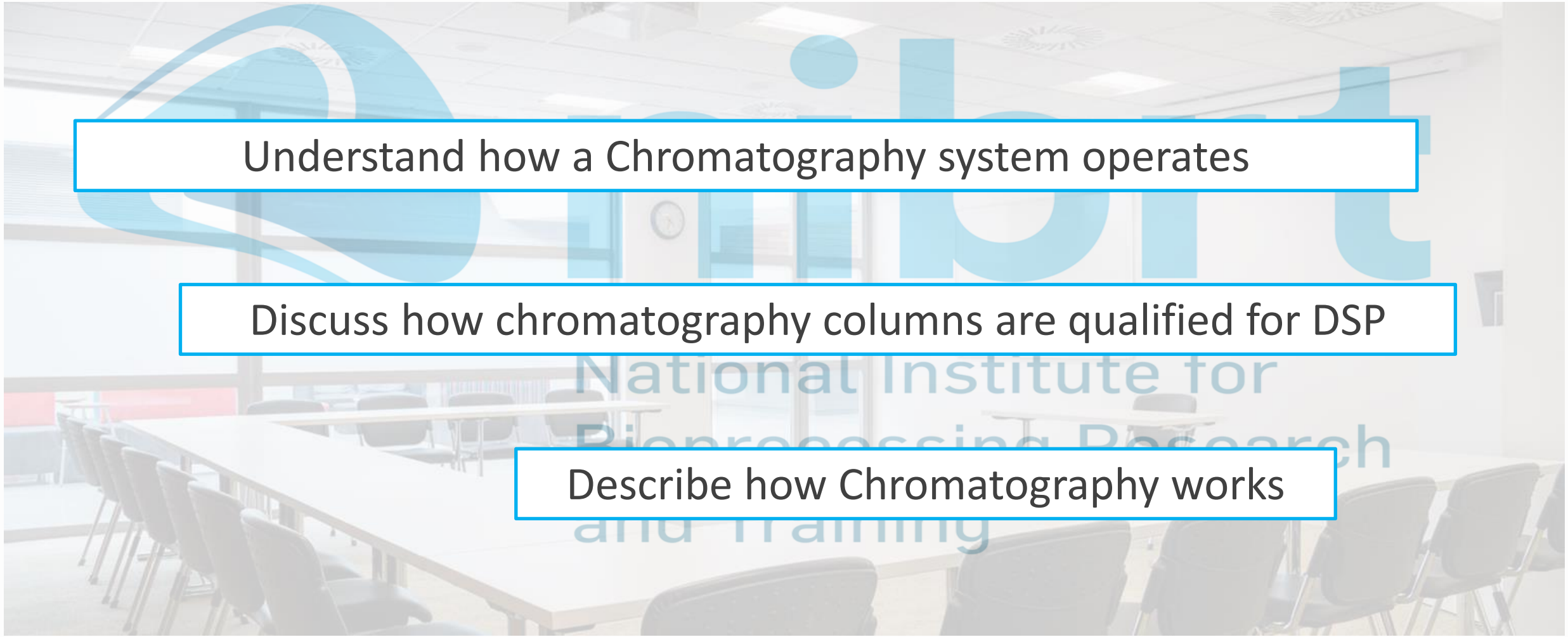
nibrt
National Institute for Bioprocessing Research and Training

Principles of Chromatography

Learning Objectives



- Understand how a Chromatography system operates
- Discuss how chromatography columns are qualified for DSP
- Describe how Chromatography works

Recommended Reading

- There are many books available through the library
- Suggested titles include:

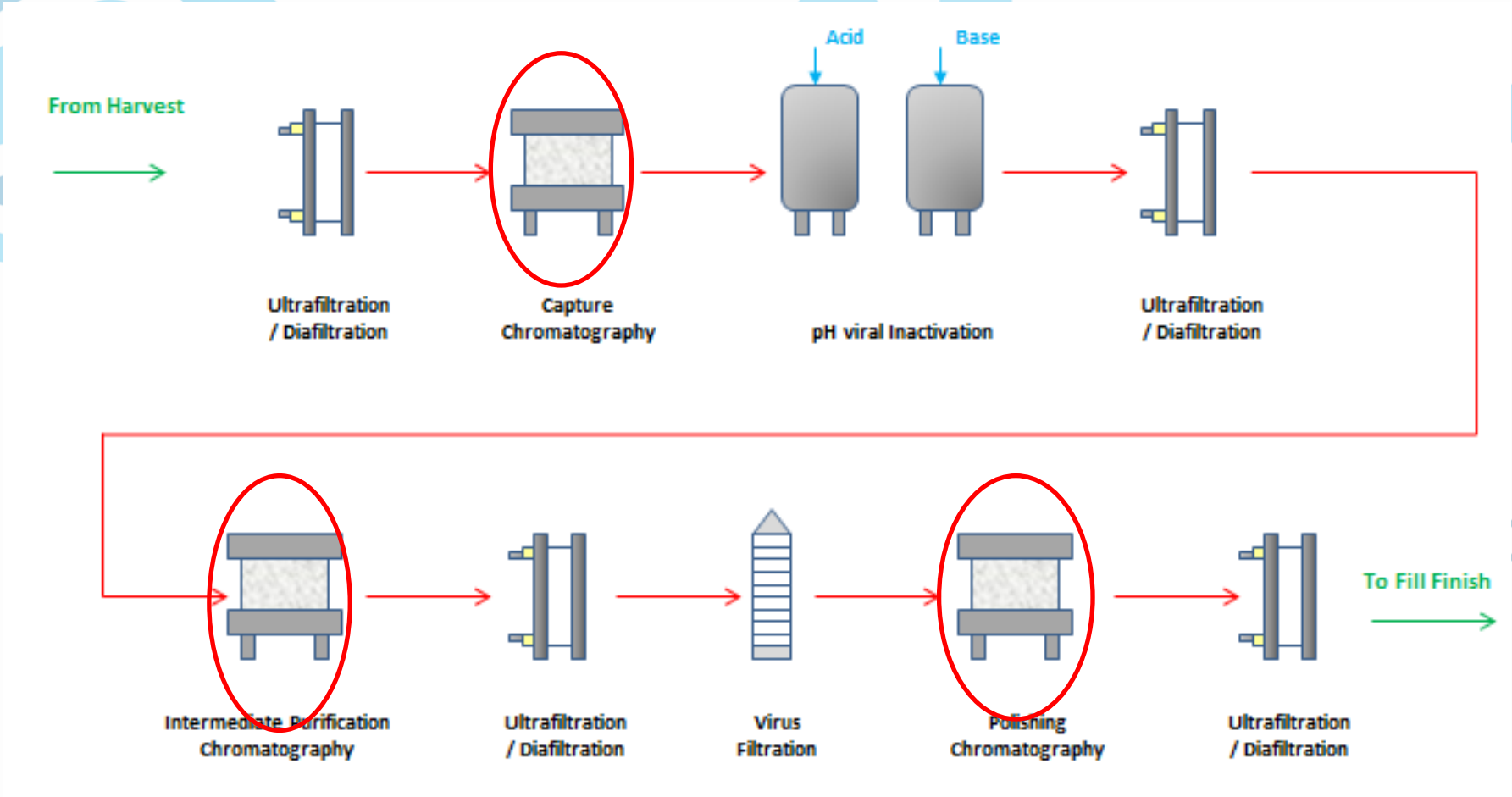
  Bioseparations Science and Engineering (2003) by Harrison, Roger G; Todd, Paul; Rudge, Scott R.; Petrides, Demetri P. Oxford ISBN-10: 0195123409 / ISBN-13: 978-01 95 12 340 http://www.amazon.co.uk/Bioseparations-Science-Engineering-Topics-Chemical/dp/0195123409/ref=sr_1_1?ie=UTF8&s=books&qid=1_232907198&sr=8-1

  Handbooks on Chromatography from GE Healthcare / Amersham / Pharmacia – **available on Moodle** – a useful series of guides to different forms of chromatography available for free download

Topics

- Chromatography Overview
- Components of a Chromatography System
- Qualifying the Column Packing

