HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

WHAT IS HPLC

- Originally referred to as ***High-Pressure Liquid Chromatography***

- Now more commonly called ***High Performance Liquid Chromatography***

- HPLC is really the automation of traditional liquid chromatography under conditions which provide for enhanced separations during shorter periods of time, *utilizing very small particles, small column diameters, and very high fluid pressures.*

HPLC MACHINE

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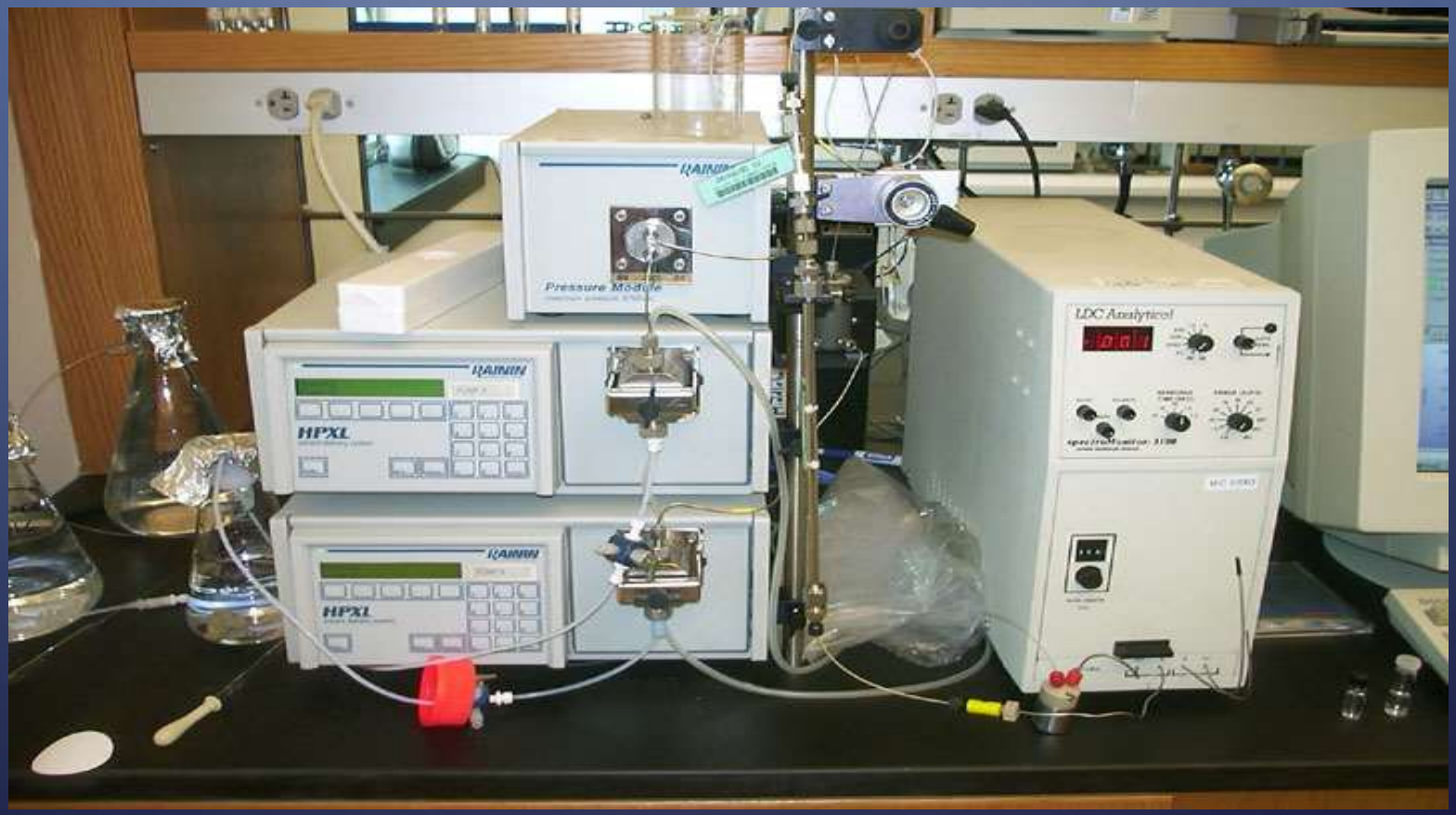


