Ministry of higher education and scientific research
AL-Mustaqbal University college
Department of medical physics

Analytical chemistry(practical)

Lecture 7

HPLC Basics

High Performance Liquid Chromatography Technique

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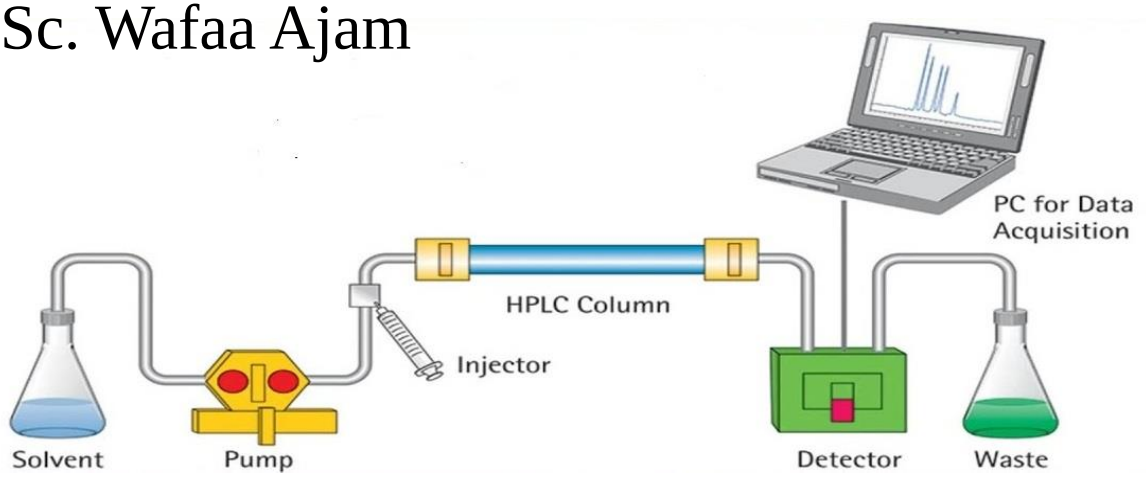
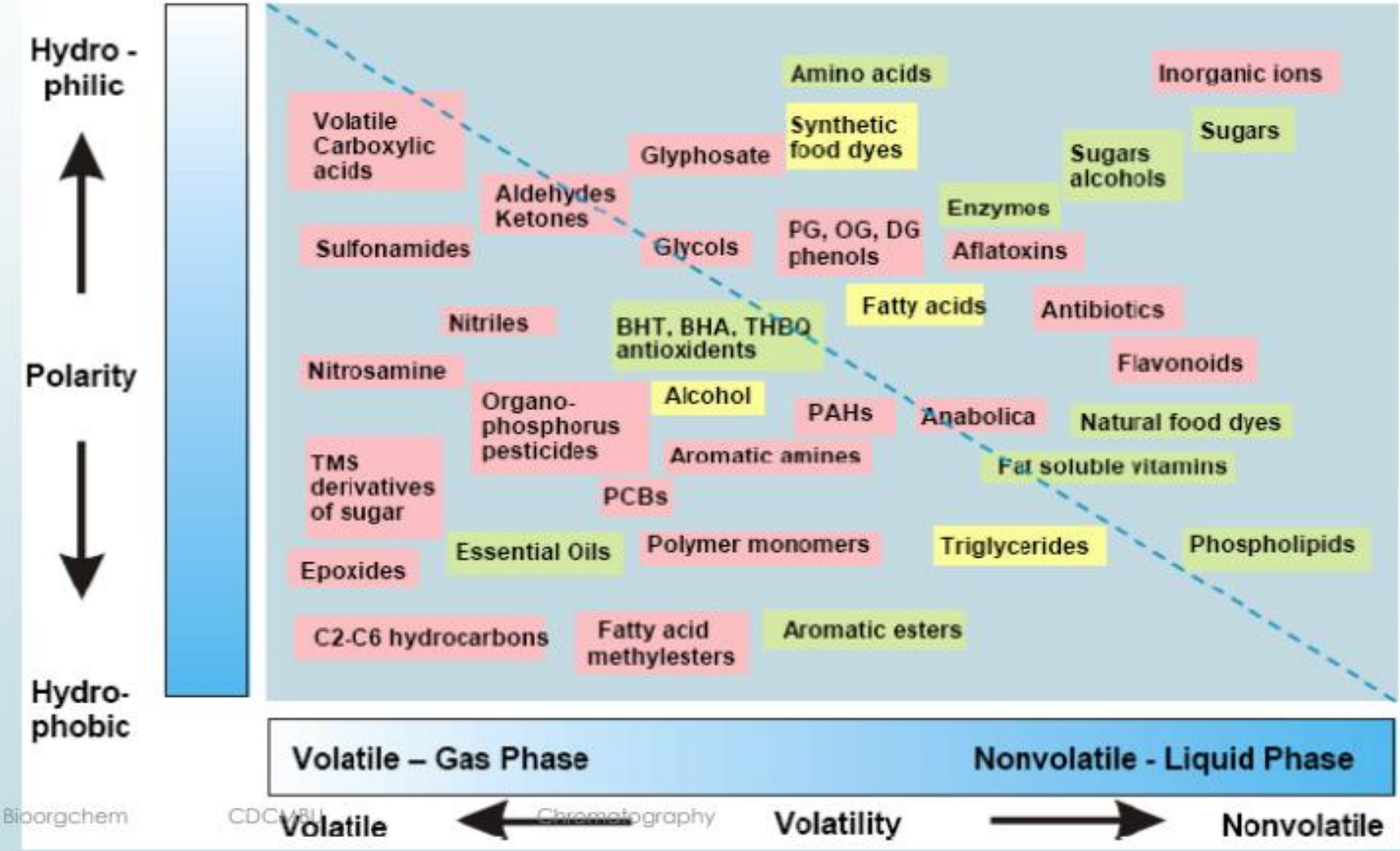


Fundamentals of (HPLC)

- ✓ Explain the general principles of HPLC analyses
- ✓ Know the major application areas of HPLC
- ✓ Identify the major components of an HPLC system and explain their principles of operation

Chromatographic Separation Techniques
Which separation technique for which compound



What is Liquid Chromatography?

