TOPIC 3

High Performance Liquid Chromatography

Course Learning Outcomes

Able to :

1. Explain the fundamental concepts & theories of separation techniques in HPLC.
2. Sketch & label the schematic diagrams in HPLC.
3. Identify the strength & limitations of each type of HPLC technique.
4. Suggest & justify the most suitable & efficient separation technique to be employed for an analysis.

Introduction

- Mobile phase is liquid.
- Analytes are first dissolved in a solvent / mobile phase, then passed through column under high pressure.
- The separation dependent upon interaction of the analytes with mobile & stationary phases.

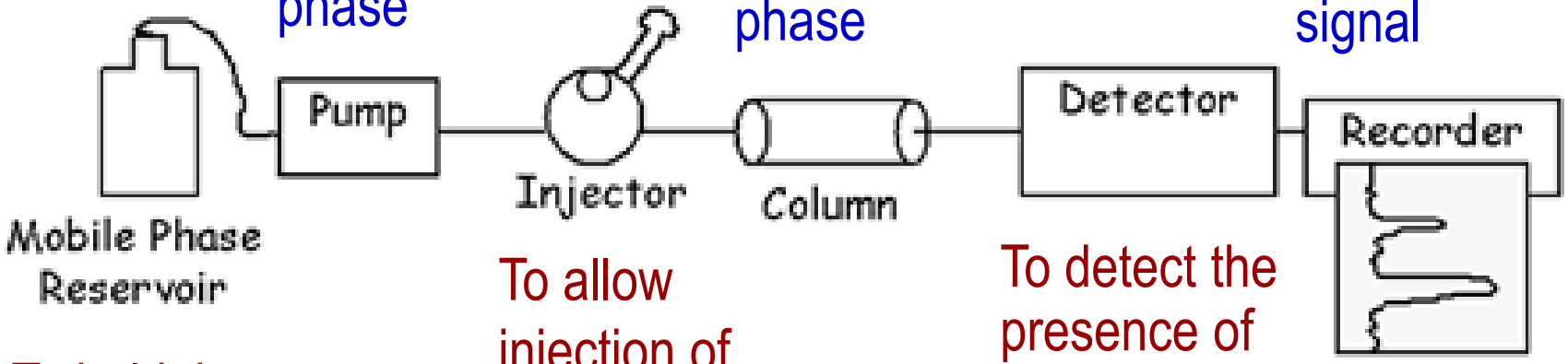
HPLC applications

Analysis of mixtures of non-volatile or thermally unstable compounds (those that cannot be analyzed by GC)

To pressurize the liquid mobile phase

Column containing the bed of stationary phase

To record the detector signal



To hold the liquid mobile phase

To allow injection of the sample mixture

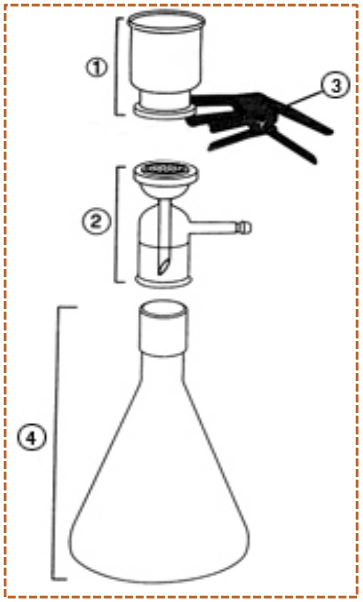
To detect the presence of components as they exit the column

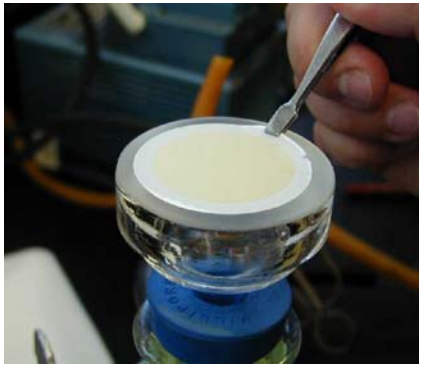
HPLC setup

Mobile phase delivery

- Consists of pump to provide the high pressure required.
- Equipped with one/more glass reservoirs.
- Require very clean & pure HPLC grade solvents to prevent column degradation with impurities & to minimize detector background signals from contaminants (usually UV transparent).
- Solvents must be filtered (to remove dust) through 0.2/0.45 µm filters & 'degassed'.

Membrane Filter Holder Assembly





- Degassing (**removal of dissolved N_2, O_2, CO_2..**) by :
  - ✓ Vacuum devices (vacuum applied to headspace above solvent).
  - ✓ Ultra sonication (high frequency vibration drives gasses out of solvent).
  - ✓ Heating (decreases solubility of gases).
  - ✓ He sparging. Sparging is a process in which dissolved gases are swept out of a solvent.

- If solvents are not degassed, gases that are soluble at high pressure (ie. 4,000 psi) become insoluble at low pressure (ie. at the end of the column or detector).
- Gas bubbles create difficulties with pumps (destabilize pressure), columns (bad separation) & detectors.
- Dissolved O_2 absorb UV radiation in the 250-200 nm wavelength range & interfering with UV detection.

Sample problem 1

Why is high pressure is needed in HPLC?

Elution

Isocratic elution

The composition of the mobile phase remains constant during the separation

- The mobile phase may comprise of a single solvent or,
- Various solvents can be mixed to give mixture with a suitable polarity.

Gradient elution

Solvent composition of the mobile phase is changed with time during the separation

- Binary gradient – 2 solvents (very common).
- Tertiary gradient – 3 solvents (quite common).
- Quaternary gradient – 4 solvents (very rarely).

- E.g. of a gradient :

  Starting at 10% methanol & ending at 90% methanol after 20 minutes.

- The two components of the mobile phase are typically termed "**A**" & "**B**";
  - ✓ **A** is the "weak" solvent which allows the solute to elute only slowly.
  - ✓ **B** is the "strong" solvent which rapidly elutes the solutes from the column.

- Solvent A is often water, while B is an organic solvent miscible with water, such as acetonitrile, methanol, THF or isopropanol.

- Organic solvent of the mobile phase is to reduce the retarding strength of the aqueous component.

- Different compounds are eluted by increasing the strength of the organic solvent.
- The sample is injected while a weaker mobile phase is being applied to the system.
- The strength of the mobile phase is later increased in increments by raising the organic solvent fraction, which subsequently results in elution of retained components.

➢ Advantages :

- ✓ Decreases the retention of the later-eluting components so that they elute faster, giving narrower (& taller) peaks for most components.

- ✓ Improves the peak shape for tailed peaks.

Pumping system

- Pumping system pumps (force) the mobile phase through the manual injector, column, detector & finally to the waste container.
- The quality of a pump for HPLC is measured by how steady & reproducible a flow it can produce.
- A fluctuating flow rate can create detector noise that obscures weak signals.

- Requirements for HPLC :
  - ✓ Pressures up to 6000 psi.
  - ✓ Pulse free, prevents remixing of solutes.
  - ✓ Control flow rate from 0.1 to 10 mL/min.

➢ Reciprocating pump

- ✓ The most used.
- ✓ Mechanical high pressure liquid pumping achieved by piston, check valves.
- ✓ Pressure surges can be somewhat overcome with pulse dampeners.
- ✓ Advantages : Provide constant flow rates & independent of solvent viscosity or column backpressure.

Reciprocating pump

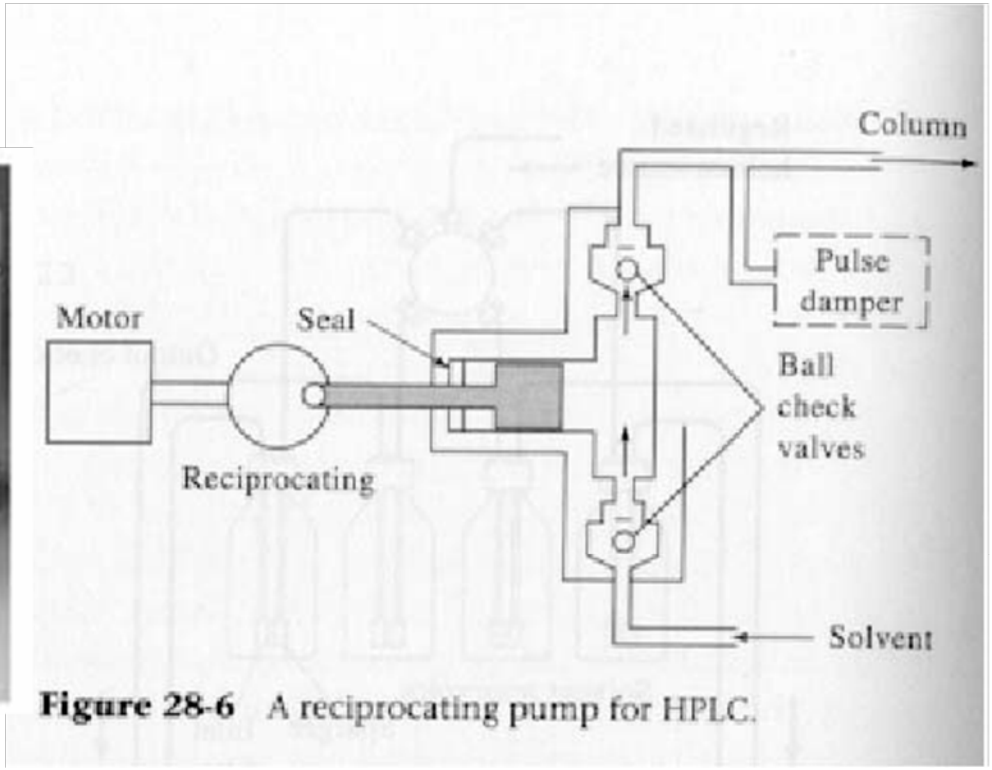


Figure 28-6 A reciprocating pump for HPLC.

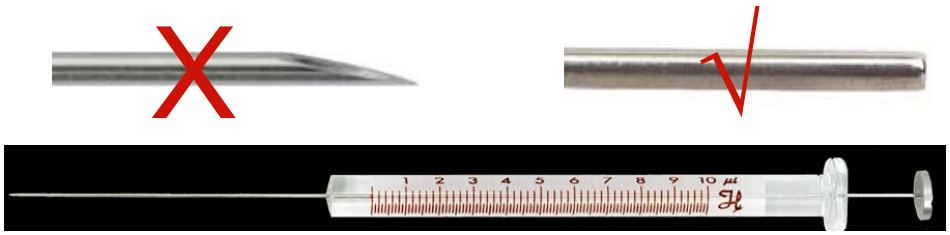
➢ Displacement pump

- ✓ Continuous pressure applied to large volume (syringe pump).
- ✓ Advantage is lack of pressure surges.
- ✓ Disadvantage is price.

Sample injection system

- **Injection port** :
  - ✓ Device for injection of sample into system.
  - ✓ Consists of a stainless steel ring with 6 different ports, 1 to the column.
  - ✓ Has interchangeable sample loops, which hold a fix volume.

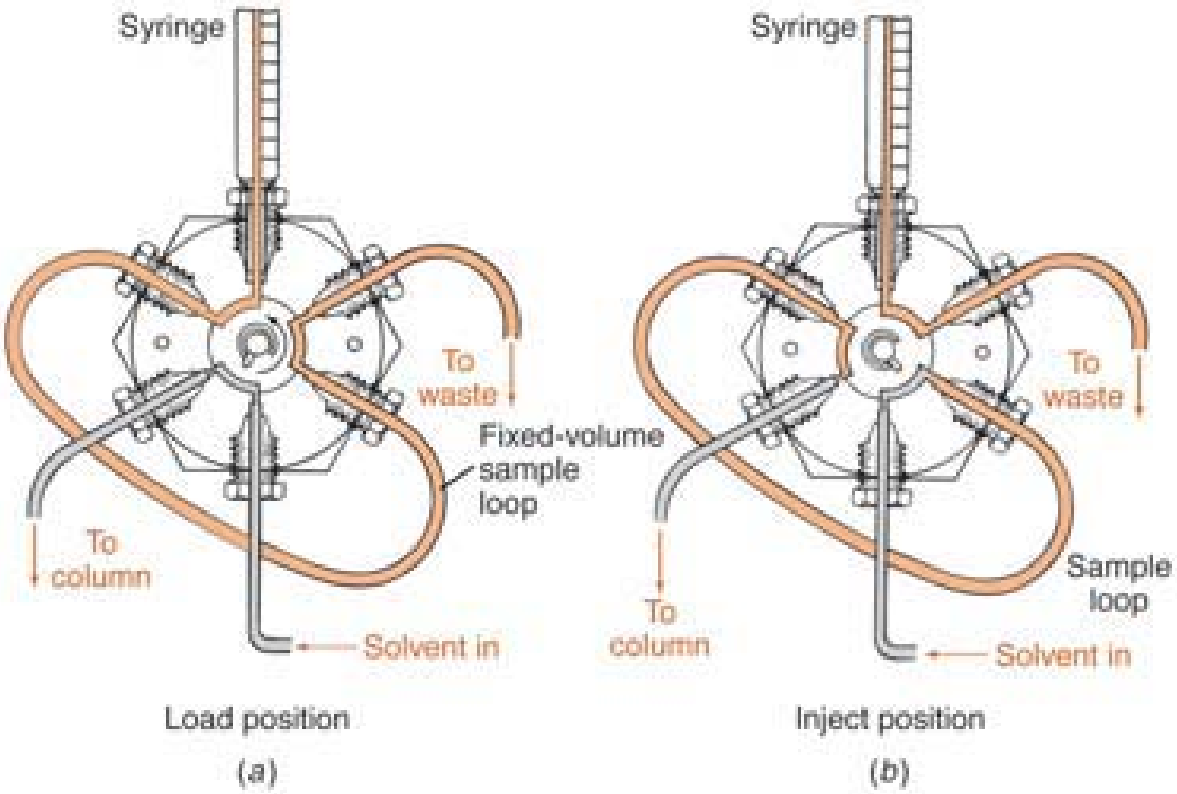
- ✓ Sample volume is no more than volume indicated on a loop.
- ✓ Samples size ranges from 2-1000 µL.
- ✓ Samples introduced manually into the valve with a syringe to fill the sample loop.
- ✓ Sample injection only with *flat end needle* (to prevent damage to the injection port).



Manual injection port

Sample loop

Injection valve



- Injection procedure :

1. Place in ***load position*** & load sample into loop.

2. Rotate to ***inject position*** – the content of the sample loop is injected into the column at high pressure.

Autosamplers

- Can inject a large number of samples automatically.
- Inject continuously variable volume 1 µL–1 mL.
- Small vials with a septum placed in a tray.
- Needle penetrates septum & withdraws the sample.
- Valve introduces the sample into the column.

Columns

1. Guard column
2. Analytical column

Guard column (precolumn)

- 3–10 cm long, placed between the injector & the analytical column.
- Contains the same packing as the analytical column.

- Function : To protect & increase the lifetime of the analytical column by :
  - ✓ Retain debris (eg. pump seal fragments), particulate matter & contaminants that would otherwise get on analytical column & foul/clog it & changing column efficiency.
  - ✓ Retains highly sorbed compounds that would be caught on & not be eluted from the analytical column.
- Must be replaced or regenerated periodically.

Sample problem 2

For the analysis of biological sample using HPLC, a guard column is normally required.

Analytical column

- Stainless steel.
- 10-30 cm long (10,15 & 25 cm).
- 4-10 mm internal diameter (3.9 or 4.6 mm).
- 3, 5 or 10 µm particle size **(d_p)**.
- 40,000-60,000 plates/m.

Eg. 5 µm ODS column (250 x 4.5 mm)

Band Broadening in LC

- van Deemter equation,

$$H = A + B/v + Cv$$

- Which of the 3 components is the largest contribution to H? Consider the following :

  - ✓ **B/v effects** – diffusion is **100x less** in liquids than in the gas & can be neglected.

- ✓ **Cv effects** – we will assume that mass transport (M.T.) effects are the largest contribution.

  - The mobile phase mass transfer coefficient :

$$C_M v = (f_M(k') d_p^2 / D_M) v$$

  - Smaller dp increases the surface area/volume ratio & thus decreases M.T. in the m.p.

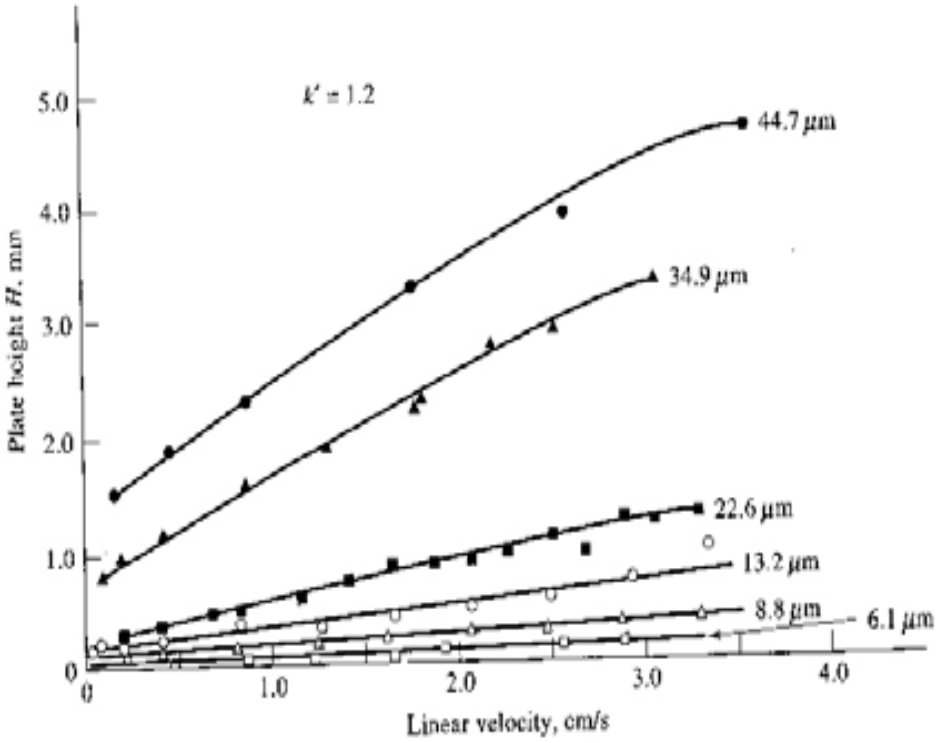


Figure 28-2 Effect of particle size of packing and flow rate upon plate height H in liquid chromatography. Column dimensions: 30 cm × 2.4 mm. Solute: N,N-diethyl-*n*-aminoazobenzene. Mobile phase: mixture of hexane, methylene chloride, isopropyl alcohol. *(From R. E. Majors, J. Chromatogr. Sci., 1973, 11, 92. With permission.)*

Reduction of dp from 45 to 6 µm results in a tenfold or more decrease in H

Sample problem 3

Discuss the effect of the particle size on the separation performance in packed column liquid chromatography.

Stationary phase

- Normally, columns are filled with silica gel because its :
  - ✓ particle shape (spherical, can be packed more homogeneously),
  - ✓ surface properties,
  - ✓ pore size (60-100 Å range)

structure help to get a good separation.

Silica structures

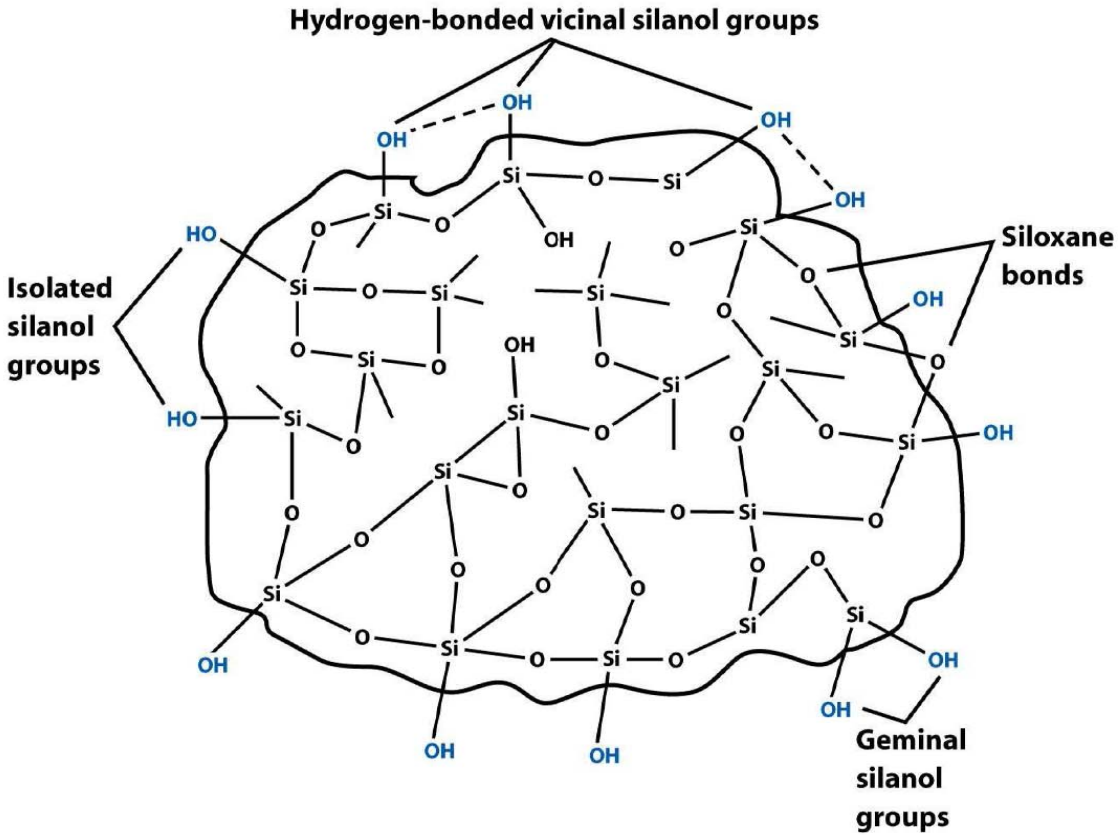


Figure 25-6
Quantitative Chemical Analysis, Seventh Edition

➢ Silica :

- ✓ Is wetted by nearly every potential mobile phase.
- ✓ Is inert to most compounds.
- ✓ Has a high surface activity which can be modified easily with water & other agents.
- ✓ Can be used to separate a wide variety of chemical compounds.

- Limitation of silica stationary phase :

  - X Operating pH range 2-8.
  - X Dissolves above pH 8. Crosslinked polymeric particles (eg., polystyrene / polymethacrylates) are used for separation of bases.
  - X Siloxane bond (Si-O-Si) to the stationary phase hydrolyses below pH 2.
    - These defects will cause changes in t_R & loss of resolution.

Detector types

- UV absorbance
  - ✓ Variable Wavelength UV-Vis detector.
  - ✓ Diode Array Detector (DAD) : measure absorbance across a broad spectrum of wavelengths simultaneously.
- Fluorescence
- Electrochemical
- Refractive Index
- Mass spectrometric

- HPLC detectors will depend on the nature of the sample.

- The most widely used detectors for HPLC are based on the absorption of ultraviolet or visible radiation.

UV region : 100-400 nm
Vis region : 400-780 nm

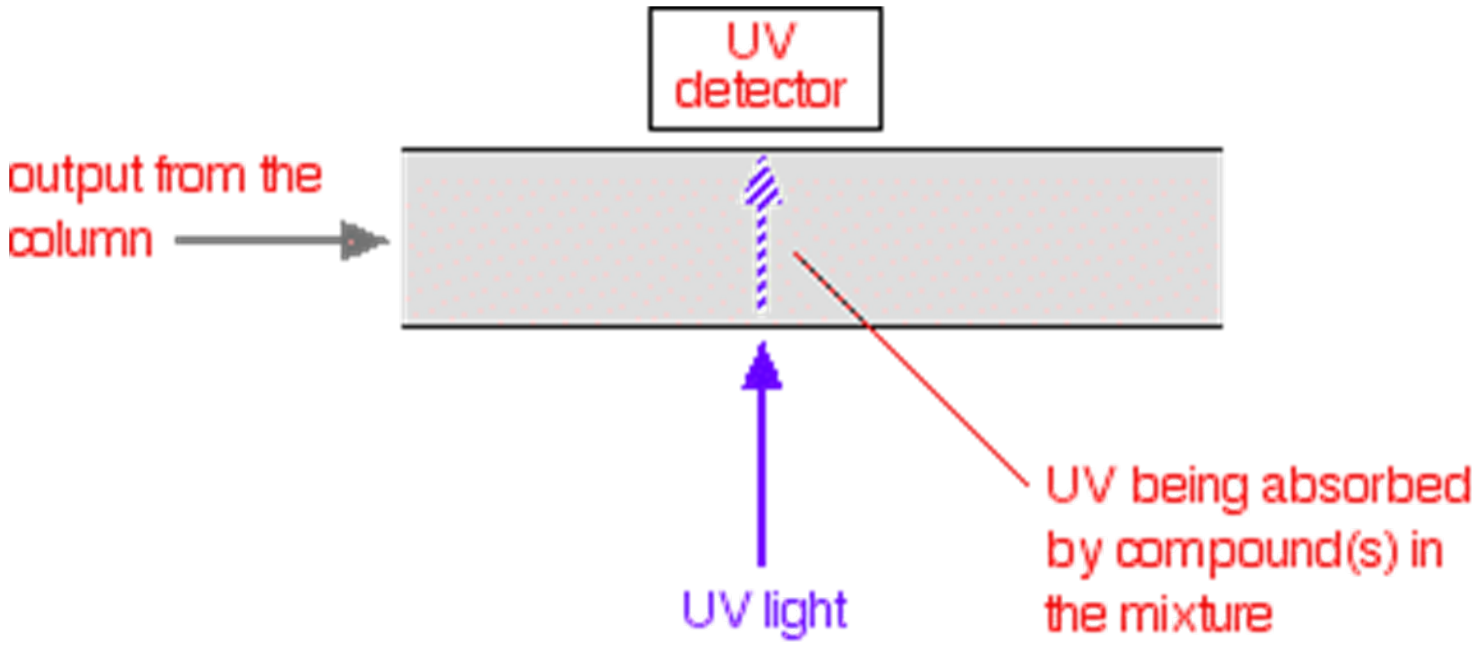
- The detectors often make use of the 254 nm & 280 nm lines from a mercury source because many organic functional groups absorb in the region.