Chromatography

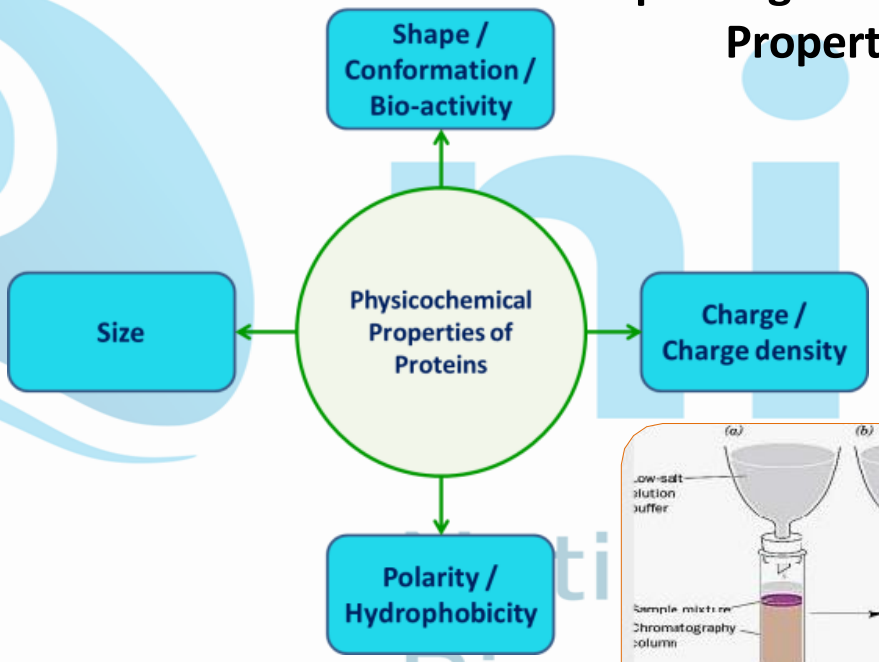


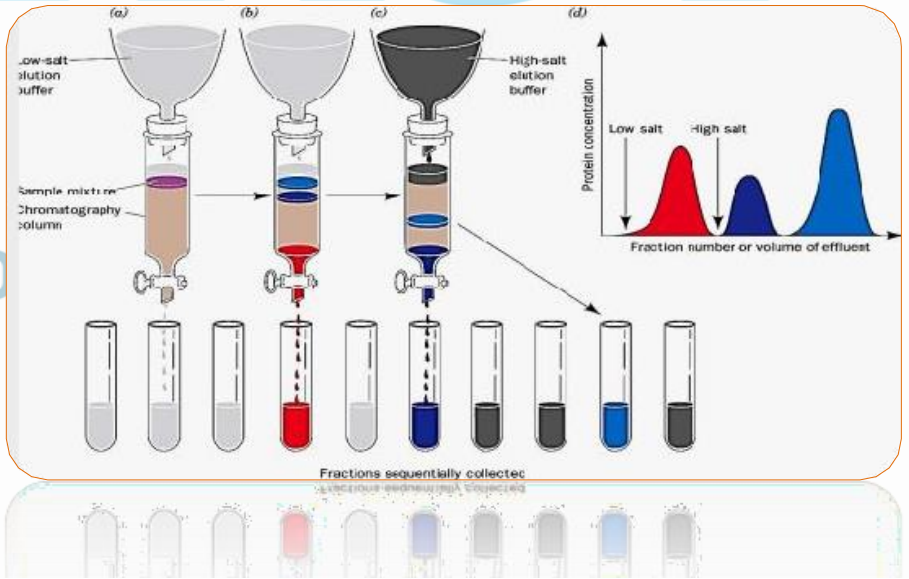
What is Chromatography?

- Chromatography is a separation technique used to purify our protein of interest.
- It is based on the affinity of the components in the mixture for a stationary phase or a mobile phase

Chromatography: An Overview

Exploiting the Physicochemical Properties of Proteins





General Principle of Chromatography

i. A crude mixture of proteins in buffer (mobile phase) prepared from an appropriate source (e.g. bulk cells grown in a fermentation system) are fed into a chromatography column
ii. The proteins interact with the column packing material (stationary phase) based on e.g. size, charge etc.
iii. Certain proteins bind to or are retained by the stationary phase
iv. Other proteins pass directly through the column (unretained)
v. The buffer is then changed to an elution buffer (different pH/salt conc. etc).
vi. This causes the retained proteins to be released from the column
vii. The released purified proteins are collected for concentration and analysis

Common Process Compounds & Methods of Purification/Removal

Component	Culture Harvest Level	Final Product Level	Conventional Method
Therapeutic Antibody	**0.5 - 6 g/l**	**1 - 10 g/l**	**UF/Chromatography**
Isoforms	**Various**	**Monomer**	**Chromatography**
Serum and host proteins	**0.1 - 3.0 g/l**	**< 0.1 - 10 mg/l**	**Chromatography**
Cell debris and colloids	10^6/ml	None	MF
Bacterial pathogens	Various	$< 10^{-6}$/dose	MF
Virus pathogens (12^+ LRV)	Various	$< 10^{-6}$/dose	virus filtration
DNA	1 mg/l	< 10 ng/dose	Chromatography
Endotoxins	Various	< 0.25 EU/ml	Chromatography
Lipids, surfactants	0-1 g/l	< 0.1-10 mg/l	Chromatography
Buffer	Growth media	Stability media	UF
Extractables/leachables	Various	< 0.1-10 mg/l	UF/ Chromatography
Purification reagents	Various	< 0.1-10mg/l	UF

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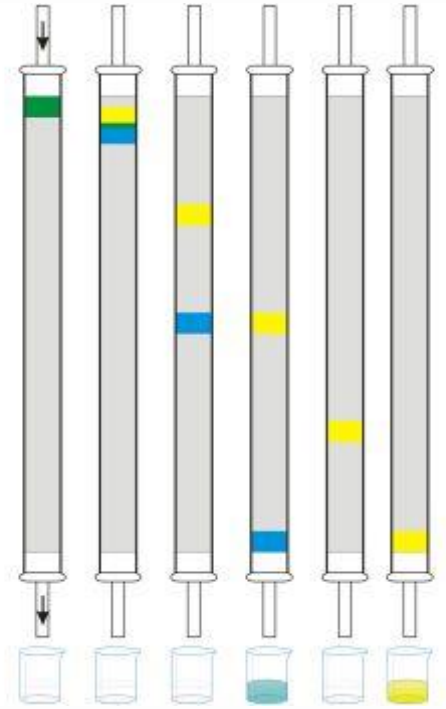
Column Chromatography

- Used to purify an individual compound from a mixture of compounds
- Vertical column packed with a stationary matrix
- Mobile phase solution used to carry protein through column



A complex sample is passed through the stationary matrix that consists of a solid support with specific characteristics defined by the support's make-up and/or chemistry.

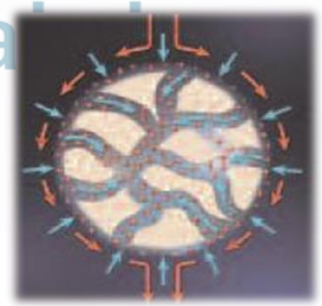
Column Chromatography Phases

1. Stationary Phase

2. Mobile Phase

Stationary Phase

- The stationary phase is a solid resin matrix made of small, bead-like particles
- The stationary phase is packed into a cylindrical column tube
- This resin has some form of chemistry attached to its surface, many kinds of resin are available



Column Packing
(stationary phase)

Stationary Phase

- The make-up and/or chemistry of the stationary phase will help separate your product from the mixture
- Stationary phase resins are expensive – a column will be packed with care and then reused for multiple batches before the resin is changed
- Prepacked columns with plastic housings are available, these systems use a single use flowpath with 'disposable' columns which are reused for multiple cycles before being discarded

'Disposable' Pre-Packed Column

Mobile Phase

- The mobile phase is the solution that carries the unpurified solutes through the stationary phase
- Depending on the properties of the stationary phase and the mobile phase, different components in the liquid phase will be retained by the column to different degrees and at different rates.