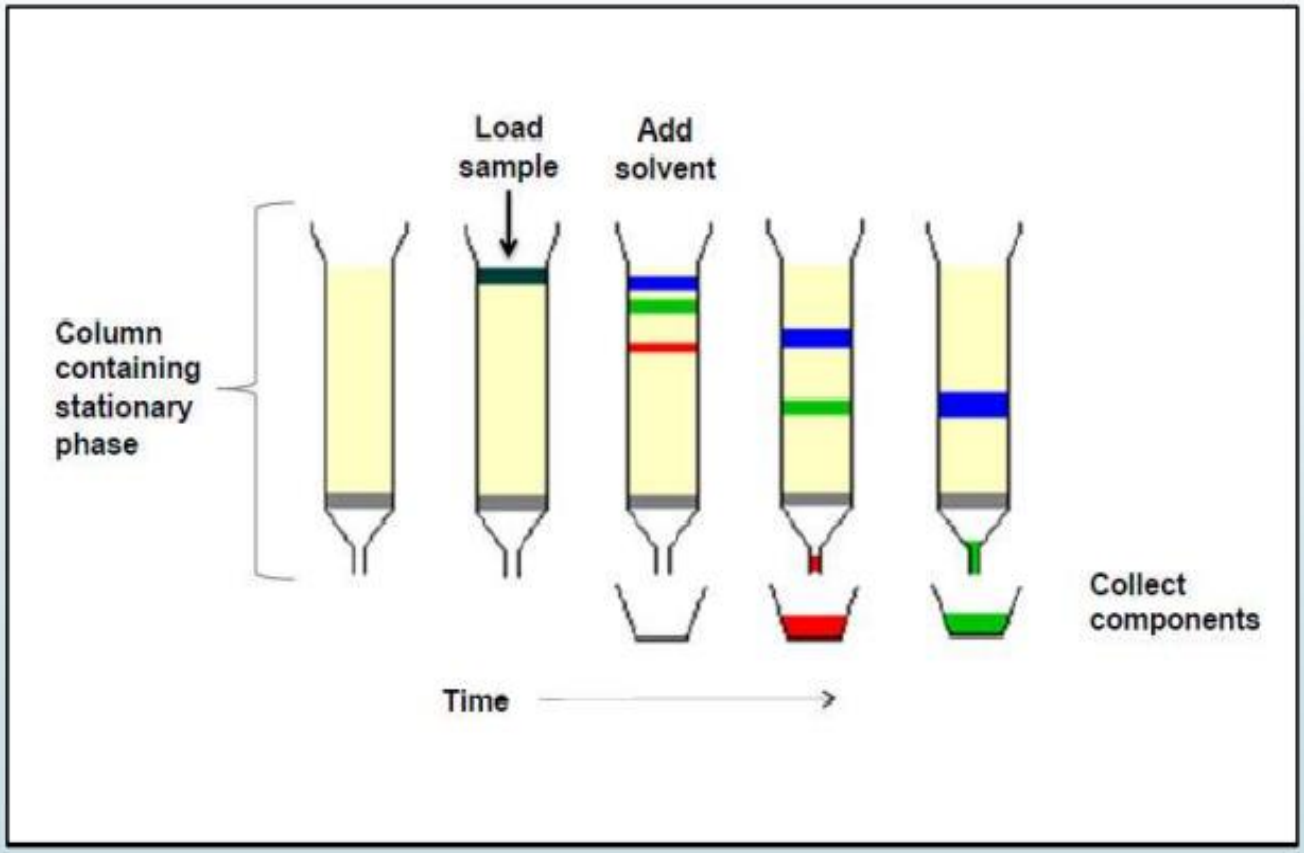
Liquid chromatography is a separation technique that involves:

•The placement (injection) of a small volume of liquid sample into a tube packed with porous particles (**stationary phase**)

•where individual components of the sample are transported along the packed tube (column) by a liquid moved by gravity.

- ✓ The components of the sample are separated from one another by the column packing that involves various chemical and/or physical interactions between their molecules and the packing particles.
- ✓ The separated components are collected at the exit of this column and identified by an external measurement technique, such as a spectrophotometer that measures the intensity of the color, or by another device that can measure their amount.