- Usually set the UV at 254 nm. This will detect any π to π* transitions. So, it would detect anything with the benzene ring.

UV-Vis absorbance detector

- Based on electronic transitions within molecules.
- Most common type of detector for LC.
- Fixed wavelength, Hg lamp 254 nm ($\pi \Rightarrow \pi^*$).

A diagram of the UV detector



- The detector sensor consists of a small cylindrical cell (normally 2.0 to 10 µl in volume) through which, the eluent from the column flows.

- UV light from a UV lamp (usually a Hg discharge lamp) emitting UV light mostly at 254 nm.

- Variable wavelength, selectable for specific wavelengths in the visible region (eg. deuterium lamp for use down to 190-400 nm). Still limited to single wavelengths.

- Analyte must absorb in the visible region (has chromophore).

You might wonder why the solvents used don't absorb UV light. They do! But different compounds absorb most strongly in different parts of the UV spectrum.

- Methanol, for eg., absorbs at wavelengths below 205 nm & water below 190 nm.

- If you were using a methanol-water mixture as the solvent, you would therefore have to use a wavelength greater than 205 nm to avoid false readings from the solvent.

UV-Vis absorbance detector
DIODE ARRAY (DAD)

- Measure absorbance across a broad spectrum of wavelengths simultaneously.
- Allows for the recording of the entire spectrum of each solute as it passed through the diode array detector.
- Utilizes a deuterium or xenon lamp that emits light over the UV spectrum range.

Sample problem 4

A sample mixture containing acetophenone, phenol, 1-hydroxyphenol & methyl benzene was analyzed using HPLC with a mobile phase system of a ACN/H_2O 80/20 & a UV detector set at wavelength 420 nm.

Quantitative analysis was done using external standard method. Discuss the reason for each of the following problems encountered & suggest an approach to overcome the problem.

i. Very low detection signals were obtained.

ii. After doing 5 replicate measurements, the peak areas obtained vary considerably.

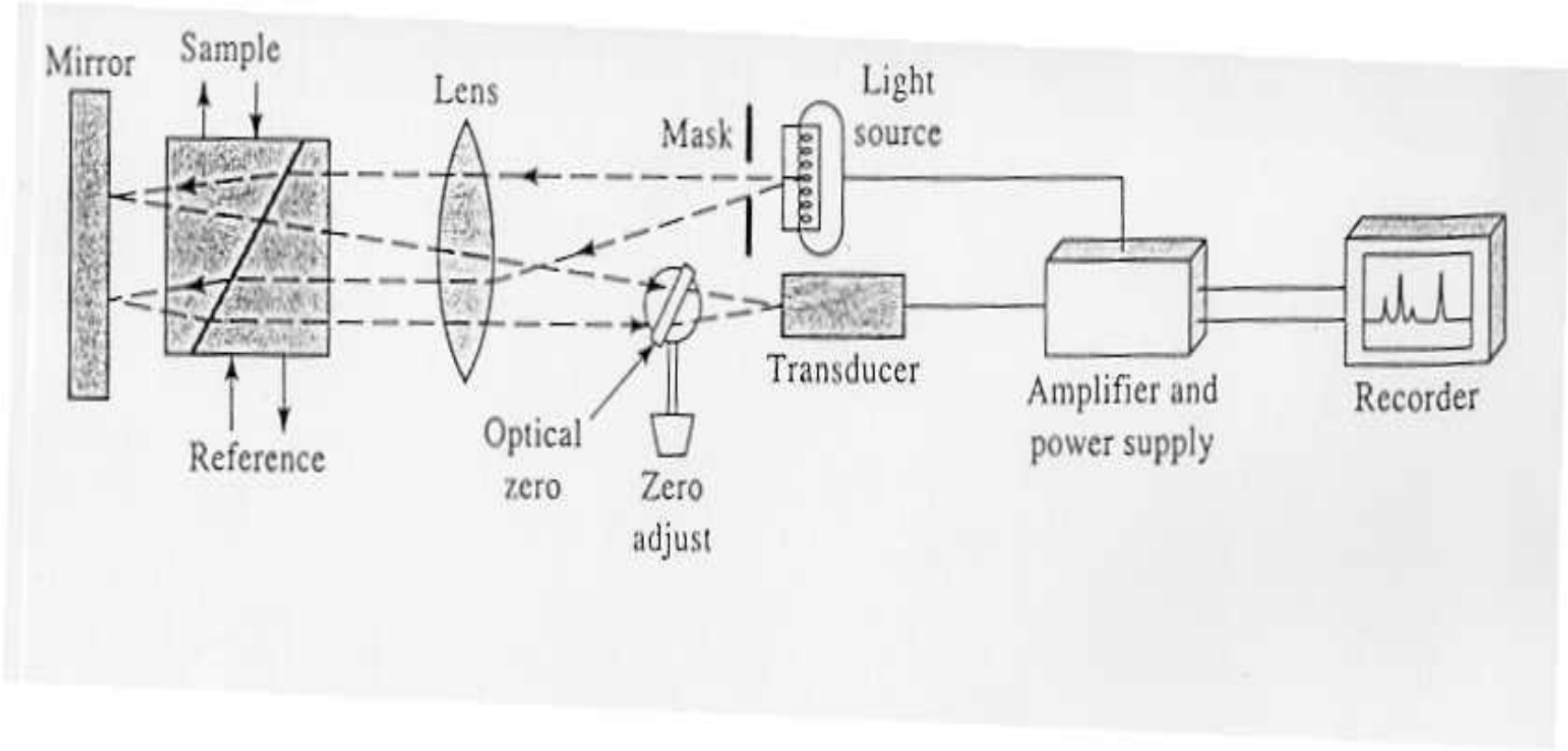
Refractive Index detector

- Based on the n, index of refraction. How much the light is bent in a substance is unique for all liquids & relative to their density.

- Advantages :
  - ✓ universal, insensitive to flow, economical, robust.

- Disadvantages :
  - X not sensitive, highly temperature dependent

Refractive Index detector



Other detectors

- **Fluorescence** :

  The most sensitive, 10-1000 times higher than that of UV detector for strong UV absorbing materials.

- **Electrochemical** :

  Based on the measurements of the current resulting from oxidation/reduction reaction of the analyte at a suitable electrode.

- **Conductivity** :

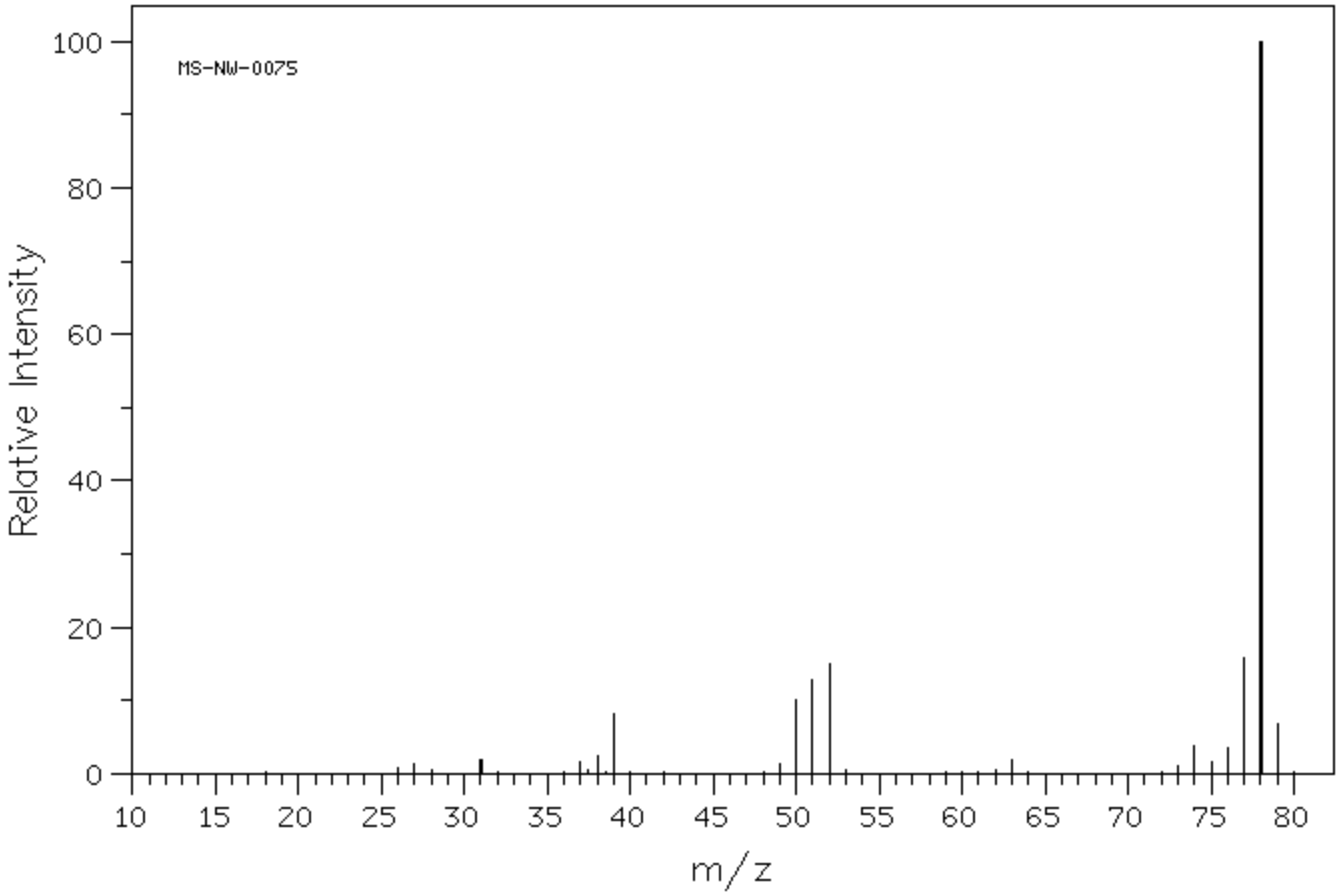
  The conductivity of the column effluent is continuously measured & the appearance of the analyte in the cell is indicated by a change in conductivity.

- **Mass spectrometry (MS)**:

  Examining a compound (in a gas phase), that will provide the structural information of each compounds match from the library of mass spectra of known compounds stored on the computer & thus can identify the compound.

  Measures the mass-to-charge-ratio (m/z) of charged analytes.

Eg. mass spectrum for benzene



It is not a simple matter to connect the MS to LC, because

- ✓ LC operates in the liquid phase whereas MS is a gas phase method.
- ✓ Analytes are non volatile & may be thermally labile but must be presented in gaseous form.

In order to allow separated components of a mixture to be passed sequentially from the LC into the MS, an interface is needed.

Sample problem 5

Identification of compound is more confirmative by using mass spectrometer as a detector compare to UV detector.

Sample problem 6

Explain the advantage of using MS detector.

Performance of HPLC detectors

HPLC detector	LOD
Absorbance	10 pg
Fluorescence	10 fg
Electrochemical	100 pg
Refractive index	1 ng
Conductivity	100 pg – 1 ng
Mass spectrometry	< 1 pg

Sample derivatization

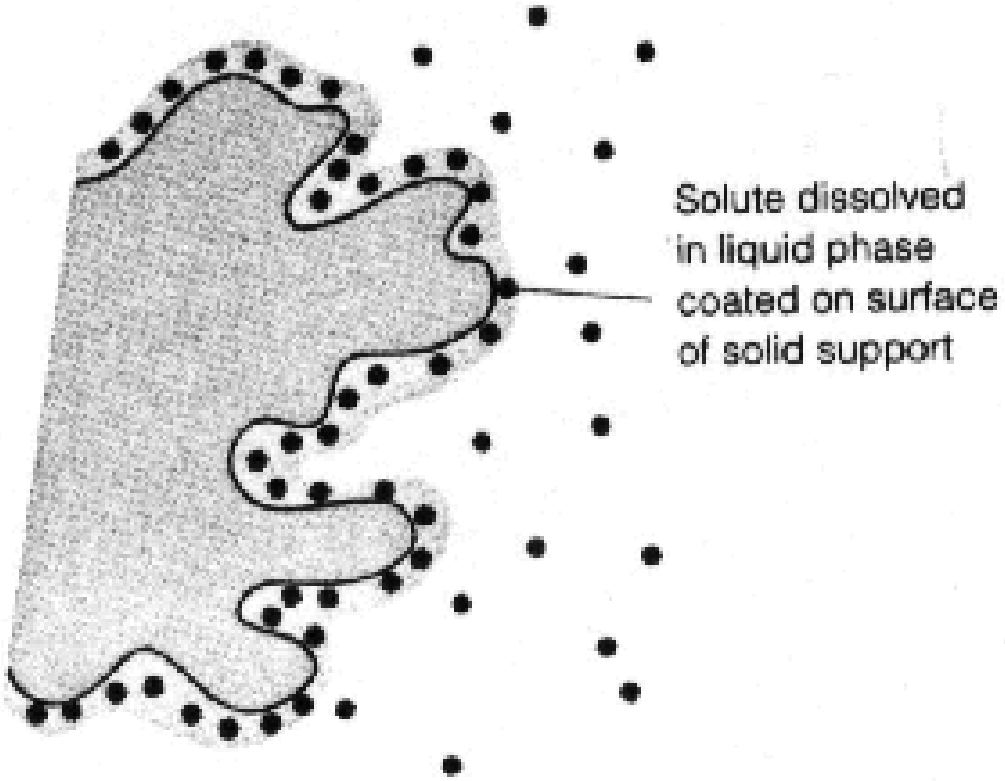
- Chemical derivatization of a sample in HPLC is undertaken to convert a non-UV/fluorescence responding analyte into one that is.
- The derivatization step may enhance the separation.

Types of liquid chromatography

1. Partition chromatography
   (separation based on polarity)
2. Adsorption chromatography
3. Size exclusion chromatography
   (separation based on molecular size)
4. Ion exchange chromatography
   (separation based on charge)

Partition chromatography

- Is a chromatographic technique in which solute are separated based on their partition between a liquid mobile phase & a liquid stationary phase coated on a solid support.



Partition chromatography

- The most widely used type of HPLC.
- Subdivided into :
  1. Liquid-liquid partition chromatography.
  2. Liquid-bonded phase chromatography.

1. Liquid-liquid partition chromatography

- ✓ The stationary phase is a solvent that is held in place by adsorption on the surface of packing particles.
- ✓ The liquid coat might itself be soluble in the mobile phase which will cause the column to **"bleed"**.
- ✓ To prevent loss of stationary phase, liquid-bonded-phase partition chromatography is used.

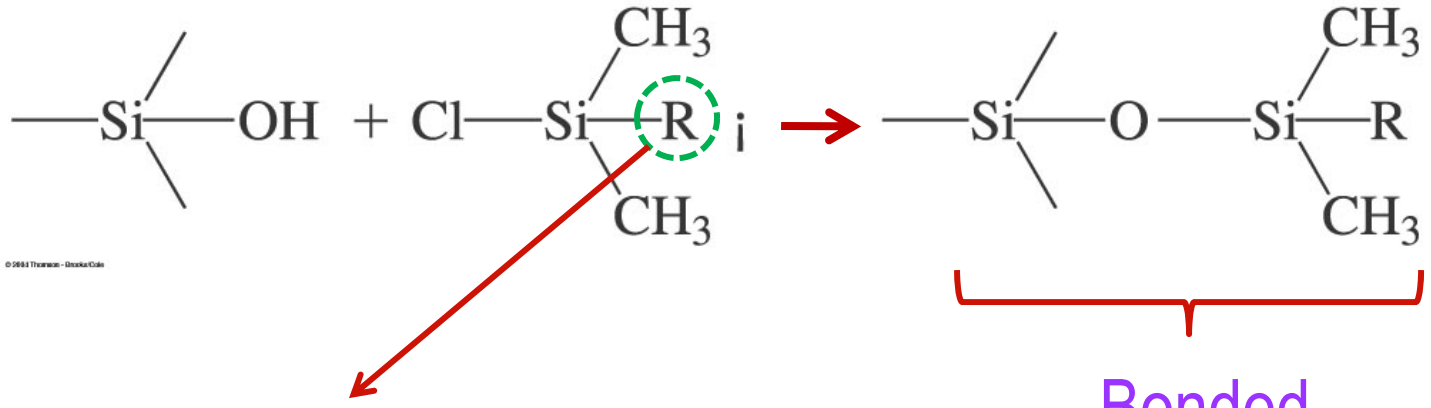
2. Liquid-bonded-phase partition chromatography

- ✓ The stationary phase is an organic species that is attached to the surface of the packing particles by chemical bonds.

Bonded phase packings

- Most bonded phase packings are prepared by reaction of an organochlorosilane with the –OH groups formed on the surface of silica particles by hydrolysis in hot, dilute hydrochloric acid.

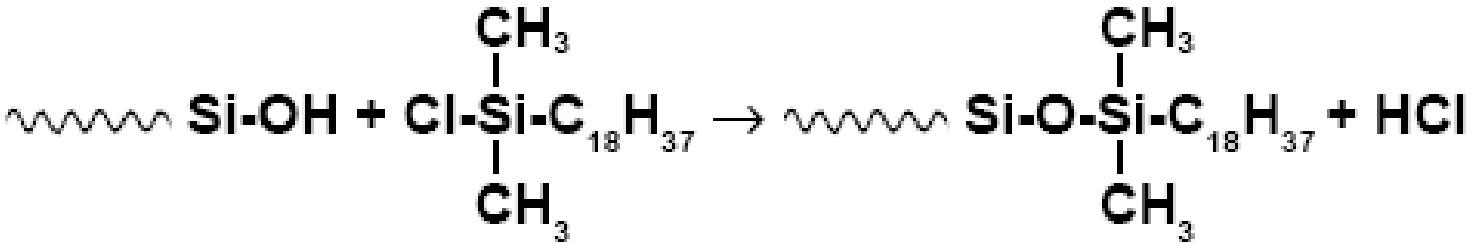
- The product is an organosiloxane.



Alkyl / substituted alkyl group

Bonded stationary phase

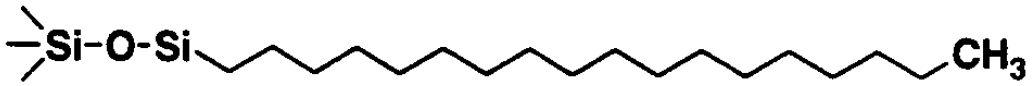
- The reaction for one such SiOH to produce C18 :

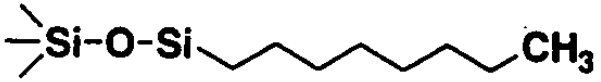


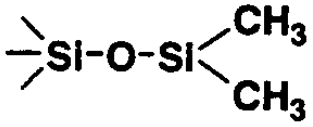
R	Structure	Polarity
Octadecyl Octyl Phenyl	$(CH_2)_{17}CH_3$ (C18) $(CH_2)_7CH_3$ (C8) $(CH_2)_3C_6H_5$	**NON POLAR**
Amino Cyano Diol	$(CH_2)_3NH_2$ $(CH_2)_3C\equiv N$ $(CH_2)_2OCH_2CH(OH)CH_2(OH)$	**POLAR**

Many different polarities for the bonded stationary phase are available

REVERSED PHASE

Octadecyl - 

Octyl - 

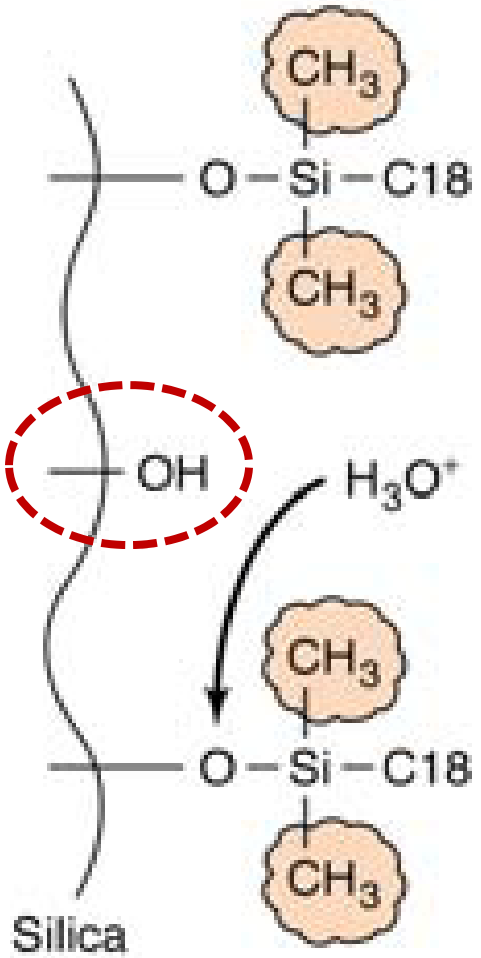
Dimethyl - 

NORMAL PHASE

Cyanopropyl - 

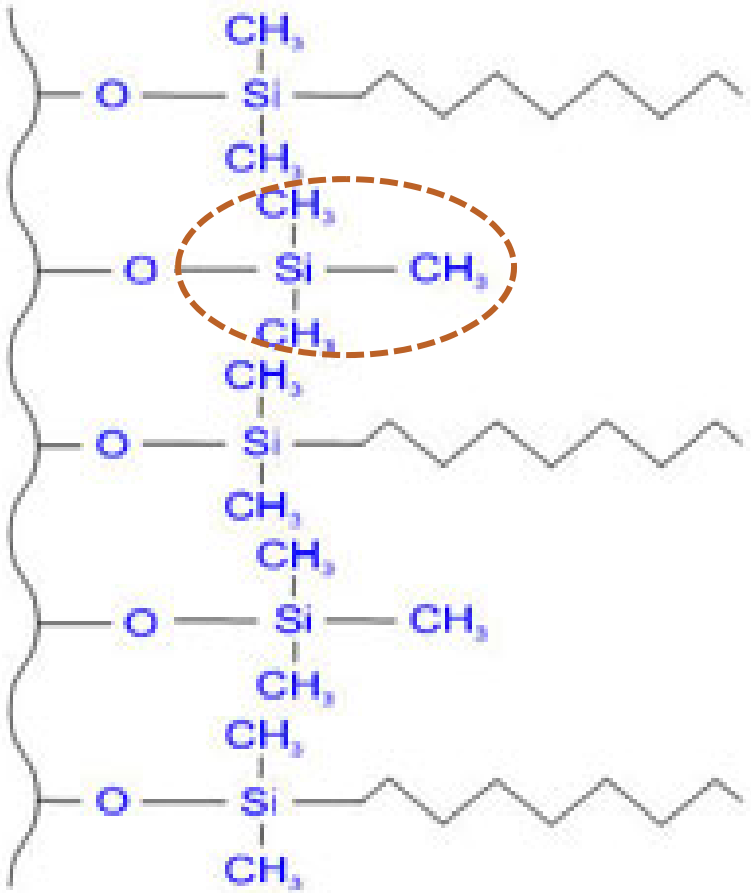
Aminopropyl - 

- It is not possible to bond all the surface silanol (Si-OH) groups.
- Unreacted silanols are capable of **adsorbing polar molecules**.



- Can be reduced by "**end-capping**" process :

  Silanols are reacted with a silylating agent e.g. trimethylchlorosilane.