Topics

- Chromatography Overview
- Components of a Chromatography System
- Qualifying the Column Packing

Main Components of Chromatography System

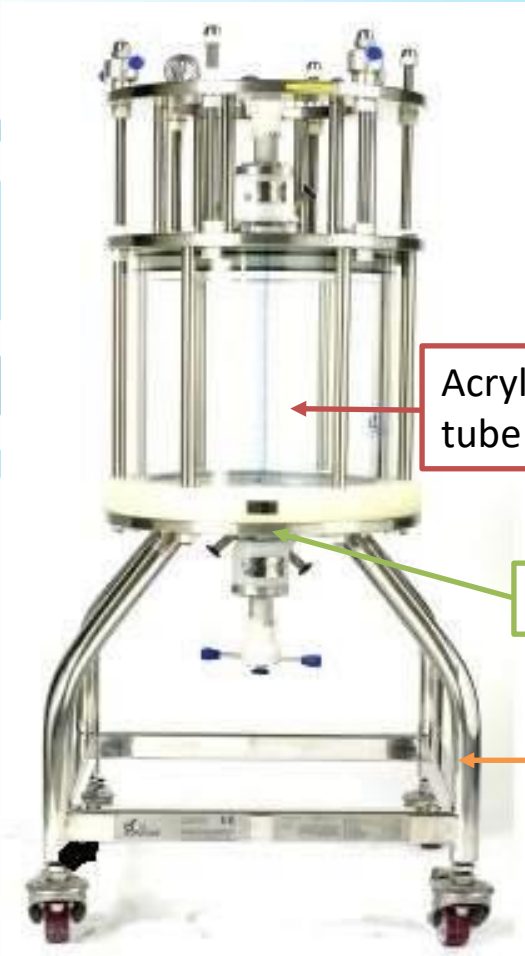
The buffer tanks hold the mobile phase.

The pump is used to generate a specified flow of the mobile phase.

The chromatography column contains the stationary phase

A detector is needed to see the separated compound bands as they elute from the column. This is generally a UV detector at 280nm.

Column Anatomy



Acrylic column tube

Bottom Adjuster

Column stand

Pall Resolute

400mm Acrylic

Top Adjuster

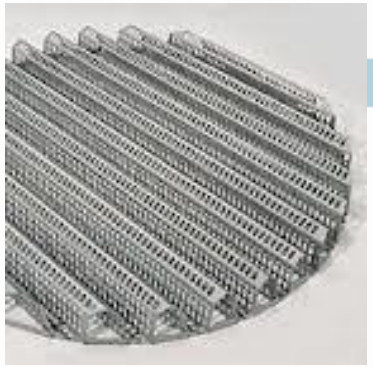
Stainless steel column tube

Pall Resolute

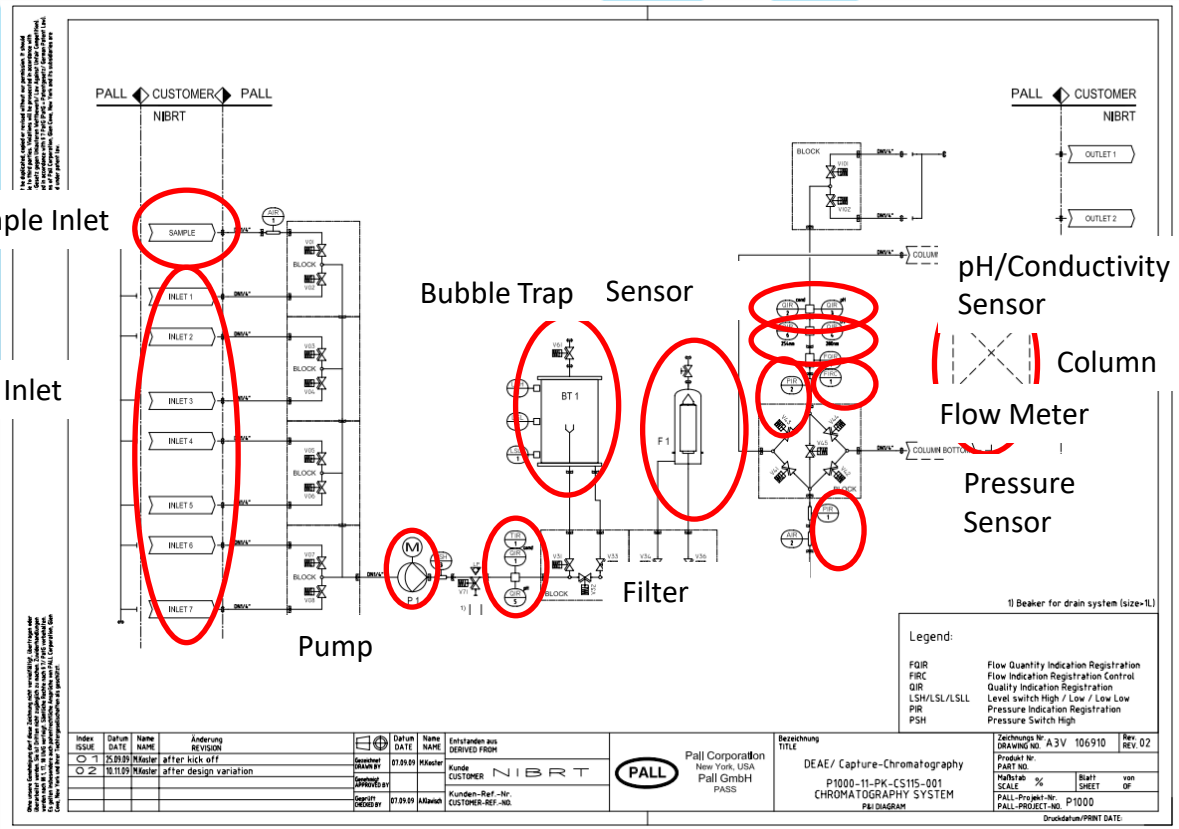
1200mm Stainless Steel

Column Bed Support

- Acts like a sieve and keeps stationary phase in column tube
- Pores must be smaller than resin beads
- Can be stainless steel or polypropylene

