HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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High Performance (or High Pressure) Liquid Chromatography (HPLC)

1. What is Liquid Chromatography?
2. What is HPLC?
3. Why we call it HPLC?
4. Basic HPLC Instrumentation.
5. How it works?
6. Applications of HPLC.

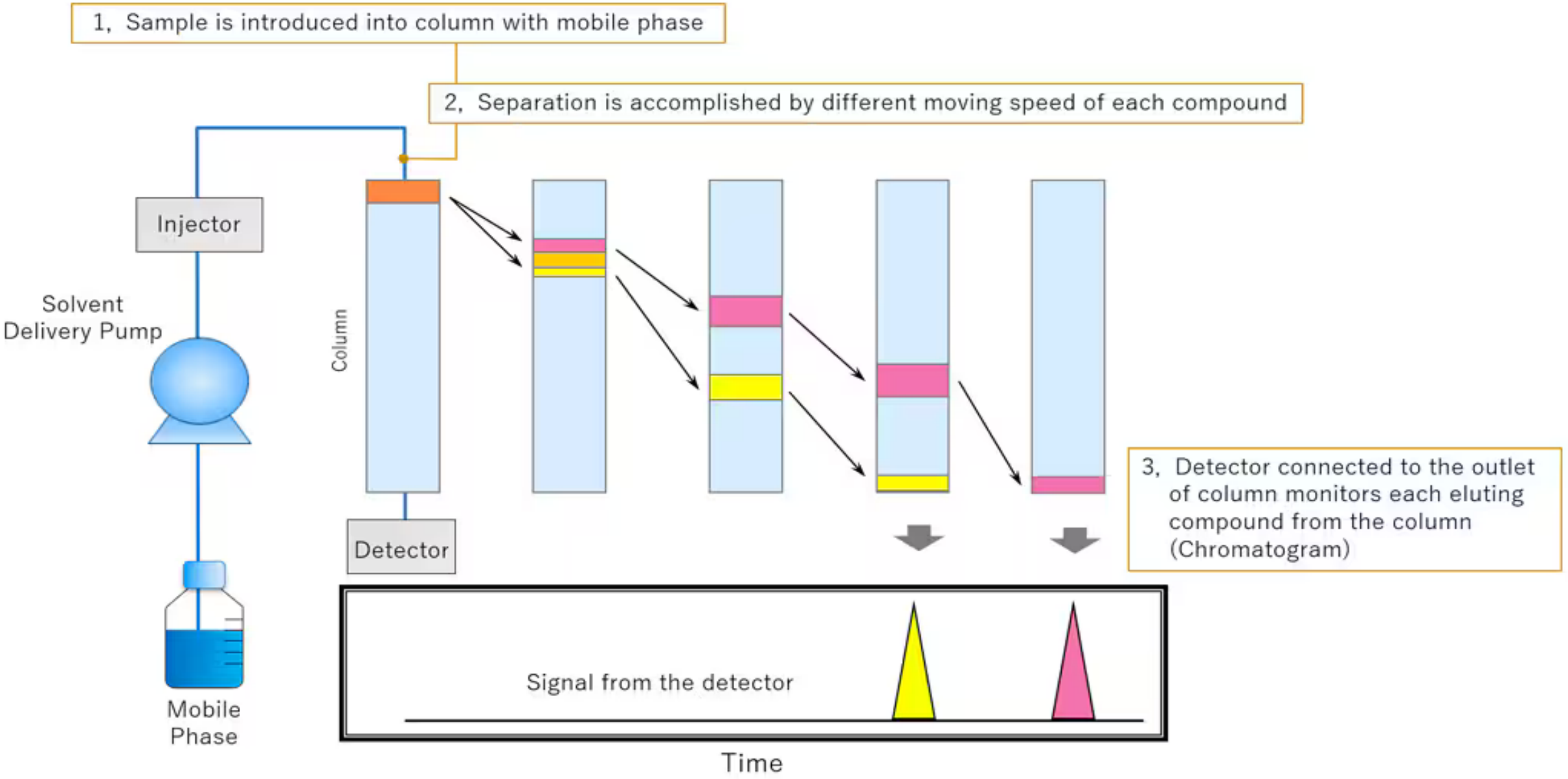
What is Liquid Chromatography?

