Pharmaceutical Analysis

(Separation methods)

Chromatography

الأستاذ الدكتور جمعه الزهوري (دكتوراه صيدلة-ألمانيا 1991)

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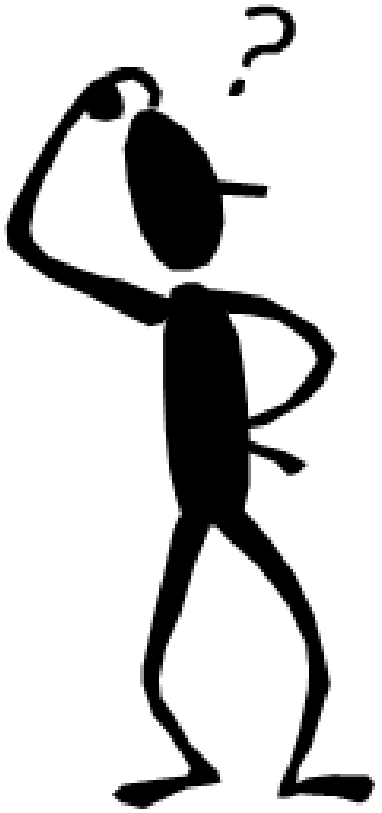
Electrophoresis

What is

Electrophoresis ?



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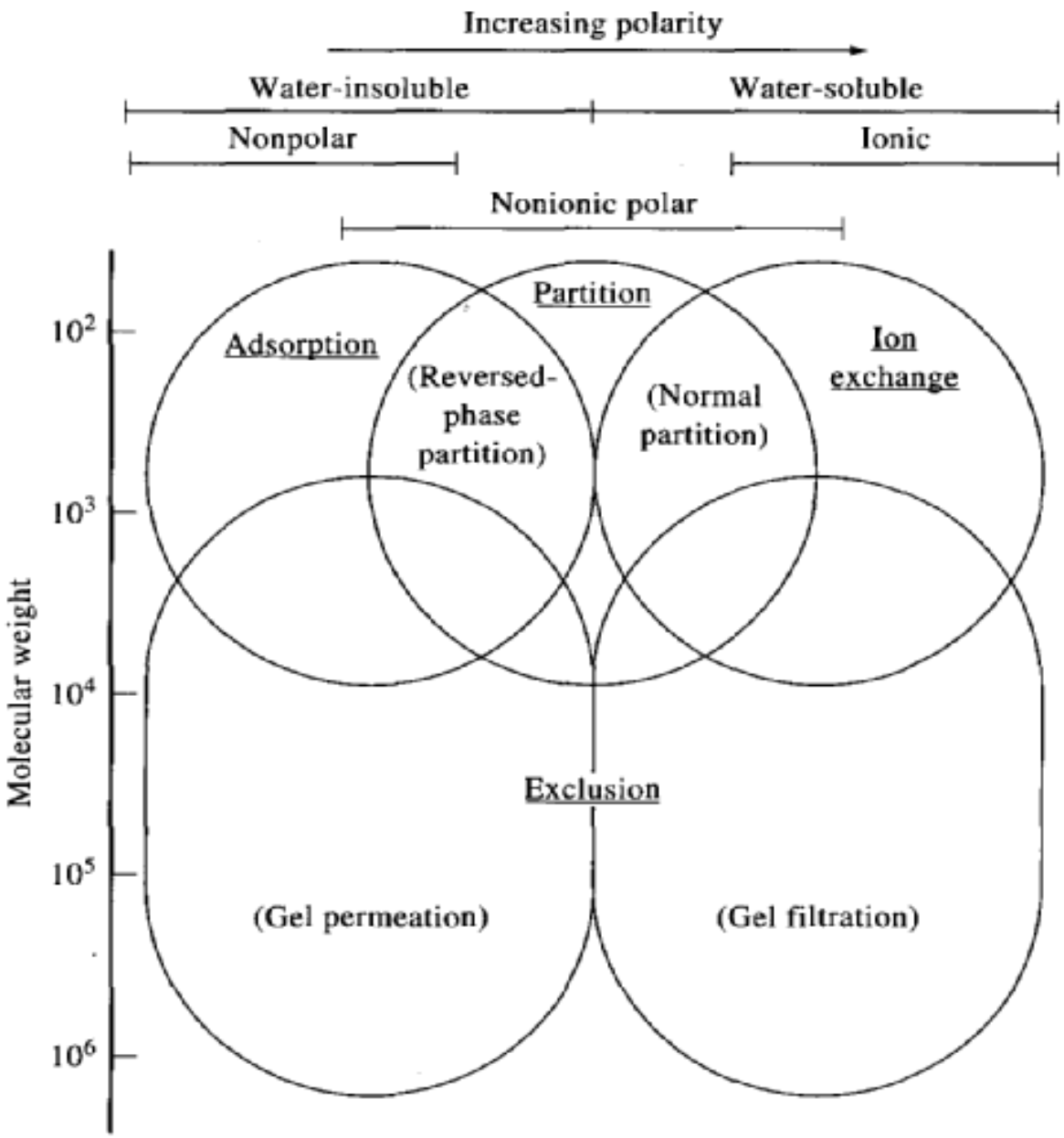


Applications of liquid chromatography.

An overview of ELECTROPHORESIS

- Electrophoresis is a separation method based on the differential rate of migration of charged species in a buffer solution across (by attraction or repulsion) in an electric field.

فصل المواد الضخمة بسبب اختلاف هجرة المواد المشحونة تحت تأثير حقل كهربائي ضمن وقاء

An overview of ELECTROPHORESIS

- The separation technique was first developed by the Swedish chemist Arne Tiselius in the 1930 for the study of serum proteins.
- Tiselius was awarded in 1948 Nobel Prize for this work.
- Electrophoresis has been applied to a variety of difficult analytical separation problems : inorganic anions and cations, amino acids ,catecholamine, drugs, vitamins ,carbohydrates, peptides, proteins, nucleic acids, nucleotides, polynucleotide and numerous other species.

- EP is suitable for sample that may be difficult to separate by LC because the principle of separation are different and not involving surface adsorption.
- A particular strength of electrophoresis is its unique ability to separate charged macromolecules of interest in the biotechnology industry and in biochemical and biological research.

An overview of ELECTROPHORESIS

- For many years , electrophoresis has been the powerhouse method of separation of proteins (enzymes, hormones, antibodies) and nucleic acids (DNA,RNA) , for which it offers unparalleled resolution.
- An electrophoretic separation is performed by injecting a small band of the sample into an aqueous buffer solution that is contained in a narrow tube or on a flat porous support medium such as paper or a semisolid gel.

An overview of ELECTROPHORESIS

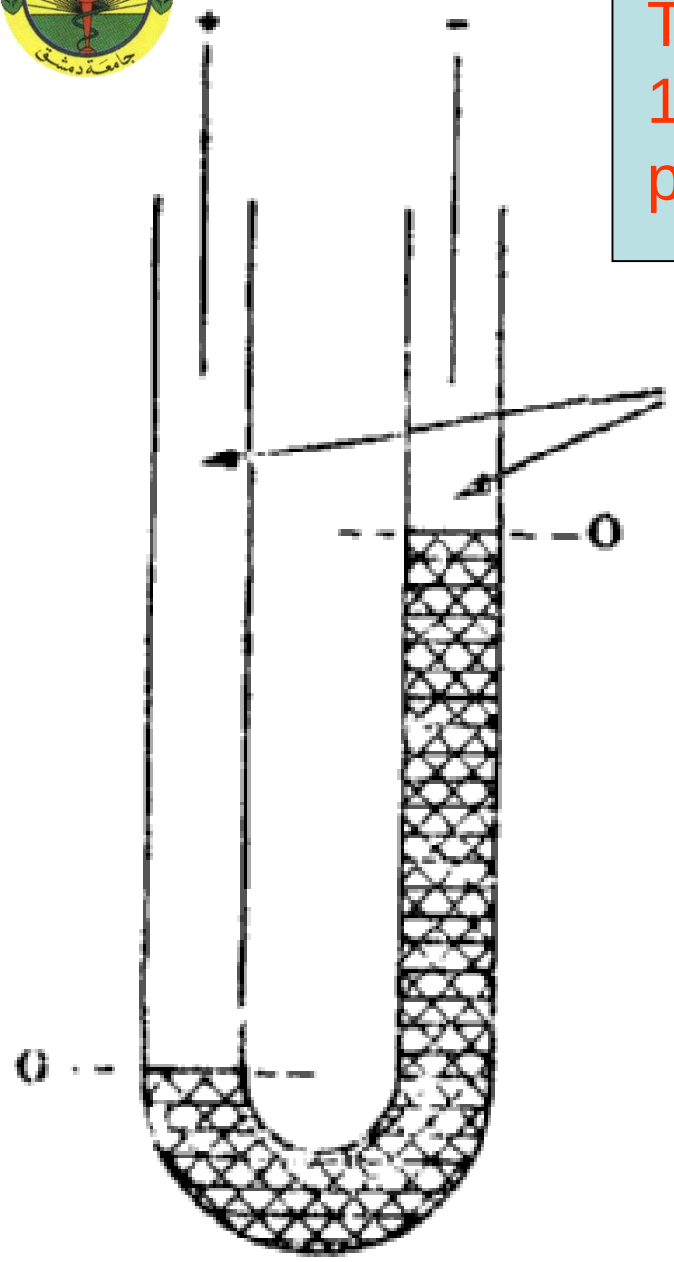
- A high dc potential is applied across the length of the buffer by means of a pair of electrodes located at either end of the buffer .This potential causes ions of the sample to migrate toward one or the other of the electrodes .
- The rate of migration of a given species depends upon its charge and also upon its size.
- Separation are then based upon differences in charge-to-size ratios for the various analytes in a sample.
- **The larger this ratio, the faster an ion migrates in the electrical field.**

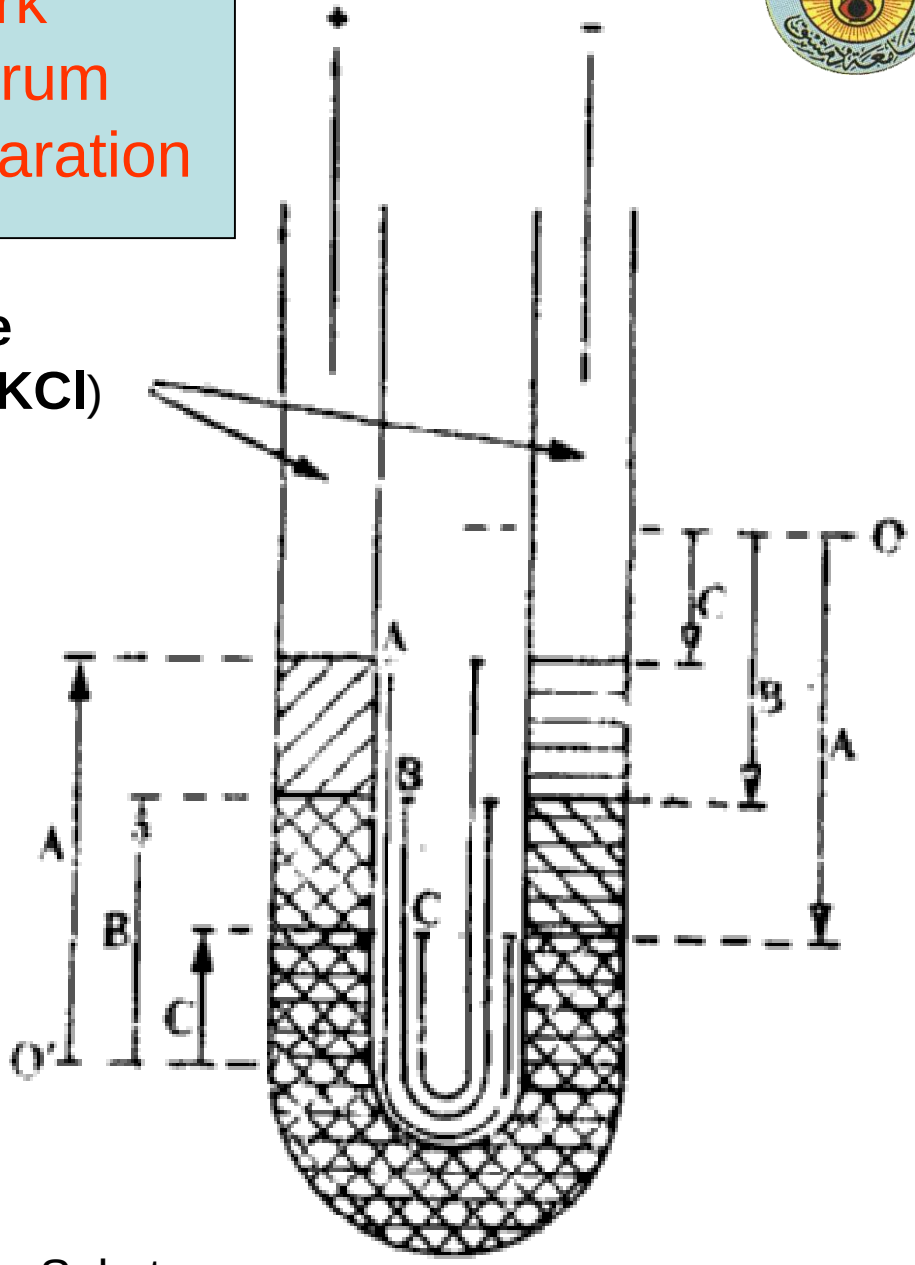
Solution

electrolyte

Tiselius work 1948 for serum protein separation

Electrolyte solution (KCl)





O= start point , A,B and C= Substances

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Electrophor

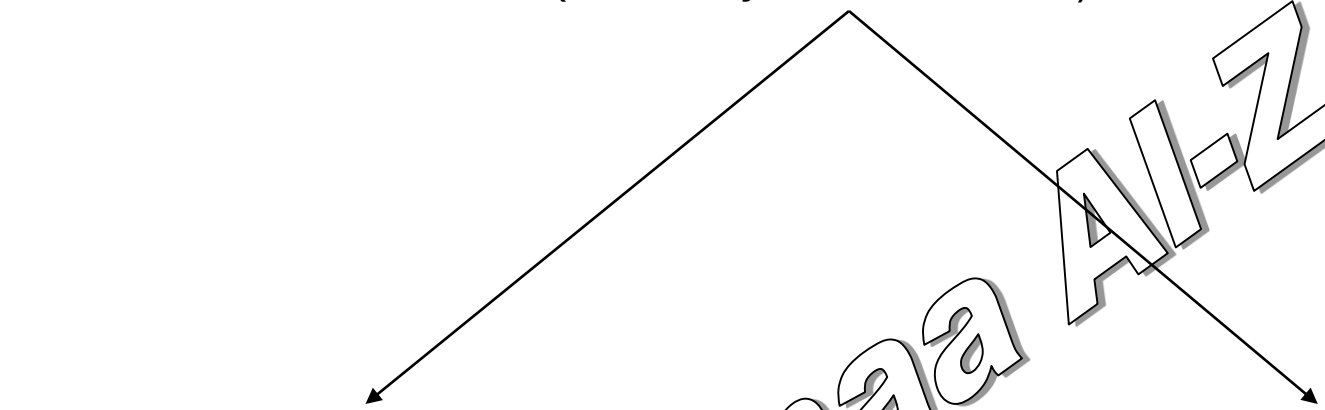
Stabilizing media methods

- Presence of a supporting medium like paper, packing, or gel.
- Resemble chromatographic methods except that migration is based on an electrical field instead of a mobile phase.
- A number of methods have been based on this approach including
  - Electrochromatography
  - Zone electrophoresis electromigration
  - Ionophoresis

Most of this unit will deal with electrochromatography -- specifically capillary electrophoresis (CE).

Types of Electrophoresis

(Nowadays classification)



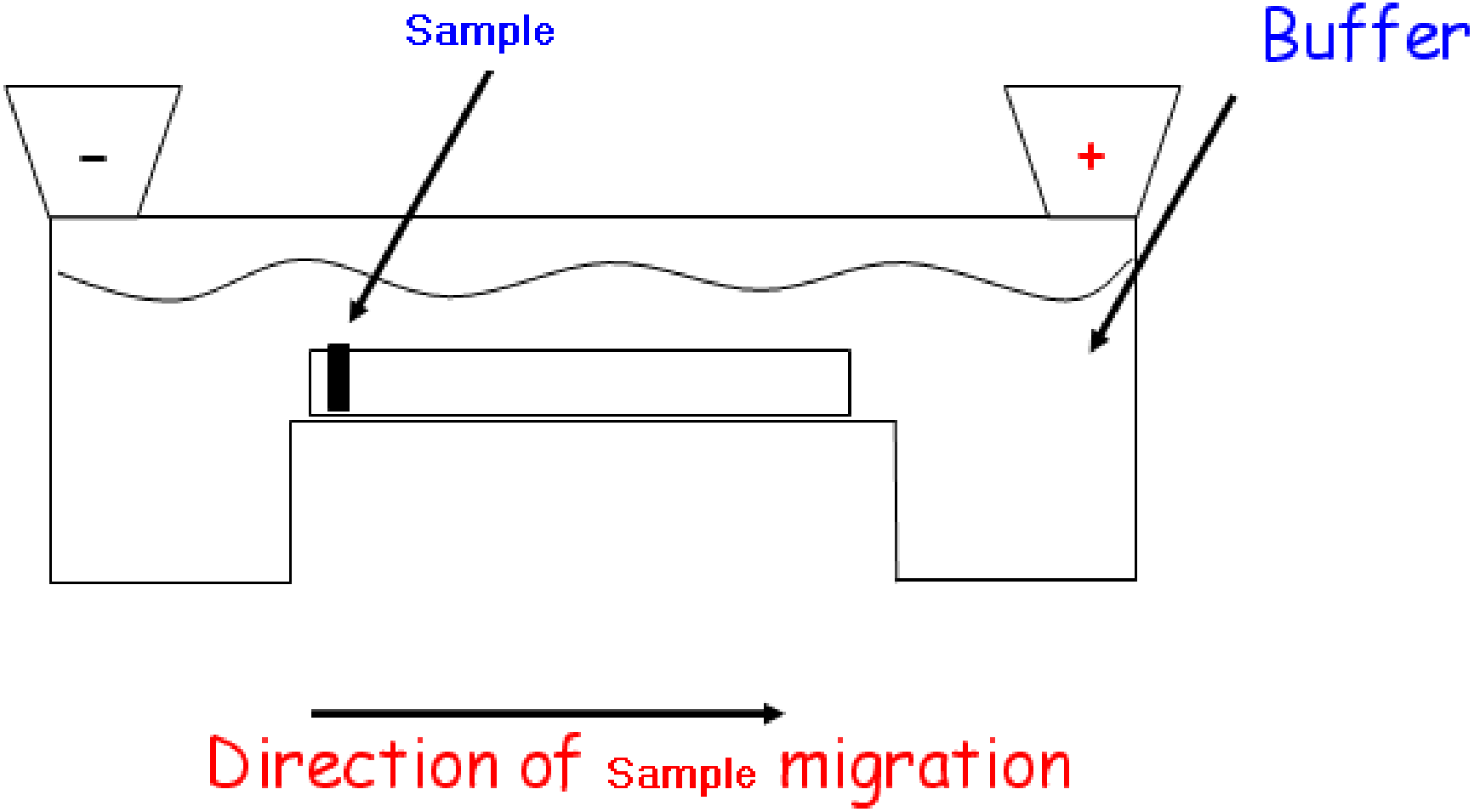
Slab electrophoresis

Capillary electrophoresis

Slab electrophoresis

Slab electrophoresis

- It is the classic method that has been used for many years to separate complex, high – molecular –weight species of biological and biochemical interest.
- Slab separations are carried out on a thin flat layer or slab (Supports) of a porous semisolid gel containing an aqueous buffer solution within its pores.
- Ordinarily this slab has dimension of a few centimeters on a side and like TLC-plate.


