Lecture 4: Basic Chromatography: HPLC (RP & IEC) & Capillary Electrophoresis

1. High performance liquid chromatography (HPLC)
2. Components of an HPLC system
3. Mechanism of reverse phase HPLC (RP HPLC)
4. Gradient Elution
5. Ion pairing agents
6. Organic modifiers
7. UV detection
8. Typical gradients
9. Other types of chromatography
10. Capillary electrophoresis (CE)

1. *High performance liquid chromatography (HPLC)*

The various techniques used for High Pressure Liquid Chromatography are:

- Reverse Phase Chromatography.
- Size Exclusion Chromatography.
- Ion Exchange (cation or anion) Chromatography.
- Hydrophobic interaction Chromatography.

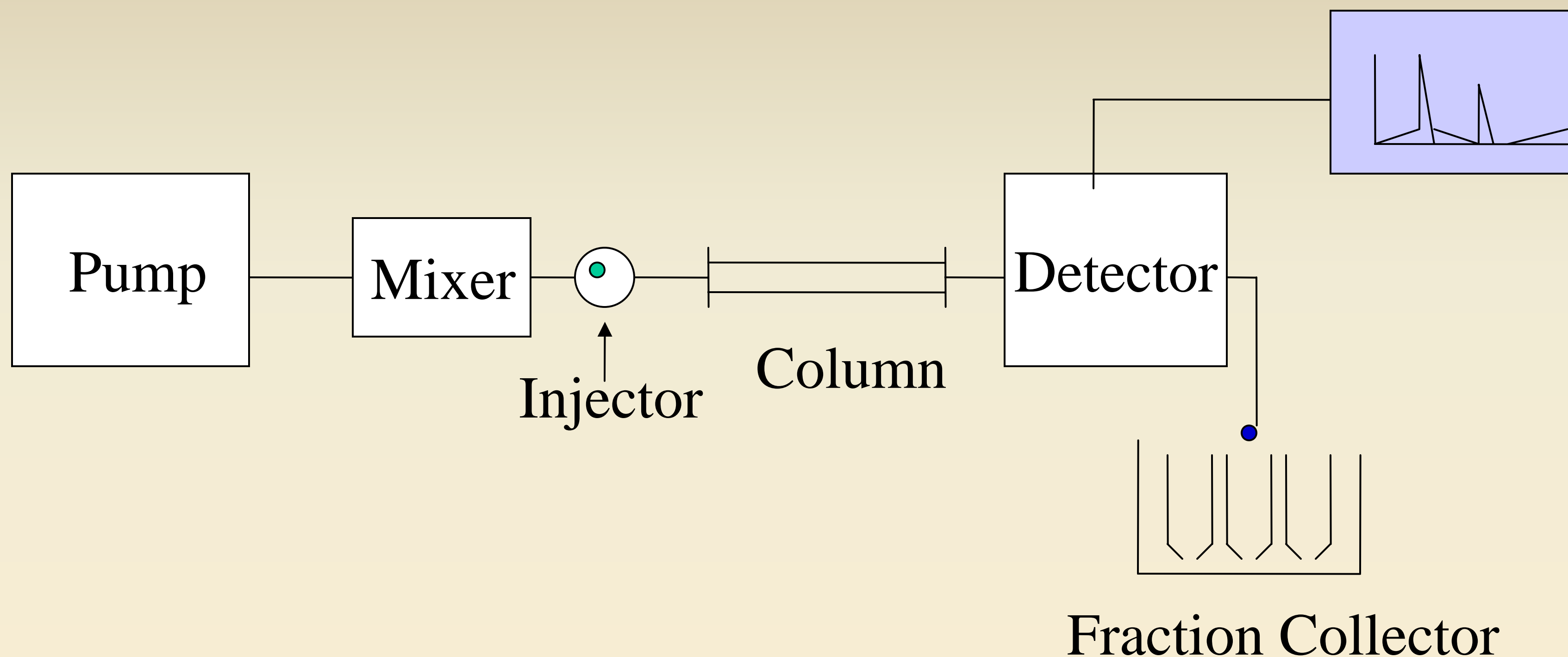
The high resolving power of Reverse Phase [RP] Chromatography has made it the dominant mode of HPLC for both analytical and preparative separation of peptides and proteins, as well as other biomolecules.

