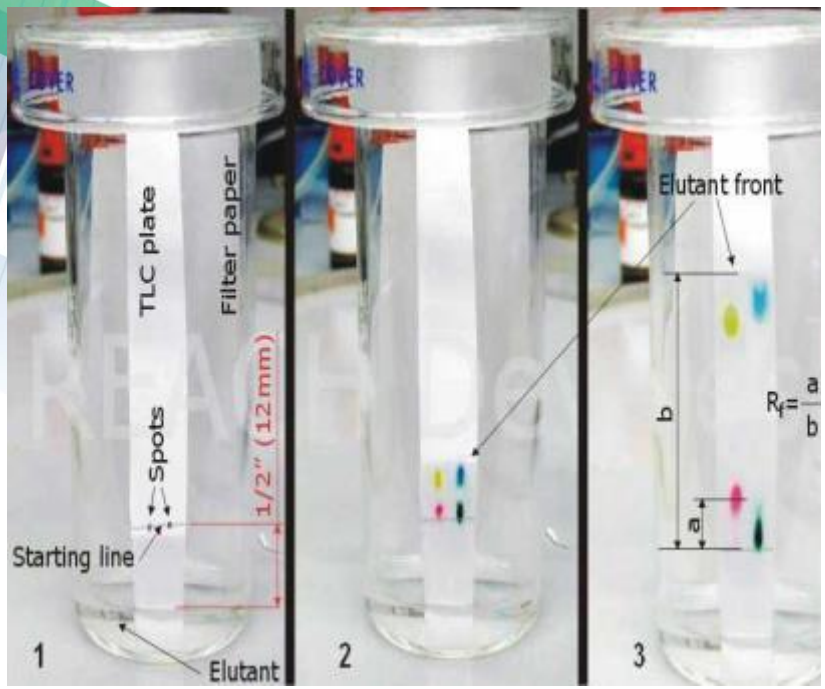
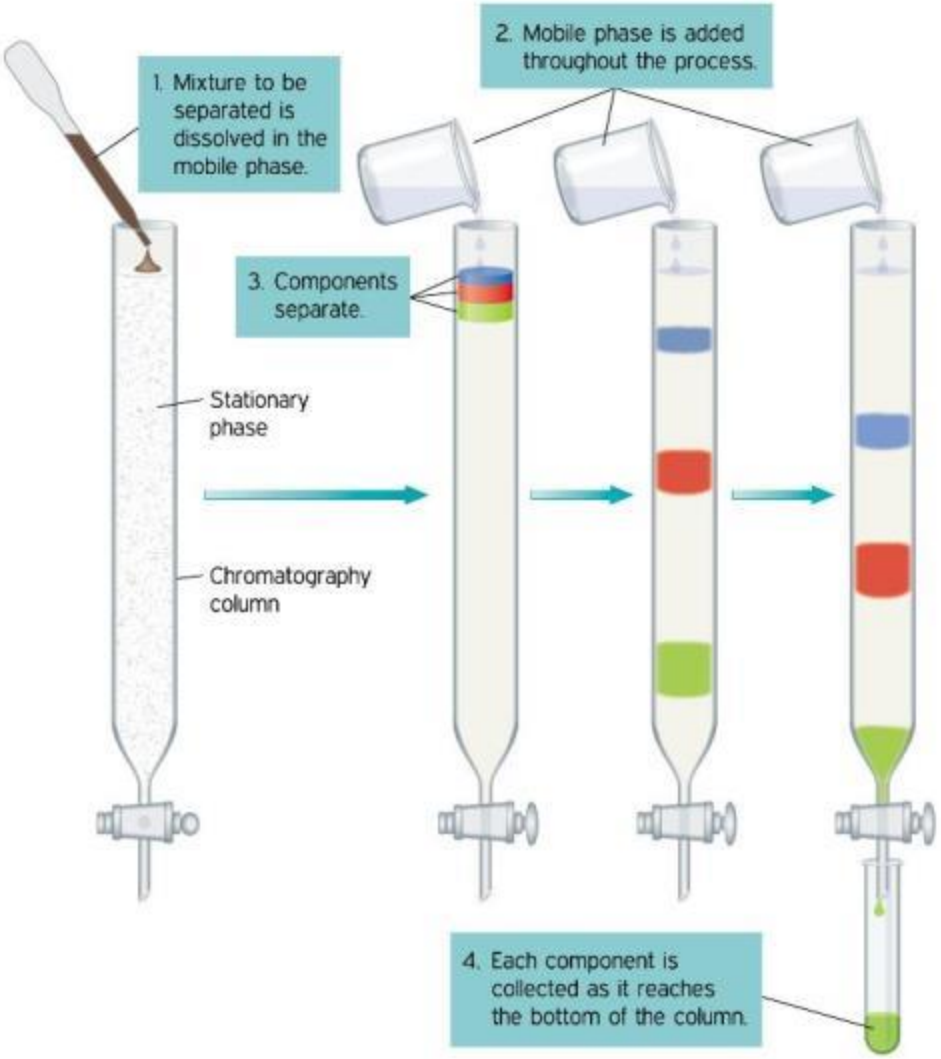
CHAPTER 5

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

Expected Outcomes

Explain the basic principles and instrumentation of HPLC
Able to state the function of each components of HPLC instrumentation
Compare characteristics of Normal phase and Reverse phase HPLC
Describe HPLC methodologies in quantitative and qualitative analysis
Explain the optimization of HPLC method
State the applications of HPLC


TLC plate
Filter paper
Spots
1/2" (12 mm)
Starting line
Elutant
1
2
Elutant front
b
a
$R_f = \frac{a}{b}$
3


1. Mixture to be separated is dissolved in the mobile phase.
2. Mobile phase is added throughout the process.
3. Components separate.
Stationary phase
Chromatography column
4. Each component is collected as it reaches the bottom of the column.

Types of chromatography

(according to the nature of **MP** and SP)

Stationary phase	**Mobile phase**	**Types**
Solid	Gas	Gas chroma. (GSC)
Solid	Liquid	Liquid chrom. (LC)
Liquid coated on a solid	Gas	Gas-liquid chroma. (GLC)
Liquid coated on a solid	**Liquid under pressure**	**High Performance liquid chrom. (HPLC)**

Principles of HPLC in Chemical Analysis

Basic separation principle

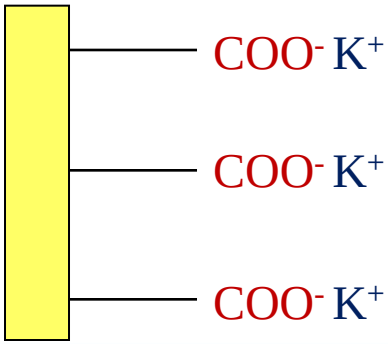
- Chromatography is a technique employed for the separation of mixtures of compounds in a sample.
- **LC is a chromatographic method, which uses the liquid as MP (eluent/solvent reservoir).**
- Separation of components occurs between mobile phase (MP, solvent) and stationary phase (SP, column packing material) under high pressure.
- Separation is based on different mechanism.

(ion-exchange, size-exclusion, adsorption, partition)

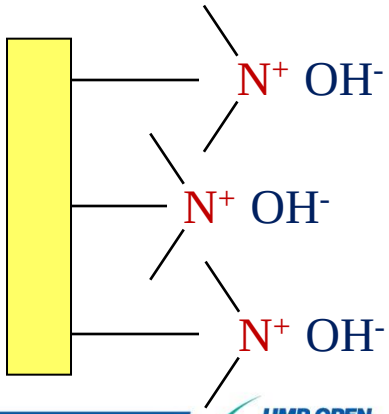
Basic Separating Principles in HPLC : Modes of Separation

Ion-exchange

- separation based on the charge properties of the molecules.
- **SP**: a resin matrix whose surface displays ionic functional groups that interact with analyte ions of opposite charge;
- **MP**: a buffered aqueous solution;
- Suitable for separation of ions and polar molecules, which are water soluble.

