1

Electrophoresis

- Separation technique based on the movement of analyte through a conductive medium in response to an applied electrical field.
- The medium is usually a buffered aqueous solution.
- In the absence of other factors, cationic species will migrate towards the cathode and anionic species towards the anode.
- The rate of migration is based the charge to size ratio of each species.

2

The approach can be subdivided into two categories - depending on the use of a stabilizing medium.

Free-Solution method.

- Absence of a supporting/stabilizing medium.
- Sample is introduced into a tube filled with a buffering liquid.
- A field is applied and species migrate based on their charge to mass ratios.
- Tiselius - 1948 Nobel prize for his development of this approach for the purification of proteins.
- Most popular method is **capillary electrophoresis.**

3

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 capillary electrophoresis.**

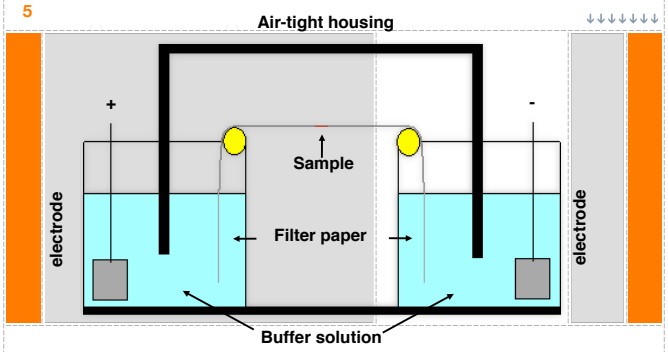
4

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.

5

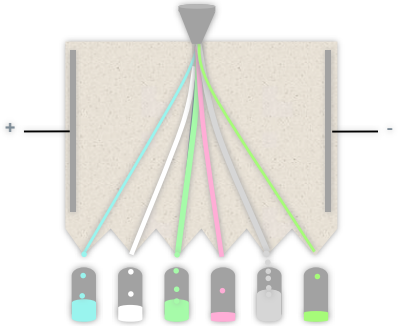


Resistance across the paper causes a voltage gradient.

Paper electrophoresis

6

It is also possible to develop a continuous separation by properly orienting the paper.



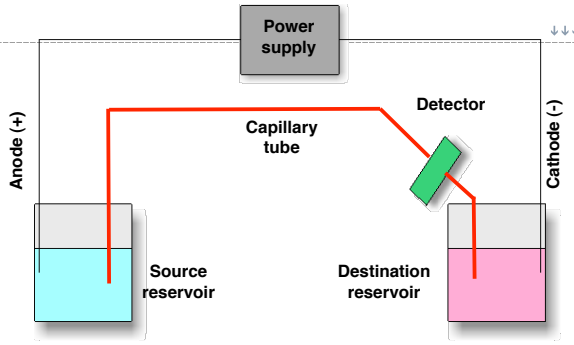
By introducing sample at the 'top', fractions can be collected at the 'bottom'.

Paper electrochromatography

Capillary Electrophoresis (CE)

Most 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 introduced in one end and migrate to the other.
- Similar to chromatography - resulting electropherogram. Both quant and qual information.



Basic equipment

CE Theory

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 velocity

ν_{ep} **- rate of solute migration.**

$$\upsilon_{ep} = \mu_{ep} E$$

Where: μ_{ep} **= solute's electrophoretic mobility**

E = magnitute of the applied field.

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

Where: **q = solute charge**

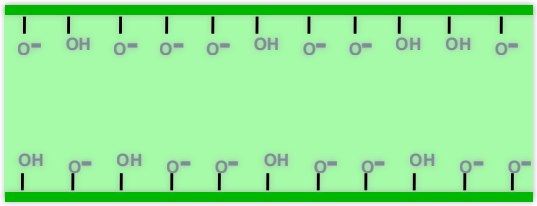
η **= buffer solvent's viscosity**

r = solute radius

Increased charge & reduced size result in greater μ_{ep} **.**

Electroosmotic mobility

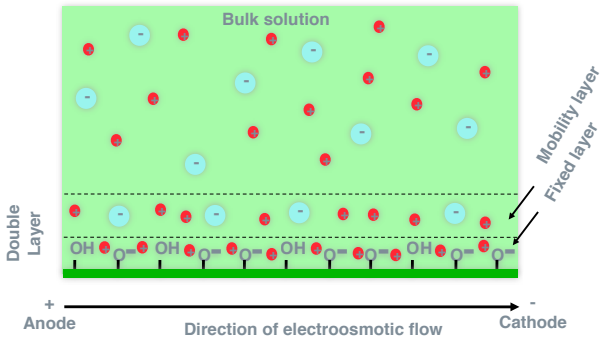
Under normal conditions, both anionic & neutral species migrate towards the cathode. This occurs because the capillary will is electrically charged - large number of silanol groups (Si-OH and SiO^-).