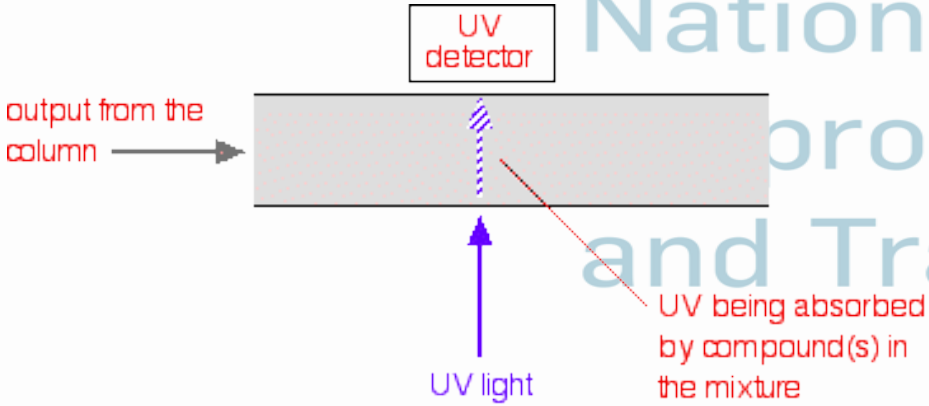


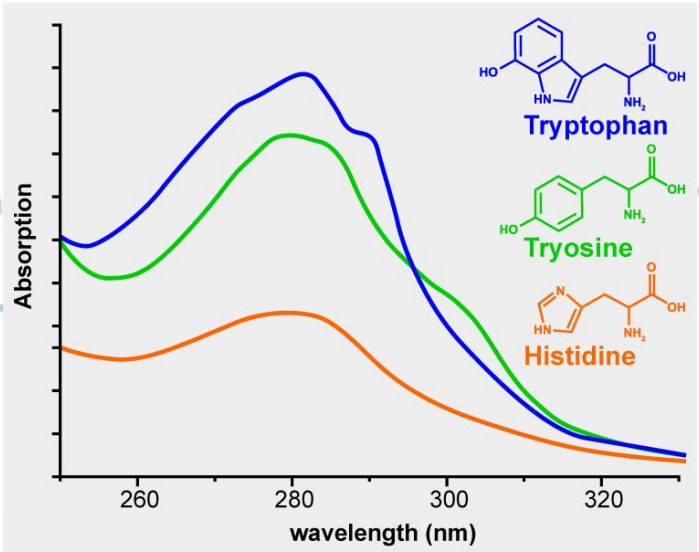
Chromatography Skid P&ID



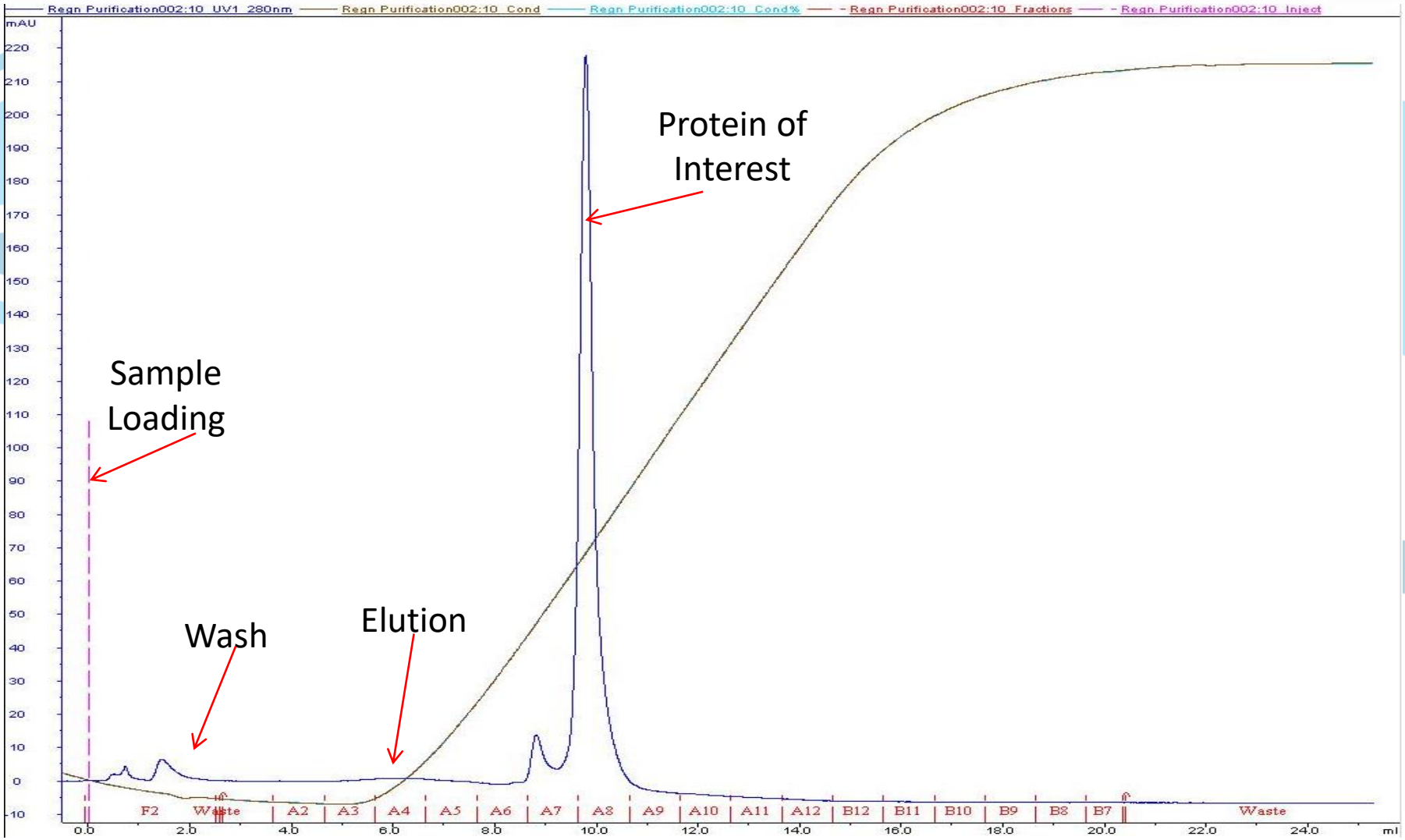
Protein Detection

- UV measurement is generally used to detect elution from the chromatography column
- The aromatic amino acid residues tryptophan & tyrosine absorb ultraviolet (UV) light at wavelength 280nm (A280).





Example of a Chromatogram



HCP target limits

- Host-cell proteins (HCPs) are **bioprocess-related impurities that may be present in intermediate or final biopharmaceutical products** such as recombinant monoclonal antibodies (MAbs).
- Although the potential clinical and genetic effects of HCPs are largely unknown, studies have shown that **HCPs may cause immune responses and adverse reactions in patients** when present at sufficient high levels.
- Consequently, FDA and EC regulations require that the level of HCP in a bioproduct be quantitatively measured during manufacturing and before approval for therapeutic use.
- The number of HCPs is expressed in parts per million (ppm), and it should be at the lowest possible level

HCP Target Limits

- For biological medicines used chronically over a lifetime (e.g. human insulin, erythropoietin or factor VIII), the level of HCP should be **not more than 10 parts per million (or 10 mg/L)**
- For other biological medicines, the permissible level of HCP should be justified by:
  i. the minimum achievable level within methodological and statistical variation
  ii. data from product manufactured for use in nonclinical and clinical studies.
- The safe level of residual HCP is suggested as **<100 parts per million (ppm)** or below detectable levels using a highly sensitive analytical method.

ppm = mg/L or µg/ml

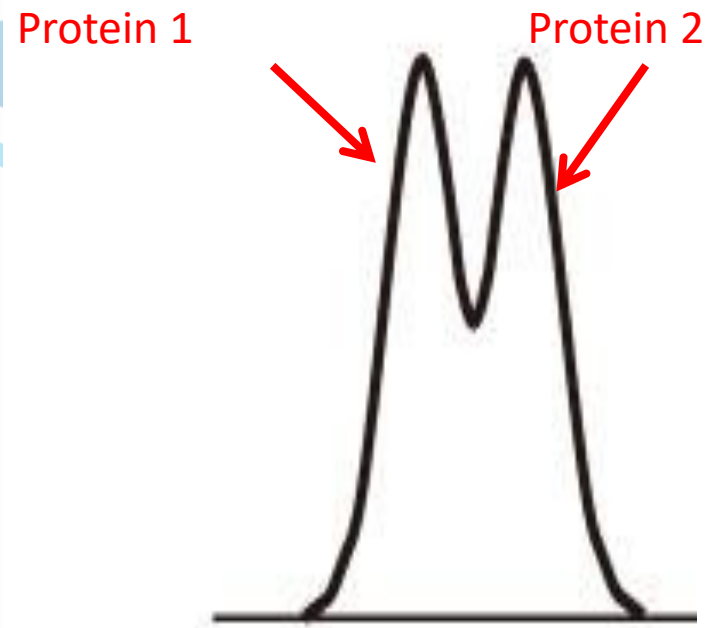
Column Capacity

- A most important parameter is the **Dynamic Binding Capacity (DBC): the available protein binding capacity of the column as a function of flow rate.**
- This depends on many factors:
  i. Properties of column matrix (bead size, binding groups etc.)
  ii. Buffer conditions, pH & ionic strength
  iii. Experimental conditions: flow rate, back pressure etc.
- To determine Dynamic Capacity by running small columns with increasing loading until the column reaches saturation (i.e. protein appears in the eluate)
  - Under these conditions, pool eluted fractions and determine total amount of bound protein which comes off after elution

Column Resolution (Rs)

- **Rs is the *relative separation between 2 peaks compared with the average base width of the two peaks***
  - It is proportional to the selectivity, efficiency and column capacity
  - *Selectivity, a function of distance between peaks & peak width (W)*
  - *Efficiency, a function of peak width & geometry*
  - *Capacity, a function of peak elution volume & total liquid volume of the column*

Resolution Factor Sample Chromatogram

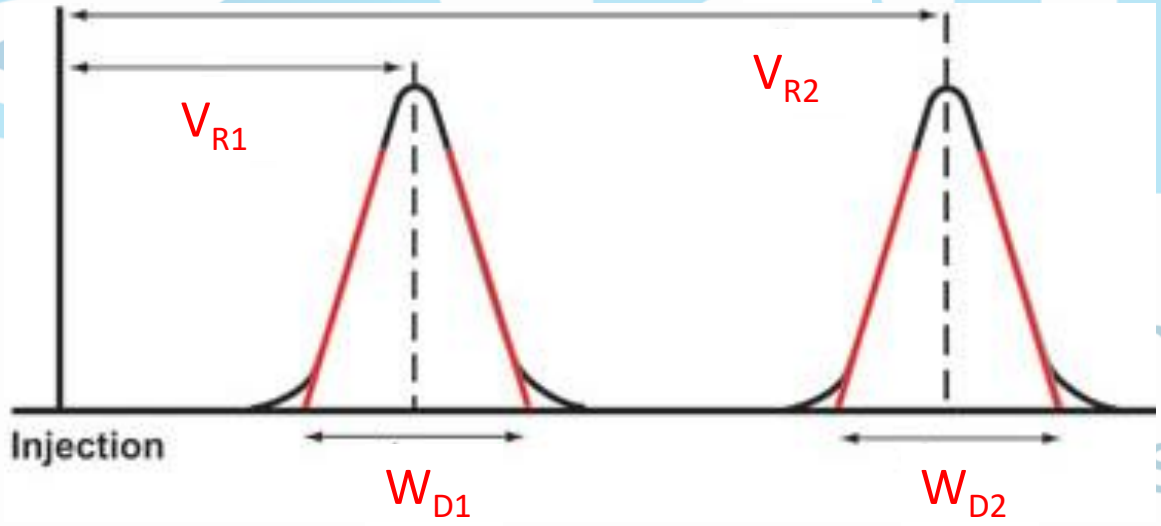


Is this acceptable?

