TEJASVI NAVADHITAMASTU

"Let our (the teacher and the taught) learning be radiant"

Let our efforts at learning be luminous and filled with joy, and endowed with the force of purpose

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CHROMATOGRAPHY -II

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

High-performance liquid chromatography (**HPLC**; formerly referred to as **high-pressure liquid chromatography**), is a technique in analytical chemistry used to separate, identify, and quantify each component in a mixture.

It relies on pumps to pass a pressurized liquid solvent containing the sample mixture through a column filled with a solid adsorbent material.

Each component in the sample interacts slightly differently with the adsorbent material, causing different flow rates for the different components and leading to the separation of the components as they flow out the column.

Principle

Resolving power of a chromatographic column is determine by distribution coefficient, column efficiency, selectivity factors and retention factors.

Resolution increases with:

1. The number of theoretical plates (N) in the column and hence plate height (H). The value of N increases with column length but there are practical limits to the length of a column owing to the problem of peak broadening.

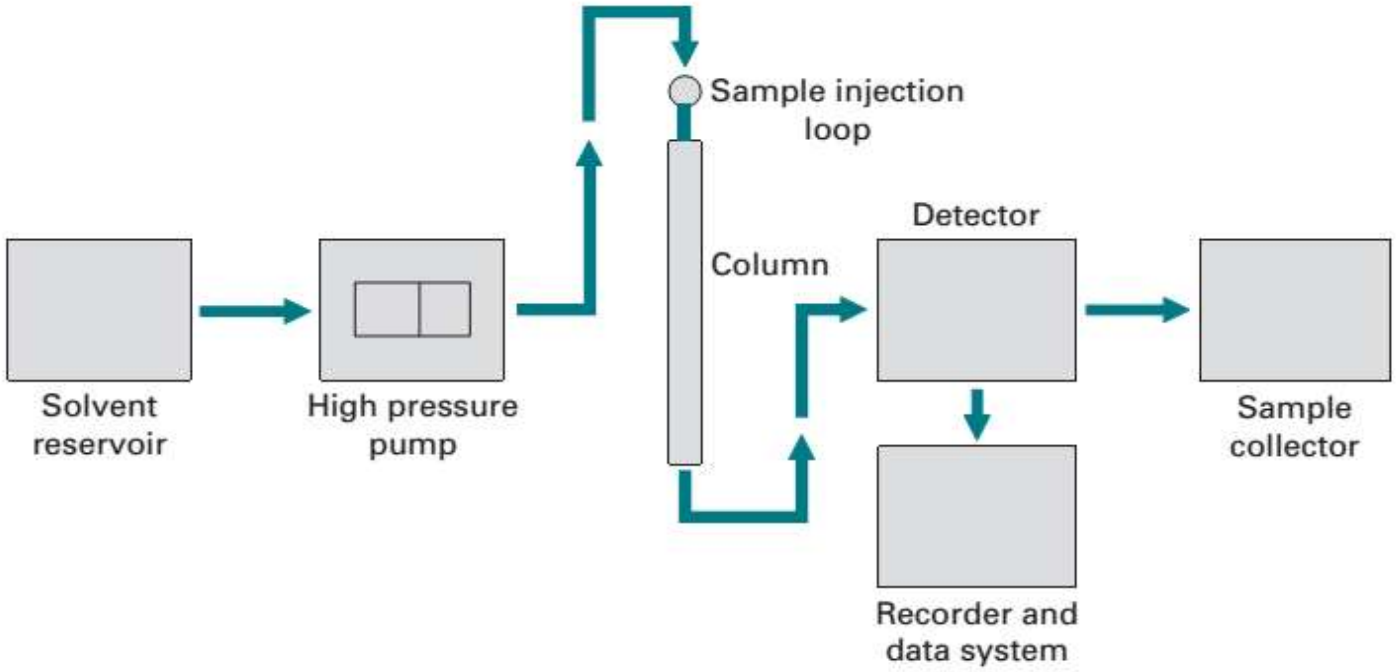
2. The selectivity of the column represented by “a”

3. The retentivity of the column as determined by the retention factor,k.

Columns

Conventional columnsused for HPLC are generally made of stainless steel and are manufactured so that they can withstand pressures of up to 50 MPa.

