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Recap: Topics under Chromatographic

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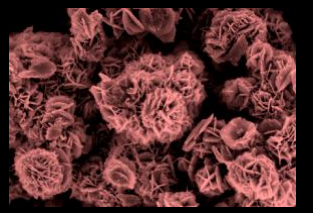
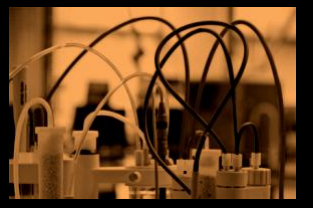
- Principle of Chromatography
- Classification of Chromatographic Techniques
- Qualitative Analysis and Quantitative Analysis
- Gas Chromatography (GC) -Principles and Instrumentation
- Liquid Chromatography (LC) – Principles and Instrumentation
- Thin Layer Chromatography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Liquid Chromatography-Principles and Instrumentation

CEB 4032/CFB3032: ANALYTICAL CHEMISTRY/ANALYTICAL INSTRUMENTATION

TS CHM DR. MOHD DZUL HAKIM WIRZAL

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Introduction to Liquid Chromatography (LC)

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- LC → most widely used analytical technique for liquid separation.

- Compare to GC (mobile phase: **gas**), LC (mobile phase: **liquid**) is relatively important because most compounds are not sufficiently volatile for GC.

- Advantages include:
  - greater sensitivity
  - ready adaptability to accurate quantitative determinations
  - ease of automation
  - suitability for separating nonvolatile species

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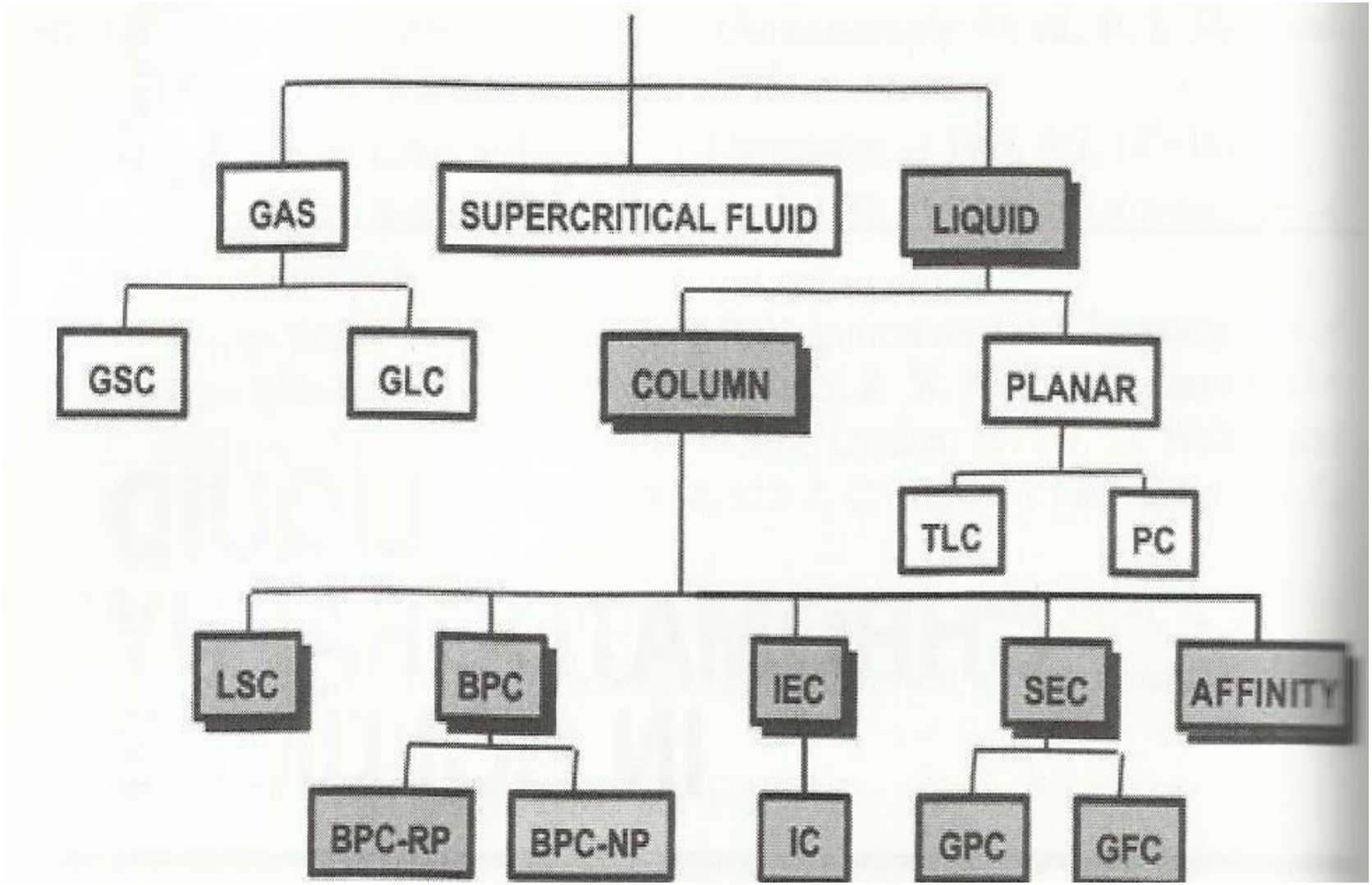
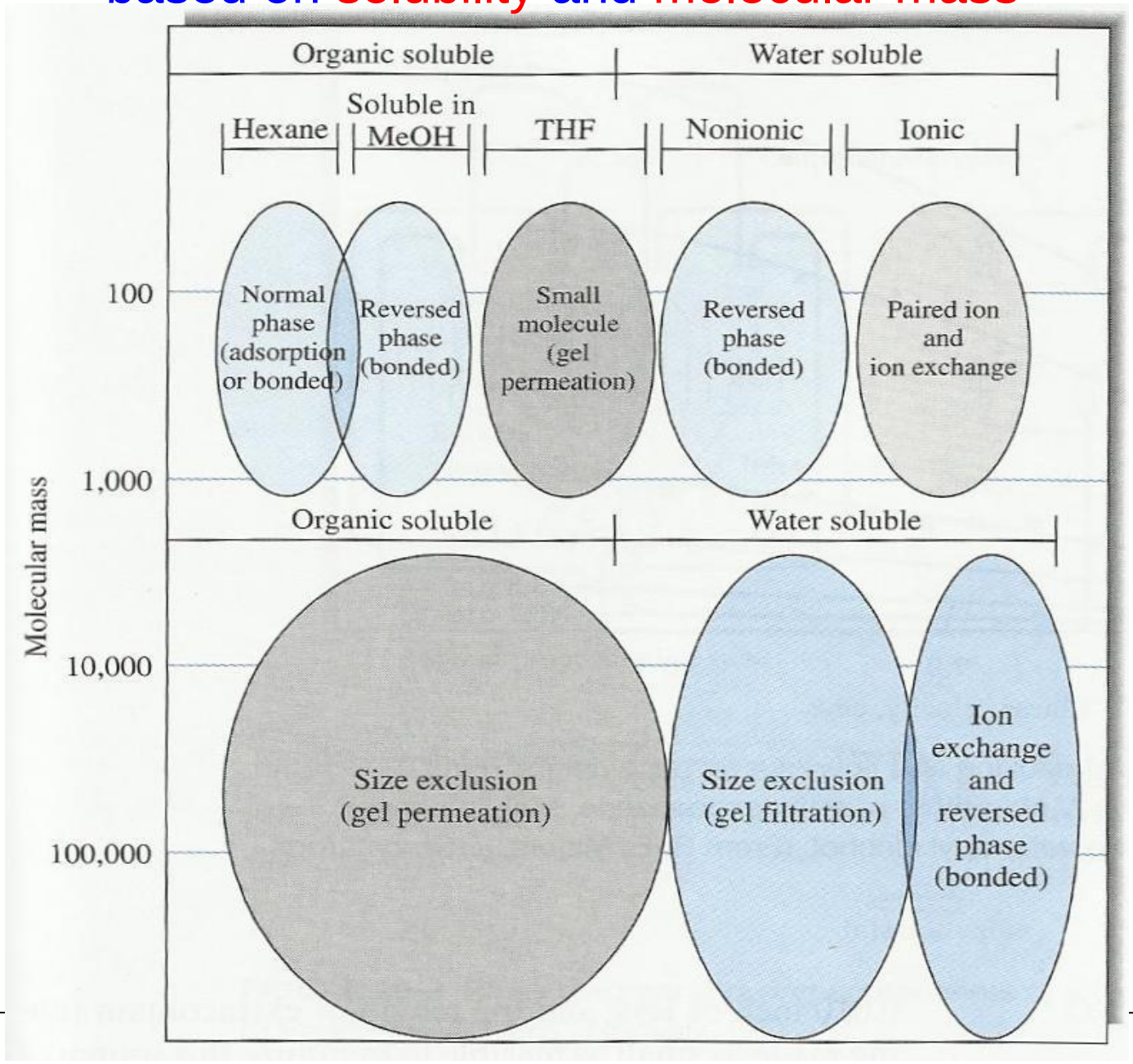
- Example samples: amino acids, proteins, nucleic acids, hydrocarbons, carbohydrates, drugs, pesticides, antibiotics, steroids and variety of inorganic substances.

- The varieties of liquid chromatographic include:
  i. Partition
  ii. Adsorption
  iii. Ion exchange
  iv. Size-exclusion
  v. Affinity

Classification of Chromatography Techniques



Selection of LC modes → Methods can be chosen based on solubility and molecular mass



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High Performance Liquid Chromatography (HPLC)

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- In the early development of LC, the increases in column efficiency could be achieved by decreasing the particle size of packings → packings with particles diameter as small as 3-10 μm was developed.
- This technology require instruments operating at high pressures, which contrasted with the classic liquid chromatography.
- Liquid chromatography (LC) → High Performance liquid chromatography (HPLC).
- Classical LC has largely been replaced by much more powerful and analytically useful form of HPLC.

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Introduction–HPLC

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- Uses of high pressure to force solvent through closed columns containing very fine particles that give high-resolution separations.
- The rate of distribution of solutes between the stationary and mobile phase in LC is largely diffusion controlled.
- Diffusion in liquids is 100 times slower that diffusion in gas.
- It is not generally feasible to use open tubular columns because the diameter of the solvent channel is too great to be traversed by a solute molecule in a short time→ packed column is used.