APPLICATIONS

HPLC can be used in both qualitative and quantitative applications, that is for both compound identification and quantification. Normal phase HPLC is only rarely used now, almost all HPLC separation can be performed in reverse phase. Reverse phase HPLC (RPLC) is ineffective in for only a few separation types; it cannot separate inorganic ions (they can be separated by ion exchange chromatography). It cannot separate polysaccharides (they are too hydrophilic for any solid phase adsorption to occur), nor polynucleotides (they adsorb irreversibly to the reverse phase packing). Lastly, incredibly hydrophobic compounds cannot be separated effectively by RPLC (there is little selectivity). Aside from these few exceptions, RPLC is used for the separation of almost all other compound varieties. RPLC can be used to effectively separate similar simple and aromatic hydrocarbons, even those that differ only by a single methylene group. RPLC effectively separates simple amines, sugars, lipids, and even pharmaceutically active compounds. RPLC is also used in the separation of amino acids, peptides, and proteins. Finally RPLC is used to separate molecules of biological origin. The determination of caffeine content in coffee products is routinely done by RPLC in commercial applications in order to guarantee purity and quality of ground coffee. HPLC is a useful addition to an analytical arsenal, especially for the separation of a sample before further analysis.

STATIONARY PHASES

- Polar ("Normal" Phase):
  - Silica, alumina
- Non-Polar ("Reversed Phase")
  - ODS Silica gel
  - C18, C8

THE MOBILE PHASE

Normal chromatography •

Hexane ; dichloromethane; isopropanol; methanol

Increasing strength

Reverse phase chromatography •

water ; methanol; acetonitrile; tetrahydrofuran (THF)

Increasing strength

DISTRIBUTION CONSTANT

All chemical reactions have a characteristic equilibrium constant. For the reaction

$Aaq + Bs \rightleftharpoons ABs(1)$

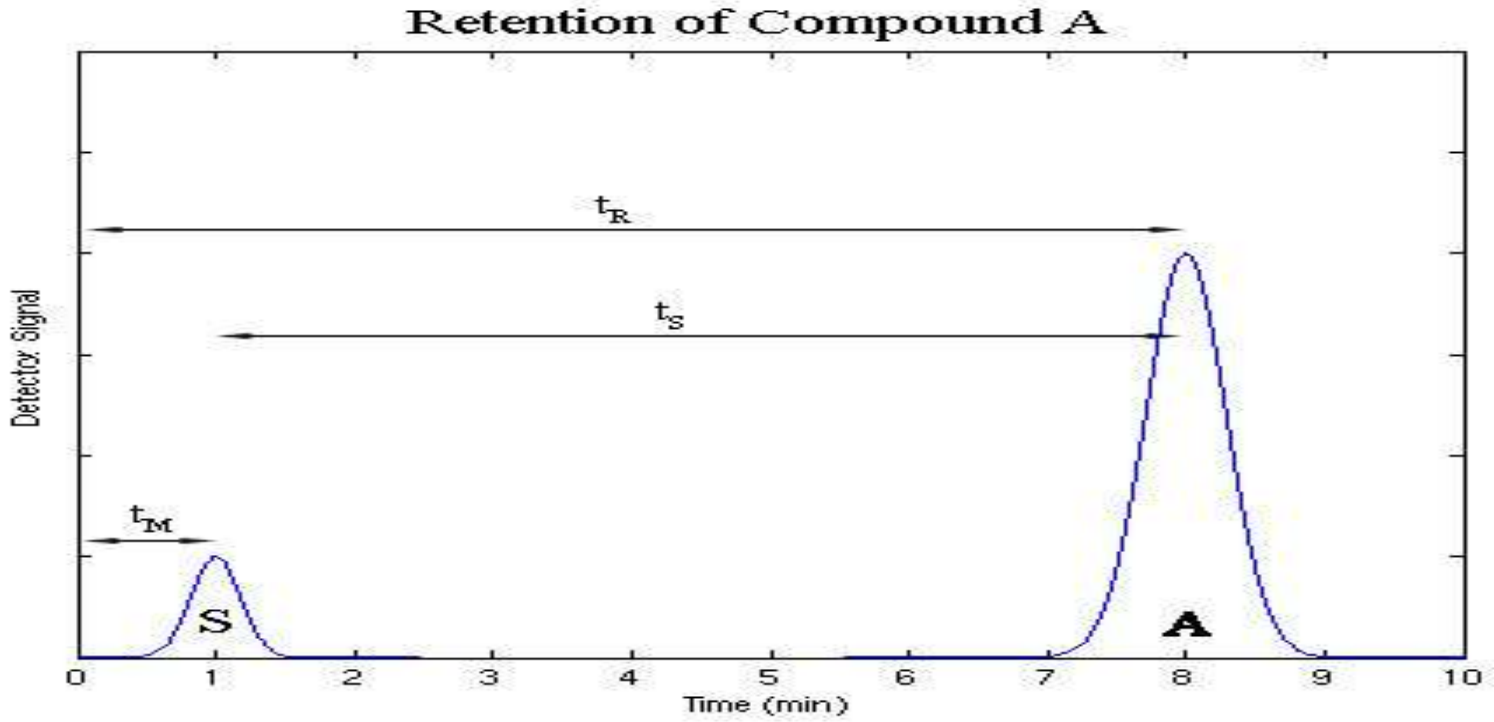
There is a chemical equilibrium constant Keq that dictates what percentage of compound A will be in solution and what percentage will be bound to the stationary compound B. During a chromatographic separation, there is similar relationship between compound A and the solvent, or mobile phase, C. This will yield an overall equilibrium equation which dictates the quantity of A that will be associated with the stationary phase and the quantity of A that will be associated with the mobile phase.