Is there good separation?

Resolution Factor

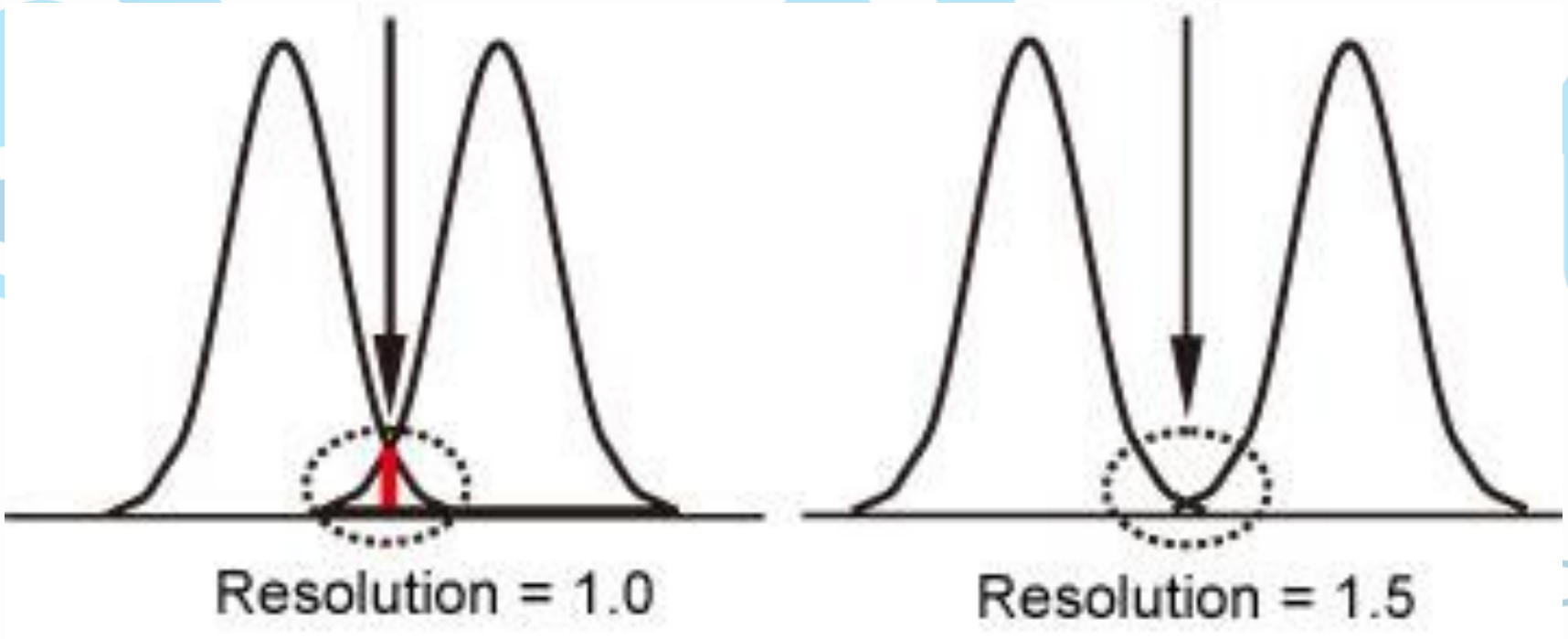
- The resolution R_f of a column provides a quantitative measure of its ability to separate two analytes.



$$Rs = \frac{2\,(V_{R2} - V_{R1})}{W_{D1} + W_{D2}}$$

- Typically resolution Factor **must be greater than 1.5**

Resolution Factor contd.



Topics

- Chromatography Overview
- Components of a Chromatography System
- Qualifying the Column Packing

What Is Column Packing?

Column packing is a process of putting stationary phase (resin) into a column.

Qualifying the Column Packing

- Once the column is packed, perform a series of standard qualification tests
- Good column packing is not sufficient to guarantee a successful chromatographic separation but it is a solid indicator of column performance
- Why Do We Test Column Efficiency?
  - GMP requires checking column efficiency
  - Check when newly packed
  - Diagnostic tool when problems occur
  - Ensures consistency

How To Measure Column Efficiency?

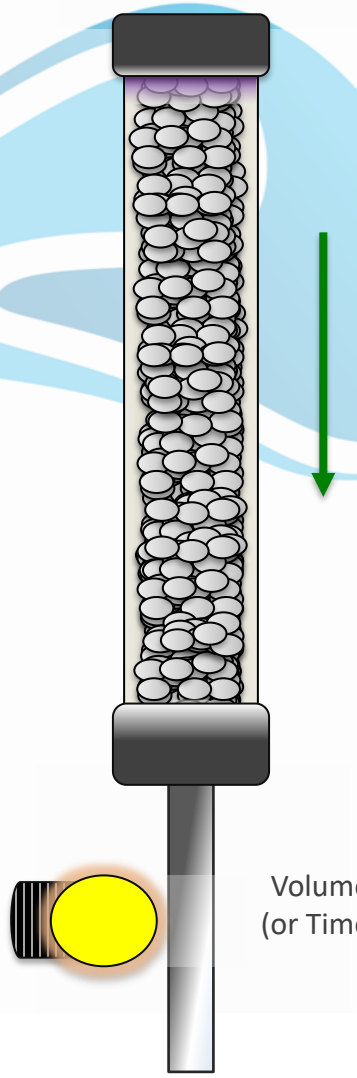
- Qualification is usually done by determining bed characterisation using pulse injections of a non-reactive marker, chosen for its ease of detection, such as NaCl
- Inject the marker at process flow rates
- Sample volume is usually a percentage of the column volume (1% is typical)
- These pulse injections are used to assess the uniformity of the packed column or bed integrity
- Based on the resultant chromatograms, a series of calculations are performed to assess the efficiency

What do we measure?

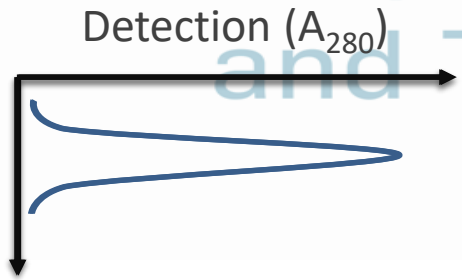
Height Equivalent to a Theoretical Plate (HETP)

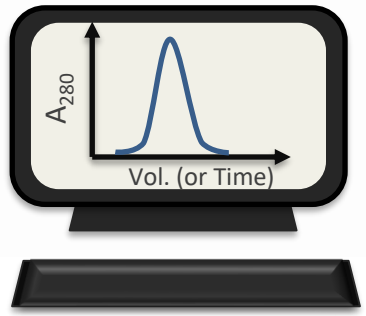
- In column chromatography, no actual plates exist
- Measure the peak widths and relate it to the length of the column
- The narrower the peaks, the more ‘plates’ can ‘fit’ onto a column
- Columns with high plate numbers are considered to be more efficient, that is, have higher column efficiency, than columns with a lower plate count

How does a band become a peak?



