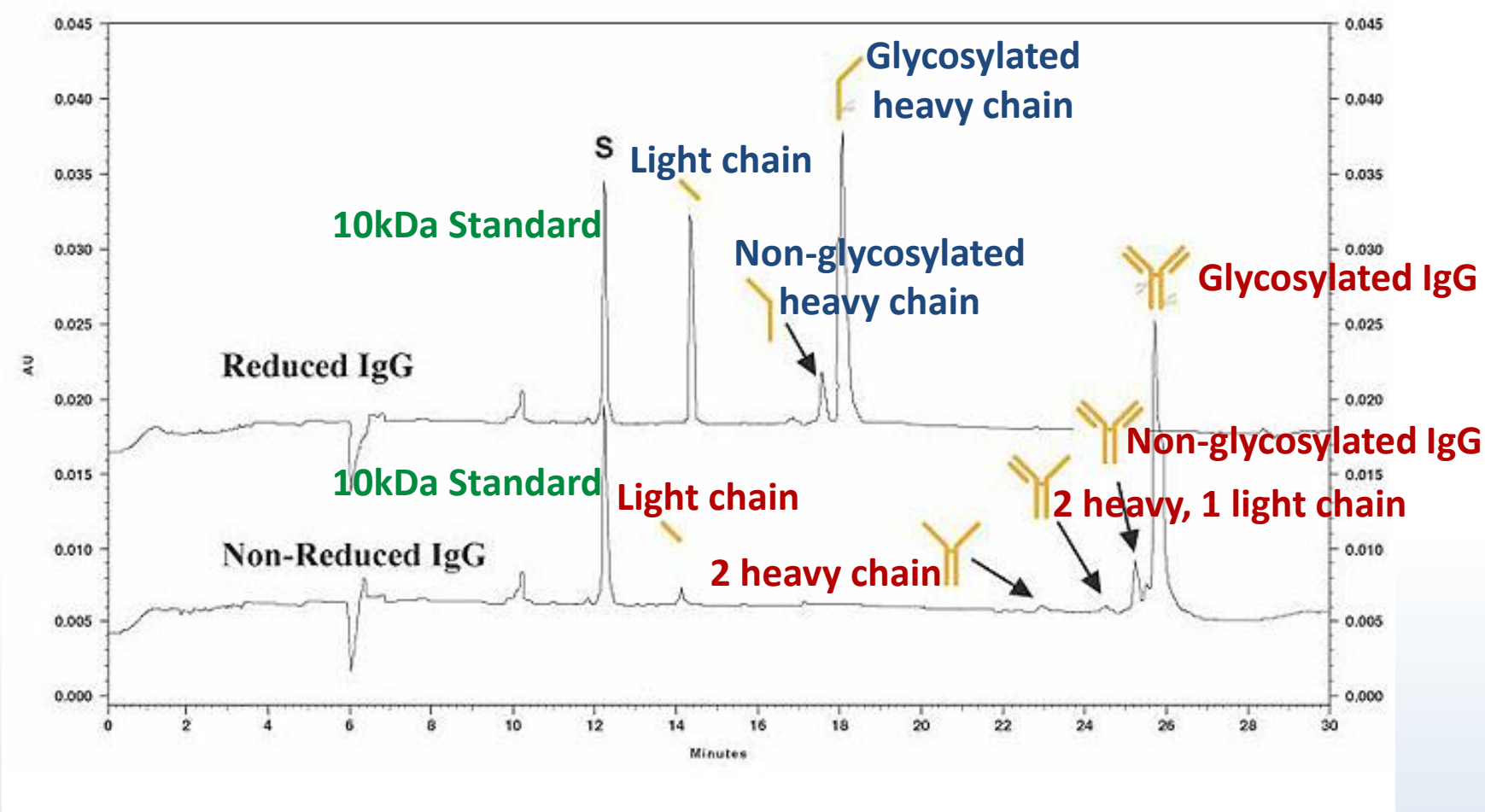
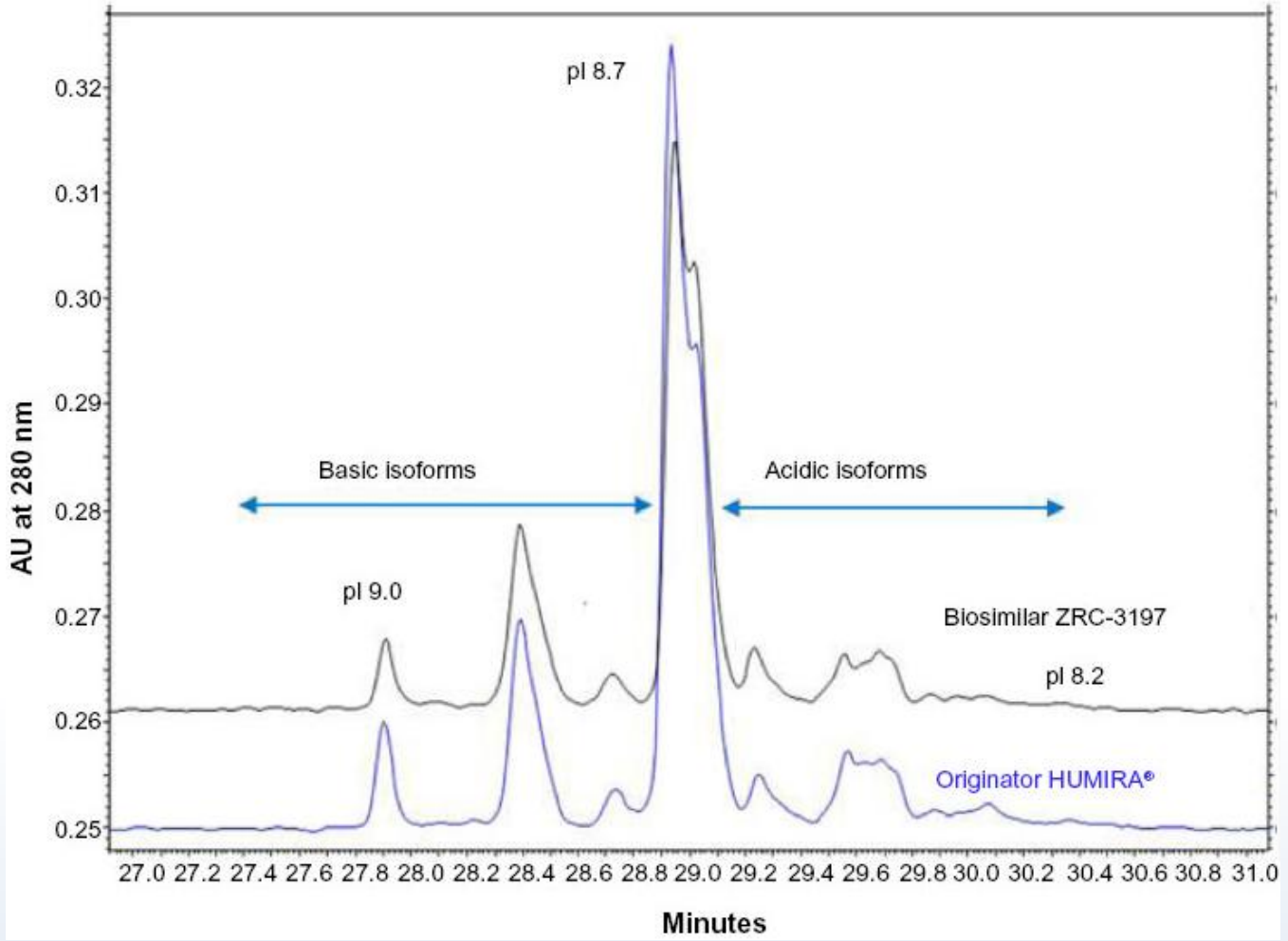


Image from Beckman Coulter

2. Analysis of charge variants by cIEF



Bandyopadhyay S, Physicochemical and functional characterization of a biosimilar adalimumab ZRC-3197, Dovepress, 2014.

Topics

- Principles of Capillary Electrophoresis
- CE Instrumentation
- Separation Modes
- Applications

Thank You