Principles of Liquid Chromatography Column



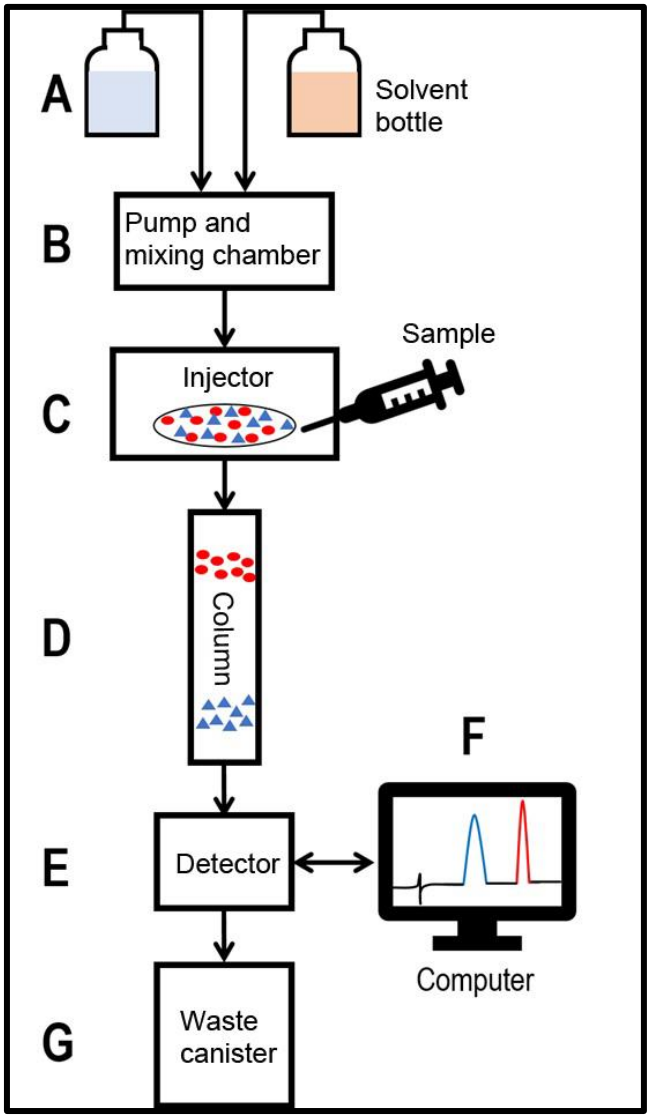
What is HPLC?

- 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.*

How HPLC technique Work?

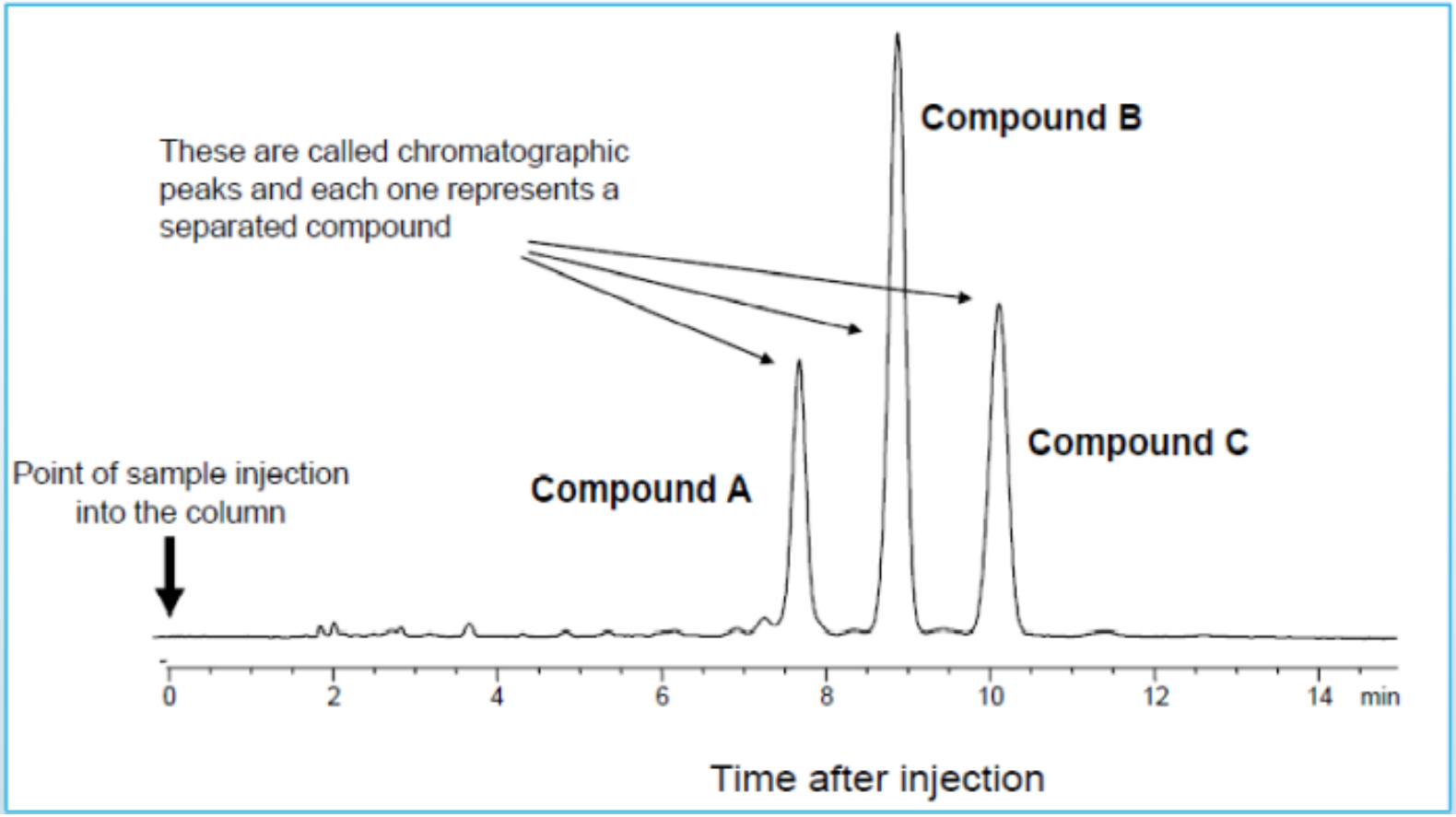
HPLC is a separation technique that involves:

- ✓ The injection of a small volume of liquid sample into a tube packed with tiny particles (3 to 5 micron (μm) in diameter called the stationary phase)
- ✓ Where individual components of the sample are moved down the packed tube (column) with a liquid (mobile phase) forced through the column by high pressure delivered by a pump .



