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FACULTY OF ENGINEERING & TECCHNOLOGY

LT.8. HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Content Outline

1. What is HPLC?
2. History of HPLC
3. Advantages of HPLC
4. Schematic of liquid Chromatography
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What is HPLC?

- The most widely used analytical separations technique
- Utilizes a liquid mobile phase to separate components of mixture
- uses high pressure to push solvent through the column
- Popularity:
  - sensitivity
  - ready adaptability to accurate quantitative determination
  - suitability for separating nonvolatile species or thermally fragile ones

History lesson

- Early LC carried out in glass columns
  - diameters: 1-5 cm
  - lengths: 50-500 cm
- Size of solid stationary phase
  - diameters: 150-200 μm
- Flow rates still low! Separation times long!
- Eureka! Decrease particle size of packing causes increase in column efficiency!
  - diameters 3-10 μm
- This technology required sophisticated instruments
  - new method called HPLC

HPLC is….

- Popularity:
  - widespread applicability to substances that are of prime interest to industry, to many fields of science, and to the public
- Ideally suited for separation and identification of amino acids, proteins, nucleic acids, hydrocarbons, carbohydrates, pharmaceuticals, pesticides, pigments, antibiotics, steroids, and a variety of other inorganic substances

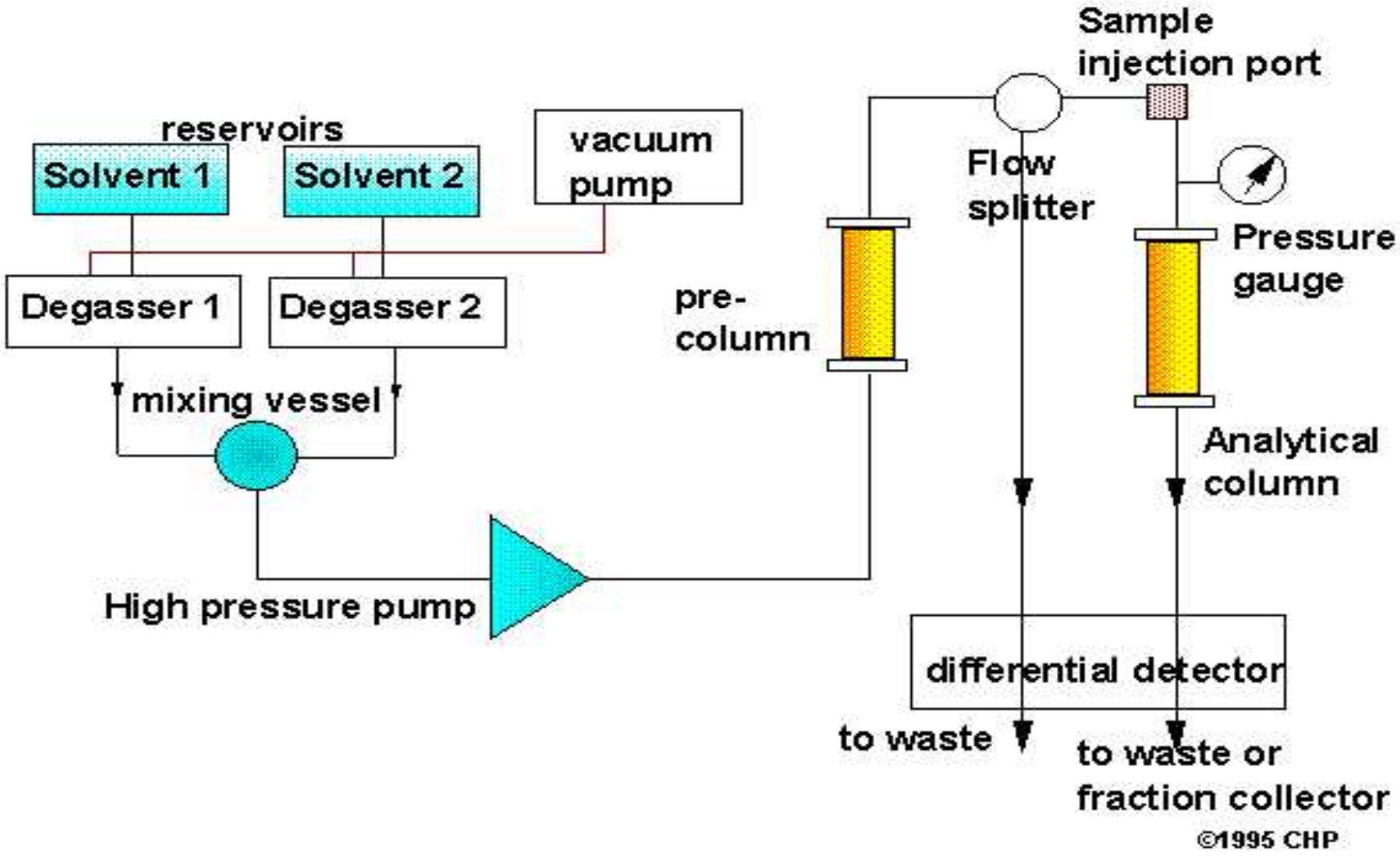
Advantages to HPLC

- Higher resolution and speed of analysis
- HPLC columns can be reused without repacking or regeneration
- Greater reproducibility due to close control of the parameters affecting the efficiency of separation
- Easy automation of instrument operation and data analysis
- Adaptability to large-scale, preparative procedures
- Advantages of HPLC are result of 2 major advances:
  - stationary supports with very small particle sizes and large surface areas
  - appliance of high pressure to solvent flow

Liquid chromatography

- Instrumentation
  - Mobile Phase Reservoir
  - Pumping Systems
  - Sample Injection Systems
  - Liquid-Chromatographic Columns
  - Detectors

Schematic of liquid chromatograph



LC column

LC injector

Types of HPLC

- Liquid-solid (adsorption) chromatography
- Liquid-liquid (partition) chromatography
- Ion-exchange chromatography
- Size exclusion chromatography

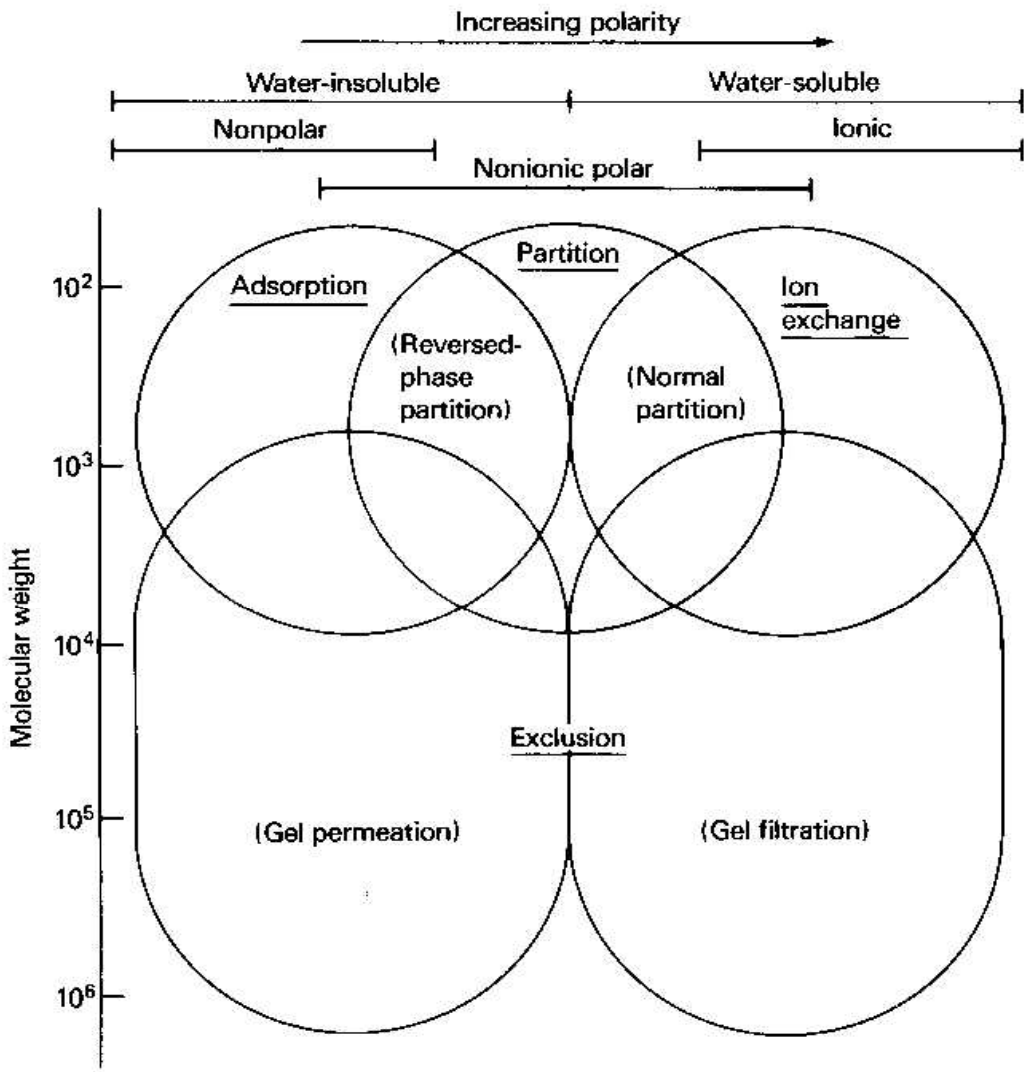


FIGURE 26–1 Applications of liquid chromatography. (From D. L. Saunders, in *Chromatography*, 3rd ed., E. Heftmann, Ed., p. 81. New York: Van Nostrand Reinhold, 1975. With permission.)