1. HPLC (cont)

The term **"Reverse Phase Chromatography"** was used because RP is a form of partition chromatography where chemically bonded phase is hydrophobic or non-polar (e.g. octadecyl group), and the starting mobile phase (e.g. water) must be more polar than the stationary phase.

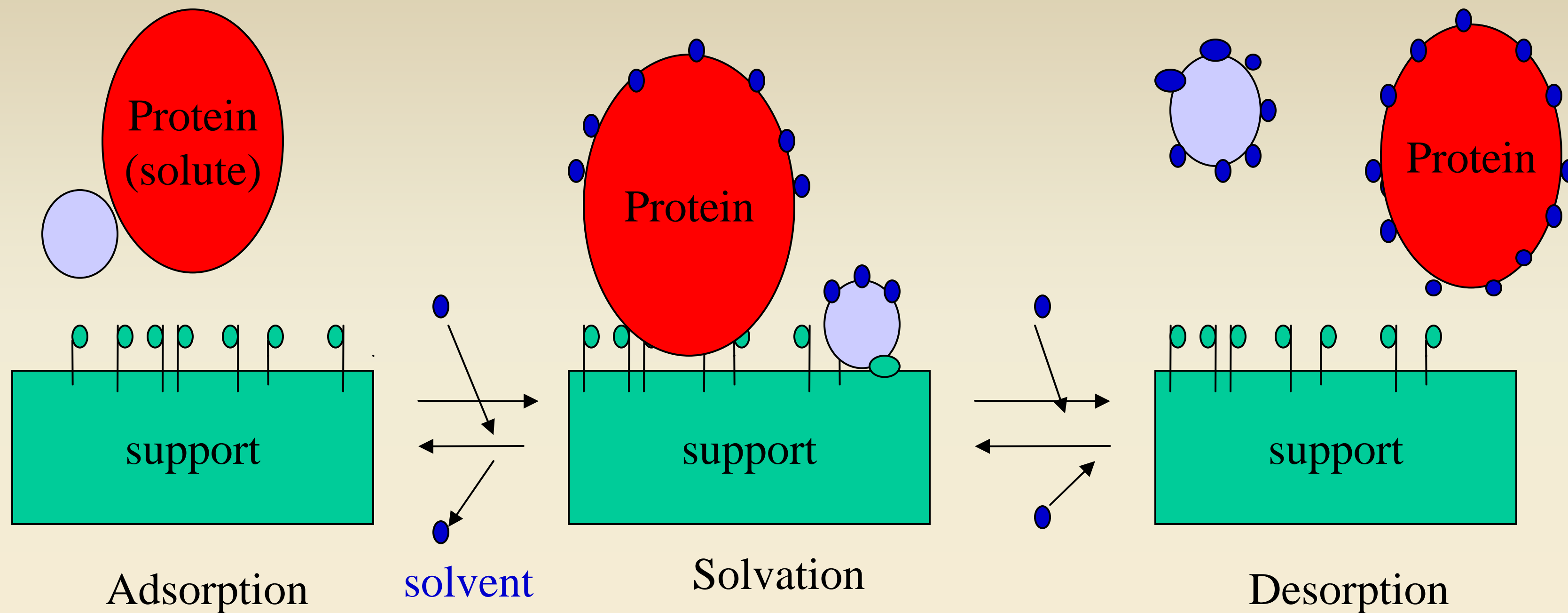
This is "reversed" from **normal phase chromatography**, where the stationary phase is polar or hydrophilic and the starting mobile phase is more non-polar or hydrophobic than the stationary phase, hence the term "Reverse Phase Chromatography".