- 2 types of partition chromatography are distinguishable based on the relative polarities of the mobile & stationary phases.

    1. **Normal Phase**
    2. **Reversed Phase**

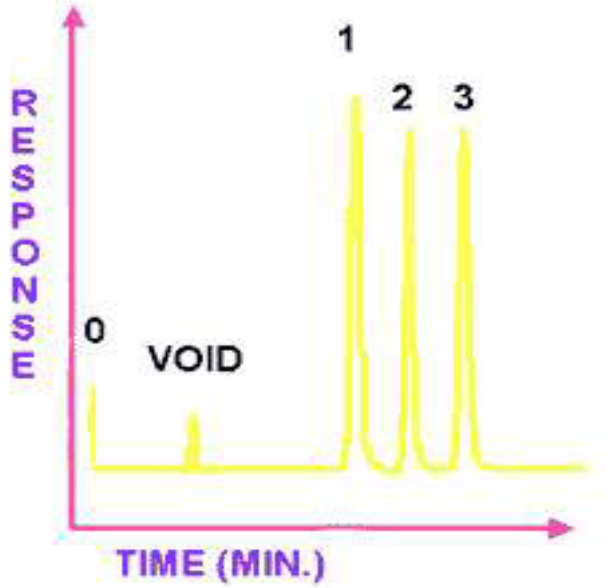
Partition HPLC : Normal Phase

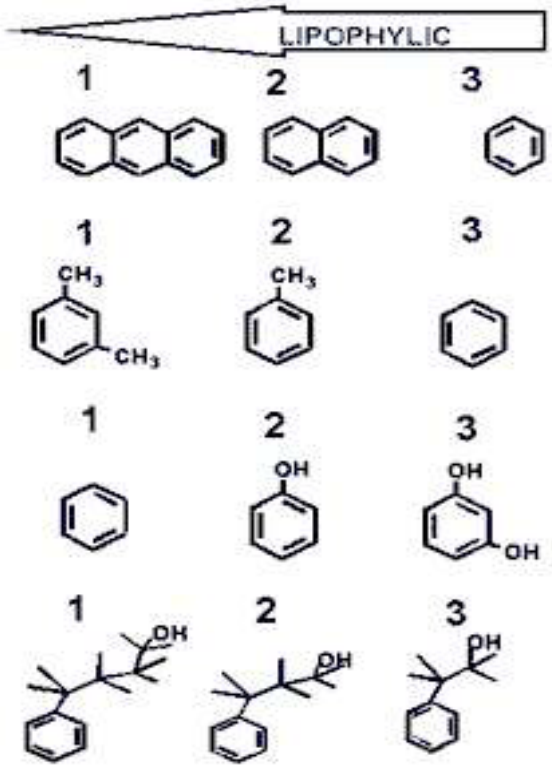
- The stationary phase is a bonded siloxane with a polar functional group (e.g. cyano < diol < amino < dimethylamino).
- The mobile phase is non-polar (e.g. hexane, methylene chloride or chloroform).
- Eluent strength is increased by adding a more polar solvent (acetone, diethyl ether).
- These phases retain polar comps in preference to non polar comps.

- **Non-polar molecules** interact least with stationary phase --> **ELUTED FIRST**.

- **Polar molecules**- retained by stationary phase --> **ELUTED LATER**.

Example :


ELUTION ORDER IN NORMAL PHASE
RESPONSE
0
VOID
1
2
3
TIME (MIN.)


LIPOPHYLIC
1
2
3
CH3
OH

Sample problem 7

i. Discuss the relative polarity of the compounds in the above analysis.

Partition HPLC : Reversed phase (RP)

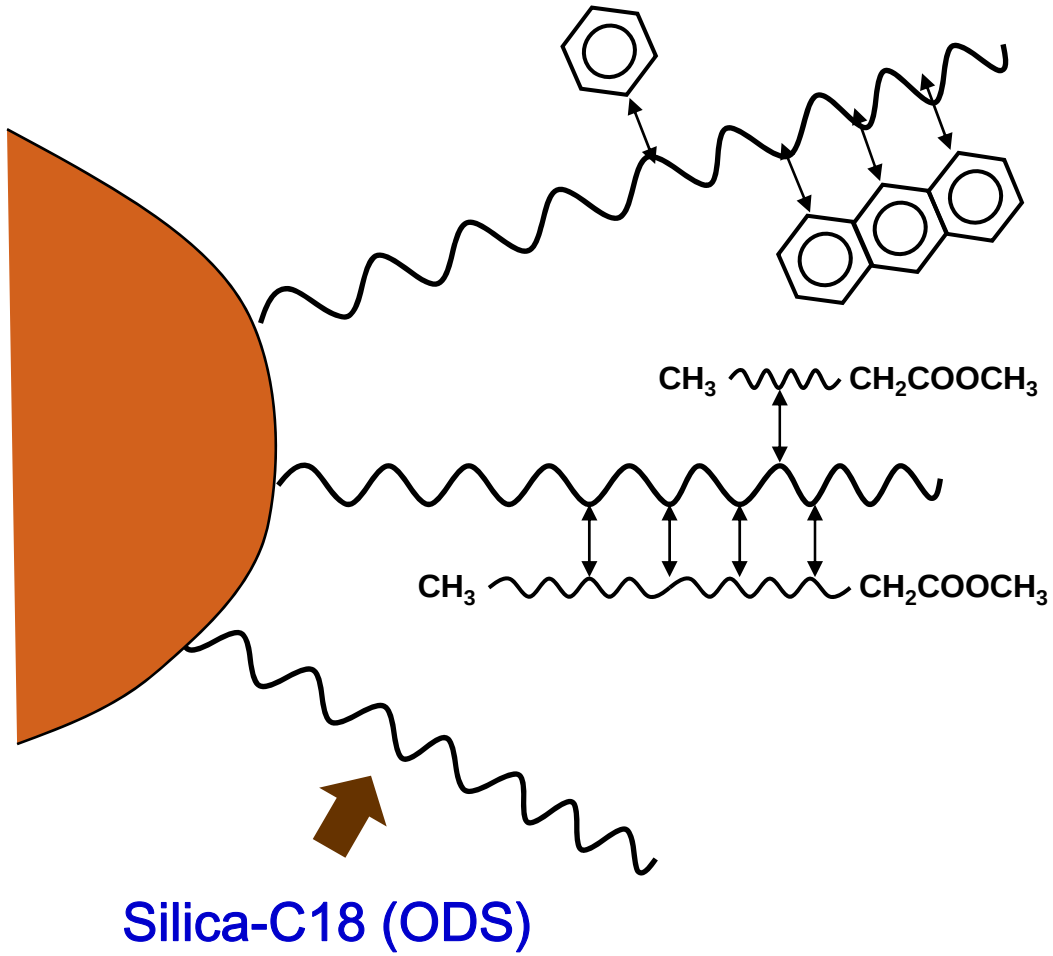
- The polarity of stationary phase is reversed (non-polar; C18 > C8 > phenyl groups).
- The mobile phase is polar (H_2O, methanol, acetonitrile, THF or usually is a mixture of H_2O with one of the organic solvents).
- The organic solvent is called the modifier.

- Metanol is used for acidic compounds.
- Acetonitrile for basic compounds.
- Tetrahydrofuran for those with large dipoles.
- Eluent strength is increased by adding a less polar solvent.

A **less polar solvent** has a **higher eluent strength**

- Hydrophobic groups (C18, C8, phenyl group) have to be chemically bonded on to matrix.
- Octadecylsilane (ODS or $C_{18}H_{37}$ or C18 chain) widely used.
- Gives non-polar (hydrophobic) interaction with sample molecules & eluents.

Hydrophobic interaction



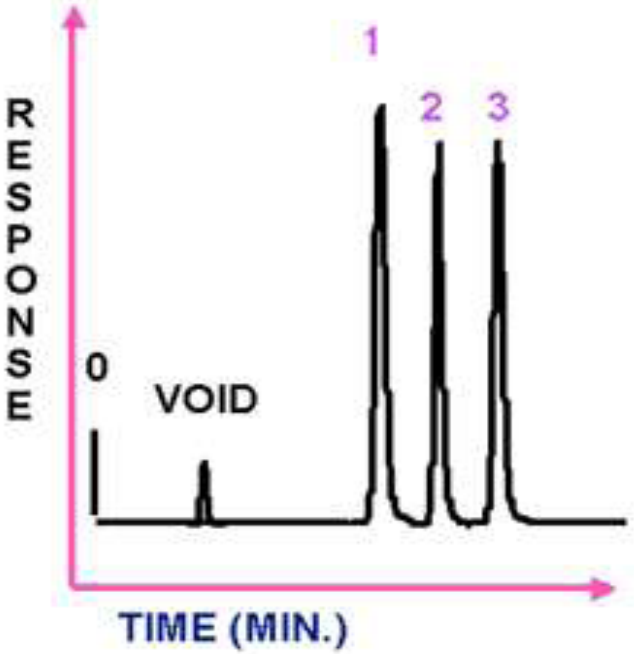
- In RP chromatography,

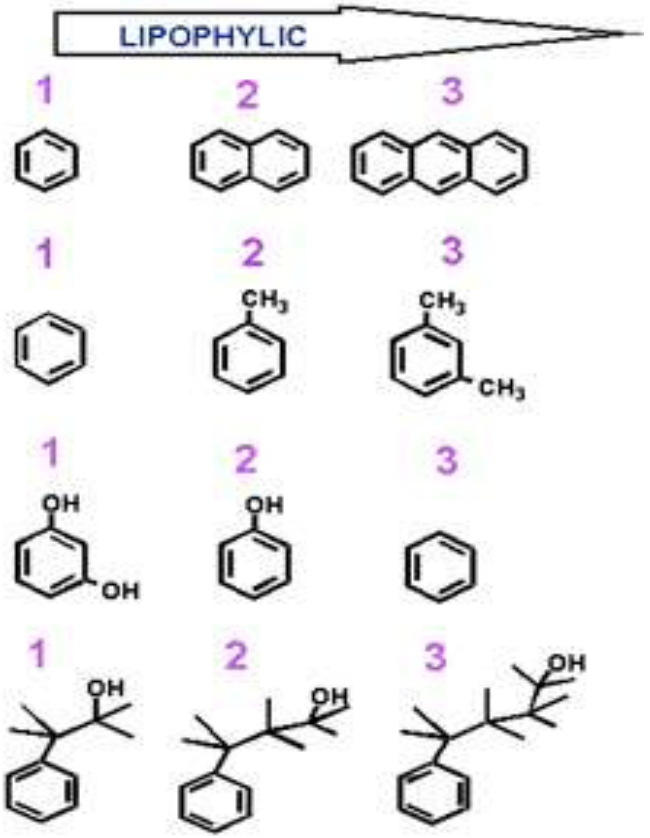
Polar sample molecules - interact least with stationary phase --> **ELUTED FIRST**.

Non-polar - retained by stationary phase -> **ELUTED LATER**.

These phases retain **non polar comps in preference to polar comps**

ELUTION ORDER IN REVERSED PHASE





- ✓ The most polar component is eluted first; increasing the polarity of the mobile phase increases the elution time.

- ✓ The affinity of the hydrophobic analyte for the hydrophobic stationary phase stronger relative to the now more hydrophilic (polar) mobile phase.

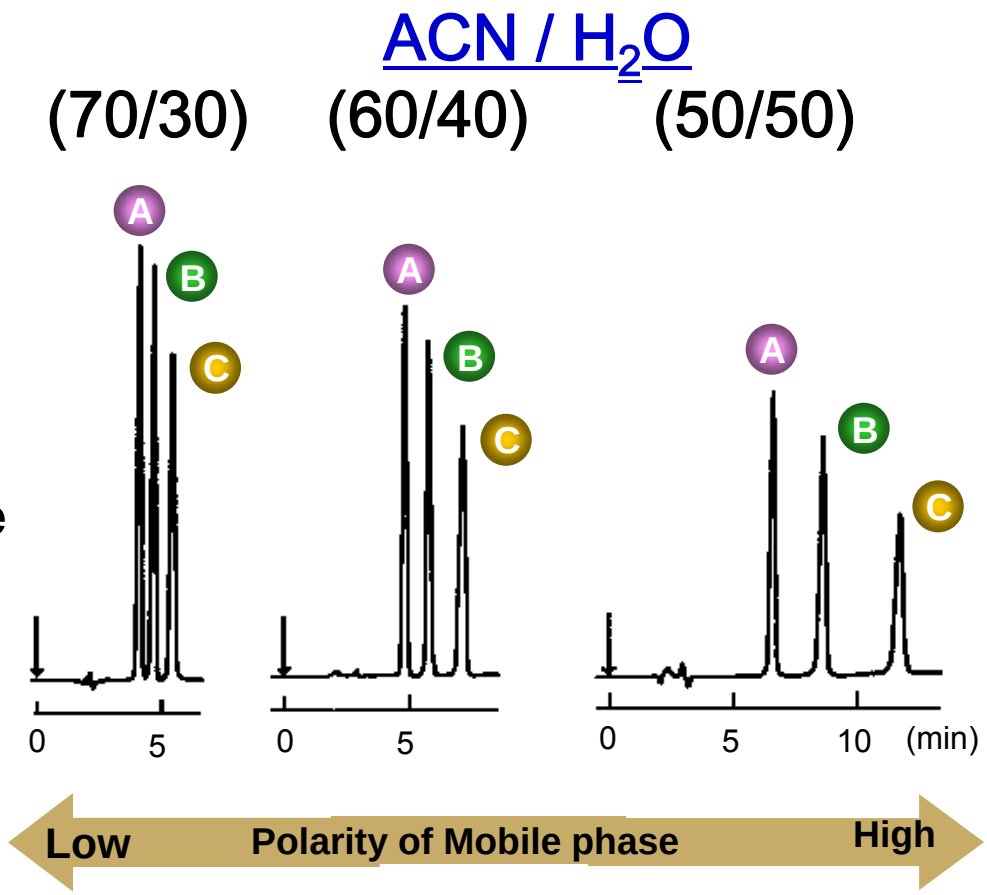
- ✓ Can decrease t_R by adding more organic solvent to the eluent.

Retention behavior in RP HPLC

Carbon chain length of sample

A < B < C

A : p-Hydroxy ethyl benzoate
B : p-Hydroxy propyl benzoate
C : p-Hydroxy butyl benzoate



Column : Finepak SIL C18

- In normal phase chromatography :

  - ✓ The least polar component is eluted first ; increasing the polarity of the mobile phase **decreases the elution time**.

Solvents polarity

water > methanol > acetonitrile > ethanol > propanol = ethyl acetate > tetrahydrofuran > butanol > chloroform > dichloromethane > **n-hexane > cyclohexane**

Polar solvents

Non-polar Solvents

Polarities of organic functional groups

Hydrocarbons < olefins < aromatic hydrocarbons < halides < sulfides < ethers < nitro compounds < esters ~ aldehydes ~ ketones < alcohols ~ amines < sulfones < sulfoxides < amides < carboxylic acids < waters

Partition Chromatography :

Method Development

(To obtain adequate separation in a reasonable time)

1. Column selection :

- **Effect of stationary phase**

  The polarity of the stationary phase is matched roughly with that of the analytes, a mobile phase of considerably different polarity is then used for elution.

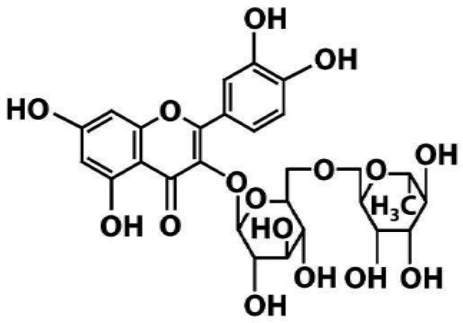
Choosing a stationary phase

Table 25-5 Selection of bonded stationary phases for HPLC

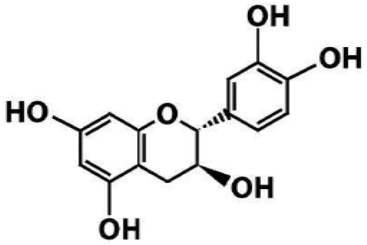
Bonded group	Polarity	Retention mechanisms	Comments
C_{18}, C_8, C_4	Nonpolar	van der Waals	C_8 does not retain hydrophobic compounds as strongly as C_{18}
Phenyl	Nonpolar	Hydrophobic and pi-pi	
Cyano	Intermediate	Hydrophobic, dipole-dipole, and pi-pi	Resolves polar organic compounds by reversed-phase or normal-phase chromatography
Amino	Polar ($-NH_2$) or ionic ($-NH_3^+$)	Dipole-dipole and H-bonding	Normal-phase or ion-exchange separations; separates carbohydrates, polar organic compounds, and inorganic ions; reacts with aldehydes and ketones
Bare silica	Very polar	H-bonding	Normal-phase separations

SOURCE: C. S. Young and R. J. Weigand, "An Efficient Approach to Column Selection in HPLC Method Development," LCGC **2002**, *20, 464.*

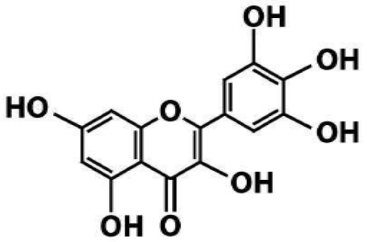
Table 25-5
Quantitative Chemical Analysis, Seventh Edition



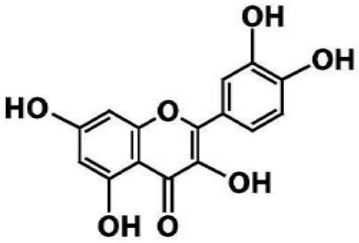
1. Rutin



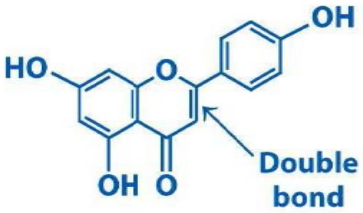
2. Chatechin



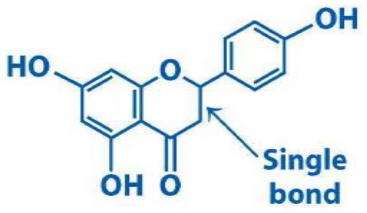
3. Myricetin



4. Quercitin



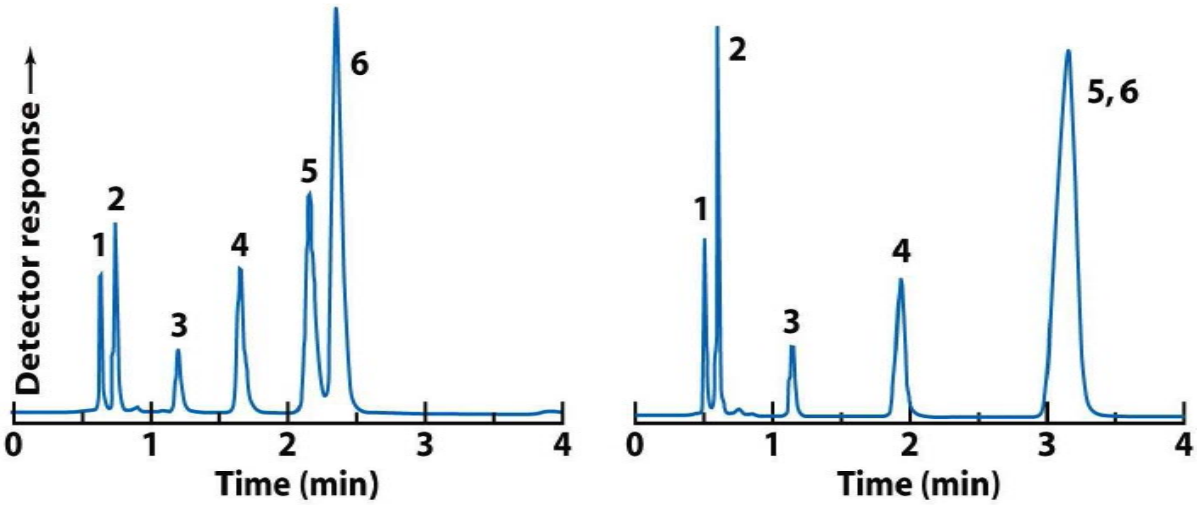
5. Apigenin



6. Naringenin

Compounds with the most similar structures are likely to be hardest to separate. Of the comps in the mixture, 5 & 6 are the most similar, differing by just one C=C bond.

Different packing materials & retention time



(a) Phenyl column (b) C18 column

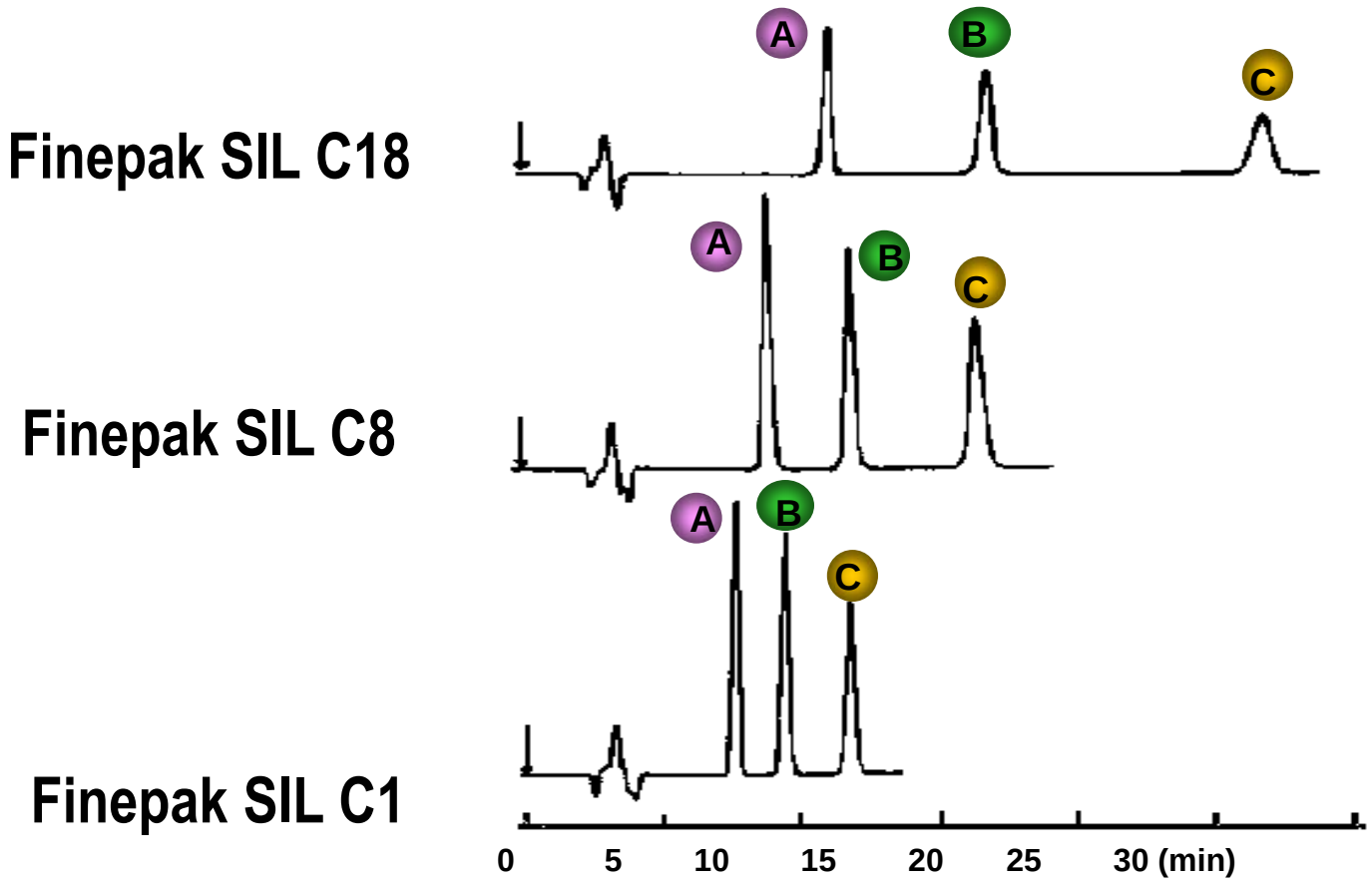
5 & 6 are resolved by a phenyl column not by a C18 column

Effect of chain length :

In RP column, **longer chain length** produce packing that are **more retentive** (more non polar),

- **thus longer t_R for non polar compounds.**

Length of packing materials carbon chains & t_R



Sample problem 8

A mixture of alcohol compounds was analyzed on a 25 cm C18 column. The mobile phase contained a mixture of 30/70% : MeOH/water. Explain how the t_R would be affected if :

The C18 column is changed to a phenyl bonded phase column.

