nibrt
National Institute for Bioprocessing Research and Training

Lecture 8 – Introduction to HPLC

Learning Objectives

- Discuss the **basic principles of HPLC**
- Describe of the various **HPLC separation mechanisms**
- List the **applications of each separation** in bioanalytics

Topics

- Overview of Chromatography
- Reverse Phase Chromatography
- Size Exclusion Chromatography
- Ion Exchange Chromatography

Chromatography

- Can be used to:

1. **Separate the pure protein** from contaminants such as viruses, DNA, other proteins, toxins, aggregates

Large scale chromatography in Downstream Processing

2. To **characterise proteins** during processing to ensure consistent production of the protein with correct sequence, folding, structure, purity and post-translational modifications

Small scale chromatography in bioanalytics

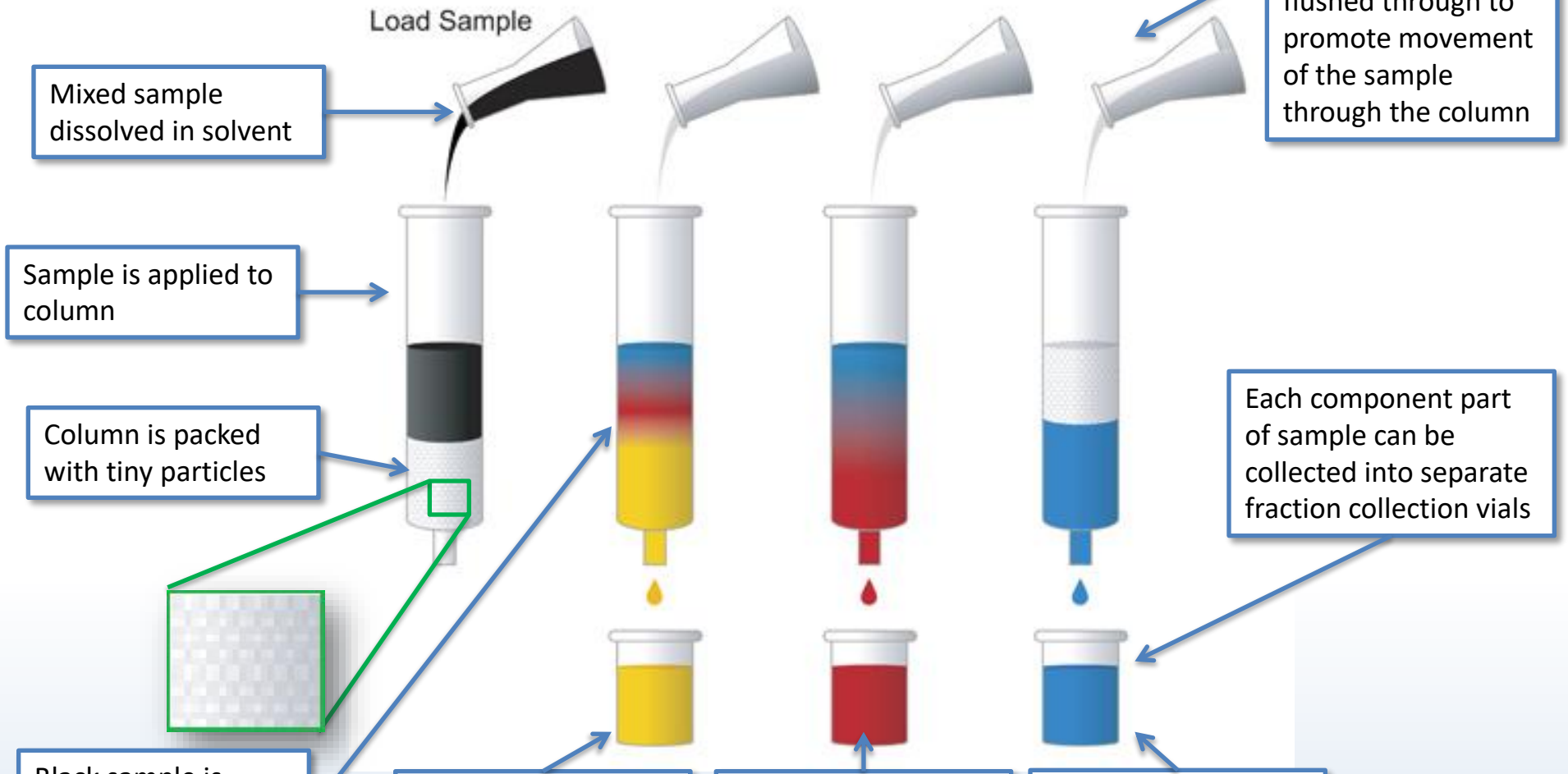
Liquid Chromatography

- Sample is dissolved in a solvent and flushed through a column packed with lots of tiny solid particles

- **Solvent** = **'mobile phase'**
- **Solid particles** = **'stationary phase'**

- The individual components of the sample separate due to:
  - higher affinity for the mobile phase
  - higher affinity for the stationary phase

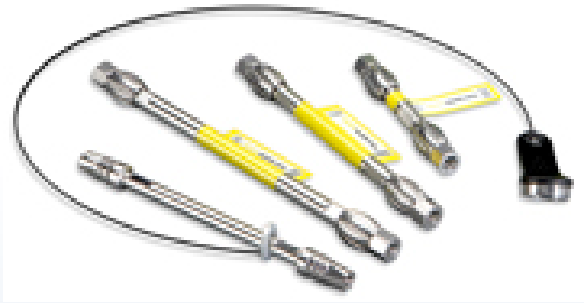
Liquid Chromatography



Modified from Waters website

High Performance Liquid chromatography
Introduction

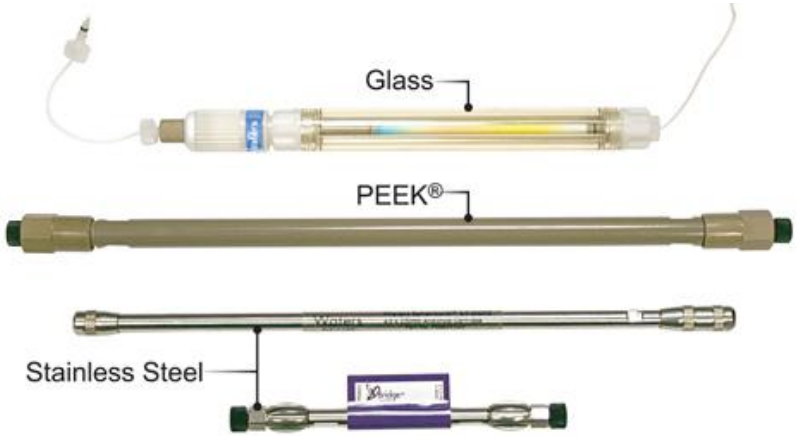
- **High pressure used to generate flow** required for LC in packed columns
- One of the most powerful tools in analytical chemistry
- Analytical or preparative



HPLC
Column

Column

- Usually glass or metal tube of sufficient strength to withstand the pressure
- Mobile phase runs through & permeates the stationary phase



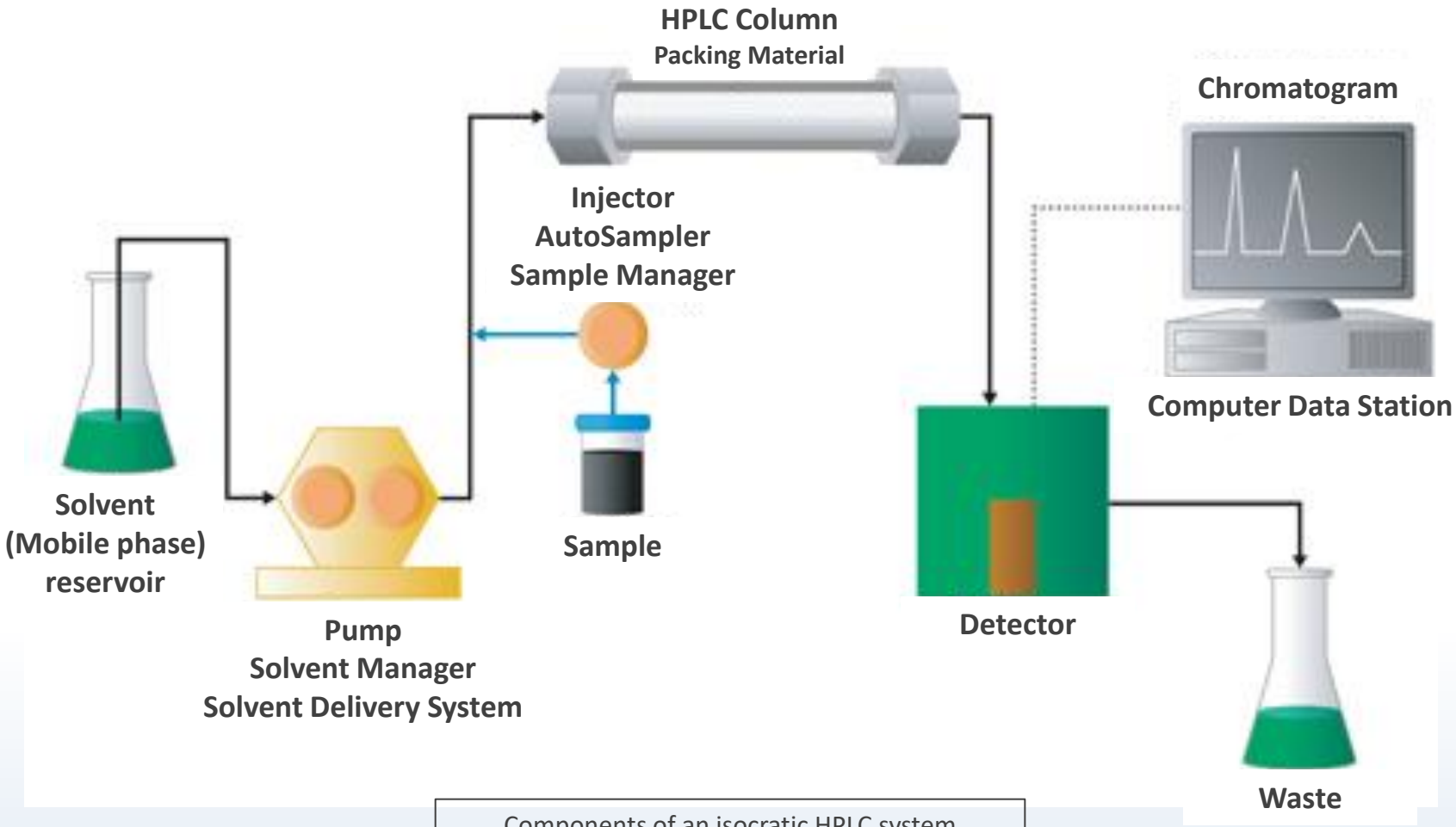
PEEK (an engineered plastic) and glass have inert surfaces but are less tolerant of high pressures than stainless steel