- ✓ These components are separated from one another by the column packing that involves various chemical and/or physical interactions between their molecules and the packing particles.
- ✓ These separated components are detected at the exit of this tube (column) by a flow-through device (**detector**) that measures their amount.
- ✓ An output from this detector is called a **"liquid chromatogram".**
- ✓ In principle, LC and HPLC work the same way except the speed, efficiency, sensitivity and ease of operation of HPLC is vastly superior.

What Does a Liquid Chromatogram Look Like?

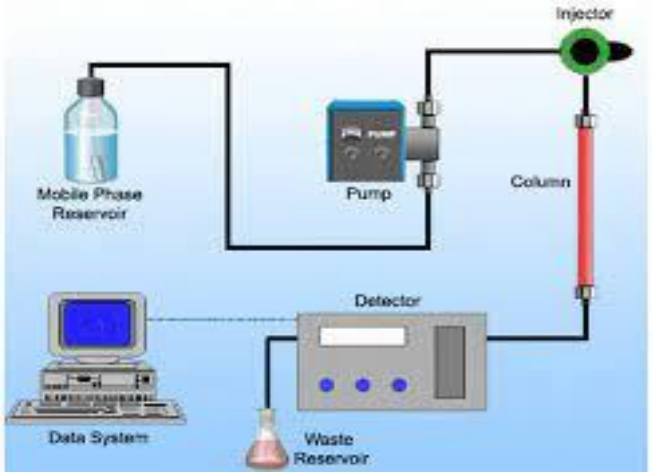


This is the chromatogram resulting from the injection of a small volume of liquid extracted from a vitamin E capsule that was dissolved in an organic solvent. Modern HPLC separations usually require 10-to 30-minutes each.

Describing the 5 major HPLC components and their functions?

1. The Pump:

- ✓ The role of the pump is to force a liquid (called the mobile phase) through the liquid chromatograph at a specific flow rate, expressed in milliliters per min (mL/min).
- ✓ Normal flow rates in HPLC are in the 1-to 2-mL/min range.
- ✓ Typical pumps can reach pressures in the range of 6000-9000 psi (400-to 600-bar).•
- ✓ During the chromatographic experiment, a pump can deliver a constant mobile phase composition (isocratic , same concentration) or an increasing mobile phase composition (gradient , different concentrations)



2. Injector:

- ✓ The injector serves to introduce the liquid sample into the flow stream of the mobile phase.
- ✓ Typical sample volumes are 5-to 20-microliters (μL).
- ✓ The injector must also be able to withstand the high pressures of the liquid system.
- ✓ An auto sampler is the automatic version for when the user has many samples to analyze or when manual injection is not practical.

3.Column:

- ✓ Considered the "heart of the chromatograph" the column's stationary phase separates the sample components of interest using various physical and chemical parameters.
- ✓ The small particles inside the column are what cause the high back pressure at normal flow rates.
- ✓ The pump must push hard to move the mobile phase through the column and this resistance causes a high pressure within the chromatograph.

4. Detector:

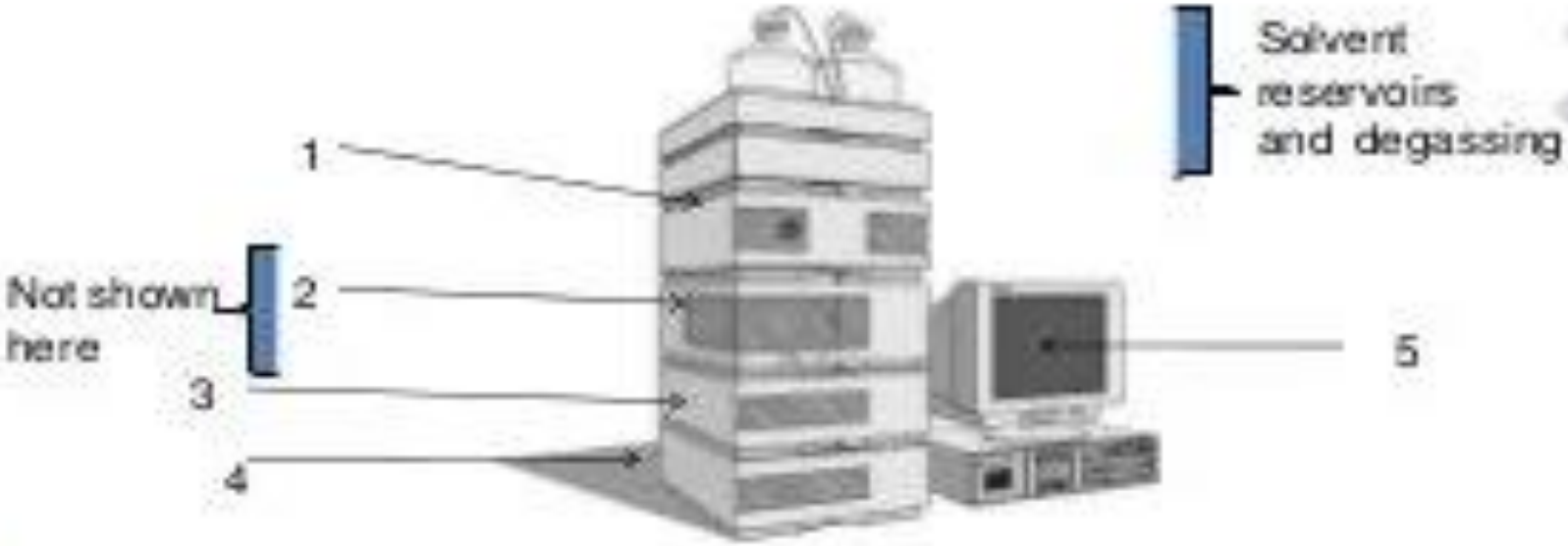
- ✓ The detector can see (detect) the individual molecules that come out (elute) from the column.
- ✓ A detector serves to measure the amount of those molecules so that the chemist can quantitatively analyze the sample components.
- ✓ The detector provides an output to a recorder or computer that results in the liquid chromatogram(i.e., the graph of the detector response).