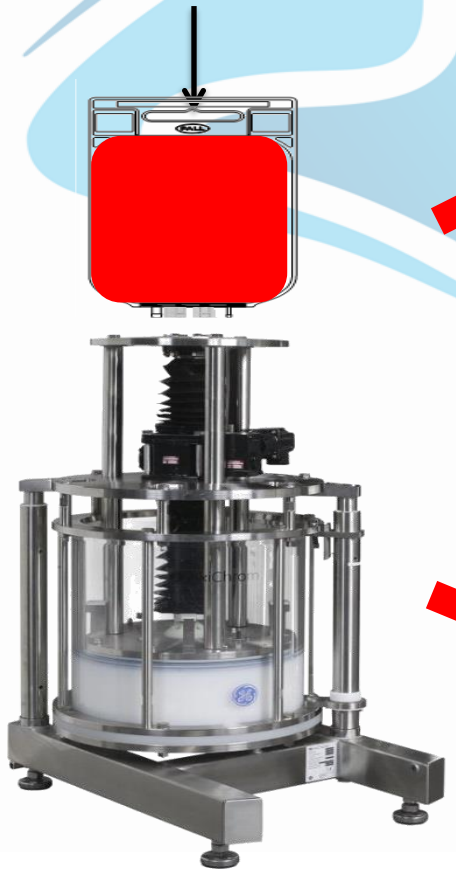
- In a **well-packed column**, the molecule of interest should stay in a narrow band of liquid.
- The molecule should move at an even flow rate, through the stationery phase and out through the bottom of the column. It then passes a detection window, where a signal (typically absorbance at 280nm) is used to detect the molecule (protein) of interest.
- In a **well-packed column**, separation of components into **narrow**, **symmetrical peaks** should occur.





Height Equivalent to a Theoretical Plate

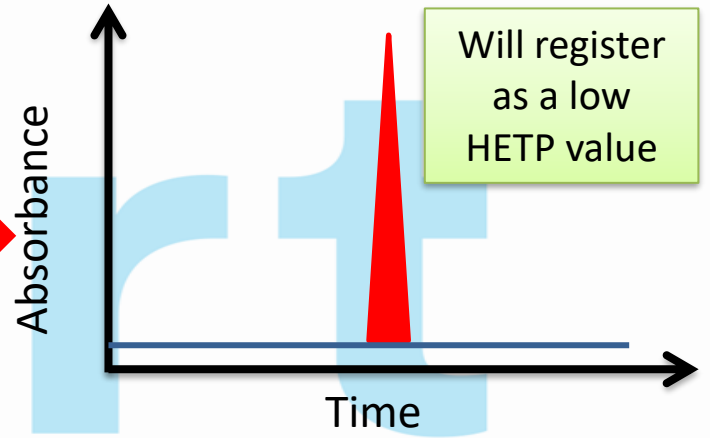
Product injected onto packed column



Well–packed column

narrow bands

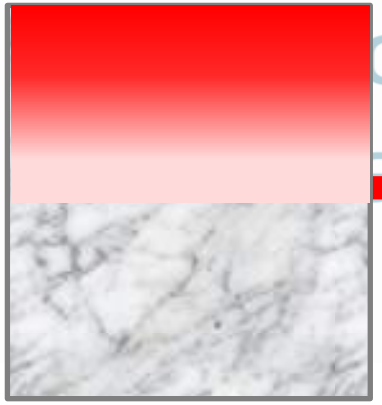
Detector
UV 280nm



Will register as a low HETP value

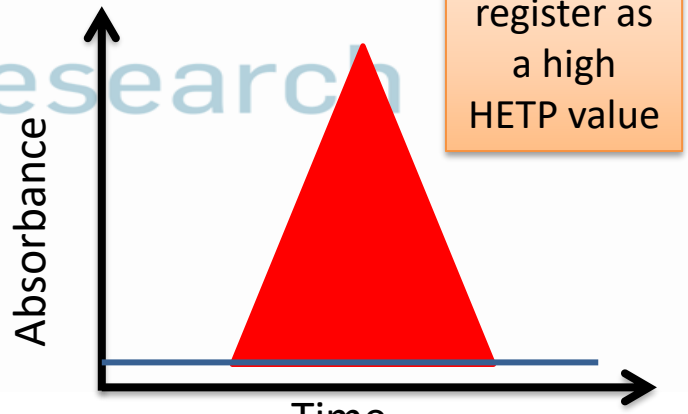
narrow peaks

Poorly packed column



broad bands

Detector
UV 280nm



Will register as a high HETP value

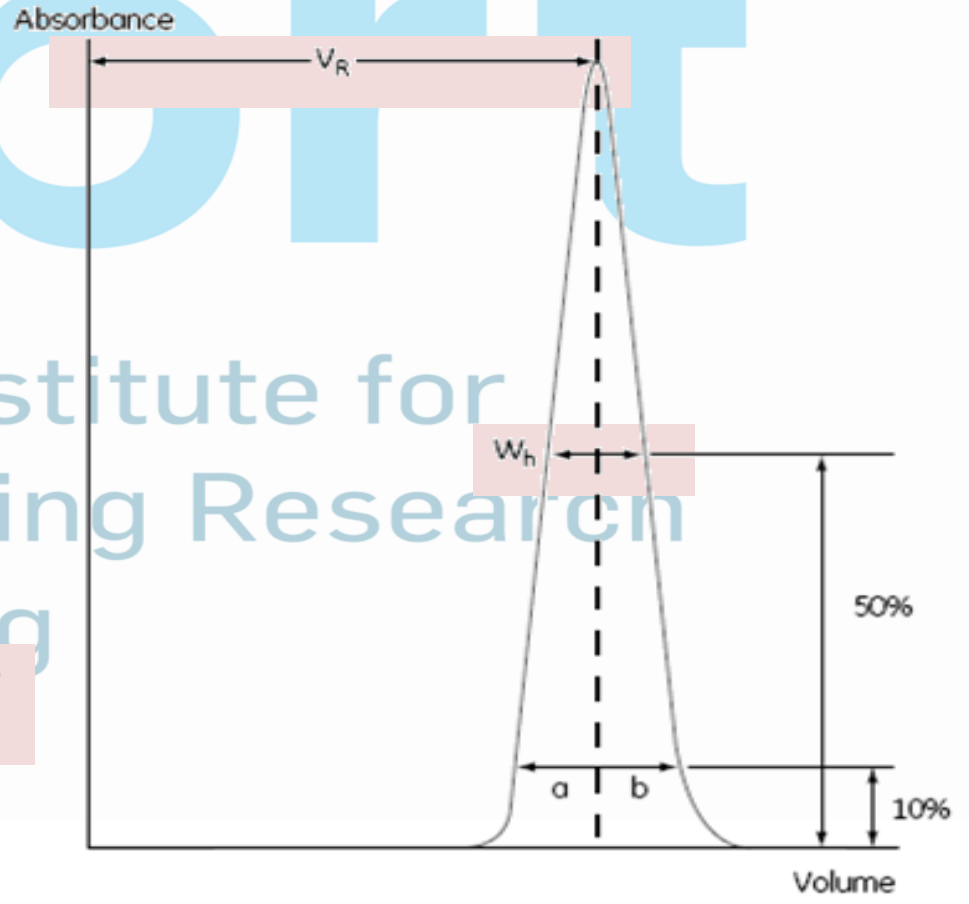
broad peaks

HETP

$$HETP = \frac{L}{N}$$

$$N = 5.54 \left[\frac{V_R}{W_h}\right]^2$$

Industry requirement is that HETP value must be within 2-3 times the average bead size in the stationary phase (in µm)



L = Bed height – height of the resin bed in the column

N = number of "plates"