Stationary phase (particles)

- Usually solid particles of very small diameter (1.7µm– 5µm)
- Particles can be coated with various substances to attract a specific type of molecule
- Should have a large surface area which is accessible to mobile phase & sample
- Common stationary phase material is **silica** (component of sand/quartz)

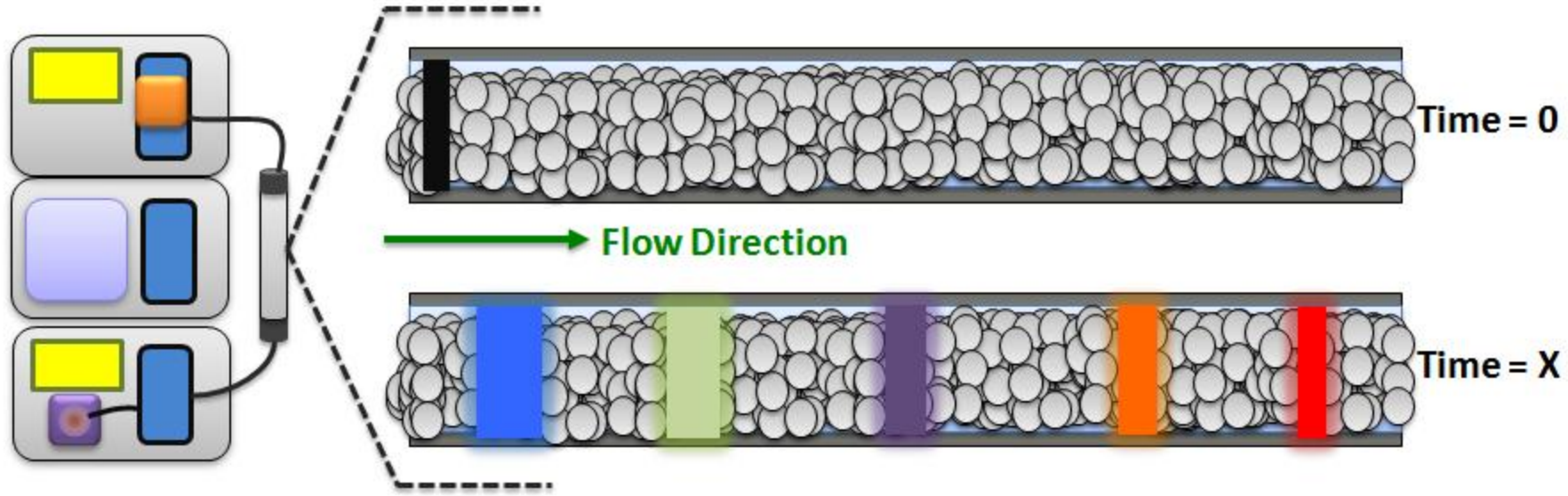
Image from Waters

HPLC
Equipment



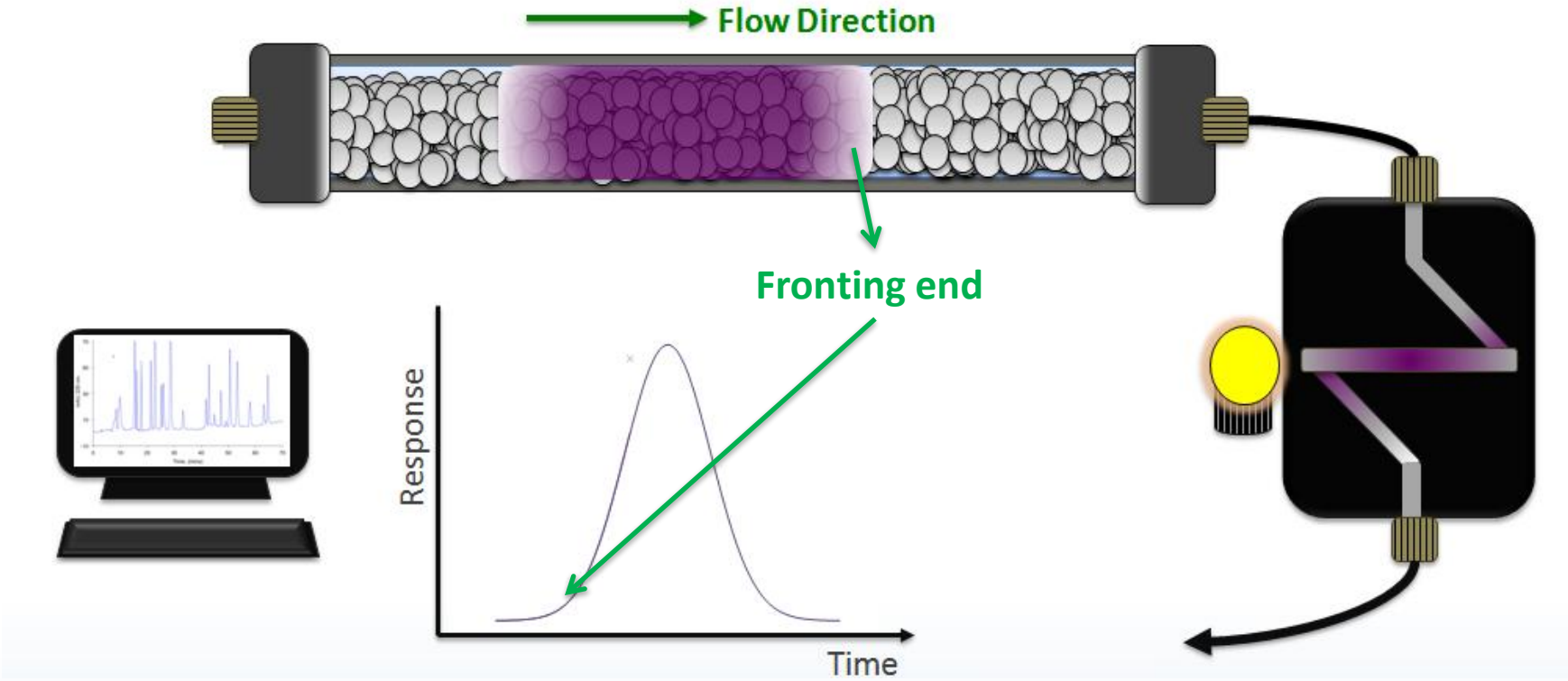
Components of an isocratic HPLC system
Adapted from Waters website

HPLC
Separation on the column



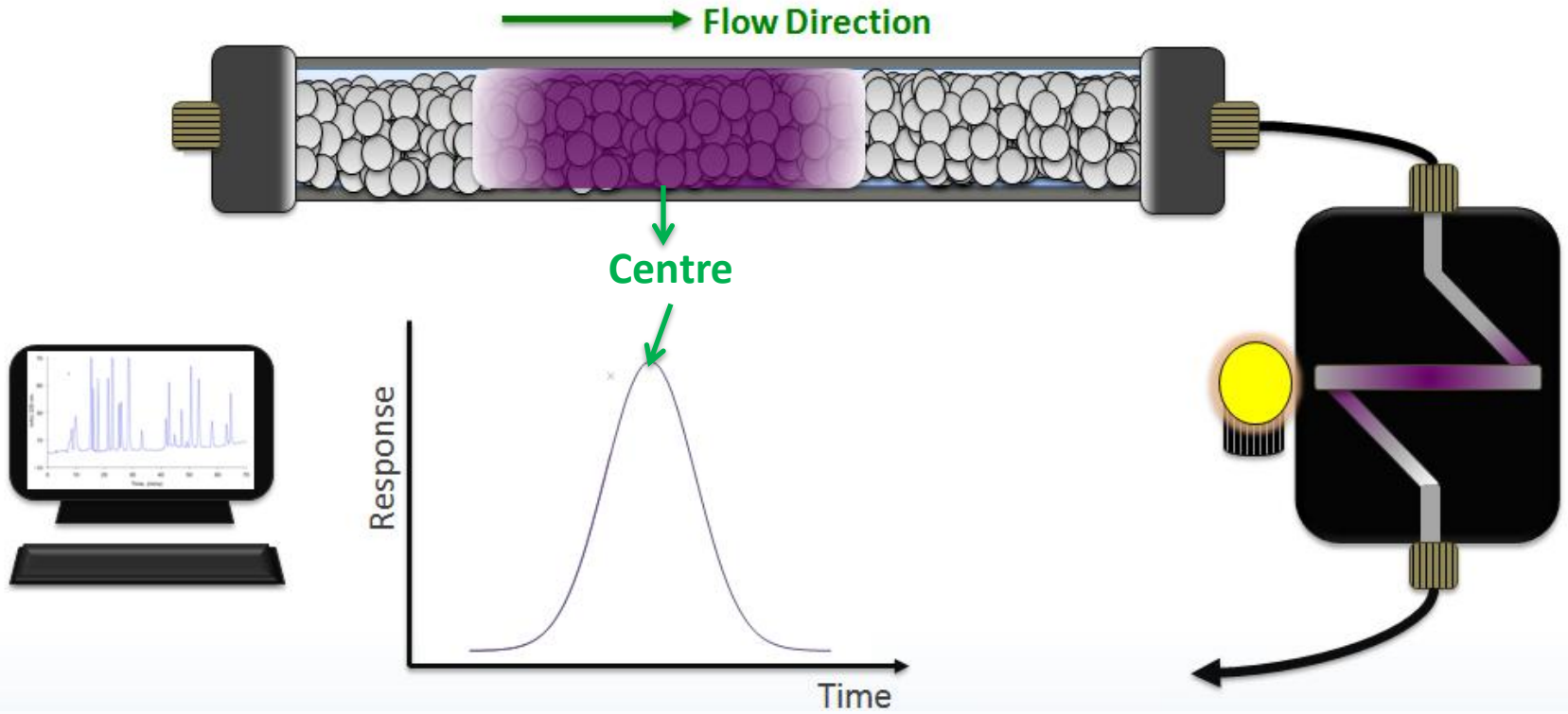
- As the mixture passes through the column the individual components separate based upon their **affinities for the stationary phase and the mobile phase**
- Affinities for stationary phase: **red** < **orange** < **purple** < **green** < **blue**
- A detector analyses the components as they elute

HPLC
How a band becomes a peak



- Bands are **more concentrated** in the **centre** than at the fronting and tailing edges, this distribution of analyte molecules reach the detector at different times generating a peak