5.Computer:

Frequently called the **data system**, the computer not only controls all the modules of the HPLC instrument but it takes the signal from the detector and uses it to determine the time of elution (**retention time**) of the sample components (**qualitative analysis**) and the amount of sample (**quantitative analysis**).

Instrumentation of HPLC

(Describing the 5 major components and their functions)



1 – Pump; 2 – Injector; 3 – Column; 4 – Detector; 5 – Computer

What is HPLC used for?

Separation and analysis of non-volatile or thermally-unstable compounds .

HPLC is optimum for the separation of chemical and biological compounds that are non-volatile.

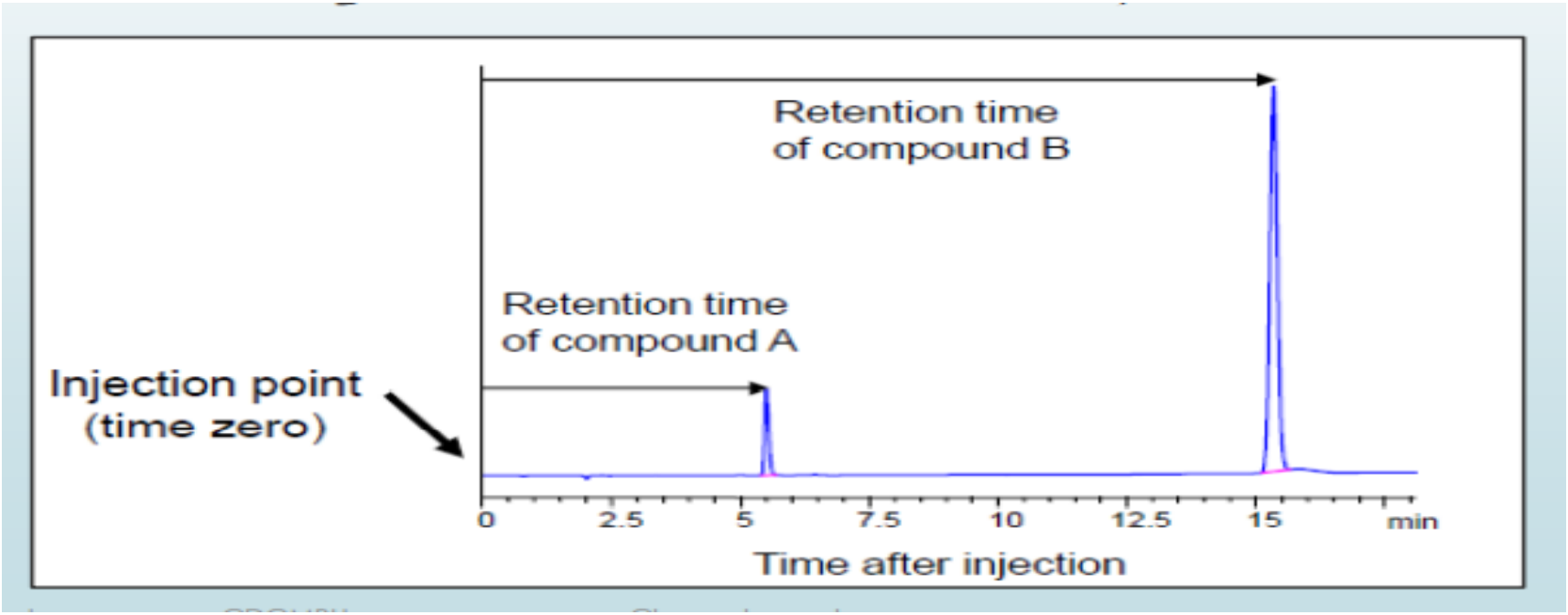
NOTE: If a compound is volatile (i.e. a gas, fragrance, hydrocarbon in gasoline, etc.), Gas chromatography is a better separation technique.

Typical non-volatile compounds are:

1. Pharmaceuticals like aspirin, ibuprofen, or acetaminophen (Tylenol)
2. Salts like sodium chloride and potassium phosphate
3. Proteins like egg white or blood protein
4. Organic chemicals like polymers (e.g. polystyrene, polyethylene)
5. Heavy hydrocarbons like asphalt or motor oil
6. Many natural products such as ginseng, herbal medicines, plant extracts
7. Thermally unstable compounds such as trinitrotoluene (TNT), enzymes , hormones.