$Amobile \rightleftharpoons Astationary$

The equilibrium between the mobile phase and stationary phase is given by the constant Kc.

$Kc = (aA)S/(aA)M \approx C_S/C_M$

Where Kc, the distribution constant, is the ratio of the activity of compound A in the stationary phase and activity of compound A in the mobile phase. In most separations, which contain low concentrations of the species to be separated, the activity of A in each is approximately equal to the concentration of A in that state. The distribution constant indicates the amount of time that compound A spends adsorbed to the stationary phase as the opposed to the amount of time A spends solvated by the mobile phase. This relationship determines the amount of time it will take for compound A to travel the length of the column. The more time A spends adsorbed to the stationary phase, the more time compound A will take to travel the length of the column. The amount of time between the injection of a sample and its elution from the column is known as the retention time; it is given the symbol tR.



The amount of time required for a sample that does not interact with the stationary phase, or has a $\mathbf{K_c}$ equal to zero, to travel the length of the column is known as the **void time**, $\mathbf{t_M}$. No compound can be eluted in less than the void time.

RETENTION TIME

- The **retention time** of a solute is taken as the elapsed time between the time of injection of a solute and the time of elution of the peak maximum of that solute.

Retention Factor

Since Kc is a factor that is wholly dependent on a particular column and solvent flow rate, a quantitative measure of the affinity of a compound for a particular set of mobile and stationary phases that does not depend on the column geometry is useful. The retention factor, k, can be derived from Kc and is independent of the column size and the solvent flow rate.