HPLC
How a band becomes a peak



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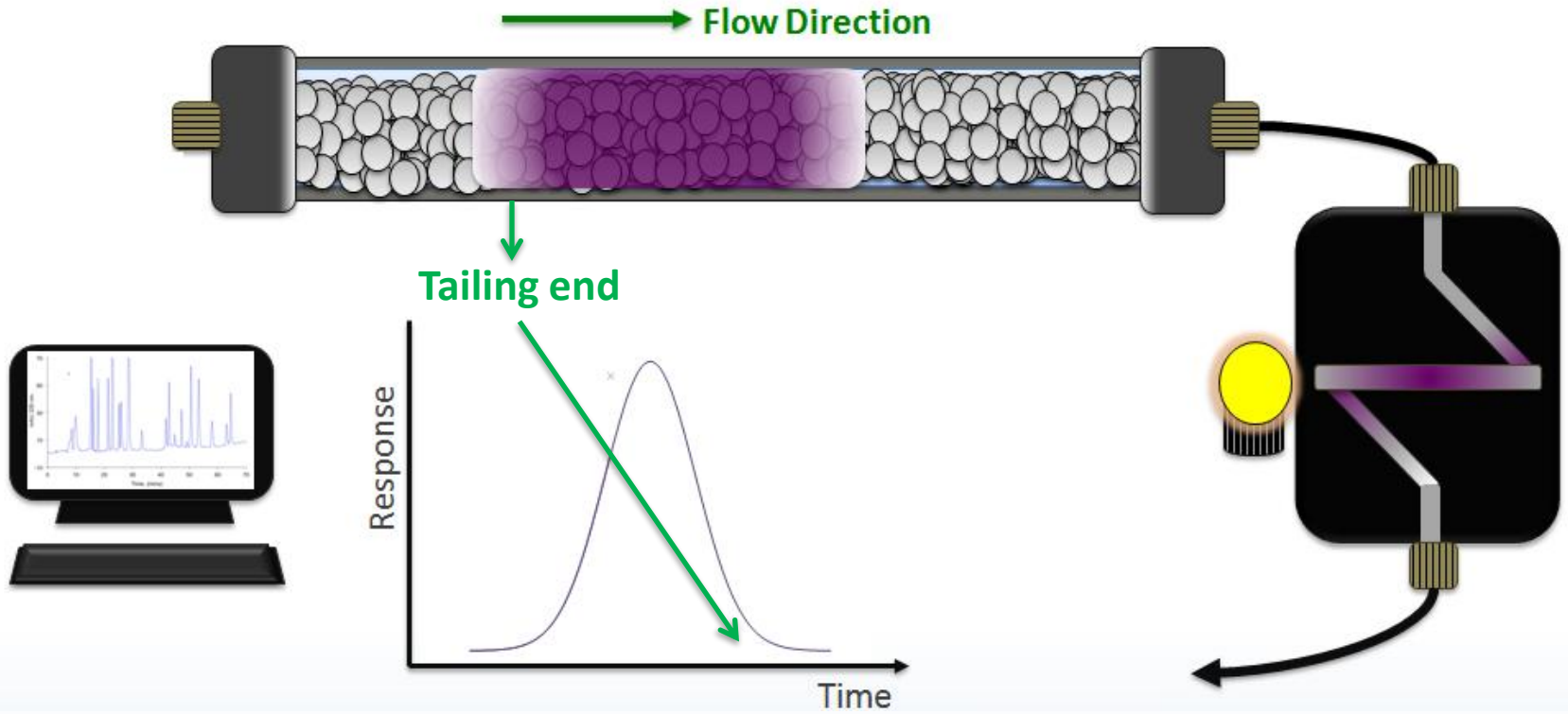

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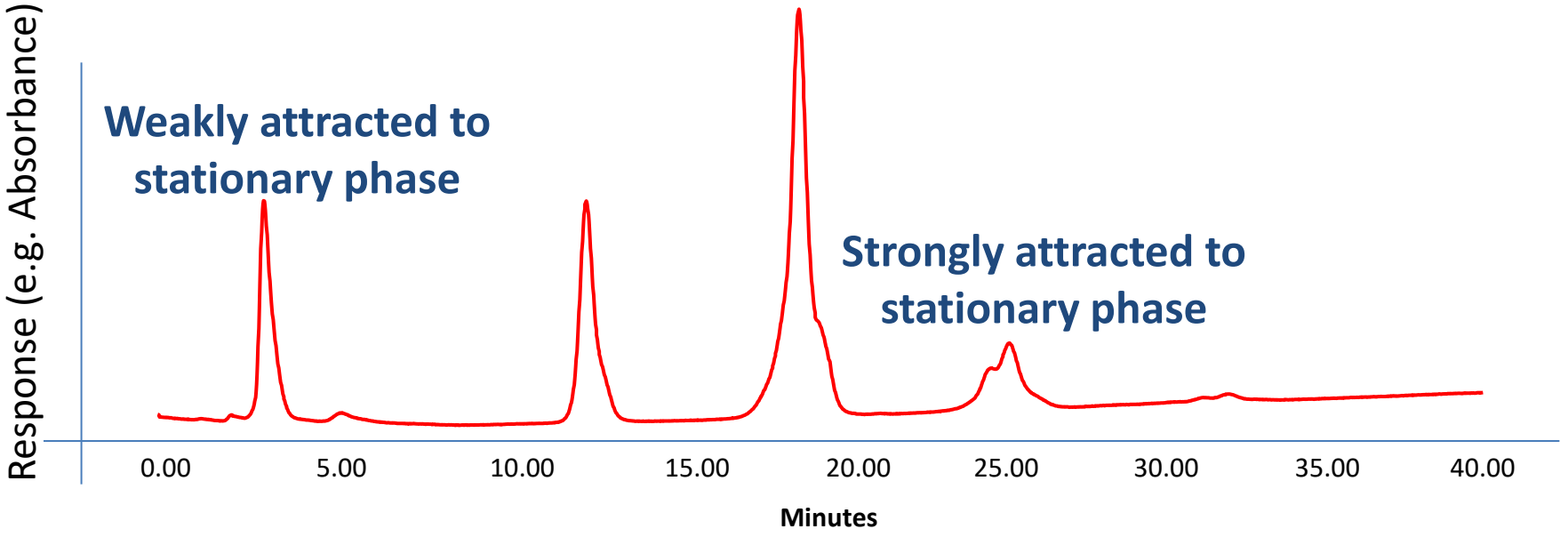
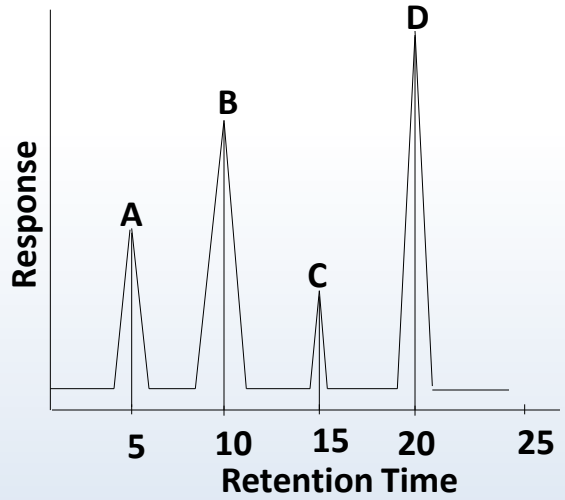
HPLC
Chromatogram