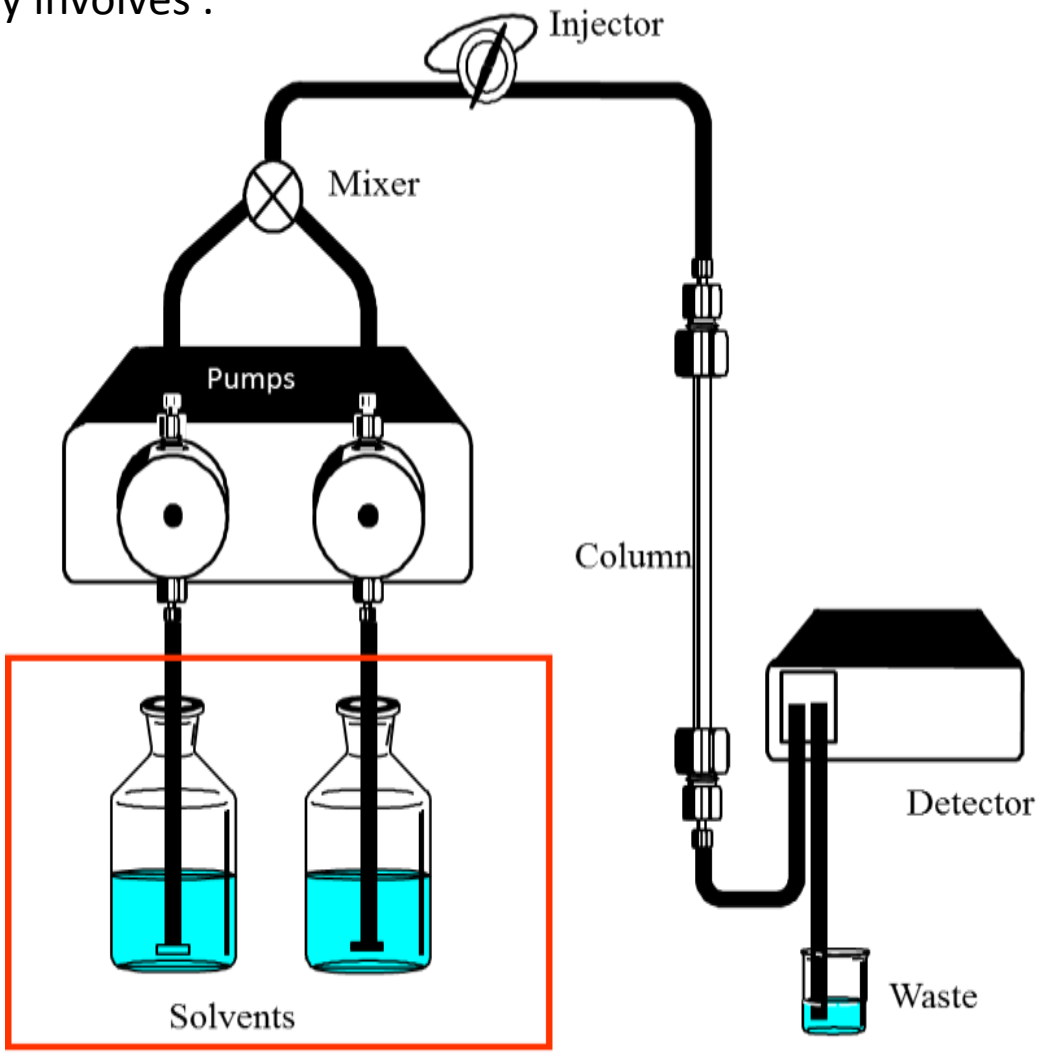
The columns are generally 3–25 cm long and approximately 4.6 mm internal diameter to give typical flow rates of 1 to 3cm^3 par minute.