Liquid Chromatography is a separation technique that involves :

- The placement (injection) of small volume of liquid sample into a tube packed with porous particle (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 chemicals and physical interaction 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 spectrophotometer that measures the intensity of the colour or by another device that can measure their amount.


1, Sample is introduced into column with mobile phase
2, Separation is accomplished by different moving speed of each compound
Injector
Solvent
Delivery Pump
Column
Detector
Mobile
Phase
3, Detector connected to the outlet of column monitors each eluting compound from the column (Chromatogram)
Signal from the detector
Time

What is HPLC ?

HPLC is a type of **liquid chromatography** which is Characterized by the use of high pressure & small particle size to push a mobile phase solution through a column of stationary phase allowing separation of complex mixtures with high resolution.

The components of complex mixture are separated by the column and detected at the exit of the column by a detector that measures their amount.

An output from this detector is called **Liquid Chromatogram.**

Why we call it HPLC ?

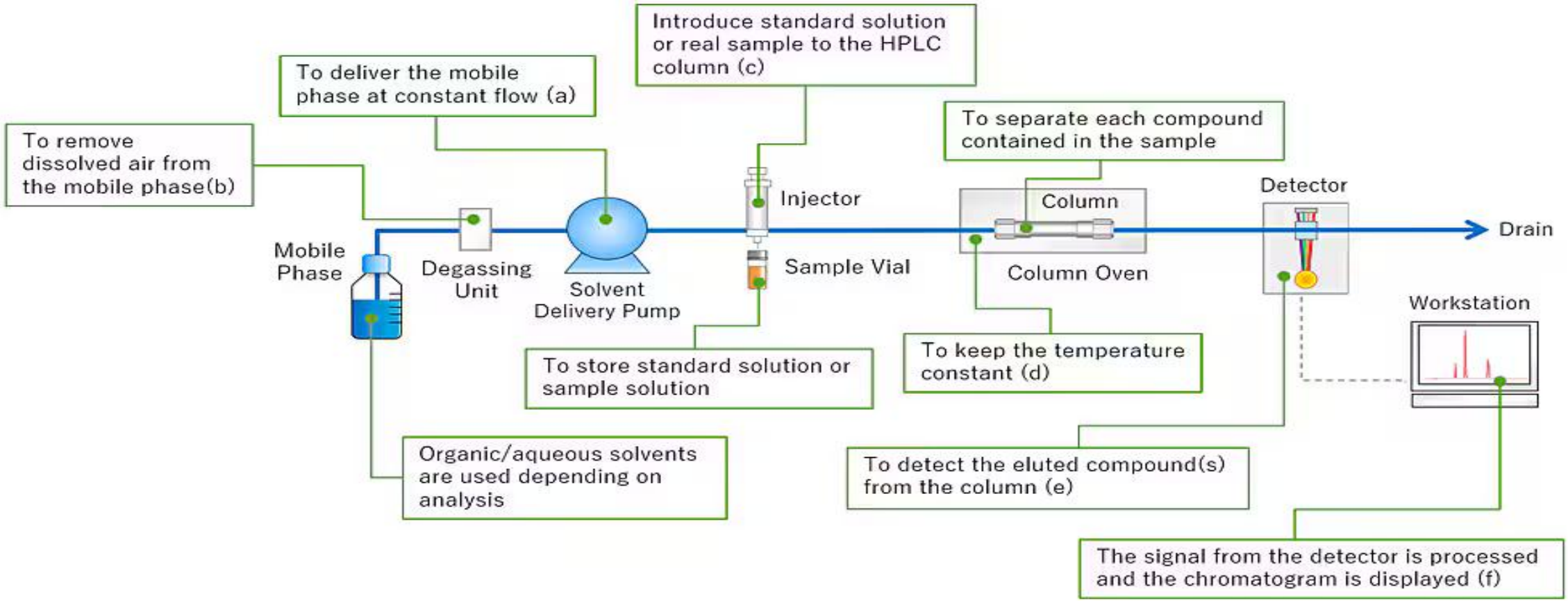
- Pressure is directly proportional to viscosity (η), column length (L), and flow rate (F). In practice, the viscosity of a fluid usually manifests itself as a resistance to flow. So if a **high-viscosity liquid** is used as the HPLC mobile phase, a **higher pressure** will be observed.