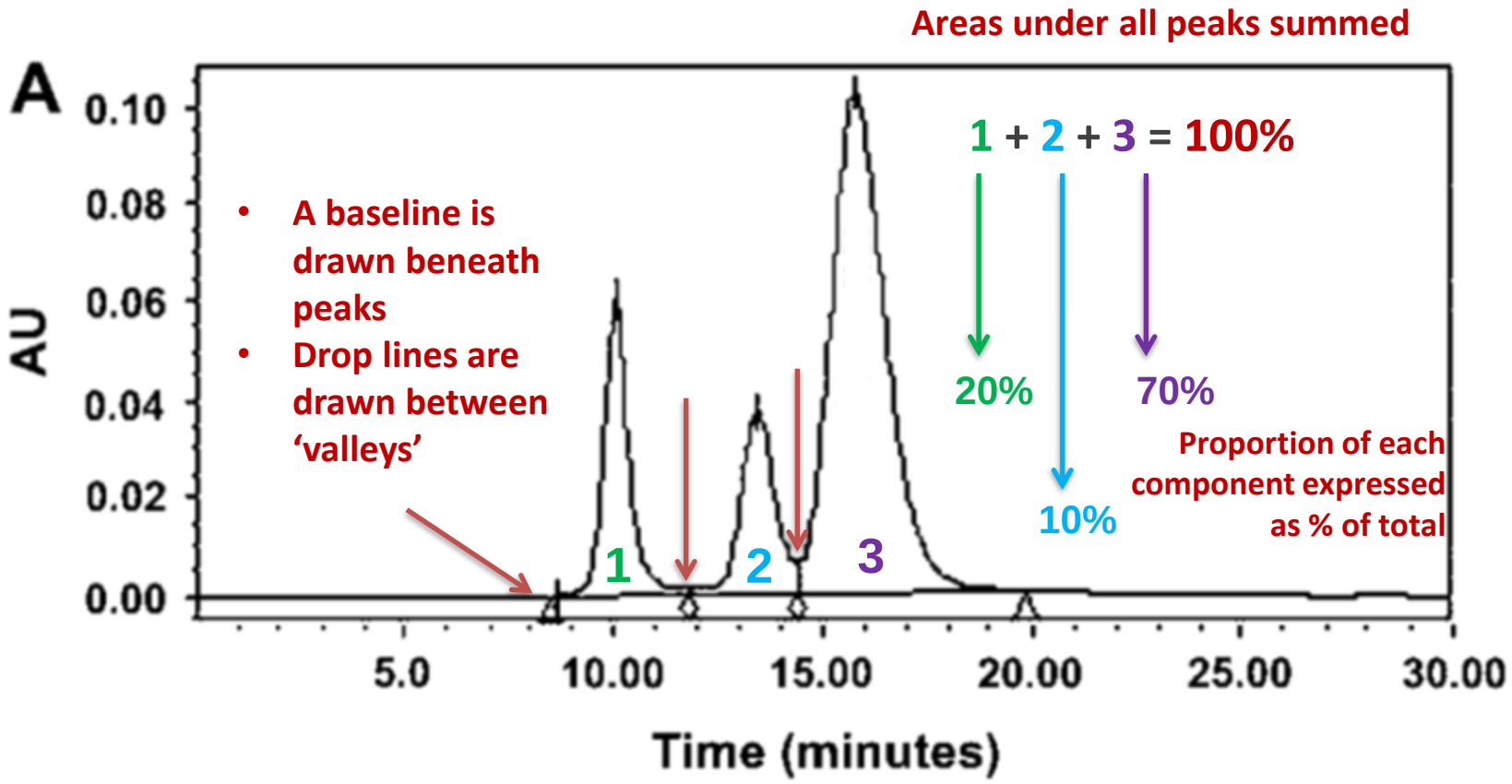
Retention time

- Time it takes sample to travel from injection port through column to detector



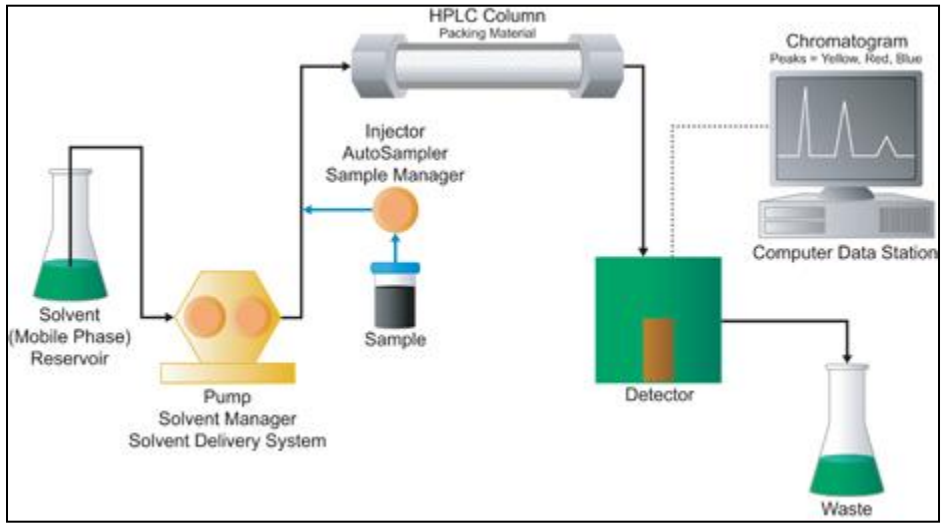
Quantitation

- Chromatograms are ‘integrated’ to quantitate sample components

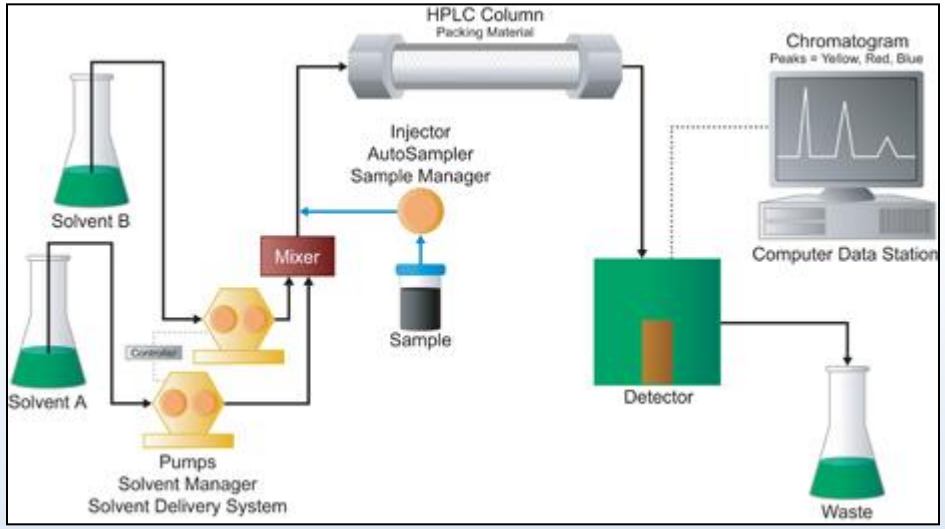


HPLC

Isocratic v Gradient Methods



Isocratic: One mobile phase is used throughout the run



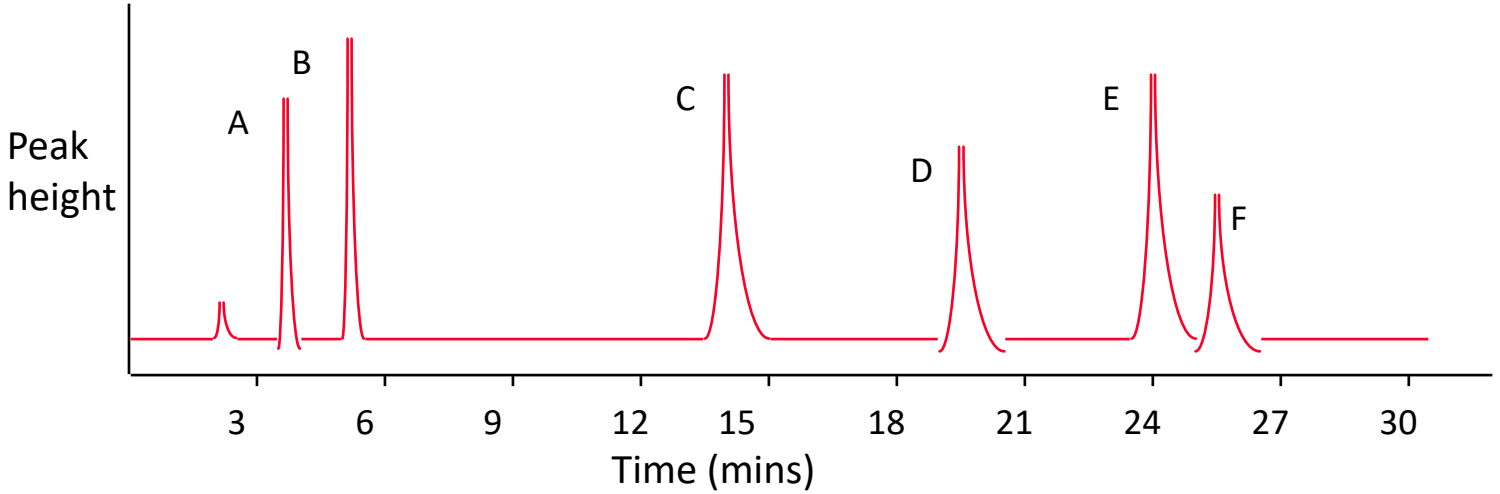
Gradient:

- Two mobile phases are used (A & B) that are mixed before hitting the column
- Allows separation of a wider range of analytes in a single run

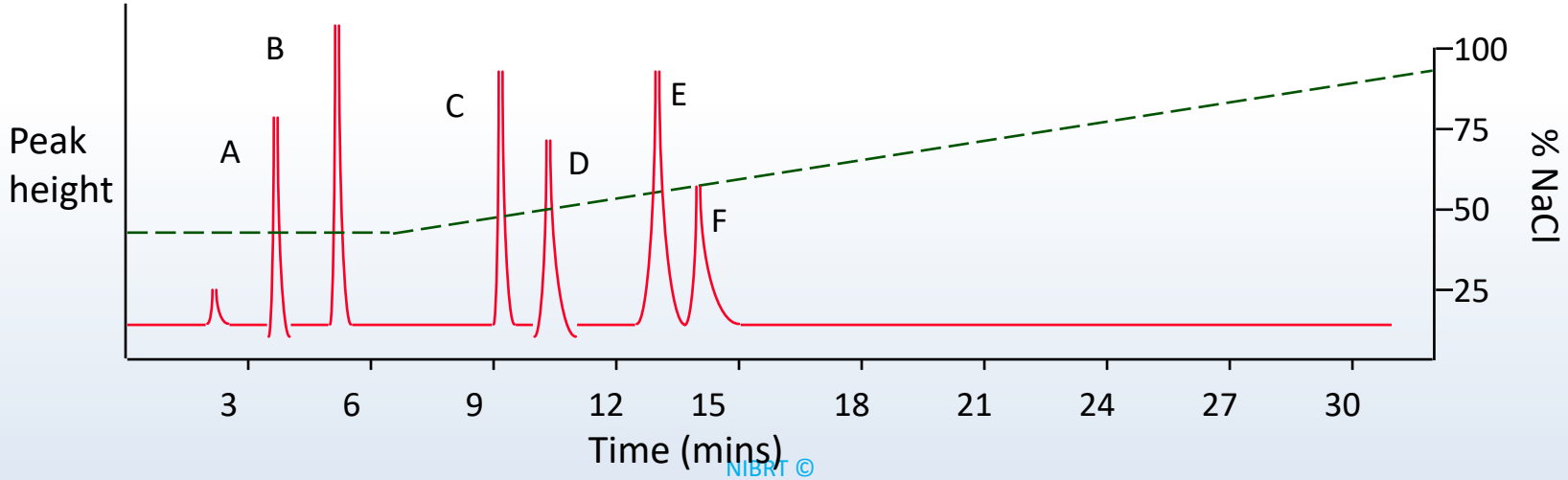
HPLC

Isocratic v Gradient Methods

- **Isocratic**: mobile phase composition remains the same throughout the run



- **Gradient**: mobile phase composition is changed during the separation. As the separation proceeds, the elution strength of the mobile phase is increased



HPLC
Mobile phases

- Water
- Organic solvents (HPLC grade)
- Buffers

Mobile phase polarity

Water > acetonitrile > methanol > ethanol > tetrahydrofuran >propanol > cyclohexane > hexane

Example: Mobile phase for RP-HPLC gradient method
Start: 90% Water + 10% Acetonitrile
End: 80% Acetonitrile + 20% Water

Degassing is required to remove dissolved oxygen to prevent band spreading and interference

Filtering of buffers is required for UPLC analysis

Sample/Analyte Characteristics

- Characteristics of chemicals can be used to create HPLC separations:
  - Polarity
  - Electrical Charge
  - Molecular Size
  - Biological affinity

Key Analytical methods
Chromatography

Isolation Method	Feature
• Normal phase chromatography (NP-HPLC)	Hydrophilicity
• Reversed phase chromatography (RP-HPLC)	Hydrophobicity
• Affinity chromatography	Biorecognition
• Size exclusion chromatography (SEC)	Size
• Ion exchange chromatography (IEX)	Charge
• Hydrophilic interaction chromatography (HILIC)	Hydrophilicity

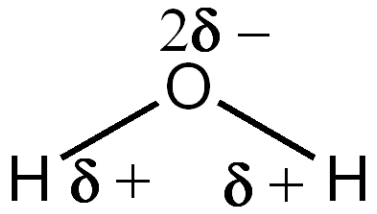
Topics

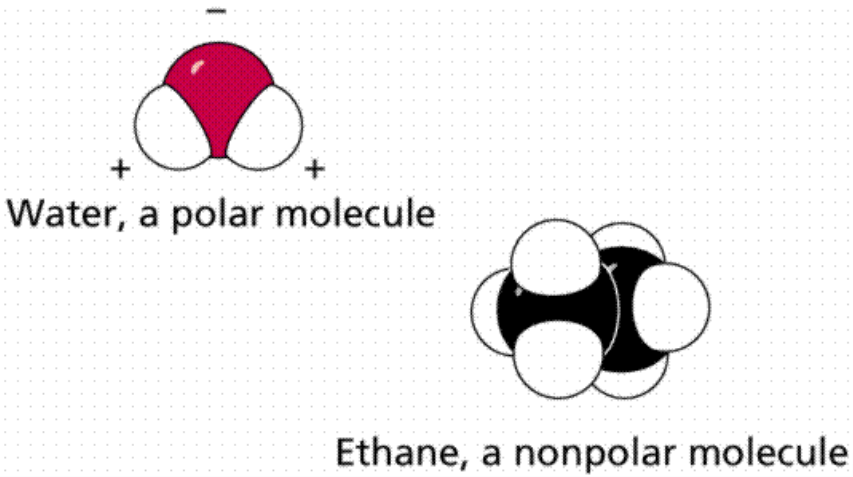
- Overview of Chromatography
- Reverse Phase Chromatography
- Size Exclusion Chromatography
- Ion Exchange Chromatography