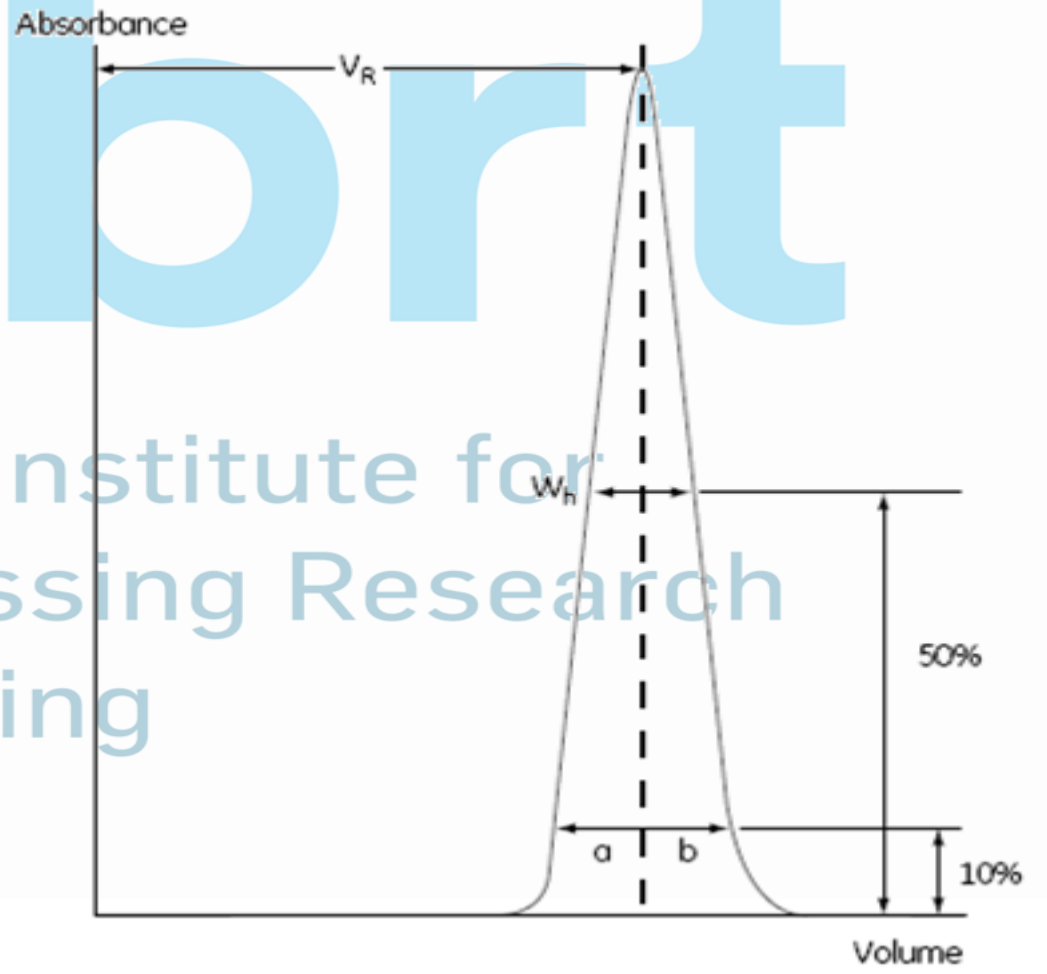
V_R = volume eluted from sample application to peak maximum

W_h = peak width measured as the width of the recorded peak at half of the peak height

Asymmetry Factor

$$A_s = \frac{b}{a}$$

Should be as close to “1” as possible, meaning that both halves of the peak are near mirror images of each other – symmetrical.
Industry standard is A_s of 0.8 to 1.5.

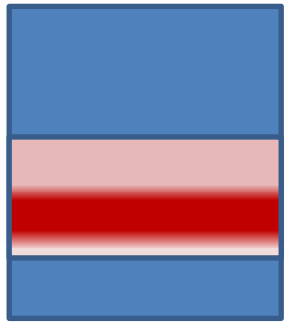


a = 1st half peak width at 10% of peak height

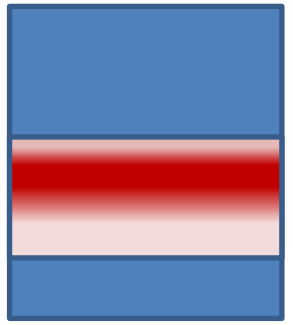
b = 2nd half peak width at 10% of peak height

Peak Asymmetry

Sample band has long tail on column



Sample band has long front on column

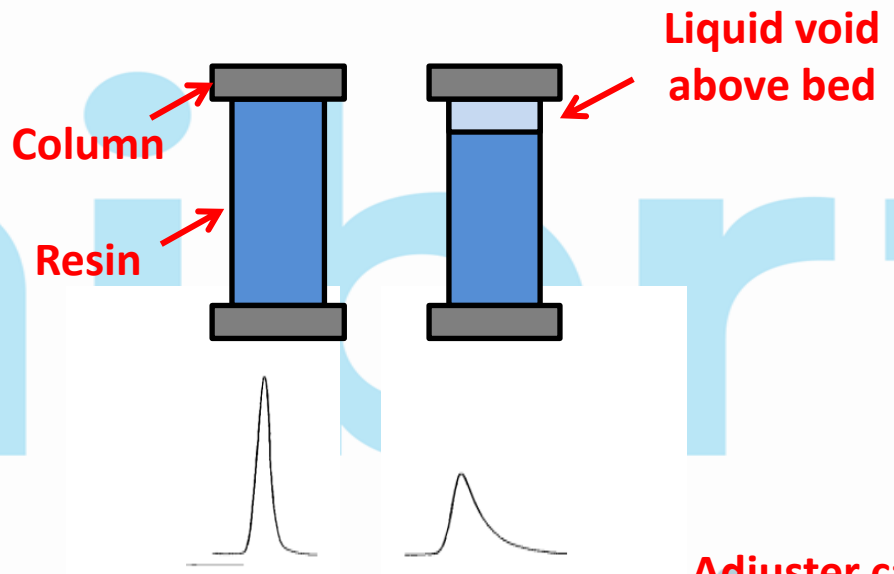


A Well Packed Column...

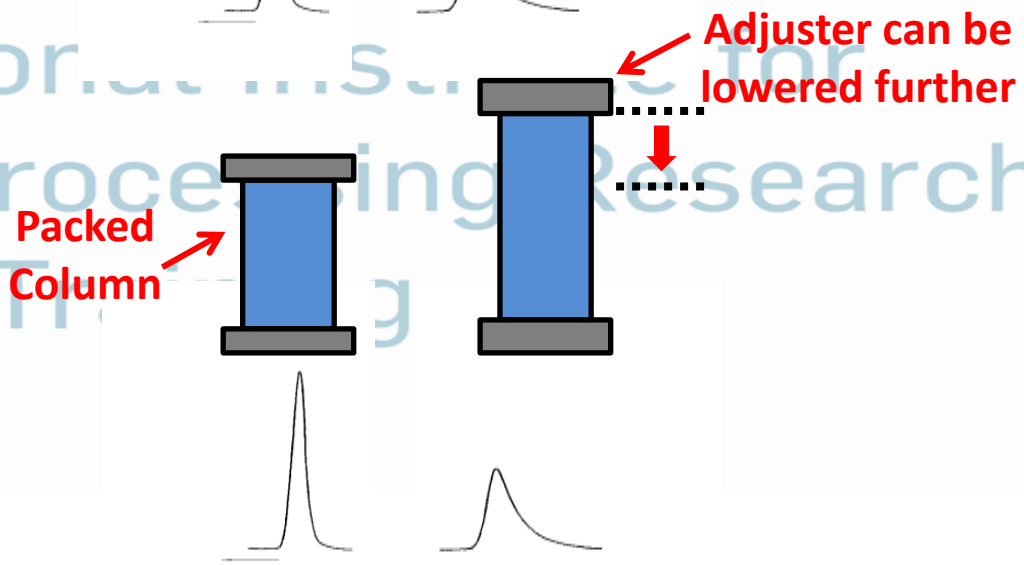
1. Performs the same way with every use
2. Has no gross bed defects
3. Has adequate column efficiency
4. Suffers minimal tailing/fronting