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Instrumentation: HPLC

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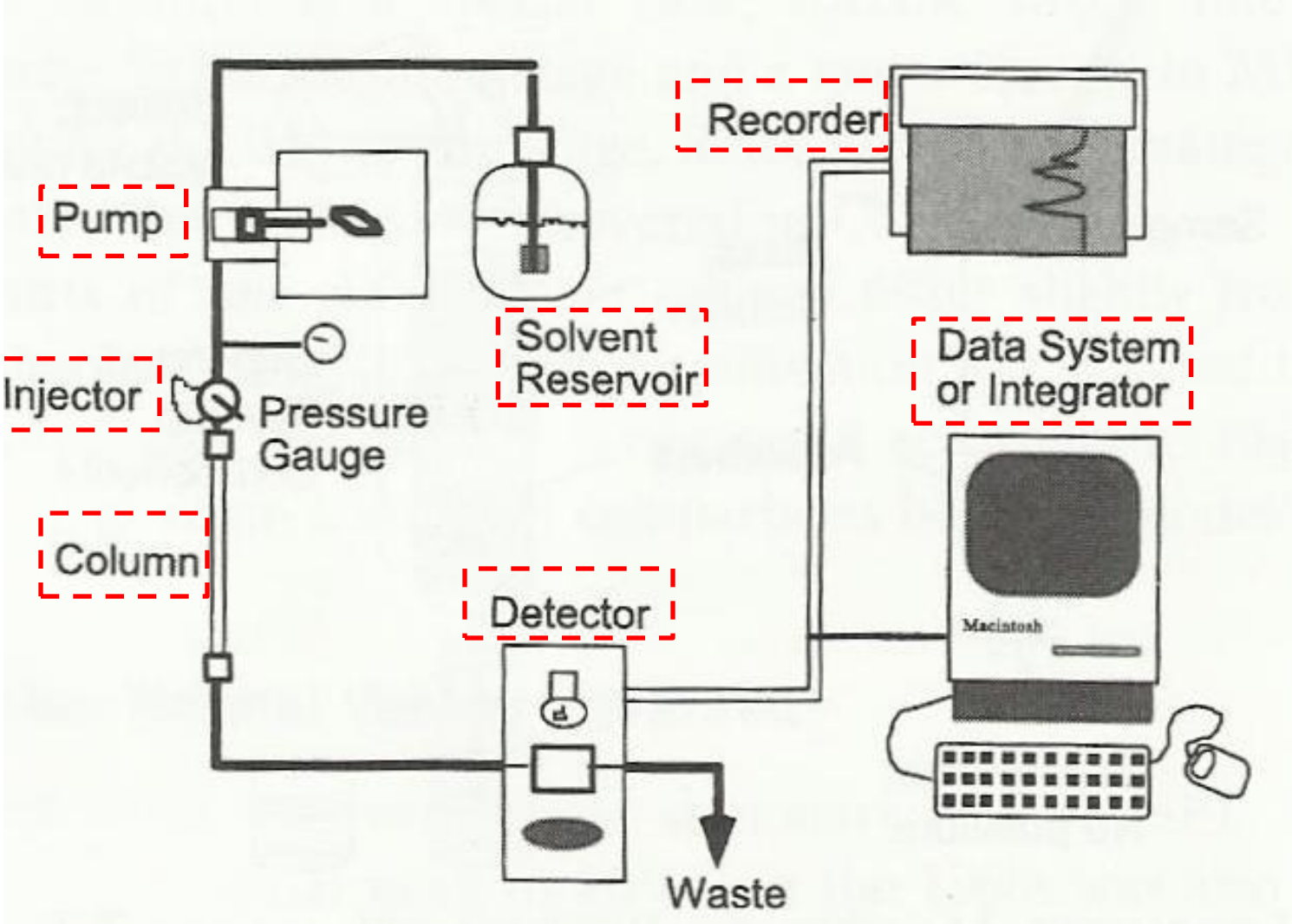
- Consists of:

1. Solvent delivery system
2. Sample injection valve
3. High pressure column
4. Detector
5. Data system

Basic Components of HPLC



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General Operation of HPLC:

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- A reservoir holds the solvent (mobile phase system).
- A high-pressure pump (solvent delivery system) is used to generate and to meter a specified flow rate of mobile phase, typically milliliters per minute.
- An injector (sample injection or autosampler) is able to introduce the sample into the continuously flowing mobile phase stream that carries the sample into the HPLC column.
- The column contains the chromatographic packing material needed to effect the separation → stationary phase.

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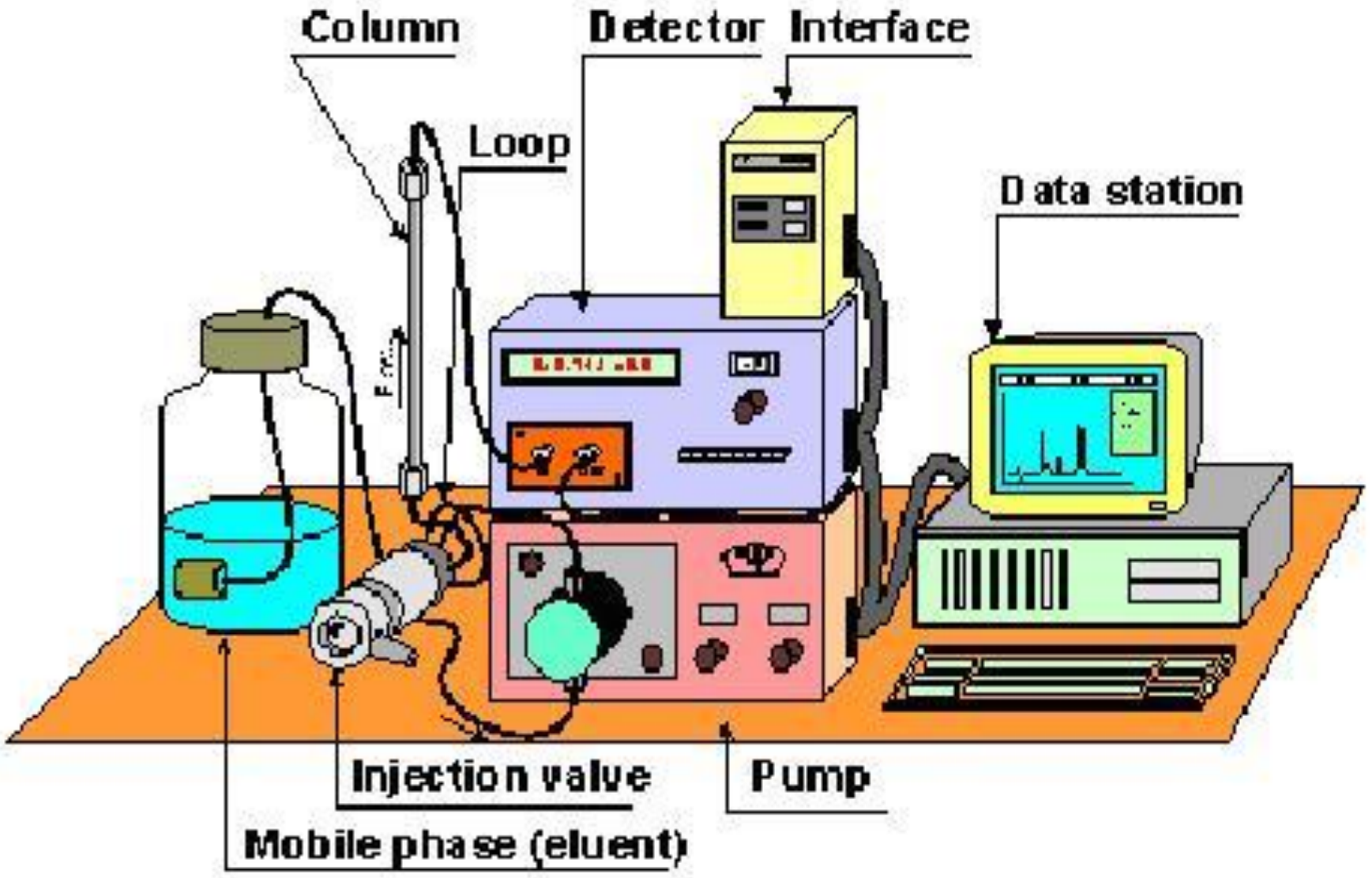
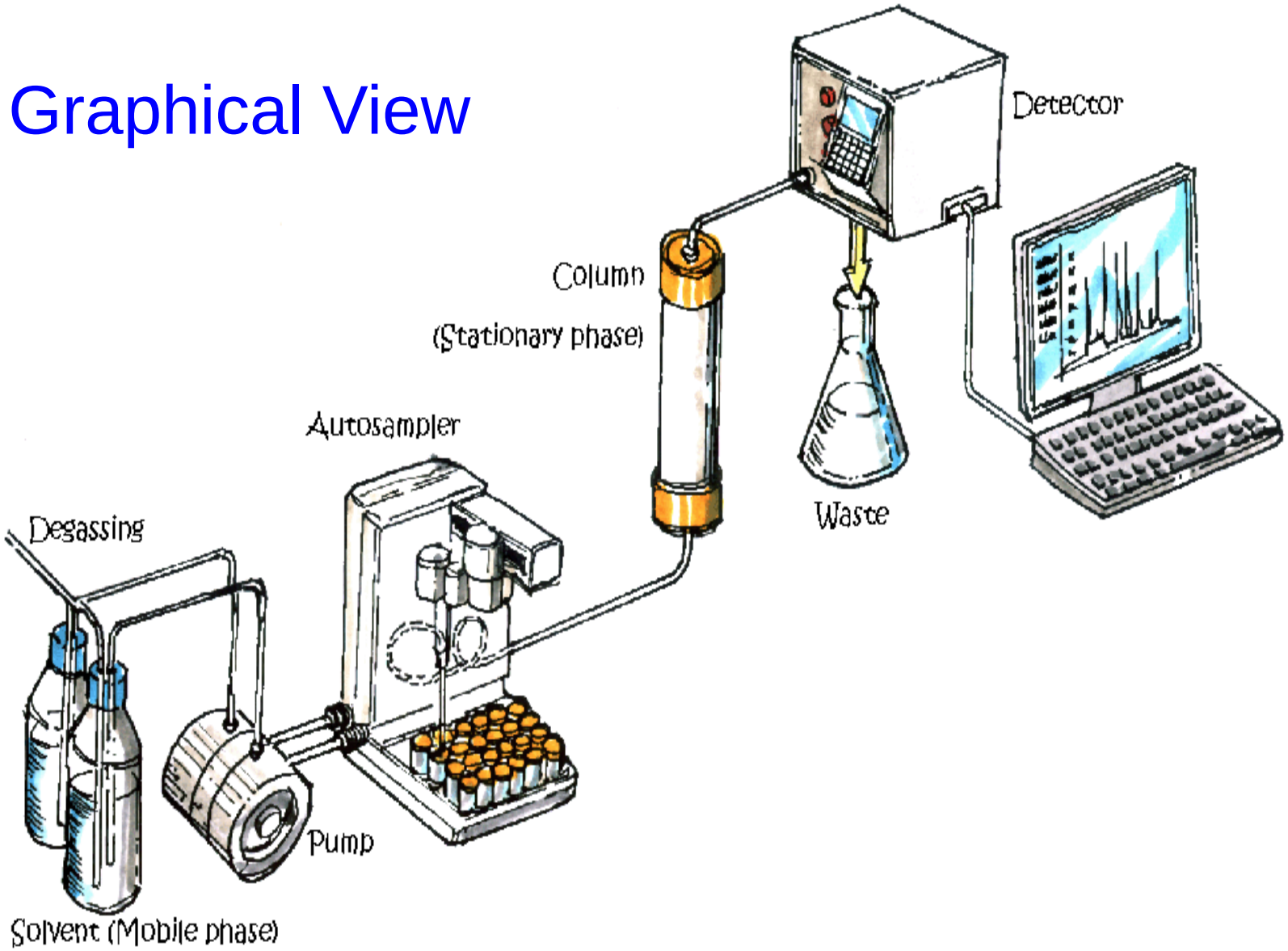
- A detector is needed to detect the separated compound bands as they elute from the HPLC column.