Polarity

Polarity:

Separation of charge across a molecule due to uneven sharing of valence electrons between atoms in a molecule





- Polar compound: more hydrophilic, e.g. water (H_2O)
- Non-polar compound: more hydrophobic, e.g. chloroform ($CHCl_3$), oils, fats

Reversed Phase HPLC

- Separation of molecules based on **hydrophobicity**
- Chemical attraction involved: **hydrophobic interactions**

Stationary Phase:

- Silica particles which have a hydrophobic (non-polar) coating

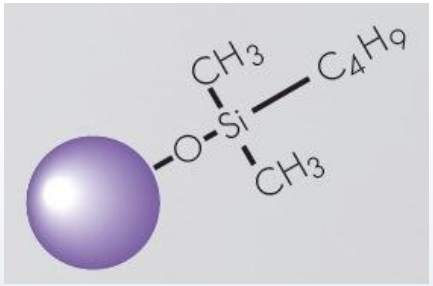
Mobile phase:

- Gradient: from mostly aqueous to mostly organic

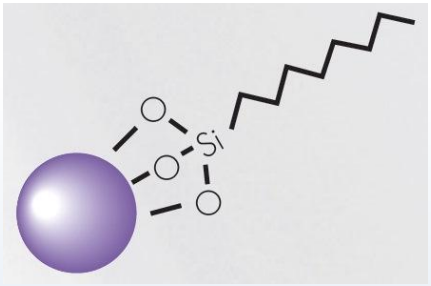
- **Hydrophobic** compounds are attracted to the to **non-polar** stationary phase in a polar mobile phase
- **Polar** compounds in the sample will be attracted to the **polar** mobile phase and move faster to create the separation

RP-LC columns

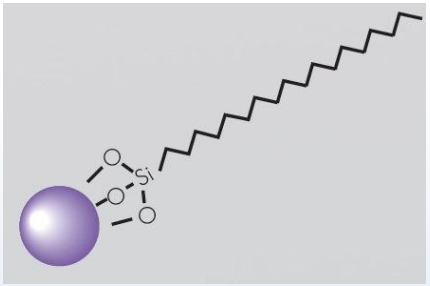
- The stationary phase is made up of hydrophobic alkyl chains ($-CH_2-CH_2-CH_2-CH_3$) that interact with the analyte e.g. C4, C8, and C18.
- C4 is usually used for **proteins**
- C18 is usually used for **peptides or small molecules**
- C4 and C8 columns are better than C18 for separation of proteins because elution takes place in lower proportion of solvent so minimise denaturation of protein



C4



C8



C18

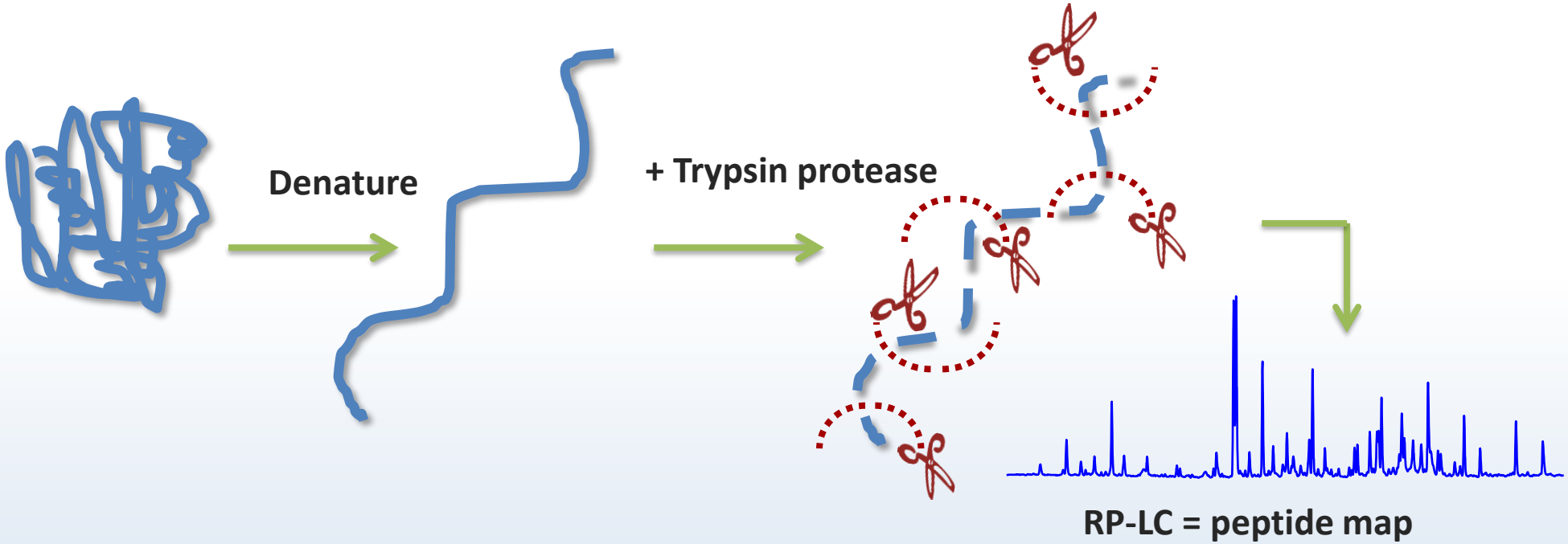
Applications of RP-LC

- Protein quantitation
- Separation of complex protein mixtures
- Detection of isoforms
- Peptide mapping

Analytical: Peptide mapping

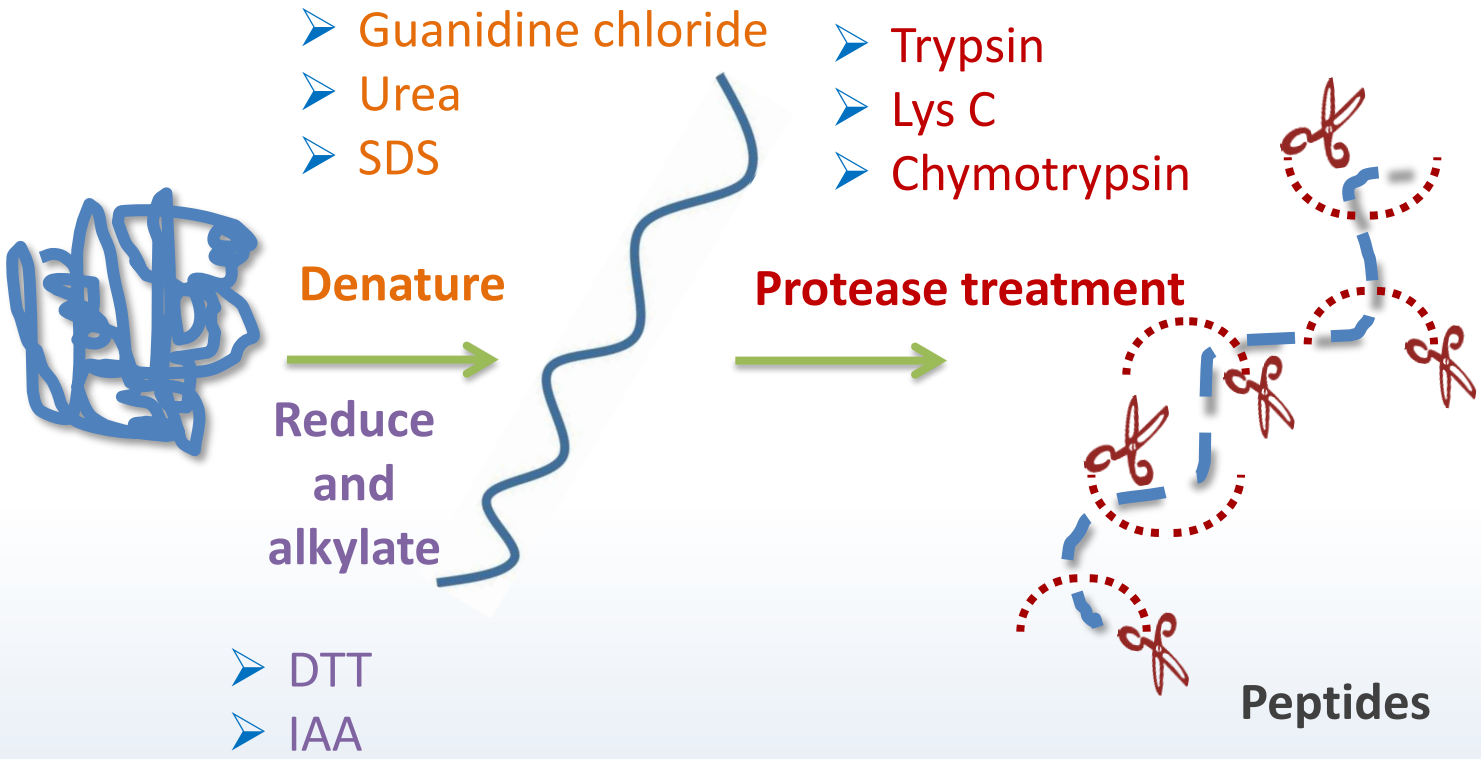
- Peptide mapping involves **digesting** a protein with a **protease** such as **trypsin** to create specific **peptides**

- Used to produce a **unique 'fingerprint'** of an individual protein when analysed by RP-LC



Protein Digestion Yields Peptides

- **Digestion** of the protein product with a **protease** to create specific **peptides**

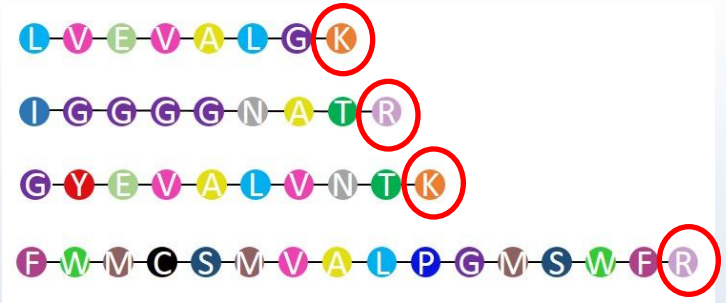


Trypsin

- Trypsin is a **serine protease** that hydrolyses specific peptide bonds:
  - at the carboxyl end of basic amino acids **arginine and lysine**