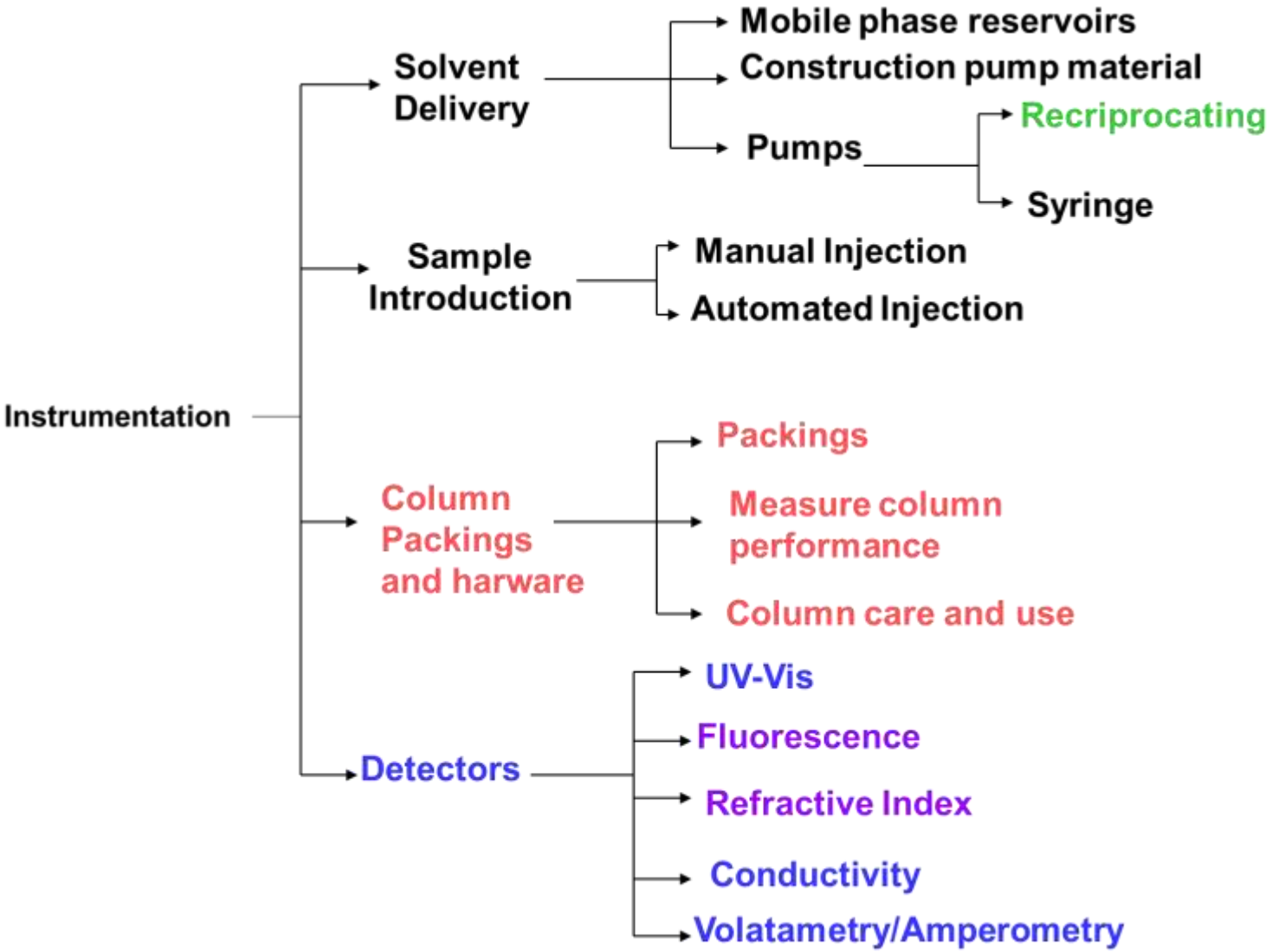
Instrumentation

The experimental set-up of HPLC mainly involves :

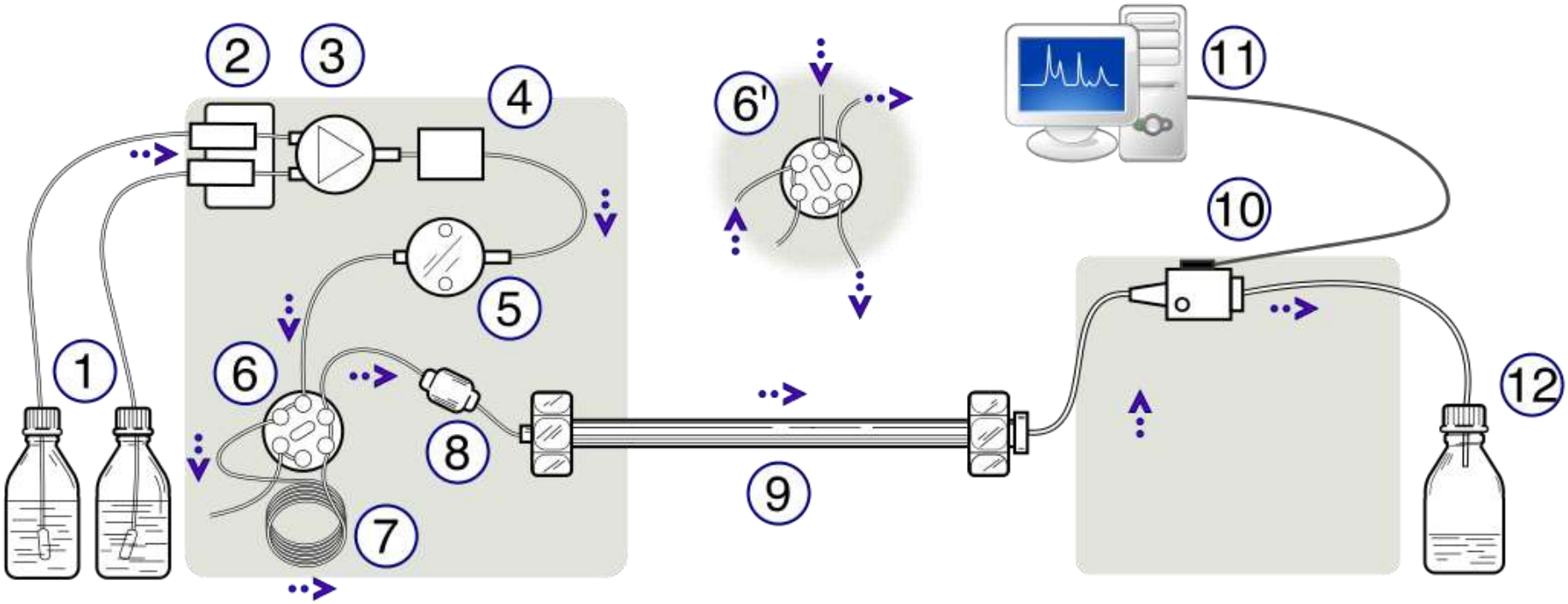
1. Solvent reservoir
2. Pump
3. Damping device
4. Sampling device
5. Column
6. Detector
7. Fraction collector
8. Recorder