- The **small particles inside the column** are what cause the high **backpressure** at normal flow rates.

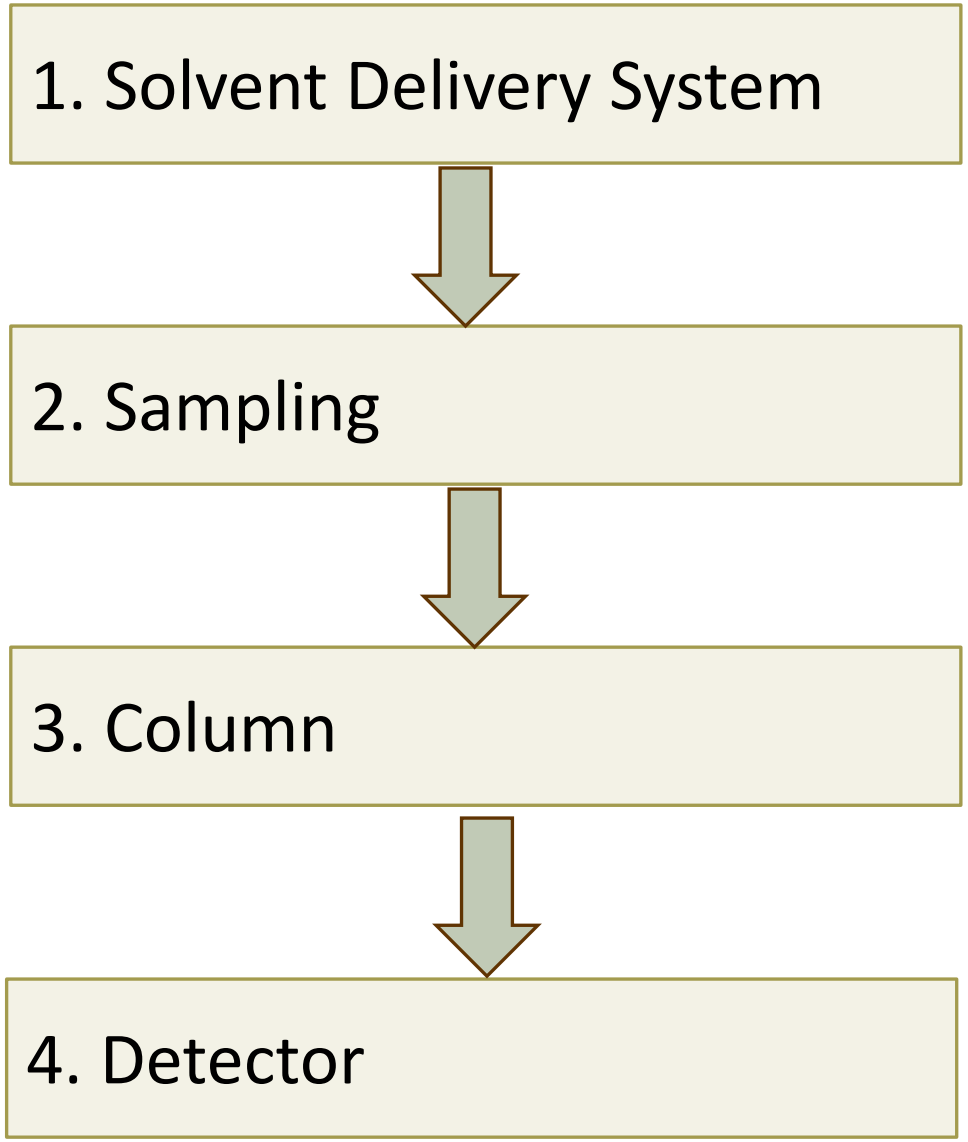
- That is why the pump must push hard to move the mobile phase through the column and this resistance causes a high pressure within the chromatogram.