$$K_C = K_C V_S / V_M$$

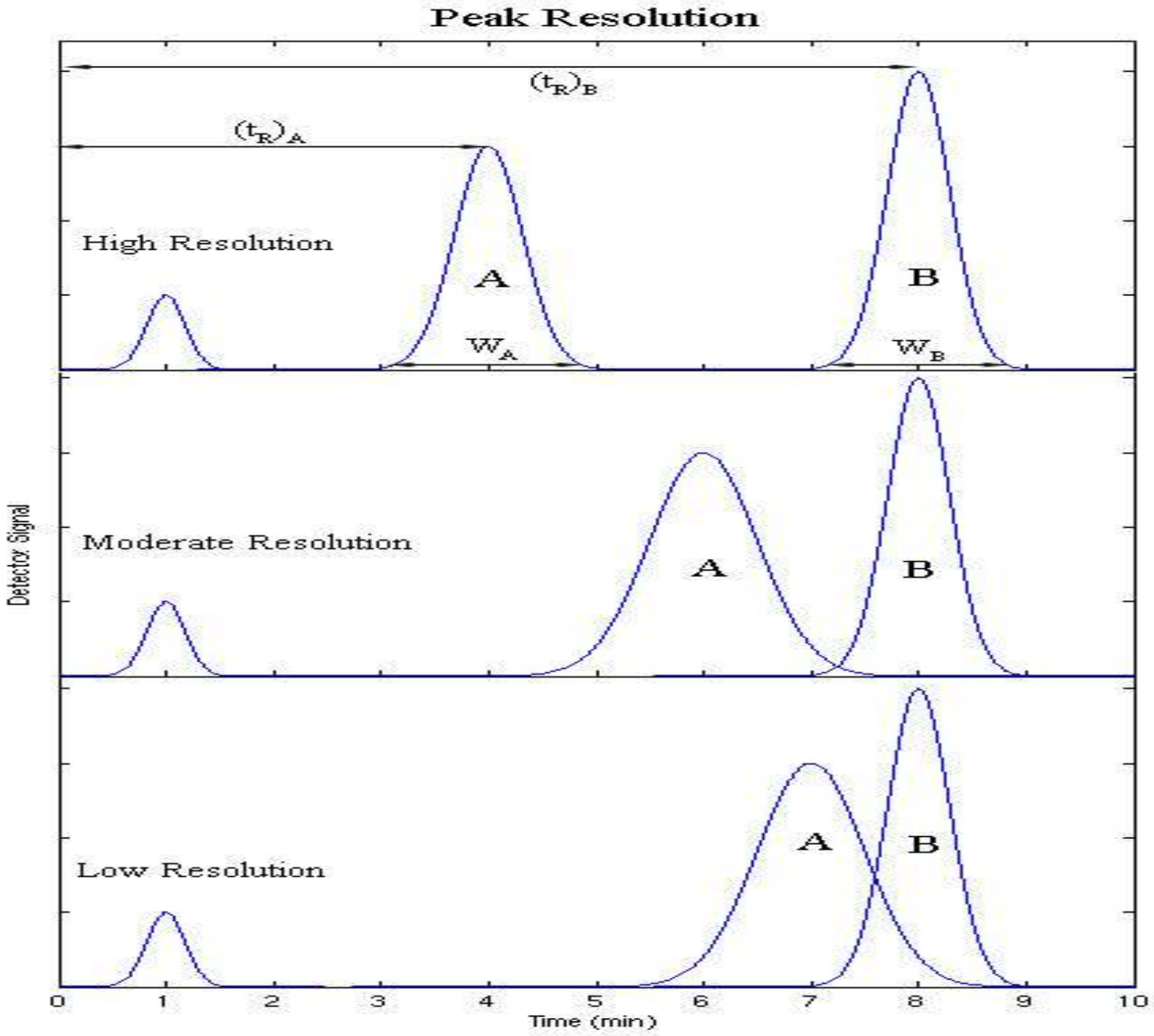
- The retention factor is calculated by multiplying the distribution constant by the volume of stationary phase in the column and dividing by the volume of mobile phase in the column.

Resolution

The resolution of a elution is a quantitative measure of how well two elution peaks can be differentiated in a chromatographic separation. It is defined as the difference in retention times between the two peaks, divided by the combined widths of the elution peaks.