Sample
Buffer
–
+
Direction of Sample migration

- The common supports include :
  - Simi solid gels (Starch gels)
  - Polyacrylamide gels
  - Polyurethane foam
  - Paper

Slab electrophoresis is capable of separating several samples simultaneously .It is currently the most widely used in biochemist and the biologist

- Samples are introduced as spots or bands on the slab, and a dc potential is applied across the slab for fixed period.
- When current is passed through the cell, the different components of a mixture move with velocities that depend on their:

  - electric charge
  - their sizes
  - and their shapes.

The Basis for Electrophoretic Separation

- The *migration velocity v* of an ion in centimeters per second in an electric field is equal to the product of the field strength E(Vcm^{-1}).and the *electrophoretic mobility* μ_e ($cm^2V^{-1}{}_S{}^{-1}$). That is

$$V = \mu_e E$$

- The electrophoretic mobility is in turn proportional to the ionic charge on the analyte and inversely proportional to frictional retarding factors.
- The electric field acts only on ions.

- The frictional retarding force on an analyte ion is determined by the size and shape of the ion and the viscosity of the medium in which it migrates.
- For ions of the same size ,the greater the the charge ,the greater the driving force and the faster the rate of migration.
- For ions of the same charge ,the smaller the ion ,the smaller the frictional forces and the faster the rate of migration.

The Basis for Electrophoretic Separation

- The ion ُs *charge-to-size ratio* combines these two effects.

Note that in contrast to chromatography, only one phase is involved in an electrophoretic separation

- As electrophoresis proceeds, the negatively charged components migrate toward the anode and the positively charged components migrate toward the cathode. The result is a series of separated bands or lines of sample constituents, such as visualized by a stain.

بحال تساوي الأيونات بالحجم فهنا الأيون ذي الشحنة الأكبر يهاجر بسرعة أكبر أما إذا تساوت الشحنات فالأصغر تكون أقل احتكاكاً وتهاجر أسرع

Neutral species are not separated

- When the separation are judged complete, the current is discontinued and the separated species are visualized by staining in much the same way as was described for TLC.
- The applied voltage is expressed in volts per centimeter. It is up to 500 v in low-voltage electrophoresis and can be several thousand volts in high-voltage electrophoresis.
- Amino acids and proteins do not migrate at the pH of their **isolectric point** , because at this point the net charge is zero and it exists as a zwitterion.

What is the isoelectric point ?

Isoelectric Points

- pH at which the protein has no NET charge

$NH_3^+ - CH_2 - COO^-$

$+1 \qquad -1$

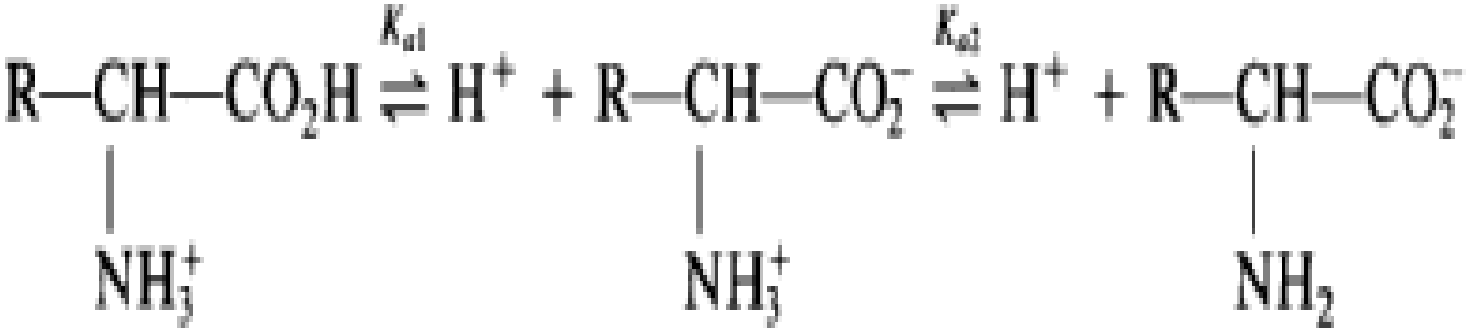
Net Charge = 0

Glycine

pI = 5.97

الحموض الأمينية لا تهاجر عند البهاء التي تكون فيه الحموض لها شحنتين Zwitterion حيث معدل الشحنة يصبح صفر وتسمى هذه النقطة بنقطة التساوي الكهربائي

Mobility is affected by the pH which influences the charge on the analyte.



Isoelectric Focusing

NH3+ - CH2 - COOH NH3+ - CH2 - COO- NH2+ - CH2 - COO-

+1 -1

pH = 2 ⟵ HCl pI = 5.97 NaOH ⟶ pH = 10

>>>H+ glycine >>>OH-

Paper electrophoresis

One type of support is paper.

- The paper is saturated with a buffering solution and a sample introduced at one point.
- A dc potential of 100-1000V is applied (current is in the milliamp range).
- Species will migrate to specific points on the paper.
- After an appropriate period of time, the paper is removed and dried
- If required, the paper is treated with a color producing agent so that the bands can be observed.

Polyacrylamide Gels

- A polyacrylamide gel forms a matrix that acts as a molecular sieve
- Components:
  - acrylamide and bis acrylamide: provide matrix
  - Tris: buffer
  - Ammonium persulfate: catalyst
  - TEMED: polymerization agent
- Native (non-denaturing) gels are run without SDS (sodium dodecyl sulfate)

The daily analysis :

- SPE
- Hb- Electrophoresis

-Gel Electrophoresis is used to :

DNA Fingerprinting

DNA Fingerprinting

DNA Fingerprinting, method of identification that compares fragments of deoxyribonucleic acid (DNA) It is sometimes called DNA typing. DNA is the genetic material found within the cell nuclei of all living things. In mammals the strands of DNA are grouped into structures called chromosomes. With the exception of identical twins, the complete DNA of each individual is unique.

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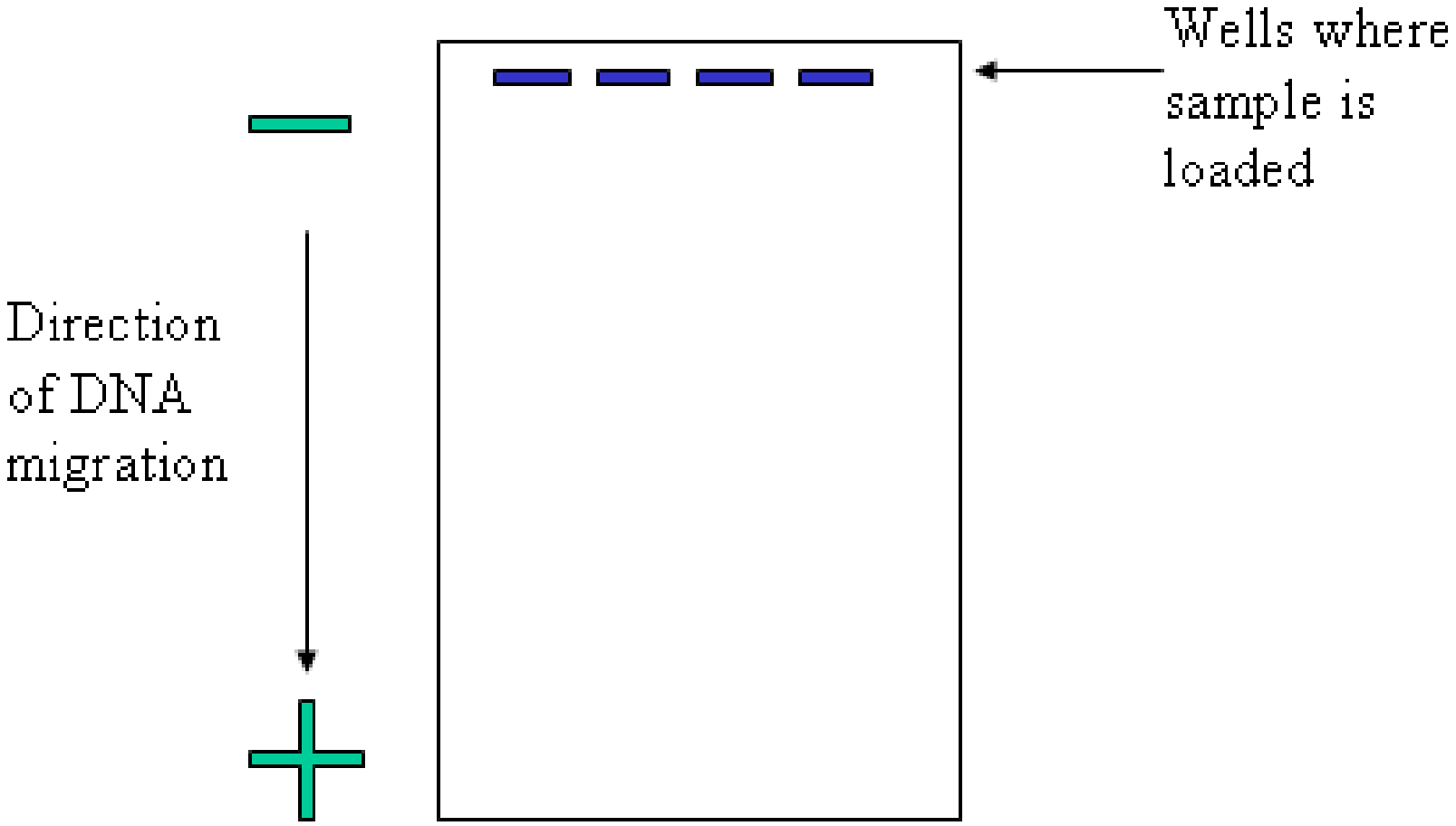
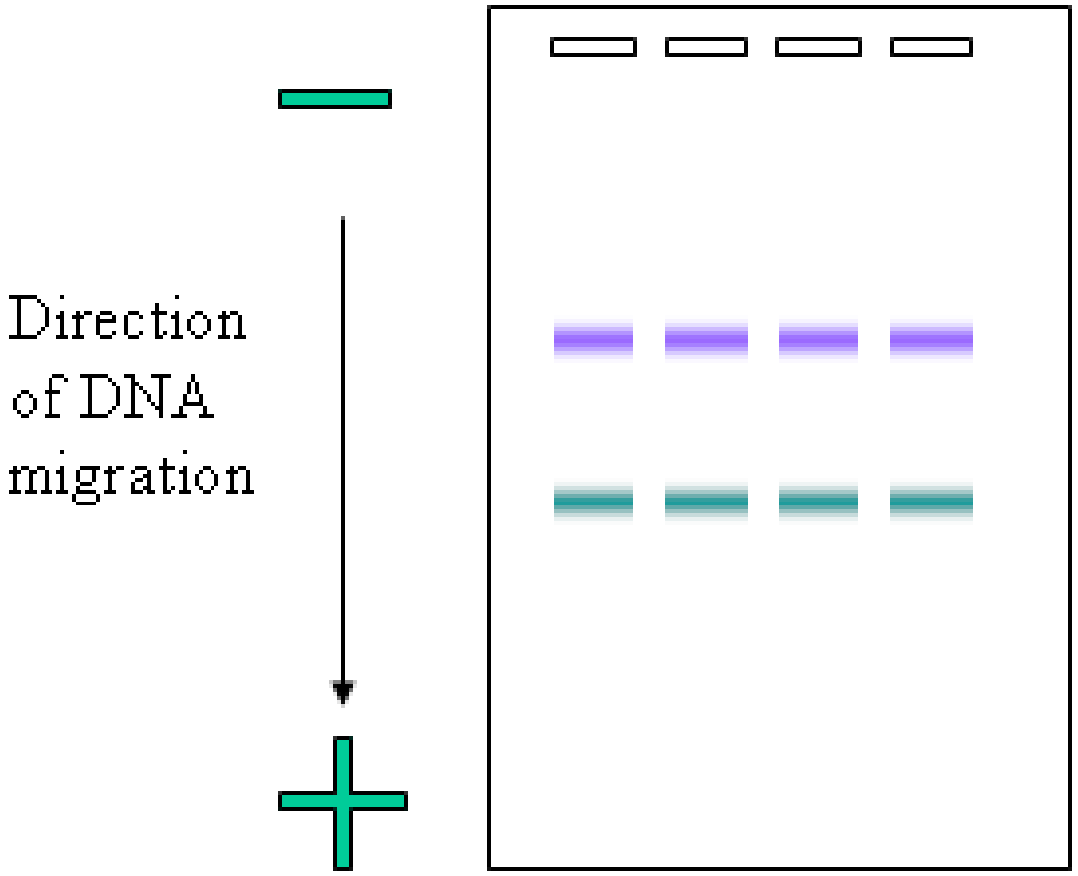
Gel electrophoresis of DNA

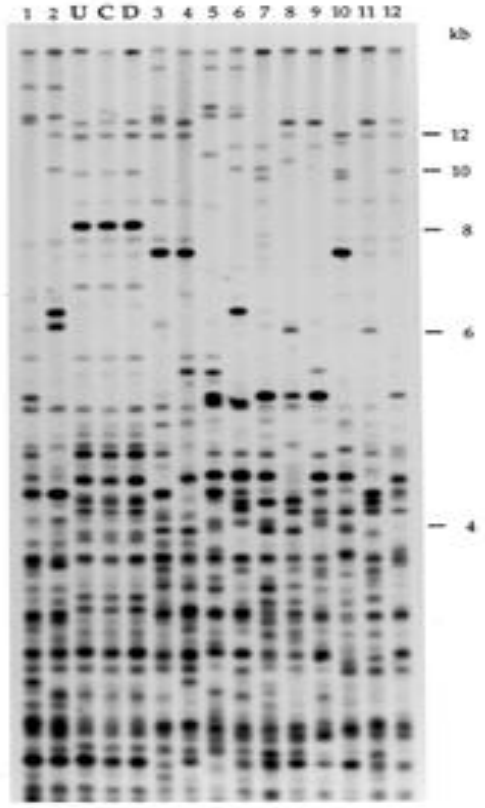


Gel electrophoresis of DNA



Historical Perspective

- DNA Fingerprinting was first developed in 1985
- The FBI now has a database called the National DNA Index System, which has data both from convicted criminals and crime scenes.