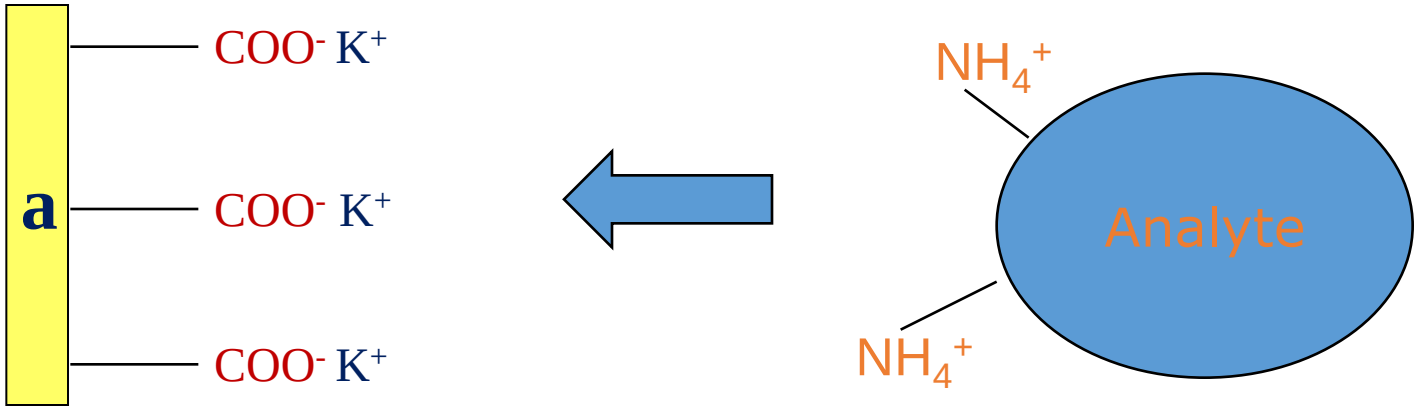


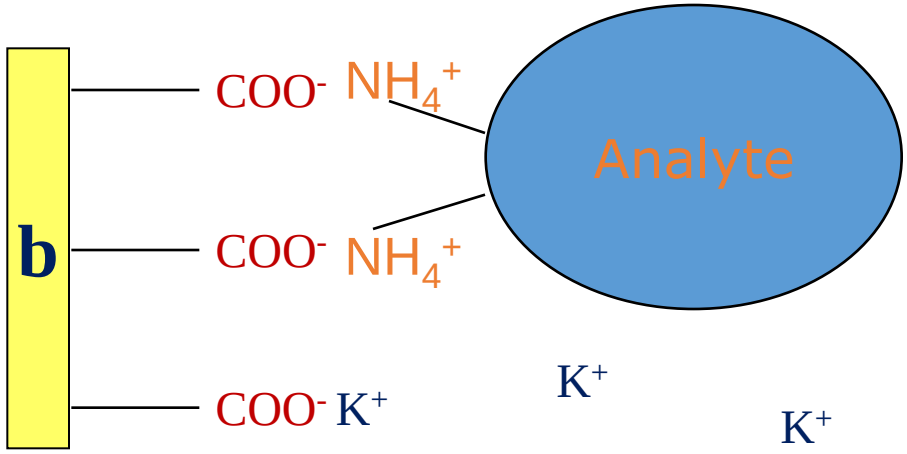
cation exchange resin



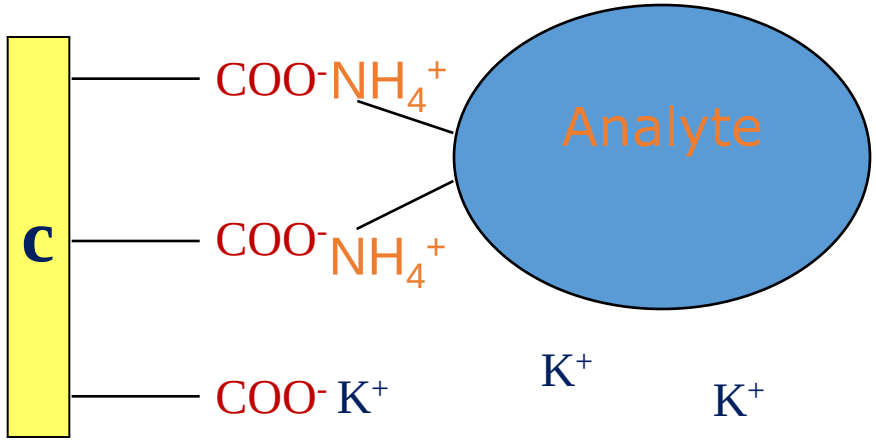
anion exchange resin

Cation Exchange Chromatography

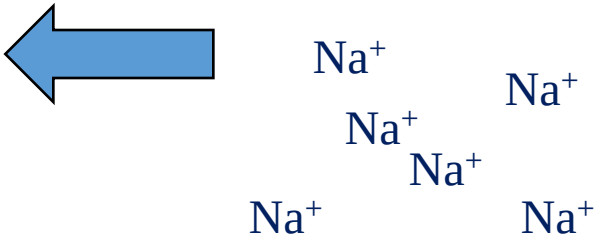


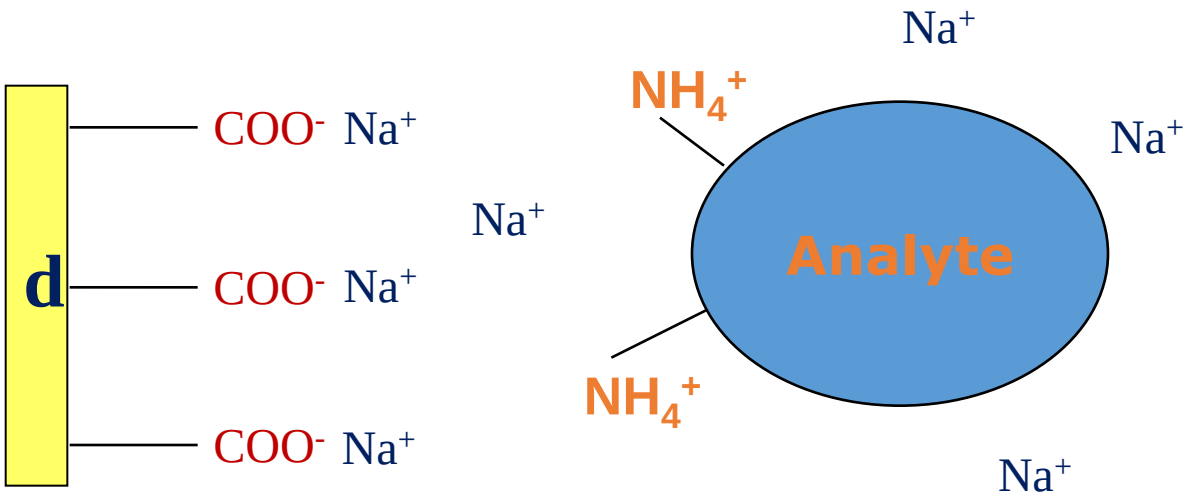


Cation Exchange Chromatography

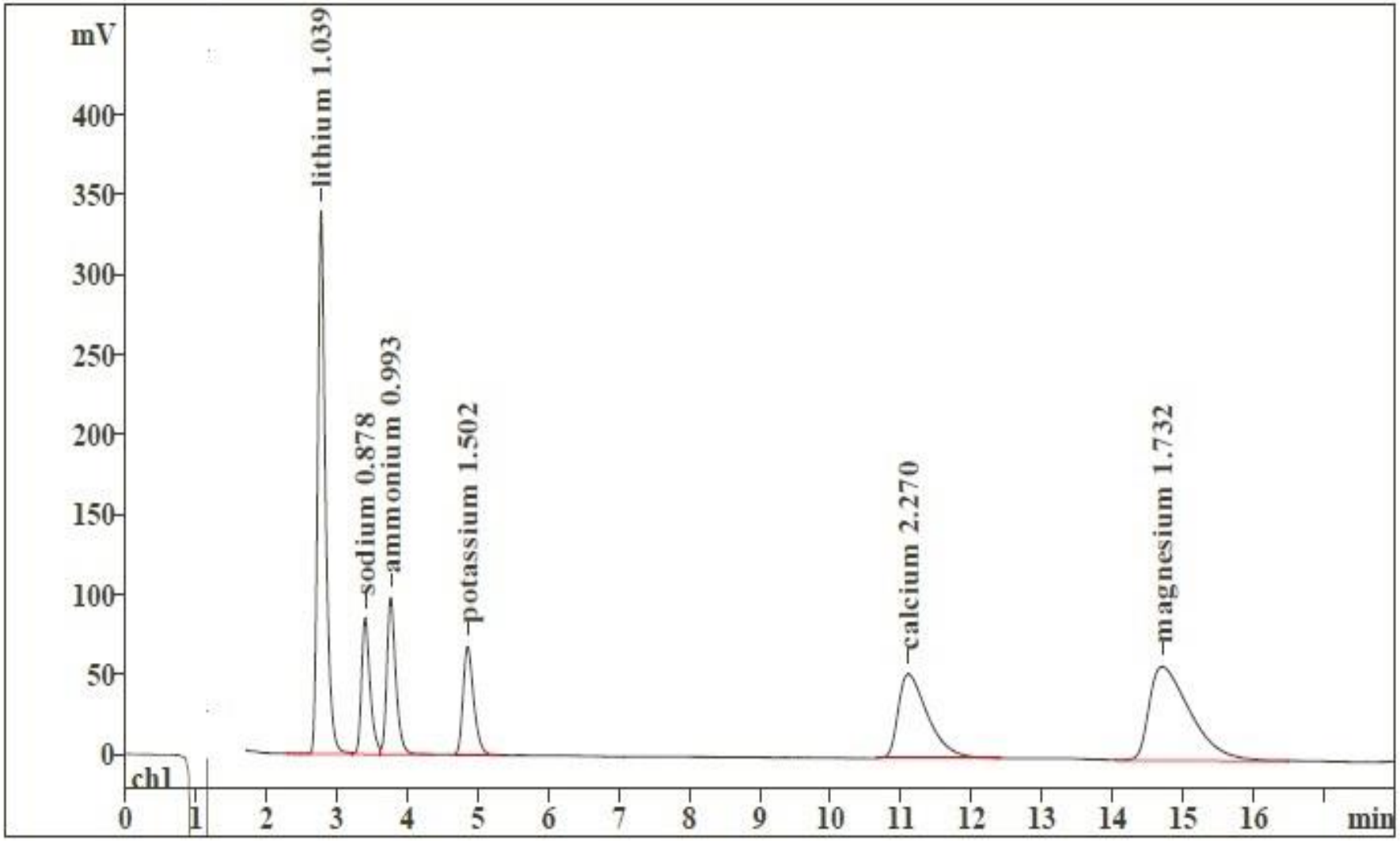


Buffer mobile phase containing Na^+.



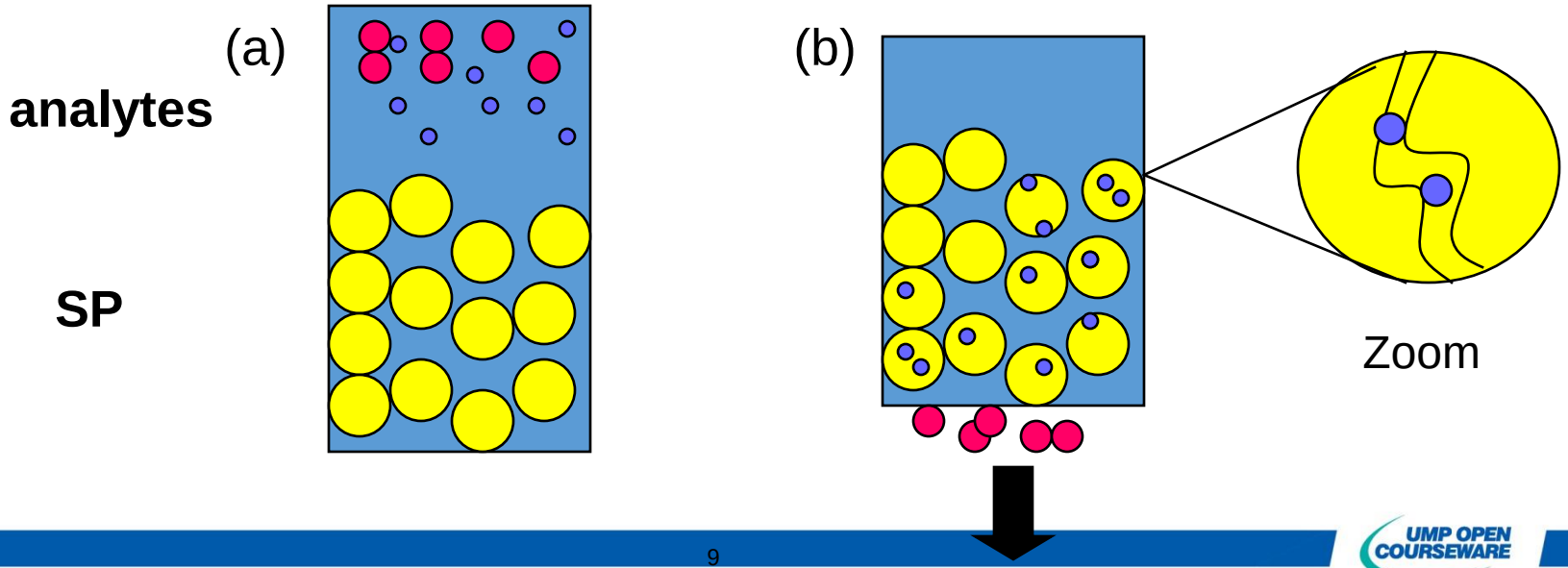


Elution of the analyte from SP.