HPLC Instrumentation and Techniques



HPLC Instrumentation and Techniques



Schematic representation of an HPLC unit. (1) Solvent reservoirs, (2) Solvent degasser, (3) Gradient valve, (4) Mixing vessel for delivery of the mobile phase, (5) High-pressure pump, (6) Switching valve in "inject position", (6') Switching valve in "load position", (7) Sample injection loop, (8) Pre-column (guard column), (9) Analytical column, (10) Detector (i.e. IR, UV), (11) Data acquisition, (12) Waste or fraction collector.

Distribution coefficients

The basis of all forms of chromatography is the distribution or partition coefficient (K_d), which describes the way in which a compound (the analyte) distributes between two immiscible phases. For two such phases A and B, the value for this coefficient is a constant at a given temperature and is given by the expression:

$$\frac{\text{concentration in phase A}}{\text{concentration in phase B}} = K_d \qquad (11.1)$$

- The distribution of a solute between the mobile and stationary phases in chromatography is described by κ, the partition coefficient, defined by:

$$\kappa = Cs\ Cm$$

  - Cs: concentration of solute in the stationary phase
  - Cm: concentration of the solute in the mobile phase.
  - The mobile phase serves to carry the sample molecules through the chromatographic column

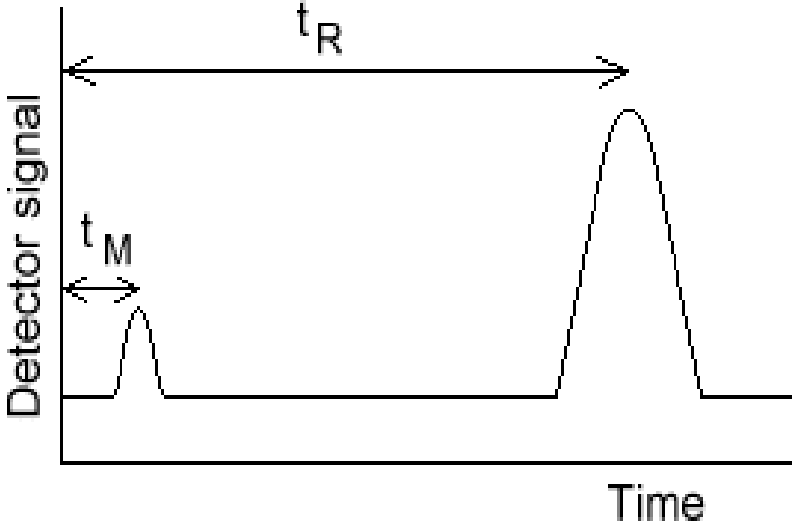
RETENTION TIME

During the sample molecules transportation through the column, each analyte is retained according to that compound's characteristic affinity for the stationary phase.

The time that passes between the sample injection and peak maximum is called the retention time.