Fingerprinting Dolly
Nature **394**, 329 - 330 (23 July 1998)

Donor Udder (U)
Derived Cells (C)
Dolly (D)
Control sheep (1-12)

Slab electrophoresis

- Typically slow.
- Sample required in µl range.
- Labor intensive.
- Difficult to automate.
- Does not yield very precise quantitative information.
- Cumbersome staining techniques

Capillary Electrophoresis

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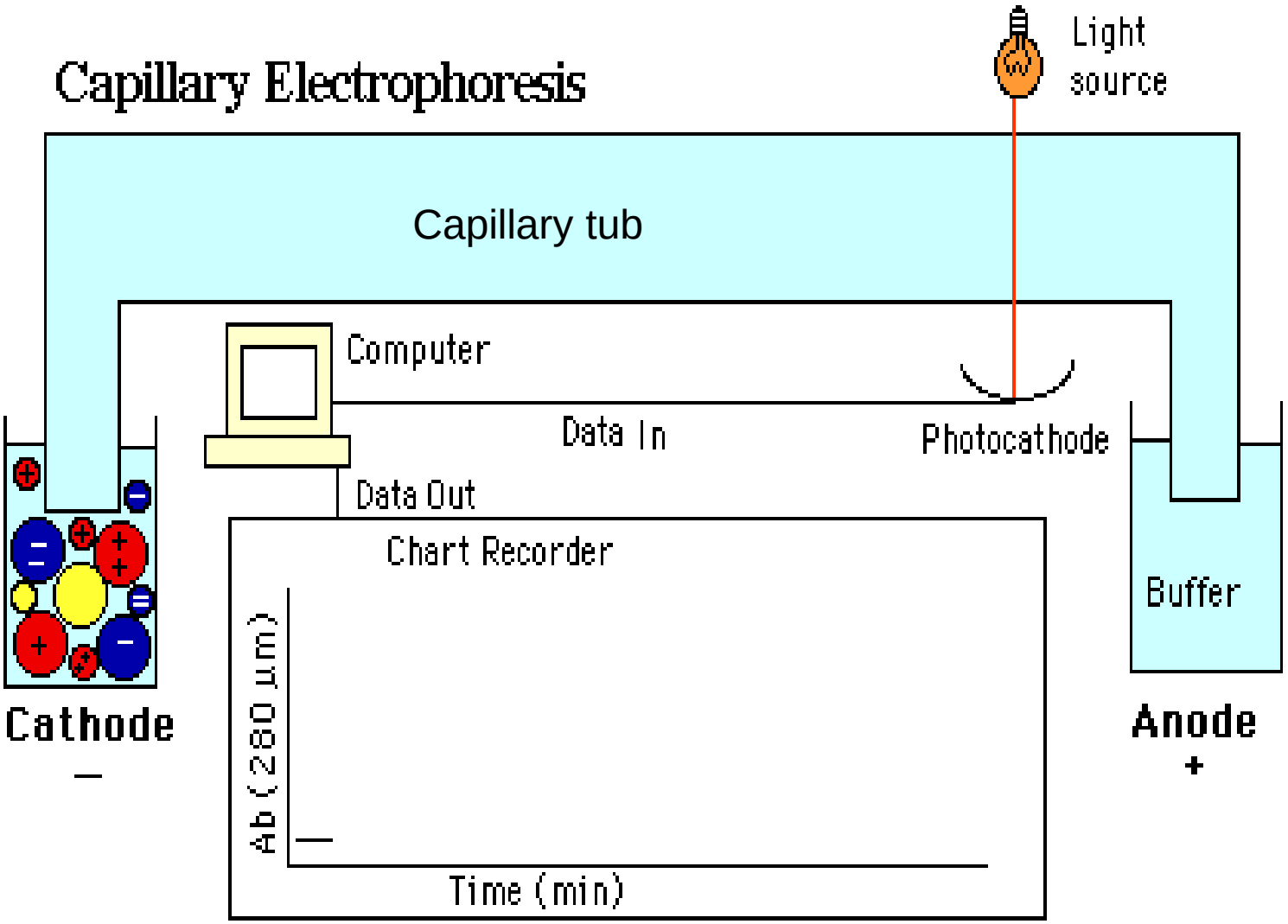
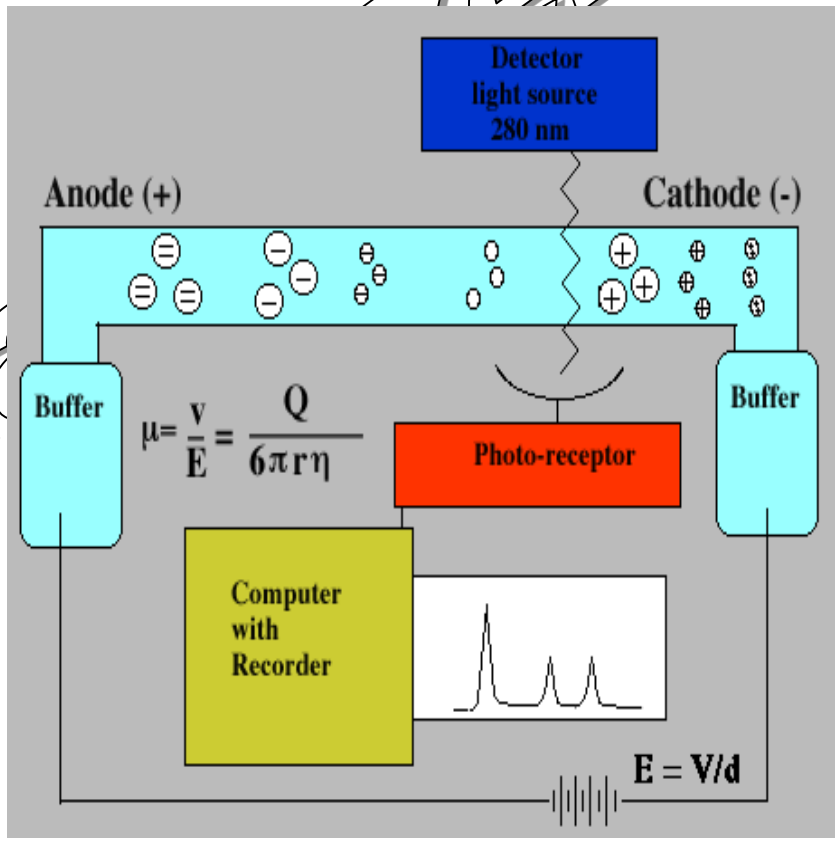


الفصل يتم على عمود شعي 20-50 سم وقطره 25-75 ميكرومتر ويلاحظ هجرة كافة الجزيئات هنا .

Basics cont.

- A photocathode is then used to measure the absorbencies of the molecules as they pass through the solution
- The absorbencies are analyzed by a computer and they are represented graphically



Capillary electrophoresis

- Capillary electrophoresis ,which is an instrumental version of electrophoresis ,has been developed and used only in the last 20 years and has become an important separation tool used by chemists and life scientists.
- This new method appears to be a satisfactory substitute for slab electrophoresis with several important advantages.

Capillary electrophoresis

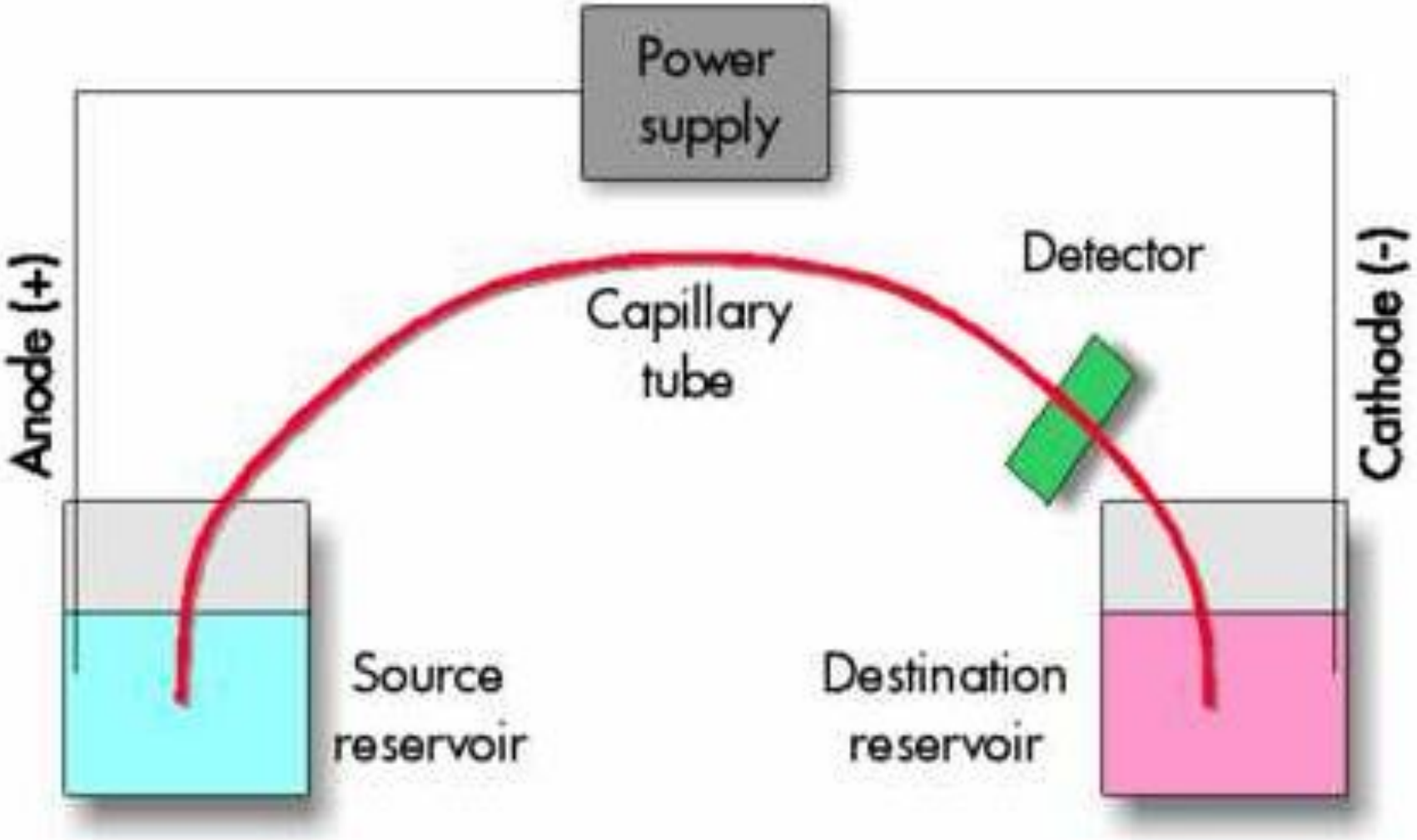
- High-speed
- High-resolution separation
- Sample required 0.1-10 nl.
- The separated species are eluted from one end of the capillary ,so quantitative detectors, like those of HPLC,can be used. .

- The first CE apparatus was designed by Hjerten (1967)
- The modern area of CE is considered to begin with many publications of Jorgensos and Lukacs (1981 -1983)
- CE had been a topic of discussion among the scientists in International Symposium on High Performance Capillary Electrophoresis 1993 (Wehr and Zhu)

Presently ,CE is :

- A versatile technique of high speed.
- High sensitivity
- Low limit of detection
- Suitable for sample that may be difficult to separate by HPLC
- New principles of separation.
- Inexpensive running cost.

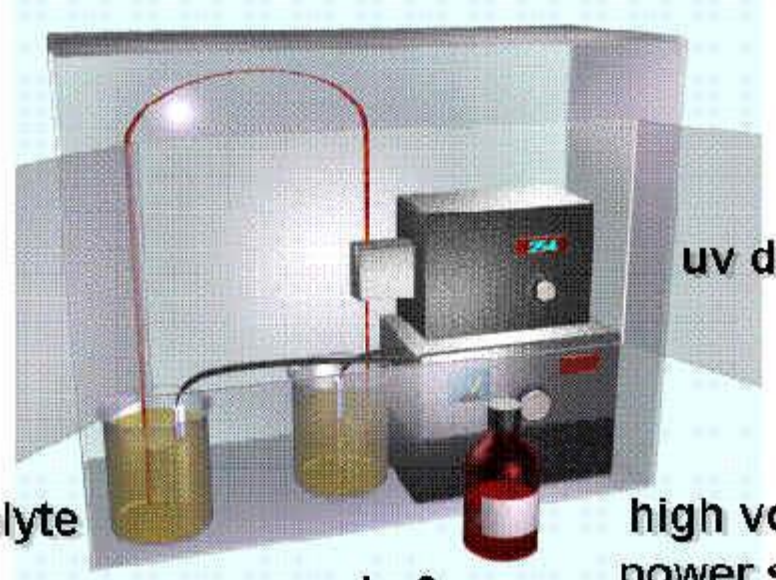
Basic equipmen



Capillary Electrophoresis – The Basics

- Electrophoresis in a buffer filled, narrow-bore capillaries
- Each capillary is about 25-75 μm in internal diameter (2 5-50 cm in length).
- When a voltage is applied to the solution, the molecules move through the solution towards the electrode of opposite charge
- Depending on the charge, the molecules move through at different speeds
  - Separation is achieved

Capillary Electrophoresis Apparatus



Capillary Electrophoresis (C

Much of the current research is in the development of CE.

- A conducting buffer is retained in a capillary tube.
- Tube ID is typically in 25-75 μm range.
- Use of a capillary tube helps overcome the problems associated with heating or interaction/degradation of the support
- It is a Free-Solution method.
- Samples are typically injected in one end and migrate to the other.
- Similar to chromatography - resulting electropherogram. Both quant and qual information.

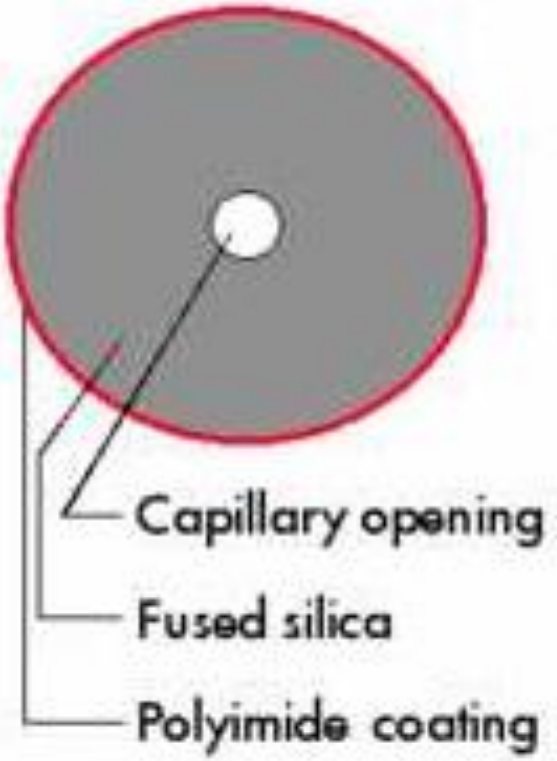
Capillary tube

ID typically 25-75 μm.

Length varies based on application but is normally in the 20-50 cm range.

The small bore and thickness of the silica are important. When a current is applied, this leads to Joule heating.

Using a small ID and having a thick wall reduces this problem.



Migration Rate in Capillary Electrophoresis

- An ion sُ migration velocity v depends upon the electric field strength.
- The electric field in turn is determined by the magnitude of the applied potential(V,in volts) and the length L over which it is applied.Thus:

$$v = \mu_e \cdot \frac{V}{L}$$

The relationship indicates that high applied potentials are desirable to achieve rapid ionic migration and a rapid separation.

It is desirable to have rapid separation ,but it is even more important to archive high –resolution separations. So we need to examine the factors that determine resolution in electrophoresis.

Plate Height in capillary Electrophoresis

- It has been shown for CE, that plate count (N) is given by :

$$N = \frac{\mu_e V}{2D}$$

D= diffusion coefficient of the solute in cm^2s^{-1}

- Contrary to Chromatography the plate count does not increase with the length of the column.

Plate Height in capillary Electrophoresis

- In slab electrophoresis, Applied Potential about 500 V.
- In Capillary E , 20000 -60000 V.
- Plate count in CE 100000 – 200000 (in HPLC 5000-20000)
- Plate counts of 3,000,000 have been reported for capillary zone E.
- Plate count of 10 000 000 have been reported for capillary gel electrophoresis of polynucleotides.

There are two factors that cause mobility of solutes.

Electrophoretic mobility

- Response to the electrical field.
- Cations migrate towards the cathode, anions to the anode and neutrals are not effected.

Electroosmotic flow

- Migration of solutes in response to the buffer solution's movement in response to the electrical field.
- Under normal conditions, the buffer moves towards the cathode. This tends to sweep all species in that direction - including anions and neutrals.

Electrophoretic Mobility

- The movement of ions solely due to the electric field, potential difference
- Cations migrate toward cathode
- Anions migrate toward anode
- Neutral molecules do not favor either

Electrophoretic mob

Migration velocity

Electrophoretic velocity - ν - how rapidly a solute migrates.

$$\nu = \mu_e E$$

Where: μ_e = solute's electrophoretic mobility
E = magnitute of the applied field.

According to stockes law

$$\mu_e = q / (6 \pi \eta r)$$

Where: q = solute charge
η = buffer solvent's viscosity
r = solute radius

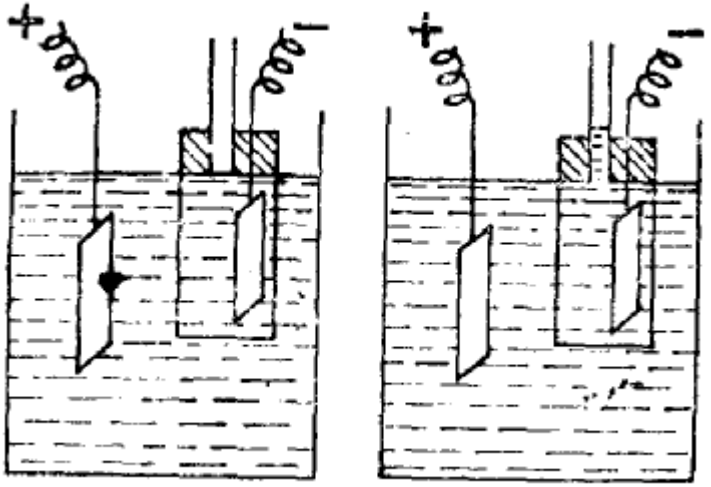
Increased charge and reduced size both result in greater μ_e .

Electrophoretic Mobility

- Properties that effect mobility
  1. Voltage applied
  2. Size and charge of the solute
  3. Viscosity of the buffer

Electroosmotic Flow

- When a high potential is applied across a capillary tube containing a buffer solution, electroosmotic flow usually occurs in which the solvent migrates.

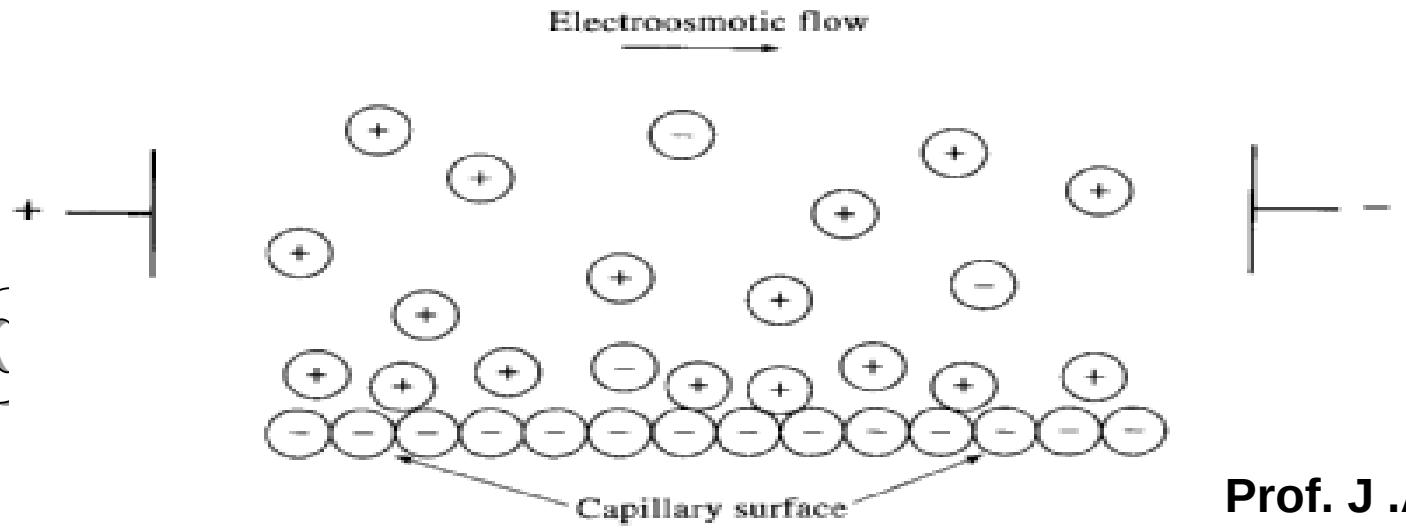


Electroosmotic Flow

- The cause of electroosmotic flow is the electric double layer that develops at the silica/solution interface.
- Above pH 3,the inside wall of a silica capillary is negatively charged due to ionization of the surface silanol group (Si-OH).
- Buffer cations congregate in an electric double layer adjacent to the negative surface of the silica capillary.