$$R_S=2[(t_R)_B-(t_R)_A]/W_B+W_A$$

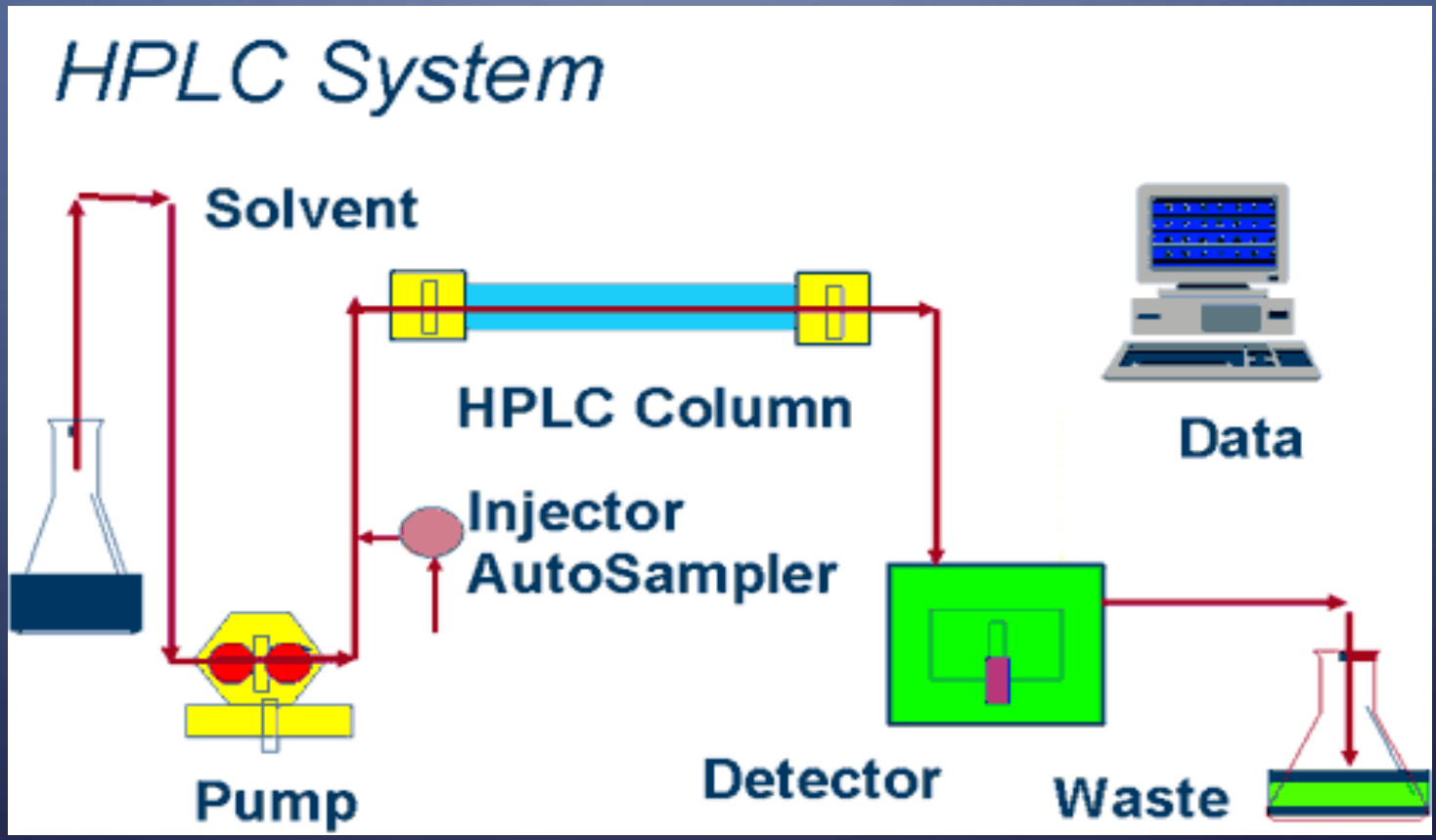
Where B is the species with the longer retention time, and t_R and W are the retention time and elution peak width respectively. If the resolution is greater than one, the peaks can usually be differentiated successfully.