Thus we call it **High Performance or High Pressure Liquid Chromatography.**

Agilent 1260 Infinity II HPLC Basic Instrumentation



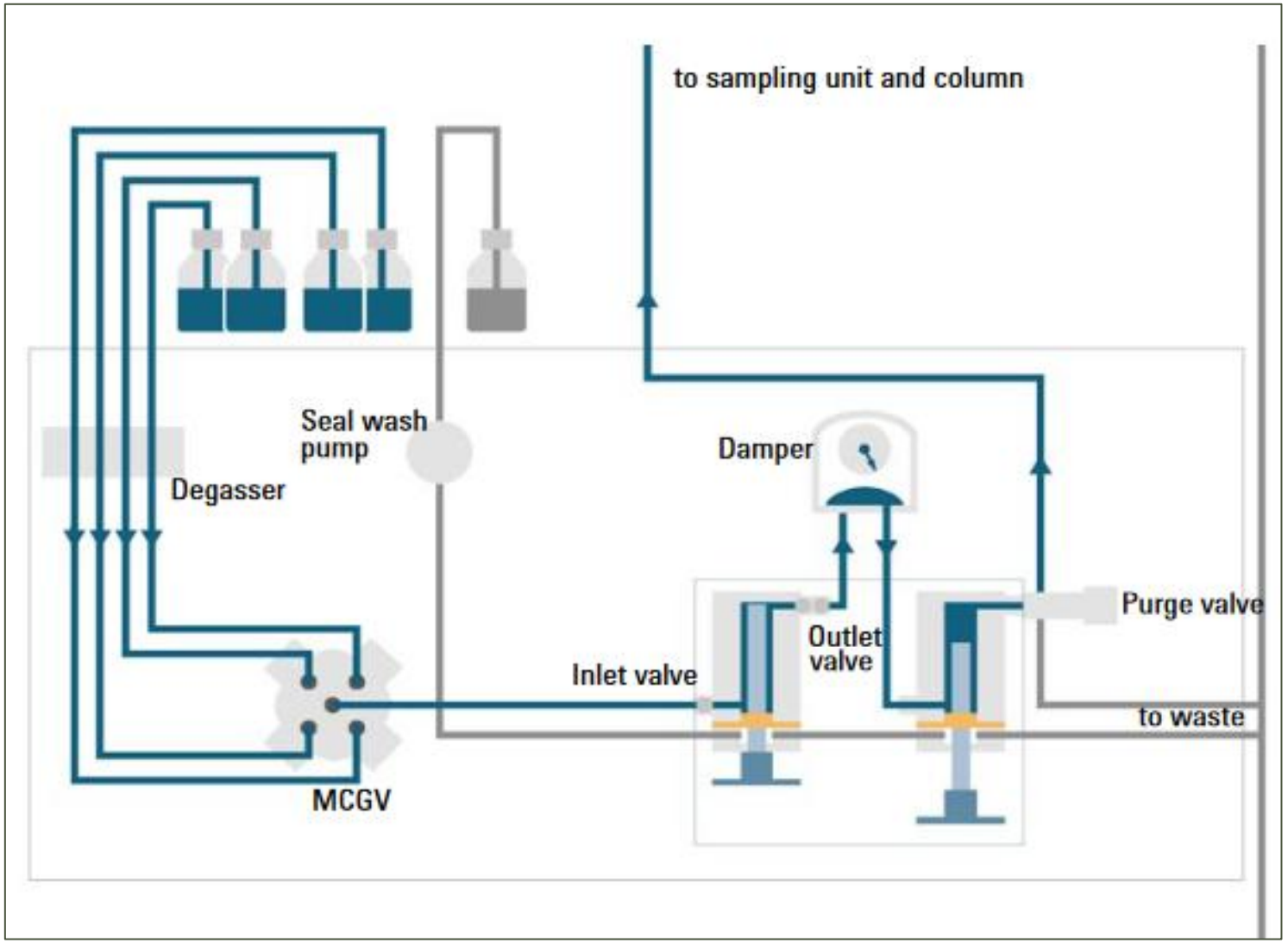
Four Modules in Agilent 1260 Infinity II HPLC



1. Solvent Delivery System

The primary function of solvent delivery system is to deliver the mobile phase (eluent) through the system as reproducibly as possible. It consists of :-

1. **Mobile Phase Reservoirs**
2. **Degasser**
3. **Multi channel gradient valve**
4. **Quaternary Pump with dampener**
5. **Purge valve**



➡ Mobile Phase Reservoir

This HPLC instrument is equipped with four glass reservoirs, each of which contain 500 ml of mobile phase. Provisions are often included to remove dust from the liquids by using **Inlet filter.** Mobile phase are selected according to the column used. In case of

Polar column – THF, Hexane, Non polar solvents.
Non Polar column – Water, Acetonitrile, Methanol, Polar solvents.