Electroosmotic Flow

- The cations in the diffuse outer layer of the double layer are attracted toward the cathode, or negative electrode, and since the cations are solvated, they drag the bulk solvent along with them.
- Electroosmosis lead to bulk solution flow.



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Electroosmotic Flow

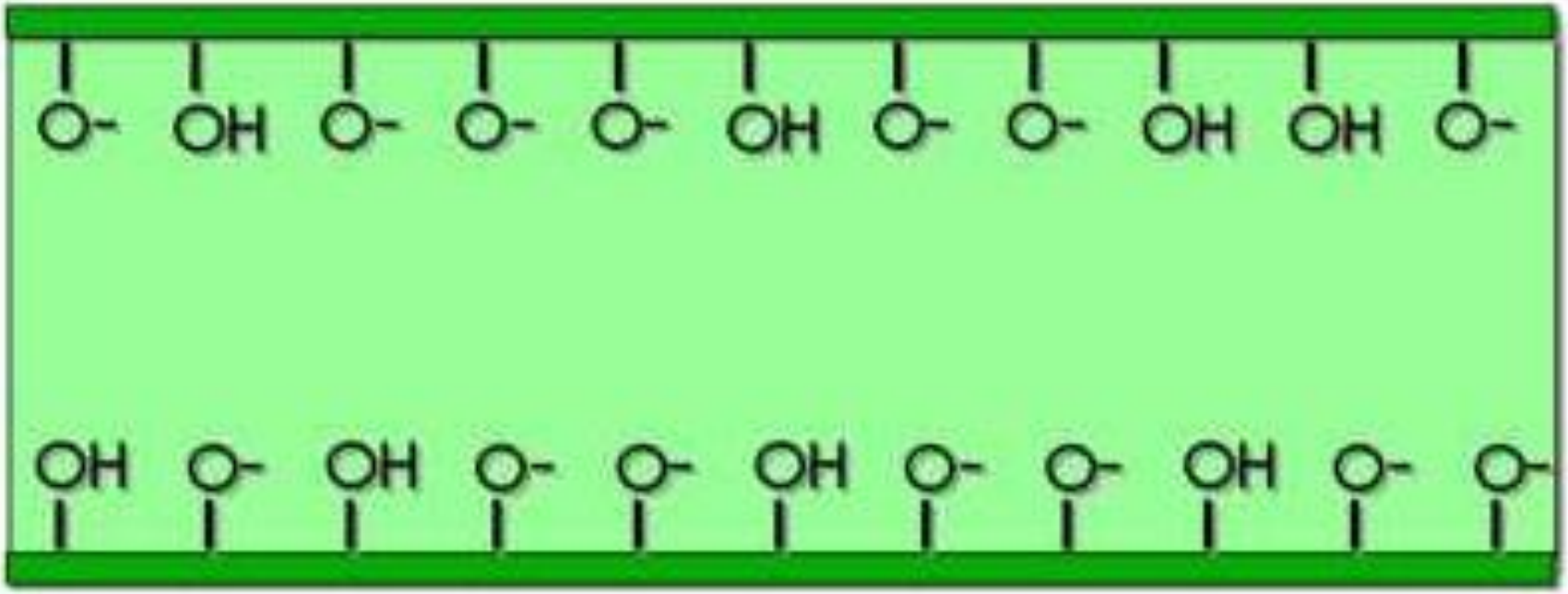
- The rate of electroosmotic flow is generally greater than the electrophoretic migration velocities of the individual ions.
- Even though analyte migrate according to their charges within the capillary ,the electroosmotic flow rate is usually sufficient to sweep all positive, neutral ,and even negative species to ward the same end of the capillary, so that all can be detected.

Electroosmotic Flow

- As the buffer sweeps toward the anode due to the electric field, osmotic flow dictates the direction and magnitude of solute ion flow within the buffer
  - All ions are then swept toward the anode.
  - Negative ions will lead the neutral ions toward the anode
  - Positive ions will trail the neutral ions as the cathode pulls them

Electroosmotic mobi

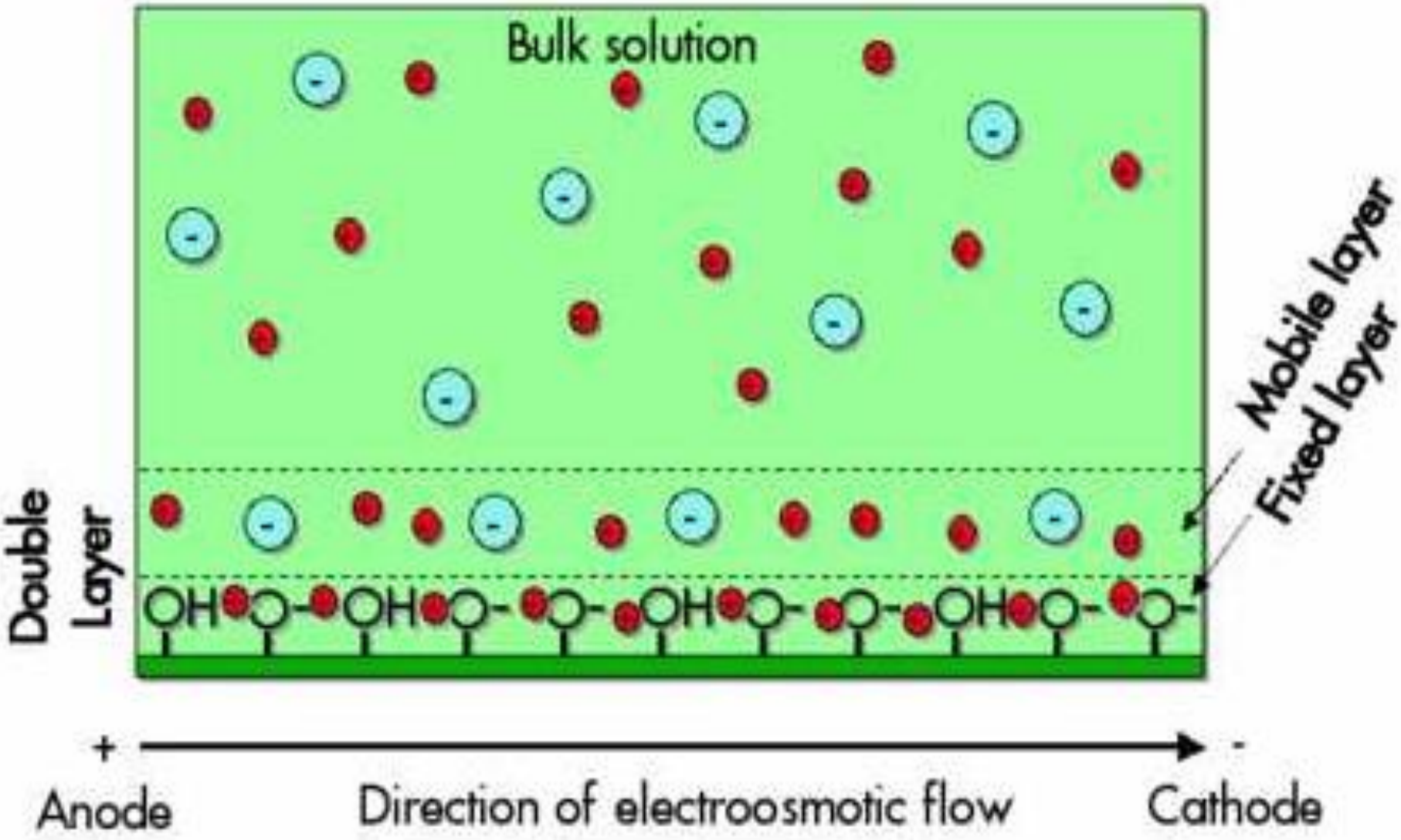
Under normal conditions, both anionic and neutral species will migrate towards the cathode. This occurs because the capillary will is electrically charged - large number of silanol groups (Si-OH).



Electroosmotic mob

- Cations are attracted towards the wall resulting in the formation of a double layer.
- The inner "fixed" layer results from cations being tightly bound to the wall.
- The second layer (mobility layer) is only loosely bound.
- Cations in the outer layer migrate towards the cathode.
- The solution is pulled along because the cations are solvated.

Double lay



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Electroosmotic flow velocity

$$\nu_{eo} = \mu_{eo} E$$

$$\mu_{eo} = \varepsilon \zeta / 4 \pi \eta$$

Where:

ε	=	buffer solution dielectric constant
ζ	=	zeta potential
η	=	buffer solution's viscosity

Zeta potential - the change in potential across a double layer.

Zeta potential

- It is directly proportional to the charge on the capillary walls. As pH increases, charge increases and zeta increases and μ_{eo} increases.

- It is proportional to the thickness of the double layer. As the ionic strength of the buffer increases, you have more cations. This will decrease the thickness of the layer.

Total mobility of sol

For any given solute, the total mobility is:

$$\nu_{tot} = \nu_{ep} + \nu_{eo}$$

$$\mu_{tot} = \mu_{ep} + \mu_{eo}$$

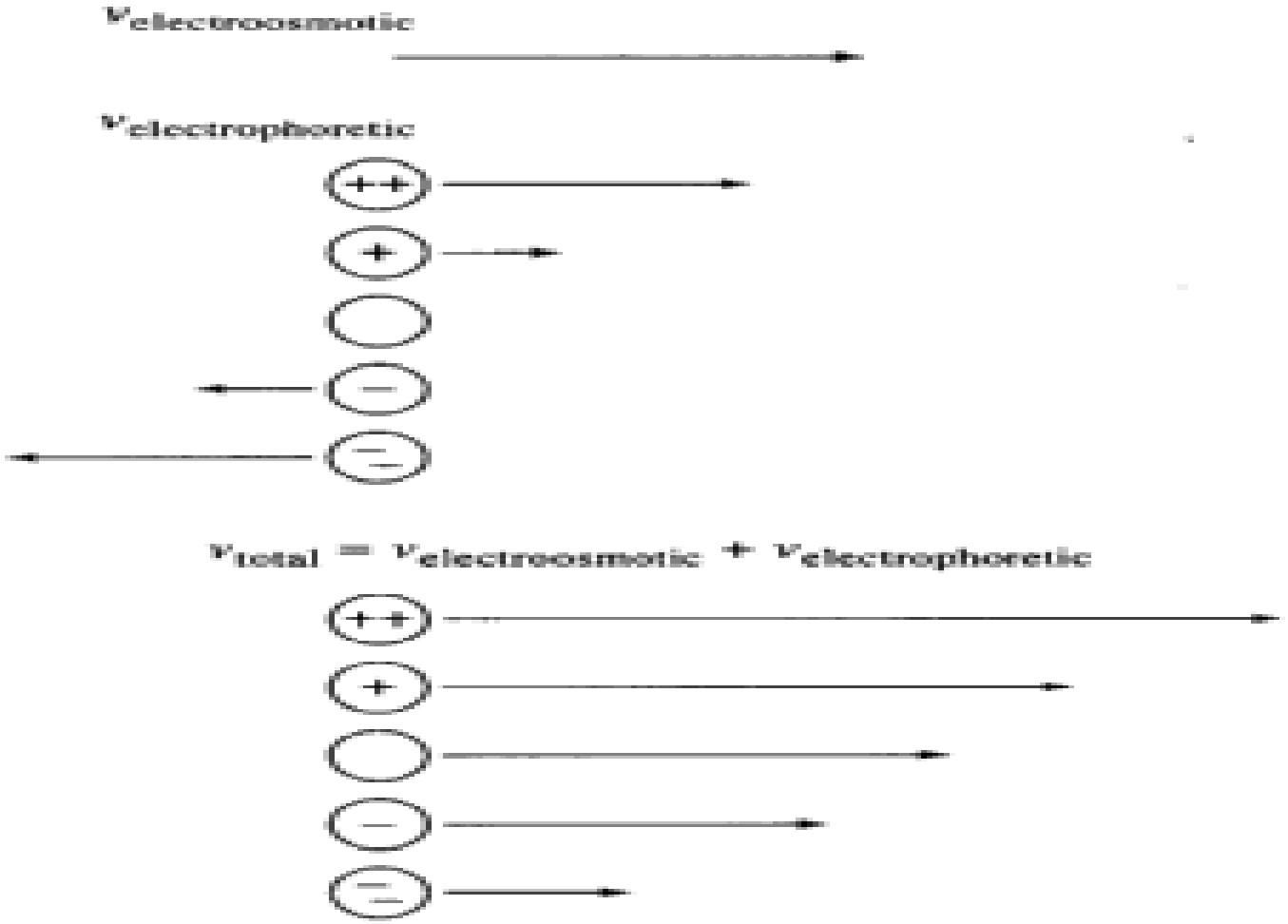
Under normal conditions

$$(\nu_{tot})_{cations} > \mu_{eo}$$

$$(\nu_{tot})_{anions} < \mu_{eo}$$

$$(\nu_{tot})_{neutrals} = \mu_{eo}$$

So cations elute first, based on their charge to size ratio - largest first. Neutrals then elute as a single band. Anions then elute based on charge to size ratio - reverse order.



Velocities in the presence of electroosmotic flow. The length of the arrow next to an ion indicates the magnitude of its velocity; the direction of the arrow indicates the direction of motion. The negative electrode would be to the right, and the positive electrode to the left of this section of solution.

Electroosmotic Flow

- Electroosmosis is often desirable in certain types of capillary electrophoresis ,but in other types is not.
- Electroosmotic flow can be eliminated by coating the inside capillary wall with reagent like trimethylchlorsilane eliminate the surface silanol group.

Migration t

$$V_{tot} = \ell / t_m$$

Where: ℓ = distance between injection & detection points.

t_m = migration time.

Since $V_{tot} = \mu_{tot} E = (\mu_{ep} + \mu_{eof}) E$

$$t_m = \ell / [(\mu_{ep} + \mu_{eof}) E]$$

The magnitute of the field is: $E = V / L$

Where V is the applied voltage and L is the length of the tube.

Migration ti

$$t_m = \frac{\ell L}{(\mu_{ep} + \mu_{eof})\, V}$$

This shows that you can decrease elution time by:

- increasing the applied voltage.
- using a shorter tube.

You can also increase the electroosmotic flow but only at the expense of resolution. This will be outlined in a bit.

The is the number of theoretical plates - similar the what is used in chromatographic methods.

$$N = (\mu_{ep} + \mu_{eof})\, V / 2D$$

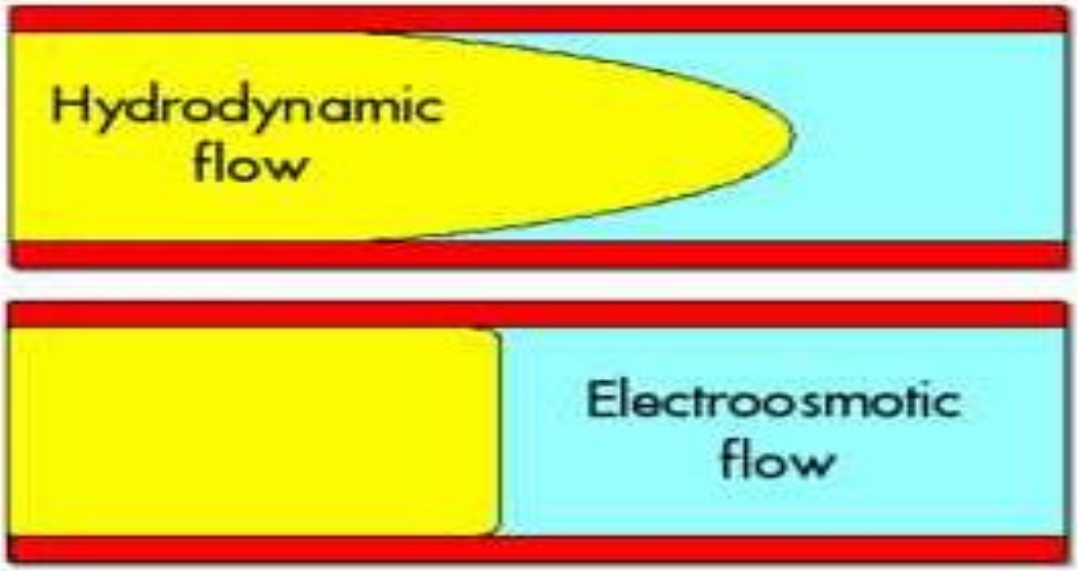
Where: D = solute diffusion coefficient.

Solutes with a large μ_{ep} (in the same direction as flow), have a greater efficiency.

Efficiency is independent of column length.

N values of 100,000 to 200,000 can be achieved.

Column length does not effect efficiency. This is because solutes tend to move as a 'plug' through the column.



- **Because ions are in the bulk solution are about to travel through the capillary without interference from the capillary itself, there is no dramatic drop in potential within the capillary**
  - **No meniscus is made**

This term is also similar to what is used in chromatography - the ratio of the capacity factors of two solutes.

In CE, the analogous expression is:

$$\alpha = \mu_{ep\,1} / \mu_{ep\,2} \qquad \alpha \geq 1$$

You can alter α by adjusting the pH of the buffer. This also will impact the resolution.