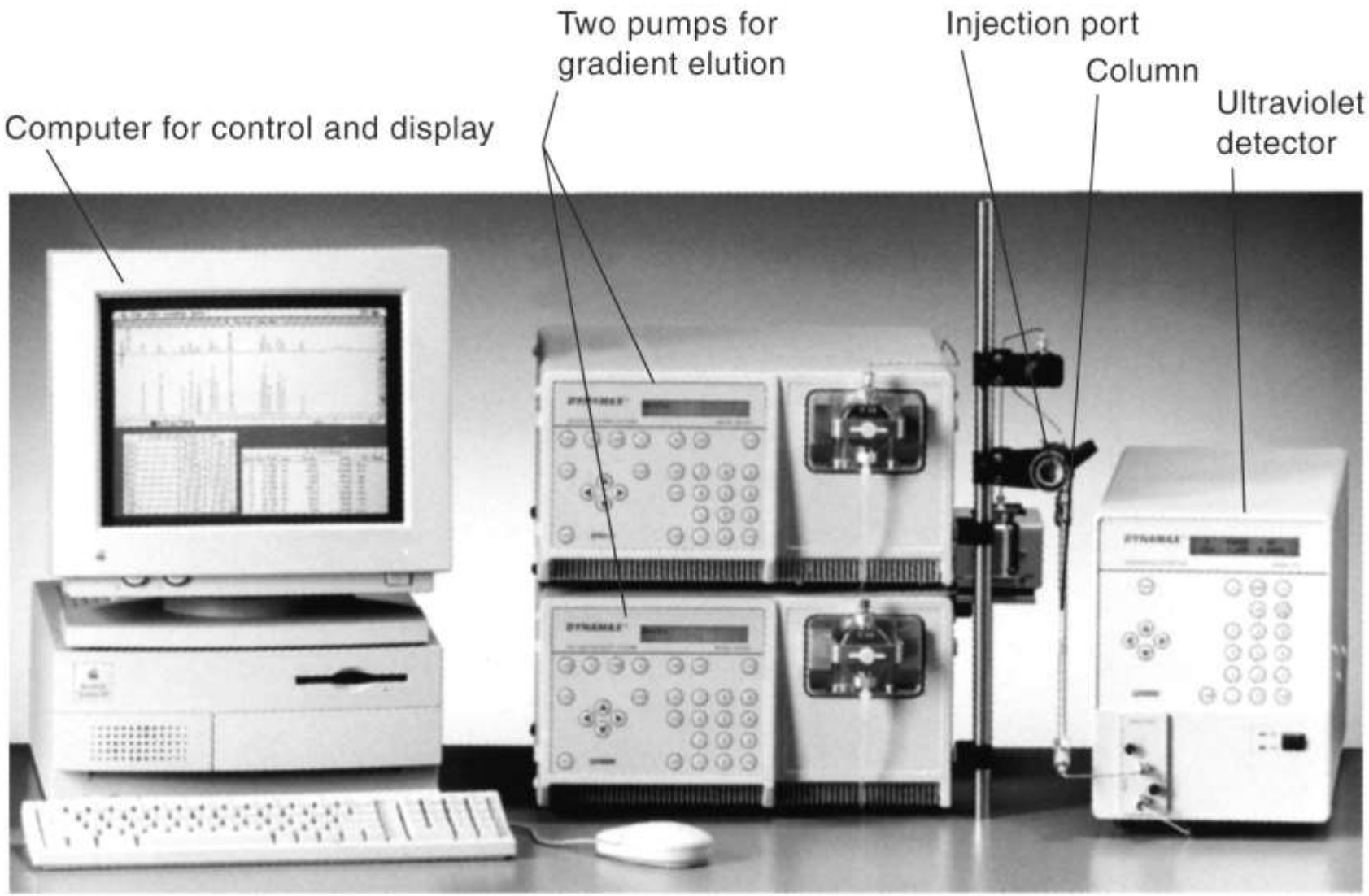

Peak Resolution
$(t_R)_B$
$(t_R)_A$
High Resolution
A
B
W_A
W_B
Moderate Resolution
A
B
Low Resolution
A
B
Detector Signal
0
1
2
3
4
5
6
7
8
9
10
Time (min)

COMPONENTS OF HPLC

- Solvent Reservoir
- Pumps
- Sample Injection System
- Columns
- Detectors
- Data Processing
- Waste

HPLC PRINCIPLE




Two pumps for
gradient elution
Injection port
Column
Ultraviolet
detector
Computer for control and display

SOLVENT RESERVOIR

- Mobile phase
- isocratic elution - single solvent separation teachnique
- gradient elution - 2 or more solvents, varied during separation
- To carry sample into the column

PUMPS

- To produce an appropriate pressure to push solvent into the sample.

- A pump capable of pumping solvent up to a pressure of 4000 psi and at flows of up to 10 ml/min

PUMP

- The **HPLC pump** drives the solvent and sample through the column. To reduce variation in the elution, the pump must maintain a constant, pulse free, flow rate; this is achieved with **multi-piston pumps**. The presence of two pistons allows the flow rate to be controlled by one piston as the other recharges. A **syringe pump** can be used for even greater control of flow rate; however, the syringe pump is unable to produce as much pressure as a piston pump, so it cannot be used in all HPLC applications.

SAMPLE INJECTION SYSTEM

sample valve
Syringe/injector

Syringe :
manual
Autoinjector

A fixed-volume loop of between 1 – 200 μl (20 μl is often used as standard)

COLUMNS

straight, 15 to 150 cm in length; 2 to 3 mm i.d.

packing - silica gel, alumina, Celite

- In the **HPLC column**, the components of the sample separate based on their differing interactions with the column packing. If a species interacts more strongly with the stationary phase in the column, it will spend more time adsorbed to the column's adsorbent and will therefore have a greater retention time. Columns can be packed with solids such as silica or alumina; these columns are called **homogeneous columns**. If stationary phase in the column is a liquid, the column is deemed a **bonded column**. Bonded columns contain a liquid stationary phase bonded to a sold support, which is again usually silica or alumina. The value of the constant **C** described in the van Deemter equation is proportional, in HPLC, to the diameter of the particles that constitute the column's packing material.