Mobile phase moves from Reservoirs to Degasser.

➡ Degasser

Degasser may consist of a vacuum pumping system, a distillation system or a system for sparging in which the dissolved gases are swept out of mobile phases.

From degasser, mobile phase moves to **MCGV(Multi Channel Gradient Valve**).

Multi channel Gradient valve

From Degasser, before moving to pump, Mobile phase moves to MCGV . In gradient elution two or more mobile phases that differ significantly in polarity enter and come out as a single output in which the ratio of two mobile phases is varied in a preprogrammed way, sometimes continuously and some time in a series of steps.

Gradient elution frequently improves separation efficiency.

Quaternary Pump with dampener

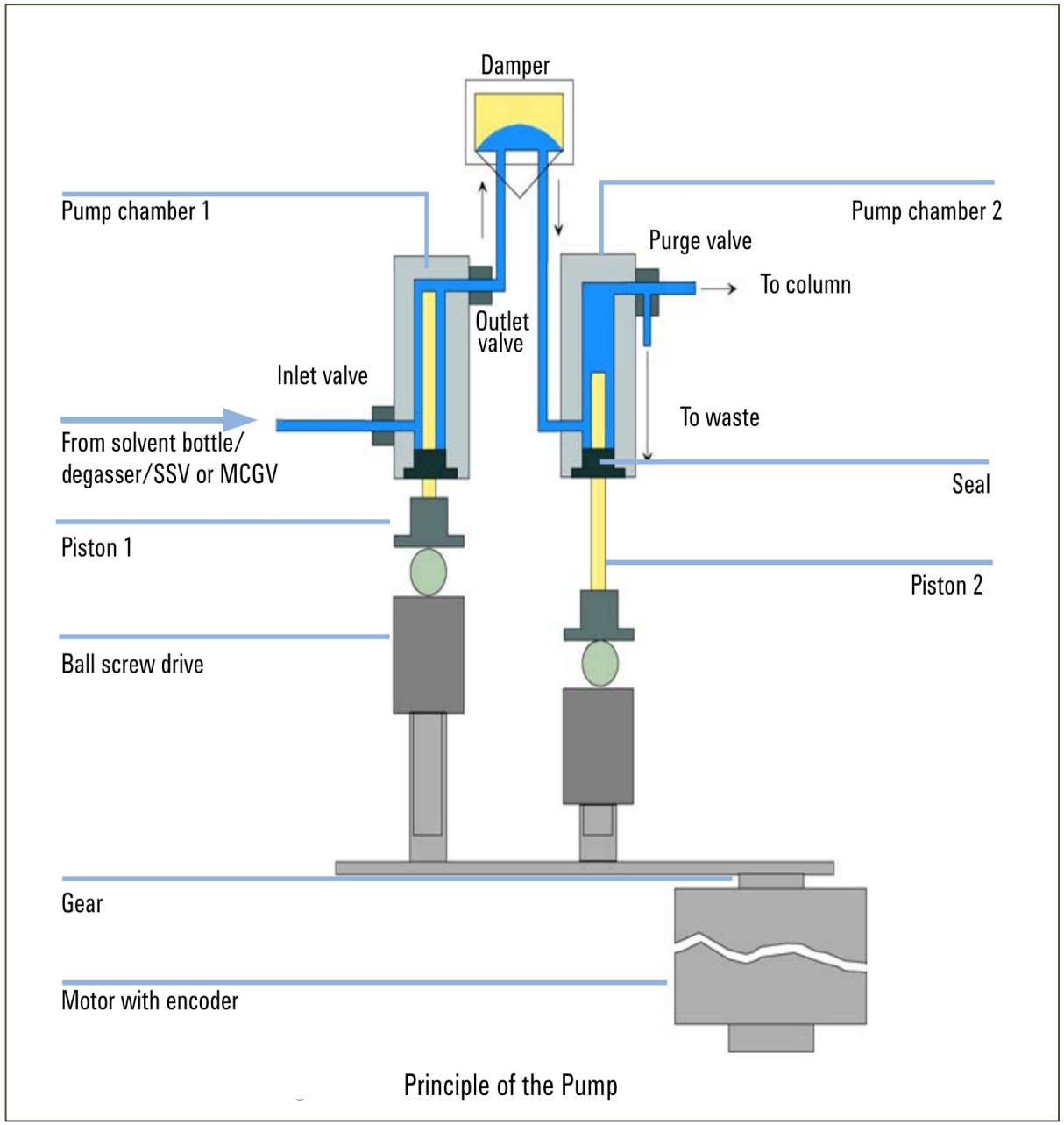
In the quaternary pump, the liquid runs from the solvent reservoir through the degasser to the MCGV and from there to the Inlet valve of pump.