1.Qualitative analysis by HPLC

The identification(ID) of individual compounds in the sample; the most common parameter for compound ID is its retention time(the time it takes)for that specific compound to elute from the column after injection); depending on the detector used, compound ID is also based on the chemical structure, molecular weight or some other molecular parameter



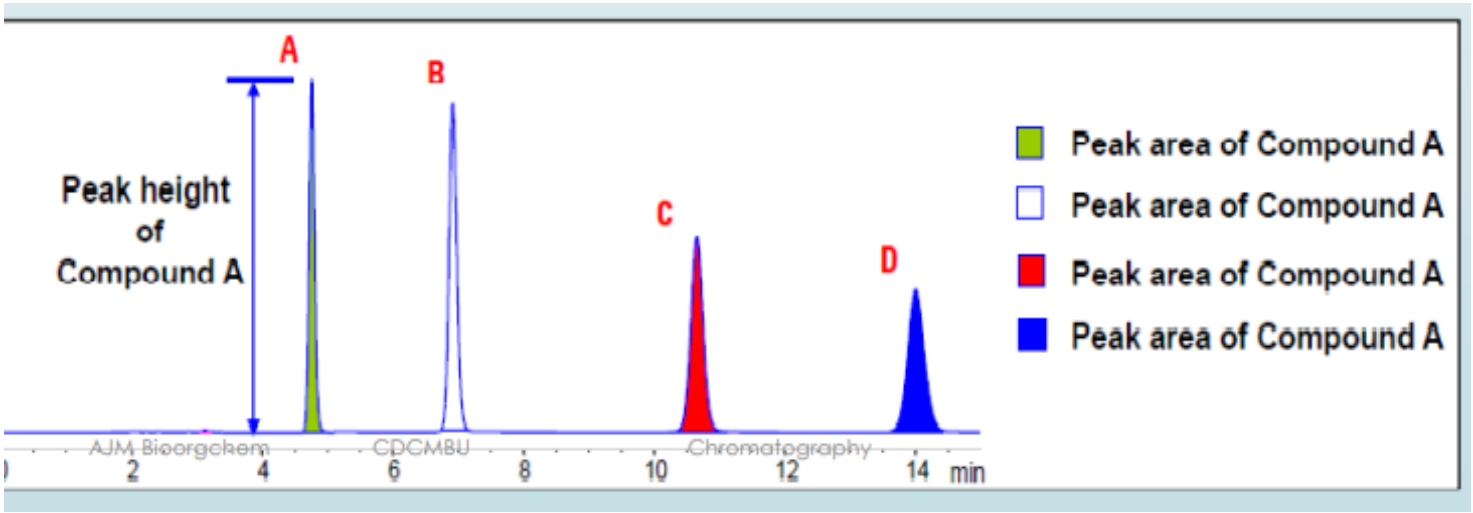
2. Quantitative analysis by HPLC

There are two main ways to interpret a chromatogram(i.e. perform quantification):

a**. determination of the peak height of a chromatographic peak as measured from the baseline;**

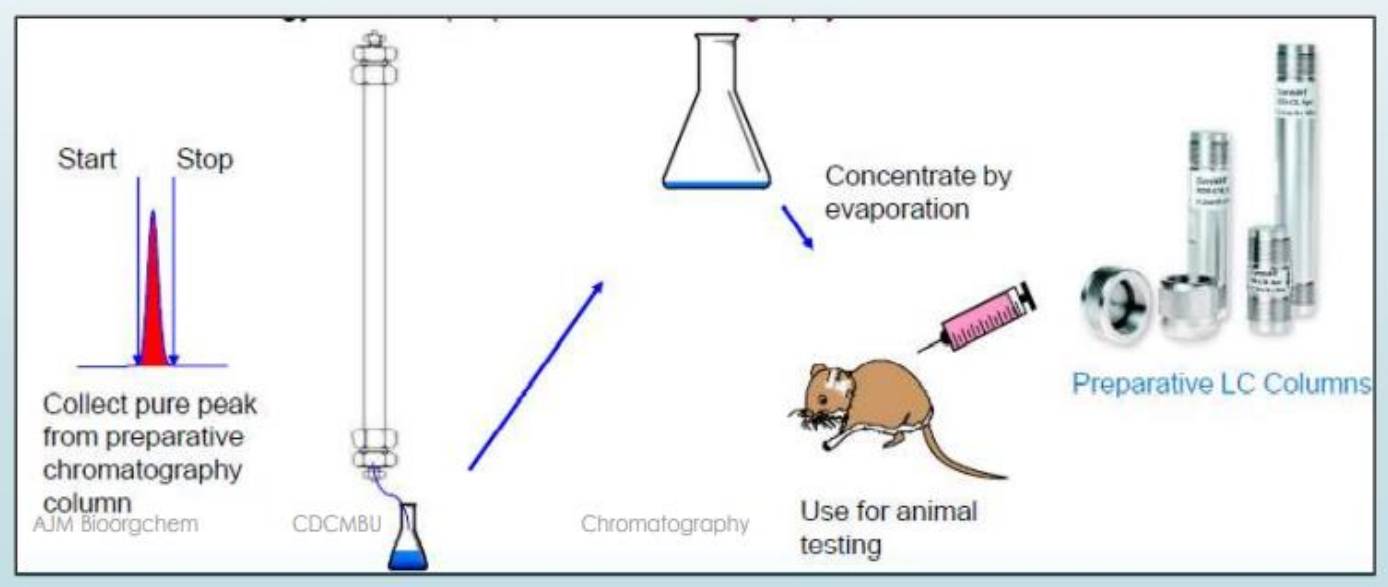
b. determination of the peak area (see figure below).

In order to make a quantitative assessment of the compound, a sample with a known amount of the compound of interest is injected and its peak height or peak area is measured. In many cases, there is a linear relationship between the height or area and the amount of sample



3. Preparation of Pure Compound(s)

- ✓ By collecting the chromatographic peaks at the exit of the detector,
- and concentrating the compound (analyte) by removing/evaporating the solvent,
- a pure substance can be prepared for later use (e.g. organic synthesis, clinical studies, toxicology studies, etc.).
- ✓ This methodology is called preparative chromatography.