PICTURE OF AN HPLC COLUMN

WHAT AFFECTS SYSTEM

Column Parameters

- Column Material
- Deactivation
- Stationary Phase
- Coating Material

Instrument Parameters

- Temperature
- Flow
- Signal
- Sample Sensitivity
- Detector

SEVERAL COLUMN TYPES (CAN BE CLASSIFIED AS)

Normal phase

Reverse phase

Size exclusion

Ion exchange

NORMAL PHASE

- In this column type, the retention is governed by the interaction of the polar parts of the stationary phase and solute. For retention to occur in normal phase, the packing must be more polar than the mobile phase with respect to the sample

REVERSE PHASE

In this column the packing material is relatively nonpolar and the solvent is polar with respect to the sample. Retention is the result of the interaction of the nonpolar components of the solutes and the nonpolar stationary phase. Typical stationary phases are nonpolar hydrocarbons, waxy liquids, or bonded hydrocarbons (such as C18, C8, etc.) and the solvents are polar aqueous-organic mixtures such as methanol-water or acetonitrile-water.

SIZE EXCLUSION

In size exclusion the HPLC column is consisted of substances which have controlled pore sizes and is able to be filtered in an ordinarily phase according to its molecular size. Small molecules penetrate into the pores within the packing while larger molecules only partially penetrate the pores. The large molecules elute before the smaller molecules.

ION EXCHANGE

- In this column type the sample components are separated based upon attractive ionic forces between molecules carrying charged groups of opposite charge to those charges on the stationary phase. Separations are made between a polar mobile liquid, usually water containing salts or small amounts of alcohols, and a stationary phase containing either acidic or basic fixed sites.

DETECTOR

- The **HPLC detector**, located at the end of the column, must register the presence of various components of the sample, but must not detect the solvent. For that reason there is no universal detector that works for all separations. A common HPLC detector is a **UV absorption detector**, as most medium to large molecules absorb UV radiation. Detectors that measure fluorescence and refractive index are also used for special applications. A relatively new development is the combination of an HPLC separation with an **NMR detector**. This allows the pure components of the sample to be identified and quantified by nuclear magnetic resonance after having been separated by HPLC, in one integrated process.

HPLC DETECTORS

- UV/Vis
- Refractive index
- Fluorescence
- Evaporative light scattering (ELSD)
- MS
- Diode Array Detector (DAD)

TYPES OF DETECTORS

Absorbance (UV with Filters, UV with Monochromators)

IR Absorbance

Fluorescence

Refractive-Index

Evaporative Light Scattering Detector (ELSD)

Electrochemical

Mass-Spectrometric

Photo-Diode Array

DATA PROCESSING

- Using specific sowtare that is connected to HPLC machine
- Receive the information from HPLC machine and present it as a graph
- The graph describes about qualitative data (Retention time) and quantitative data (area under curve)

THE FACTORS WHICH INFLUENCE THE HPLC PERFORMANCE

Internal diameter of column1

1- the smaller in diameter, the higher in sensitivity

2. Pump pressure

- the higher in pressure, the higher in separation

3. Sample size

4. The polarity sample, solvent and column

5. Temperature

- the higher in temperature, the higher in separation

APPLICATION OF HPLC

1. Pharmaceuticals industry

To control the drug stability

Quantity of drug determination from pharmaceutical dosage forms, ex. Paracetamol determination in panadol tablet

Quantity of drug determination from biological fluids, ex: blood glucose level

2. Analysis of natural contamination

- Phenol & Mercury from sea water

3. Forensic test

- Determination of steroid in blood, urine & sweat.
- Detection of psychotropic drug in plasma

APPLICATION OF HPLC

4. Clinical test

- Monitoring of hepatic chirosis patient through aquaporin 2 in the urine.

5. Food and essence manufacture

- sweetener analysis in the fruit juice
- preservative analysis in sausage.