Resolu

$$R = \frac{0.177\,(\mu_{ep2} - \mu_{ep1})\,V^{1/2}}{(\mu_{avg} + \mu_{eof})^{1/2}\,D}$$

- ✔ Increasing the applied voltage and decreasing μ_{eof} will improve resolution.
- ✔ Remember, increasing μ_{eof} will improve analysis time and efficiency.

Instrumentation

- The instrumentation for capillary electrophoresis is simple.
- A buffer-filled fused-silica capillary extends between two buffer reservoirs that also hold platinum electrodes.
- Sample introduction is performed at one end and detection at the other.

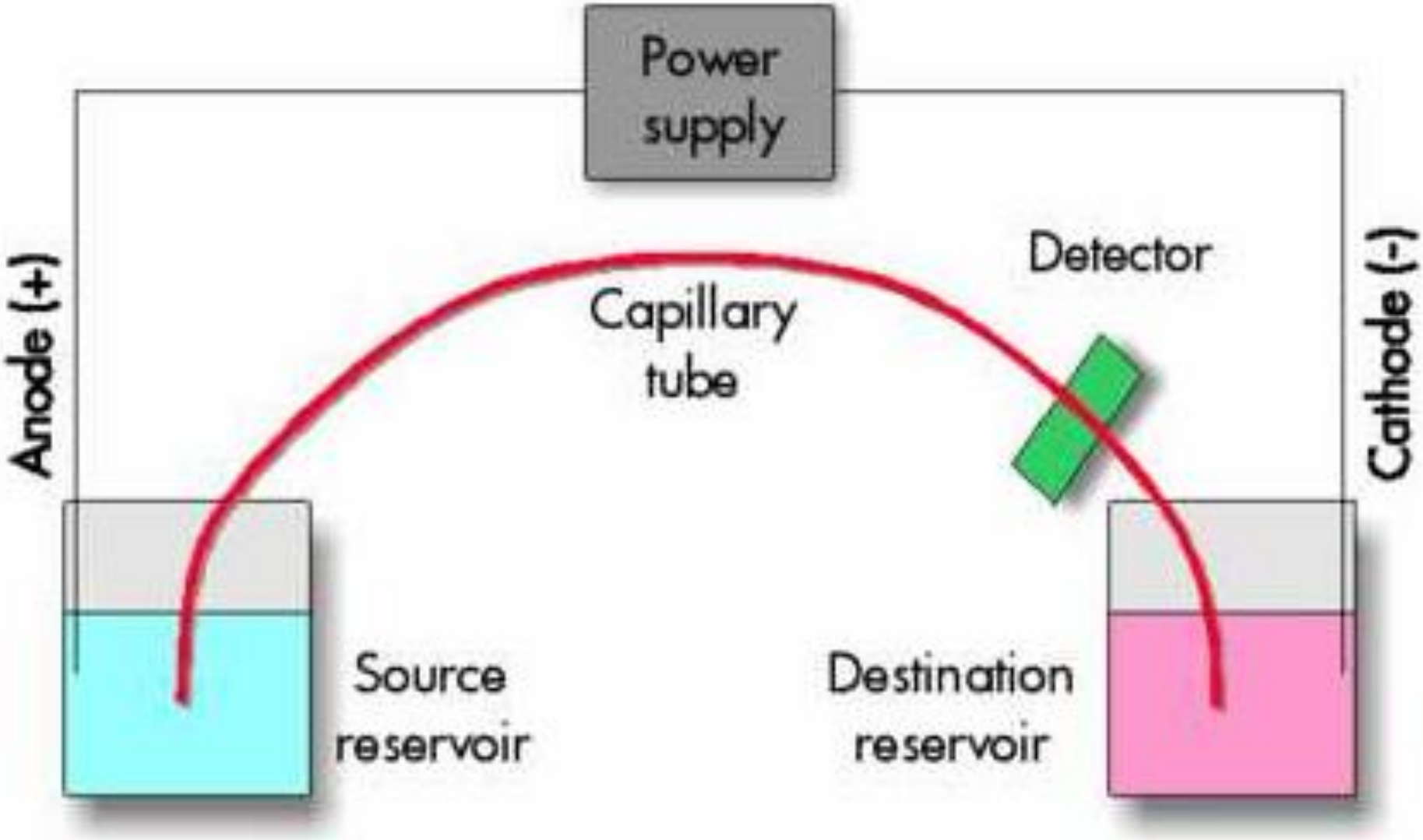
The basic instrumentation is pretty simple.

- Power supply
- Anode compartment
- Cathode compartment

Both with buffer reservoirs

- Capillary tube
- Detector
- Sample vial

Basic equipm



Sample introduction.

The tube is initially filled with buffer solution.

The sample is introduced by dipping one end into the sample and then using causing sample to enter the tube.

Electrokinetic injection

Applying a potential to cause the sample to move in to the tube (least popular method).

Hydrodynamic

Pressure injection - the sample vial is pressurized.

Siphoning - the sample is pulled into the tube.

Sample Introduction

- The most common sample introduction methods are electrokinetic injection and pressure injection .(hydrodynamic)
- One end of the capillary and its electrode are removed from their buffer compartment and placed in a small cup containing the sample.
- A potential is then applied for a measured time, causing the sample to enter the capillary by a combination of ionic migration and electroosmotic flow.
- The capillary end and electrode are then placed back into the regular buffer solution for the duration of the separation.

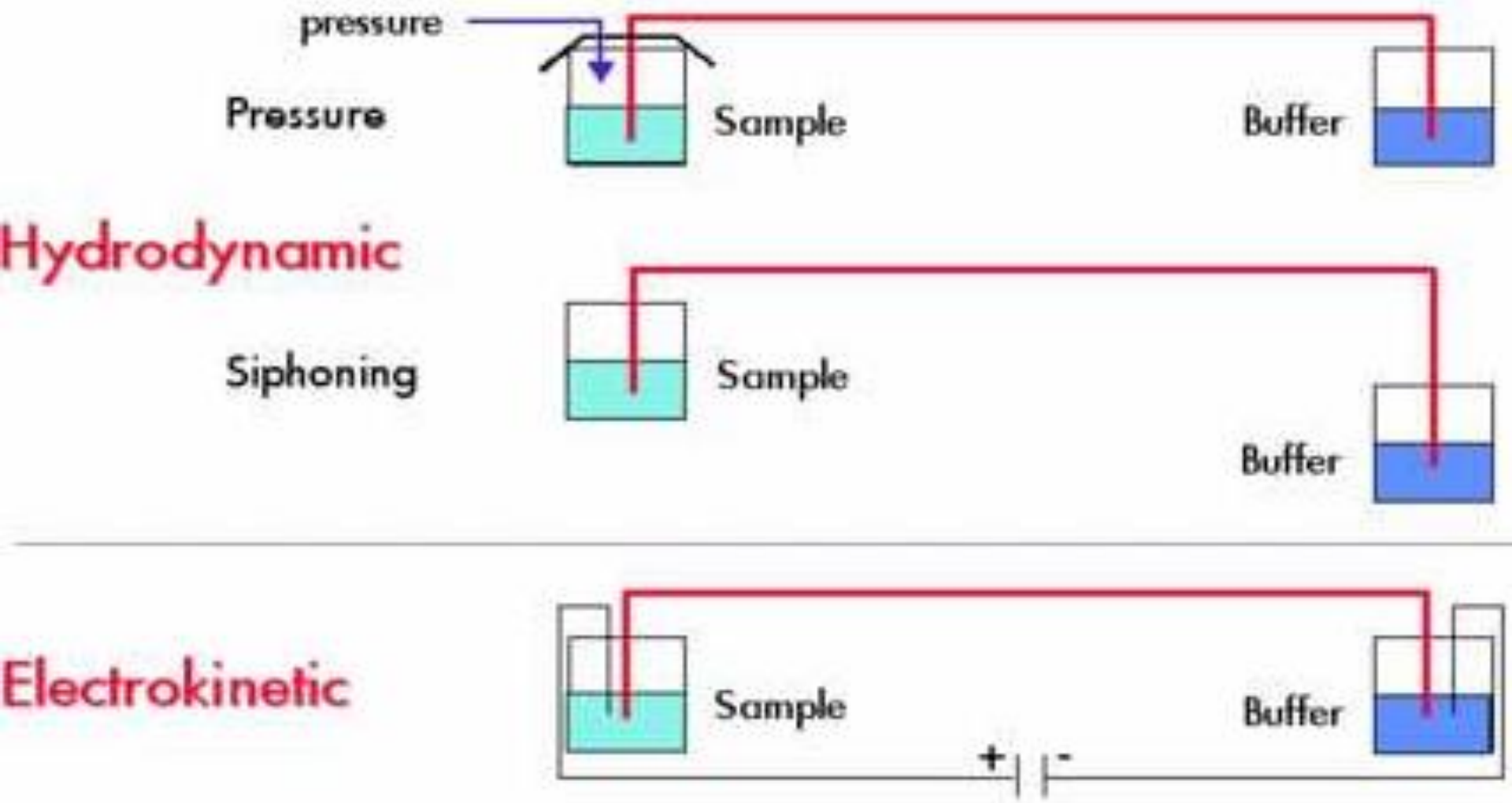
Sample Introduction

- With pressure injection, the sample introduction end of the capillary is also placed momentarily into a small cup containing the sample , and a pressure difference is then used to drive the sample solution into the capillary.
- The pressure difference can come from applying a vacuum at the detector end, by pressurizing the sample, or by elevating the sample end.
- Pressure injection does not discriminate due to ion mobility, but cannot be used in gel-filled capillaries.

Sample Introduction

- For both electrokinetic injection and pressure injection ,the volume injection is controlled by the duration of the injection.
- Injection of 5 to 50 nl are common.

Sample introduc



Voltage applic

Migration (separation) begins once an electrical field is applied.

You want to use a large electrical field

- Shorter analysis times
- Better separations
- Improved resolution.

When using narrow-bore capillary tubes, it is possible to applies voltages up to 40,000 V. That actual voltage applied is based on the application.

Currents are in the microampre range.

Detection

- Because the separated analyte move past a common point in most types of capillary electrophoresis ,detectors are similar in design and function to those described for HPLC.
- One difference in behavior of detectors is encountered, however, because in capillary electrophoresis each ion migrates at a rate determined by its electrophoretic mobility. so peak area somewhat dependent upon retention times.
- In contrast, in HPLC area independent of retention times.

Detec

Several methods have been used.

Approaches taken are similar to what is used in capillary LC.

UV/Vis absorption	Must have absorbing chromophore
Indirect absorbance	Universal
Fluorescence	Must be fluorescent (or have fluorscent label)
Laser fluorescence	Must be fluorescent (or have fluorscent label)
Radiometric	Must be radioactive (or radioactive label)
Mass spectroscopy	Universal or selective based on MS method
Amperometric	Solute must under reduction or oxidation
Conductometric	Universal

Detect

	Detection Limit		On-Column Detection
	Moles injected	Molarity	
UV/Vis absorption	10^{-13} - 10^{-16}	10^{-5} - 10^{-7}	Yes
Indirect absorbance	10^{-12} - 10^{-15}	10^{-4} - 10^{-5}	Yes
Fluorescence	10^{-15} - 10^{-17}	10^{-7} - 10^{-9}	Yes
Laser fluorescence	10^{-18} - 10^{-20}	10^{-13} - 10^{-16}	Yes
Radiometric	10^{-17} - 10^{-19}	10^{-10} - 10^{-12}	Yes
Mass spectroscopy	10^{-16} - 10^{-17}	10^{-8} - 10^{-10}	No
Amperometric	10^{-18} - 10^{-19}	10^{-7} - 10^{-10}	No
Conductometric	10^{-15} - 10^{-16}	10^{-7} - 10^{-9}	No

Absorbance Methods

- Both fluorescence and absorbance detectors are widely used in capillary electrophoresis, although the later are more common because they are more generally applicable.
- In order to keep the detection volume on the nL scale or smaller, detection is performed on-column.

Absorbance Methods

- In this case a small section of the protective polyimide coating is removed from the exterior of the capillary by burning, dissolution, or scraping.
- The section of the capillary then serves as the detector cell.
- Unfortunately ,the path length for such measurements is no more than 50-100μm, which restricts detection limits in concentration term, because such small volumes are involved, however, mass detection limits are equal to or better than those for HPLC.

Absorbance Methods

- In order to improve the sensitivity of absorbance measurements, several techniques have been suggested for increasing the path length of the measurements.

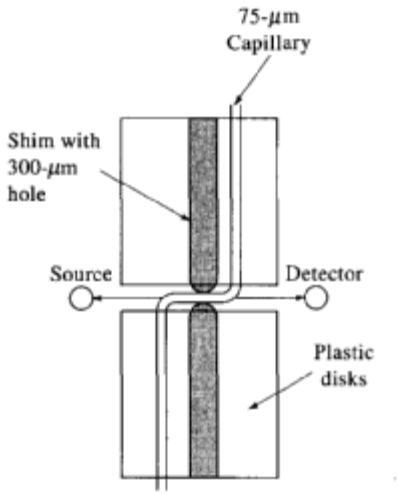
  1- Z-shape

  2- Bubble formed

  3- silver coating

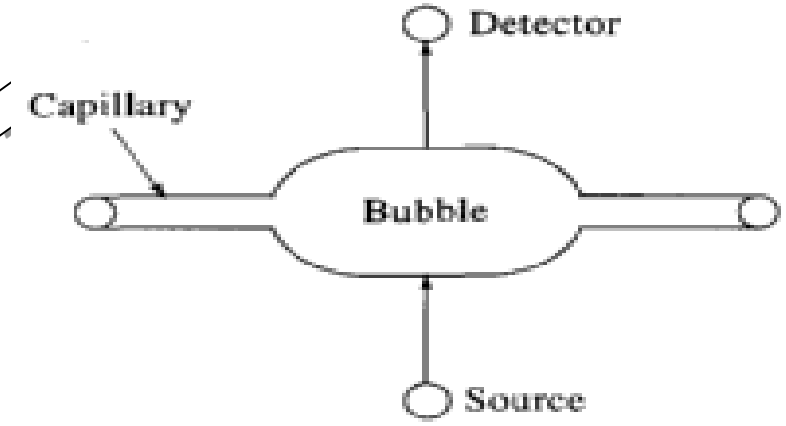
Absorbance Methods

- **Z shape** : involves bending the end of the capillary in to a “Z”shape giving a path length of 3 mm.
- The sensitivity is smaller than expected ,probably because of inadequate focusing of the light.



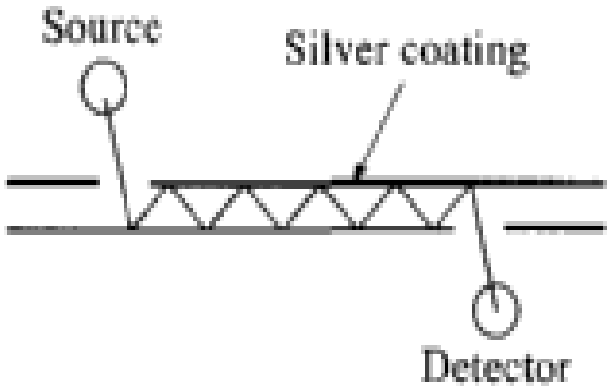
Absorbance Methods

- **Bubble formed** : Here a bubble is formed near the end of the capillary .
- The bubble for a 50-µm capillary has an inside diameter of 150 µm, thus giving a threefold increase in path length.



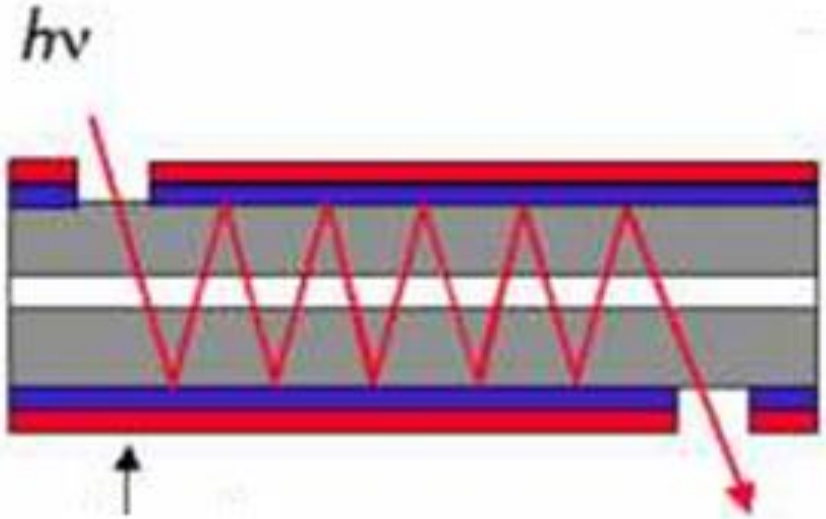
Absorbance Methods

- **Silver coating** : In this technique, a reflective coating of silver is deposited on the end of the capillary.
- In this case the radiation beam undergoes numerous reflections until it exits the capillary.



Because of the small volumes, one must get 'creative' to obtain a measurable response -- while still in the capillary tube.

Simple
Z flowcell



Silver
coating

Indirect detection :

- ***It has been used for detection of species that are difficult to detect because of low molar absorptivities without derivatization.***
- ***An ionic chromophere is placed in the electrophoresis buffer. The detector then receives a constant signal due to the presence of this substance.***
- ***The analyte displaces some of these ions, just as in ion-exchange chromatography, so that the detector signal decrease during the passage of an analyte band through the detector. so***

Indirect detection :

So , The analyte is then determined from the decrease in absorbance.