Once a column is chosen, 2 factors to achieve efficient separations with baseline resolution (**R_s > 1.5**) :

- ✓ **Retention @ capacity factor, *k*' (a polarity term)**
- ✓ **Separation factor, α (a selectivity term)**

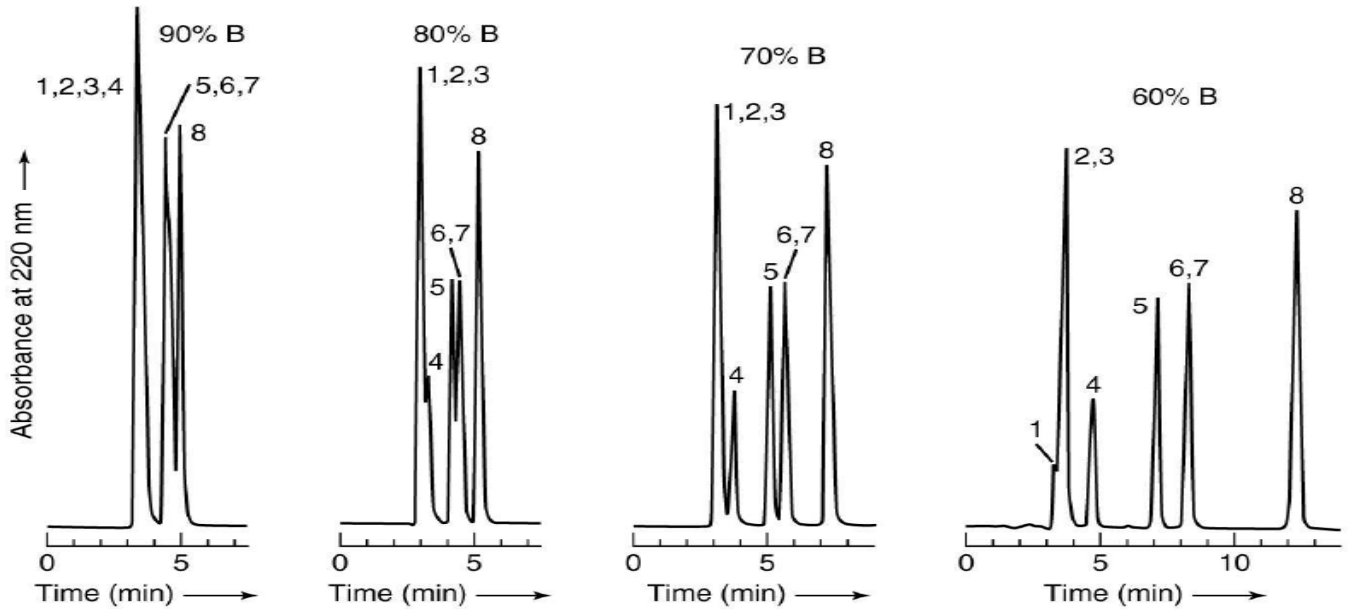
is based upon choice of mobile phase, especially the polarity.

Retention @ capacity factor, *k'*

- ✓ *k'* used to described the migration rate of solutes on column.
- ✓ Reasonable separation, *k'* = 0.5-20 (provide adequate resolution for many comps).
- ✓ *k'* too small, the first peak is distorted by the solvent front.
- ✓ *k'* too great, the run takes too long.

$$k'_A = \frac{(t_R)_A - t_M}{t_M}$$

E.g. : Isocratic separation of 8 aromatic compounds by RP HPLC



t_M is the time when the solvent front is observed near 3 min

- ✓ If a solvent front not observe, we can estimate :

$$V_M \sim \frac{Ld_c^2}{2} \quad \text{or equivalently,} \quad t_M \sim \frac{Ld_c^2}{2F}$$

L = length of the column (cm)
d_c = column diameter (cm)
F = flow rate (mL/min)

Sample problem 9

A mixture of 4 relatively non polar drugs was analyzed on a 25 cm C18 column (3 µm particle size). The mobile phase contained a mixture of 40% MeOH & 60% water. A flow rate of 0.8 mL/min was used. The column dead volume was measured to be 1.5 mL/min.

Calculate the elution time (in min) of a non-retained solute.

Selectivity factor, α

- ✓ **α** described the separation of 2 species (A & B) on the column.

$$\alpha = \frac{K_B}{K_A}$$

$$\alpha = \frac{k'_B}{k'_A} \quad \text{(Retention factor)}$$

$$k'_A = \frac{(t_R)_A - t_M}{t_M} \quad and \quad k'_B = \frac{(t_R)_B - t_M}{t_M}$$

$$\alpha = \frac{(t_R)_B - t_M}{(t_R)_A - t_M} \quad \text{(Retention time)}$$

2. Effect of solvent strength :

- Solvent that interact strongly with solutes are termed '**strong solvent**' or '**polar solvents**'.

- In RP HPLC, **water is the weakest solvent**, but the **most polar solvent**.

- The polarity of the mobile phase can be changed by :
  - ✓ Mixing solvents that have different **polarity index (P')**.
  - ✓ ***k'* gets larger** with increase in P' for RP chromatography.
  - ✓ ***k'* gets smaller** with increase in P' for normal phase chromatography.

Solvent	Refractive Index	Viscosity (cP)	Boiling Point (°C)	Polarity Index (P)	Eluent Strength (ε^o)
Fluoroalkanes	1.27-1.29	0.4-2.6	50-174	**<-2**	**-0.25**
cyclohexane	1.423	0.90	81	**0.04**	**-0.2**
N-hexane	1.327	0.30	69	**0.1**	**0.01**
1-chlorobutane	1.400	0.42	78	**1.0**	**0.26**
Carbon tetrachloride	1.457	0.90	77	**1.6**	**0.18**
i-propyl ether	1.365	0.38	68	**2.4**	**0.28**
toluene	1.494	0.55	110	**2.4**	**0.29**
Diethyl ether	1.350	0.24	35	**2.8**	**0.38**
tetrahydrofuran	1.405	0.46	66	**4.0**	**0.57**
chloroform	1.443	0.53	61	**4.1**	**0.40**
ethanol	1.359	1.08	78	**4.3**	**0.88**
Ethyl acetate	1.370	0.43	77	**4.4**	**0.58**
dioxane	1.420	1.2	101	**4.8**	**0.56**
methanol	1.326	0.54	65	**5.1**	**0.95**
acetonitrile	1.341	0.34	82	**5.8**	**0.65**
nitromethane	1.380	0.61	101	**6.0**	**0.64**
Ethylene glycol	1.431	16.5	182	**6.9**	**1.11**
water	1.333	0.89	100	**10.2**	**large**

- Effect of solvent on selectivities :

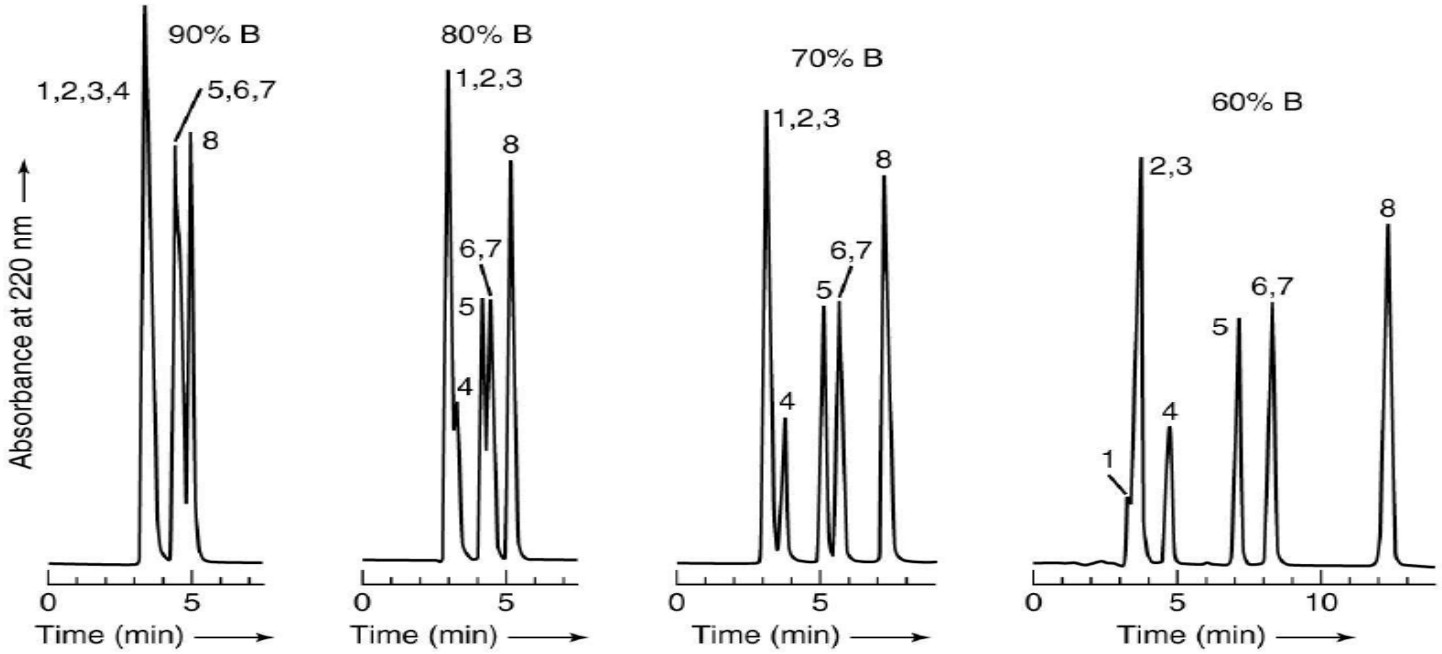
  - ✓ To optimize separations, first determine **required polarity** to get *k*' in correct range.

  - ✓ Then, systematically try **different solvents**, keeping total polarity (& thus *k*') the same.

Isocratic elution

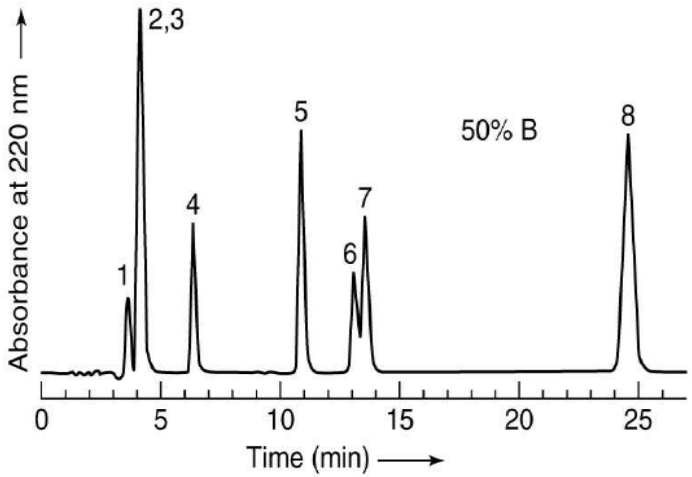
- Various solvents can be mixed to give mixture with a suitable polarity for the expt, which depend on the ratio of the solvents combined to form the solvent mixture.

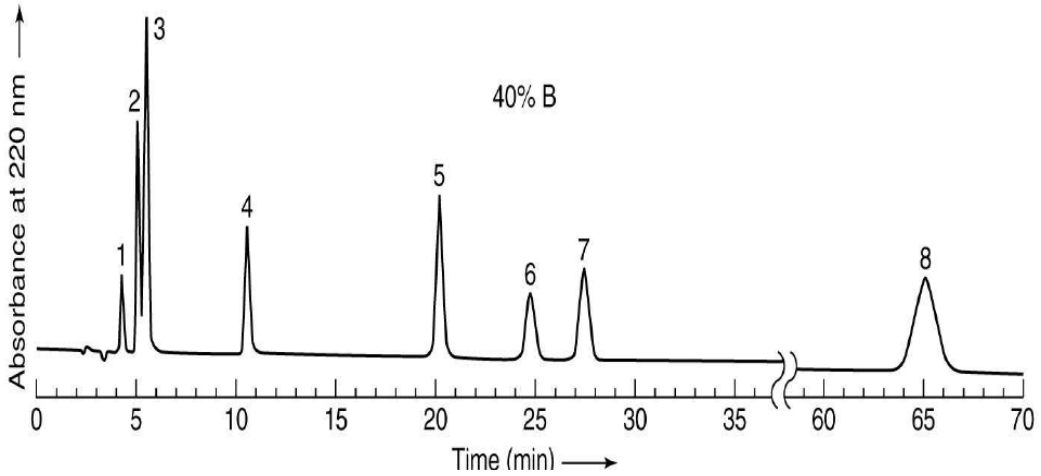
E.g. : Isocratic separation of 8 aromatic compounds by RP HPLC

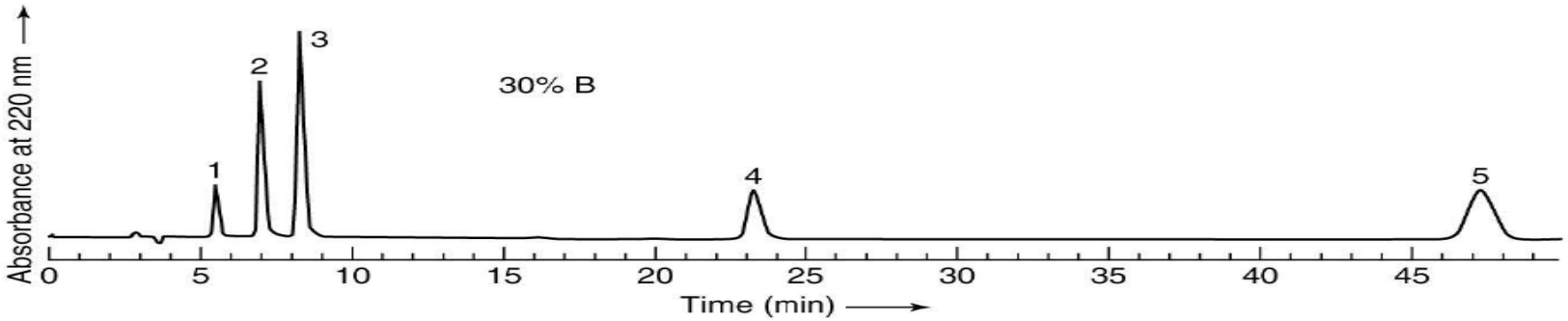


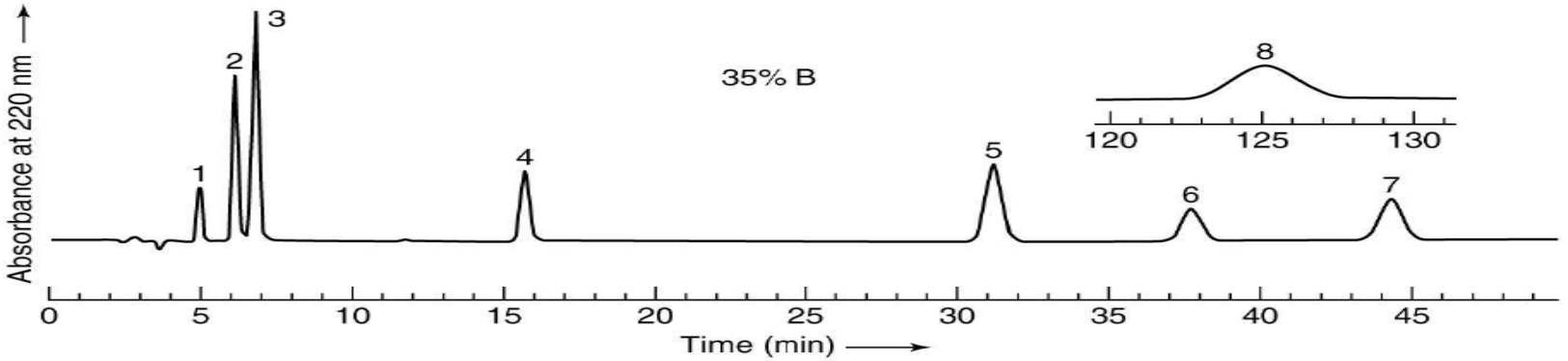
Solvent A – aqueous buffer

Solvent B – acetonitrile


Absorbance at 220 nm
2,3
1
4
5
6
7
8
50% B
0
5
10
15
20
25
Time (min)


Absorbance at 220 nm
3
2
1
4
5
6
7
8
40% B
0
5
10
15
20
25
30
35
60
65
70
Time (min)


Absorbance at 220 nm
3
2
1
4
5
30% B
0
5
10
15
20
25
30
35
40
45
Time (min)


Absorbance at 220 nm
3
2
1
4
5
6
7
8
35% B
120
125
130
0
5
10
15
20
25
30
35
40
45
Time (min)

- ✓ The initial expt was done at high concentration of ACN (90% B) to ensure the elution all components.
- ✓ Then B was successfully lowered to separate all the components.
- ✓ Eluent containing 40% B did not separate peak 2 & 3.
- ✓ 30% B took too long to elute peak 8.

- ✓ Therefore, 35 % B was selected.
- ✓ With 35% B, peak 1 is elute at 4.9 min & peak 8 eluted at 125 min.

- ✓ t_M = 2.7 min
- ✓ Peak 1, *k*' = 4.9 - 2.7/2.7 = **0.8**
- ✓ Peak 8, *k*' = 125 - 2.7/2.7 = **45**
- ✓ For ***k*' > 20**, gradient elution is indicated.

- General elution problem :
  - ✓ For a complex mixture, isocratic conditions can often be found to produce adequate separation of early-eluting peaks or late-eluting peaks, but not both.

- Based on the observations of the above isocratic runs, a gradient program for decreasing separation time by changing mobile phase composition over time during the separation can be used.

Gradient elution

- Used in case of general elution problem :
  - ✓ Mixtures of compounds with a *wide range of polarities*.
  - ✓ To move strongly retained components of the mixture faster, but having the least retained components well resolved.

- E.g., in RP,
  - start with *low solvent strength* (high polarity of mobile phase) to separate the least retained comps &
  - gradually *increase the solvent strength* to elute faster the retained comps (less polar comps).

- E.g. of a gradient :

  Starting at 10% methanol & ending at 90% methanol after 20 minutes.

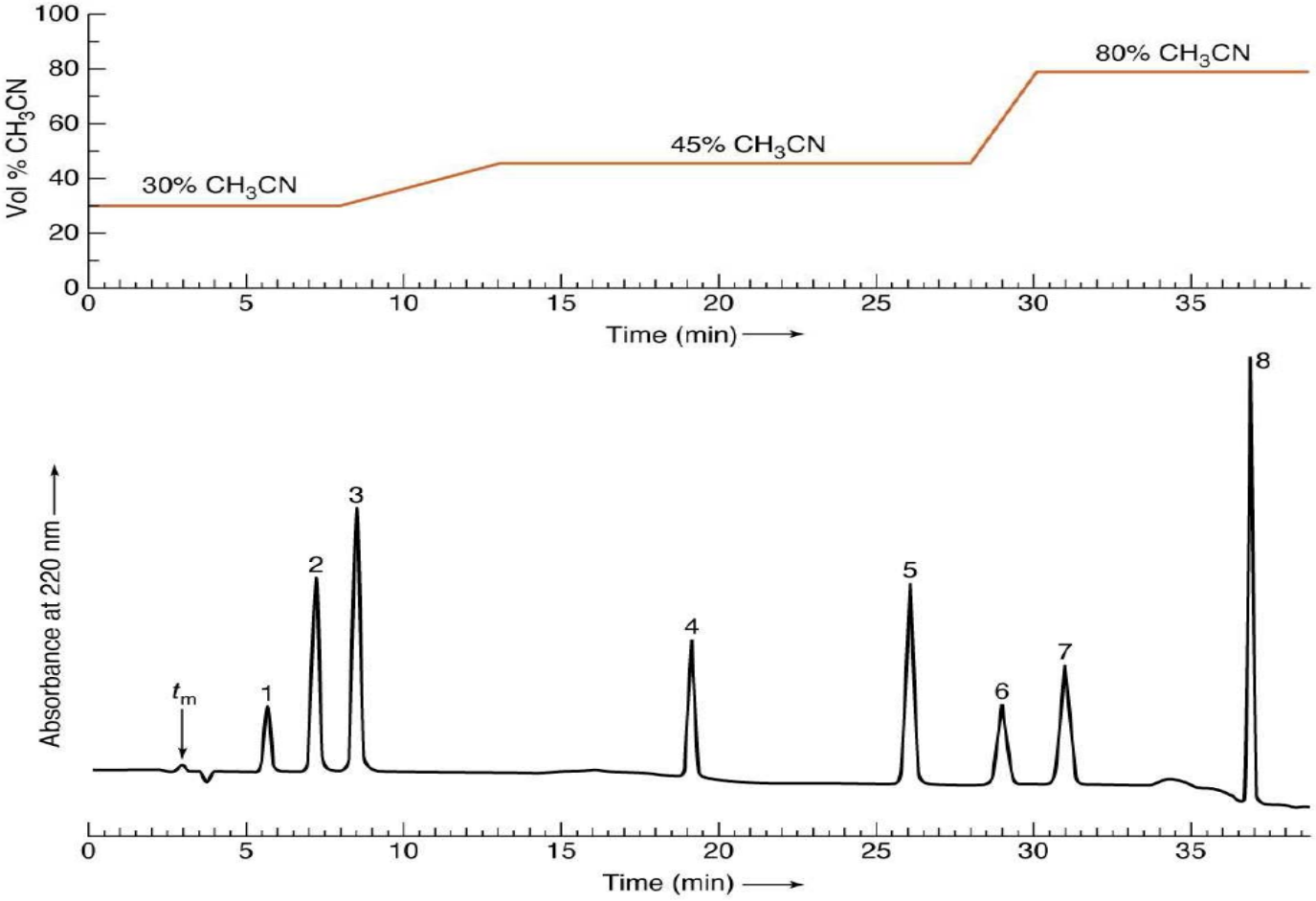
- The two components of the mobile phase are typically termed "A" and "B";

  - ✓ **A** is the "weak" solvent which allows the solute to elute only slowly.

  - ✓ **B** is the "strong" solvent which rapidly elutes the solutes from the column.

- Solvent A is often water, while B is an organic solvent miscible with water, such as acetonitrile, methanol, THF or isopropanol.

- Organic solvent of the mobile phase is to reduce the retarding strength of the aqueous component.


100
80
60
40
20
0
Vol % CH3CN
30% CH3CN
45% CH3CN
80% CH3CN
0
5
10
15
20
25
30
35
Time (min)
Absorbance at 220 nm
tm
1
2
3
4
5
6
7
8
0
5
10
15
20
25
30
35
Time (min)

- ✓ Resolved all peaks in 38 min.
- ✓ Peak 1-3 were separated with a low eluent strength (30% B) for 8 min.
- ✓ The eluent strength was then increased steadily over 5 min to 45% B & held for 15 min to elute peak 4 & 5.
- ✓ Finally, the solvent was changed to 80% B over 2 min & held there to elute the last peaks.

Sample problem 10

A mixture of 4 relatively non polar drugs was analyzed on a 25 cm C18 column (3 µm particle size). The mobile phase contained a mixture of 40% MeOH & 60% water. A flow rate of 0.8 mL/min was used. The column dead volume was measured to be 1.5 mL/min.

i. 2 of the drugs in the mixture were found to have very long retention time. If you wished to speed up the elution of the 2 drugs without affecting the elution of the earlier peaks, suggest a suitable gradient approach for the separation. Explain.

ii. Briefly explain what would happen to the t_R of the compounds if a 25 cm C8 column (3 µm packing) was used instead of the C18 column without changing other conditions.

Sample problem 11

When using a C18 stationary phase is it more beneficial to increase or decrease mobile phase composition polarity during elution? Explain.

Sample problem 12

A mixture of alcohol compounds was analyzed on a 25 cm C18 column. The mobile phase contained a mixture of 30% MeOH & 70% water. Explain how the retention would be affected if :

The composition of the mobile phase changed to 70% MeOH & 30% water.

Sample problem 13

Discuss the reason of the mentioned problem & suggest an approach to overcome the problem.

In a separation using a C18 column with a mobile phase of ACN/water : 80/20, the first 2 peaks eluted very close to t_M.

Sample problem 14

A test mixture consisting of benzene, methylbenzene, phenol & acetophenone is to be separated on a C18 column with a mobile phase of 50/50 MeOH/water.

i. Describe the order of elution for these compounds.

ii. How would you change the composition of the mobile phase so as to reduce the retention of the compounds?

iii. If the C18 bonded phase contained unreacted silanol groups, how would the t_R be affected? Briefly explain the process to reduce the concentration of unreacted silanols.

Sample problem 15

Give a reason for the mentioned problem & suggest an approach to overcome the problem.

In normal phase separation of 5 comps using isocratic mixture of hexane:acetone (80:20), the retention time for the last 2 peaks were too long.