ADVANTAGES

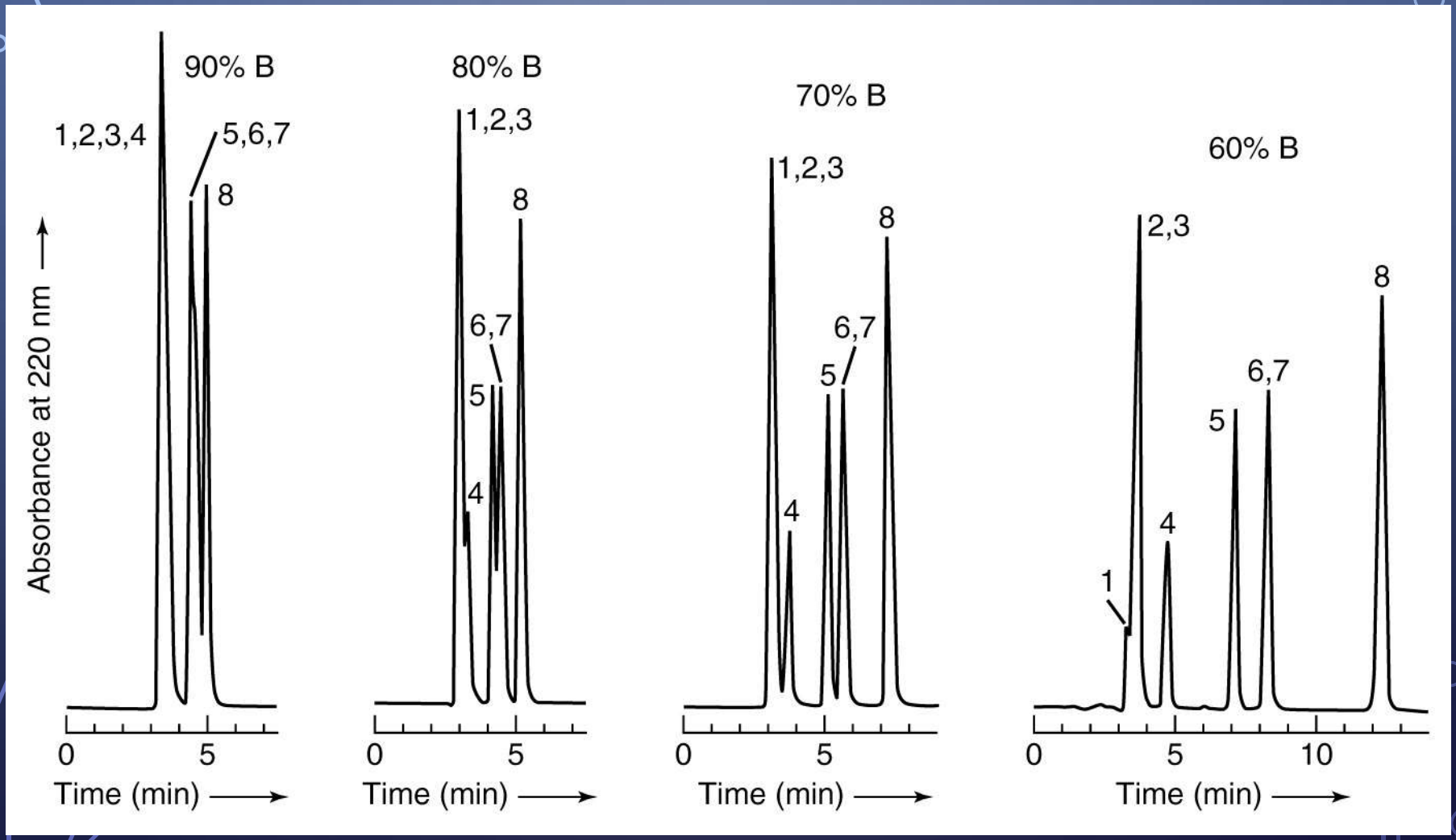
1-Needs a small sample with a high accuracy and precis

2-Non-destructed sample during operation compared to GC.

DISADVANTAGES

- Need a skill to run the instruments
- Solvents consuming

HPLC CHROMATOGRAM



Predict the order of elution from first to last of the following morphinane compounds from an ODS column in an acetonitrile/buffer mixture pH 8 (10 : 90).