HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

By-

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What is High-performance liquid chromatography (HPLC)?

- High-performance liquid chromatography or commonly known as HPLC, is an analytical technique used to separate, identify or quantify each component in a mixture.
- The mixture is separated using the basic principle of column **chromatography** and then identified and quantified by spectroscopy.
- In the 1960s, the column chromatography LC with its low-pressure suitable glass columns was further developed to the HPLC with its high-pressure adapted metal columns.
- HPLC is thus basically a highly improved form of column liquid chromatography.
- Instead of a solvent being allowed to drip through a column under gravity, it is forced through under high pressures of up to 400 atmospheres.

Principle of High-Performance Liquid Chromatography (HPLC)

- The separation principle of high performance liquid chromatography works on the distribution of samples between a mobile phase or eluent and a stationary phase packed on the column.
- HPLC chromatographic peaks in the elution curve are affected mainly by flow rate, particle size, diffusion rate, and thickness of the stationary phase.
- Unfortunately, the smaller the particle size, the greater is the resistance to the flow of the mobile phase for a given flow rate.
- This resistance creates a backpressure in the column that is directly proportional to both the flow rate and the column length and inversely proportional to the square of the particle size.

... Principle of High-Performance Liquid Chromatography

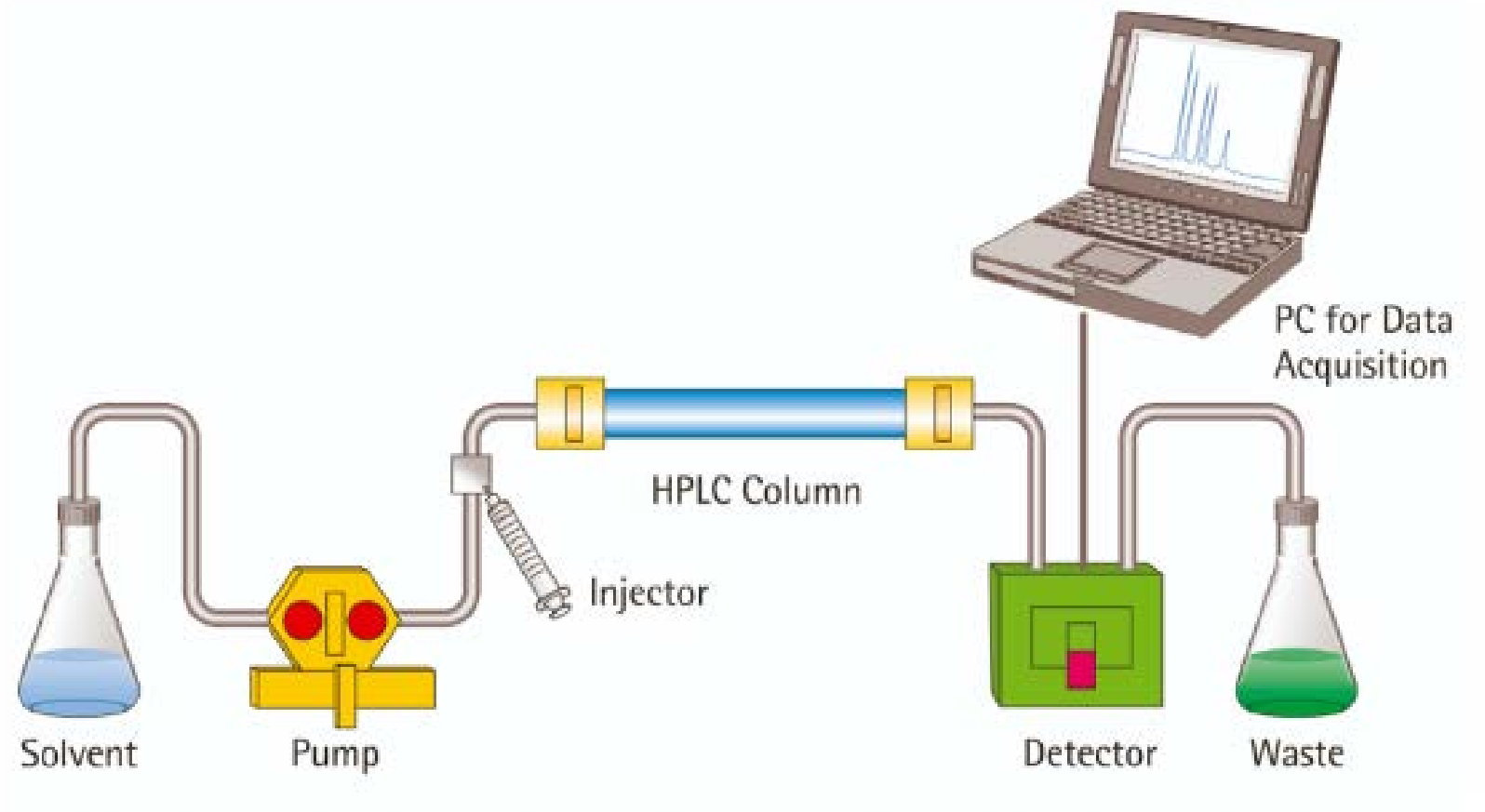
- The back-pressure may be sufficient to cause the structure of the matrix to collapse, thereby actually further reducing eluent flow and impairing resolution.
- This problem has been solved by the development of small particle size stationary phases, generally in the region of 5-10 mm diameter with a narrow range of particle sizes, which can withstand pressures up to 40 MPa.
- This development, which is the basis of HPLC that was originally and incorrectly referred to as high-pressure liquid chromatograph.