Sample problem 16

A gradient elution for a normal phase separation of a series of comps with a wide range of polarities starts with 80/20 hexane/acetone & ends with 50/50 hexane/acetone.

Sample problem 17

In a gradient elution normal phase separation, what should be changed in regard to acetone (starting solvent) & hexane when the following situation develop?

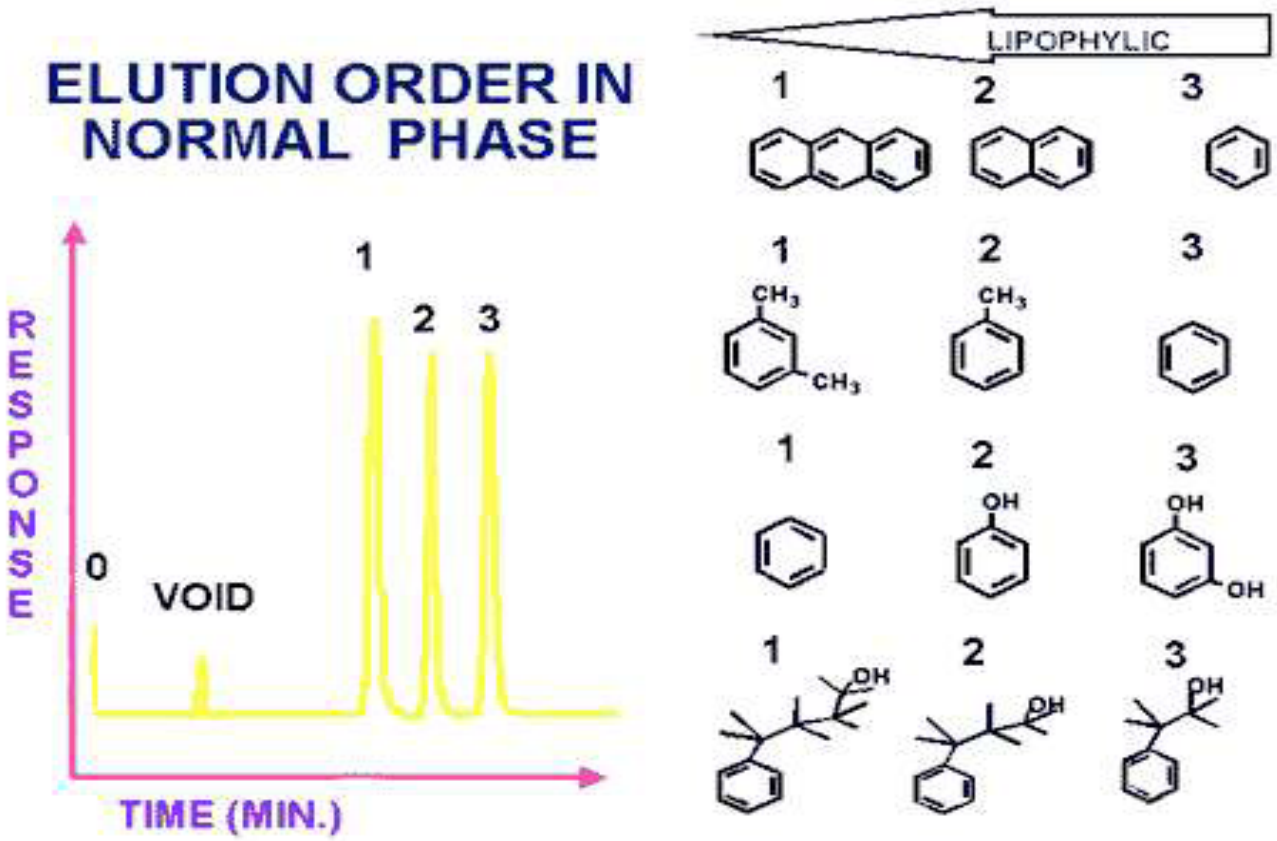
i. The peaks are bunched together near t_M.

ii. Peaks continue to elute for a considerable time after completion of the gradient.

Sample problem 18

On a normal phase LC column, a compound had a t_R of 15 min. Which solvent, hexane or diethyl ether would increase the t_R? Explain why.

E.g. :


ELUTION ORDER IN NORMAL PHASE
RESPONSE
0
VOID
1
2
3
TIME (MIN.)
LIPOPHYLIC
1
2
3
CH3
CH3
OH
OH
OH

Sample problem 19

ii. Predict the effect on the t_R of comps 1 & 3 if the mobile phase is changed from 80/20 hexane/acetone to 50/50 hexane/acetone.

Typical Applications of High-Performance Partition Chromatography

Field	Typical Mixtures Separated
Pharamaceuticals	Antibiotics, sedatives, steroids, analgesics
Biochemicals	Amino acids, proteins, carbohydrates, lipids
Food products	Artificial sweeteners, antioxidants, aflatoxins, additives
Industrial chemicals	Condensed aromatics, surfactants, propellants, dyes
Pollutants	Pesticides, herbicides, phenols, polychlorinated biphenyls (PCBs)
Forensic chemistry	Drugs, poisons, blood alcohol, narcotics
Clinical medicine	Bile acids, drug metabolites, urine extracts, estrogens

Ion-pair chromatography (IPC)

- IPC is a method for improving the separation of charged/ionized (cation & anion) & very polar analytes on a reversed phase column.

- Use of IP reagents can enhance peak shape & t_R when common remedies such as modifying eluent ratios or changing stationary phase fail.

- IP reagent is an ionic compound containing a counter ion of opposite charge to the analyte.

- Has ability to increase retention of highly polar compounds on RP analytical column.

- Typical IP reagent contain a :
  - ✓ **non polar portion**, such as a long chain of aliphatic hydrocarbon,
  - ✓ **polar portion**, such as an acid or base.
- The polar portion interact with the charged group on the analyte, forming an **"ion-pair"**.
- The non polar portion interacts with the RP media.

- **Mobile phase** : aqueous buffer containing organic solvent (MeOH/ACN) & IP containing a counter ion of opposite charge to the analyte.

- Typically :

  - ✓ Alkyl amines or tetra alkyl amines salt are added to ion pair with acids.

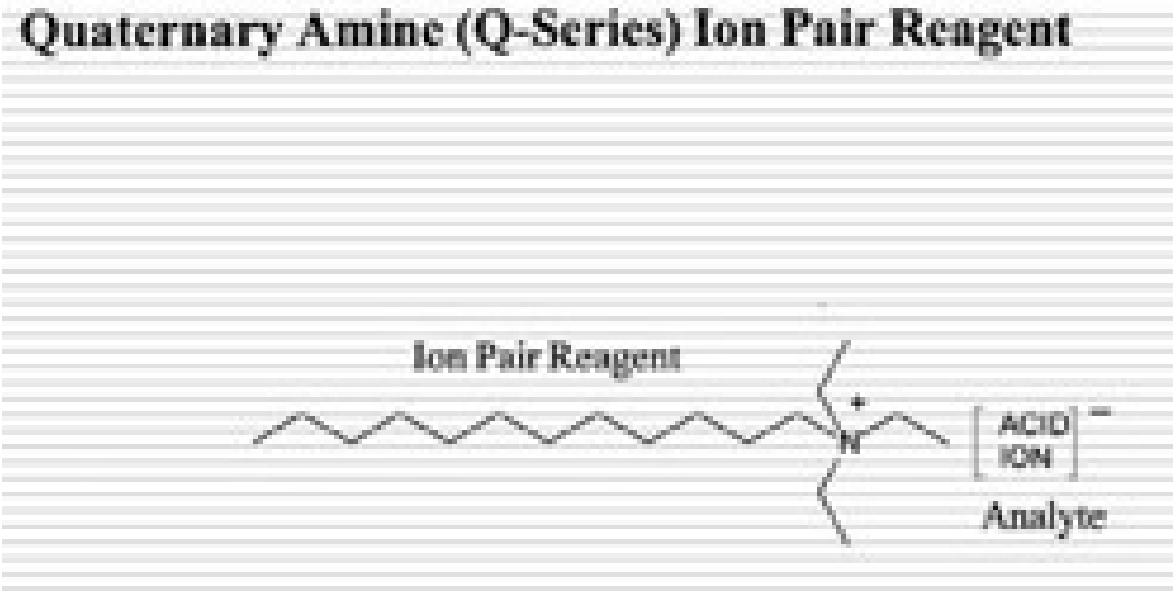
  - ✓ Alkyl sulfates, sulfonates or phosphates sodium salt are used to ion pair with bases.

- Mechanism of separation :

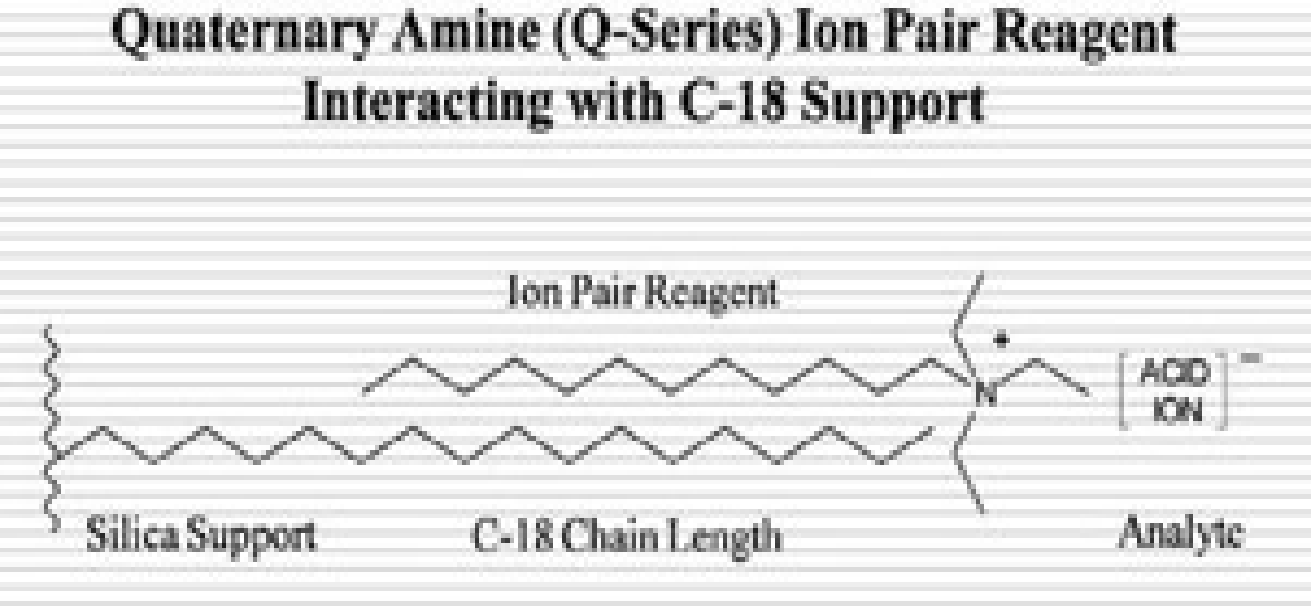
  - ✓ The counter ion combines with the analyte ion to form an "**ion-pair**" (neutral species/uncharged) that is retained by the RP packing.

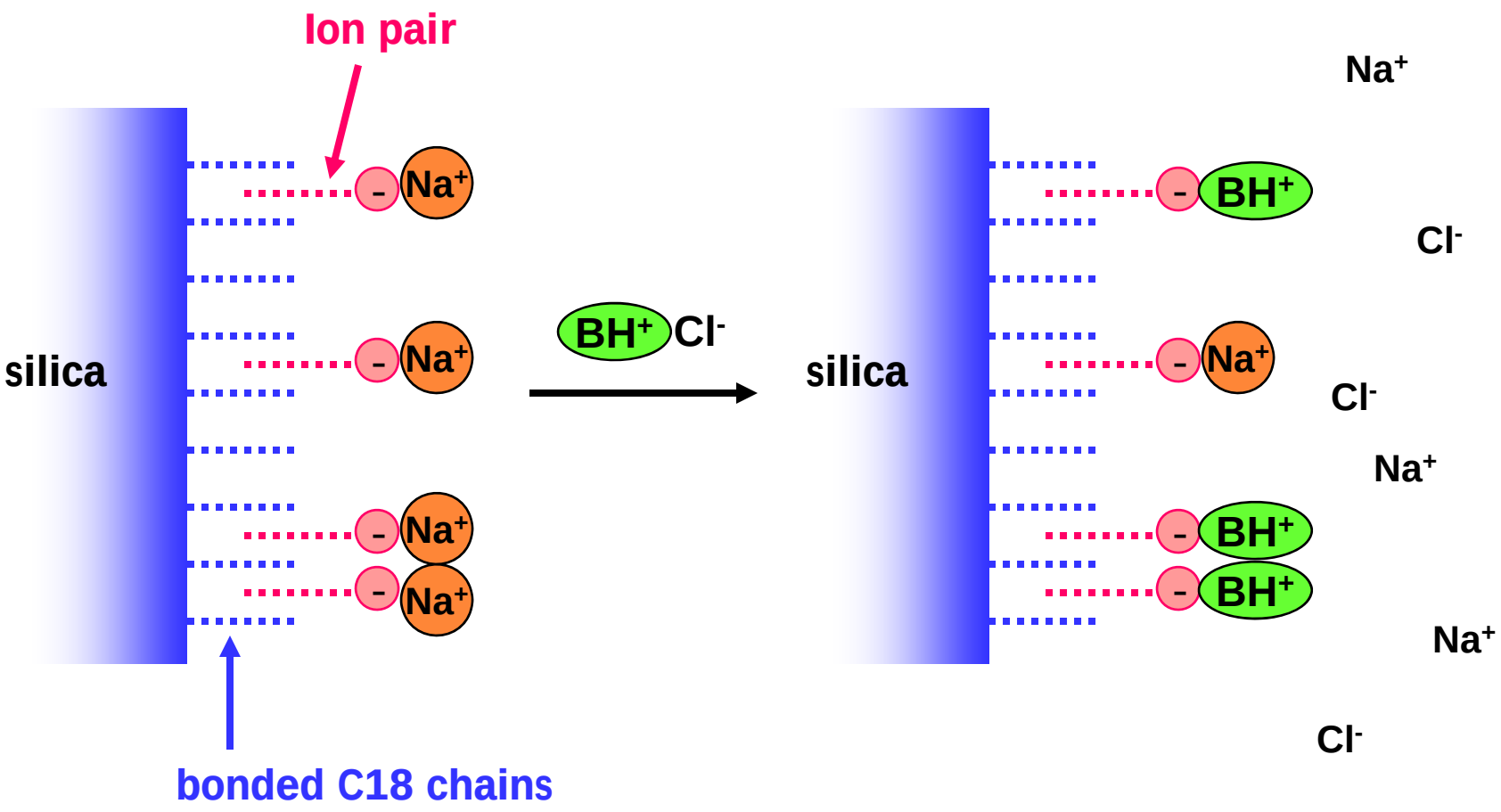
  - ✓ Elution of the ion-pairs is then accomplished with an aqueous solution of methanol or other water soluble organic solvent.

Quaternary amine ion pair reagent

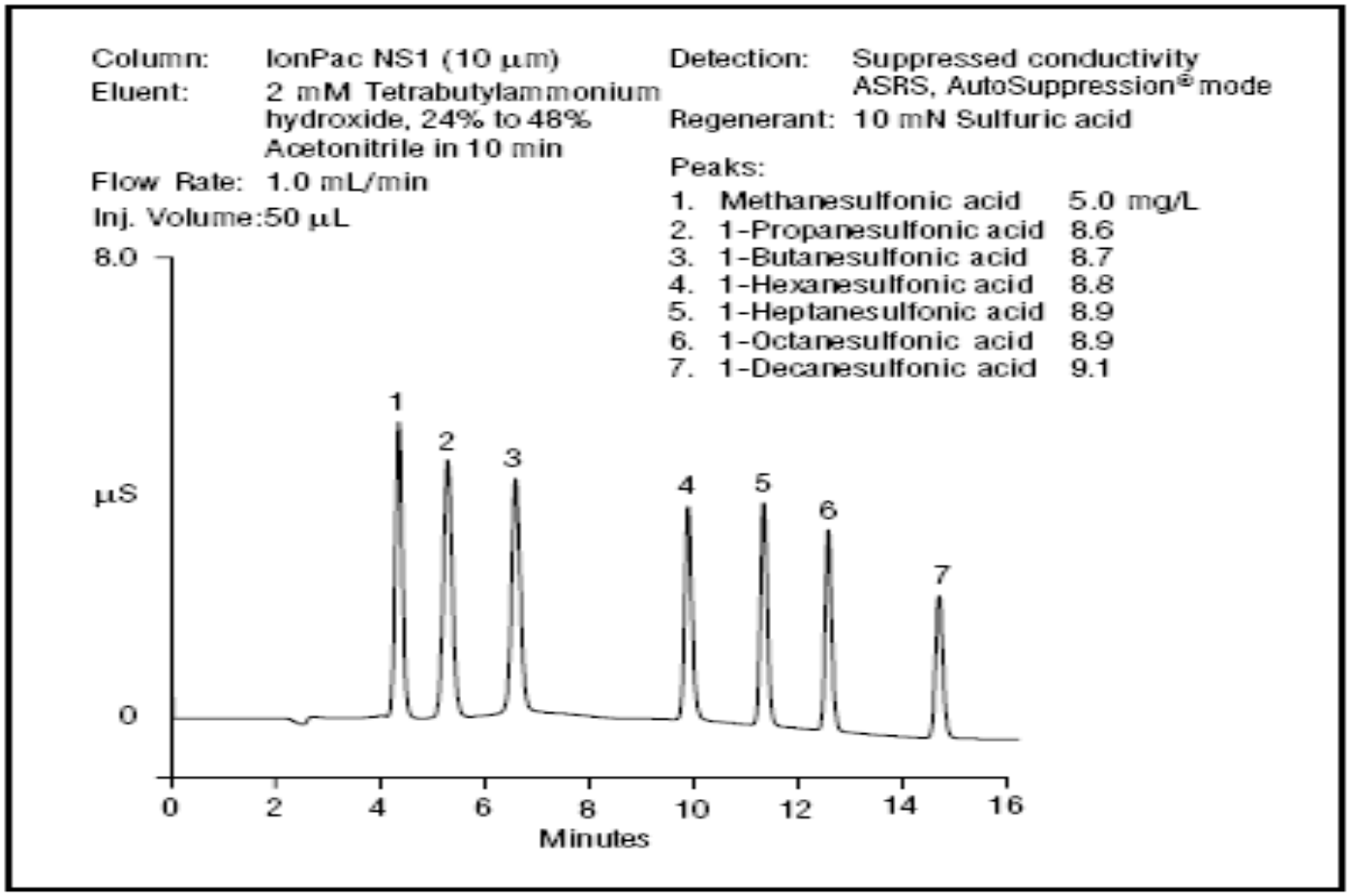


Quaternary amine ion pair reagent interacting with C18 support




Ion pair
silica
Na+
Na+
Na+
Na+
BH+ Cl-
silica
BH+
Na+
BH+
BH+
Na+
Cl-
Cl-
Na+
Na+
Cl-
bonded C18 chains

Separation of acids using IPC



Separation of bases using IPC

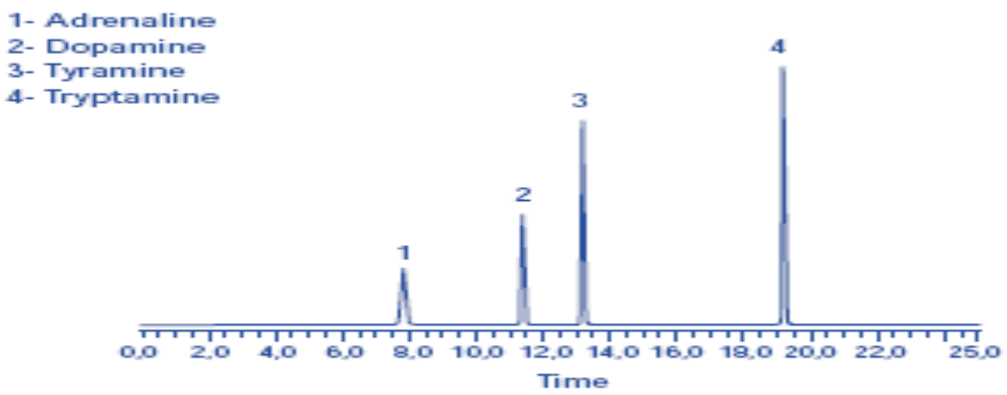


Figure 4. Mixture of biogenic amines resolved by IPC

Columns: Discovery™ C18 Column (250 x 4.0 mm) ID, 5 µm (Cat. No 04971-40)
Eluent: acetonitrile: heptanesulfonic acid buffer pH 2.4:
Buffer concentration: 0,005 M heptanesulfonic acid sodium salt (Cat. No 51832) + 0,01 M phosphoric acid (Cat. no 79606)
Weigh-in: ~ 2 mg in 10 ml acetonitrile/phosphoric acid (0.01 M) 1:9
Acetonitrile gradient: t=0 min : 6%, t=5 min : 6%, t=18 min : 25%
Flow: 1.5 ml/min
Detection: 220 nm
Injection volume: 20 µl
Temperature: ambient
Detector: UV 1000
Pump: P 4000

SUPELCO

Sample problem 20

Problems are encountered when using RP HPLC to analyze solutes that are very polar & readily ionized. Briefly discuss an approach that can be applied to overcome these problems by using similar technique.

Sample problem 21

Consider a separation of a water soluble vitamins mixture.

Column : XPERTEK Inertsil, ODS2, 5 µm, 4.6x150 mm
Mobile phase : 5mM IPCC-05 + 0.1% H_3PO_4 in 80% ACN
Flow rate : 1 mL/min

i. What type of HPLC separation is mentioned?

ii. What is the purpose of adding 5 mM IPCC reagent into the mobile phase system.

Sample problem 22

Give a reason for the mentioned problem & suggest an approach to overcome the problem.

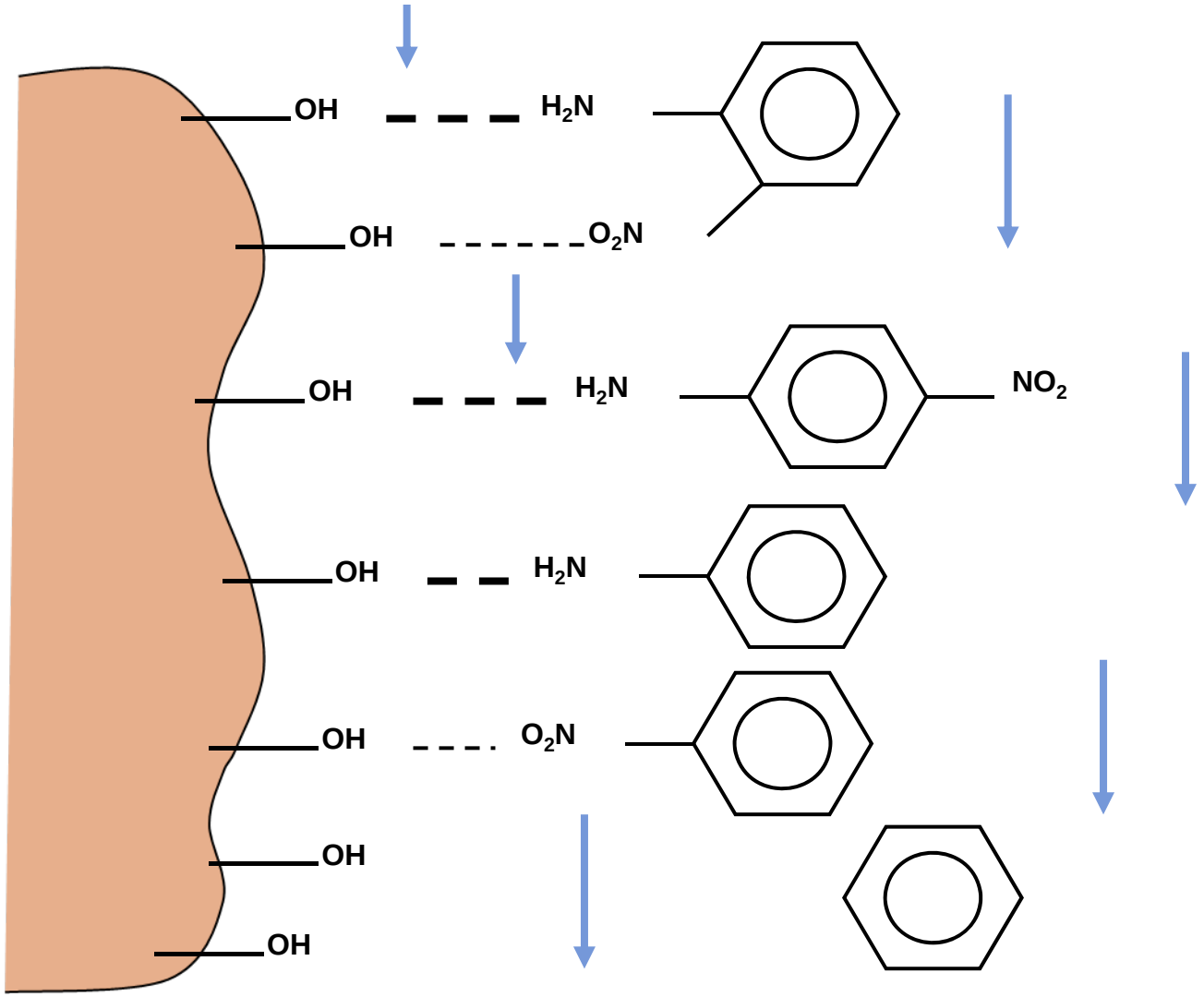
You are given a task to separate anions in a water sample. In your lab, the liquid chromatograph available can only run partition chromatography. When the separation of anions in the sample was carried out using RP chromatography with $MeOH/H_2O$ (50/50) as a mobile phase, no peaks was observed.