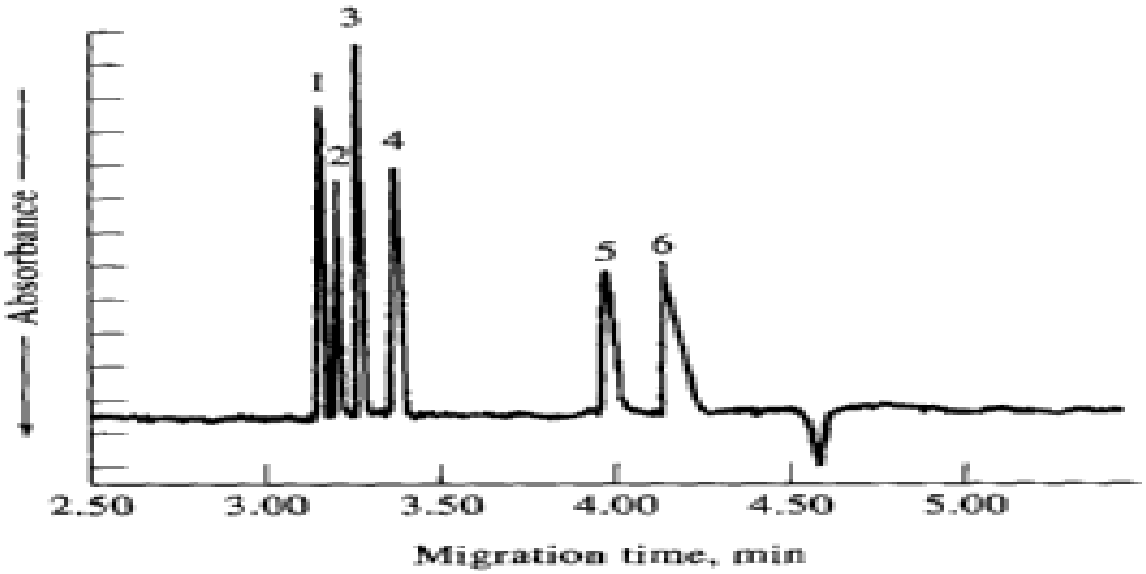
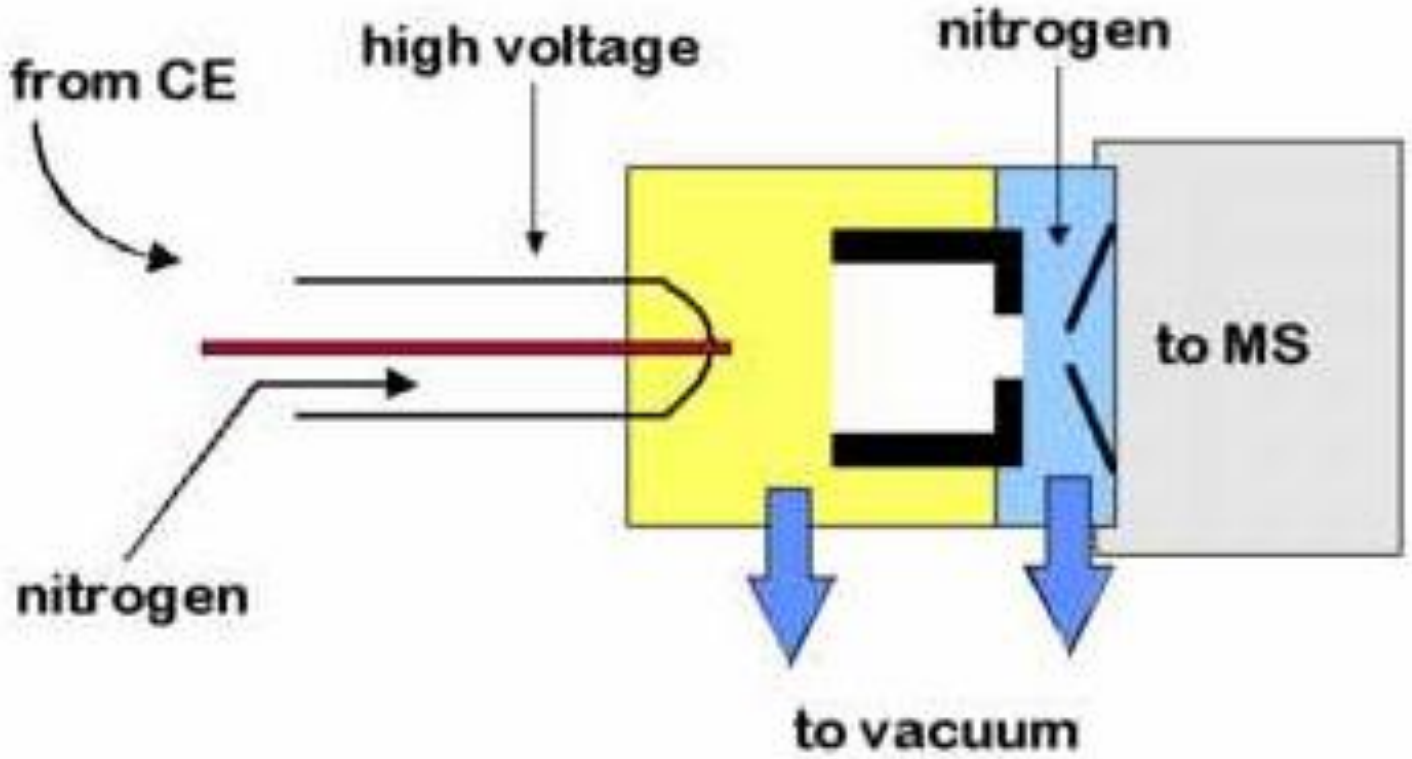


Figure 30-6 Electropherogram of a six-anion mixture by indirect detection with 4-nM chromate ion at 254 nm. Peak: (1) bromide (4 ppm), (2) chloride (2 ppm), (3) sulfate (4 ppm), (4) nitrate (4 ppm), (5) fluoride (1 ppm), (6) phosphate (6 ppm).

Mass spectroscop

Attempts have been made to interface a CE to a MS. This is similar to what is done with LC-MS systems. It will be covered in the 'Hyphenated Methods' unit.



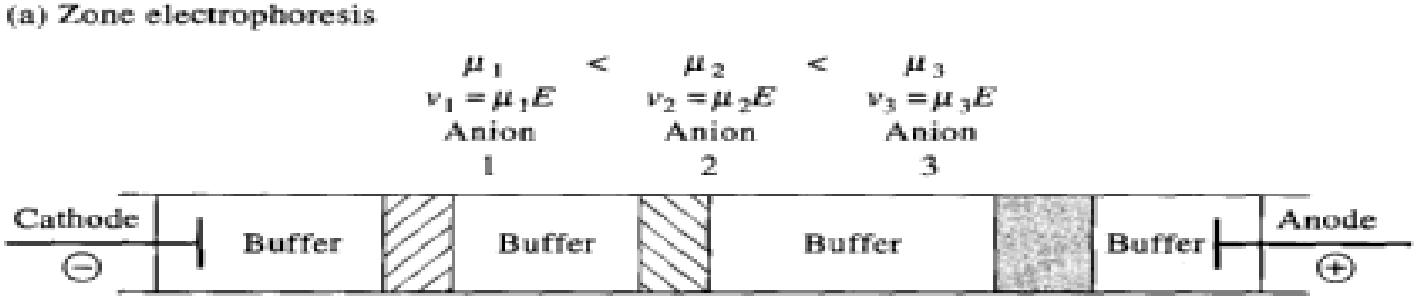
Application of Capillary Electrophores

Capillary electrophoresis separation are performed in several ways called modes. It is noteworthy that these modes were first employed in slab electrophoresis and were subsequently adapted for capillary electrophoresis

- Capillary zone electrophoresis (CZE)
- Capillary isoelectric focusing (CIEF)
- Micellar electrokinetic capillary chromatography (MECC)
- Capillary gel electrophoresis (CGE)
- Capillary electrochromatography
- Chiral separations
- Capillary iso-tachophoresis (CITP)

Capillary Zone Electrophoresis (CZE)

- The buffer composition is constant throughout the region of the separation.
- The applied potential causes the different ionic compounds of the mixture to each migrate according to its own mobility and to separate into zones .



Capillary Zone Electrophoresis

- Completely resolved zones have regions of buffer between them;
- The situation is analogous to elution column chromatography ,where regions of mobile phase are located between zones containing separated analytes.

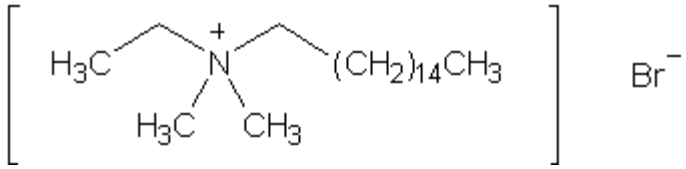
Separation of small ions :

1- Cation separation

- The analyte move in the same direction as the electroosmotic flow.
- Thus, for cation separations the walls of the capillary are untreated, and the electroosmotic flow and the cation movement is toward the cathode.

2- anions separation :

- The electroosmotic flow is usually reversed by treating the wall of the capillary with an alkyl ammonium salt ,such as acetyl trimethylammonium bromide.
- The positively charged ammonium ions became attached to the negatively charged silica surface and, in turn, create a negatively charged double layer of solution, which is attracted toward the anode, thus reversing the electronnsmotic flow.



- Separation of Molecular Species :
- Herbicides ,pesticides and pharmaceuticals that are ionic or can be derivatized to yield ions have been separated and analyzed by CZE.
- In case of neutral carbohydrate ,the separation are preceded by formation of negatively charged borate complex. This complex are readily formed if a borate buffer is used as the separation medium.

Sodium borate $Na_2B_4O_7$

Capillary zone electrophor

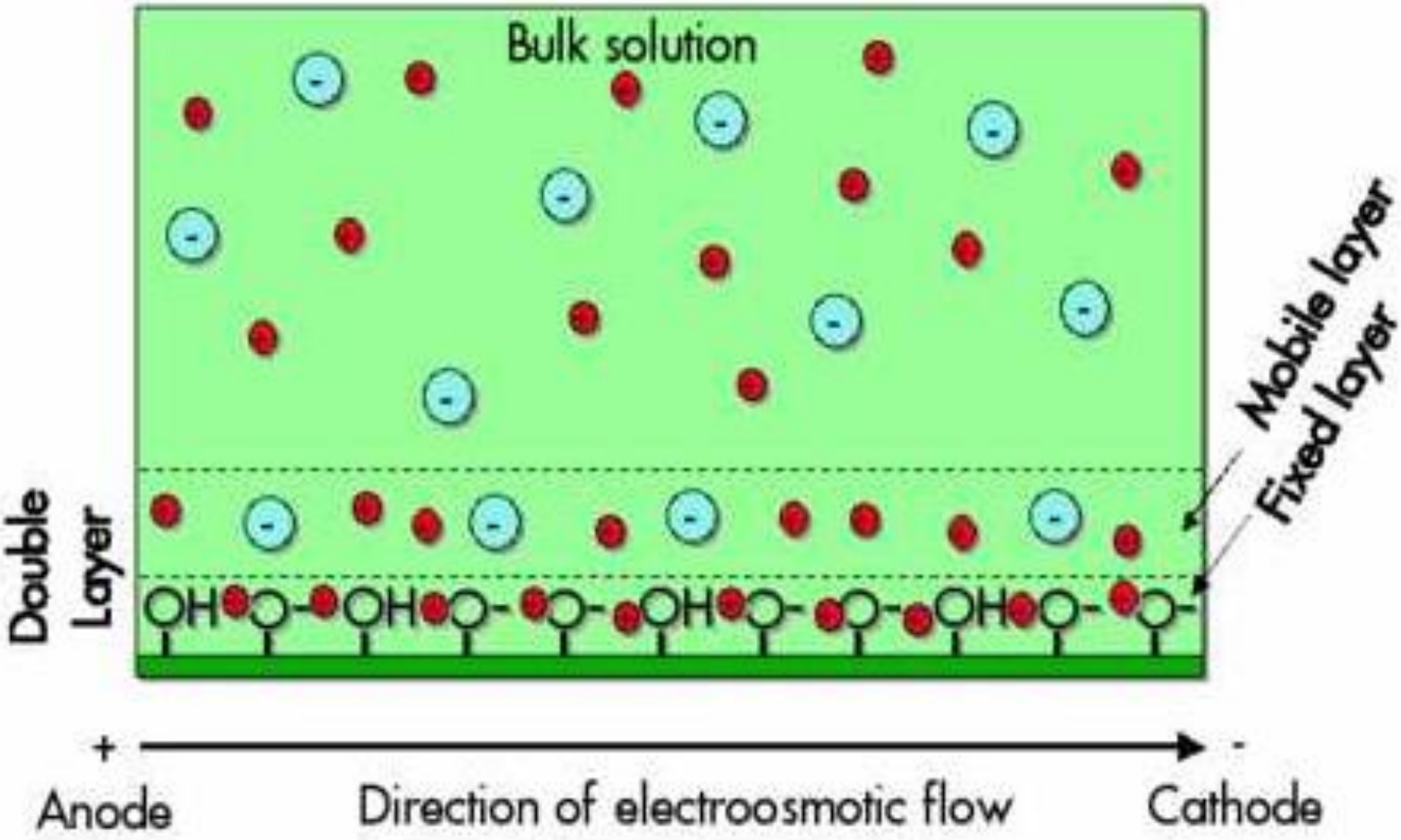
The simplest form of the technique.

- A capillary tube is filled with the buffer solution, sample loaded and the ends of the tube placed in reservoirs containing additional buffer solution.
- Under normal conditions, the 'sample' end of the tube is the anode and the solutes migrate toward the cathode
- As outlined earlier, cations elute first - with smaller (more highly charged) species eluting before larger (less charge) species.
- Neutrals elute next as a single band.
- Anions elute last - reverse order of cations

Capillary zone electrophore

- The elution order can be reversed by the addition of an alkylammonium salt.
- The ammoniuim 'head' will be attracted to the capillary wall.
- The 'tails' of the salt will form form a hydrophobic layer resulting in additional ammonium 'heads' point towards the solution.
- This, in effect, causes the capillary surface to become positive.

Double lay



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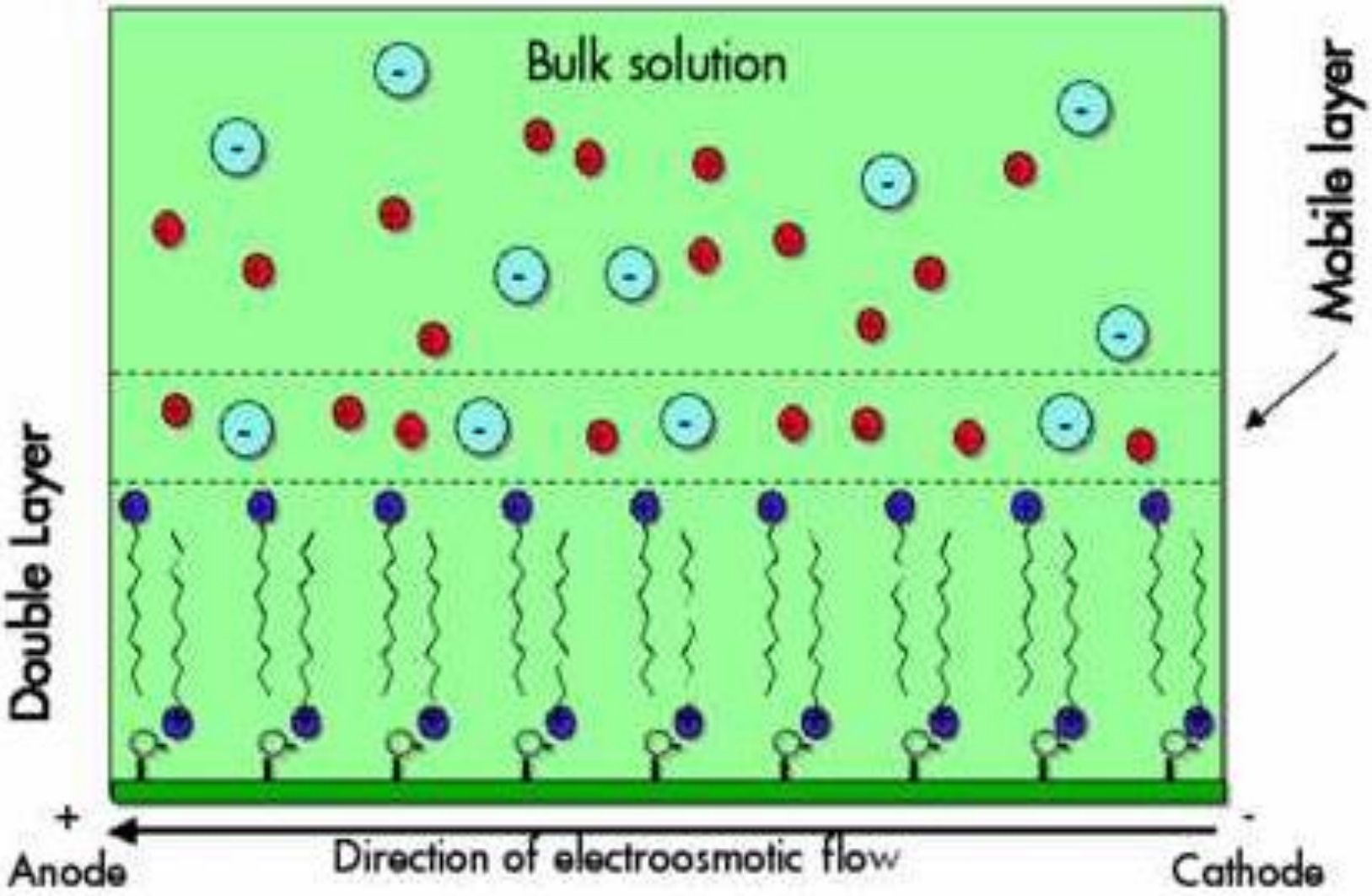
Capillary zone electrophoresis



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Capillary Gel Electrophoresis (CGE)

- CGE is generally performed in a porous gel polymer matrix, the pores of which contain a buffer mixture in which the separation is carried out.
- Gel provide a molecular sieving action that retarded the migration of analyte species to various extents depending upon the pore size of the polymer and size of the analyte ions. This type of sieving action is particularly helpful in separating macromolecules such as proteins, DNA fragments that have the same charge but differ in size.

Capillary Gel Electrophoresis

- Currently, most macroscale electrophoresis separations are carried on a gel slab.

  (polyacrylamide (CH_2=CH-CO-NH_2)
- Some capillary electrophoretic separation of speisies that differ in size are also performed in gels contained in capillary tubes.
- Other gels that have been used for capillary gel electrophoresis include:

  agarose , a polysaccharide extracted from a marine alga, methyl cellulose, and polyethylene glycol.