2. Components of an HPLC system



Basic Components of an HPLC System

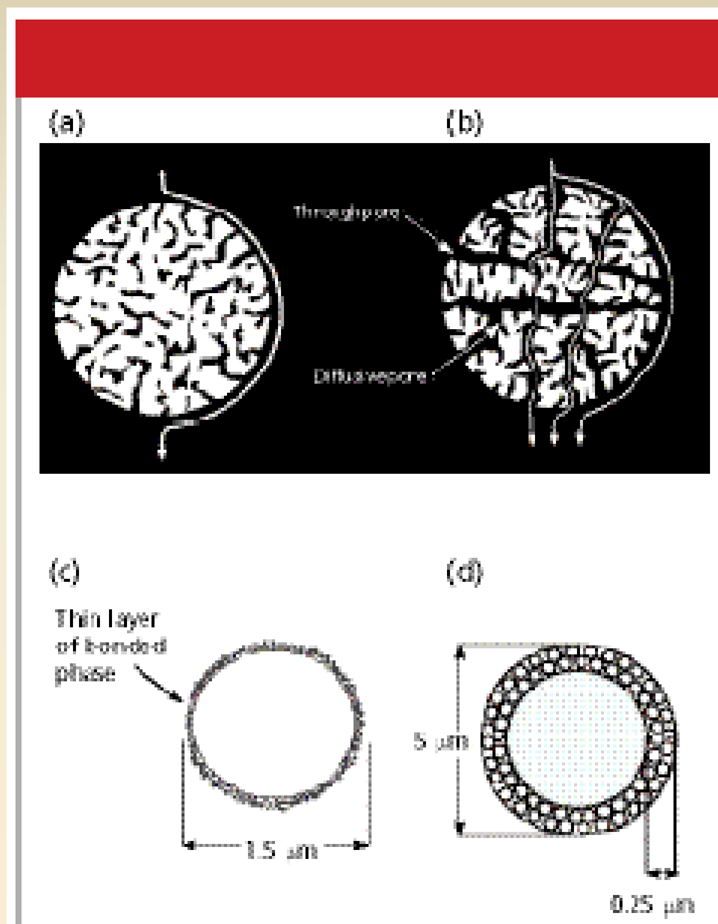
3. Mechanism of reverse-phase HPLC



Peptides and proteins are adsorbed onto the **hydrophobic surface of the column** and remain there until the concentration of the **organic modifier** is high enough to elute the molecules from the hydrophobic surface. The elution order is related to the increasing hydrophobic nature of the solute, the more soluble a solute is in water or the more hydrophilic the solute, the faster it will be eluted.

3. Mechanism of reverse-phase HPLC (cont)

Silica gel with chemically bonded phases is the packing of choice because of excellent efficiency, rigidity, and ability to be functionalized. 3– 5 mm diameter particles are the norm, smallest ~ 1.5-mm. Smaller particles shortens path length of the diffusion process, improves mass transfer, and provides better efficiency. Smaller particles decrease column permeability resulting in increase in backpressure, which is inversely proportional to the particle diameter squared.



Schematics of various particle types, incl. (a) totally porous, (b) perfusion, (c) nonporous, and (d) superficially porous particles.

3. Mechanism of reverse-phase HPLC (cont)

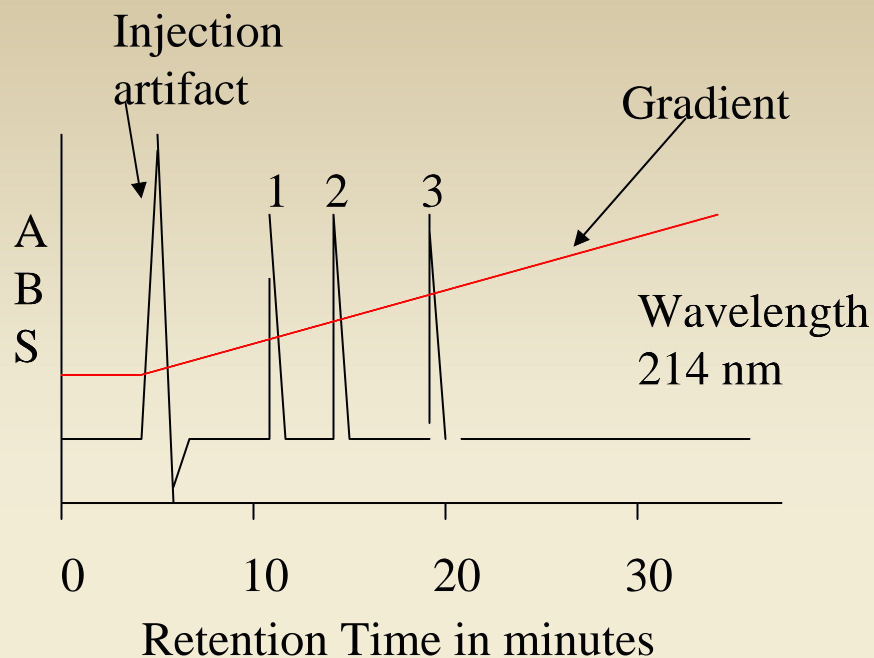
a.Totally Porous packing is dominated by diffusive pores. The surface area of the particle is contained within the pores. A reduction in particle size improves both the interparticle mass transfer and the intraparticle mass transfer. In a porous particle, solutes transfer from moving mobile phase into the stagnant mobile phase within the pores to interact with the stationary phase, then solute molecule must diffuse out of the particle and continue its journey down the column. Such a transfer occurs as the differential separation process proceeds and the solute is eluted from the column.