Adsorption chromatography (Liquid-solid chromatography)

- Stationary phase : Silica or alumina particles (polar).
- Mobile phase : A non polar solvent (e.g. hexane) modified with a small amount of polar solvent (e.g. acetone, diethyl ether, methylene chloride).
- Detector : Normally UV.

Mechanisme of the separation :

- ✓ Analytes compete with mobile phase for the binding sites on the stationary phase.
- ✓ Analytes in the liquid sample interacts with adsorption sites on solid surface.
- ✓ Polar groups (-OH) on solid surface form dipolar interaction (e.g. H bonds) with sample.
- ✓ Analytes are separated based on repeated adsorption-desorption steps onto the solid support.


OH
H_2N
OH
O_2N
OH
H_2N
NO_2
OH
H_2N
OH
O_2N
OH
OH

- Often employed for **relatively non-polar hydrophobic materials.**

- The main advantage:

  - ✓ **separation of isomers (*cis & trans*)** which can have very different physiosorption characteristics due to steric effect in the molecules.

Sample problem 23

How is adsorption chromatography able to separate isomer compounds unlike other LC techniques?

Sample problem 24

Discuss the reason for the mentioned problem & suggest an approach to overcome the problem.

1-chlorophenol & 2-chlorophenol were not separated on a bonded siloxane with diol functional groups stationary phase.

Sample problem 25

Consider the separation of 5 benzene comps : n-propylbenzene, n-buthylbenzene, 2-chlorobuthylalcohol, 3-chlorobuthylalcohol & benzamide. (June 2009)

Column : Allshere, silica 5 um, 150x4.6 mm

Mobile phase : (A) hexane, (B) acetone

Gradient : (Time, %B), (0,40), (2,40), (15,90), (18,90)

Flow rate : 1 mL/min

Detector : UV at 254 nm.

i. Discuss the mechanism of the above separation.

ii. Of the above compound, which one would you expect to elute first from the column. Justify your answer.

iii. Suggest a better gradient approach that may improve the resolution of peak 1 & 2. Justify your answer.

iv. What may possible happen to the chromatogram if you change the above column to a bonded siloxane with diol functional groups stationary phase without changing the other conditions. Explain.

Size Exclusion Chromatography (SEC)

- Used for large molecular weight or macromolecule compounds e.g. **proteins & polymers**.
- Good separation of large molecules from the small molecules.

- 2 types of SEC :
  - ✓ Gel permeation chromatography (GPC)
  - ✓ Gel filtration chromatography (GFC)

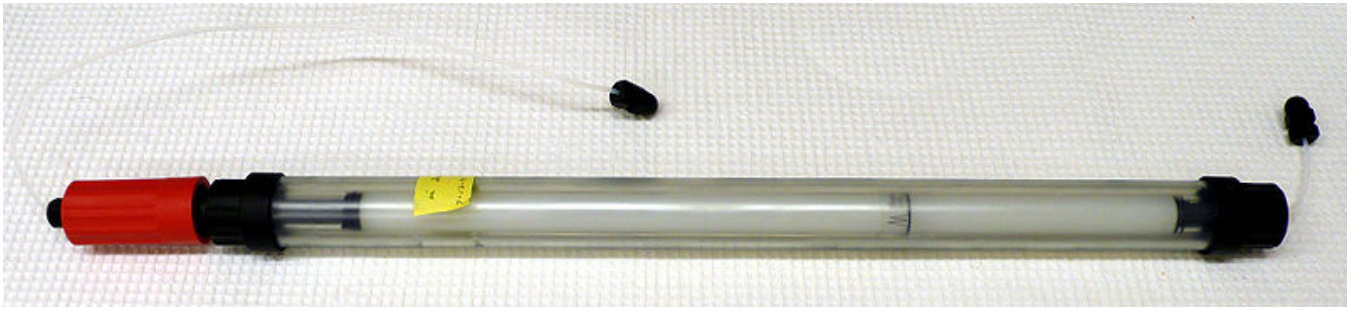
Gel permeation chromatography (GPC)

- Stationary phase :
  - ✓ Cross-linked porous polystyrene (hydrophobic packing)
- Mobile phase :
  - ✓ Organic solvent (THF, $CHCl_3$, DMF)
- Analyte :
  - ✓ Polymers

Gel Filtration Chromatography (GFC)

- Stationary phase :
  - ✓ Polar silica gel (hydrophilic) / porous polymer
- Mobile phase :
  - ✓ Polar aqueous solvent/buffer solution
- Analyte :
  - ✓ Water soluble (proteins, nucleic acids, carbohydrates)

- Stationary phase :
  - ✓ Pore sizes (40-2500 Å).



Column consists of a hollow tube tightly packed with extremely small porous polymer beads designed to have pores of different sizes

- Detector : Refractive index

SEC : Separation mechanism

- The main difference between SEC & other chromatographic methods is that a separation is not achieved through any kind of interaction (e.g. partitioning) but achieved by sieving action simply by classifying the molecules based on their **size** & **shape** in solution.

SEC : Separation mechanism

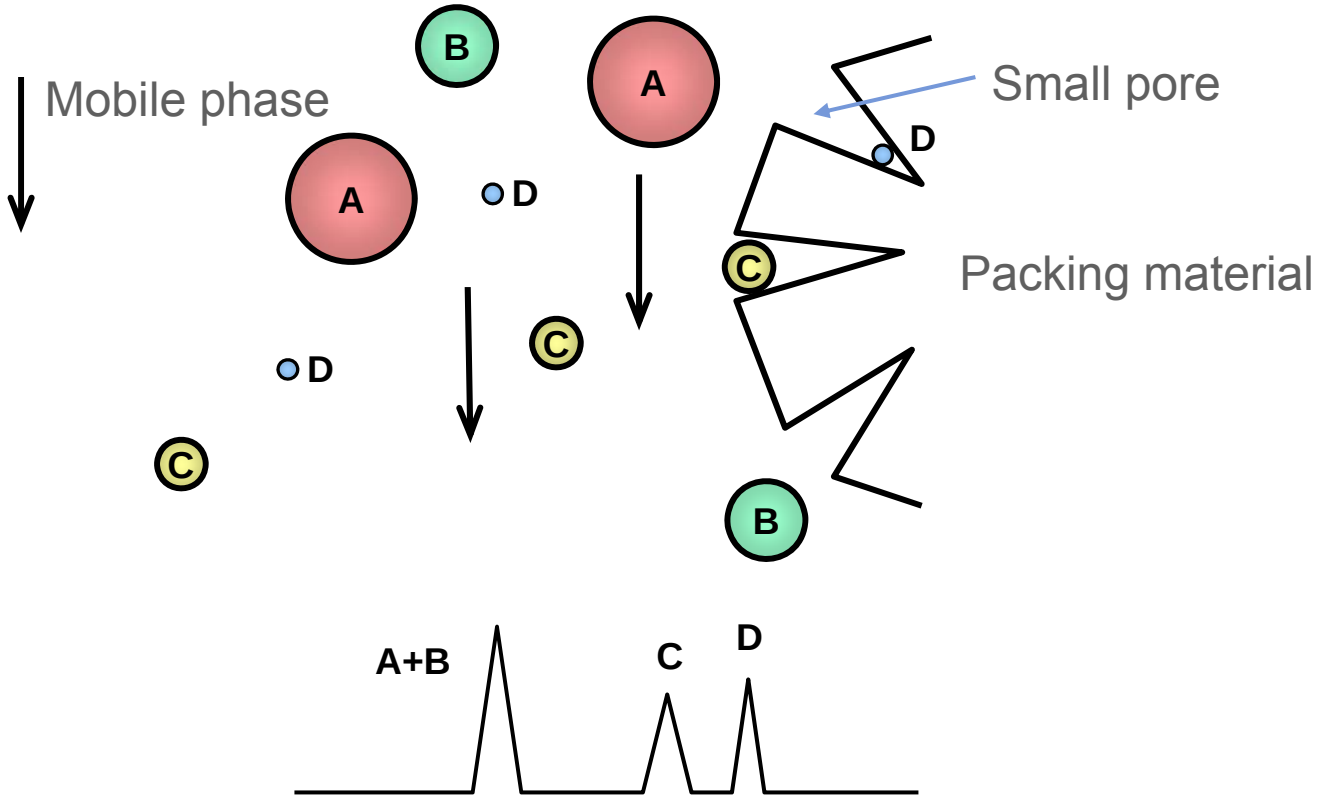
- **Order of elution :**

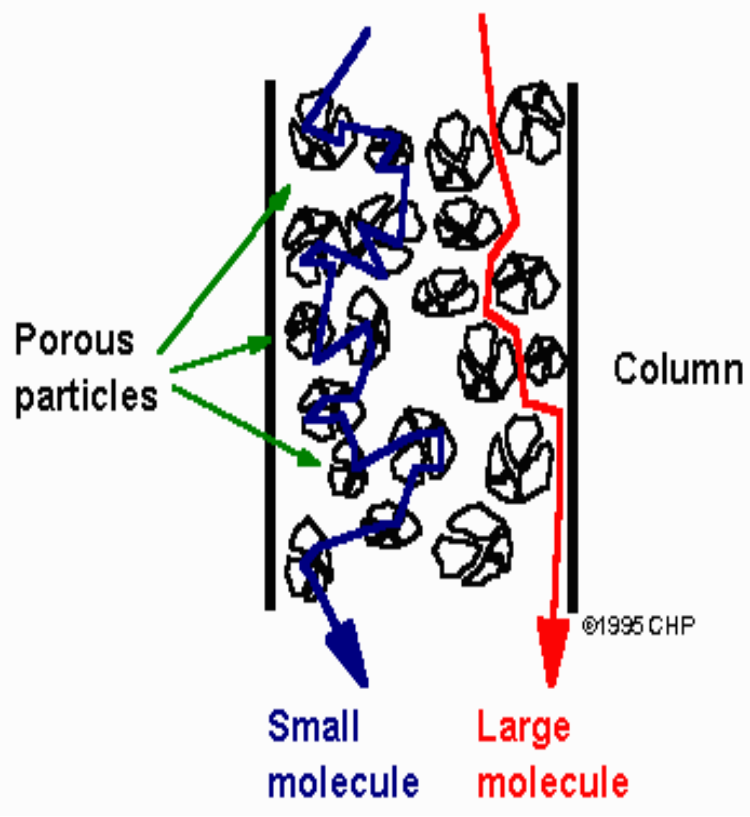
1. **Large molecules (higher M_w)** which cannot enter the pores excluded from pores - not retained, first eluted (exclusion limit).

2. **Intermediate molecules** – selectively permeate the pores based on their relative sizes - retained, intermediate elution times.

3. **Small molecules** completely permeate into pores - strongly retained, last eluted (permeation limit).

SEC separation mechanism





Molecules that are smaller than the pore size can enter the particles & therefore have a longer path & longer transit time compared to the larger molecules that cannot enter the particles.

- Each size exclusion column has a range of molecular weights that can be separated.

- The **exclusion limit (no retention)** defines the molecular weight at the upper end of this range & is where molecules are too large to be trapped in the stationary phase & elute together.

Properties of commercial packings for SEC

Type	Particle size, μm	Average pore size, Å	Molecular weight exclusion limit *
Polystyrene divinylbenzene	10	10^2 10^3 10^4	700 (0.1 to 20) x 10^4 (1 to 20) x 10^5
Silica	10	125 300 500 1000	(0.2 to 5) x 10^4 0.03 to 1) x 10^5 (0.05 to 5) x 10^5 (5 to 20) x 10^5

* Molecular weight above which no retention occurs

- The **permeation limit (most retention)** defines :

  - ✓ The M_w at the lower end of the range & is where molecules of a small enough size can penetrate into the pores of the stationary phase completely.

  - ✓ All molecules below this M_w are so small that they elute as a single band.

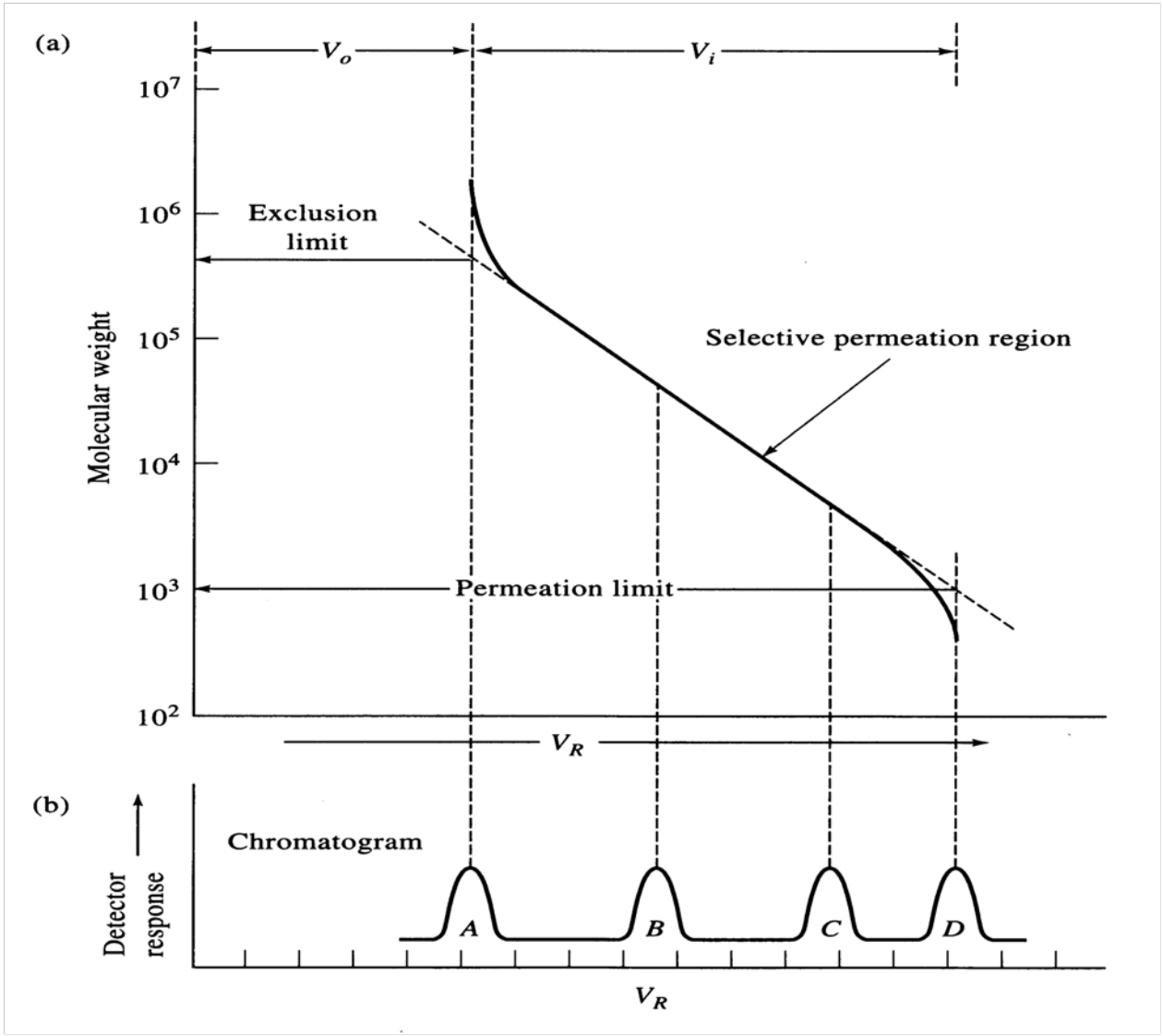
- Molecules larger than the pore size cannot enter the pores & elute together as the first peak in the chromatogram is called **total exclusion limit**.

- Molecules that are smaller than the pore size can enter all pores,
- & have the longest residence time on the column,
- & elute together as the last peak in the chromatogram.
- This last peak in the chromatogram determines the **total permeation limit**.

- Molecules that can enter the pores will have an average residence time in the particles that depends on the molecules size & shape.

- Different molecules therefore have different total transit times through the column.

- This portion of a chromatogram is called the **selective permeation region**.


(a)
V_o
V_i
10^7
10^6
10^5
10^4
10^3
10^2
Exclusion limit
Molecular weight
Selective permeation region
Permeation limit
V_R
(b)
Chromatogram
Detector response
A
B
C
D
V_R

Factors limit the choice of analytes

- Only sample with M_w in the range can be separated (in the selective permeation region).
- If the M_w of the analytes exceeded the range, it will not be retained & eluted immediately.
- If the M_w of the analytes are below the range, it will be retained & eluted later with no separation.

Sample problem 26

Discuss the reason of the mentioned problem & suggest an approach to overcome the problem.

i. When a Bio-gel 150 with a M_w range of 22,000-250,000 is used for size exclusion chromatography packing, the first 3 comps were separated well. However, the last 2 comps were eluted as a single peak.

ii. When a Bio-gel 150 with a M_w range of 15,000-150,000 is used for size exclusion chromatography packing, protein A (M_w 185,000) & protein B (M_w 158,000) are not retained & eluted together as a single peak.

Sample problem 27

A laundry detergent contain a low % of water soluble polyacylate (M_w is several thousand) as well as various organic & inorganic chemicals of much lower M_w. Describe a suitable type of LC to separate the polyacrylate from the other chemicals.

Ion Exchange Chromatography (IEC)

- IEC is used for the separation of inorganic ions (cations & anions).
- Stationary phase : Polystyrene polymer crosslinked with divinylbenzene (resin).
- Detector : Conductivity.

Ion exchange resin beads

➢ Principles :

- ✓ Separations based upon exchange of ions (cations or anions) on an ion exchange stationary phase (resin) column.

- ✓ Ions of the opposite charge are attracted to the charges immobilized on the stationary phase (resin) by electrostatic force. E.g. for cation analysis, positively charged ions bind to negatively charged resin.

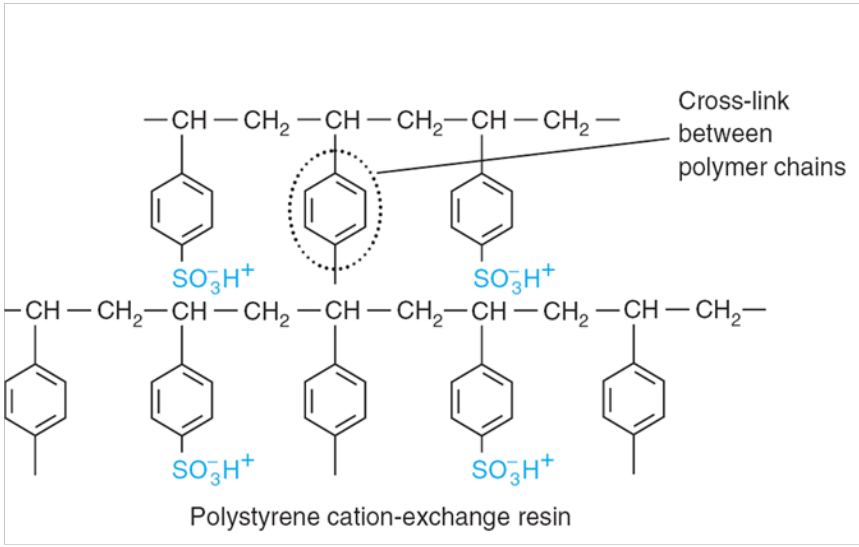
- IEC can be subdivided :
  - ✓ **Cation exchange chromatography**
  - ✓ **Anion exchange chromatography**

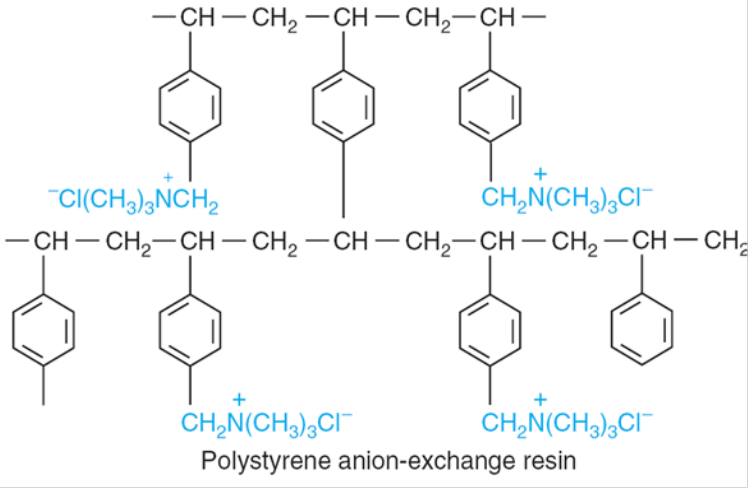
Cation Exchange Chromatography (Analysis of Cation)

- Resins contain acidic functional group added to the aromatic ring of the resin.

- For **cation analysis (positively charged ions)**, exchange is with ionizable cations on a cation exchange resin :
  - ✓ Support-R-$SO_3^-H^+$ (sulphonic acid) or
  - ✓ Support-$RCOO^-H^+$ (carboxylic acid).

Structures of styrene-divinylbenzene cross-linked ion exchange resins





Cation exchangers :
$-SO_3^-$, $-CO_2^-$

Anion exchanger :
$-NR_3^+$

Polystyrene resins - Ion exchange groups

Type	Active group	pH range of operation
Strongly acidic cation exchanger	Sulphonic acid RSO_3^-	1-14
Weakly acidic cation exchanger	Carboxylic acid $RCOO^-$	5-14
Strongly basic anion exchange	Quarternary ammonium ion $RN(CH_3)_3^+$	1-12
Weakly basic anion exchanger	Amine group $RC_2H_4N(C_2H_5)_2$	1-9

Mechanism of ion-exchange chromatography for the separation of cation :

$$n\text{RzSO}_3^-\text{H}^+_{(s)} + \text{M}^{n+}_{(aq)} \rightleftharpoons (\text{RzSO}_3)_n\text{M}_{(s)} + n\text{H}^+_{(aq)}$$

$$n\text{RzCO}_2^-\text{H}^+_{(s)} + \text{M}^{n+}_{(aq)} \rightleftharpoons (\text{RzCO}_2)_n\text{M}_{(s)} + n\text{H}^+_{(aq)}$$

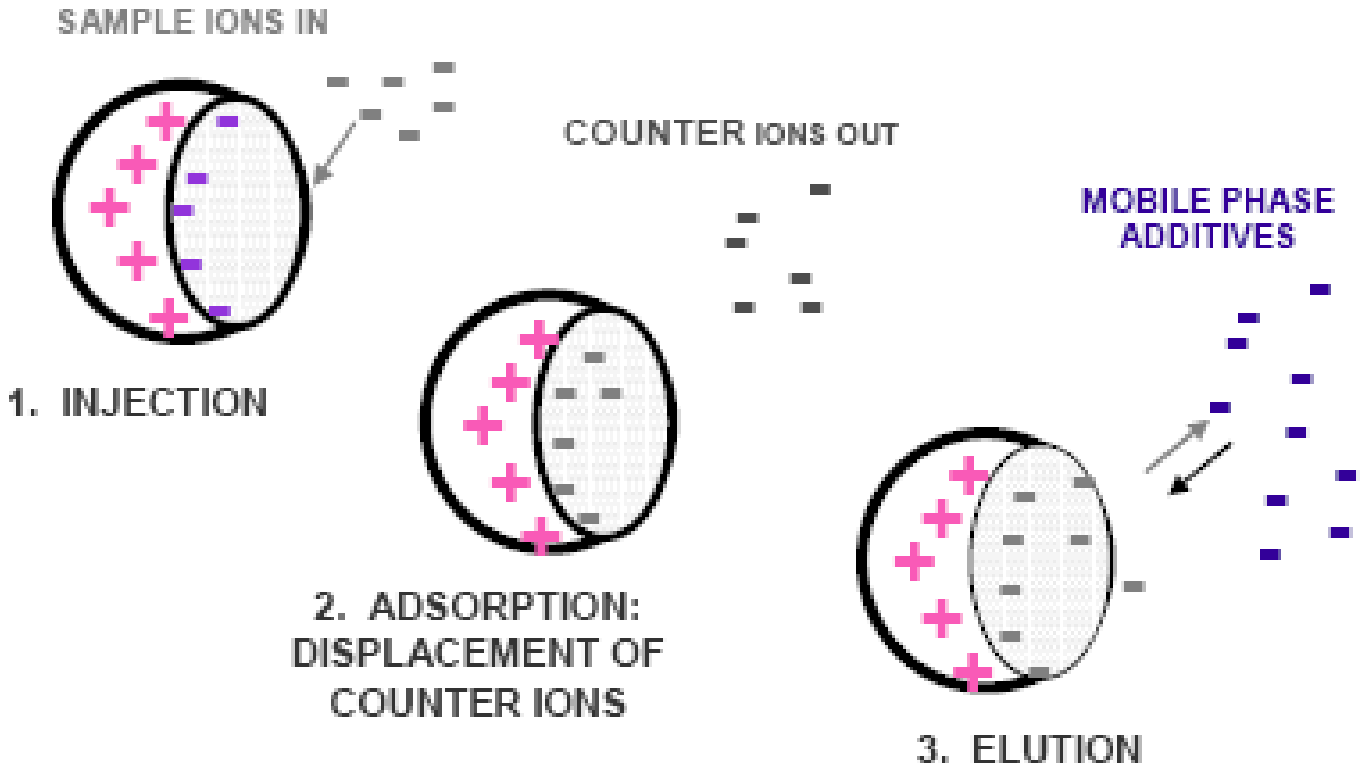
The exchange process :

⇀ The analyte attached to the stationary phase (retained in the column).