The requirements for quat. Pump include (1) The generation of pressure of up to **6000 psi**, (2) Pulse free output, (3) Flow rates ranging from **0.1- 10 ml/min**, (4) Resistance to corrosion by a variety of solvents.

The pump assembly comprises two substantially identical piston units having a ball screw drive and a pump head . The motor drives the two ball screw having different circumferences (Ratio 2:1) allowing the first piston to move at twice the speed of second Piston. The solvent enters the pump head close to the bottom limits and leave it at its Top.

The outlet diameter of the piston is smaller than the inner diameter of the pump head chamber allowing the sample to fill the gap in between.

The outlet of the first piston unit is connected through the outlet valve to the damping unit to reduce pressure fluctuations and outlet of damping unit is connected to second piston unit.

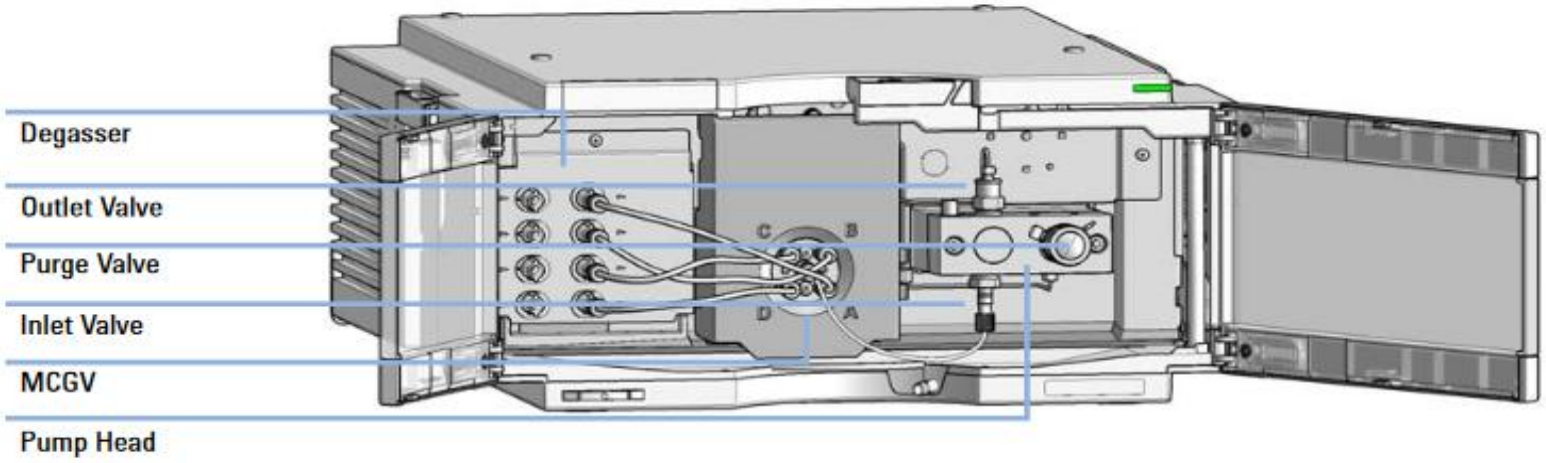


Principle of the Pump

Purge valve

Here, a Purge Valve is attached with the outlet of Quat. Pump to flush out the solvent lines within the pump by diverting the flow to waste , essentially removing any air bubbles or residual solvent from the system before starting an analysis.