Electroosmotic mobility

- 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 layer



Electroosmotic mobility

Electroosmotic flow velocity

$$\upsilon_{eof} = \mu_{eof} E$$

$$\mu_{eof} = \frac{\varepsilon\zeta}{4\pi\eta}$$ (electroosmotic mobility)

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, ζ and μ_{eof} increase.

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.

Electroosmotic mobility

Total mobility of solutes

For any given solute, the total mobility is:

$$\nu_{tot} = \nu_{ep} + \nu_{eof}$$
$$\mu_{tot} = \mu_{ep} + \mu_{eof}$$

ν	= velocity
μ	= mobility
eof	= electroosmotic
ep	= electrophoretic

Under normal conditions

$$(\nu_{tot})_{cations} > \mu_{eof}$$
$$(\nu_{tot})_{anions} < \mu_{eof}$$
$$(\nu_{tot})_{neutrals} = \mu_{eof}$$

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.

Migration time

$$\upsilon_{total} = \frac{\iota}{t_m}$$

Where: ℓ = distance between inj. & detection points.
t_m = migration time.

Since $\upsilon_{total} = \mu_{total} E = (\mu_{ep} - \mu_{eof}) E$

$$t_m = \frac{\iota}{(\mu_{ep} - \mu_{eof}) E}$$

The magnitude of the field is: $E = \frac{V}{L}$

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

u_{ep} - u_{eof} is comparable to the adjusted retention volume.

Migration time

$$t_m = \frac{\iota L}{(\mu_{ep} - \mu_{eof}) V}$$

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 at the expense of resolution. This will be outlined in a bit.

Efficiency

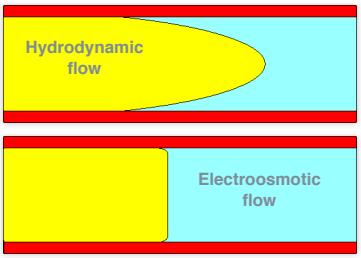
The number of theoretical plates - similar the what is used in chromatographic methods.

$$N = \frac{(\mu_{ep} - \mu_{eof})V}{2D}$$

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.



Efficiency

Selectivity

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

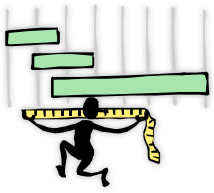
In CE, the analogous expression is:

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

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

Resolution

$$R = \frac{0.177(\mu_{ep_2} - \mu_{ep1})\sqrt{V}}{\sqrt{(\mu_{avg} - \mu_{eof})D}}$$



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

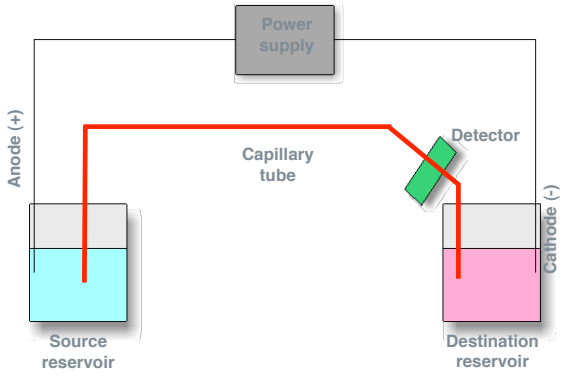
Instrumentation

The basic instrumentation is pretty simple.

- Power supply
- Anode compartment
- Cathode compartment

Both with buffer reservoirs

- Capillary tube
- Detector
- Sample vial

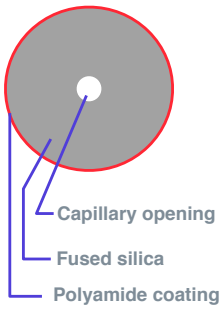


Basic equipment

Capillary tubes

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.



Basic equipment

Sample introduction.

- The tube is initially filled with buffer solution. Sample is introduced by dipping one end into the sample and then causing sample to enter the tube.