mV
400
350
300
250
200
150
100
50
0
lithium 1.039
sodium 0.878
ammonium 0.993
potassium 1.502
calcium 2.270
magnesium 1.732
chl
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
min

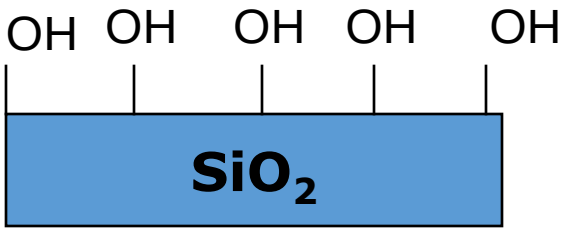
Size exclusion

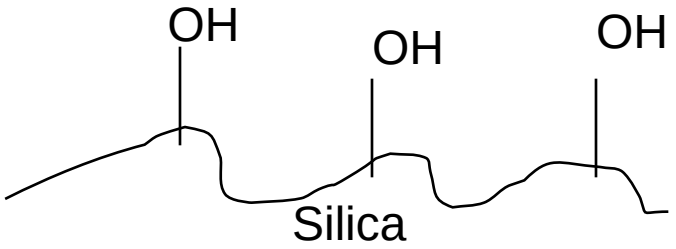
- separation according to molecular size
- **SP**: material having specific pore size controller
- **MP**: aqueous solution
- suitable to large molecules/macromolecular complexes eg. polymers



Adsorption

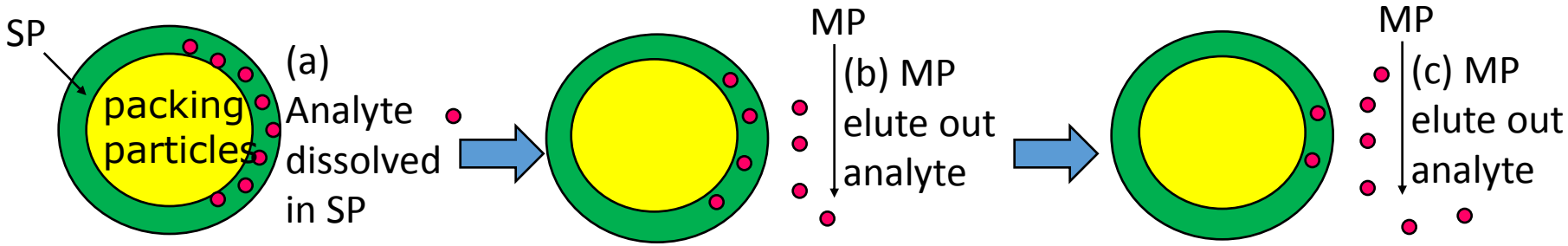
- separation based on sorption and desorption processes.
- **SP**: solid having unmodified surface, which is very polar, such as silica or SiO_2)
- **MP**: solvents, hexane, EA, $CHCl_3$ and MeOH;
- Not suitable for the separation of strong polar compounds





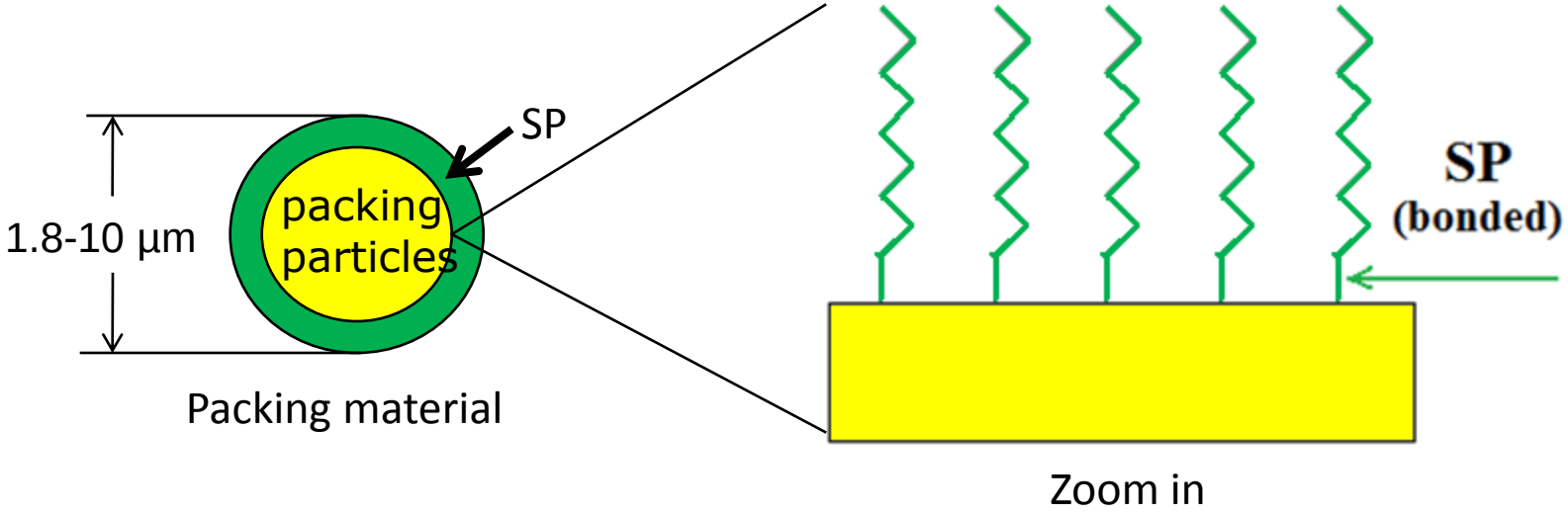
Partition

- separation based on the difference of the dissolution of solutes between MP and SP;
- **SP**: liquid coated or bonded on the packing particles;
- **MP**: solvents such as hexane, EA, $CHCl_3$, MeOH, ACN and ultrapure H_2O;
- Suitable for separating various compounds in the mixture, and has broad application.



Partition Chromatography (PC)

- Bonded phase PC
- SP is a liquid chemical group that covalently bonded to silica packing particles, so as to avoid losing SP and increase the thermal stability of SP.



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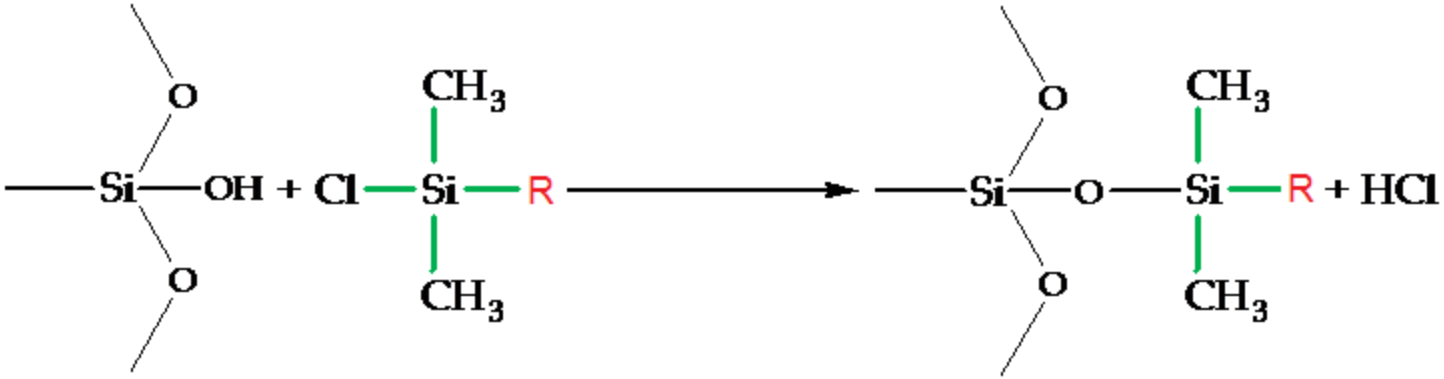
Partition Chromatography (PC)

- How to bond SP on Silica-gel?

  Formed by the reaction of silica particles with an organo-silane of the general formula $Si(CH_3)_2RCl$.



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What is R?

- If R is a polar functional group – stationary phase is polar.

- Example
  - cyano ($-C_2H_4CN$)
  - amino ($-C_3H_6NH_2$)
  - diol ($-C_3H_6OCH_2CHOHCH_2OH$)

- If R is a non-polar functional group – stationary phase is non-polar.

- Example
  - N-octyl (-C_8)
  - N-octyldecyl (C_{18})

Basic Separating Principles in HPLC : Predicting Elution Order by MP

- Separation in HPLC is basically governed by manipulating the **polarity** of **both SP** & **MP**.

Mobile Phase Selection

- Elution order of HPLC is governed by polarity.
- Retention times are controlled by polarity of mobile phase.