- The mobile phase exits the detector and can be sent to waste.

Graphical View

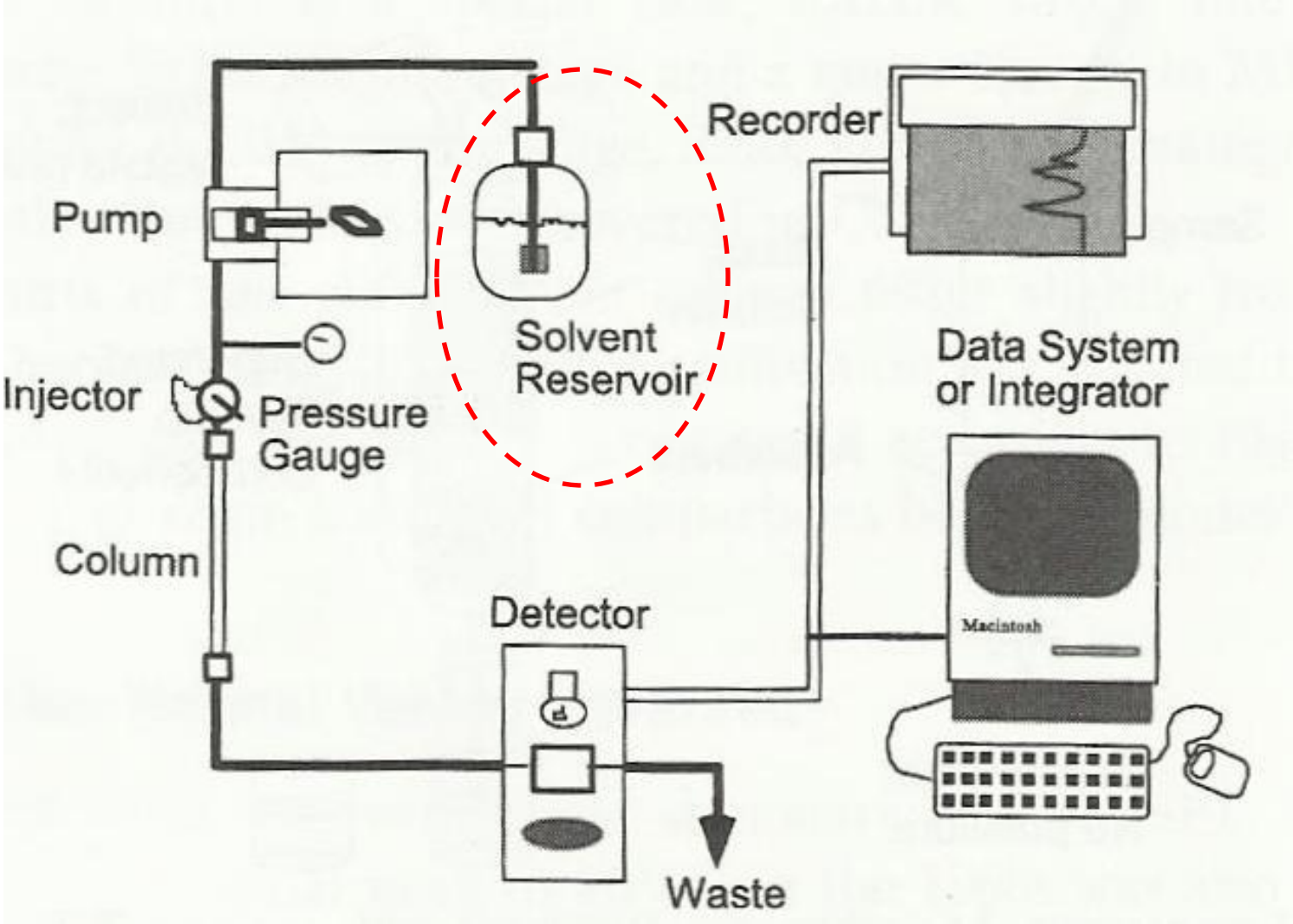


Graphical View



Modern HPLC

Solvent Delivery (Mobile Phase) System



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Solvent Delivery (Mobile Phase) System

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- Contains a pump to provide the high pressure required.
- The solvent reservoirs can be filled with a range of solvents of different polarities.
- The solvents must be pure and be degassed to avoid formation of gas bubbles for proper check valve function when enter piston chamber.

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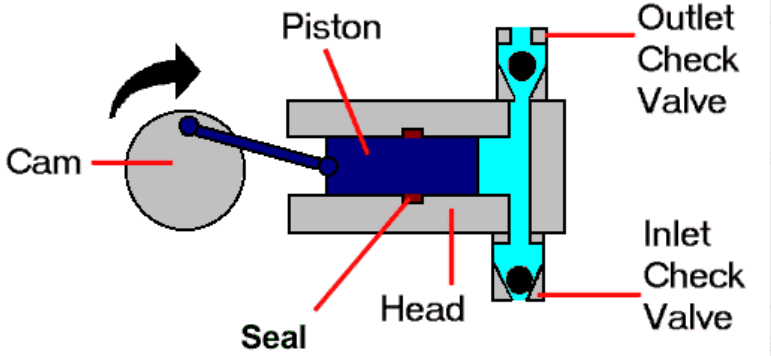
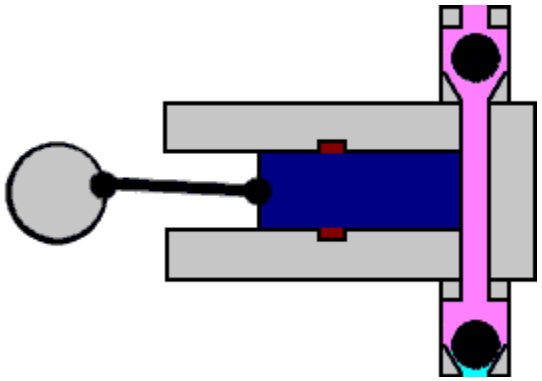
- Typical flowrate → 1-2 ml/min.
- Solvent used for HPLC should be of "HPLC" grade → extends the pump life by preventing scoring and reduces contamination of plugging of the column.
- The most commonly used pump for HPLC is the reciprocating pump → has small cylindrical piston chamber that is alternately filled with mobile phase and emptied via movement of the piston.
- Advantages: small internal volume, capable of high output pressure, constant flow rates, independent of solvent viscosity or column backpressure.

HPLC pump


Piston
Outlet
Check
Valve
Cam
Inlet
Check
Valve
Head
Seal




Waters
515 HPLC Pump

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Solvent Reservoir

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Solvent Selection

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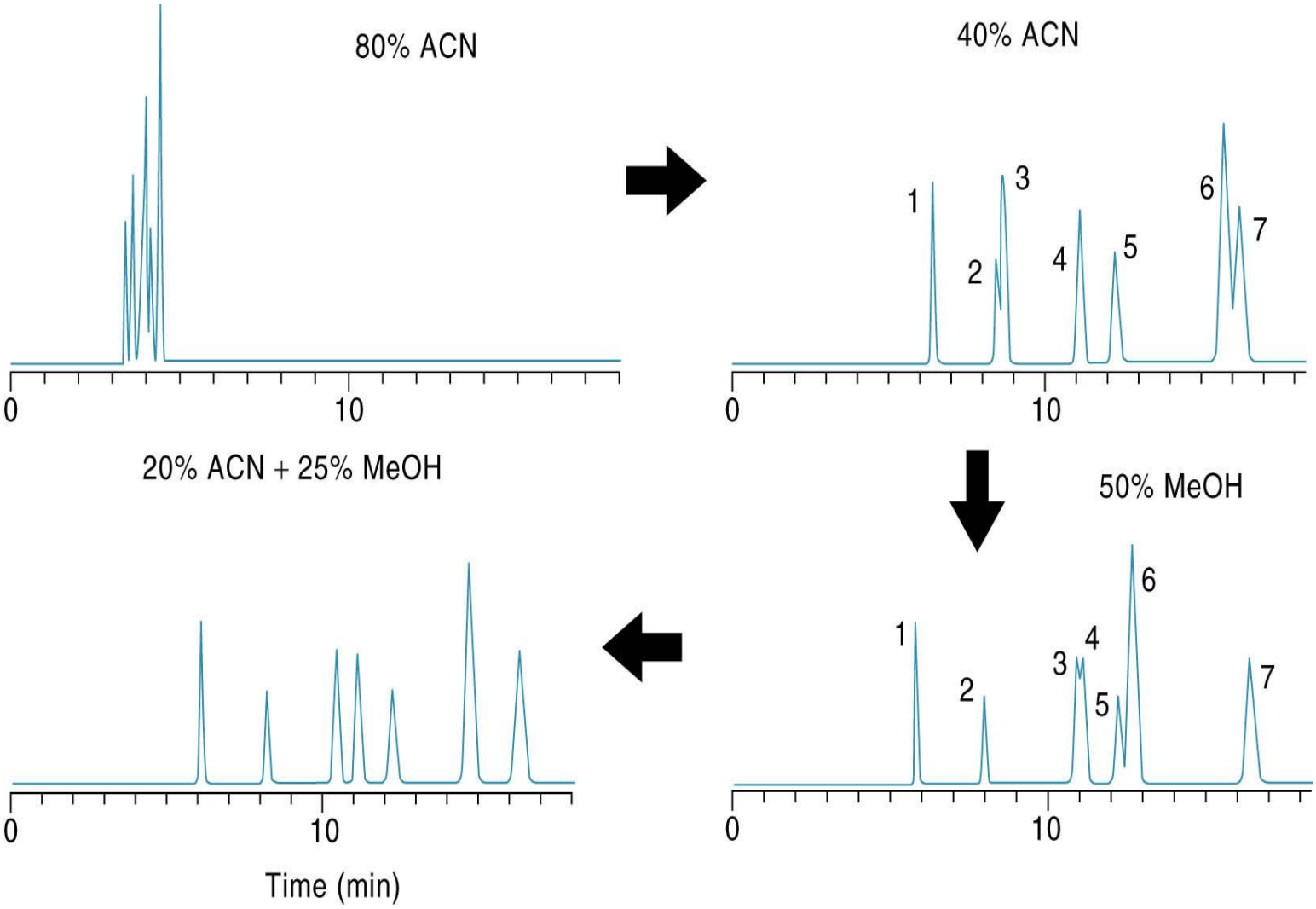
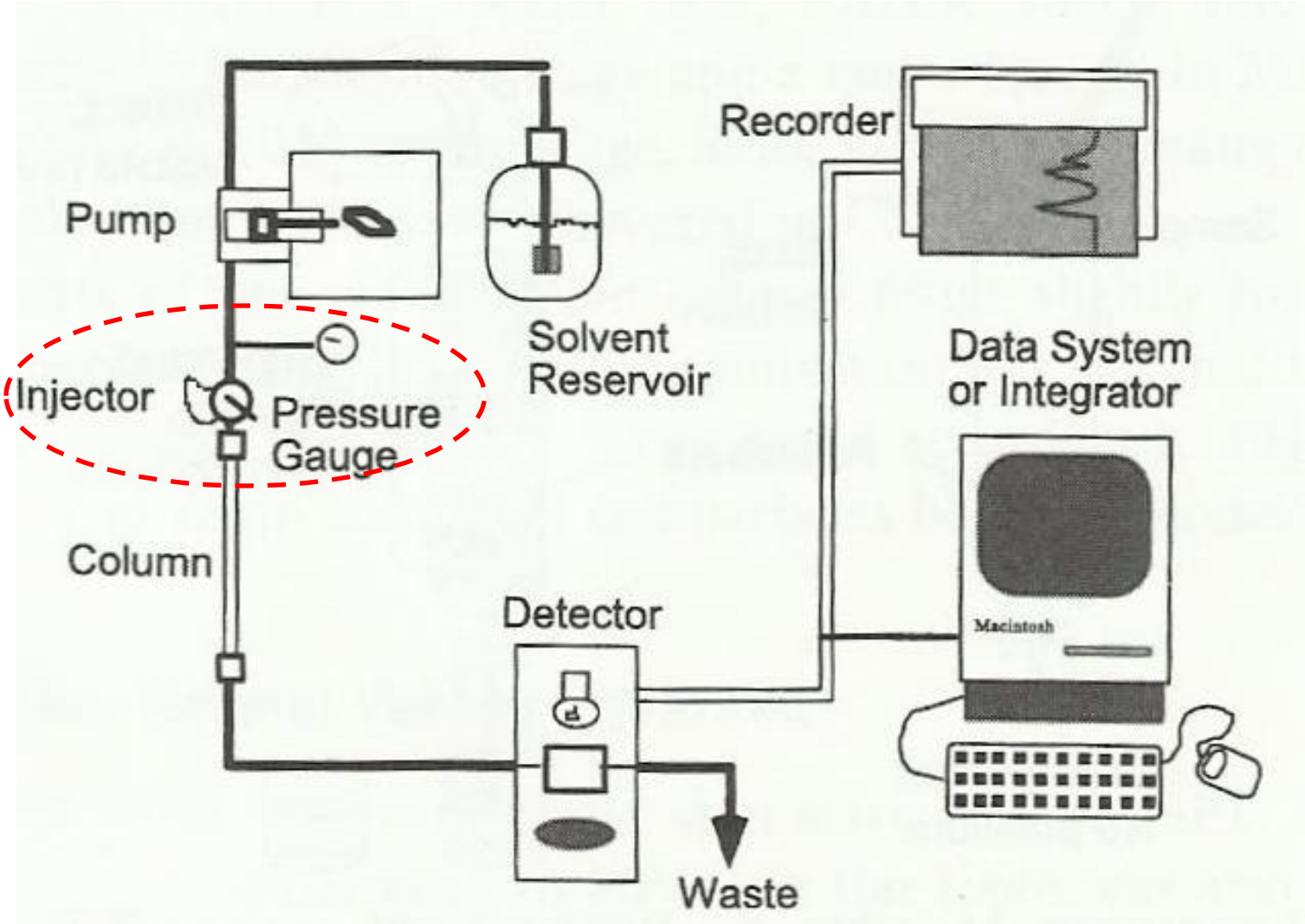
- Pure solvent will not provide sufficient separation of a range of compounds, and a blend of two or more solvents is used.
- Example: 2 solvents → A and B (strong).
- If we have incomplete separation, a change in %B from 35 to 40 or 45% will often result in better resolution.