The retention time, tr, is given in seconds by:

tr = ts + tm



ts: time the analyte spends in the stationary phase

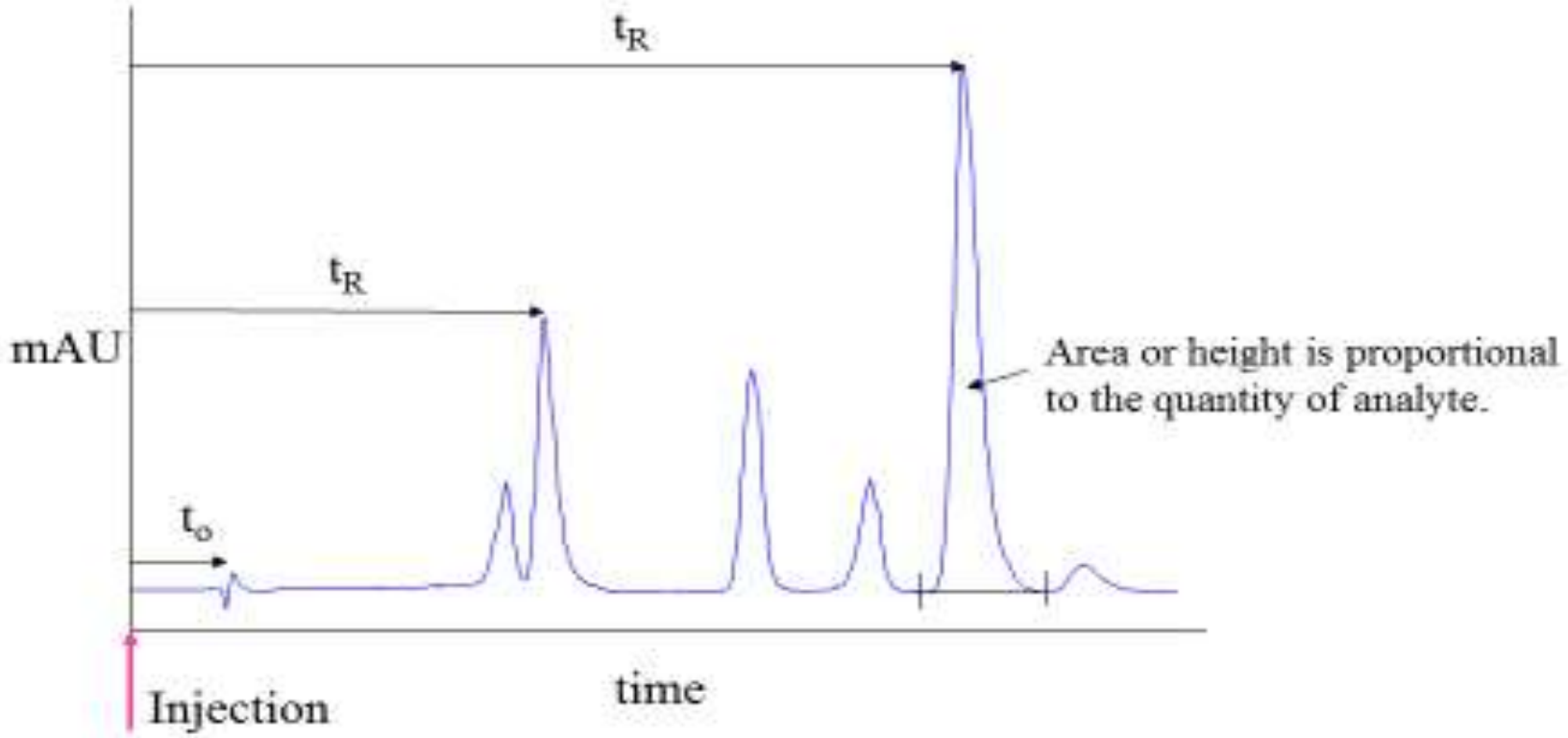
Tm: time spent in the mobile phase it is often referred to as the dead, or void time, as all components spend tm in the mobile phase

The area underneath each peak is proportional to the amount of corresponding analyte in solution.

THE CHROMATOGRAM

t_o - elution time of unretained peak

t_R- retention time - determines sample identity



FAST PROTEIN LIQUID CHROMATOGRAPHY

FPLC was developed and marketed in Sweden by Pharmacia in 1982 and was originally called **fast performance liquid chromatography** to contrast it with HPLC or high-performance liquid chromatography.

Fast protein liquid chromatography (FPLC), is a form of liquid chromatography that is often used to analyze or purify mixtures of proteins.

As in other forms of chromatography, separation is possible because the different components of a mixture have different affinities for two materials, a moving fluid (the "mobile phase") and a porous solid (the stationary phase).