Basic Separating Principles in HPLC : Polarity of MP

Mobile phase/solvent	Polarity index (P')
Cyclohexane	0.04
n-hexane	0.1
carbon tetrachloride	1.6
i-propyl ether	2.4
toluene	2.4
diethyl ether	2.8
tetrahydrofuran	4.0
ethanol	4.3
ethyl acetate	4.4
dioxane	4.8
methanol	5.1
acetronitrile	5.8
water	10.2

Principles of HPLC in Chemical Analysis (Summary)

HPLC:

- **HPLC** is a kind of LC, which uses **high pressure** to drive liquid **MP** through a column of **SP**.
- Separation is basically governed by manipulating the **polarity** of both SP & MP.
- Elution order of HPLC is governed by **polarity of analyte.**

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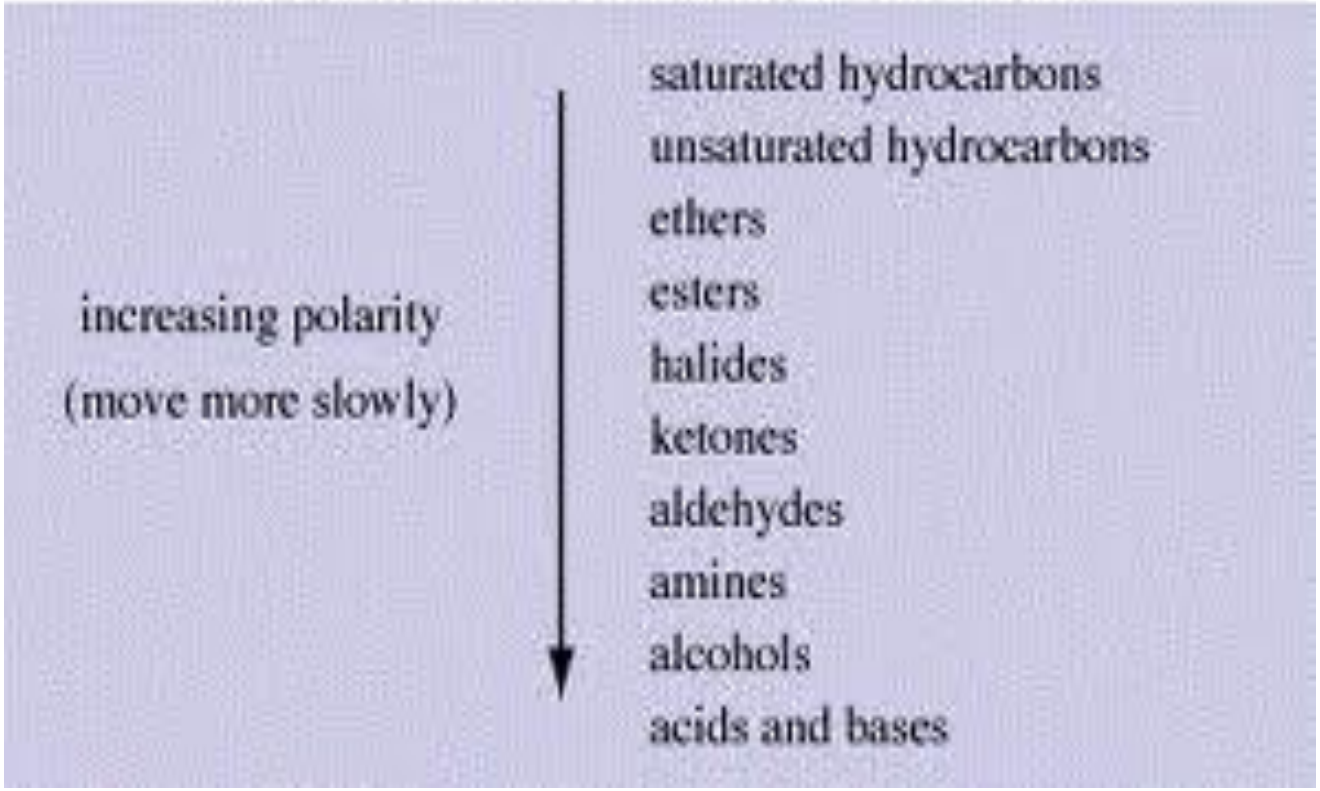
The expected elution order of organic classes.



Principles of HPLC in Chemical Analysis (Summary)

- **Bonded phase PC**
- SP is a liquid chemical group that covalently bonded to silica packing particles, so as to avoid losing SP and increase the thermal stability of SP.

Polar	cyano (-C_2H_4CN) amino (-$C_3H_6NH_2$) diol (-$C_3H_6OCH_2CHOHCH_2OH$)
Non-polar	octyl (-C_8H_{17}) octyldecyl (-$C_{18}H_{37}$)

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5.2 Characteristics of Normal Phase and Reverse Phase HPLC

Normal-phase HPLC (NP-HPLC)

- Polar stationary phase
- Non/less polar mobile phase

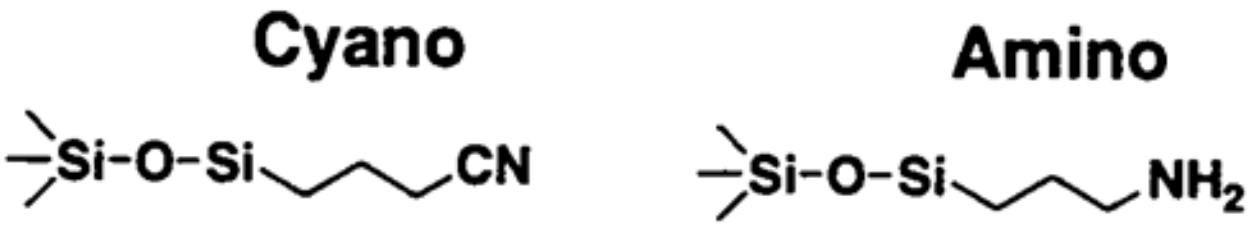
  Note: mixture of non/less polar solvents could be used as MP. E.g. are hexane, EA, $CHCl_3$, ether, dichloromethane

- Least polar solutes are first to elute from the column

Characteristics of Normal Phase and Reverse Phase HPLC

SP in NP-HPLC (2):

- Organic moieties with cyano-silane or amino-silane functional groups have replaced reactive silanol groups (Si-OH) on the silica surface.



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Characteristics of Normal Phase and Reverse Phase HPL C

Reversed-phase HPL C (RP-HPLC)

- Non/less polar stationary phase
- Polar mobile phase

  E.g. are MeOH, ACN, Ultrapure water

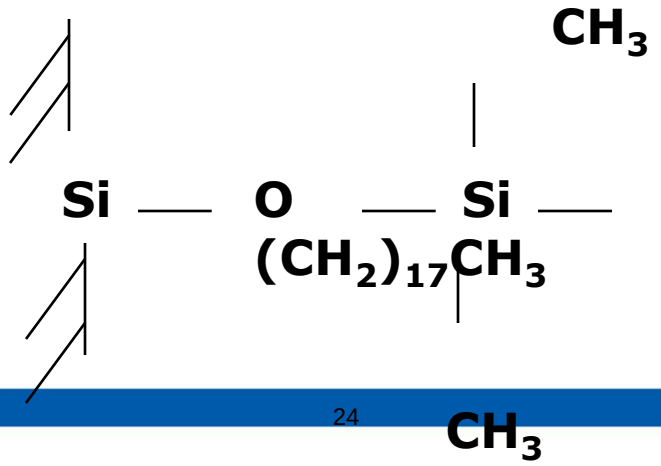
- Most polar solutes are first to elute from the column

Characteristics of Normal Phase and Reverse Phase HPL C

SP in RP-HPLC

It uses a polar mobile phase and a non-polar stationary phase.

The silanol groups (Si-OH) present in silica is treated with an organochlorosilane:



ODS bonded groups

You are given one samples contains an analytes A-alchohol

B-alkane

C-ester

Predict the elution order for both NP and RP

HPLC. Your answer should suggest suitable

1. SP
2. MP
3. Elution order

Basic Separating Principles in HPLC : Predicting Elution Order

Exercise 1:

Predict the order of elution for the separation of

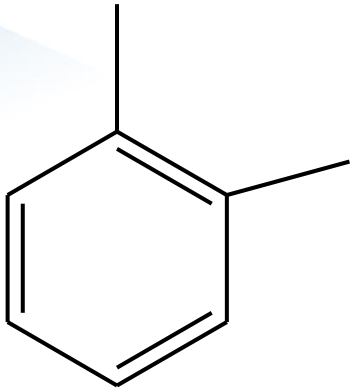
- $CH_3C(O)CH_3$
- $CH_2=CH_2CH_3$ and
- C_3H_7OH

using a C8 bonded phased stationary phase.

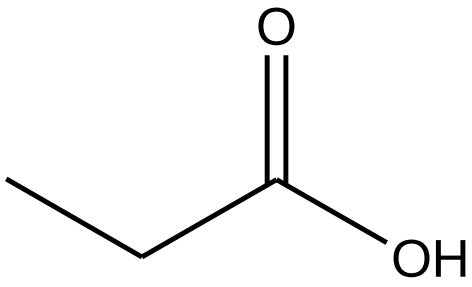
Explain your answer.

- Exercise 2

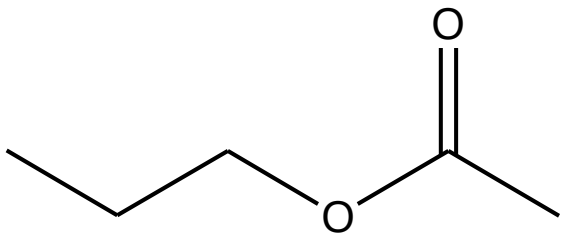
A student set up a HPLC separation of the following compounds is run through a column with C_2H_4-CN functional group attached to the siloxane backbone and n-hexane as the mobile phase.What is the order of elution for these compounds? Explain your reasoning.



o-xylene



propionic acid



propyl acetate

- Discuss the advantages of Reverse Phase HPLC compared to Normal Phase HPLC analysis

Solution to exercise 1:

- C8 is non-polar so non-polar molecule will then be retent longest. So the alkene will be eluted last, followed by ketone and alcohol.
- Propanol
- Propanone
- Propene

Characteristics of Normal Phase and Reverse Phase HPL C

Reversed-phase HPLC

Advantages

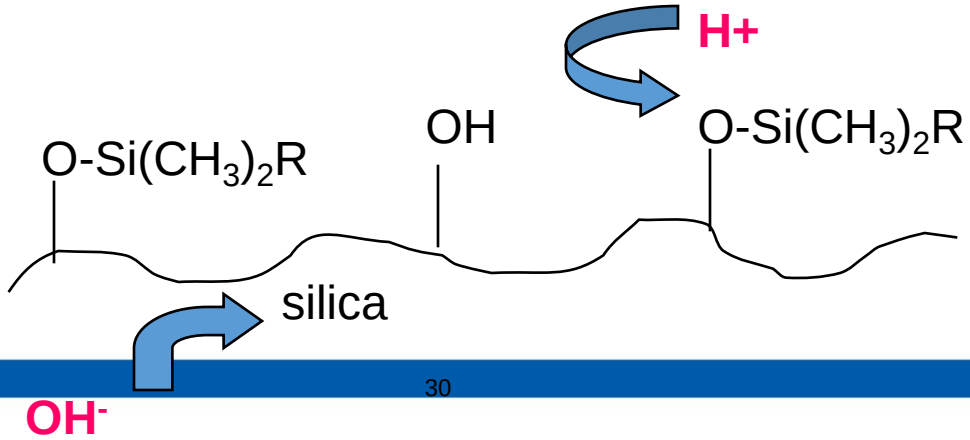
- The mode has a very broad scope that allows samples with wide ranges of polarity to be separated.
- The mode is generally experimentally easier, faster and more reproducible than other LC modes.
- It can be applied to the separation of ionic or ionizable compounds by the use of ion-pairing techniques.