Tailing Causes

1. Liquid void above bed

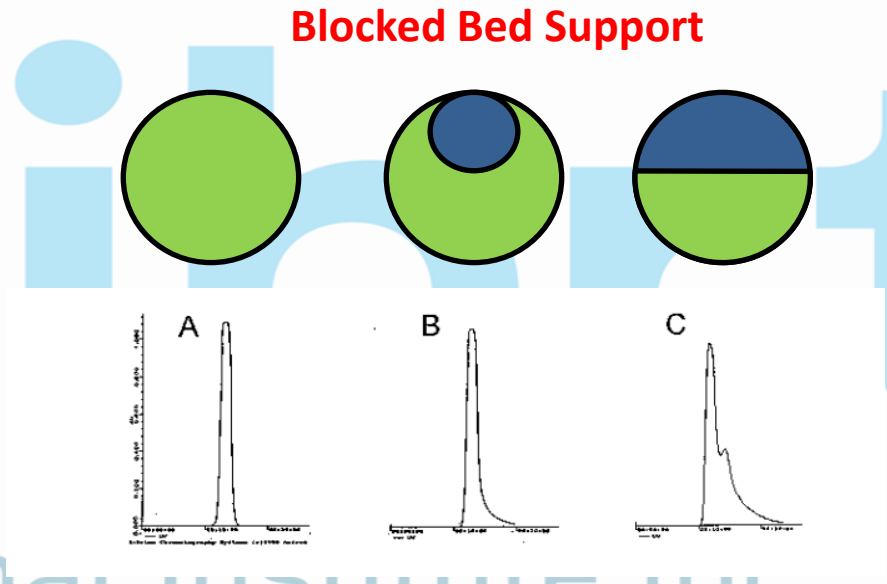


2. Column packed too loosely

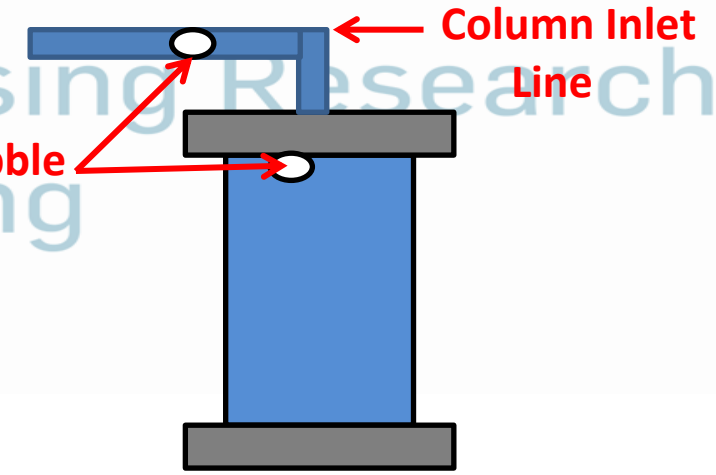


Tailing: Causes

3. Blocked bed support



4. Air bubble



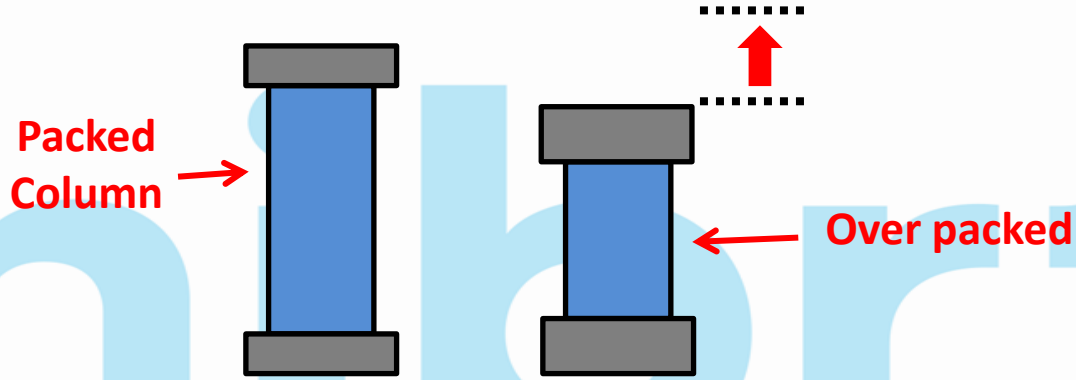
Tailing Recommendations

1. Replace the bed supports

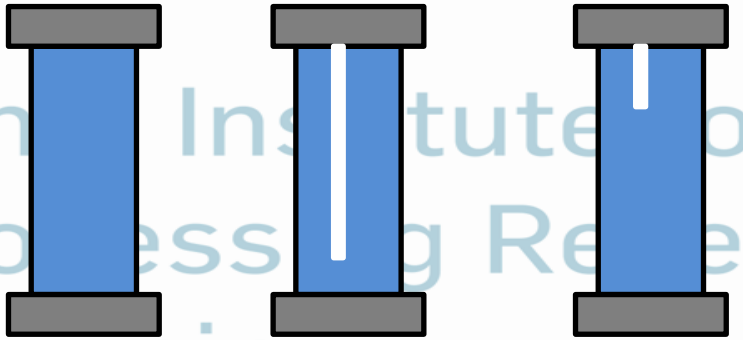
2. Repack – lower the adjuster further

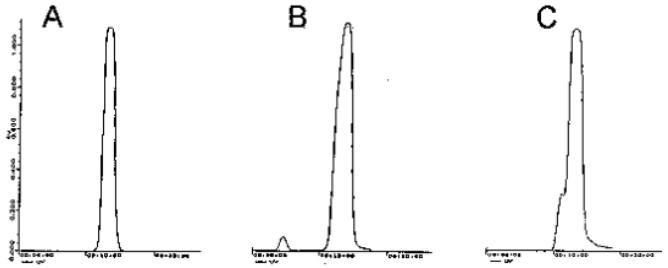
Fronting Causes

1. Column over packed



2. Channelling





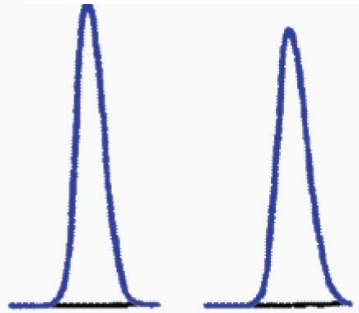
Fronting Recommendations

- Should repack

Which Column Would You Use?

Which chromatogram looks acceptable?

A_s = 1.0 – 1.05

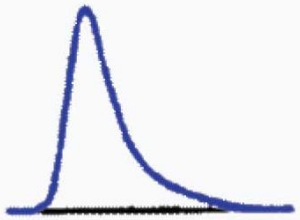


Excellent

A_s = 1.1

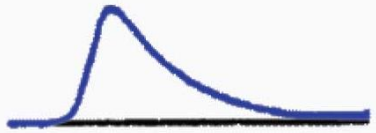
Acceptable

A_s = 3.0



Not acceptable

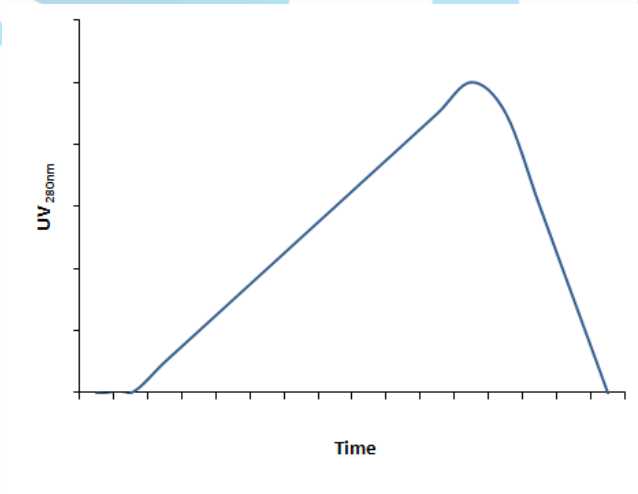
A_s = 4.0

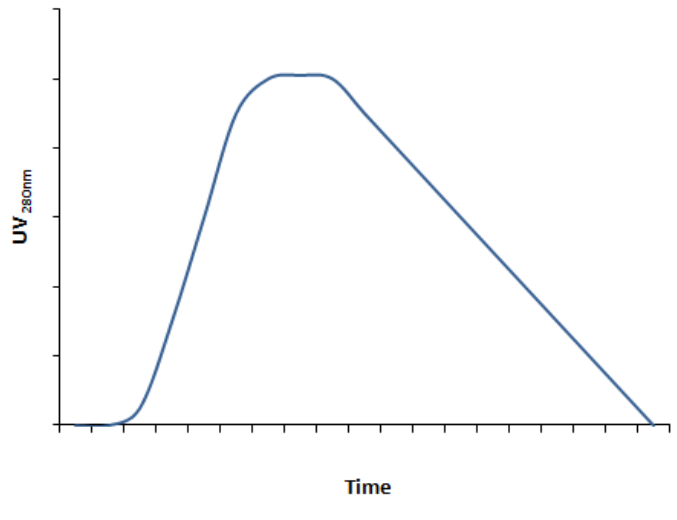


Not acceptable

Column Packing

Q: Match the following terminology with chromatograms below.
Fronting, Tailing





Fronting

Tailing

Summary

- Chromatography – separation of molecules based on their individual physicochemical properties – size, change, polarity, affinity
- Partition of mixtures between the column mobile and stationary phases
- Analysing chromatography runs involves examination of column capacity, selectivity, resolution and peak asymmetry
- Use HETP to compare column performance
- In scale-up, keep bed height, linear flow rates and sample concentration constant increase column diameter, volumetric flow rate and sample load

Sample Questions

- SAQ: Explain how a Chromatography system operates
- SAQ: What is meant by column efficiency and how is it measured?
- LAQ: How are chromatography columns ‘qualified’ for DSP?
- What key measures are used to qualify columns?

Topics

- Chromatography Overview
- Components of a Chromatography System
- Qualifying the Column Packing

Thank You