- This generates several peptide fragments that serve as a protein ‘fingerprint’
  - Protein should always have same peptide fragmentation

Cleavage Products:

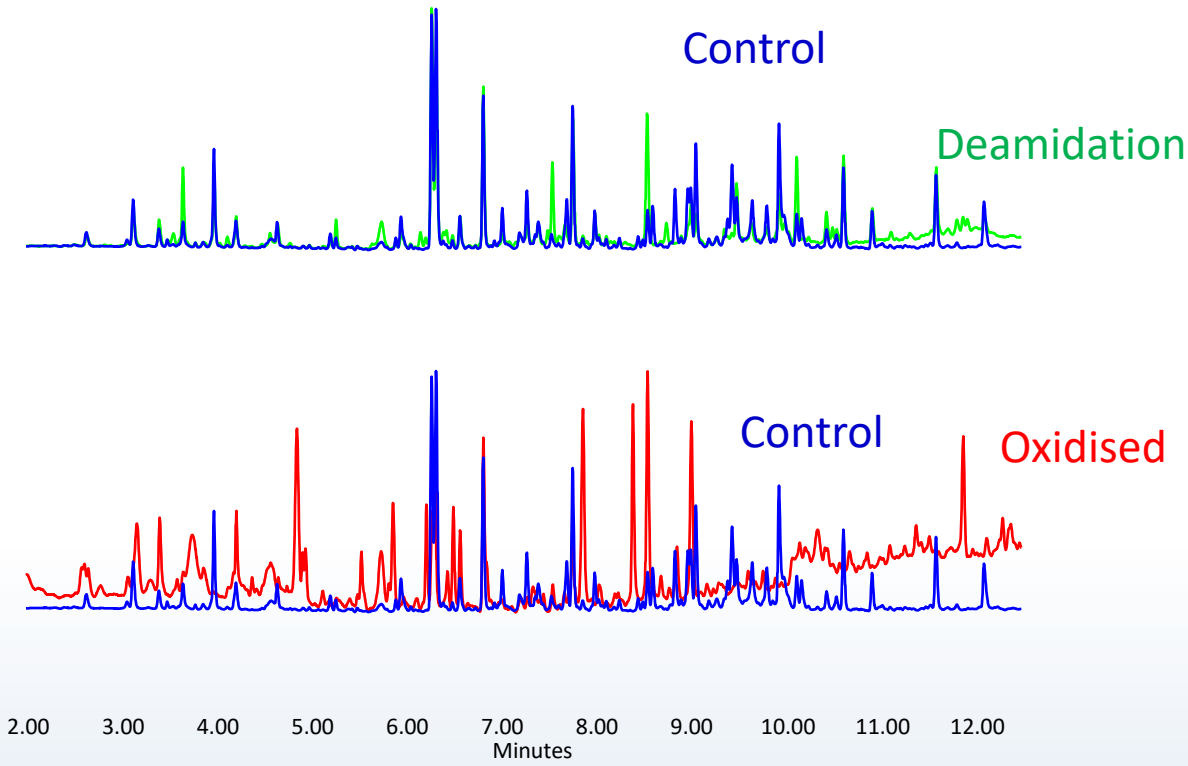


Interpreting the Peptide Map

- Peptide maps tend to be complex with many peaks
- Serve as a unique **'fingerprint' or 'map'** for the protein
- The fingerprint is **compared** to a **reference standard**, and the chromatograms can be **overlaid** or mirrored **('butterfly plot')** for **comparison**
- Can also be compared to **'theoretical'** peptide map, which can be calculated based on the known amino acid sequence and the protease used

Peptide mapping of BSA

Overlaying of results:



Topics

- Overview of Chromatography
- Reverse Phase Chromatography
- Size Exclusion Chromatography
- Ion Exchange Chromatography

Size Exclusion Chromatography

- Separation of molecules based on **size**
- **No** chemical attraction involved

Stationary Phase:

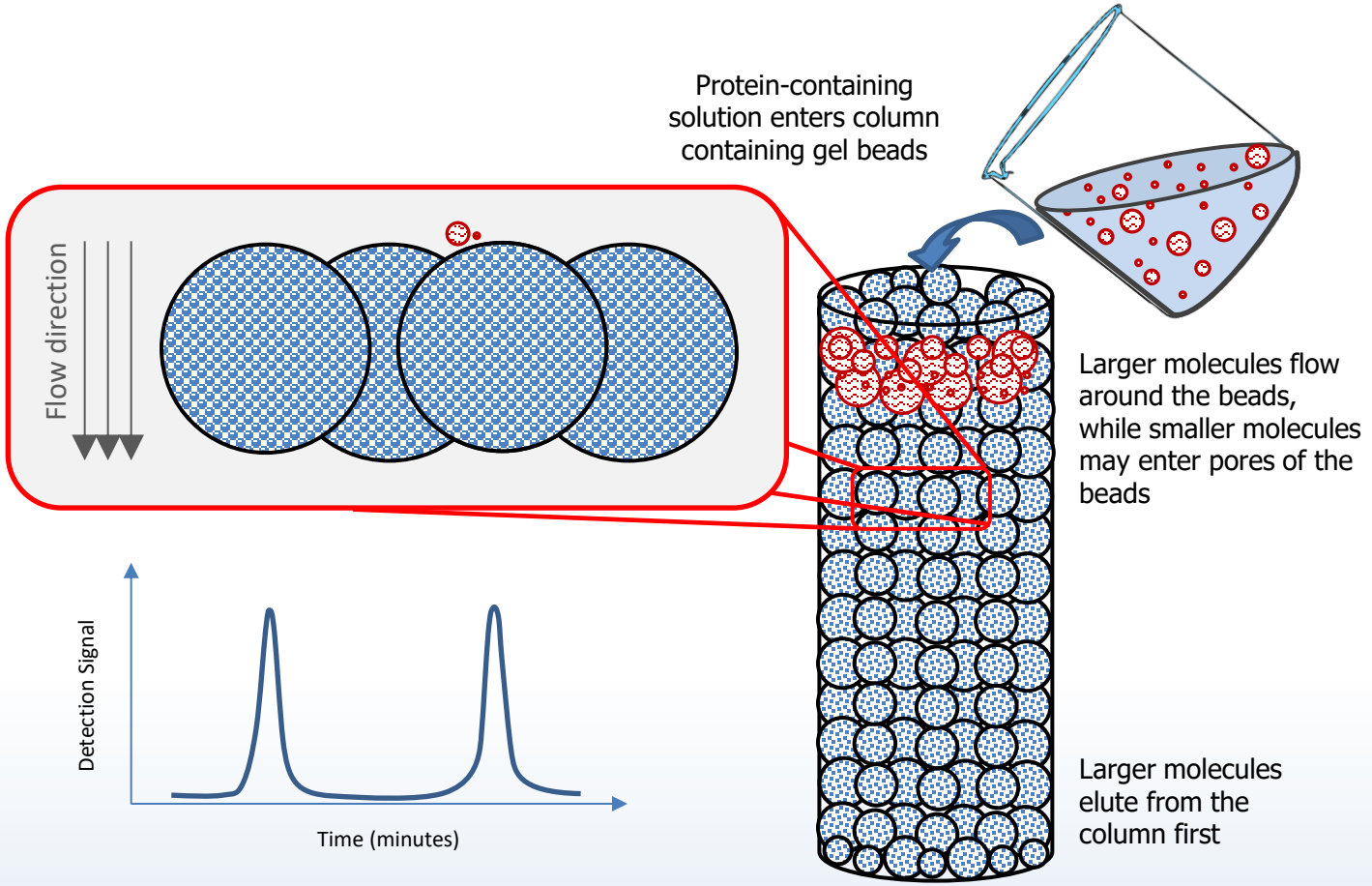
- Silica particles which have **pores** which are of a **defined size**

Mobile phase:

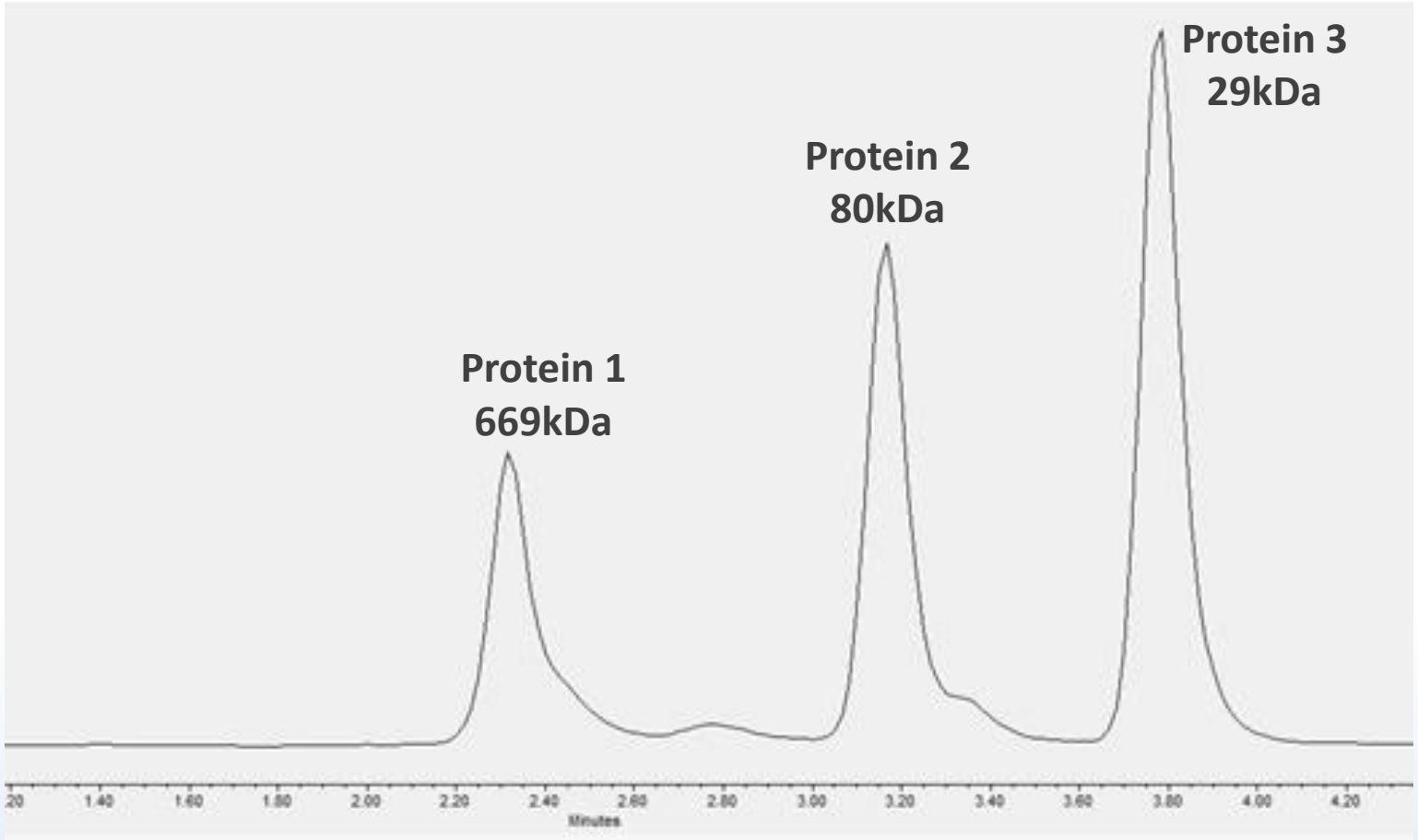
- Aqueous buffer to control pH and ionic strength of protein solution