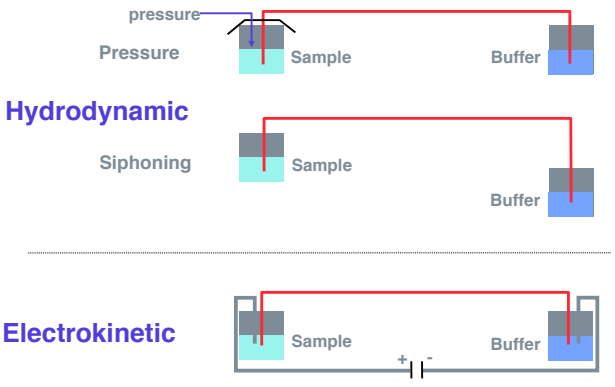
Electrokinetic injection method

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

Hydrodynamic injection method

- Pressure injection - the sample vial is pressurized.
- Siphoning - the sample is pulled into the tube.

Sample Introduction



Voltage application

Migration begins when 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 microamp range.

Detection

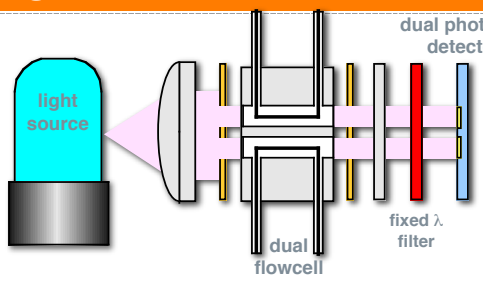
Approaches taken are similar to what is used in capillary LC.

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

Detection

	Detection Limit Moles injected	On-Column Molarity	Detection
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

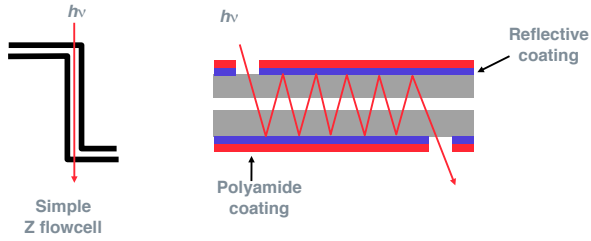
UV/Vis



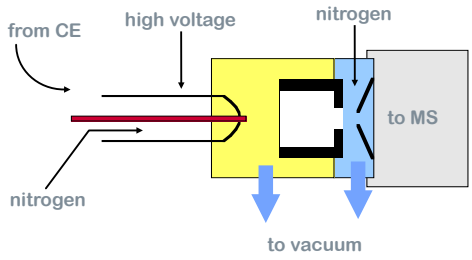
If the filter is replaced by a monochrometer, you end up with a variable wavelength UV/Vis system. This is similar to what is used for LC detection - at the capillary level though.

UV/Vis

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



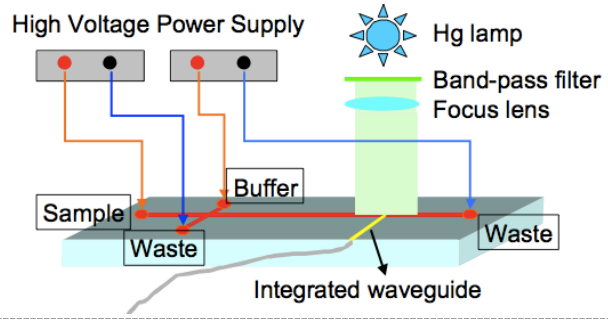
Mass spectroscopy



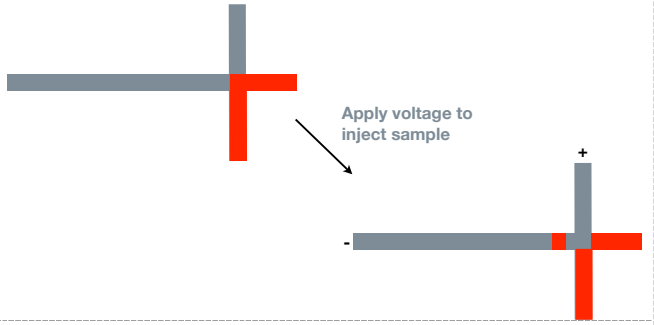
Microcapilllary and Multichannel EC

- Push to build instruments as small as possible (IC chip level)
- Build multi-channel cells to conduct MANY assays at the same time.
- Much of the effort is directed towards DNA/RNA/PCR type work (aka CSI stuff)
- I'm just going to show you a few examples.

Glas 'chip' approach/



Glass 'chip' approach



Variations of CE

Many variations of the approach have been used to deal with specific types of problems. We'll look at just a few examples.

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

The simplest form of the technique.