Columns

- Conventional columns used for HPLC are generally made of stainless steel and are manufactured so that they can withstand pressures of up to 50MPa.
- The columns are generally 3–25 cm long and approximately 4.6mm internal diameter to give typical flow rates of 1-3cm^3 min^{-1}.
- Microbore or open tubular columns have an internal diameter of 1–2mm and are generally 25-50 cm long. They can sustain flow rates of 5-20mm^3 min+.
- Microbore columns have three important advantages over conventional columns:
  - reduced eluent consumption due to the slower flow rates;
  - ideal for interfacing with a mass spectrometer due to the reduced flow rate; and
  - increased sensitivity due to the higher concentration of analytes that can be used.

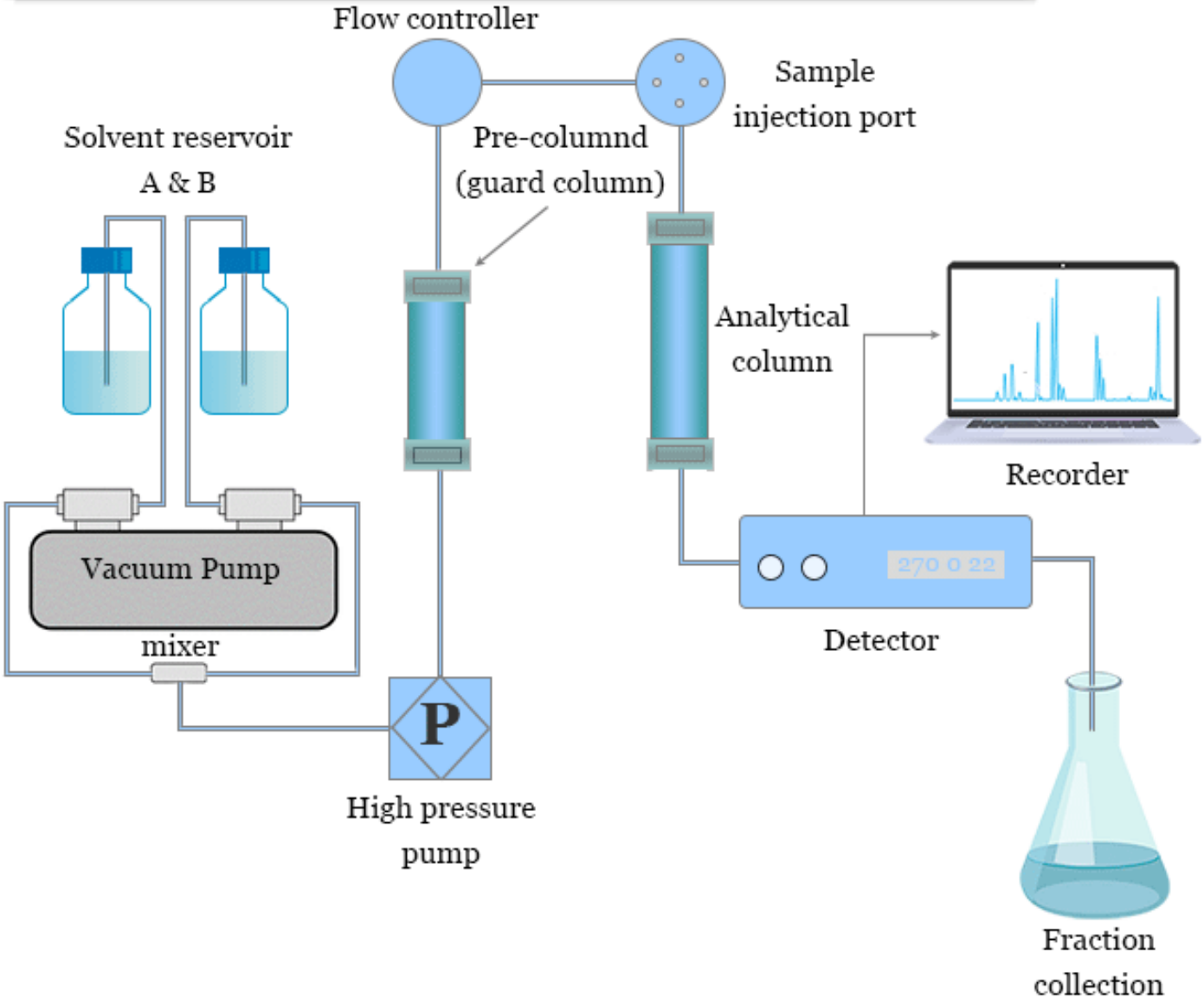
Matrices and stationary phases

- Two main forms of matrix/stationary phase material are available, based on a rigid solid structure.
- Both forms involve approximately spherical particles of a uniform size to minimise space for diffusion and hence band broadening to occur.
- They are made of chemically modified silica or styrene/divinyl benzene copolymers.
- The two forms are:
  - Microporous supports: In which micropores ramify through the particles that are generally 5-10 μm in diameter.
  - Bonded phases: In which the stationary phase is chemically bonded onto an inert support such as silica.



Components of an isocratic HPLC system. For gradient elution two reservoirs and two pumps are used with liquid-phase mixing before entry to the sample injection loop.

High Performance Liquid Chromatography



Application of sample

- The most common method of sample introduction is by use of a loop injector .
- This consists of a metal loop, of fixed small volume, that can be filled with the sample.
- The eluent from the pump is then channelled through the loop by means of a valve switching system and the sample flushed onto the column via the loop outlet without interruption of the flow of eluent to the column.
- Repeated application of highly impure samples such as sera, urine, plasma or whole blood, which have preferably been deproteinated, may eventually cause the column to lose its resolving power.
- To prevent this occurrence, a guard column is often installed between the injector and the column.
- This guard column is a short (12 cm) column of the same internal diameter and packed with material similar to that present in the analytical column.
- The packing in the guard column preferentially retains contaminating material and can be replaced at regular intervals.