b.The diffusive pores are the same type present in the porous particles, the through - pores allow mobile phase to pass through the packing increasing the rate of mass transfer in mobile phase. When compared with a porous packing of the same particle and pore size, perfusion packings give better efficiency for large molecules

3. Mechanism of reverse-phase HPLC (cont)

c. Nonporous packings (1.5–2.5 mm) allows faster rates (few minutes) of mass transfer and separations for both large and small molecules. Unfortunately, the thin layer of stationary phase limits the capacity of the packing, making it unsuitable for preparative separations.

d. Superficially porous packings are similar to nonporous silica but the particle size (5 mm) and surface area are larger resulting in lower pressure drop and increased sample capacity. Recommended for larger biomolecules.



Buffer A: 0.1% TFA water
Buffer B: 0.1% TFA acetonitrile

Time	% A	% B
0	100	0
3	100	0
30	70	30

#	Peptide Sequence	MW	RT	Hydrophobicity
1	RGGGGIGIGK	871.0	10.5	-0.180
2	RGGGGIGLGK	871.0	15.0	-0.250
3	RGGGGLGLGK	871.0	20.5	-0.320

Retention Time: The time between injection and the appearance of the peak maximum.

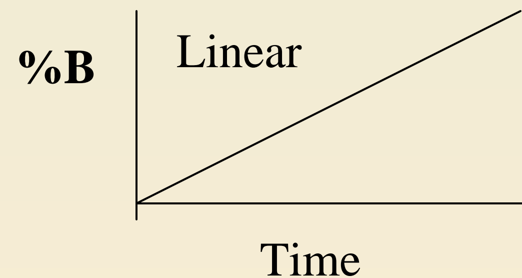
4. Gradient elution

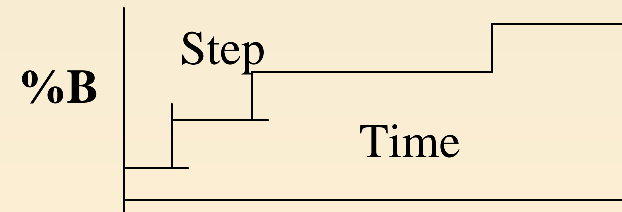
Technique for decreasing separation time by increasing mobile phase strength over time during the chromatographic separation. Gradient can be linear or stepwise.

Binary, ternary and quaternary solvent gradients can be used. The most widely used is the linear binary gradient.

Types of reversed phase column:

Type	MW in Da
C_{18}	< 5,000
C_{8}	5,000 – 10,000
C_{4}	> 10,000





5. Ion-pairing agents

Ion-pairing agents are ionic compounds that contain a hydrocarbon chain that imparts a certain hydrophobicity so that the ion pair can be retained on a reversed-phase column.