FPLC- A modification of HPLC

- FPLC was introduced by PHARMACIA (Sweden) at 1982. (Pharmacia's smart system). FPLC = Fast Protein Liquid Chromatography
- FPLC is basically a "protein friendly" HPLC system in which stainless steel components replaced with glass and plastic.
- The chance of denaturation is high because of their stainless steel made instruments which elevates the inner temperature and resulting denaturation of sample (protein) under investigation.
- Also many ion-exchange separations of proteins involve salt gradients; thought these conditions could result in attack of stainless steel system.
- FPLC is an intermediate between classical column chromatography and HPLC.

Principle

In FPLC the mobile phase is an aqueous solution, or "buffer". The buffer flow rate is controlled by a positive-displacement pump and is normally kept constant, while the composition of the buffer can be varied by drawing fluids in different proportions from two or more external reservoirs.

The stationary phase is a resin composed of beads, usually of cross-linked agarose, packed into a cylindrical glass or plastic column.

FPLC resins are available in a wide range of bead sizes and surface ligands depending on the application.

In the most common FPLC strategy, ion exchange, a resin is chosen that the protein of interest will bind to the resin by a charge interaction while in buffer A (the running buffer) but become dissociated and return to solution in buffer B (the elution buffer).

FPLC is generally applied only to proteins; however, because of the wide choice of resins and buffers it has broad applications.

In contrast to HPLC the buffer pressure used is relatively low, typically less than 5 bar, but the flow rate is relatively high, typically 1-5 ml/min.

FPLC can be readily scaled from analysis of milligrams of mixtures in columns with a total volume of 5ml or less to industrial production of kilograms of purified protein in columns with volumes of many liters.

When used for analysis of mixtures the effluent is usually collected in fractions of 1-5 ml which can be further analyzed, e.g. by MALDI mass spectrometry.

When used for protein purification there may be only two collection containers, one for the purified product and one for waste.[2]

Procedure:

- A mixture containing one or more proteins of interest is dissolved in 100% buffer A and pumped into the column.
- The proteins of interest bind to the resin while other components are carried out in the buffer.
- The total flow rate of the buffer is kept constant; however, the proportion of Buffer B (the "elution" buffer) is gradually increased from 0% to 100% according to a programmed change in concentration (the "gradient").
- At some point during this process each of the bound proteins dissociates and appears in the effluent.
- The effluent passes through two detectors which measure salt concentration (by conductivity) and protein concentration (by absorption of ultraviolet light at a wavelength of 280nm).
- As each protein is eluted it appears in the effluent as a "peak" in protein concentration and can be collected for further use.[1]

Comparison between HPLC & FPLC

HPLC	FPLC
Column is made up of steel	Plastic or glass columns are used
Pressurized pump generates pressure 0-550 bar (14.6-8000 psi)	It is 0-40 bar in case of FPLC
Standard analytical column of 4-5 mm and 10-30 cm in length is used	Microbore column of dimension 1-2mm*10-25cm is widely used
Flow rate is in between 0.010-10ml/min	Flow rate is in between 1-499 ml/hr
Not suitable for thermolabile compounds or protein separation	Very reliable in separating and purifying proteins
Can separate any molecule	Used only for proteins
Follow adsorption chromatography	Follow ion exchange and gel filtration chromatography
Sample loading capacity is low (0.5 ml)	Sample loading capacity is high (upto 50 ml)
Stationary phase is generally made up of silica	Stationary phase is generally made up of agarose

ULTRA PERFORMANCE LIQUID CHROMATOGRAPHY (UPLC)

1. **Ultra Performance** Liquid Chromatography or **Ultra Pressure** Liquid chromatography.
2. It improves in three areas:
    1. **Resolution,**
    2. **Speed,**
    3. **Sensitivity.**
3. It can withstand **high system back-pressure.**
4. Special analytical columns **UPLC BEH C18** packed with **1.7µm** particles are used in connection with system.
5. The factor responsible for development of UPLC technique was **evolution of packing material** used to effect the separation.
6. The technology takes full **advantage** of chromatographic principles to run separations using **columns packed with smaller particles.**
7. It **decreases analysis time** and **solvent consumption**.

PRINCIPLE

- The principle of UPLC is based on **Van Deemeter equation** which describes the relationship between flow rate and HETP or column efficiency.
- A completely new system design with advanced technology in the pump, auto sampler, detector, data system, and service diagnostics was required.
- The ACQUITY UPLC system has been designed for low system and dwell volume.
- Achieving small particle, high peak capacity separations requires a greater pressure range than that achievable by HPLC system.