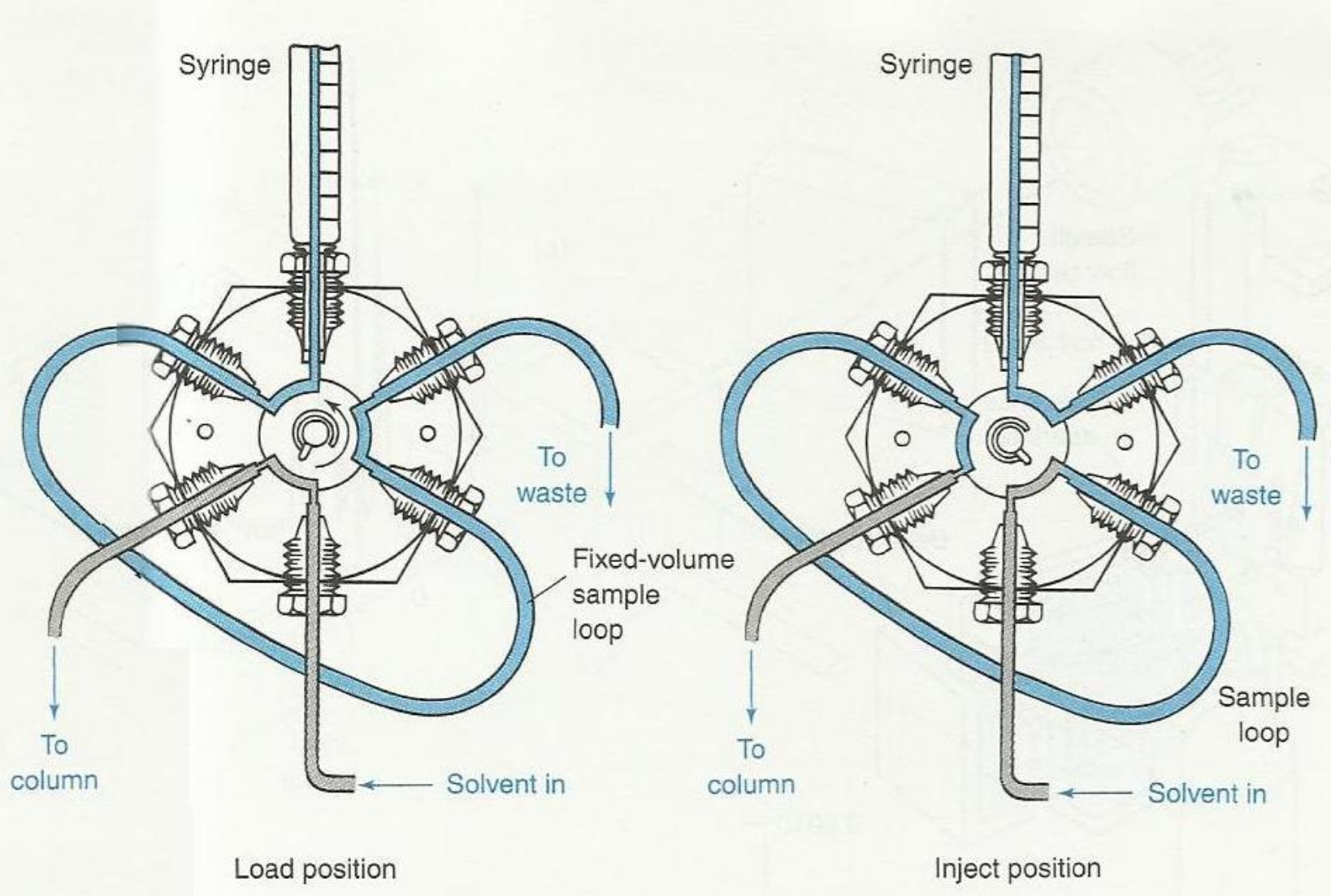


Hypothetical series of method development experiments, beginning with strong mobile phase of 80% acetonitrile-water.

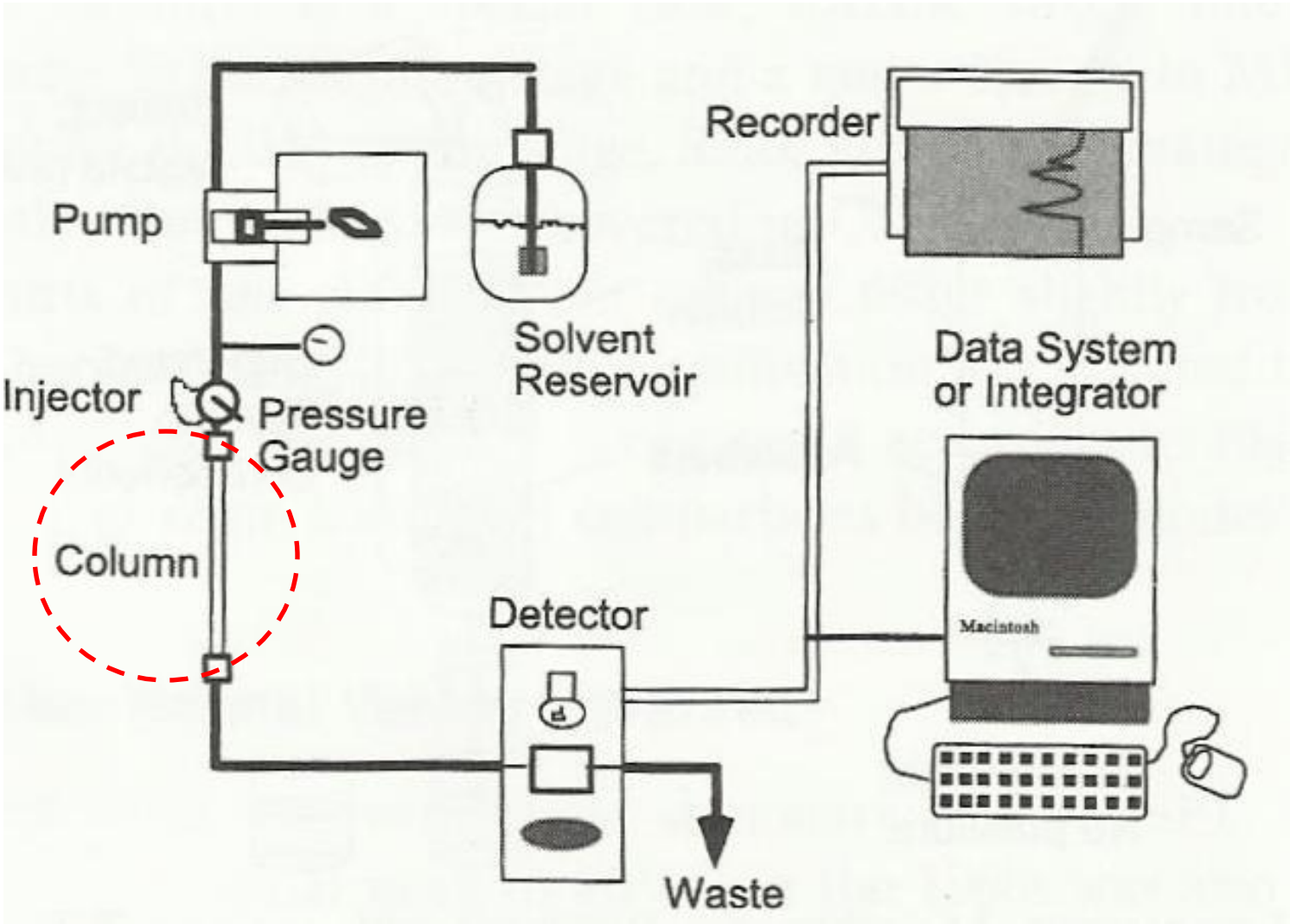
Sample Injection System



Sample Injection System



Column


Recorder
Pump
Solvent
Reservoir
Data System
or Integrator
Injector
Pressure
Gauge
Column
Detector
Macintosh
Waste

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Columns

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- Usually constructed from stainless steel or plastic columns that are 5-30 cm in length, with inner diameter of 1-5 mm.
- Expensive and easily degraded by dust or particles in the sample or solvent.
- The entrance to the main column is protected by a short guard column containing same stationary phase as the main column.
- Fine particles and strongly absorbed solutes are retained in the guard column which is periodically replaced.