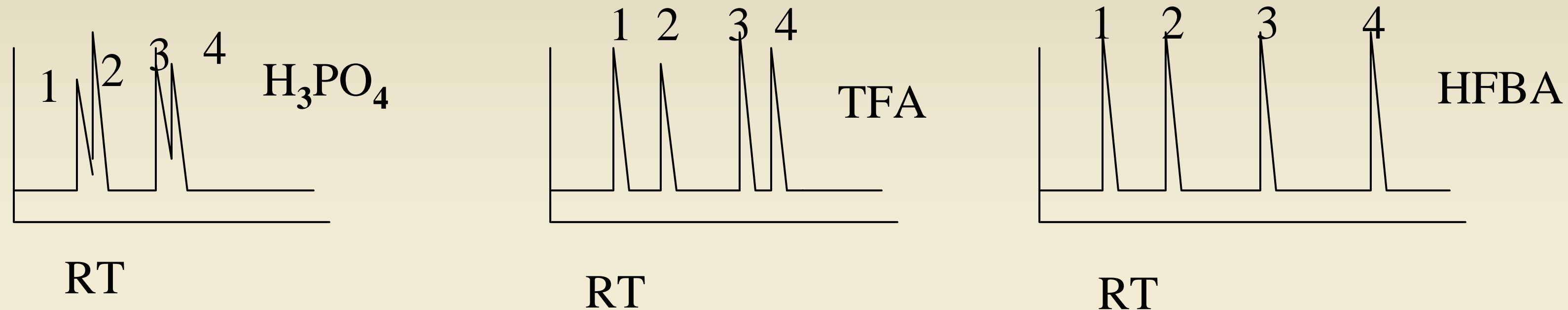
Ion Pairing agents are added at concentrations of 0.05 to 0.2.

All ion-pairing agents are potentially capable of ion-pairing with the positively charged basic residues of peptides or proteins, thus reducing hydrophilicity and increasing their retention time.

Hydrophobic counterions such as TFA and HFBA in addition to ion-pairing with the positively charged solute also increase the affinity of the solute (peptide or protein) for the hydrophobic stationary phase.

While hydrophilic counterions such as H_3PO_4 following ion-pair formation with positive charged residues would be unlikely to interact with the stationary phase.

5. Ion-pairing agents (cont)



-Trifluoroacetic acid (TFA).
-Heptafluorobutyric acid (HFBA).
-Hexafluoroacetone (HFA).
-Formic Acid (FA)
-Phosphoric Acid.
-Hydrochloric Acid.
-Triethylamine Phosphate (TEAP).

The longest retention times are observed with HFBA and the shortest with HCl. Also the highest resolution is obtained with HCl and HFBA.

6. Organic modifiers

Additive that changes the character of the mobile phase. In RP chromatography, water is the weak solvent, and acetonitrile, the strong solvent is added gradually to generate a gradient.

Acetonitrile.
Isopropanol.
Methanol.
Ethanol

Acetonitrile is the reverse phase solvent of choice because the UV cut off for acetonitrile is190 nm, allowing detection at lower wavelengths. It is less viscous than methanol, thus causing less fluctuations in pressure. Less buble formation occurs when it is mixed with water. It has also better selectivity for peptides and proteins. Isopropanol is used either alone or in combination with acetonitrile to elute large or hydrophobic proteins.

7. UV detection

Peptides and proteins are detected by UV absorption at wave length from **210-220** nm which detect the amide bond. The aromatic side chains of tyrosine, phenylalanine and tryptophan absorb light in the **250 to 290** nm ultraviolet range.

The two most widely used wavelength are 214 and 280 nm

8. Typical gradients

When dealing with an unknown mixture, although time consuming the following is a good starting gradient. Once you find the are where your compounds of interest elute, you can greatly shorten the analysis time.

Buffer A: 0.1% TFA water
Buffer B: 0.1% TFA acetonitrile

Time	%A	%B
0	100	0
5	100	0
75	30	70
85	0	100
95	0	100
96	100	0
120	100	0

9. Other types of chromatography

Size Exclusion chromatography:

Mainly used for very large proteins.

Cation exchange chromatography:

Very useful when peptides or proteins contain an inordinate number of negatively charged residues (Asp and Glu). Also useful for proteins containing posttranslational modifications that make them more hydrophilic.

Mixed mode chromatography:

uses a mixture of reverse-phase and cationic or anionic sorbents.

For Chromatography in a hurry: New faster shorter columns are available from every manufacturer. These columns claim to do separation in a tenth of the time needed by conventional columns. However their peak resolution is not as good as conventional columns from the same manufacturers.