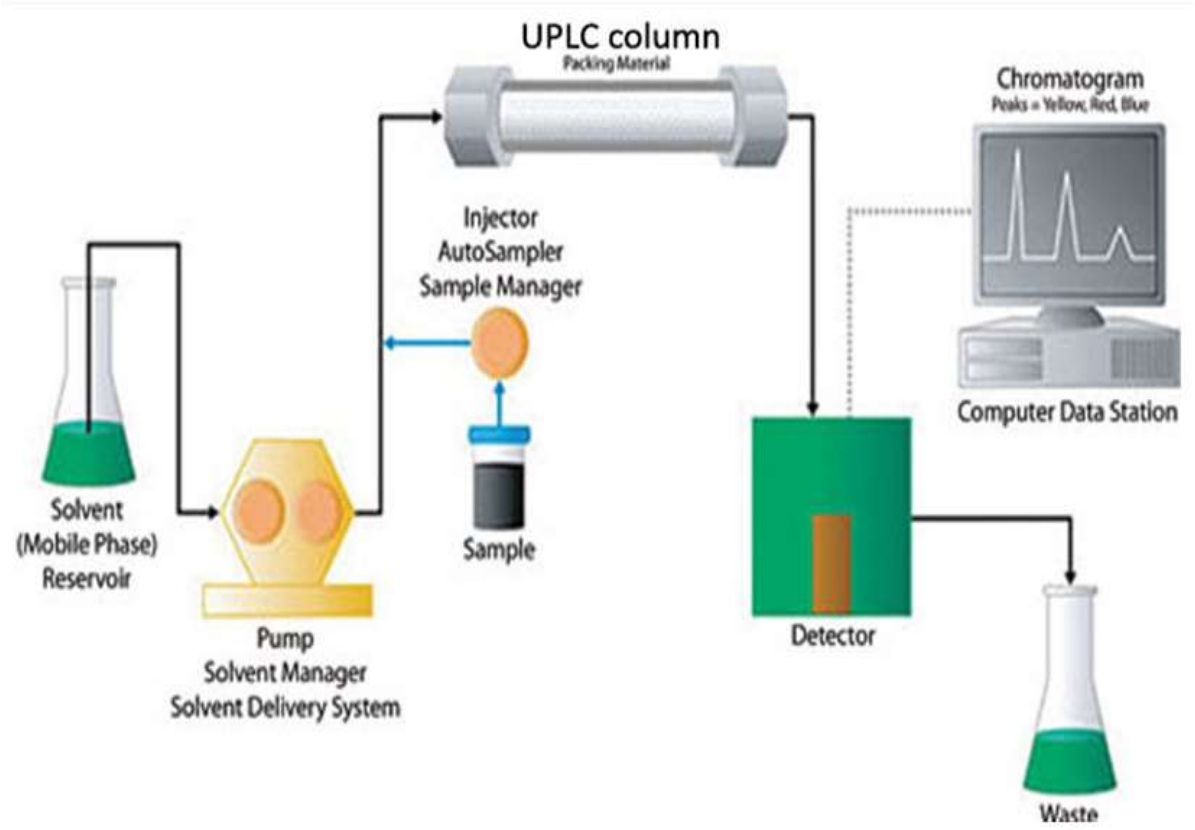
Comparison between HPLC & UPLC

CHARACTERISTICS	HPLC	UPLC
Particle size	3-5µm	Less than 2µm
Maximum back pressure	35-40 Mpa less	103.5 Mpa more
Column	Alltima C18	Acquity UPLC BEH C18
Column dimension	150 X 3.2 mm	150 X 2.1 mm
Column temperature	30 °C	65 °C
Volume injection	5µL (Std. In100% MeOH)	2µL (Std.In100% MeOH)
Sample throughput	less	more
Sample preparation	simple	tedious
Column coagulation	Does not takes place	Takes place
Analysis time	more	less
Sensitivity	less	higher

Parameters	HPLC Assay	UPLC Assay
Column	XTerra,C18,50 × 4.6mm	AQUITY UPLC BEH C18,50 ×2.1mm
Particle size	4µm particles	1.7µm particles
Flow rate	3.0 ml per min	0.6 ml per min
Injection volume	20 µl	3 µl partial loop fill or 5 µl full loop fill
Total run time	10 min	1.5 min
Theoretical Plate count	2000	7500
Lower limit of quantization	0.2 µg/ml	0.054µl/ml
Total solvent consumption	Acetonitrile:10.5ml, water:21ml	Acetonitrile:0.53ml, water:0.66ml
Delay volume	720 µl	110 µl
Column temperature	30 °C	65 °C
Maximum back pressure	35-40 Mpa less	103.5 Mpa, more
Resolution	Less	High
Method development cost	High	Low