Examples of Different Instruments and Configurations

Modular HPLC System – basic configuration with isocratic pump, manual injector, variable wavelength detector, and hand-held controller

Modular HPLC System – high-end configuration with quaternary pump, autosampler, column thermostat, diode array detector, and computer with control and data analysis SW

Integrated HPLC System "all parts in one box" – different configurations possible, here with gradient pump, autosampler, column oven, VWD, and computer with control and data analysis SW (not shown on picture)

Let's Look at Individual Tools models ?

Pump Module–types:

1. **Isocratic pump** -delivers constant mobile phase composition; •solvent must be pre-mixed; •lowest cost pump

2. **Gradient pump** -delivers variable mobile phase composition; •can be used to mix and deliver an isocratic mobile phase or a gradient mobile phase.

a. Binary gradient pump –delivers two solvents.

b. Quaternary gradient pump –four solvents.

Gradient vs. Isocratic Conditions

Isocratic

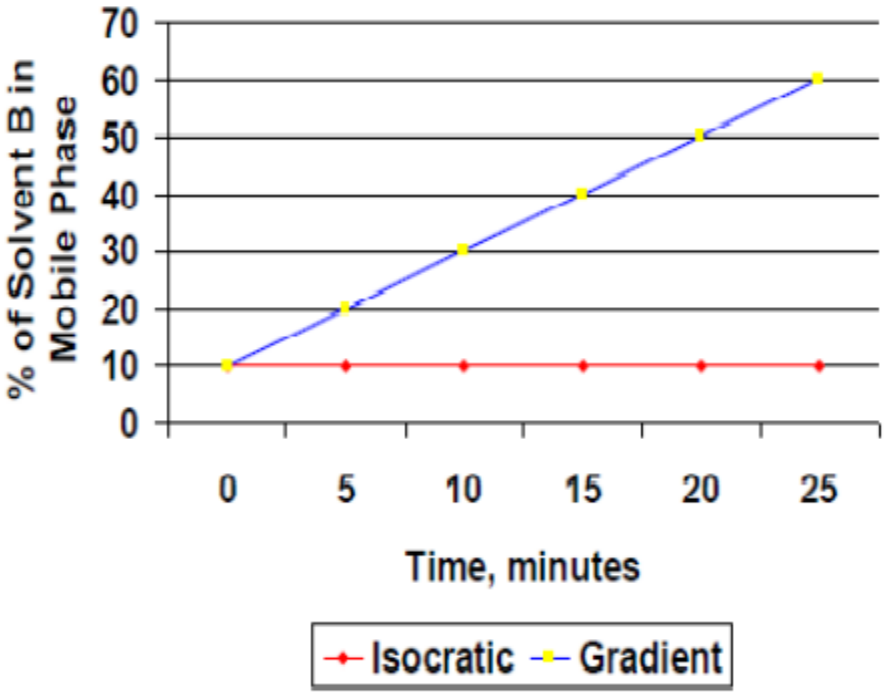
mobile phase solvent composition remains constant with time

- Best for simple separations
- Often used in quality control applications that support and are in close proximity to a manufacturing process

Gradient

mobile phase solvent ("B") composition increases with time

- Best for the analysis of complex samples
- Often used in method development for unknown mixtures
- Linear gradients are most popular (for example, the "gradient" shown at right)



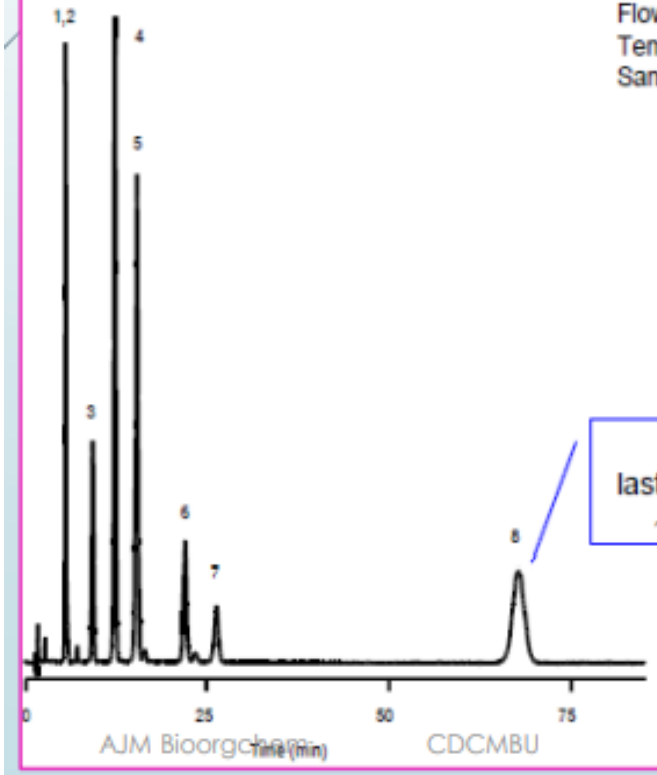
We think Mobile Phase Gradients in HPLC better than Isocratic?