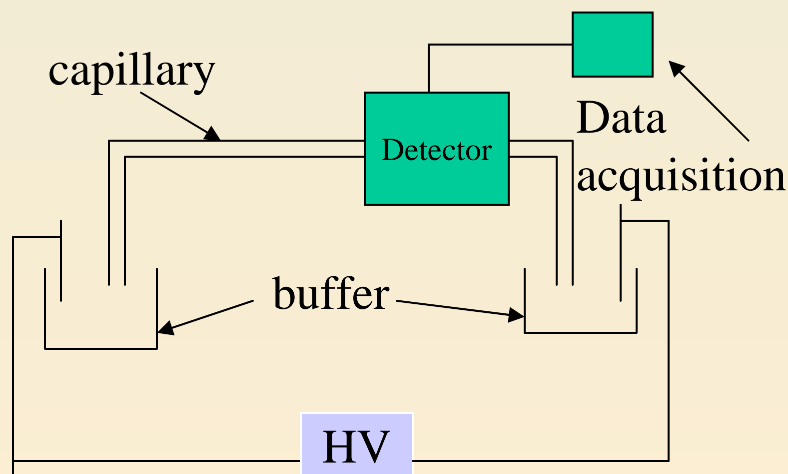
Waters IS (Intelligent Speed) columns are supposed to be 10 times more speedy than the conventional XTerra C_{18} column.

Applied Biosystems Poros columns also have a series of fast columns.

For purification of very hydrophilic Proteins or peptides: which are usually glycosilated, phosphorylated or contain a high percentage of basic, acidic or polar residues, use HFBA as a pairing agent. HFBA is the ion pairing reagent of choice. In some cases both HFBA and a mixed mode column (C_{18} or C_4 + Cation or C_{18} or C_4 + Anion) could separate such compounds. This regimen is also successful for separation of glycolipids and oligosaccharides.

10. Capillary electrophoresis (CE)

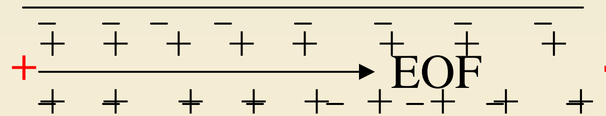
CE is a cross between gel electrophoresis and high pressure liquid chromatography. Separation is based on size to charge ratio. It uses high voltage generating electroosmotic [EOF] and electrophoretic flow of buffer solutions and ionic species, respectively within the capillary.



The basic instrument is made of a fused silica capillary, a controllable high voltage power supply, two electrode assemblies, two buffer reservoirs, a UV detector and a data acquisition system. The ends of the capillary are placed in the buffer. After filling the capillary with buffer the sample can be introduced by dipping the end of the capillary in the sample solution. In CE nothing is retained so the analogous term to retention time is migration time.

10. CE (cont)

The fused silica capillaries have ionizable silanol group in contact with the buffer within the capillary. The pI of the silica is about 1.5. The degree of ionization is controlled by the pH of the buffer. The negatively charged wall attracts positively charged ions from the buffer, creating an electrical double layer. When voltage is applied across the capillary, cations in the diffuse portion of the double layer migrate in the direction of the cathode carrying water with them. The result is a net flow of buffer solution in the direction of the negative electrode.



The EOF makes possible the simultaneous analysis of cations, anions and neutral species in a single analysis. **At neutral to alkaline pH, the EOF is sufficiently stronger than electrophoretic migration, such that all species are swept towards the negative electrode.**

The order of migration is: cations, neutrals, and anions

10. CE (cont)

Zwitterionic molecules such as peptides are easily separated. At high pH the EOF is large and the peptide is negatively charged. Despite the peptide migration towards the positive electrode, the EOF is overwhelming and the peptide migrates toward the negative electrode. At low pH the peptide is positively charged EOF is very small. Thus peptide electrophoretic migration and EOF are towards the negative electrode. In silica capillaries most solutes migrate towards the negative electrode regardless of charge when the buffer pH is is above7.0. At acidic pH, most zwitterions and cations will also migrate towards the negative electrode.

Buffers for capillary electrophoresis

Buffer	*pH range*	*Zwitterionic buffer*	*pH range*
Phosphate	1.14 - 3.14	MES	5.15 – 7.15
Acetate	3.76 – 5.76	PIPES	5.80 – 7.80
Borate	8.14 – 10.14	HEPES	6.55 – 8.55
		Tricine	7.15 – 9.15

10. CE (cont)

Advantage of CE:

-Separation takes minutes rather than hours.
-Uses much less reagents.
-Better separation of peptide with similar hydrophobicity index.

Disadvantage:

-Requires much more skill and technical ability.