SYSTEM COMPONENTS / INSTRUMENTATION

1. **Solvent reservoirs**
2. **Auto sampler**
3. **Van guard column**
4. **Tubing's**
5. **Column**
6. **Detectors :**
   - I. UV detectors
   - II. Fluorescent detector
   - III. Refractive index detector
   - IV. Light scattering detector
   - V. Electrochemical detector
   - VI. Mass spectrometric detector



ADVANTAGES OF UPLC

- **Shortening analysis time** up to **nine** times.
- Provides the **selectivity, sensitivity**, and **dynamic range** of LC analysis.
- Maintains **resolution** performance.
- Fast **resolving power** quickly quantifies related and unrelated compounds.
- Operation **cost** is reduced.
- Less **solvent consumption.**
- Improves the quality of data , resulting in more definitive map.
- Separation on UPLC is performed at very high pressures up to 15000psi.

DISADVANTAGES OF UPLC

- Due to increased pressure **requires more maintenance** and **reduces the life of the columns.**
- In addition, the **phases** of less than **2 µm** are generally **non-regenerable** and thus have limited use.

SUMMERY

- FACTORS AFFECTING PRFORMANCE OF UPLC: Pressure, Column Particle size of packing, Temperature.
- Smaller particles gives improved separations, mostly following expected trends.
- Reduction in sample size, significantly show reduction in flow rate.
- UPLC sets new standard in the science of chromatography. Working range with 15000 to 16000 psi pressure and column packed with size less than 2µm.
- Due to very narrow and sharp peaks, more number of peaks may appear in less time which may facilitate in analysis of complex mixtures and it may give more information regarding sample to be analyzed.

RAPID RESOLUTION LIQUID CHROMATOGRAPHY : RRLC

- Rapid Resolution Liquid Chromatography (RRLC) has become an increasingly useful approach to achieve higher throughput, improve sensitivity and reduce costs.
- Because of their lower particle sizes they operate at higher pressure (600 Bar) levels than normal columns (400 Bar).
- “Rapid Resolution” LC system enables faster analysis (theoretically up to 20x) than with conventional HPLC while maintaining equivalent resolution.
- This is achieved by using sub-2 micron column particle chemistry and high flow rates. Often higher temperatures are employed to minimize system back-pressure.
- Typically RRLC (Rapid Resolution Liquid Chromatography)columns have lower particle size - 1.8 microns compared with 2 to 10 micron conventional columns.

COLUMNS IN RRLC

- Thermostatted Column Compartment SL
- Temperature range: 10 °C below ambient to 100 °C
- Two independent heat exchangers allow pre-column heating and post-column cooling for lowest detection limits 400.

ADVANTAGES

- High resolution chromatography – 90,000 plates in 4 minutes
- Ultra-fast separations – up to 20 times faster
- Full compatibility with existing HPLC methods
- More detection capabilities – from UV-visible and ELSD through LC/MS
- Near-zero sample carryover – for uncompromised data quality
- Highest system flexibility – for automated method development

PARAMETERS IN HPLC AND RRLC

	HPLC	RRLC
Column ID(mm)	2.1- 4.6	2.1- 4.6
Particle size (µm)	3,5	1.8
Pressure (psi)	3000	9000
Flow rate (ml/min)	0.6-1.2	0.2- 2.0
Temperature (^{0}c)	upto 40	upto 100

APPLICATIONS OF RRLC

FASTER ANALYSES WITH RRLC

Quaternary Amines by HPLC-CAD

System: Agilent 1200 RRLC
Column: Shiseido MG C18, 4.6 x 250 mm (5 µm)

Mobile Phases A: Water, 0.1% Formic acid
B: Acetonitrile
Flow Rate : 1.00 mL/min
Gradient : T= 0 min 10% B, T= 15 min 90% B, T= 20 min 90% B, T= 22 min 10% B.

Column Temperature: 40 °C

Injection Volume: 2 µL

Run Time: 25.00 minutes

Corona CAD: N2 Pressure: 35.0 psi

Filter: High

Nebulizer Temperature: 30 °C

Quaternary Amines by RRLC-CAD

System: Agilent 1200 RRLC
Column: Waters UPLC BEH C18, 2.1 x 50 mm (1.7 µm)

Mobile Phases A: Water, 0.1% Formic acid
B: Acetonitrile
Flow Rate : 0.65 mL/min
Gradient: : T= 0 min 10% B, T= 3 min 90% B,T= 4 min 90% B, T= 4.5 min 10% B

Column Temperature: 50 °C (pre-col.), 30°C (post-col.)
Injection Volume: 2 µL

Run Time: 6.00 minutes

Corona *ultra*2 Pressure: 35.0 psi: N

Filter: High

Nebulizer Temperature: 30 °C

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