Capillary gel electrophor

- The capillary tube is filled with a polymeric, porous gel.
- Solutes migrate based on both their electrophoretic movility and their size.
- Sort of a cross between electrophoresis and size exclusion chromatography.
- This approach has been used to separate DNA fragments - they all have similar charge to size ratios but vary by size.
- The same method can be applied to the separation of other large biomolecules like protein and oligonucleotides.

Capillary Isotachophoresis

- In CITP all analyte bands ultimately migrate at the same velocity. (iso = same , tach = speed)
- In any particular application, either cations or anions can be separated ,but not both at the same time.
- In a separation, the sample is injected between two buffers.
- A leading one containing ions of a higher mobility than any of the analyte ions and a terminating one with ions of a lower mobility than the sample ions.

Capillary Isotachophores

- For example ,in separating anions, fast –moving chloride ions might be contained in the leading buffer ,and slow-moving heptanoate ions in the terminating buffer.
- For a separation of anions, the leading electrolyte solution is connected to the anode, and the terminating one to the cathode.

$CH_3(CH_2)_5COO^-$

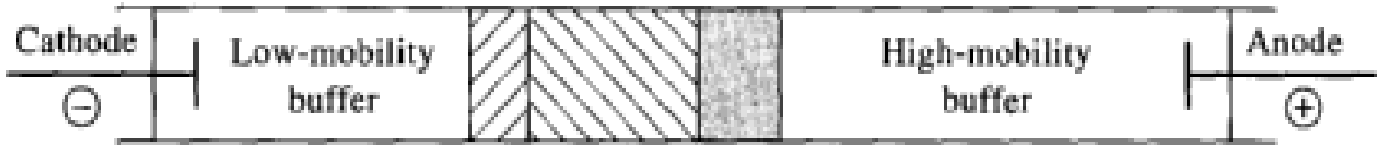
Capillary Isotachophoresis

- When the potential field is first applied in an isotachophoretic separation, analyte ions migrate as in zone electrophoresis, each ion with its unique velocity .
- This difference in migration rate results in the separation of the various analyte species in to adjacent bands, with the fastest species located in a band directly adjacent to the leading buffer and the slowest just ahead of terminal buffer .After the bands have formed, they then move at the same velocity.

Capillary Isotachophoresis

- When equilibrium is reached in an isotachophoretic experiment, a condition is reached in which each sample component is migrating in a band that is sandwiched between a band that contains the next slower-moving ions and the next-faster moving band.

(b) Isotachophoresis



Capillary Isoelectric Focuusi

- Capillary isoelectric focusing (CIEF) is used to separate amphiprotic species, such as amino acids and proteins that contain a weak carboxylic acid group and weak base amino group.
- In isoelectric separation is performed in a buffer mixture that varies in pH continuously along its length. This pH gradient is prepared from a mixture of several different ampholytes in an aqueous solution.

Capillary Isoelectric Focusing

- Ampholytes are amphoteric compounds usually containing carboxylic and aminogroups.
- Ampholyte mixtures having different pH ranges can be prepared or are available from several commercial sources.

Capillary Isoelectric Focusin

- To perform an isoelectric focusing experimental in a capillary tube, the analyte mixture is dissolved in a dilute solution of the ampholytes, which is then transferred to the tube.
- One end of the capillary is then inserted in a solution of strong base, such as sodium hydroxide ,that also holds the cathode.
- The other end of the tube is immersed in a solution of a strong acid such as phosphoric, that also holds the anode.

Capillary Isoelectric Focusing

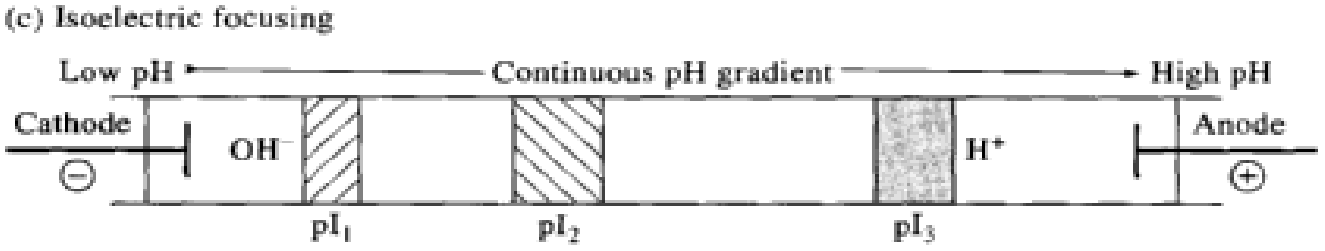
- When the potential is applied ,hydrogen ions begin to migrate from the anode compartment toward the cathode. Hydroxide ions from the cathode begin to move in the opposite direction.
- If a component of the ampholyte or the analyte has a net negative charge ,it migrates toward the positive anode.

Capillary Isoelectric Focusing

- As it migrates it passes into continuously lower pH regions, where progressive protonation of the species occurs, which lowers its negative charge. Ultimately it reaches the pH where its net charge is zero (its isoelectric point). Migration of the species then ceases.
- This process goes on for each ampholyte species and ultimately provides a continuous pH gradient throughout the tube.

Capillary Isoelectric Focusing

- Analyte ions also migrate until they reach their isocratic point .These processes then result in the separation of each analyte into a narrow band that is located at the pH of its isoelectric point.



Capillary Isoelectric Focusing

- Note that isoelectric focusing separations are based upon differences in equilibrium properties of the analyte (Ka,Kb) rather than on differences in rates of migration.
- Once each analyte has migrated to a region where it is neutral ,the positions of bands become constant and no longer change with time.

- **Mobilization of Focused Bands :**

In order to detect the focused bands in a capillary isoelectric focusing separation, it is necessary to move ,or mobilize ,the contents of the capillary so that bands pass the detector located at one end, and that can be accomplished through:

1- Application of a pressure difference, just as for sample loading.

2- By simply changing the solution in the electrode compartment.

Capillary isoelectric focusing

- Capillary adaptation of the well-established technique for separating amphoteric species such as proteins.
- Relies on the formation of a pH gradient by the use of zwitterionic molecules (ampholytes) - these are generally synthetic aliphatic polyamino, polycarboxylic or polysulphonic acids.
- Application of an electrical field results in the formation of a pH gradient.
- High resolution is obtained since amphoteric species will be focused at the optimum pH - overcoming diffusion.

Capillary Electrochromatography

- CEC is a hybrid of capillary electrophoresis and HPLC that offers some of the best features of each of the two techniques.
- Advantages:

1- like HPLC ,it is applicable to separation of unchared species.

2- like CE ,it provides highly efficient separations on microvolumes of sample solution without the need for a high-pressure pumping system.

- Packed Column Electrochromatography :

  In this method a polar solvent is usually driven by electroosmotic flow through a capillary that is packed with RP HPLC paking.

Separation depend upon distribution

Capillary Electrochromatograp

- **Micellar Electrokinetic Capillary Chromatography:**

This technique involved introduction of surfactant ,such as sodium dodecyle sulfate(SDS) ,at a concentration level at which micelles form.

Micelles form in aqueous solutions when the concentration of an ionic species having a long chain hydrocarbon tail is increased above a certain level called the critical micelle concentration (CMC), .At this point the ion begin to form spherical aggregates made up to 40 to 100 ions whose hydrocarbon tails are in the interior of the aggregate and whose charged ends are exposed to water on the outside.

R- NaO_3S

Capillary Electrochromatogra

Micellar Electrokinetic Capillary Chromatography:

Micelles constitute a stable second phase that is capable of absorbing nonpolar compounds into the hydrocarbon interior of the particles ,thus solubilizing the nonpolar species.

Solubilization is commonly encountered when a greasy material or surface is washed with a detergent solution.

Micellar Electrokinetic Capillary Chromatography:

- For most applications to data, the surfactant has been SDS. The surface of an anionic micelle of this type has a large negative charge, which gives it a large electrophoretic mobility toward the positive electrode.
- Most buffers, however, exhibit such a high electroosmotic flow rate toward the negative electrode that the anionic micelles are carried toward that electrode also, but at a much reduced rate.

Micellar Electrokinetic Capillary Chromatography

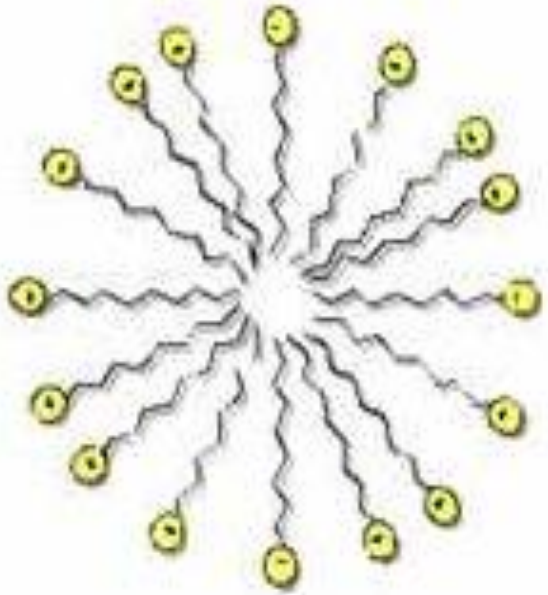
- **Thus during an experiment ,the buffer mixture consists of a faster-moving aqueous phase and a slower-moving micellar phase.**
- **When a sample is introduced into this system, the components distribute themselves between the aqueous phase and the hydrocarbon phase in the interior of the micelles.**
- **The mechanism of separation is depends upon differences in distribution coefficients for analyte between the mobile aqueous phase and the hydrocarbon pseudostationary phase**

Micellar electrokine capillary chromatography

CZE is not able to separate neutral species. MECC can overcome this limitation.

The method relies on the addition of a surfactant (such as sodium dodecylsulfate)

At high enough surfactant concentrations, micelles will form - consisting of 40-100 surfactant molecules



Micellar electrokinetic capillary chromatography

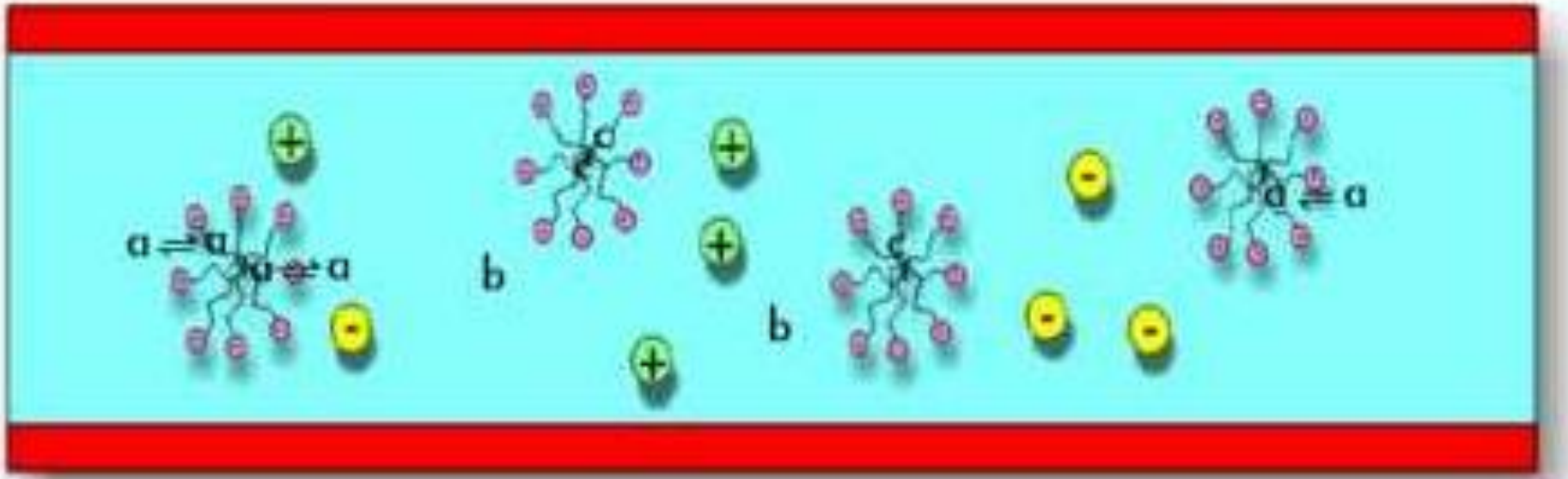
Because the micelles are negatively charged, they migrate towards the cathode - less velocity that for cations.

Neutral species will partition between the micelles and the buffer similar to what is seen in HPLC.

For neutrals to be separated, they must have some solubility in both the micelle and the solution. If not, they still will elute as a single band.

Micellar electrokinetic capillary chromatography

a = soluble in both
b = not soluble in micelle
c = not soluble in buffer



Capillary Electrochromatograph

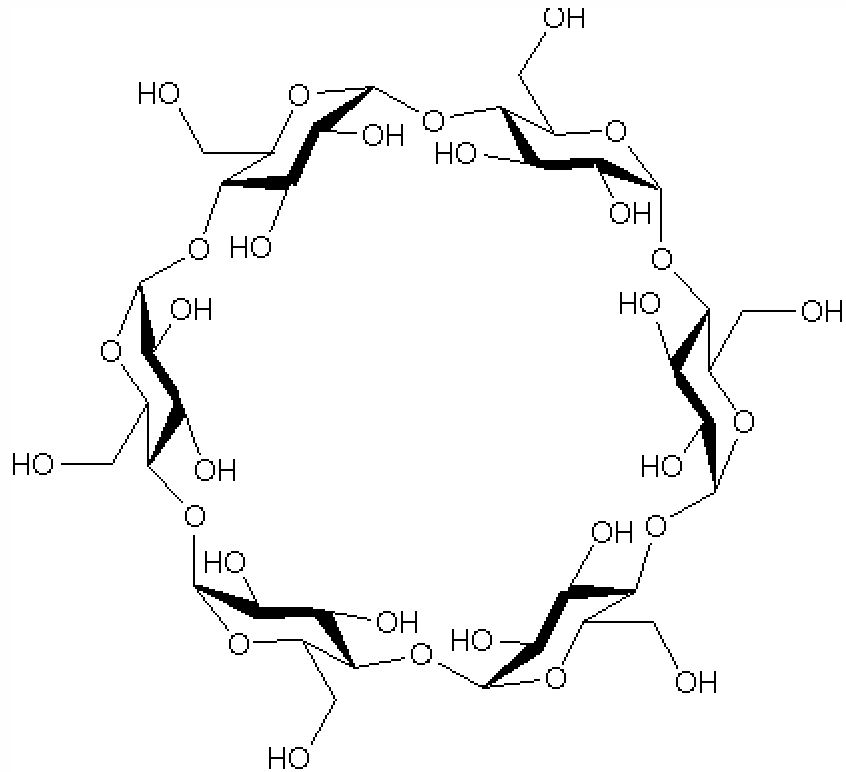
Another hybrid method for separating neutral species.

- The capillary tubings is packed with 1.5-3.0 μm silica particles coated with a bonded, nonpolar stationary phase - or a phase bound directly to the tubing wall.
- It is similar to micelle type approaches and also analogous to HPLC separations.
- The movement of the buffer solution due to electroosmotic flow is is 'mobile' phase.
- Separation of neutrals is based on their ability to partition between the buffer and the stationary phase.
- Unlike HPLC, high-pressure pumping is not required and better efficiency is possible - with a shorter analysis time.

It is possible to separate enantiomers using CE.

The most common approach is to add cyclodextrins into the buffer solution (a 1-100 mM).

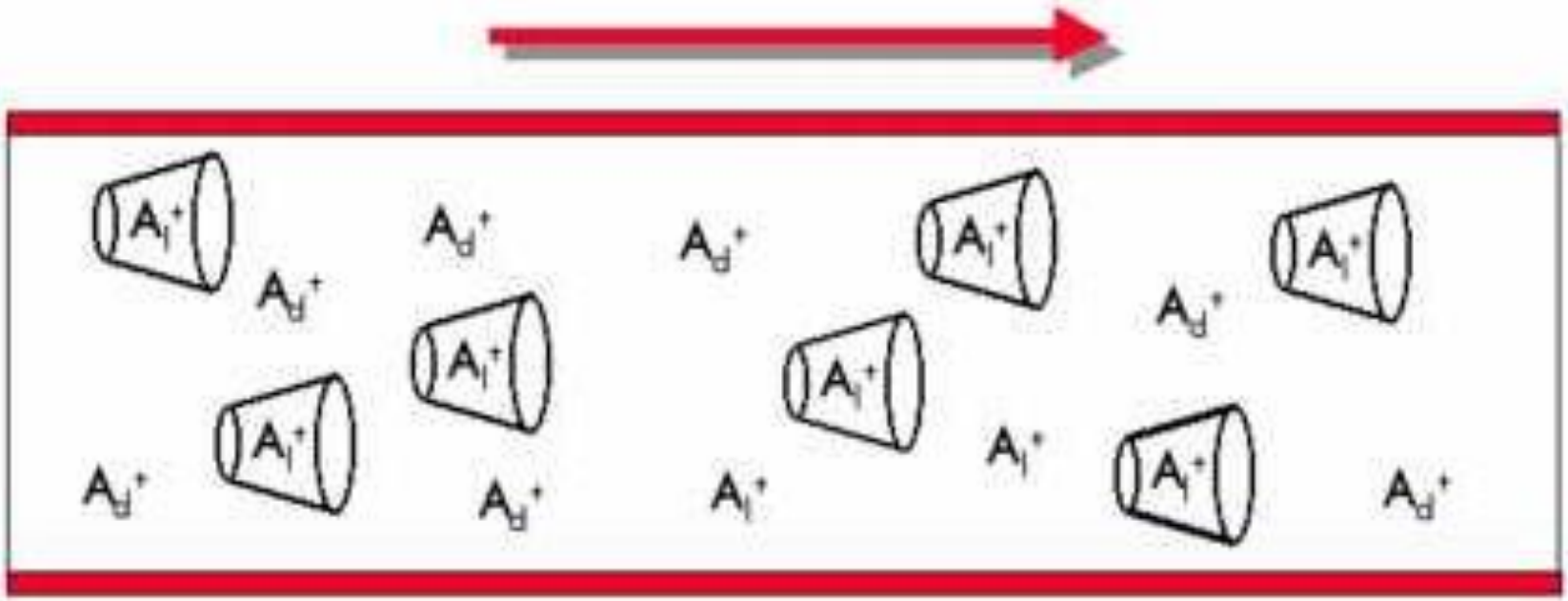
Common cyclodextrins include: β-cyclodextrin, chemically modified cyclodextrans like dimethylated or hydroxypropylated forms.