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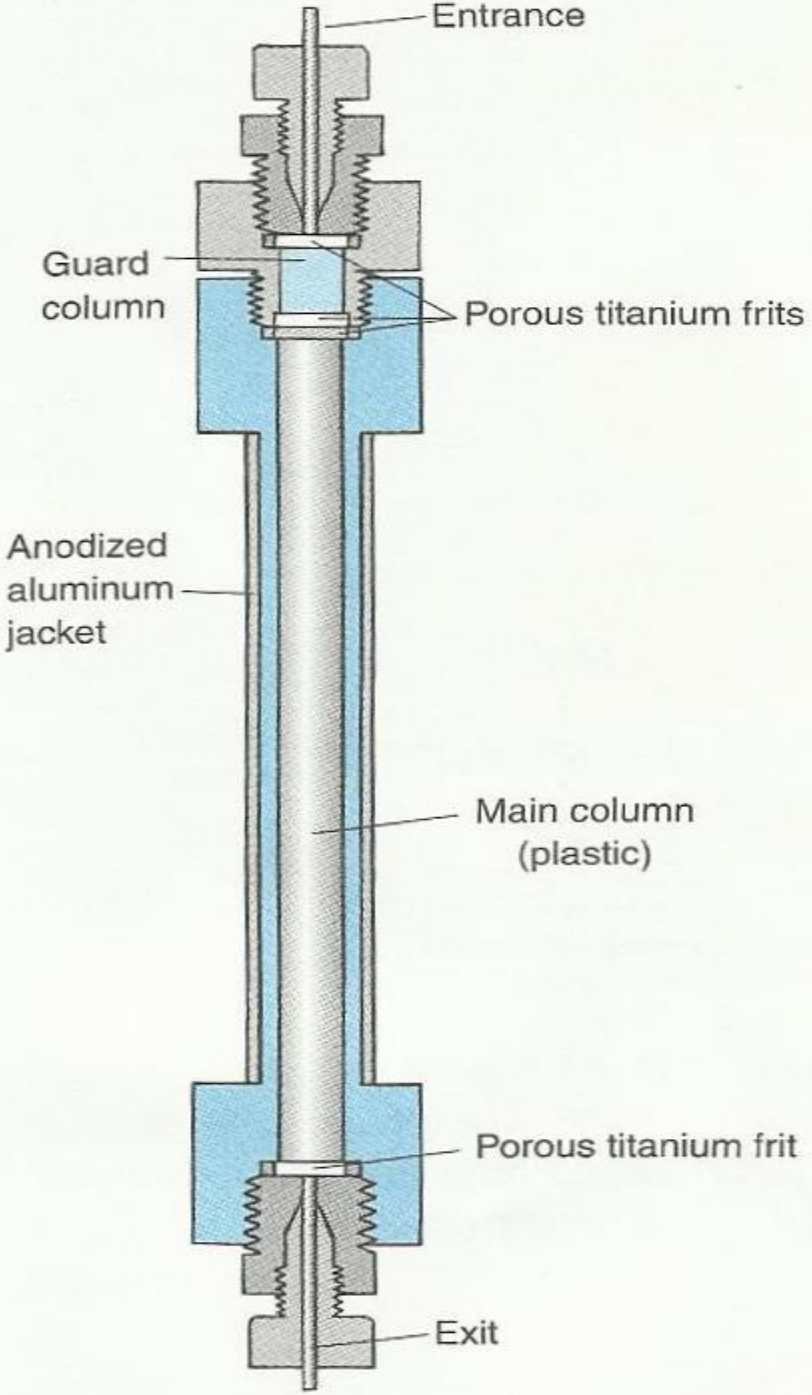

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HPLC column with replaceable guard column to collect irreversibly adsorbed impurities.

Titanium frits distribute the liquid evenly over the diameter of the column.

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Effect of Column Temperature

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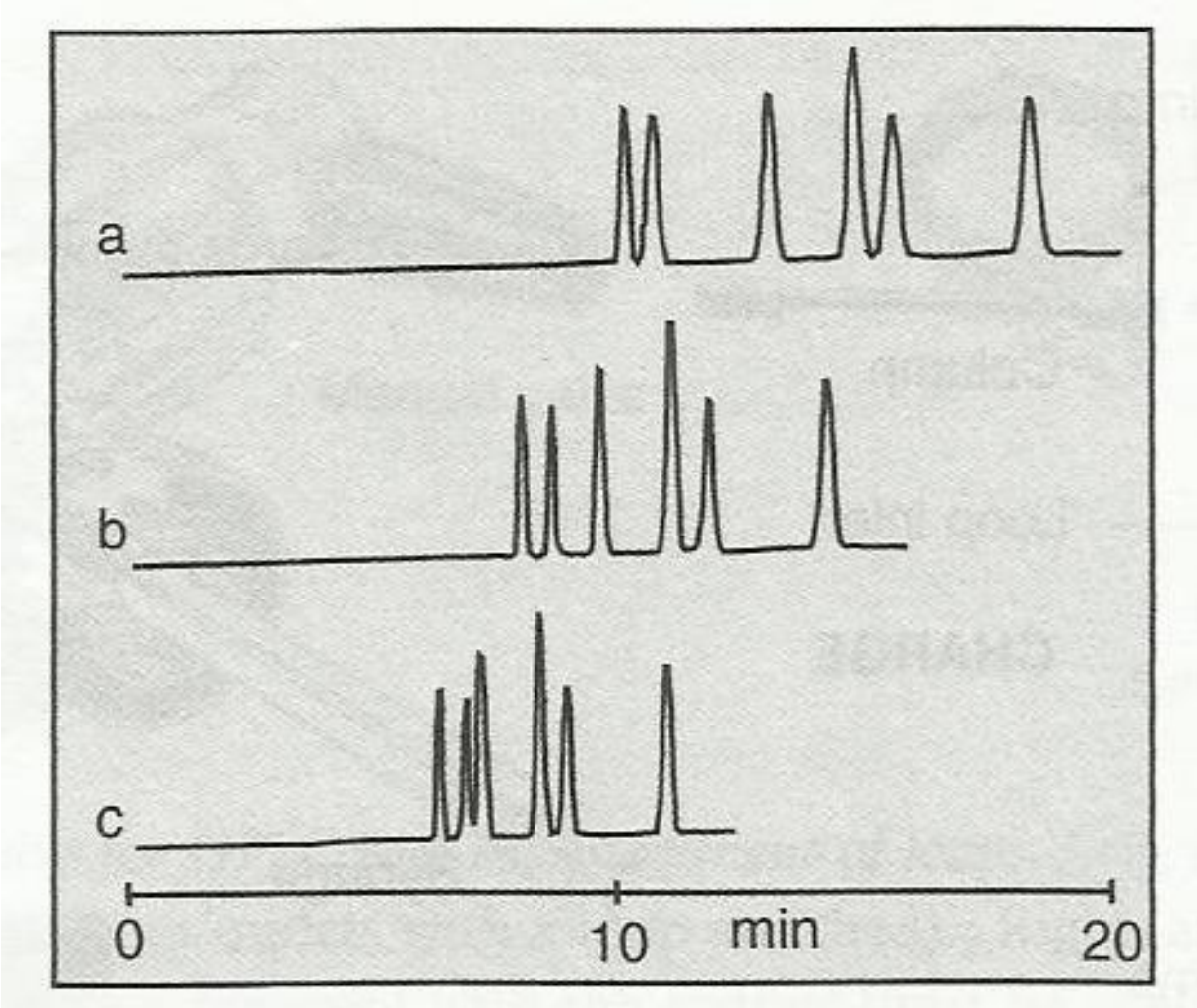
- Heating a column →decreases the viscosity of the solvent →reducing the required pressure or permitting faster flow.

- Increased temperature

  → decreases retention times by speeding the diffusion of solutes.

  → poor resolution, degrade the stationary phase and decrease column lifetime.

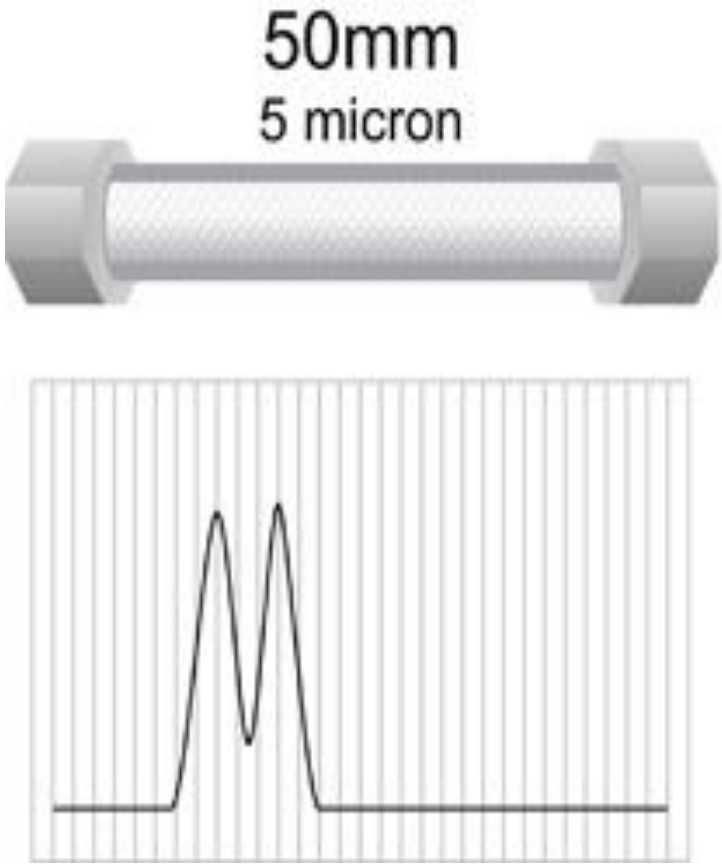


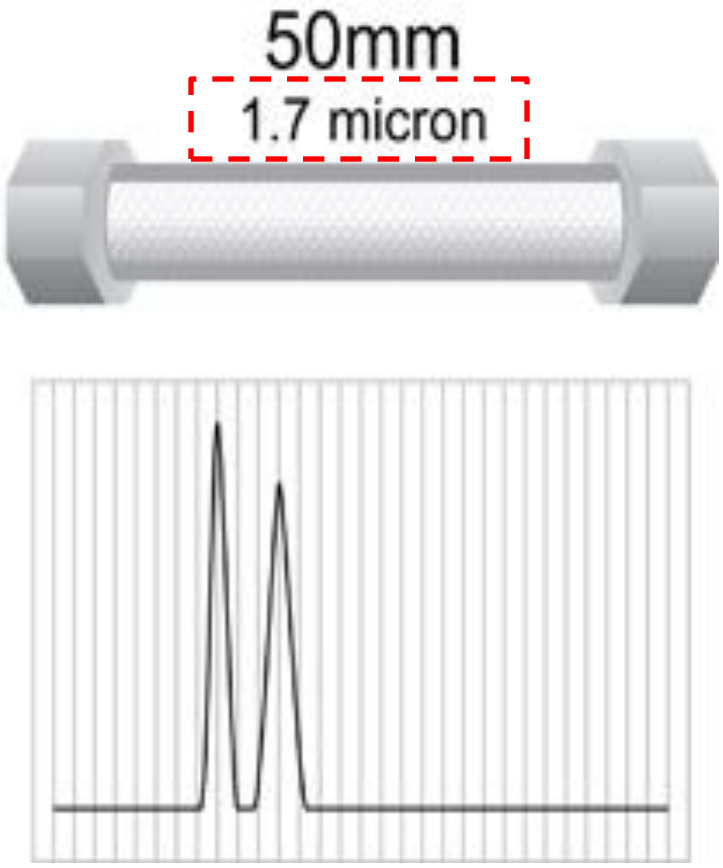
Effect of column temperature upon compound separation. Analysis of a compound mixture using the same mobile phase flow rate at three different temperatures (a) 25 °C, (b) 35 °C , (c) 45 °C