Characteristics of Normal Phase and Reverse Phase HPL C

Reversed-phase HPLC

Disadvantages

- For silica bonded phases, stable columns can be maintained at pH 2-10. Below pH 2 the bonded groups will be hydrolyzed, and above pH 10, the silica is appreciably soluble in the mobile phase.

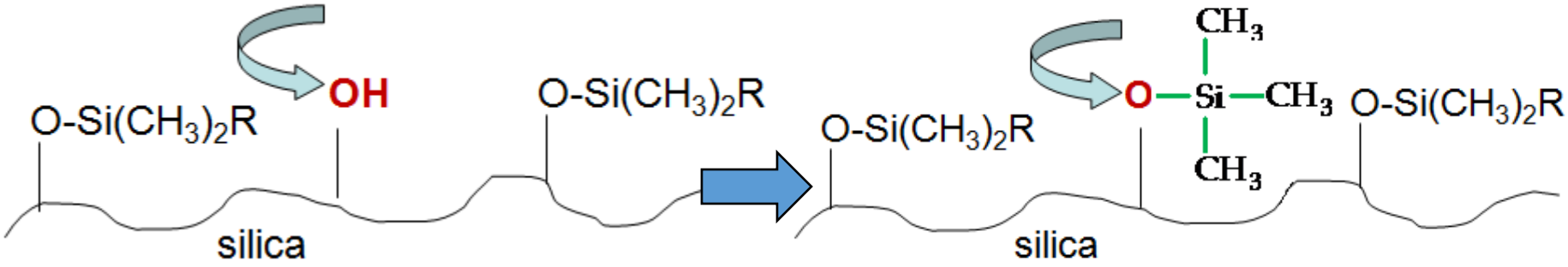


Characteristics of Normal Phase and Reverse Phase HPL C

Reversed-phase HPLC

Disadvantages

- The presence of unreacted silanol groups on the silica surface can often cause poor peak shape and non-reproducible behavior between columns due to solute adsorption.



5.3 Quantitative and Qualitative Analysis

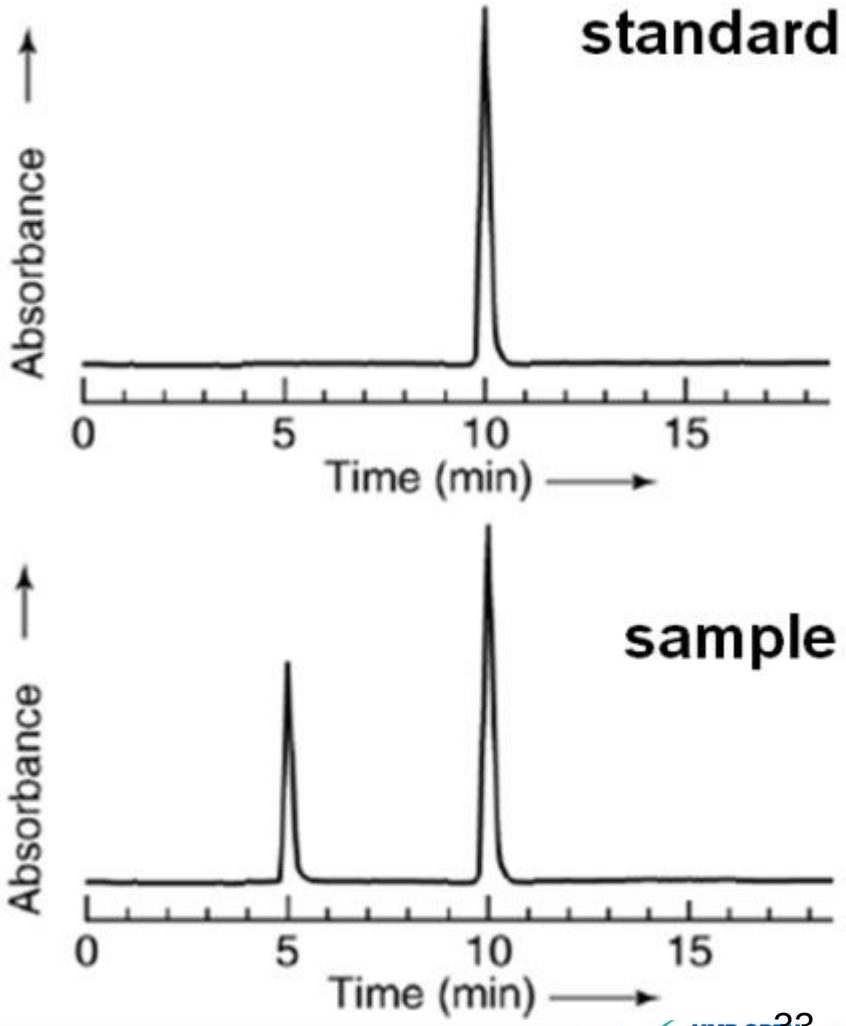
- Retention Time Matching
- Standard Curve Method
- Internal Standard Method

Methodologies in Qualitative and Quantitative Analysis

Qualitative=ID

Retention Time Matching

- T_r of standard and sample are matched when both are run under the same conditions.



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Methodologies in Qualitative and Quantitative Analysis

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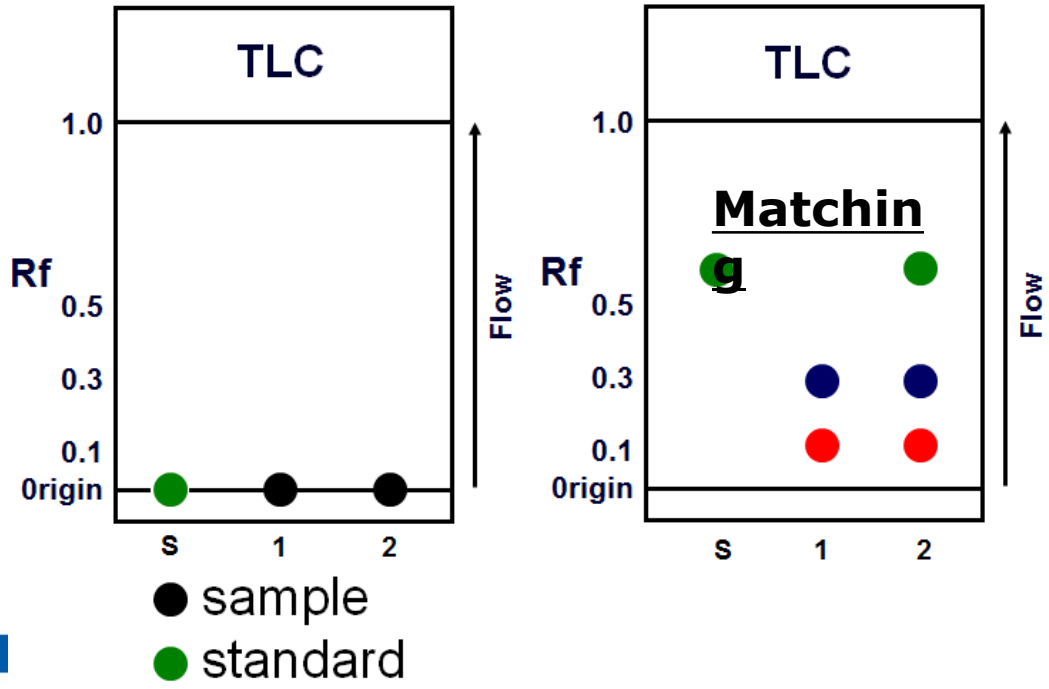
Disadvantages

- As std and sample are injected consecutively, simultaneous analysis is not possible. Which chromatography allows simultaneous analysis?



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Methodologies in Quantitative and Qualitative Analysis

Quantitative=amount

Standard curve method

- prepare a set of standard solutions containing a pure analyte
- obtain a series of chromatograms
- plot a calibration curve of **peak area/height versus concentration**
- determine the concentration of unknown sample from the calibration curve

Methodologies in Quantitative and Qualitative Analysis

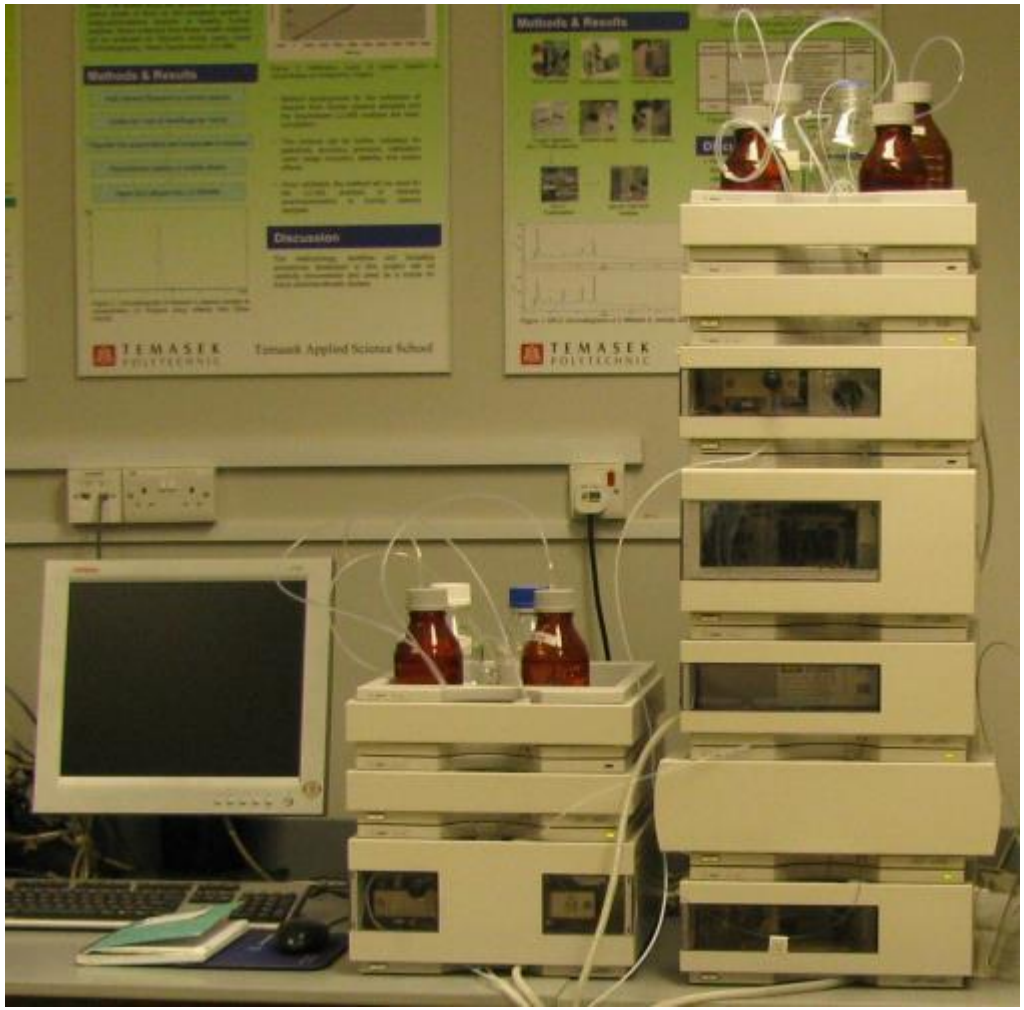
Quantitative=amount

Internal standard method

- prepare a set of standard solutions containing a pure analyte
- Spike a known amount of an internal standard into the standard and sample solutions
- obtain a series of chromatograms
- plot a standard curve of **peak area ratio versus concentration**
- determine the concentration of unknown sample from the standard curve