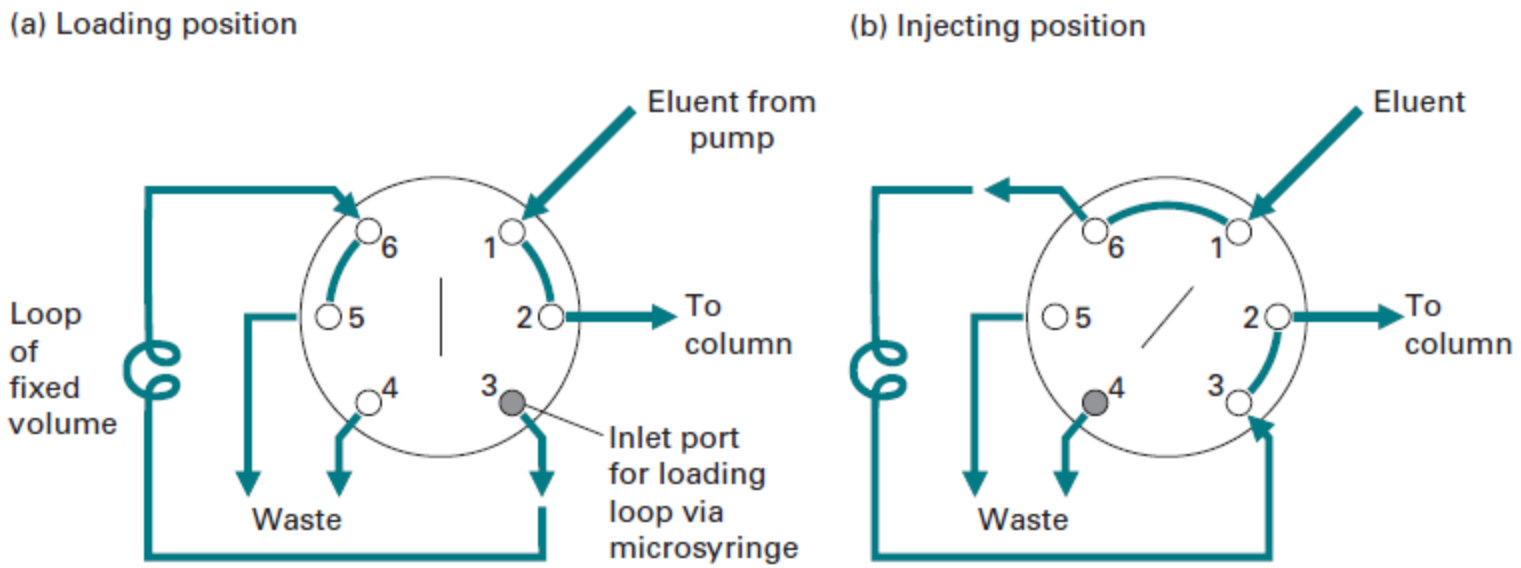


Fig. 11.5 HPLC loop injector. (a) The loop is loaded via port 3 with excess sample going to waste via port 5. In this position the eluent from the pump passes to the column via ports 1 and 2. (b) In the injecting position eluent flow is directed through the loop via ports 1 and 6 and then onto the column.

Mobile phases

- 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.
- All eluents for use in HPLC systems must be specially purified because traces of impurities can affect the column and interfere with the detection system.
- Pure eluents for use in HPLC systems are available commercially, but even with these a 15mm microfilter is generally introduced into the system prior to the pump.
- 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

- The main features of a good pumping system are that it is capable of outputs of at least 50 MPa.
- 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.
- Constant
- displacement pumps maintain a constant flow rate through the column irrespective of changing conditions within the column.

Detectors

- The most commonly used detectors are:
- Variable wavelength detectors: These are based upon ultraviolet–visible spectrophotometry.
  - These types of detector are capable of measuring absorbances down to 190nm and can give full-scale deflection for as little as 0.001 absorbance units.
  - All spectrophotometric detectors use continuous flow cells with a small internal volume (typically 8mm^3) and optical path length of 10mm which allow the continuous monitoring of the column eluate.

...Detectors

- **Scanning wavelength detectors:** These have the facility to record the complete absorption spectrum of each analyte, thus aiding identification.
- Such opportunities are possible either by temporarily stopping the eluent flow or by the use of diode array techniques, which allow a scan of the complete spectrum of the eluate within 0.01 s and its display as a 3D plot on a VDU screen in real time.
- **Fluorescence detectors:** These are extremely valuable for HPLC because of their greater sensitivity (10–12 g cm^{-3}) than UV detectors.
- However, the technique is limited by the fact that relatively few analytes fluoresce.
- Pre-derivatisation of the test sample can broaden the applications of the technique.
- **Mass spectrometer detectors:** These enable the analyte to be detected and its structure determined simultaneously.

...Detectors

- **NMR spectrometer detectors:** These give structural information about the analyte that is complementary to that obtained via HPLC–MS.
- **Refractive index detectors:** These rely on a change in the refractive index of the eluate as analytes emerge from the column.
- The great advantage is that they will respond to any analyte in any eluent, changes in refractive index being either positive or negative.
- **Evaporative light-scattering detectors (ELSD):** These rely on the vaporisation of the eluate, evaporation of the eluent and the quantification of the analyte by light scattering.
- The eluate emerging from the column is combined with a flow of air or nitrogen to form an aerosol; the eluent is then evaporated from the aerosol by passage through an evaporator and the emerging dry particles of analyte irradiated with a light source and the scattered light detected by a photodiode.
- The intensity of the scattered light is determined by the quantity of analyte present and its particle size.