Separation of Herbicides on ZORBAX StableBond-C18

Isocratic Elution

70% water/30% Acetonitrile

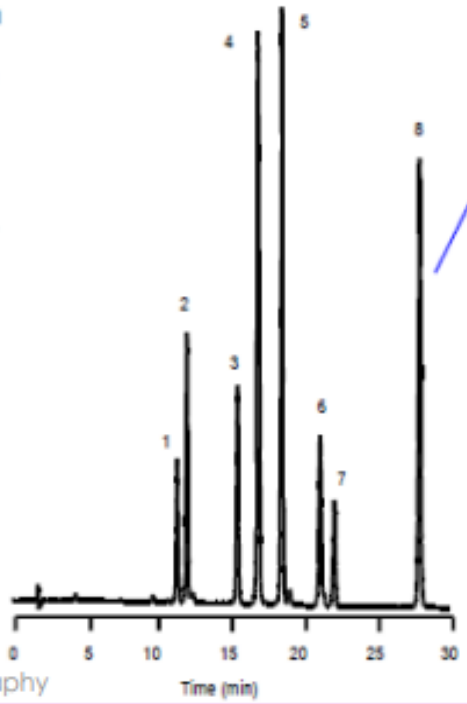
Column:	ZORBAX SB-C18 4.6 x 150 mm, 5 µm
Mobile Phase:	A: H_2O with 0.1% TFA, pH 2 B: Acetonitrile
Flow Rate:	1.0 mL/min
Temperature:	35°C
Sample:	1. Tebuthiuron 2. Prometon 3. Prometryne 4. Atrazine 5. Bentazon 6. Propazine 7. Propanil 8. Metolachlor



Note: last peak eluted in ~70 minutes

Gradient Elution

20 – 60% Acetonitrile in water in 30 min.



Note: last peak eluted in ~ 28 minutes and it is sharper

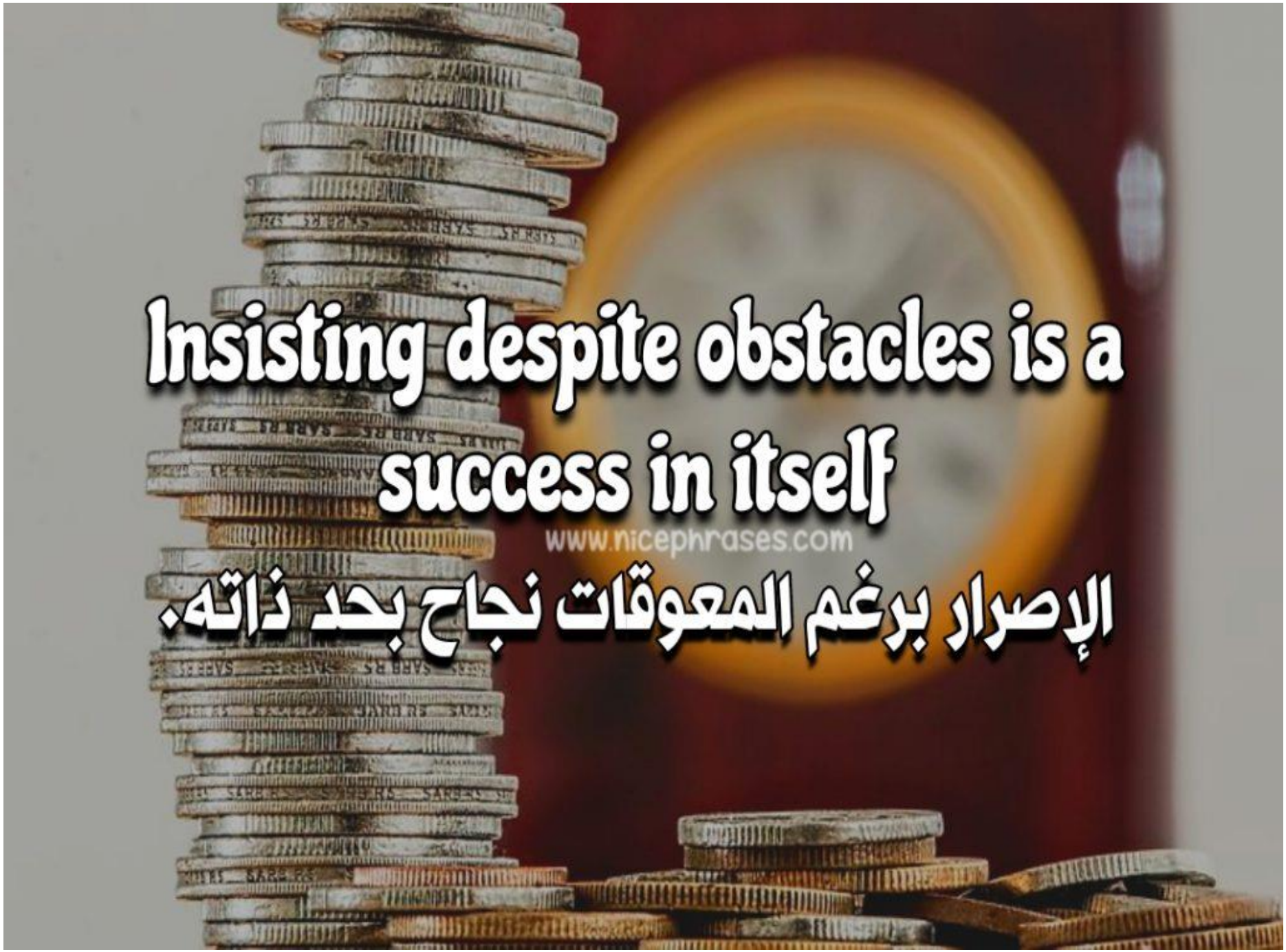
Major differences between HPLC & GC

HPLC

- Mobile phase changes
- Constant temperature
- Compounds partition from the mobile phase based on solubility.
- Elution is generally time or volume dependent

GC

- Mobile phase is constant
- Increasing temperature
- Compounds partition from the mobile phase based on volatility.
- Elution is generally temperature dependent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