5.4 Components of HPLC instrumentation

1) **MP** supply system
2) Pump
3) Sample injection system
4) **Column**
5) **Detector**
6) Workstation



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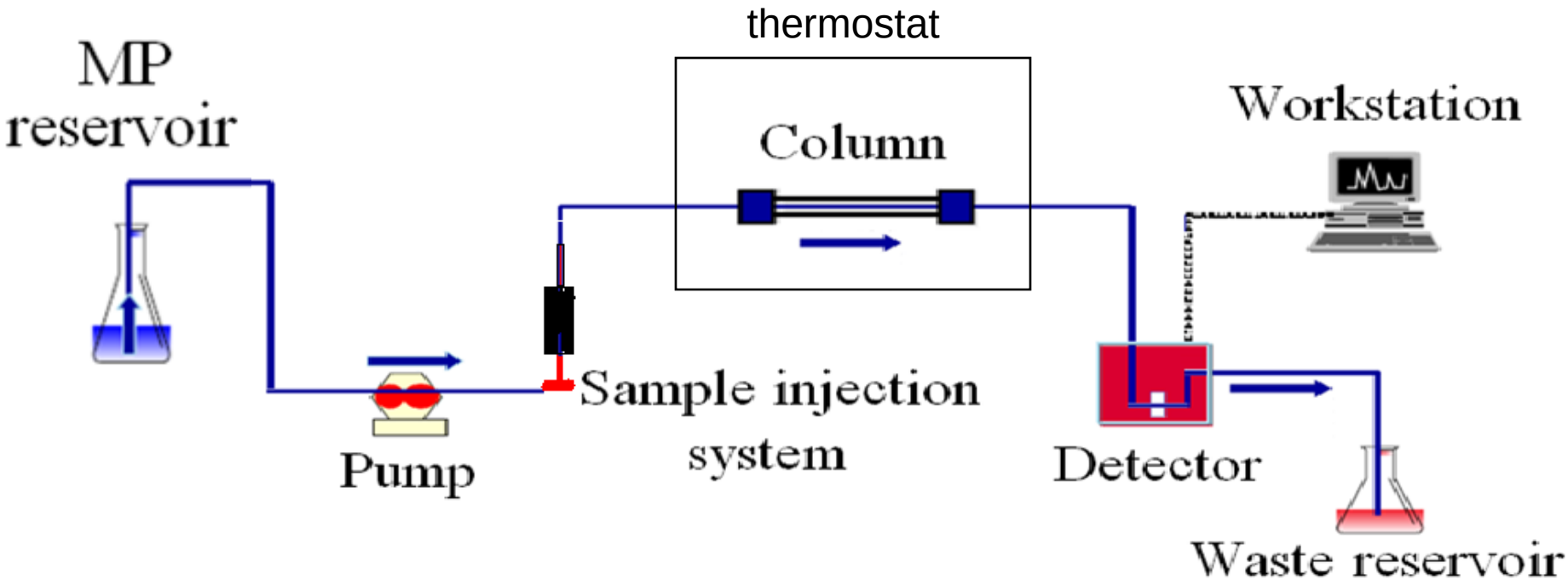
HPLC instrumentation



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HPLC instrumentation

1) MP supply system

known as MP (solvent) reservoirs

Function: to provide MP/solvent (s) for the run

A. MP/Solvent reservoir(s):

- One: Filled with single solvent or the mixture of the solvents of different polarities
- >1: Filled with several solvents of different polarities, respectively

HPLC instrumentation

1) MP supply system

B. Solvent(s):

- The solvent(s) used must be high pure;
- The solvent(s) must be filtered before use;
- The solvent(s) must be degassed before use;
- HAc, FA, H_3PO_4, TFA or ammonium acetate, phosphate, and ion-pairing chemicals which are soluble in MP can be used to control pH value.

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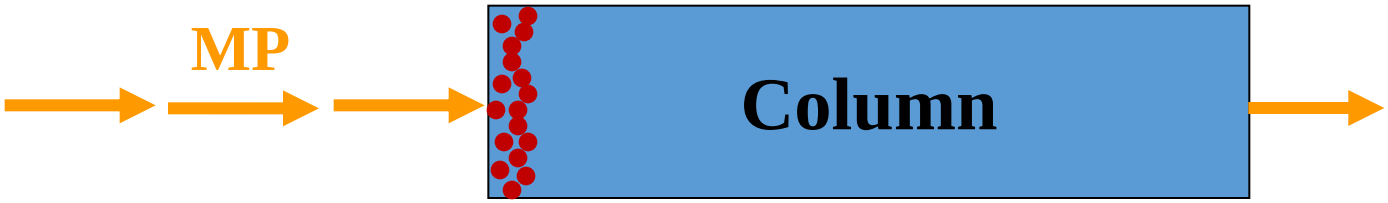
HPLC instrumentation

Filtration:

- To remove small particulate matter such as dust and insoluble salt, which will greatly damage the pump and collect on the top of the column.
- Micropore filter membrane (0.45μm usually and 0.2μm for buffer salt solution) is commonly used for filtration.



small particulate matter **clogged column**

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HPLC instrumentation

1) MP supply system

C. Degasser:

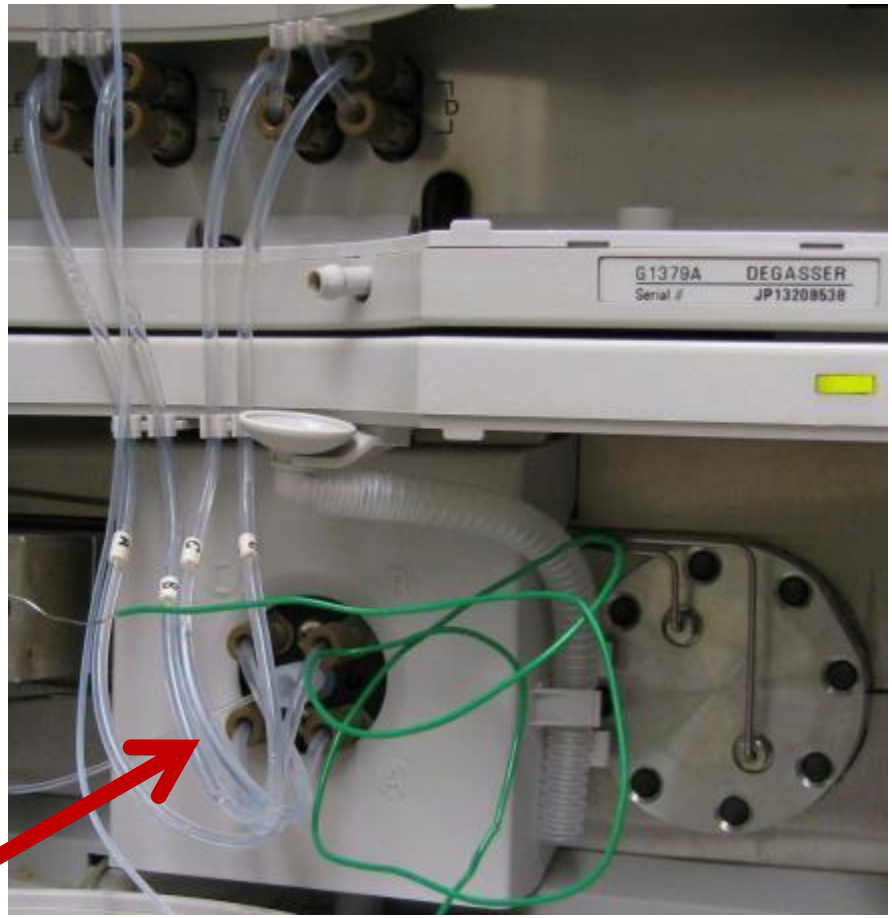
To remove dissolved gases in MP such as N_2 and O_2 which may lead to the formation of gas bubbles when MP enters the detector resulting in distortion of signals. Gas bubbles within the column can also lead to very high pressure and unstable pressure.

HPLC instrumentation

1) MP supply system

D. Mixing chamber

Place where the solvents are mixed in correct compositions.