Effect of Particle Size in the Column Packing

- The efficiency of a packed column increases as the size of the stationary-phase particles decreases.
- Typical particle sizes → 3-10 μm
- Small particles:
  - → better resolution
  - → provide more uniform flow through the column and reduce multiple diffusion path.
  - → less distance solute diffuse in the mobile phase

Example:





Greater separation efficiency
(better resolution)

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Stationary Phase

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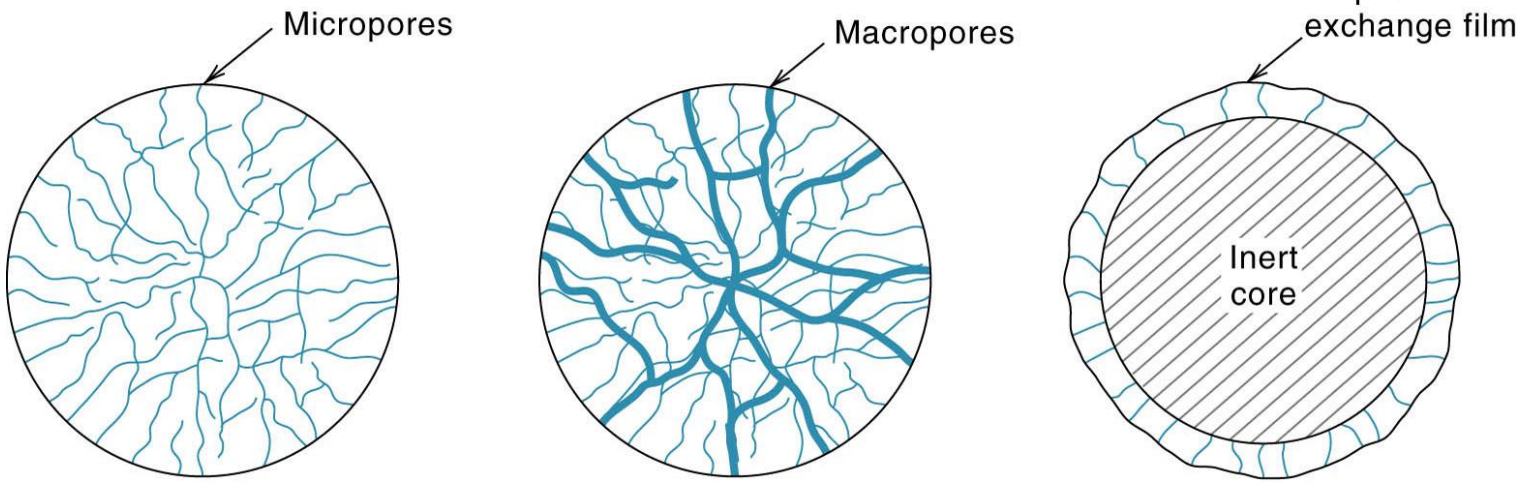
- Spherical particles have been developed that can be packed more homogeneously and provide improved efficiency.
- The particles are high purity silica, 5-10 μm in diameter, pore sized 60-100 Å range.
- Most HPLC is performed in the liquid-liquid (partition chromatography) mode → liquid stationary phase are either coated on the particles or chemically bonded.

Structural Types of Particles Used in HPLC



(a) Microporous particle (b) Perfusion particle (c) Nonporous particle

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Microporous Particles

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- The most common used particles are microporous or diffusive particles permeable to solvent and have high surface area.

- The use of small particles minimizes the diffusion pathlength.

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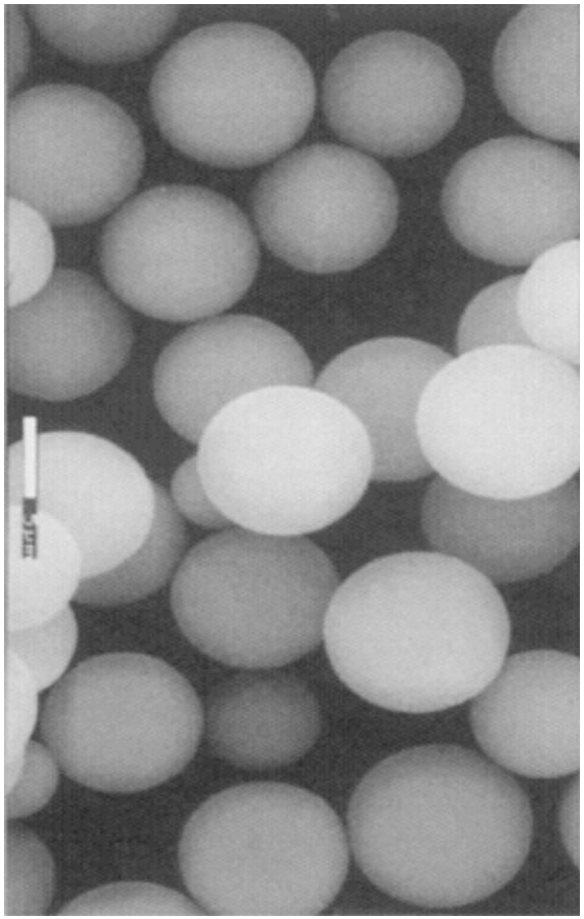
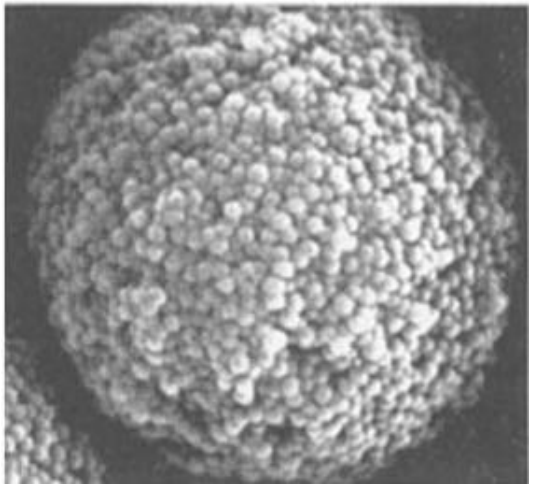
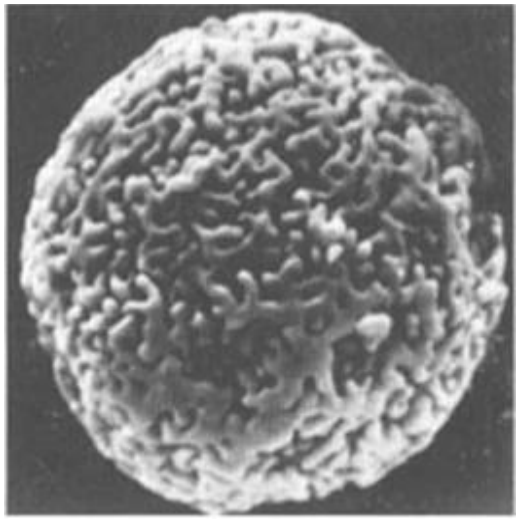


Spherical porous silica particles, 10 mm, 800X magnification.
Particles are fully porous with 100 Å pores.

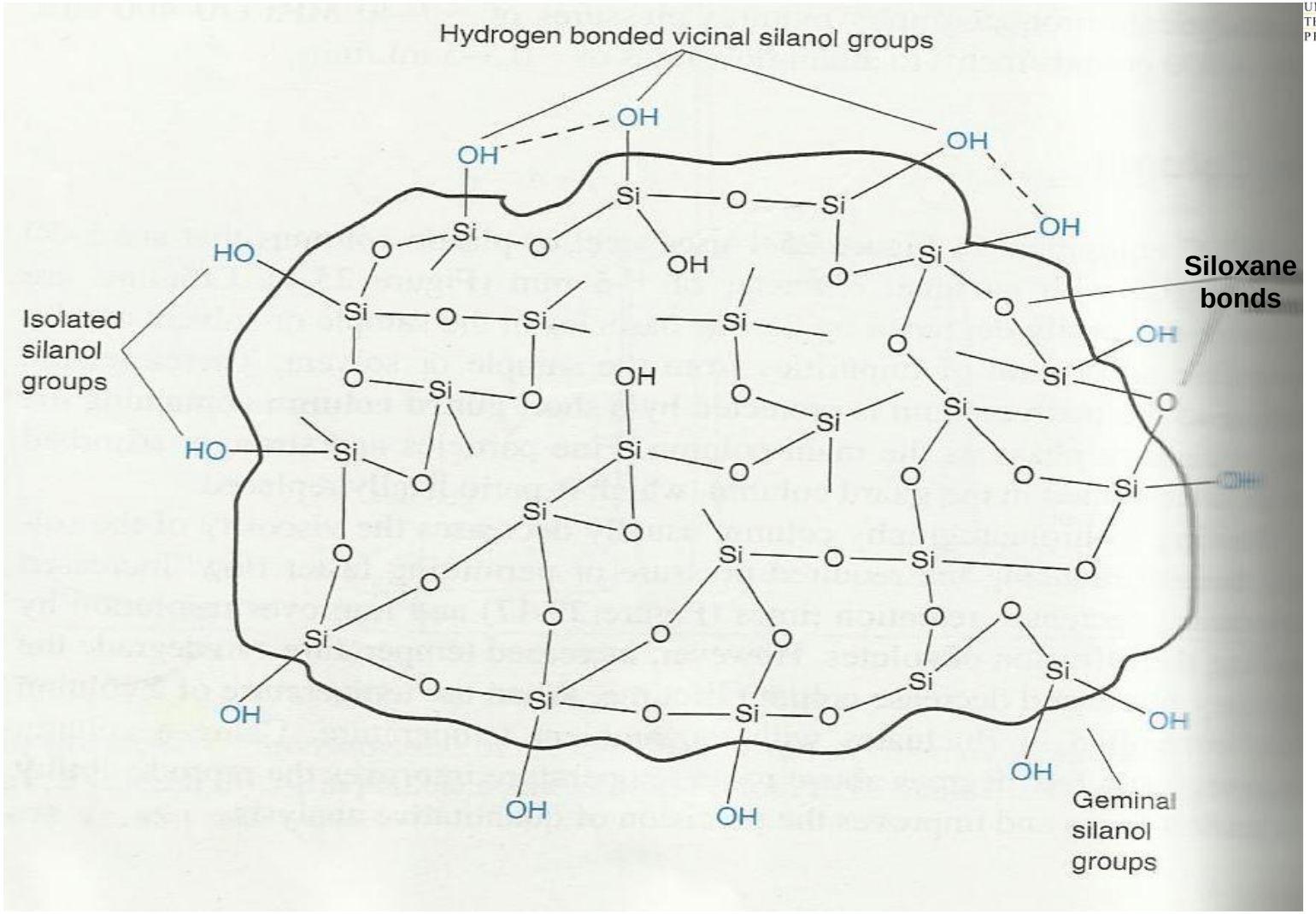


Zorbax porous silica microsphere particle, 50% porosity, 100 Å pores.