- **Small** proteins **penetrate pores** in the column & progress through the column more slowly
- **Large** proteins **cannot penetrate the pores** and flow through the column faster

Size Exclusion Chromatography



Size exclusion chromatography
Protein Mix

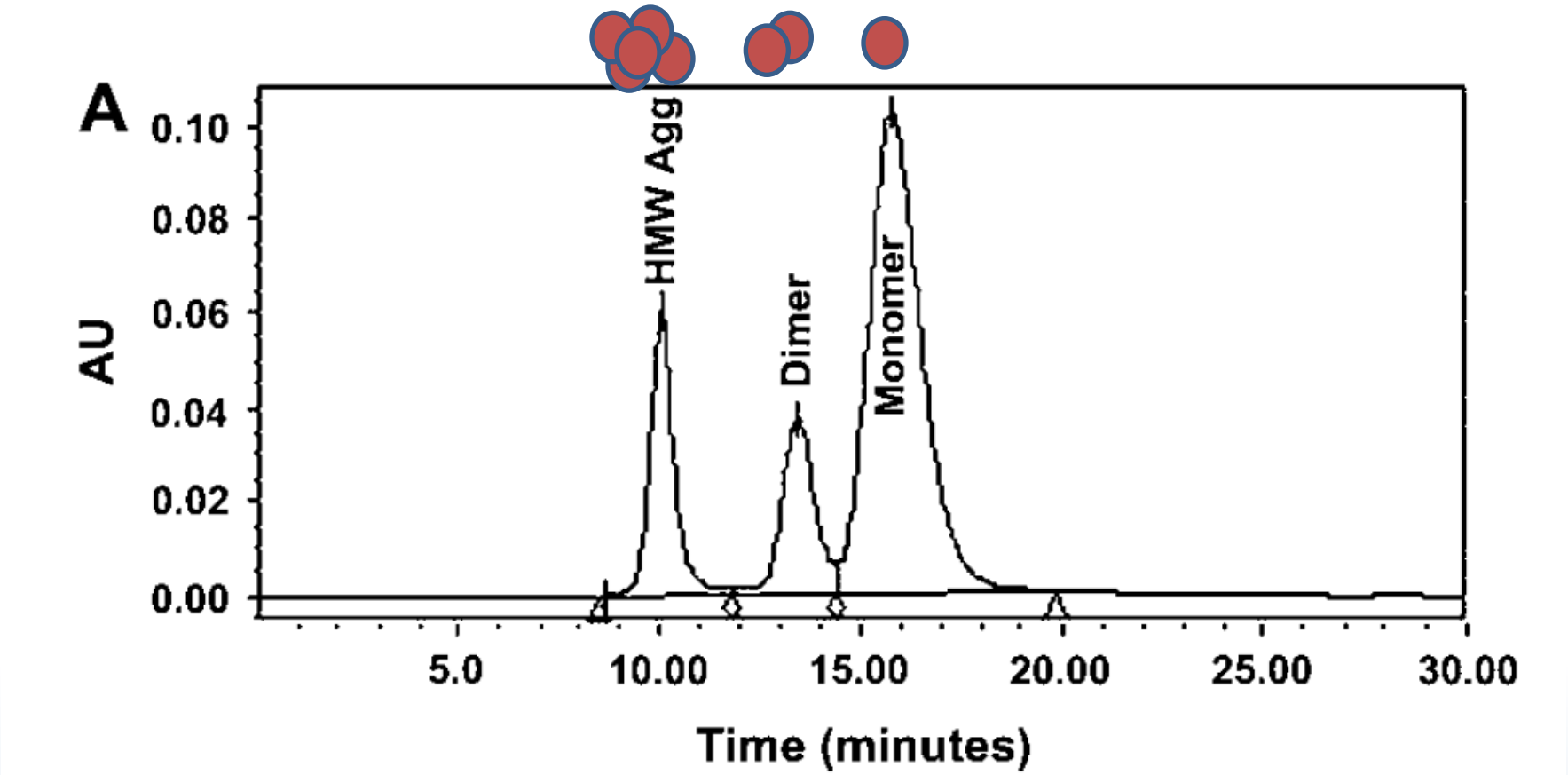


Applications of SEC

- SEC is currently the **primary method** of aggregate detection in biotherapeutic production

  - Fast (sample analysis, approx. 30min)
  - Efficient
  - Quantitative
  - High precision

Detection of protein aggregation with SEC

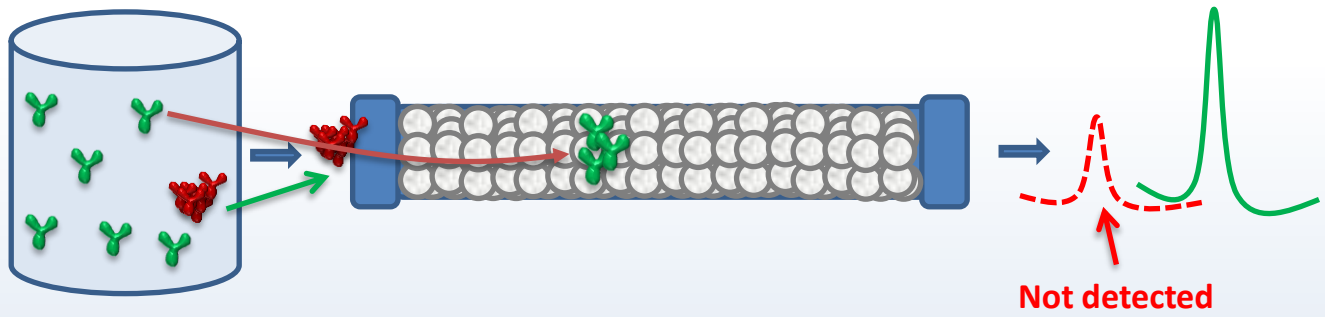


SEC Chromatogram of a therapeutic antibody sample containing monomer (eluted last), dimer (eluted second last) and high molecular weight (MW) aggregates (eluted first)

Berkowitz (2006) *AAPSJ* 8 (3): 590-605

SEC Limitations

- Failure to detect larger aggregates
  - Collection of larger aggregates at column frits or guard columns; excluded from analysed portion of sample, not detected
  - Can co-elute with void volume
  - UV detection not as sensitive as light scattering for large aggregates



SEC Limitations

SEC is subject to inaccuracies in the detection of protein aggregates

- Formulation changes for protein during analysis
  - Dilution of protein in high ionic strength buffers
  - Buffer/solvent conditions may produce false positives or mask true aggregation state of protein

- Must use orthogonal methods to further characterise aggregation during drug development

Topics

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Ion Exchange Chromatography

- Separation of molecules based on **charge**
- Chemical attraction involved: ionic interactions

Stationary Phase:

- Particles (e.g. agarose) to which positive or negative charge has been applied

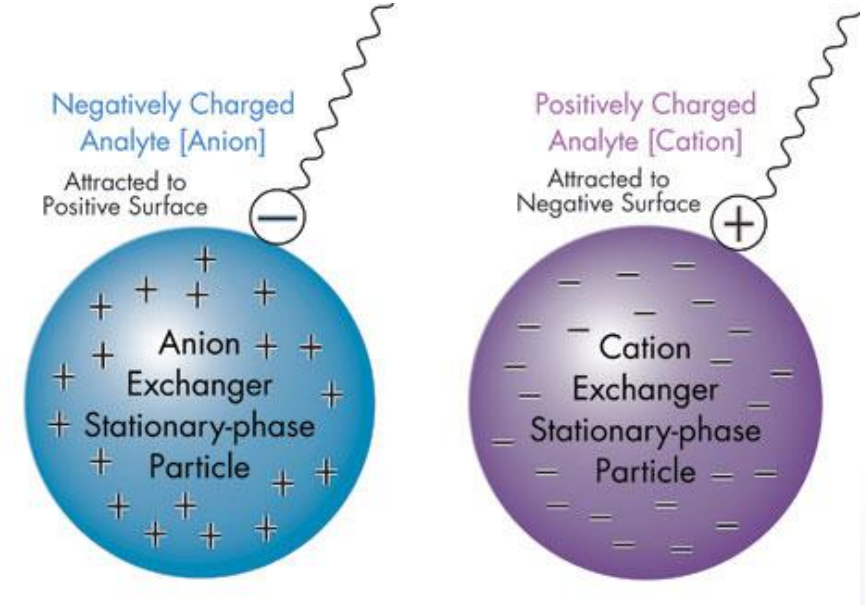
Mobile phase:

- Aqueous buffer

- Charged compounds with the opposite charge to stationary phase will be attracted to the particle surface (ion–exchange) and be retained

Ion exchange chromatography

- Separations method that depends on charge
- Stationary phase particle has a charge (positive or negative)
- Ionised compounds with the opposite charge will be attracted to the particle surface (ion–exchange) and be retained



- Separation depends on the reversible adsorption of charged solute molecules to immobilized ion exchange groups of opposite charge

Image from Waters website

Binding can be controlled using pH

pH > pI, the protein has a net negative charge
pH < pI, the protein will have a net positive charge