Mixing chamber

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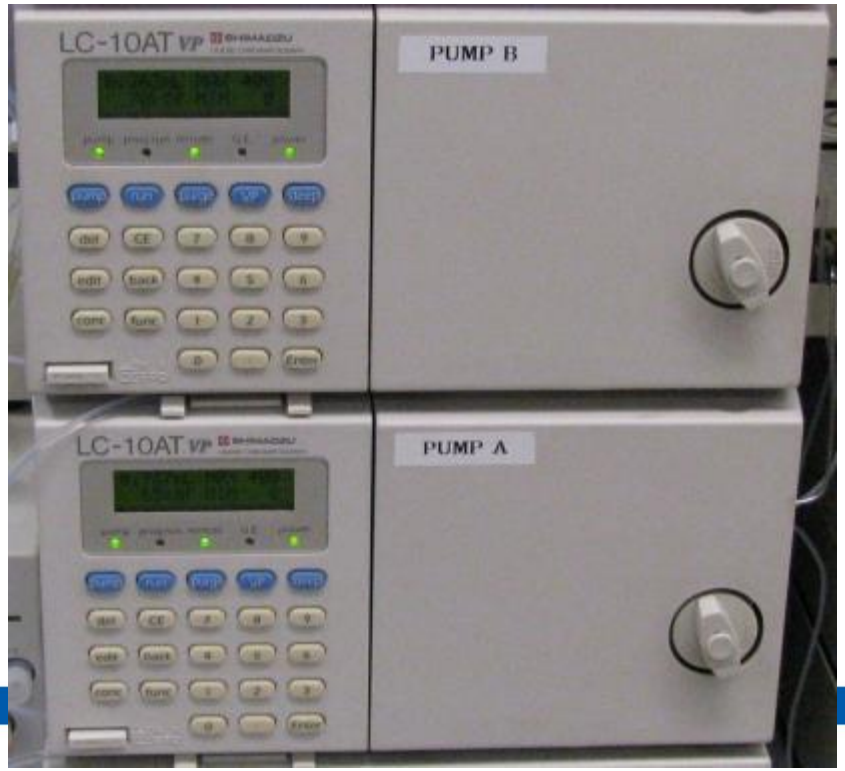
HPLC instrumentation

2) Pump

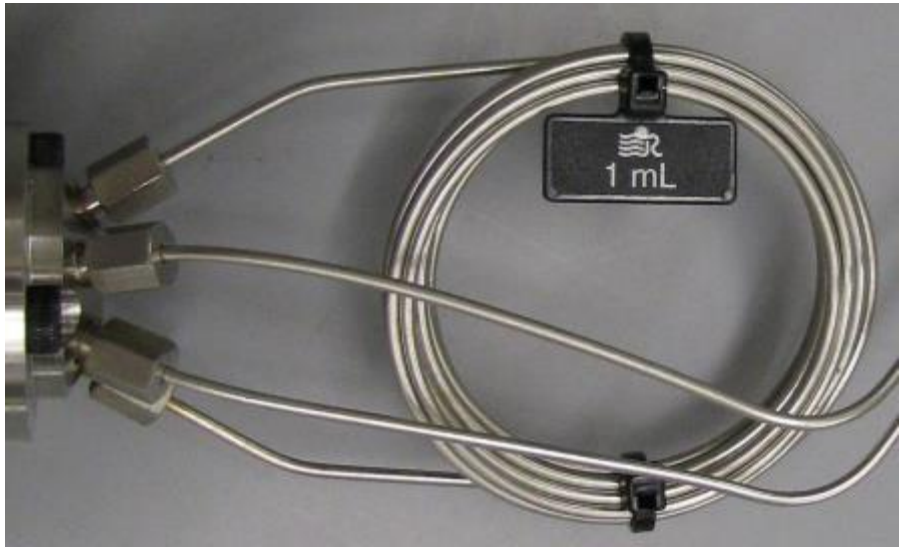
Function: to provide the high pressure (driving force) required for the run, which is the core component of HPLC.



HPLC instrumentation

3) Sample Injection System

- Six-port valve and sample loop



Sample loop

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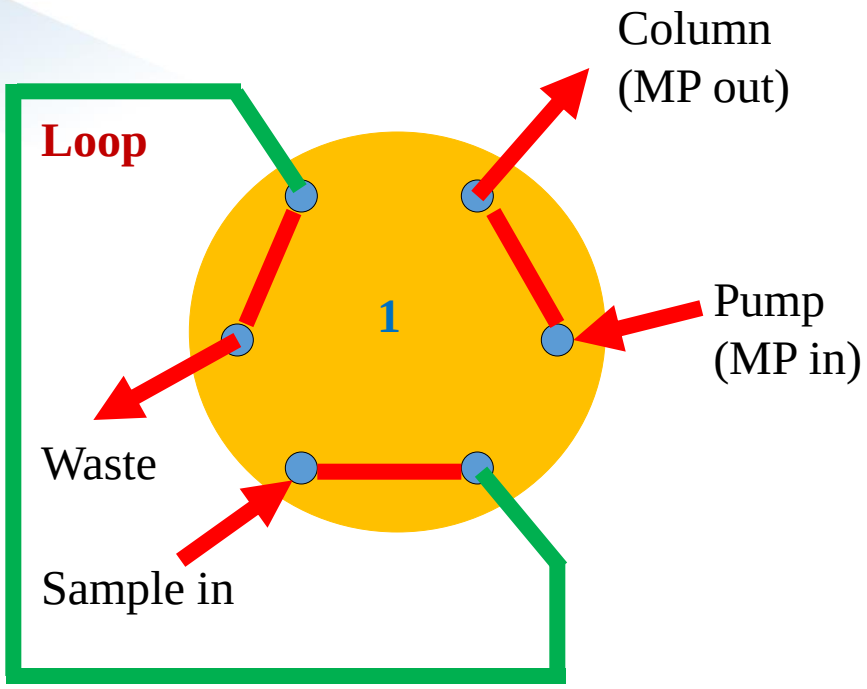
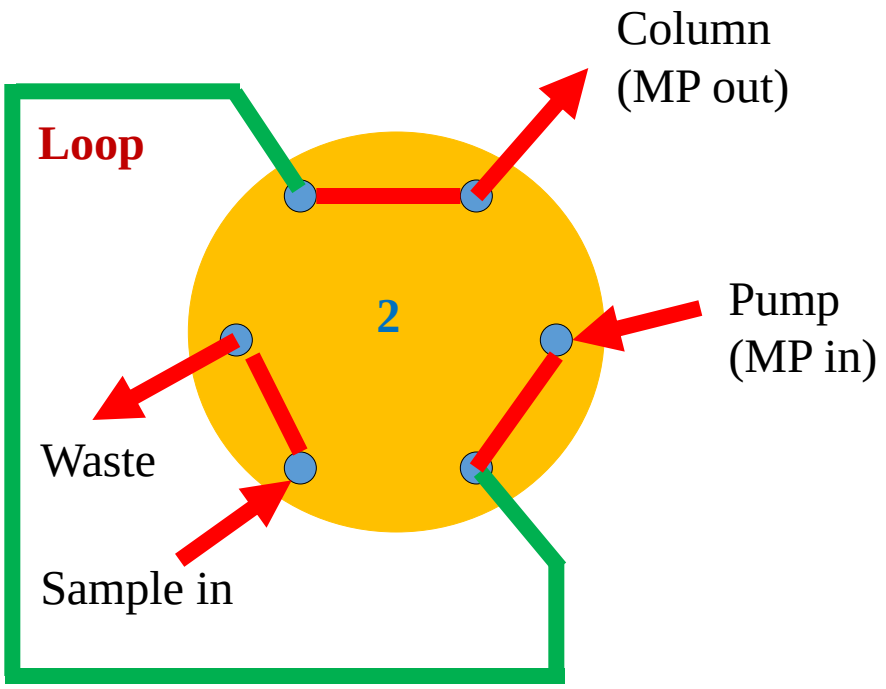
HPLC instrumentation



Load Position



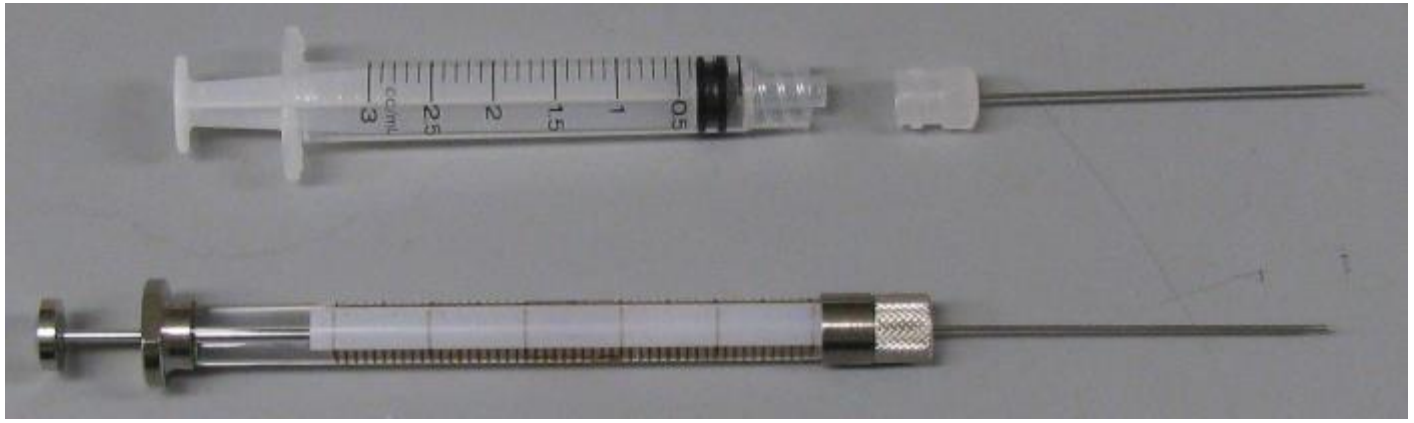
Inject Position

46

HPLC instrumentation

3) Sample Injection System

- Syringe & Needle



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HPLC instrumentation

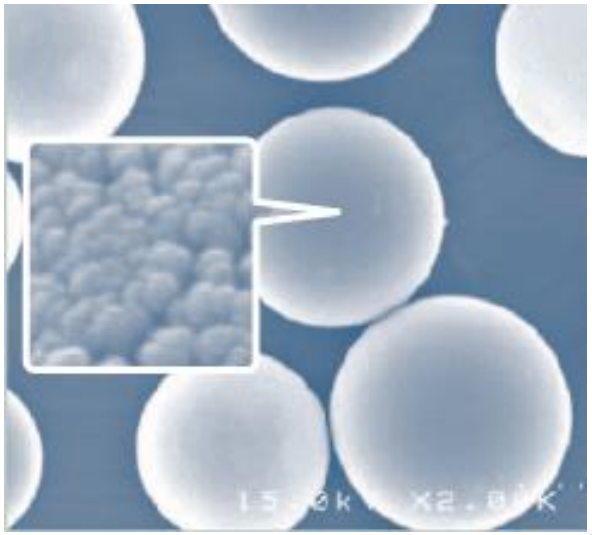
4) Column: Analytical column

- Function: to separate the analyte in the run
- External packing is usually constructed from stainless steel tubing. Why?

HPLC instrumentation

4) Column: Analytical column

- Common dimensions of HPLC column
  - packings (∅): 1.8~10 μm



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HPLC instrumentation

- Analytical columns with internal diameter of around 5 mm packed with 5-μm particles offer a good compromise between
  - sample capacity
  - column efficiency
  - applied pressure
  - volume of MP used

50

HPLC instrumentation

4) Column: Guard column

- To avoid the formation of clogged column induced by small insoluble particles from sample/MP

Same properties as analytical column

HPLC instrumentation

5) Detector: Types of detector

1. Ultraviolet-visible Detector (UV-VIS)
   Diode array detector (DAD)/Photodiode array detector (PDA)
2. Refractive index detector (RI)
3. Mass spectrometer detector (MSD)

5.5 Optimization of HPLC Analysis

Factors that affect a HPLC analysis

- Flow rate of mobile phase
- Type of column
- Type of detector

Factors that affect HPLC analysis

Flow Rate of Mobile Phase

- As flow rate increases, retention time decreases.
- If the flow rate is too high, some of the compounds may elute at the same time. This leads to poor separation efficiency.
- The flow rate must be adjusted and optimized to effect good separation of the solutes in a sample.