Xerogel silica particles
70% porosity, 100 Å pores.

Schematic Structure of Silica Particle



Surface area → up to 8 μmol of silanol group (Si-OH) per square meter.

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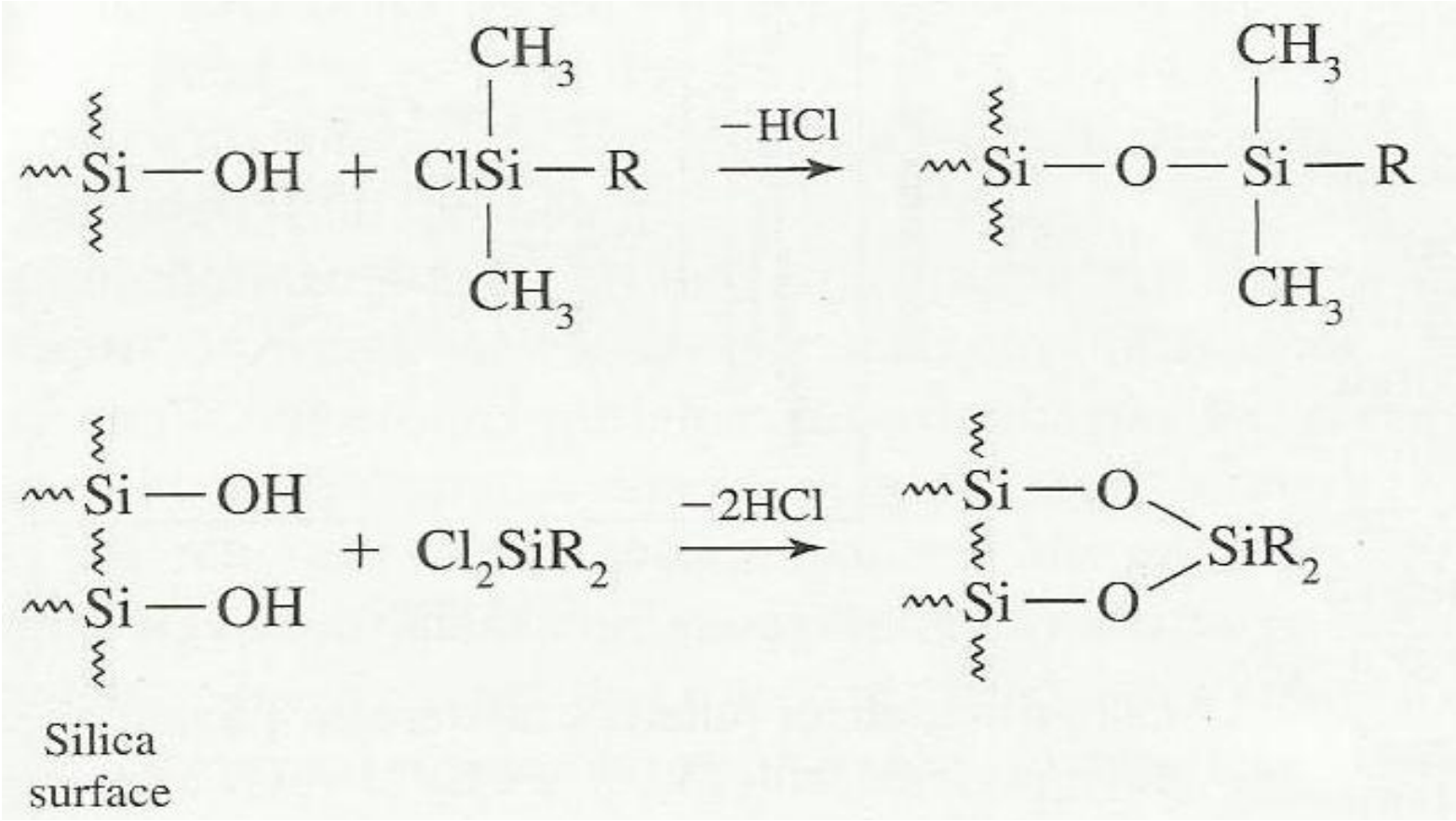
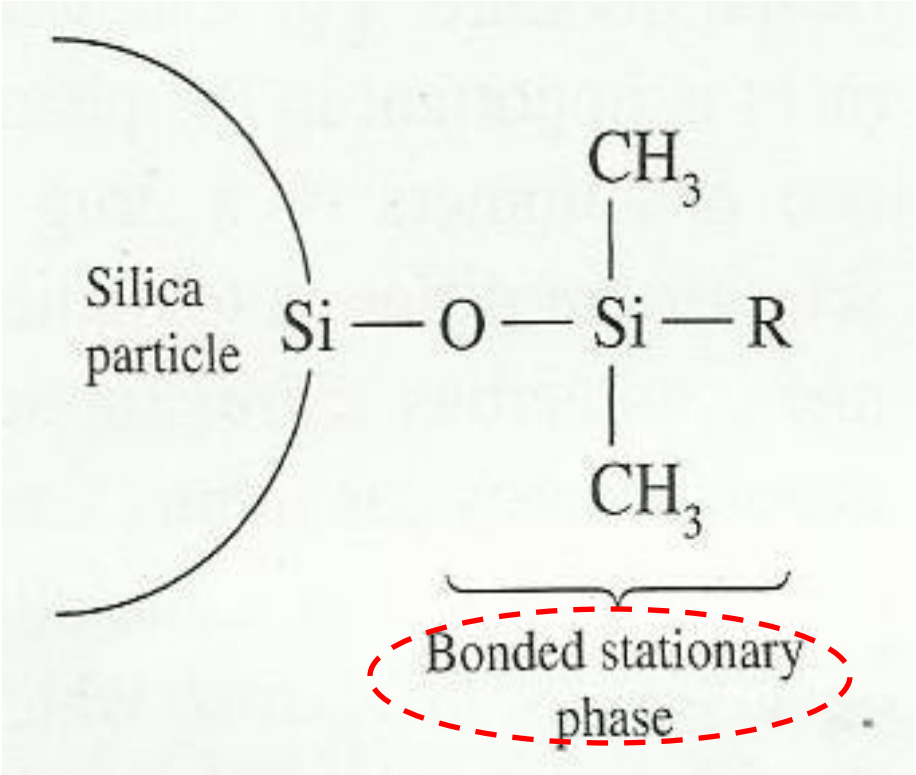
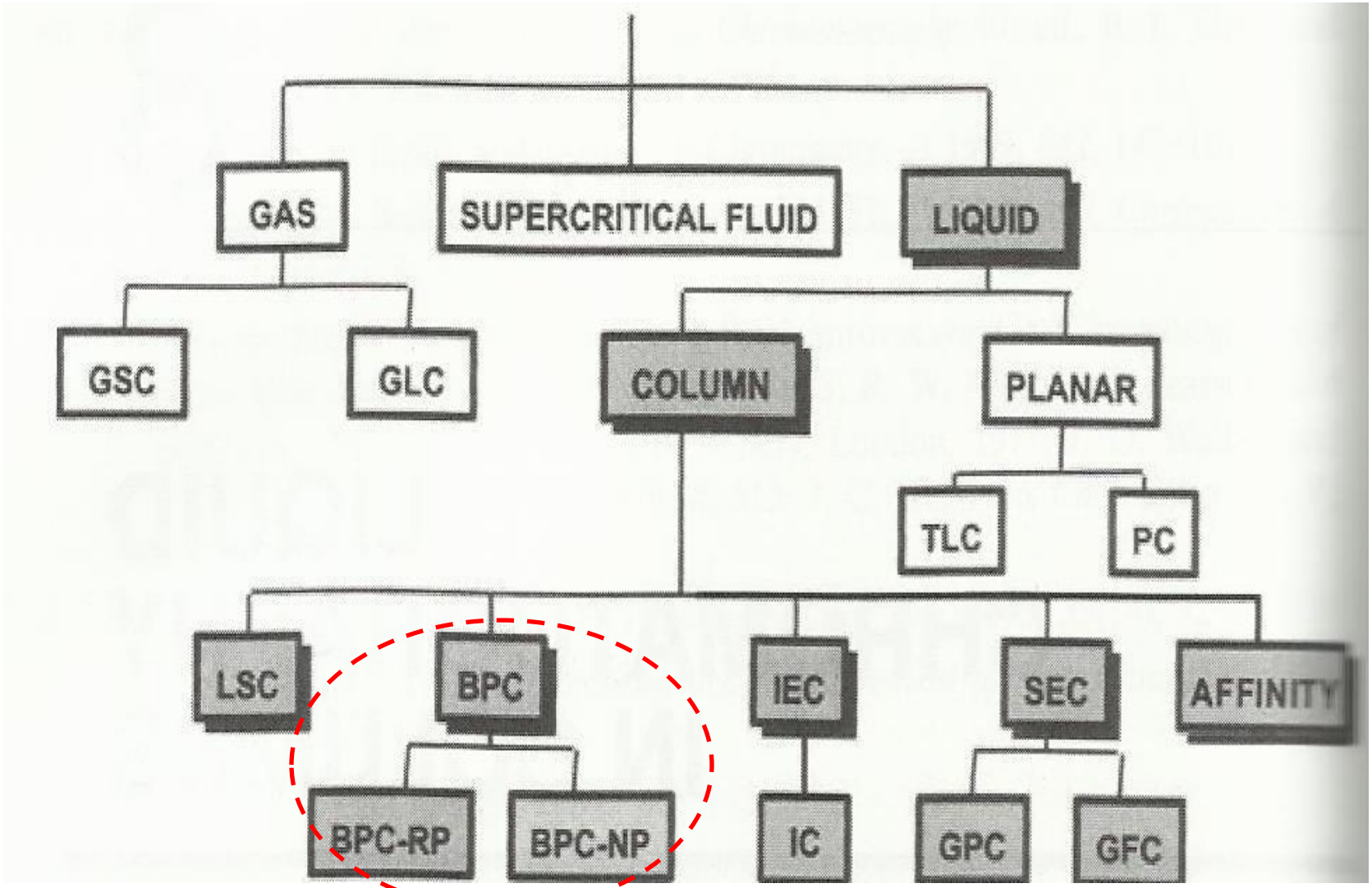
- Silica particles have surface silanol groups – SiOH. These are used for chemical bonding of stationary phases by silination reactions with chlorosilanes:





Common polar phases		Common nonpolar phases	
$R = (CH_2)_3NH_2$	Amino	$R = (CH_2)_{17}CH_3$	Octadecyl
$R = (CH_2)_3C{\equiv}N$	Cyano	$R = (CH_2)_7CH_3$	Octyl
$R = (CH_2)_2OCH_2CH(OH)CH_2OH$	Diol	$R = (CH_2)_3C_6H_5$	Phenyl