Instrumentation

Main components in an HPLC system include the solvent reservoir, or multiple reservoirs, a high-pressure pump, a column, injector system and the detector.

Columns

Two types of columns are available: Conventional column made up of stainless steel capability of withstanding pressure of 50 MPa. These have length of 3-25 cm and can give typical flow rates of 13 cm^3 min^{-1}. (ii) Microbore or open tubular columns have an internal diameter of 1–2 mm and are generally 25-50 cm long. They can sustain flow rates of 520 mm^3 min^{-1}.

Matrices and stationary phase

They are made of chemically modified silica or styrene/divinylbenzene copolymers. The two forms are:

• Microporous supports: In which micropores ramify through the particles that are generally 510 mm in diameter.

• Bonded phases: In which the stationary phase is chemically bonded onto an inert support such as silica

Mobile phase

The choice of mobile phase to be used in any separation depends on the type of separation to be achieved. Isocratic elution may be made with a single pump, using a single eluent or two or more eluents premixed in fixed proportions. Gradient elution generally uses separate pumps to deliver two eluents in proportions predetermined by a gradient programmer. It is also essential that all eluents be degassed before use otherwise gassing (the presence of air bubbles in the eluent) tends to occur in most pumps. Gassing, which tends to be particularly bad for eluents containing aqueous methanol and ethanol, can alter column resolution and interfere with the continuous monitoring of the eluate. Degassing of the eluent may be carried out in several ways – by warming, by stirring vigorously with a magnetic stirrer, by applying a vacuum, by ultrasonication, and by bubbling helium gas through the eluent reservoir.

Pumps

Pumping systems for delivery of the eluent are one of the most important features of HPLC systems. The main features of a good pumping system are that it is capable of outputs of at least 50 MPa and ideally there must be no pulses (i.e. cyclical variations in pressure) as this may affect the detector response. There must be a flow capability of at least 10 cm^3 min^{-1} and up to 100 cm^3 min^{-1} for preparative separations.

Detectors

It is used for detection of analyte in the sample. Detectors should have high sensitivity to detect even minute amount of analytes.

Variable wavelength detectors:

These are based upon ultraviolet–visible spectrophotometry. These types of detector are capable of measuring absorbances down to 190 nm and can give full-scale deflection (AUFS) for as little as 0.001 absorbance units. They have detection sensitivity of the order of 5 $\times 10^{-10}$ g cm–3 and a linear range of 10^5.

Scanning wavelength detectors: These have the facility to record the complete absorption spectrum of each analyte, thus aiding identification.

Fluorescence detectors: These are extremely valuable for HPLC because of their greater sensitivity (10–12 g cm–3) than UV detectors but they have a slightly reduced linear range. However, the technique is limited by the fact that relatively few analytes fluoresce.

Electrochemical detectors: These are selective for electroactive analytes and are potentially highly sensitive. Two types are available, amperometric and coulometric, the principles of which are similar.

Variants of HPLC

Ultra High Performance Liquid Chromatography (uHPLC):

Where standard HPLC typically uses column particles with sizes from 3 to 5µm and pressures of around 400 bar, uHPLC use specially designed columns with particles down to 1.7µm in size, at pressures in excess of 1000 bar. The main advantage of an uHPLC is speed. These systems are faster, more sensitive, and rely on smaller volumes of organic solvents than standard HPLC, resulting in the ability to run more samples in less time.

Fast protein liquid chromatography (FPLC):

FPLC is a system similar to high-performance liquid chromatography that is used to separate or purify proteins and other biomolecules from complex mixtures. The main difference between FPLC and HPLC is the standard working pressure. FPLC columns can only be used up to maximal pressures of 3-5 MPa. If the pressure of the HPLC can be limited, nearly every FPLC column may also be used in an HPLC system

Test your understanding

Fast protein Liquid chromatography (FPLC) can be used upto maximal pressure of

a. 3-5 Mpa

b. 3-5 kpa

c. 100 Mpa

d. None of these

HPLC can be made to operate like Fast Protein Liquid chromatography

a. By reducing working pressure

b. By increasing working pressure in excess of 50 Mpa

c. By operating at 50 Mpa

d. None of these

High performance liquid chromatography (HPLC) cannot be used to

a. separate types of organic pesticides

b. identify the various pigments from a leaf extract

c. determine the mercury content of a fish sample.

d. determine the caffeine content of coffee samples.

Expand the term HPLC……

What is the differing factor between HPLC and normal liquid column chromatography?