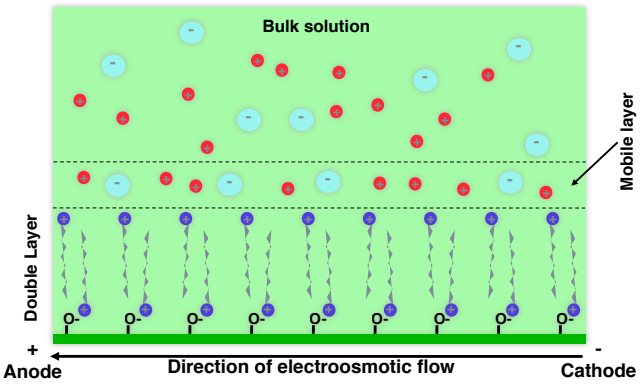
- Tube is filled with the buffer, sample loaded and the ends of the tube placed in the reservoirs.
- Under normal conditions, the 'sample' end of the tube is the anode and the solutes migrate toward the cathode
- As outlined earlier, cations elute first - with smaller (more highly charged) species eluting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

Capillary zone electrophoresis

- 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.

Capillary zone electrophoresis

Capillary zone electrophoresis

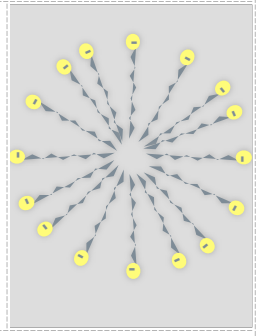


Capillary isoelectric focusing

- 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).
- 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.
- Use pressure for create flow to force off column after focusing.

Micellar electrokinetic capillary chromatography

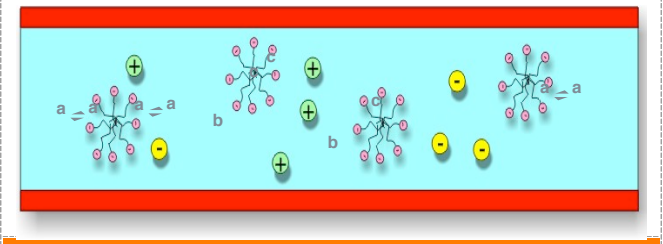
- CZE is not able to separate neutral species. MECC can overcome this limitation.
- The method relies on the addition of a surfactant (such as sodium dodecylsulfate)
- At high enough surfactant concentrations, micelles will form - consisting of 40-100 surfactant molecules



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.

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



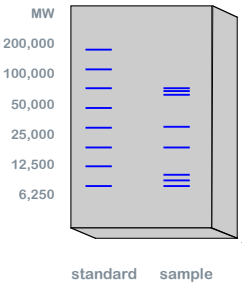
Micellar electrokinetic capillary chromatography

Capillary gel electrophoresis

- ☑ Capillary tube is filled with a polymeric, porous gel.
- ☑ Solutes migrate based on both their electrophoretic mobility and their size.
- ☑ Sort of a cross between electrophoresis and size exclusion chromatography.
- ☑ Approach has been used to 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.

Electrophoresis

- 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.'



Capillary Electrochromatography

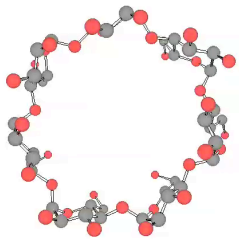
Another hybrid method for separating neutral species.

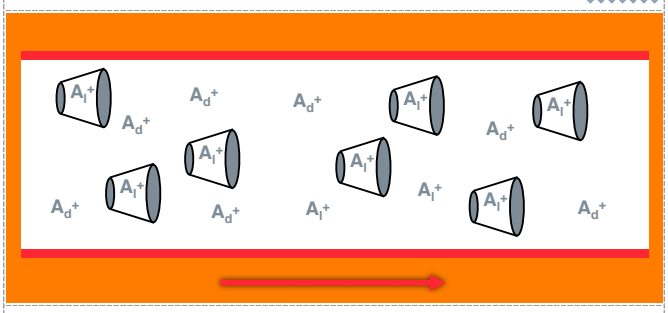
- Tubing is packed with 1.5-3.0 µm bonded, nonpolar phase - or phase is bound to the tubing wall.
- Similar to micelle approaches and also analogous to HPLC separations.
- Movement of the buffer solution due to electroosmotic flow acts as the 'mobile' phase.
- Neutrals partition between the buffer and the stationary phase.
- Unlike HPLC, high-pressure pumping is not required and better efficiency is possible - with a shorter analysis time.

Chiral separations

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.





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

Chiral separations