Classification of Liquid Chromatography Techniques



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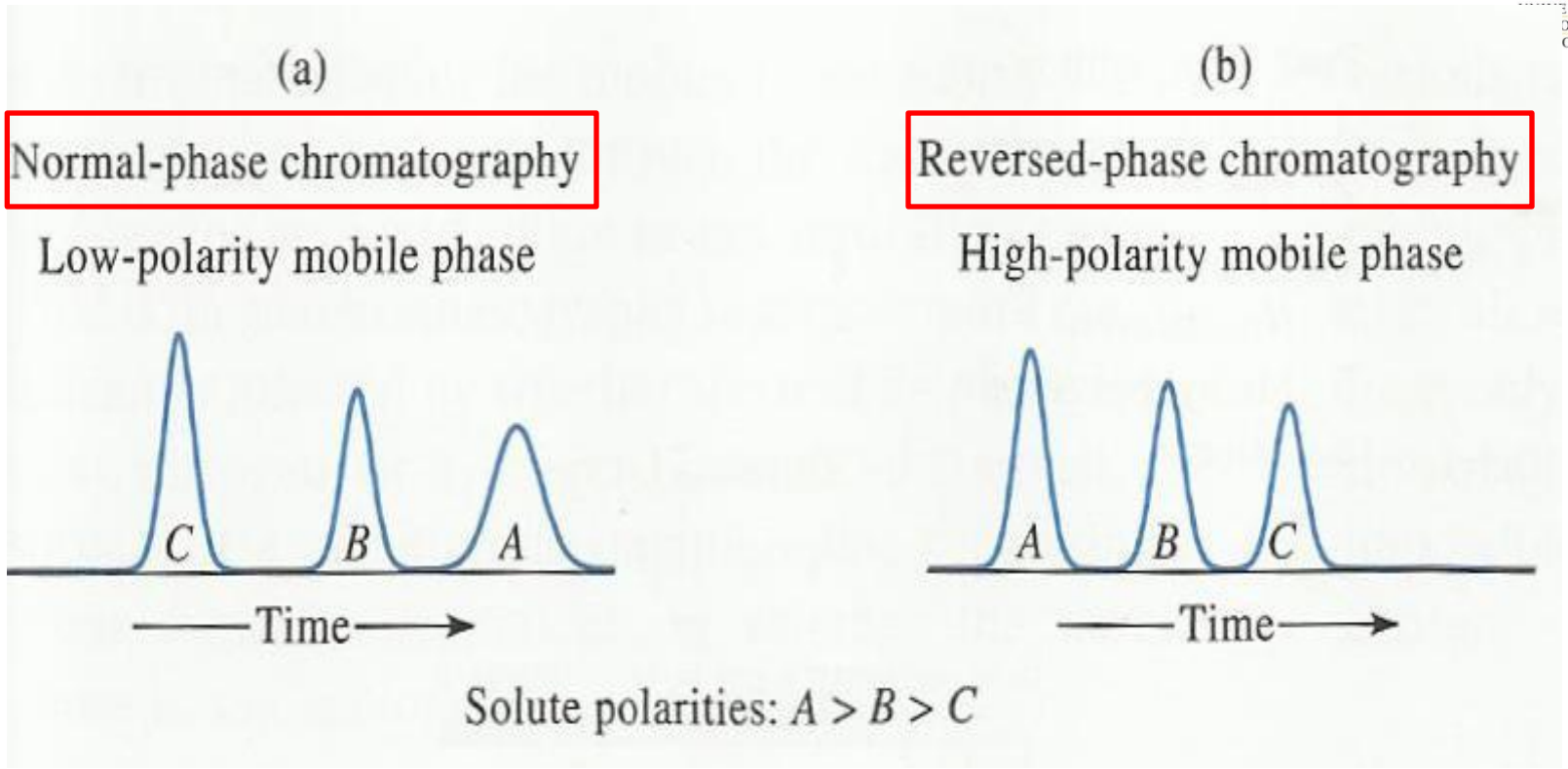
- Normal-phase chromatography
  - consists of polar stationary phase
  - more polar solvent has higher eluent strength.

- Polar phase for normal phase chromatography in increasing order of polarity include cyano, amino and dimethylamino.

- Reversed-phase chromatography
  - consists of nonpolar stationary phase
  - less polar solvent has higher eluent strength.

- The most common nonpolar bonded phases (for reverse-phase chromatography) are C18 and C8, with C18 the most popular, C8 is intermediate in hydrophobicity and C18 is very nonpolar.



Relationship between polarity and elution times for normal-phase and reversed phase chromatography

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Perfusion Packing

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•Packings are larger than the microporous ~ 12µm

•Used in higher flowrate and give better efficiency for large molecules such as protein.

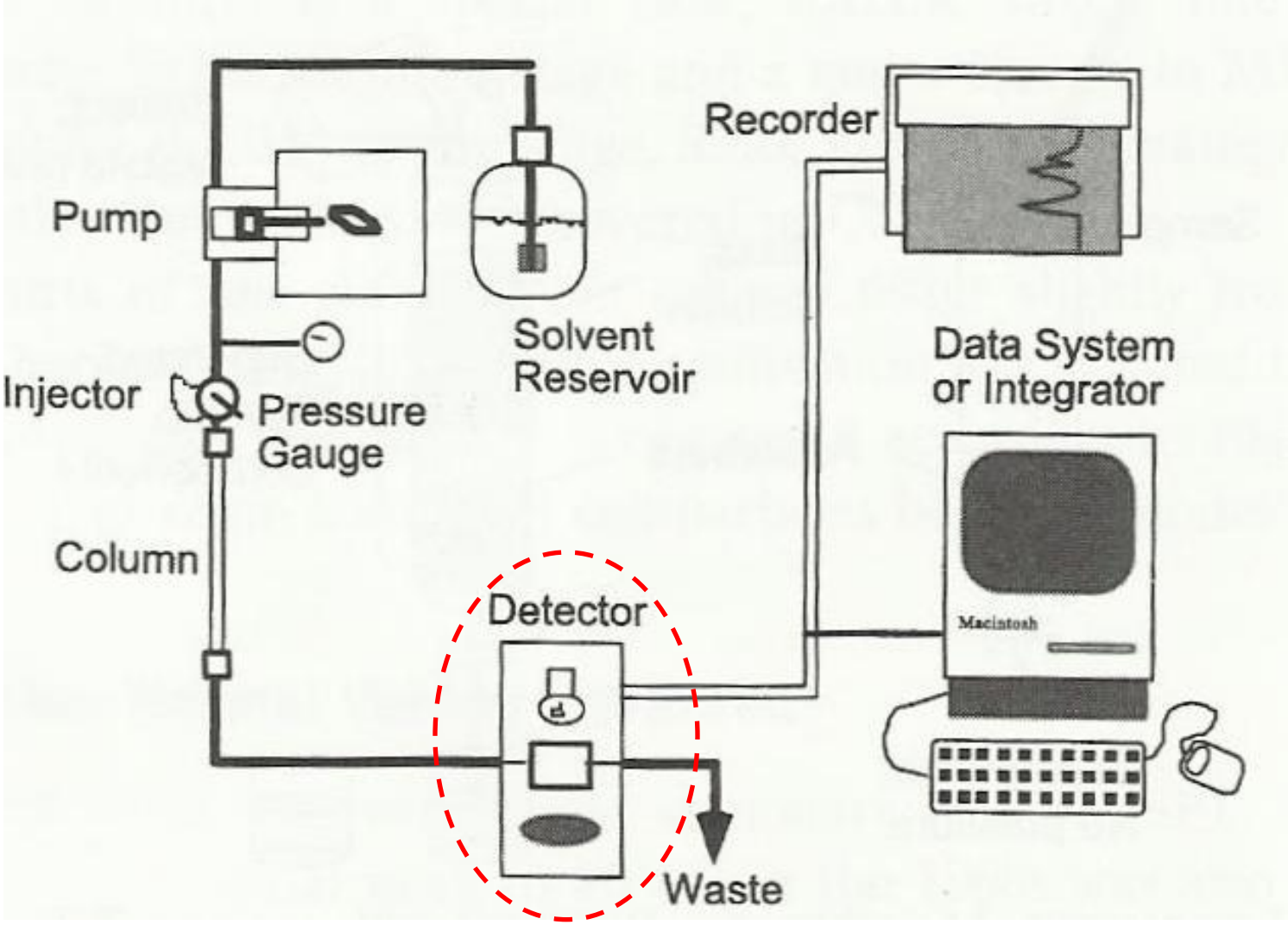
Nonporous Packing

▪Silica or resin → much smaller particle size,

1.5 – 2.5 µm.

▪Useful for separating complex peptide mixtures and are used in ion chromatography.

Detector


Recorder
Pump
Solvent
Reservoir
Data System
or Integrator
Injector
Pressure
Gauge
Column
Detector
Macintosh
Waste

Detectors

- Detectors with high sensitivity are required in HPLC.

- An ideal detector of any type is:
  - sensitive to low concentrations of every analyte.
  - does not broaden the eluted peaks.
  - insensitive to changes in temperature and solvent temperature.

- Widely used detectors are refractometer detector and UV detector.

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Differential Refractometer Detector

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- Universal detector → response to almost all solute
- Very sensitive to temperature and pressure changes.
- Detection limit: 1-10 ppm → not useful for trace analysis.
- Can not be used effectively with gradient elution due to a change in baseline → impossible to match exactly the sample and the reference while the solvent composition is changing.

Gradient elution: steady changes of mobile phase concentration during chromatography run

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Ultraviolet Detector

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- Most common detector, better sensitivity, 0.01 ppm
- Not temperature sensitive
- Relatively inexpensive
- Sensitive to a large number of organic compounds.
- Can be used with gradient elution.
- Can not be used with solvents that have significant absorption in the UV or with sample components that do not absorb in the UV.

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Photodiode Array Detector

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- High quality detector provide full-scale absorbance range.
- Most sensitive scale, an absorbance of 0.0005 would give a 100% signal.
- Good for gradient elution with non-absorbing solvents

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Comparison of HPLC Detectors

Detector	Approximate limit of detection[a] (ng)	Useful with gradient?
Ultraviolet	0.1–1	Yes
Refractive index	100–1 000	No
Evaporative light-scattering	0.1–1	Yes
Electrochemical	0.01–1	No
Fluorescence	0.001–0.01	Yes
Conductivity	0.5–1	No
Mass spectrometry	0.1–1	Yes
Fourier transform infrared	1 000	Yes

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HPLC Method Development

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- Mode of HPLC →liquid-solid adsorption or liquid-liquid partition (most commonly used).

- Liquid-liquid partition process →sensitive to small MW differences and so are preferred for the separation of members of a homologous series (alkane, alkene).

- Liquid-solid adsorption process →sensitive to steric effects and are preferred for the separation of isomers having different steric configurations (*cis, trans*).

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- General rule:
  i. highly polar materials are best separated using partition chromatography.
  ii. very nonpolar materials are separated using adsorption chromatography.

- Steps in method development:
  1. determine goal
  2. select method of sample preparation
  3. choose detector
  4. column selection
  5. mobile phase/ solvent selection

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END OF HPLC CHAPTER