Factors that affect HPLC analysis

Type of Column

- Polarity of SP matced with an analyte
- This would effect greater interaction between the analyte and the column leading to good separation.
- The selection of a specific column (stationary phase) depends on whether or not the planned separation is possible or logical with a given mechanism.

Factors that affect HPLC analysis

Type of Column

1. Particle size: prefer **smaller particle**

→ Higher plate number

→ Narrower peaks

→ Higher pressure required to move the eluent through the column

- Manufactured columns have about 50,000 plates/m if packed with 5 μm particles, and about 25,000 plates/m if packed with 10μm particles.

Factors that affect HPLC analysis

Type of Column

2. Particle shape

Columns packed with spherical particles required less pressure for a given eluent velocity.

Factors that affect HPLC analysis

Parameter Increase	Retention Time
Column Length	Increase
Column Internal Diameter	Increase
Column Particle size	Decrease

Different Modes of Liquid Chromatography

There are two mobile - phase elution methods:

- Isocratic elution
- Gradient elution

constant composition

composition changes over time

Other than the polarity of SP and MP, we [illegible] methods to achieve good separation.

Different Modes of Liquid Chromatography

Isocratic elution

A single mobile phase composition is in use for the entire separation.

Q: How to resolve peaks 1 to 3?

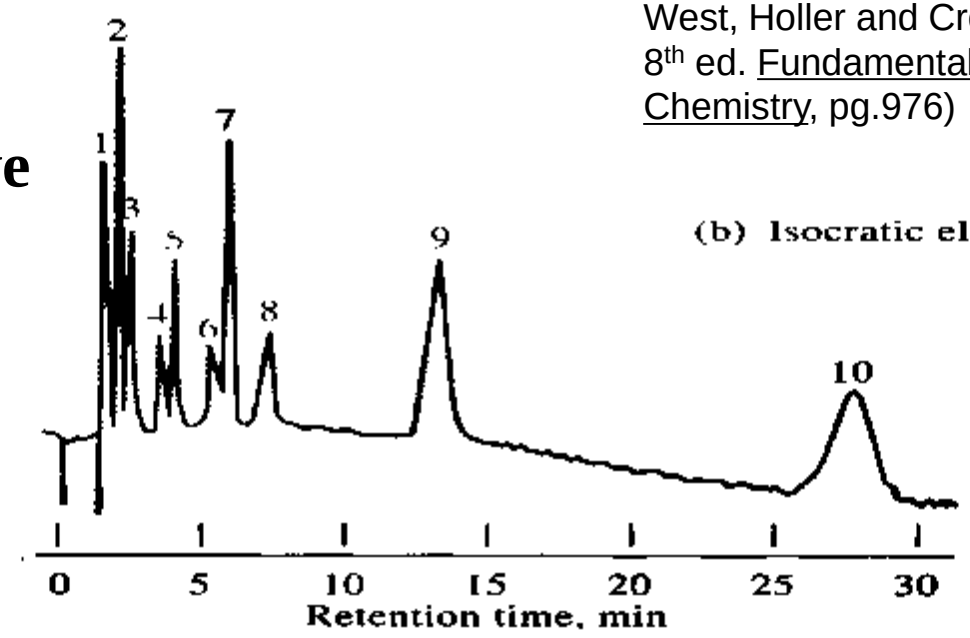


Diagram extracted from: Skoog, West, Holler and Crouch, 2004, 8th ed. Fundamentals of Analytical Chemistry, pg.976)

4.3.2 Different Modes of Liquid Chromatography

Gradient elution

- The composition of the mobile phase changes with time during the separation, usually by mixing two solvents with different eluting powers in continually changing proportions
- Gradient elution allows early eluting peaks to be adequately separated without the later eluting peaks becoming too dispersed.

A gradient elution in HPLC is one in which the composition of the solvent is changed continuously or in a series of steps.

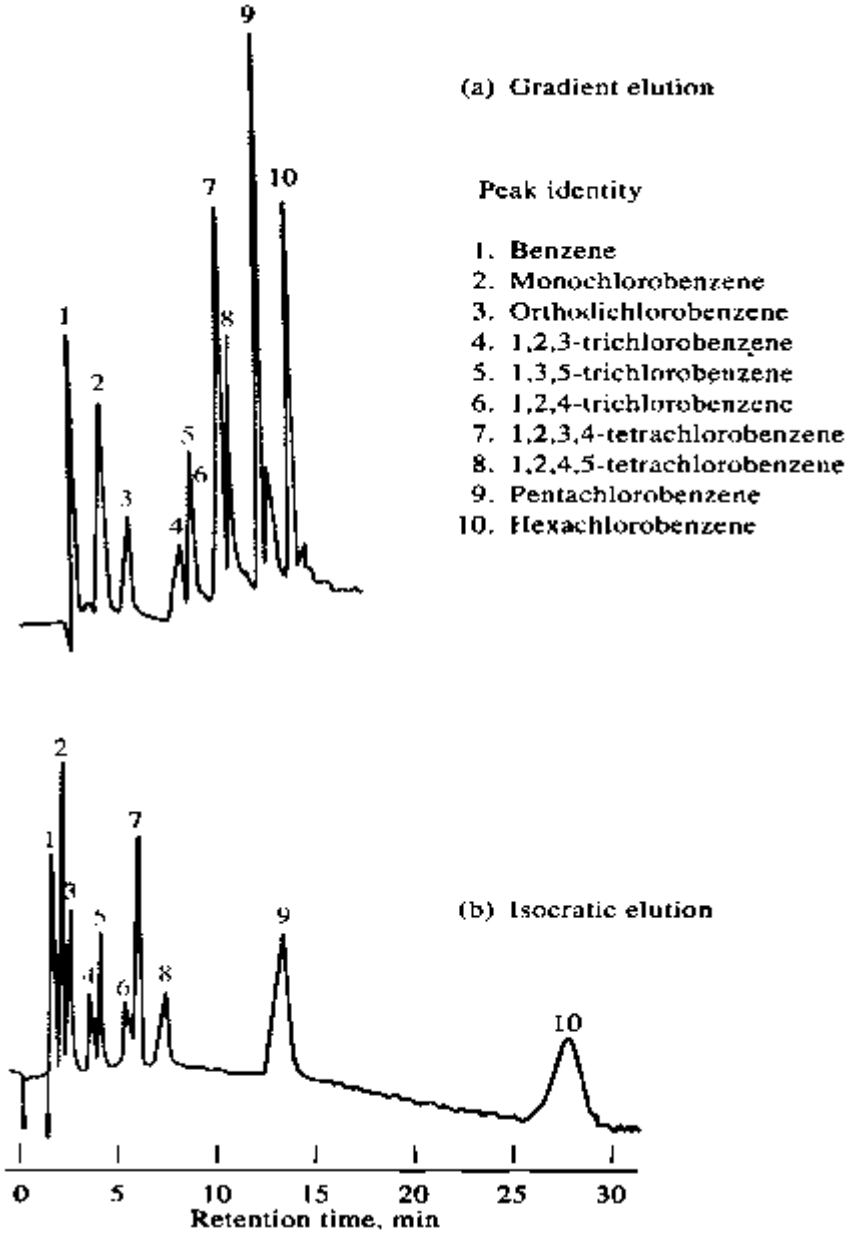


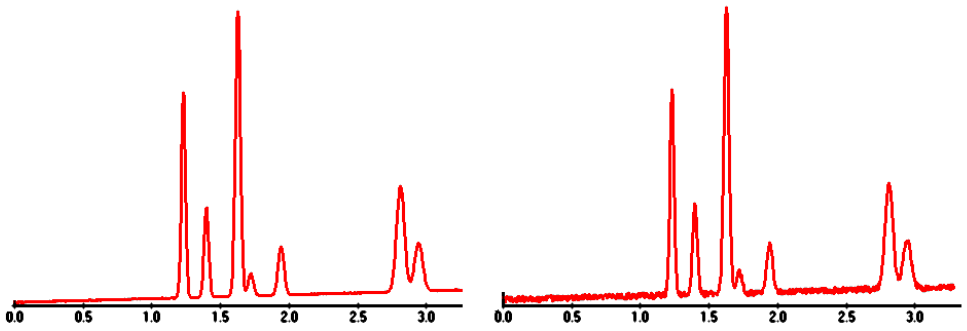
Diagram extracted from: Skoog, West, Holler and Crouch, 2004, 8th ed. Fundamentals of Analytical Chemistry, pg.976)

Common Problems in HPLC Analysis

Column Troubleshooting

1. Noisy Baseline

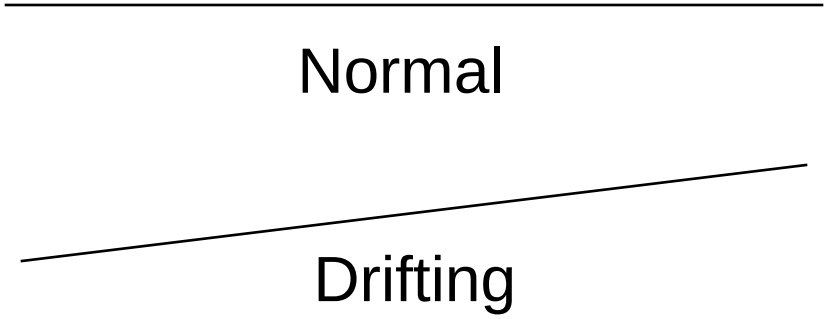
- Possible causes :
  - Dirty flow cell
  - Detector lamp failing
  - air bubbles passing through detector
  - temperature effects on detector



Common Problems in HPLC Analysis

2. Drifting baseline

- Possible causes :
  - Gradient elution
  - Temperature unstable (RI detector)
  - Contamination in mobile phase
  - Contamination in system



Common Problems in HPLC Analysis

3. Ghost peaks - peaks which appear even when no sample is injected

- Possible causes :
  - dirty mobile phase

4. Unusually high pressure

- Possible causes :
  - air pockets trapped in the column
  - pump malfunction
  - clogged column

Common Problems in HPLC Analysis

5. Fronting peaks

- Possible causes
  - Column overload

6. Negative peaks

- Possible causes
  - absorbance of sample is less than mobile phase

5.6 Applications of HPLC

- Chemistry and biochemistry research
- Quality control
- Environmental control
- Federal and state regulatory agencies
- Pharmaceutical industries