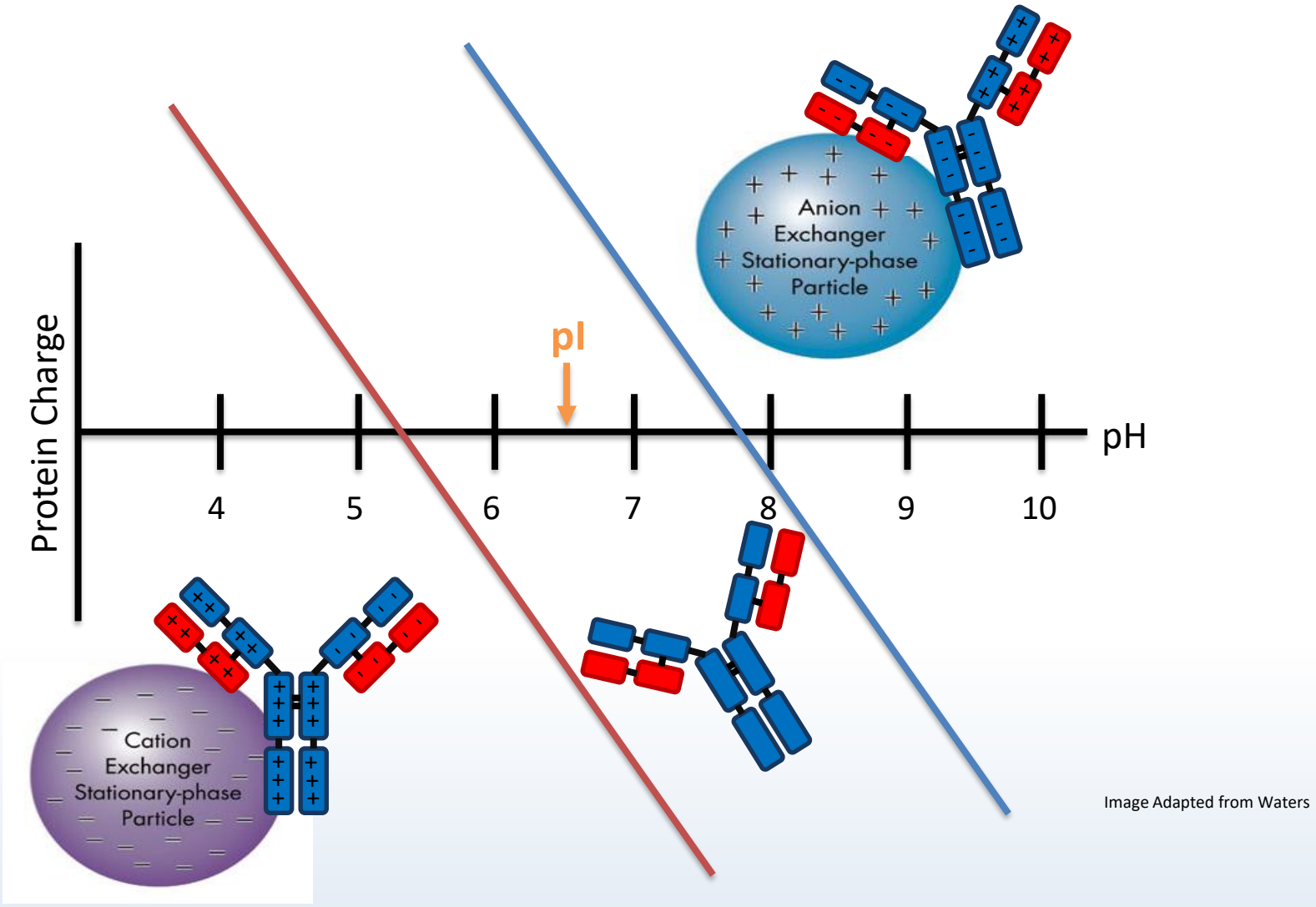
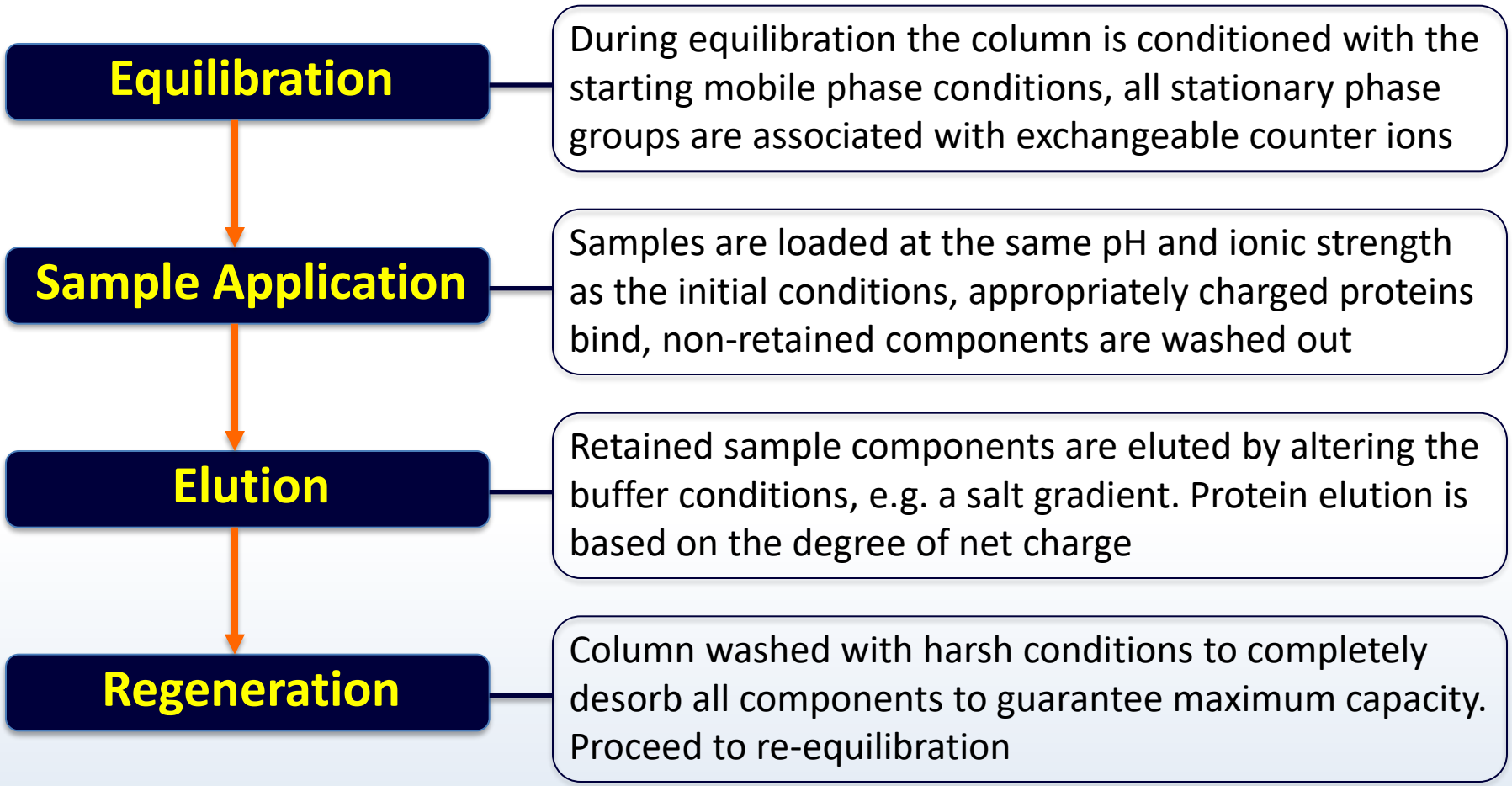


Image Adapted from Waters

Ion exchange chromatography

Separation mechanism

Ion exchange separations consist of four main steps:



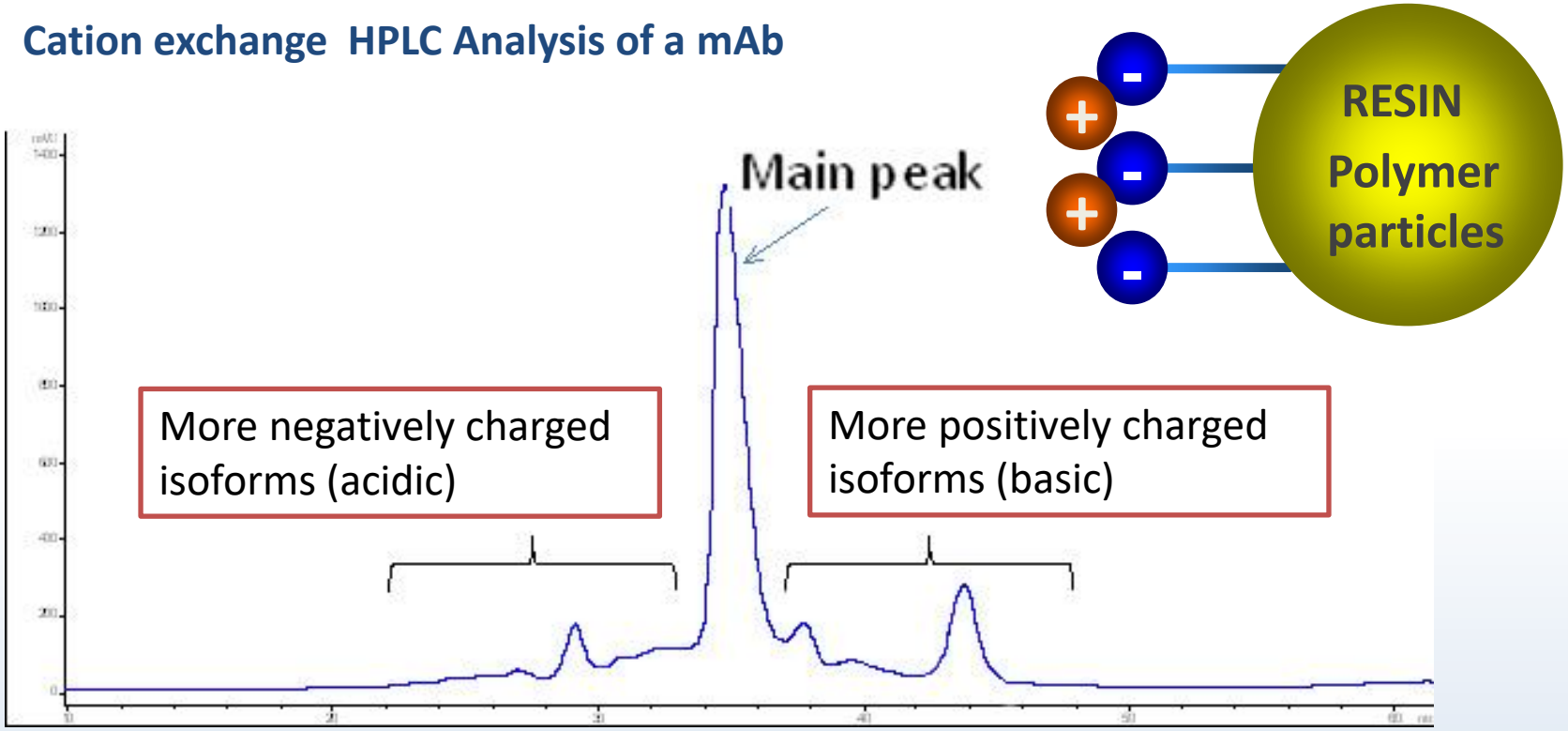
Applications of IEX

- Examination of charge variants

- Monitoring chemical changes to proteins during processing
  - Chemical change could result in change in overall charge of protein and lead to change in retention time
  - Oxidation, deamidation

Applications of IEX

- Identifying isoforms (charge variants) of the biopharmaceutical



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Thank You