α-Cyclodextrin

Chiral separation

The enantiomer that is more strongly attracted to the cyclodextrin will tend to migrate more rapidly.

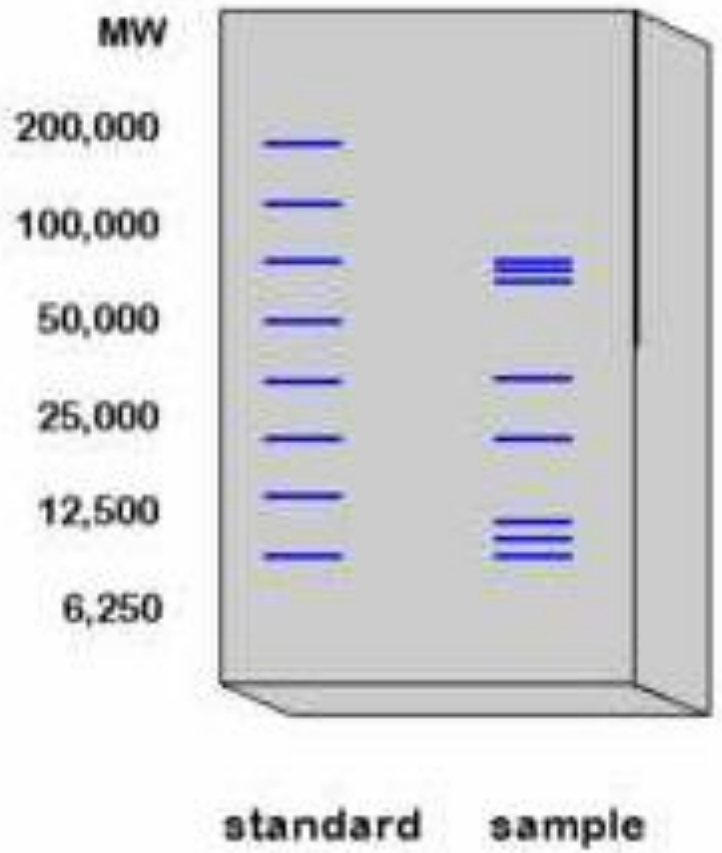


Electrophore

Qualitative analysis can be conducted by comparing the patterns produced to standards.

This example is a molecular weight determination of proteins but other materials can be evaluated.

This approach is used in genetic 'fingerprinting.'



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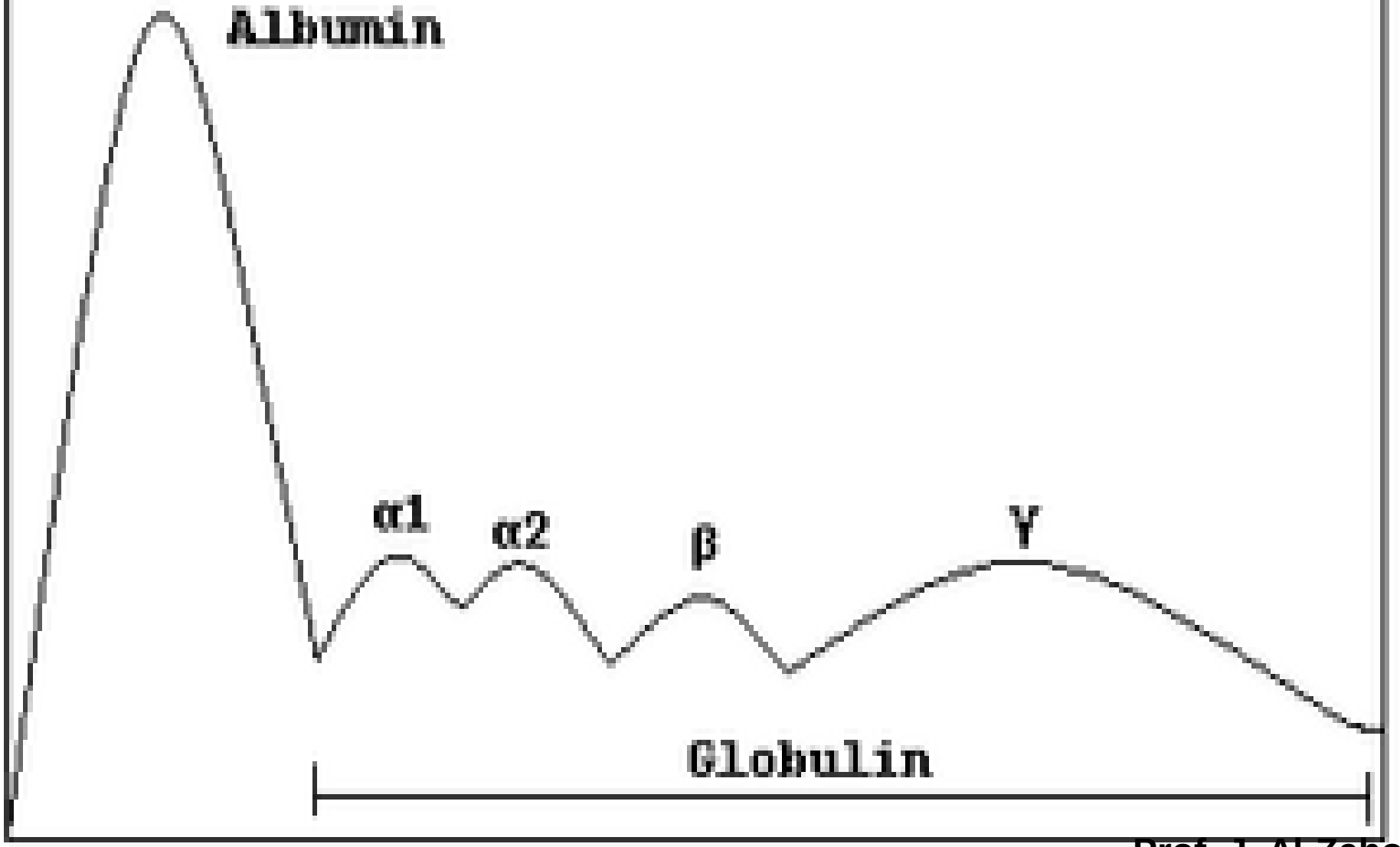

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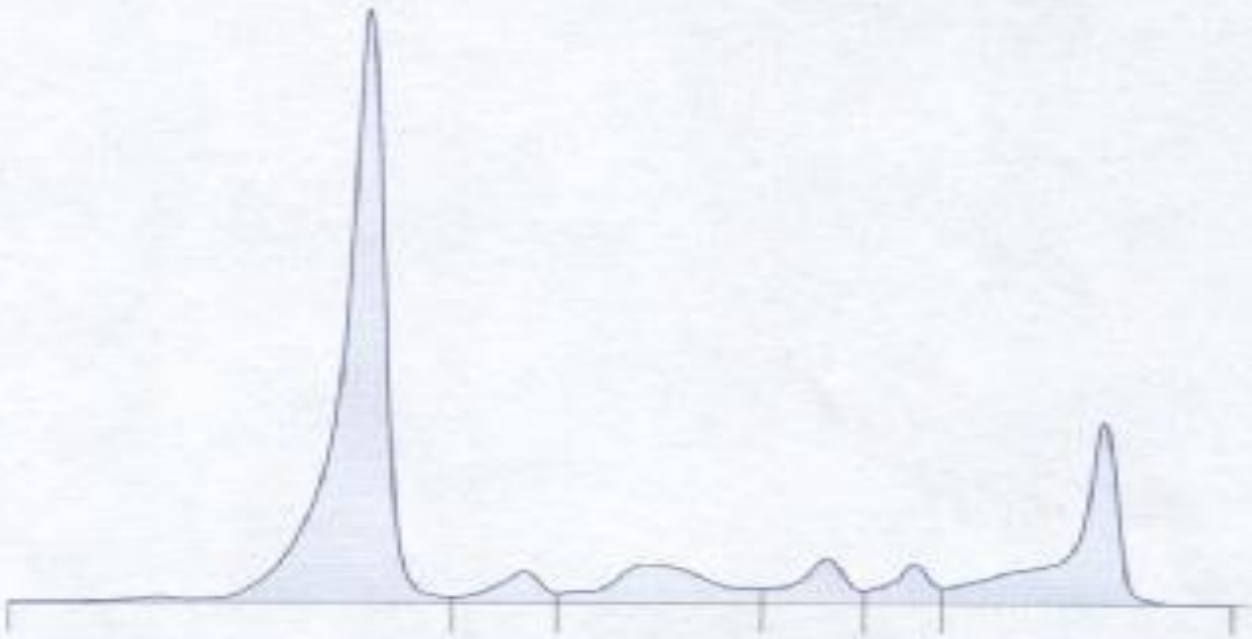

Protein Electrophoresis

Fractions	%	Ref. %	g/dl	Ref. g/dl
Albumin	**58.8**	55.8 - 66.1	4.35	4.02 - 4.76
Alpha 1	**3.5**	2.9 - 4.9	0.26	0.21 - 0.35
Alpha 2	**9.3**	7.1 - 11.8	0.69	0.51 - 0.85
Beta 1	**5.0**	4.7 - 7.2	0.37	0.34 - 0.52
Beta 2	**3.7**	3.2 - 6.5	0.27	0.23 - 0.47
Gamma	**19.7**	11.1 - 18.8	1.46	0.80 - 1.35

A/G **1.43** T.P. : **7.4**

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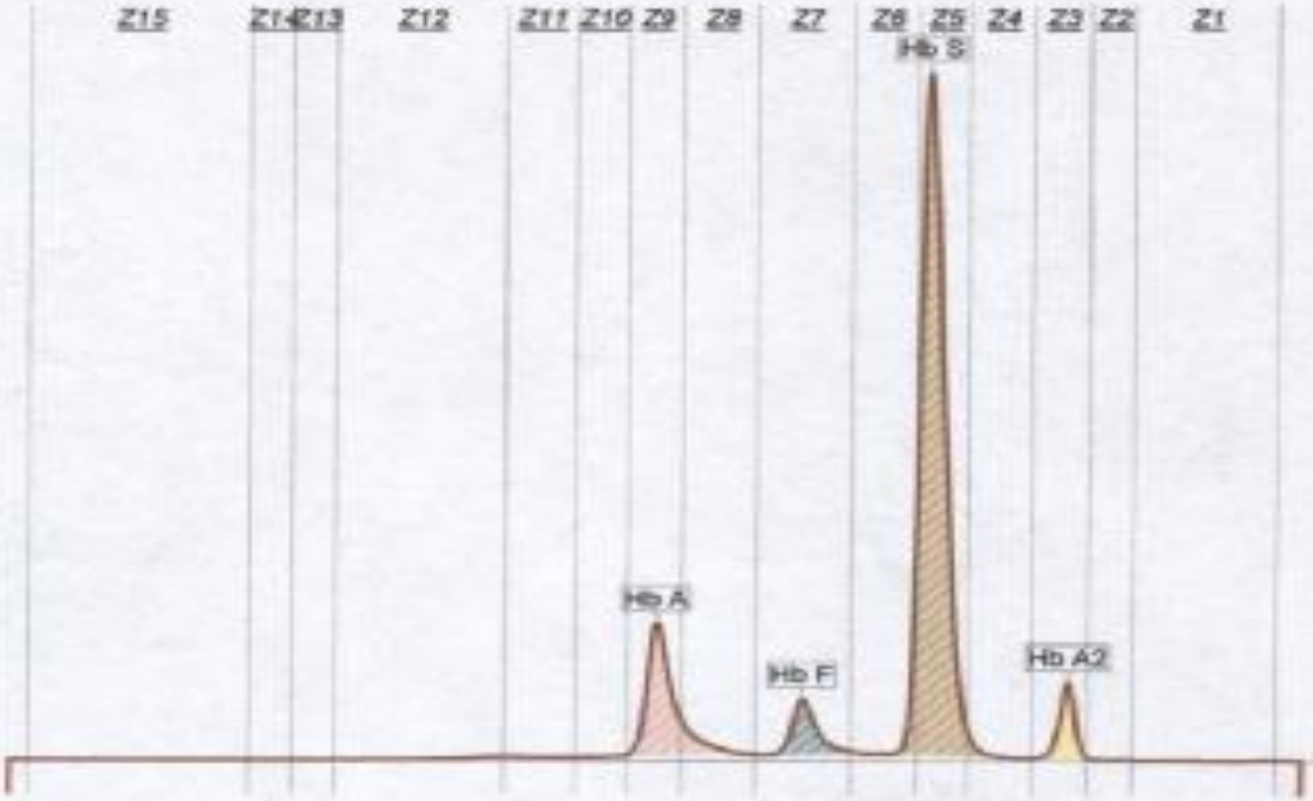

Hb Electrophoresis

Fractions	%	Ref. %
Hb A	15.3	
Hb F	6.1	
Hb S	73.3	
Hb A2	5.3	

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Applications

- Analysis of carbohydrates
- inorganic anions/metal ions
- DNA profiling
- Protein
- Chiral compound
- Vitamins
- Drugs (Beta-blocker)
- pesticides

- Advantages
  - Fast
  - Small Sample
  - Relatively inexpensive
  - Automated
  - Broad spectrum of application from small ions to macromolecules.
- Disadvantages
  - Joule Heating

ounds	Sample matrix	Electrolytes	D
		Capillary Electrophoresis	
Analysis of Biomolecules			
Simple Mixture Analysis			
Amines			
Tetramethyl ammonium bromide, tetramethyl ammonium perchlorate, tetrapropyl ammonium hydroxide, tetrabutyl ammonium hydroxide, and trimethylphenyl ammonium iodide	—	0.1 mM KCl in 50% methanol	MS
Polyamines	—	Formic acid, 0.01% ED, 5% EG	Fluorescence
Alkylamines	—	0.5 M PB	Fluorescence
Histamine	Wine	0.1 M BB, 0.2 M KCl, pH 9.5	Post column fluorescence
Putrescine, cadaverine, spermidine, and spermine	—	0.005 M BB with 0.1% ED, 2% SDS, and 5% EG	Post column fluorescence
Acids			
Formic, acetic, propionic, and butyric	—	0.025 M Na veronal, pH 8.6	Indirect UV 225 nm
Malonic, lactic, aspartic, glutamic glucornic, hydrochloric, and phosphoric	—	0.02 M Benzoic acid, histidine, pH 6.2, 0.1% triton	Indirect UV 254 nm
Benzoic, benzilic, and naphthoic	—	0.1 M Tris–acetic acid, pH 8.6, 20% dextran	UV 205 nm
Picric, cinnamic, and sorbic	—	0.01 M KCl, pH 5.6, ME: 0.01 M HCl	UV 254 nm
Hippuric and gibberilic	—	0.1 M Ammonium acetate, MeCN (1:9)	—
Carbohydrates			
Neutral	—	0.2 M Boric acid, KOH, pH 5.0	UV 240 nm
Oligosaccharides	—	0.01 M Na_2HPO_4, 0.01 M $Na_2B_4O_7$, pH 9.4	Fluorescence
Cyclodextrins	—	0.03 M Benzoic acid, Tris, pH 6.2	Indirect UV 254 nm
Amino acids			
All	—	1.0 mM PB, pH 5.31 or 7.12	—
All	—	0.2 mM Na salicylate, 0.04 mM Na_2CO_3, NaOH, pH 9.7	Indirect fluorescence
Debsyl derivatives	Urine	0.02 M PB, pH 7.0, MeCN (1:1)	—
Fluorescamine derivatives	—	10% Propanol, pH 10.16	Fluorescence

Micellar electrokinetic chromatography

Simple mixture analysis			
Alkyl amines	–	0.01 M Na_2HP_4, 0.006 M $Na_2B_4O_7$, 0.05 SDS, pH 7.0	Fluorescence
Amino acids (AA)	–	0.05 M BB, pH 9.5, MeOH, 2% THF, 0.05 M SDS	Fluorescence
AA-PTH derivatives	–	0.1 M BB, 0.05 PB, 0.1 SDS, pH 7, 4.3 M urea	UV 260 nm
Protein mixtures	–	BB, pH 10.5, 0.8 M SDS	UV 220 nm

...ure analysis			
Alkaloids	—	MeCN–10 mM Tris, pH 8.3 (80:20)	UV 2...
Antidepressants	—	MeCN/50 mM NaH_2PO_4 (pH 2.3)/water (60:20:20)	UV 210 nm
Benzamide	—	MeCN/10 mM MES, pH 3.0	UV 200 nm
Norgestimate drugs	—	MeCN/THF/25 mM Tris–HCl, (pH 8.0)/water (35:20:20:25)	UV 225 nm
Steroid hormones	—	MeCN/10 mM borate, pH 8.0 (65:35)	UV 205 nm
Parabens	—	MeCN/25 mM MES, pH 6.0 (80:20)	UV 250 nm
PAHs	—	MeCN/25 mM Tris, pH 9.0	Fluorescence
Explosive compounds	—	MeOH/10 mM MES	UV 254 nm
Chiral mixture analysis			
Amino acids, dansyl and dinitrophenyl derivatives	—	MeOH/PB (15:85) and MeOH/15 mM TEA, pH 4.7 (15:85), β-CD	UV
β-Blockers	—	MeCN/4 M acetate, pH 3.0 (80:20), imprinted polymers	UV
Benzoin and temazepam	—	HAS, 2-PrOH/4 mM PB, pH 7, β-CD, and others	UV and others
Barbiturates	—	MeOH/5 mM PB, pH 7.9 (80:20), β-CD	UV
2-Phenylpropionic acid and warfarin	—	PB with CDs	UV
Dichloroprop.		PB with vancomycin	UV


Thank you