↽ The mobile phase ion replaces the analyte & the analyte will be eluted out.

ION EXCHANGE

INSIDE A PORE IN THE STATIONARY PHASE



Strong acid cation resins

- Strong acid resins are so named because their chemical behavior is similar to that of a strong acid (remain ionized even in strongly acidic solution).

- The resins are highly ionized in both the acid ($R\text{-}SO_3H$) & salt ($R\text{-}SO_3Na$) form of the sulfonic acid group.

- The H & Na forms of strong acid resins are highly dissociated & the exchangeable Na^+ & H^+ are readily available for exchange **over the entire pH range**.

- Consequently, the exchange capacity of strong acid resins is **independent of solution pH**.

Weak acid cation resins

- In a weak acid resin, the ionizable group is a **carboxylic acid (COOH)** as opposed to the sulphonic acid group (SO_3H) used in strong acid resins.

- These resins behave similarly to weak organic acids that are weakly dissociated.

- The degree of dissociation of a weak acid resin is strongly influenced by the solution pH.

- Weak-acid exchangers gradually lose its charge below pH 5 & therefore cannot retains the cation & lose their cation-exchange capacity.

- At low pH values, the weak-acid exchangers will "hold-on" to the protons too strongly for exchange to occur.

- Also, the weak-acid cation exchangers will not completely remove the cations of very weak bases, while strong-acid resin will.

Sample problem 28

Give a type of resin known as a “strong-acid resin” in cation exchange chromatography. What will happen to t_R of analytes if the pH used is less than 4? Explained briefly.

Sample problem 29

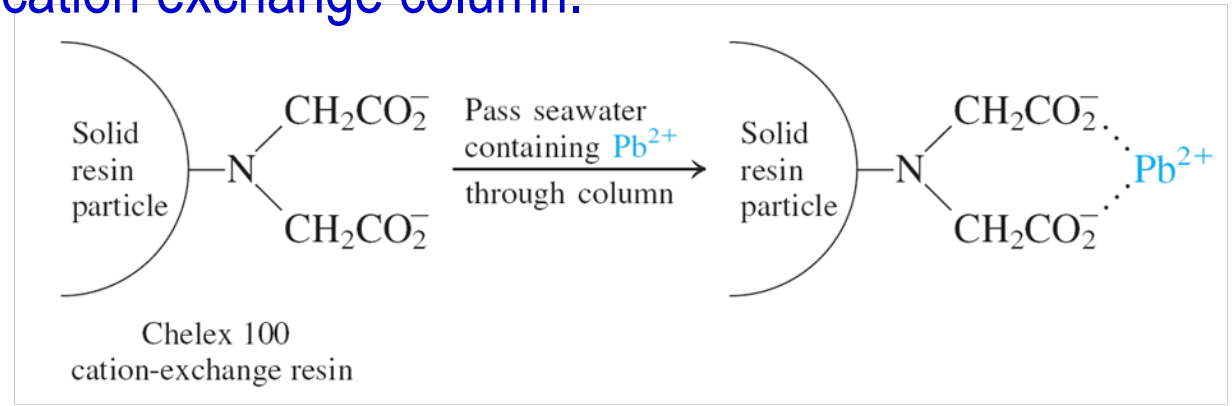
Give a type of resin known as a “weak-acid resin” in cation exchange chromatography. What will happen to t_R of analytes if the pH used is less than 4? Explained briefly.

Sample problem 30

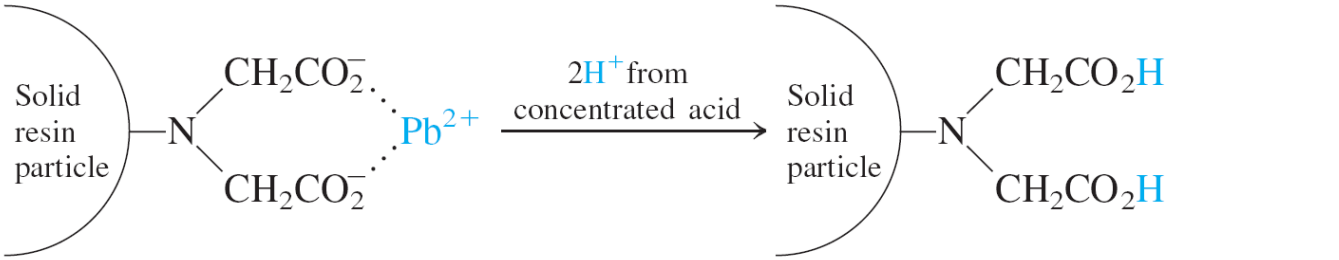
For a separation of organic acids using ion exchange chromatography, the comps were separated using carboxylic acid $–COO^-H^+$ resin as the stationary phase at pH 4. The comps were not separated due to overlapping of peaks.

Analysis of cations

Metals in natural waters can be preconcentrated with a cation exchange column.



The cations can then be displaced into a small volume of solution by eluting the column with concentrated acid.



Ion-exchange selectivity

- Ion-exchangers favor for binding ions of higher charge, decreased hydrated radius & increased polarizability.
- The equilibrium constant is called **selectivity coefficient,** *K*.
- Order of selectivity :

$$Pu^{4+} \gg La^{3+} > Y^{3+} > Sc^{3+} > Al^{3+} \gg Ba^{2+} > Pb^{2+} > Sr^{2+} > Ca^{2+} > Ni^{2+} > Cd^{2+} > Cu^{2+} > Co^{2+} > Zn^{2+} > Mg^{2+} \gg Tl^{+} > Ag^{+} > Cs^{+} > Rb^{+} > K^{+} > NH_4^{+} > Na^{+} > H^{+} > Li^{+}$$

ION EXCHANGE

CATIONS

RETENTION & ELUTION STRENGTH

MONO-VALENT

$Li^{+} > H^{+} > Na^{+} > NH_4^{+} > K^{+} > Rb^{+} > Cs^{+} > Ag^{+}$

DI-VALENT

$Be^{++} > Mn^{++} > Mg^{++} > Zn^{++} > Co^{++} > Ni^{++} > M^{++} > Ca^{++} > Sr^{++}$

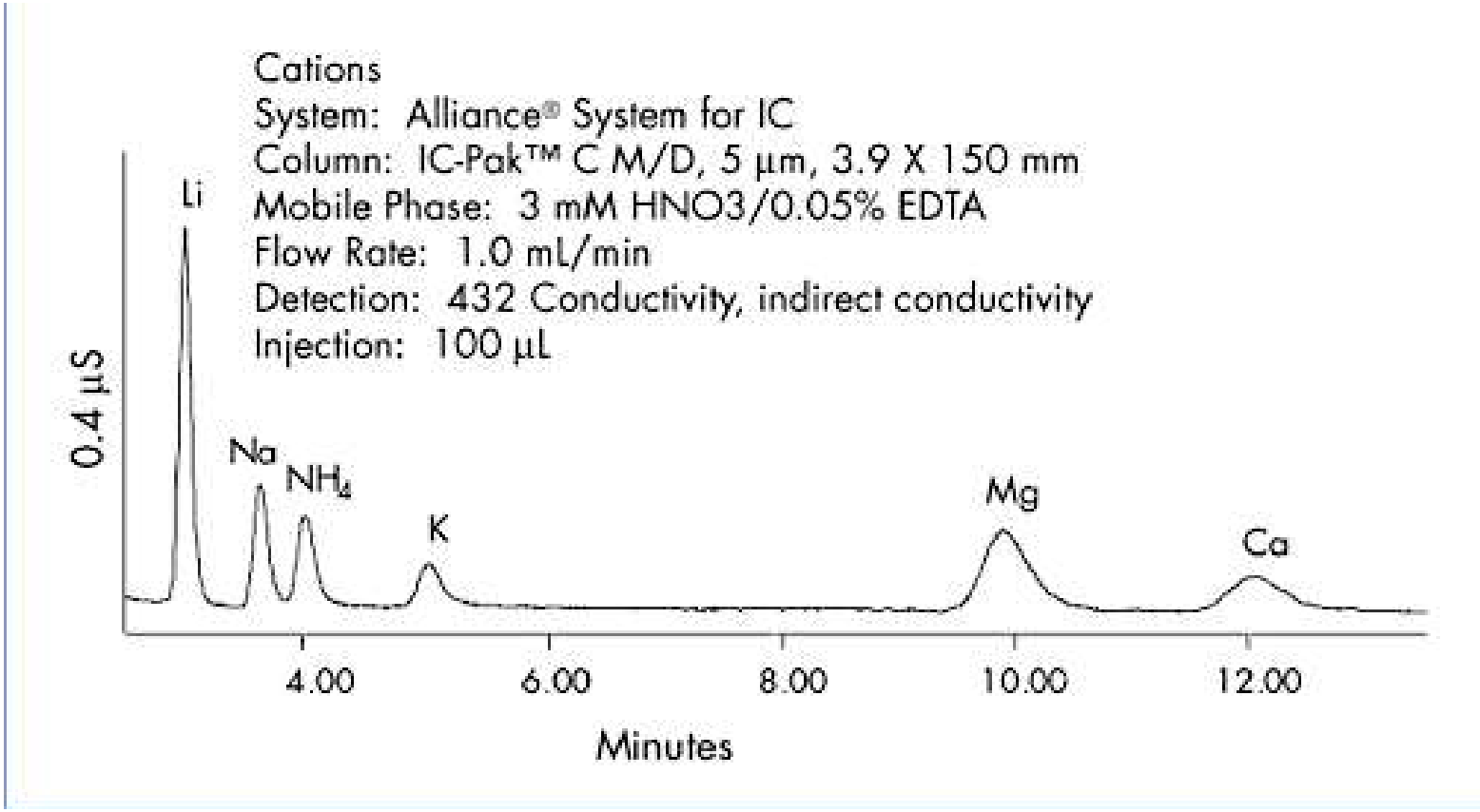
TRI-VALENT

$Al^{+++} > M^{+++} > Ce^{+++}$

transition metals

Transition metals

Analysis of cations



Sample problem 31

Explain why Ca ion was last eluted in this separation?

Anion Exchange Chromatography (Analysis of Anion)

- Basic group on the resin in which the **hydroxyl anions** make up the anion exchange resin.

- For **anion analysis**, exchange is with ionizable anions on an anion exchange resin :

  - ✓ Support-$N(CH_3)_3^+OH^-$, tertiary amine : strong base.
  - ✓ Support-$NH_3^+OH^-$, primary amine : weak base.

Hydroxyl group exchange with anion

$$n\mathrm{RzNR_3^+OH^-} + \mathrm{A}^{n-} \rightleftharpoons (\mathrm{RzNR_3})_n\mathrm{A} + n\mathrm{OH^-}$$

$$n\mathrm{RzNH_3^+OH^-} + \mathrm{A}^{n-} \rightleftharpoons (\mathrm{RzNH_3})_n\mathrm{A} + n\mathrm{OH^-}$$

Strong base anion resins

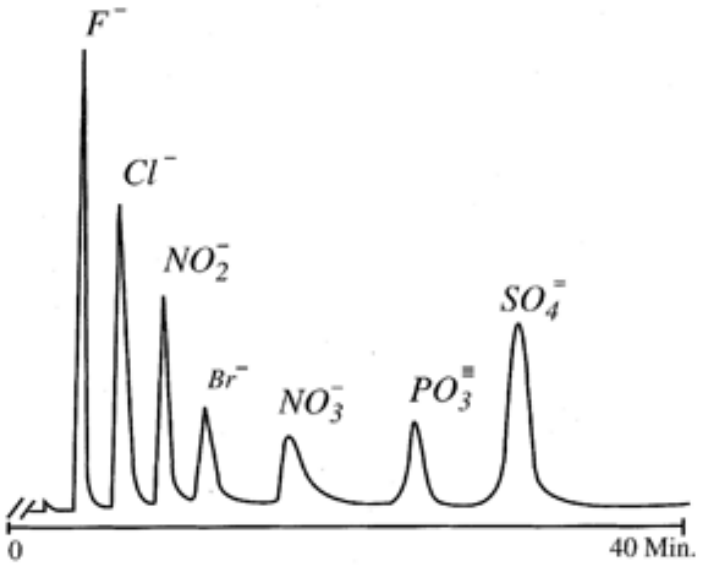
- Like strong acid resins, strong base resins are highly ionized (remain cationic at all values of pH) & therefore, can be used over the entire pH range.

- These resins are used in the hydroxide (OH) form.

Weak base anion resins

- Weak base resins are like weak acid resins in that the degree of ionization is strongly influenced by pH.
- They are deprotonated in moderately basic solution & lose their ability to bind anions.
- Consequently, weak base resins exhibit minimum exchange capacity above a pH of 9.

Analysis of anions



Order of increasing affinity for anion exchangers, increasing selectivity :

$F^- < OH^- < OAc^- < HCO_3^- < Cl^- < NO_2^- < HSO_3^- < CN^- < Br^- < NO_3^- < H_2PO_4^- < HSO_4^- < I^-$

ION EXCHANGE

ANIONS

RETENTION & ELUTION STRENGTH

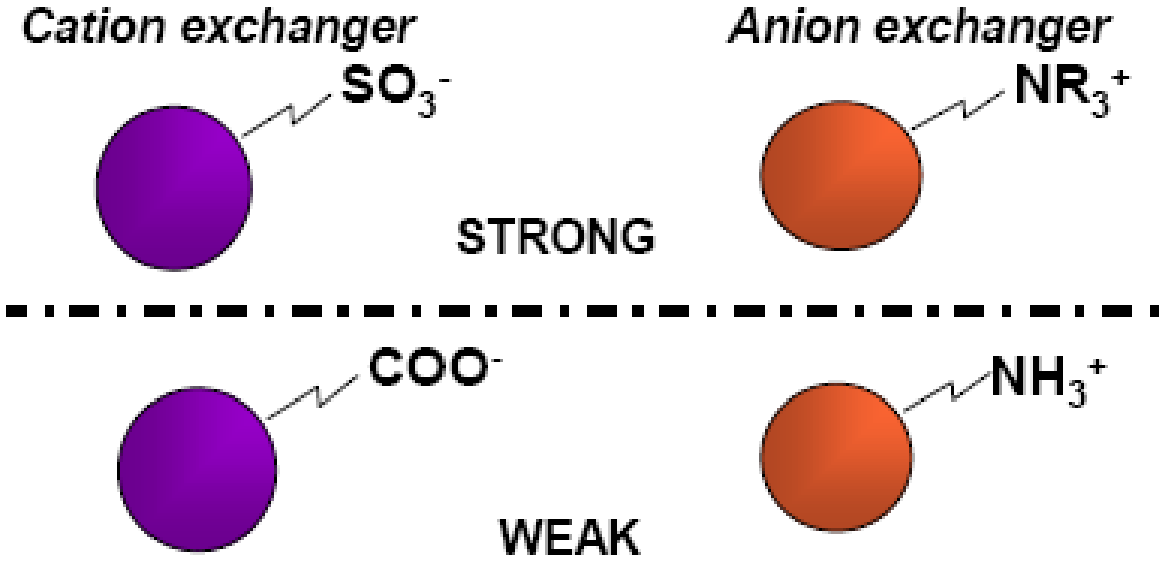
F^-, OH^- > OAc^- > $H_2PO_4^-$ > HCO_3^- > Cl^- > NO_2^- >

HSO_3^- > CN^- > Br^- > NO_3^- > I^-

$A^{---} > A^{--} > A^-$

Ion Exchange Theory

Strong vs. Weak Exchange Materials

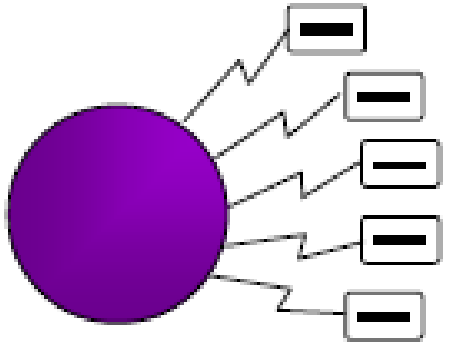


Strong Exchangers stay ionized as pH varies between 2 and 12.
Weak exchangers can lose ionization as a functionof pH.

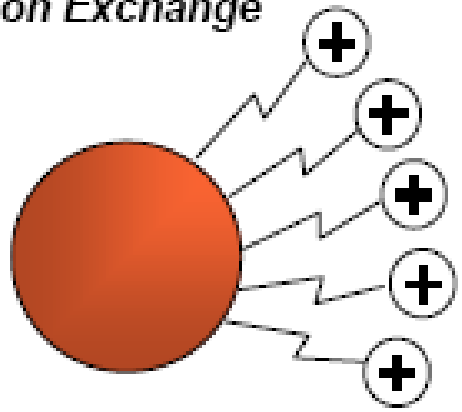
Ion Exchange Theory

Cation Exchange vs Anion Exchange

Cation Exchange



Anion Exchange

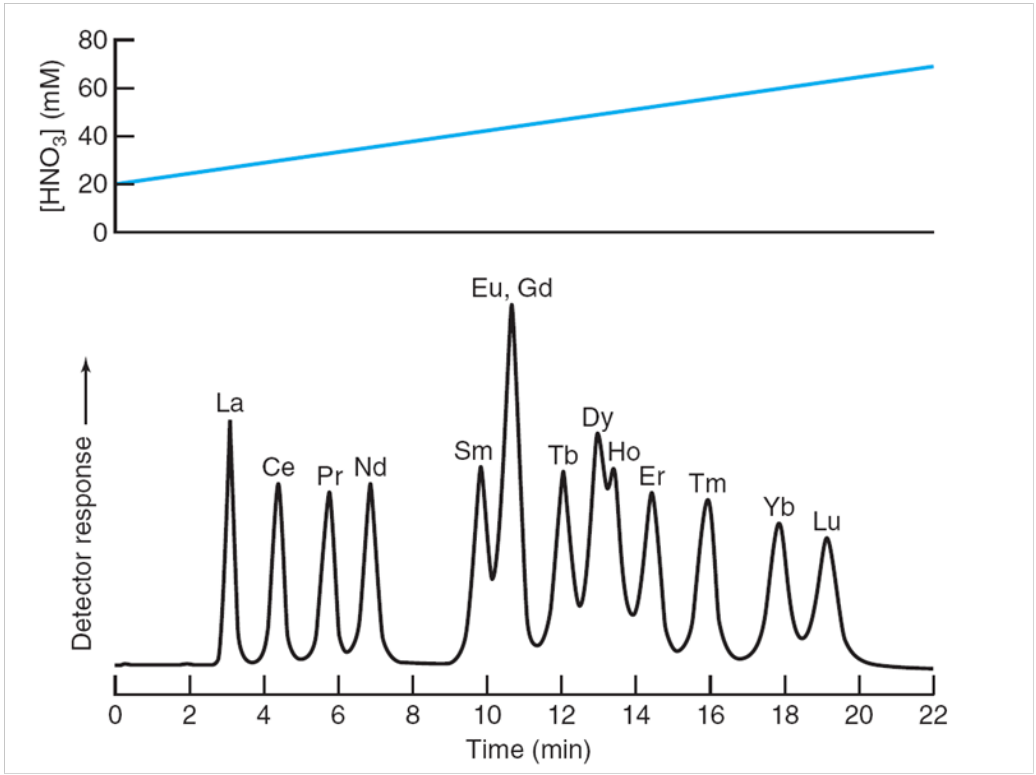


Cation exchange columns have a negative charge to attract cations.
Anion exchange columns have a positive charge to attract anions

IEC : Steps of separation

- Solutes are injected and bound;
- The column is washed to equilibrate it in the starting buffer of low ionic strength;
- The bound molecules are eluted off or separate one ion from another using a **gradient elution** of a buffer which steadily increases the ionic strength of the eluent solution or changing the pH.

- An $[H^+]$ gradient was used for a cation-exchange separation.
- More strongly bound cations require a higher $[H^+]$ to be eluted.



Sample problem 32

How is gradient is employed in ion exchange chromatography?

Guidelines for the principal categories of ion exchange

Analyte Type	*Weak* ACID e.g., pK_a ~ 5		*Strong* ACID	*Weak* BASE e.g., pK_a ~ 10		*Strong* BASE
Charge State vs. pH*	No charge at pH < 3	− [anion] at pH > 7	− [anion] Always Charged	+ [cation] at pH < 8	No Charge at pH > 12	+ [cation] Always Charged
Stationary Phase Particle	*Strong* Anion Exchanger		*Weak* Anion Exchanger e.g., pK_a ~ 10		*Strong* Cation Exchanger	*Weak* Cation Exchanger e.g., pK_a ~ 5	
Charge State vs. pH*	+ Always Charged		+ at pH < 8	No Charge at pH > 12	− Always Charged	No Charge at pH < 3	− at pH > 7
Mobile Phase pH Range							
to Retain analyte [capture]	pH > 7		pH < 8		pH < 8	pH > 7	
to Release analyte [elute]	pH < 3		pH > 12		pH > 12	pH < 3	

- To retain a strongly basic analyte [always positively charged], **use a *weak-cation* exchange** stationary phase particle at pH>7; this assures a negatively charged particle surface.

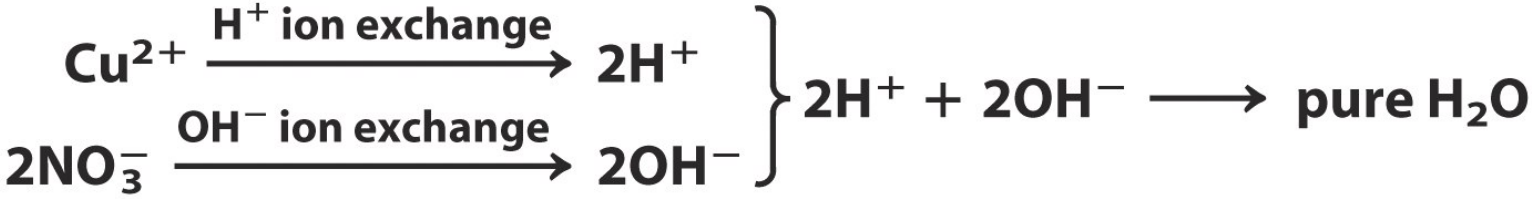
- **To release or elute the strong base**, **lower the pH of the mobile phase below 3**; this removes the surface charge and *shuts off* the ion-exchange retention mechanism.

- Do not use a strong-cation exchanger to retain a strong base; both remain charged & strongly attracted to each other, making the base nearly impossible to elute.

Application area of IEC :

- Ion analysis :
  - ✓ Separation & analysis of biological substances (peptides, proteins, enzymes, antibodies, nucleotides & olygonucleotides);
  - ✓ Separation of ionizable compounds.
- Purification of water : Deionized water

Purification of water : Deionized water (DI)



- DI is prepared by passing water through an anion exchange resin in its OH^- form & a cation exchange resin in its H^+ form.
- E.g. $Cu(NO_3)_2$ is present in the solution.
- The cation exchange resin binds Cu^{2+} & replaced it with $2H^+$.
- The anion exchange resin binds NO_3^- & replaced it with OH^-.
- The eluate is **pure water**.

IEC : Detection

- By measuring the conductivity of the solution.
- Since the mobile phase contains ions that create a background conductivity, making it difficult to measure the conductivity due only to the analyte ions as they exit the column.

- This problem can be greatly reduced by selectively removing the mobile phase ions after the analytical column & before the detector, i.e. by using a **suppressor column.**

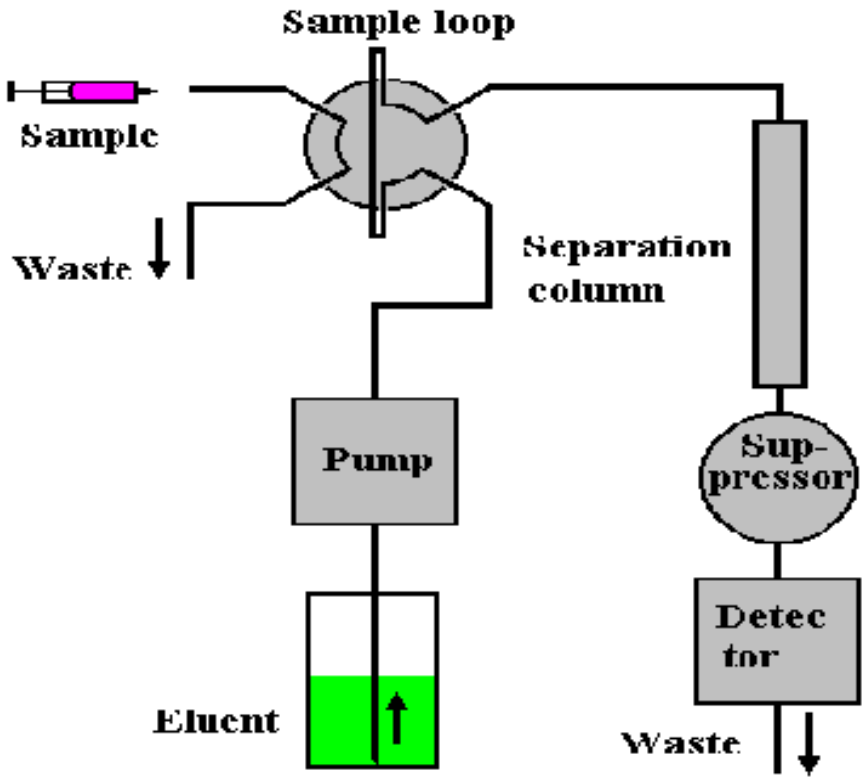
  - ✓ Suppressor column is a column packed with a second ion-exchange resin of opposite charge to the analytical column & placed after the analytical column.

- ✓ Using a suppressor column,
  - the mobile phase ions from the analytical column will be removed before reaching the detector by converting the ions of the mobile phase to a molecular species.

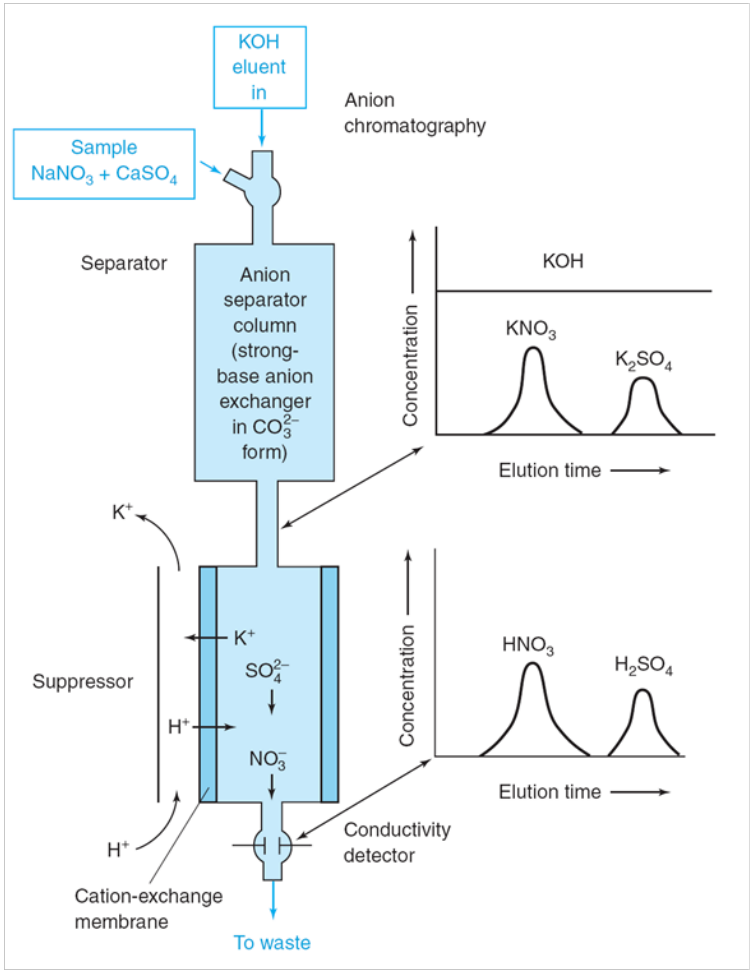
Ion Chromatography

- A high-performance version of ion-exchange chromatography, with a key modification that removes eluent ions & exchanges the analyte ion for H^+ (cations) or OH^- (anions) before detecting analyte ions.

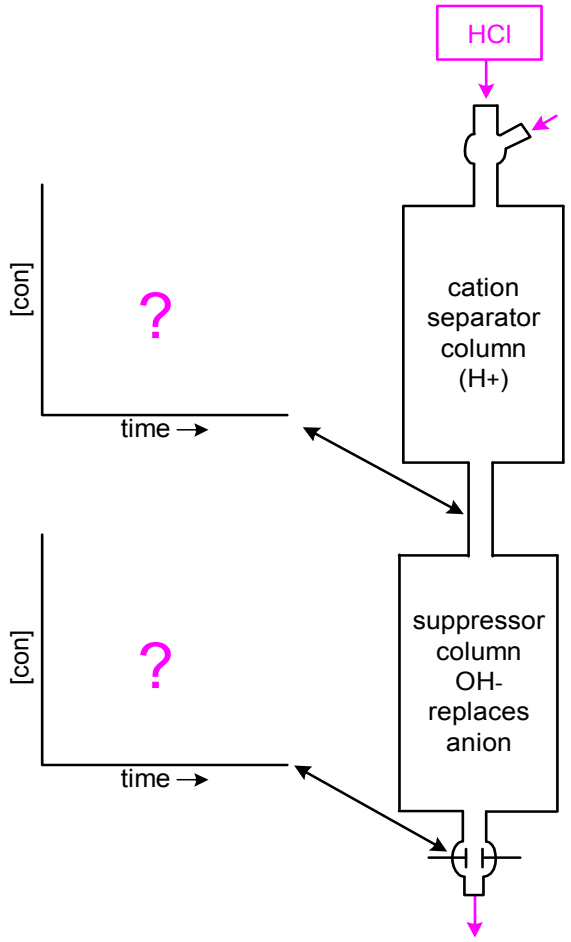
Schematic diagram of ion chromatography set-up



Suppressed-ion anion chromatography



Suppressed-ion cation chromatography



- Consider a sample containing $NaNO_3$ & $CaSO_4$ injected into an anion exchange column in the carbonate form, followed by elution with KOH.

- NO_3^- & SO_4^{2-} equilibrate with the resin & are slowly displaced by the OH^- eluent.

- Na^+ & Ca^{2+} cations are not retained & simply wash through.

- KNO_3 & K_2SO_4 are eluted from the separator column.

- These species cannot be easily detected because the solvent contains a high conc. of KOH, which has high conductivity compare to the analytes.

- To solve the problem, the solution next passes through a suppressor, in which cation are replaced by H^+.

- H^+ exchange with K^+, through a cation exchange membrane in the suppressor.

- H^+ diffuses from high conc. outside the membrane to low conc. inside the membrane.

- K^+ diffuses from high conc. inside to low conc. outside.
- K^+ is carried away outside the membrane, so its conc. is always low on the outside.
- The net result is that KOH eluent, which has high conductivity, is converted into H_2O, which has low conductivity.
- Analyte is converted to the corresponding acid, HNO_3 of H_2SO_4 with high conductivity & can be detected.

- For anion analytes, a mixture of MA & NaOH emerge from the column & react in the suppressor cation column as follow :

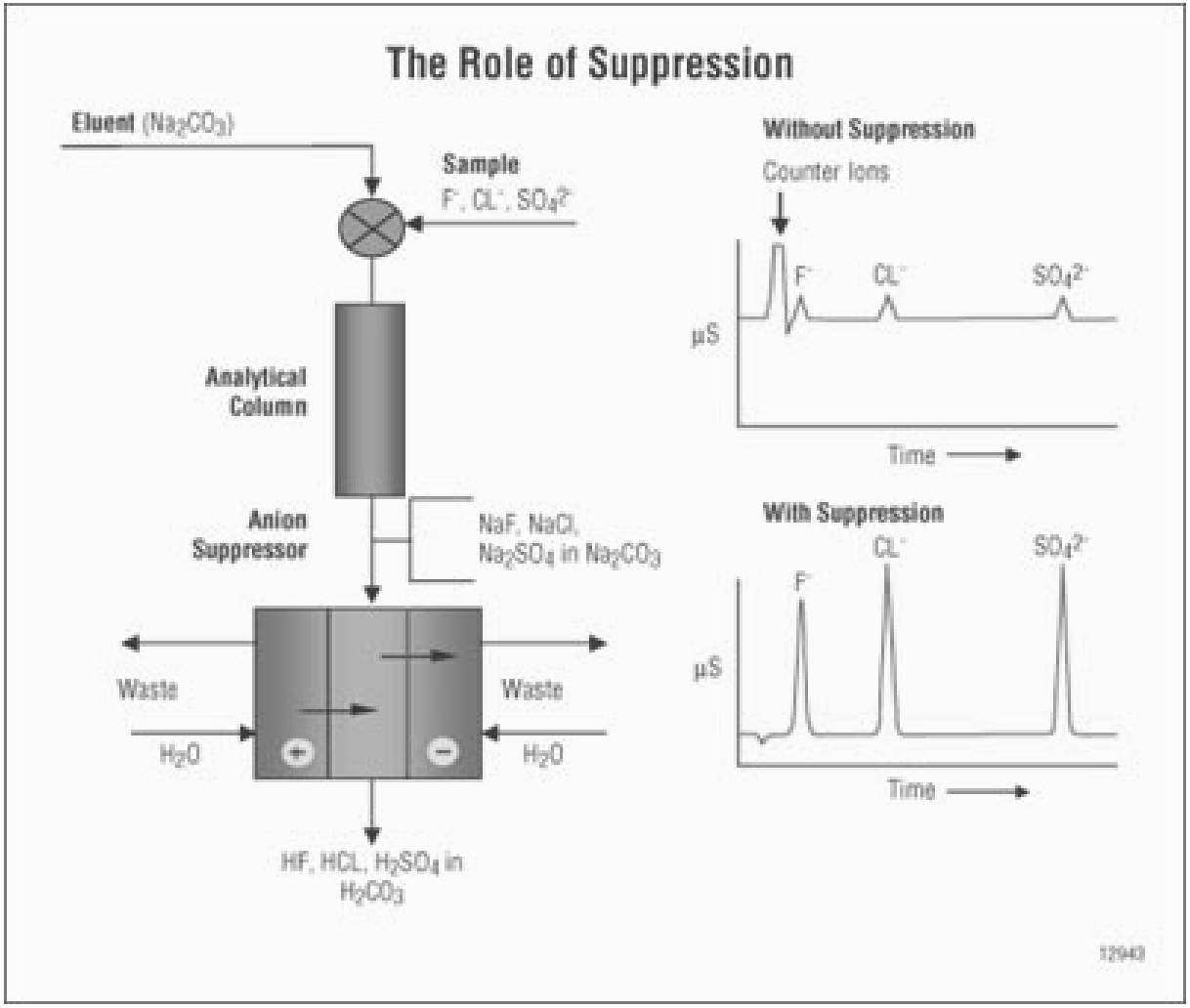
$$RSO_3^-H^+ + M^+ + A^- \rightarrow RSO_3^-M^+ + H^+ + A^-$$

$$RSO_3^-H^+ + Na^+ + OH^- \rightarrow RSO_3^-Na^+ + H_2O$$

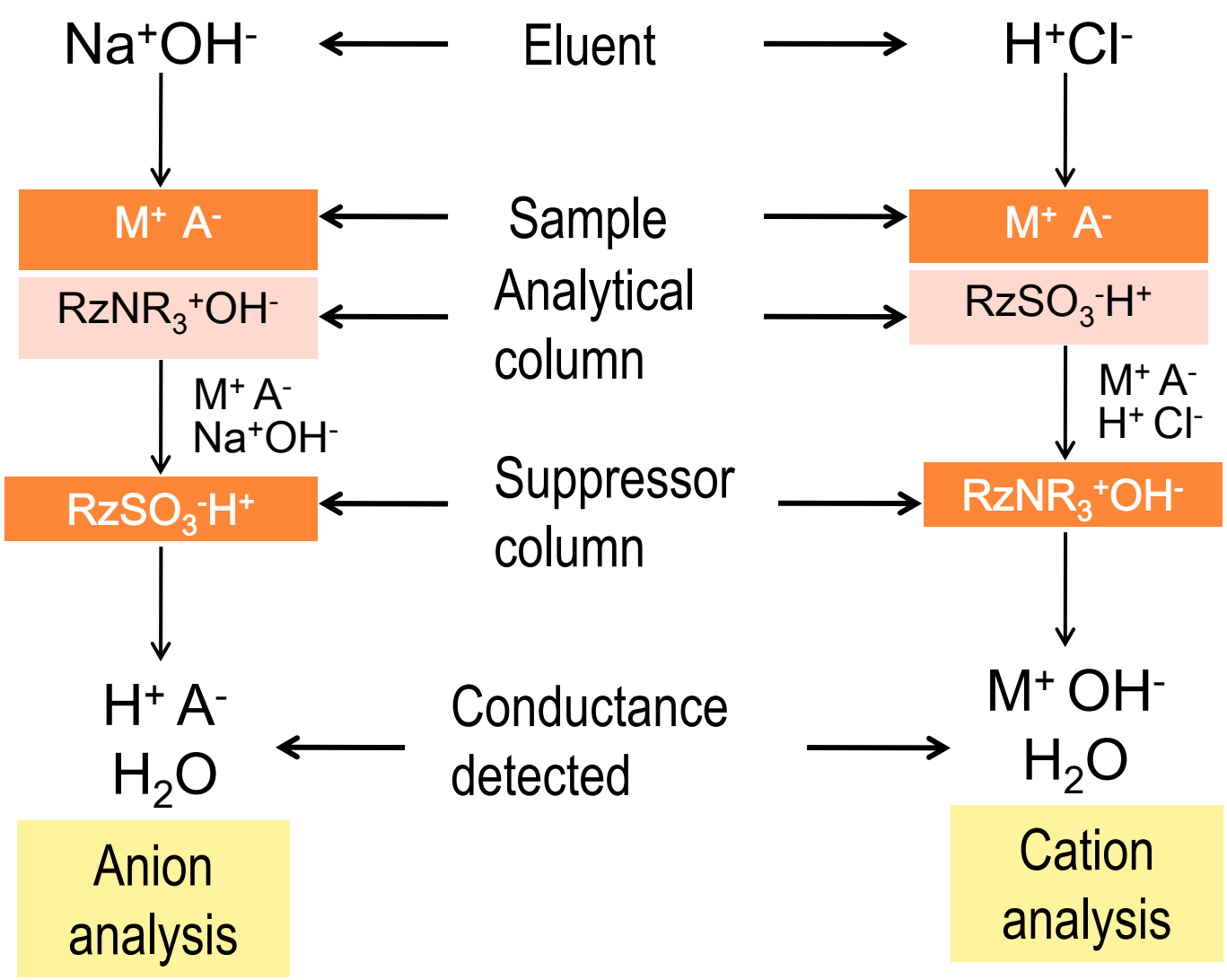
- For cation analytes, a mixture of MA & HCl emerge from the column & react in the suppressor anion column as follow :

$$RNR_3^+OH^- + M^+ + A^- \rightarrow RNR_3^+A^- + M^+ + OH^-$$

$$RNR_3^+OH^- + H^+ + Cl^- \rightarrow RNR_3^+Cl^- + H_2O$$


The Role of Suppression
Eluent (Na_2CO_3)
Sample
F^-, CL^-, SO_4^{2-}
Analytical Column
Anion Suppressor
NaF, NaCl, Na_2SO_4 in Na_2CO_3
Waste
H_2O
Waste
H_2O
HF, HCL, H_2SO_4 in H_2CO_3
Without Suppression
Counter Ions
F^-
CL^-
SO_4^{2-}
µS
Time
With Suppression
CL^-
SO_4^{2-}
F^-
µS
Time
12940

Principle of ion chromatography



Sample problem 33

For a separation of metal ions (Mg, Ca, Na) in a water sample, suggest the :

Reaction the occur in the suppressor column

Sample problem 34

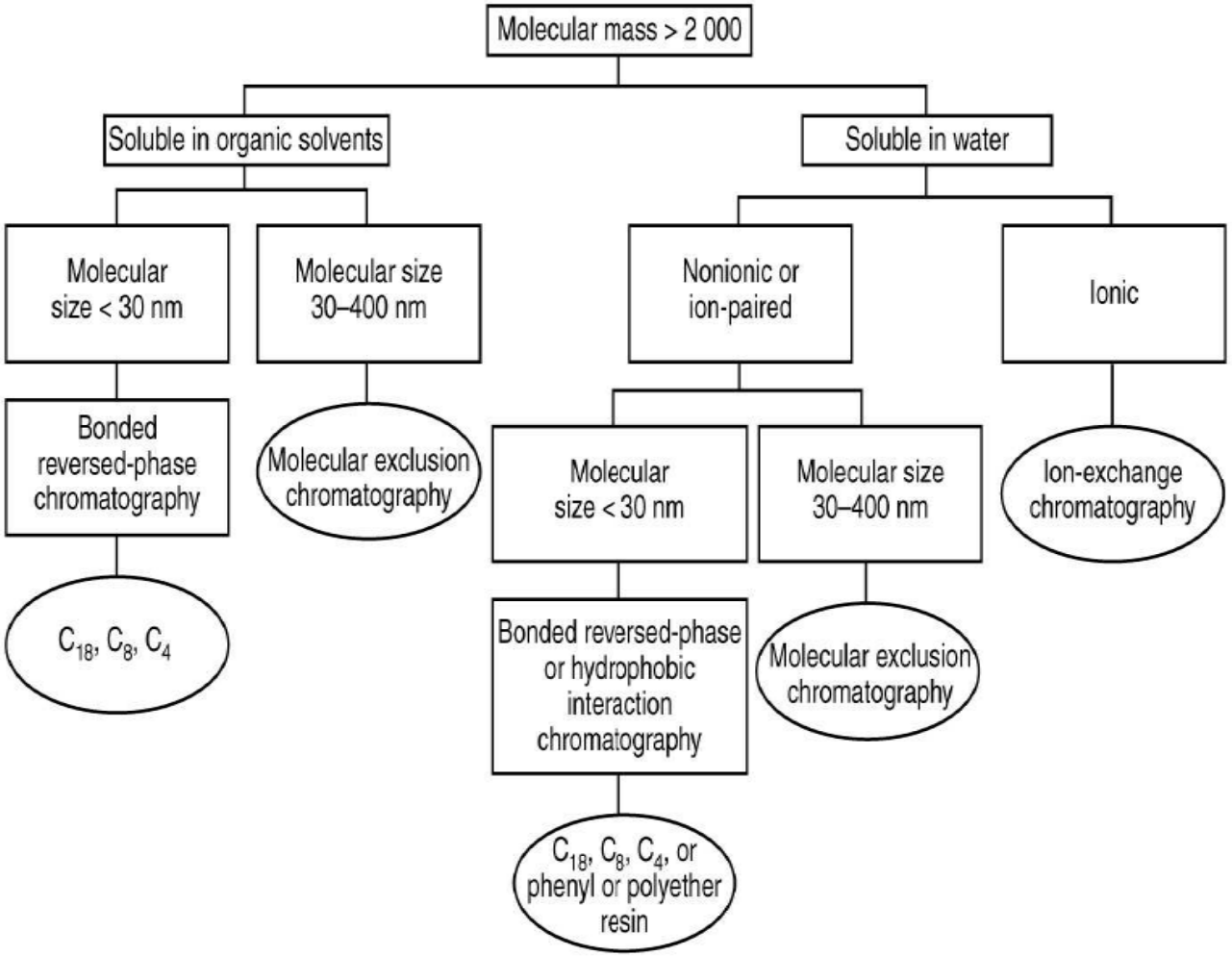
Given three analytes, **Anion A^-**, **Anion B^{2-}** & **Anion C^{3-}** which were separated using a strong anion exchange resin.

i. Suggest a stationary phase that can be used in the above separation.

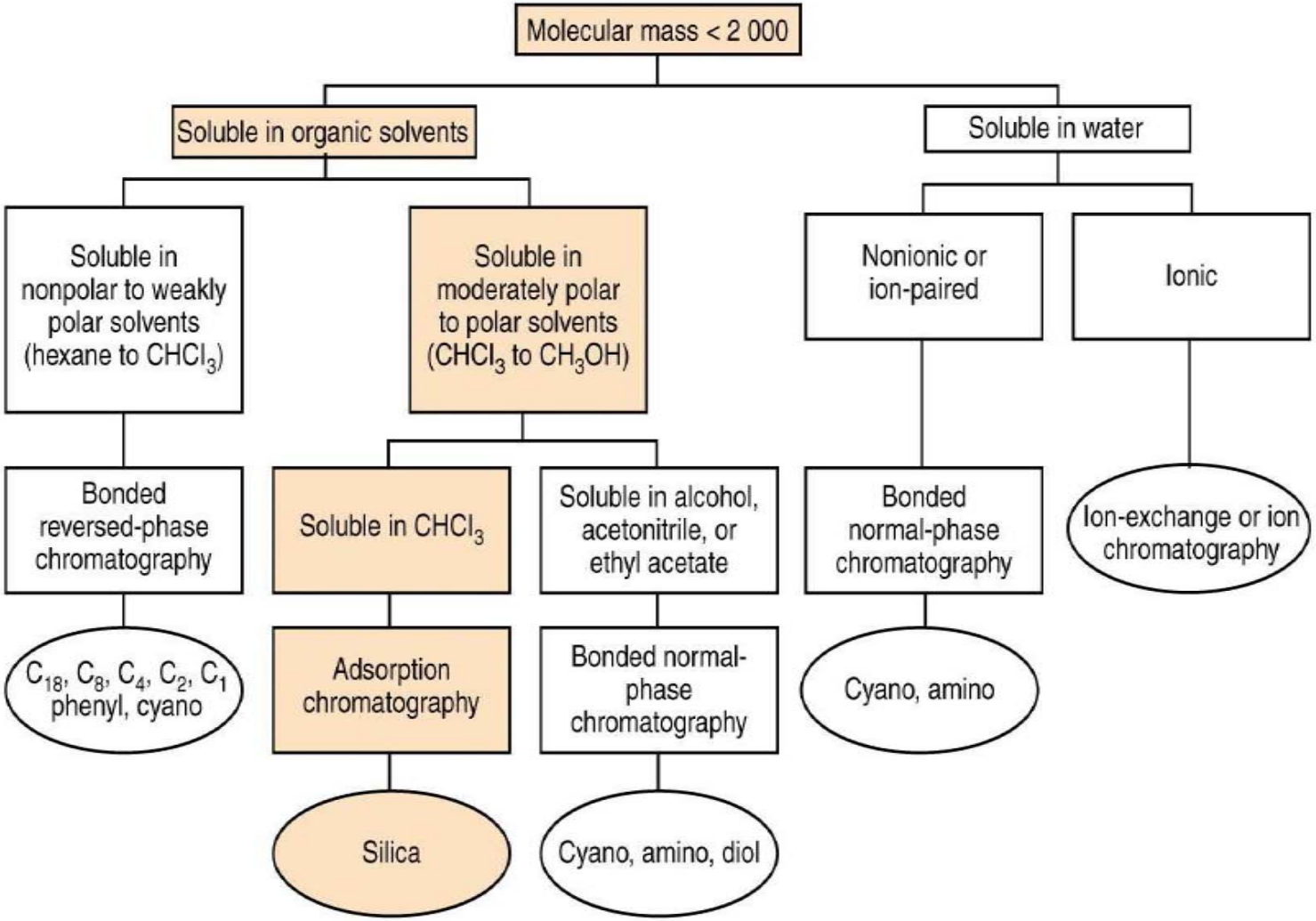
ii. Suggest a suitable mobile phase that can be used in the above separation.

iii. Predict the order of elution of the above anion mixture. Explain.

HPLC applications



HPLC applications



THE END