Purging is necessarily done for each mobile phase channel with flow rate 5ml/min for 2-5 min to ensure smooth flow before starting analysis of sample.

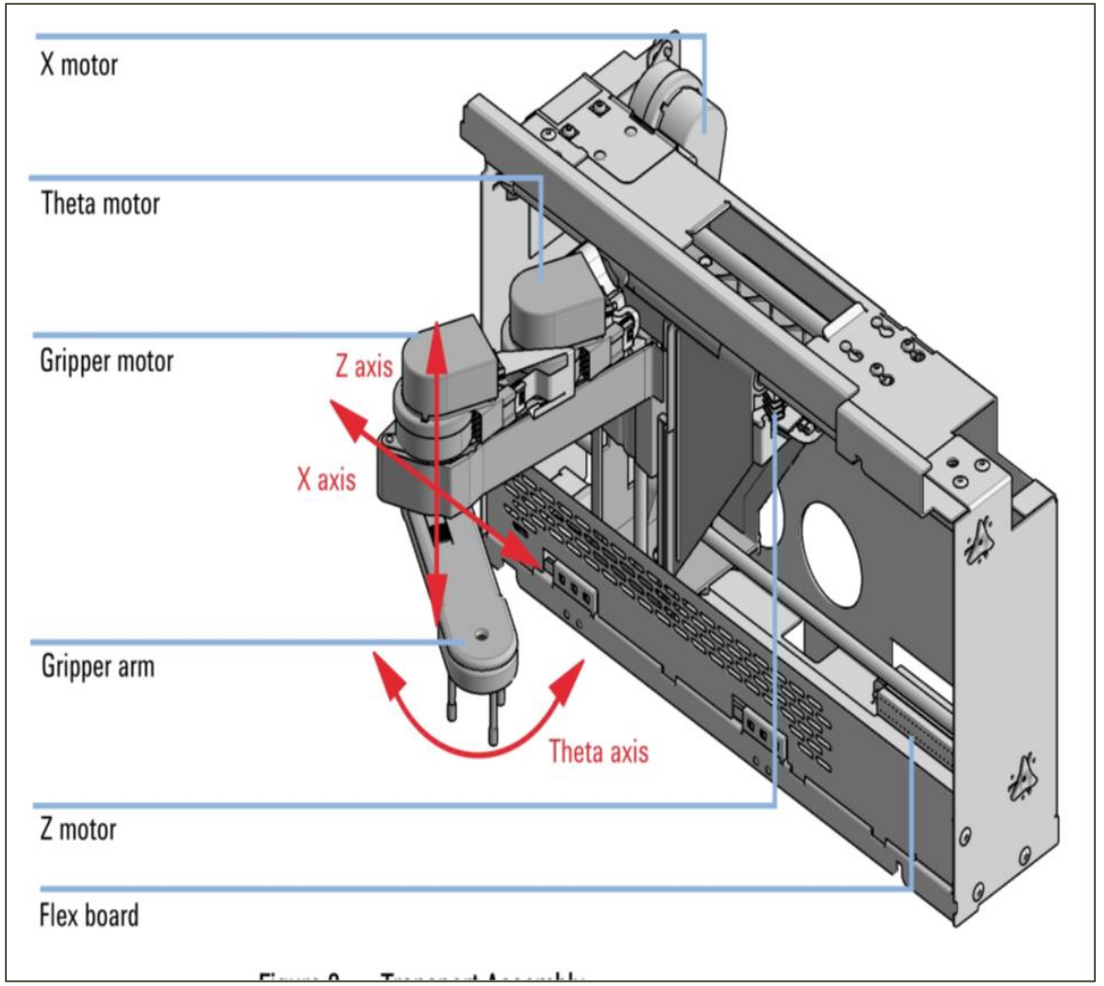


An Overview of whole Solvent Delivery System

2. Sampling/ Sample Injection System

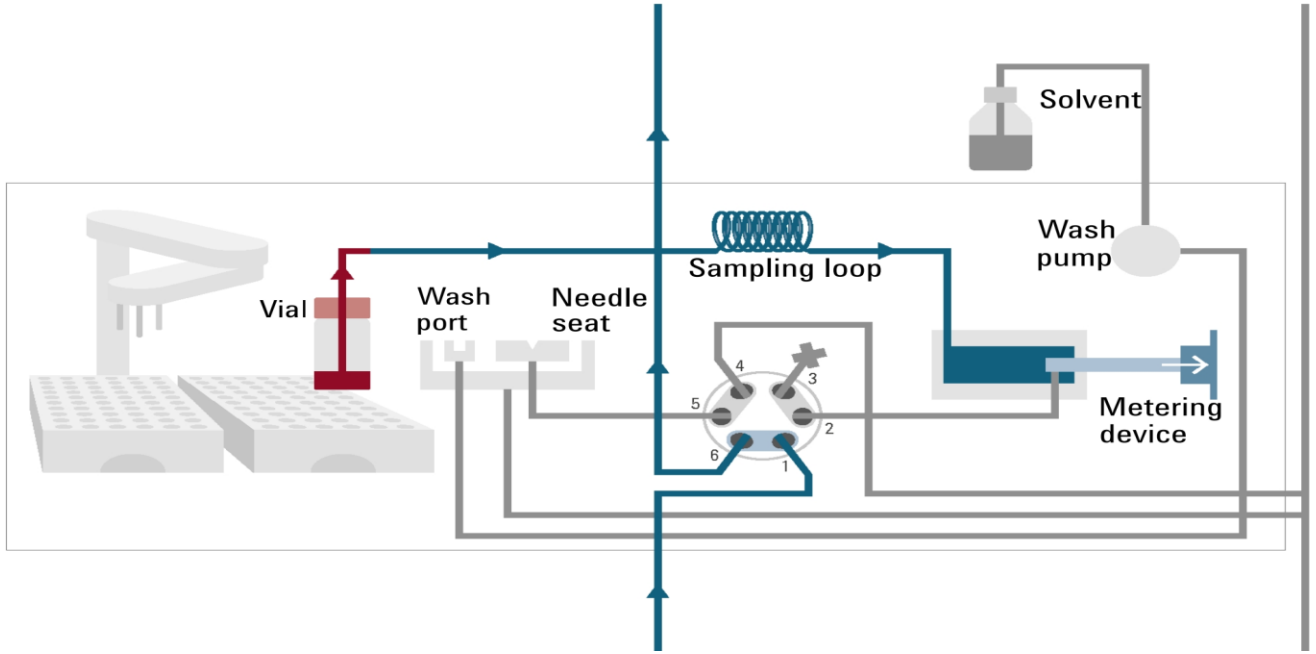
The function of next module of the system is to introduce the sample into the flowing mobile phase stream prior to the column so that it may be carried to the column and subsequently separated. The injector may be operated either manually via micro syringe or automatically from a vial held in a sample carousel (or tray) using a syringe assembly controlled by a stepping motor and automatically actuated valves.

The 1260 Infinity II HPLC system incorporate an Autosampler controlled by Agilent control software with sample-rack of 2× 66 vials**. The autosampler's transport mechanism uses an X-Z-Theta movement to optimize vial pick up and return.** Vials are picked up by the gripper arm and positioned below the needle station. The gripper transport mechanism, the needle station and the hydraulic unit are driven by motors. Movement is monitored by optical sensors and optical encoders to ensure correct operation.



Transport Assembly

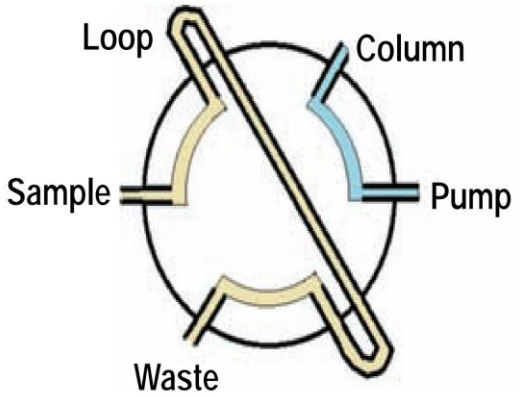
Load and Inject Positions in an Injection valve



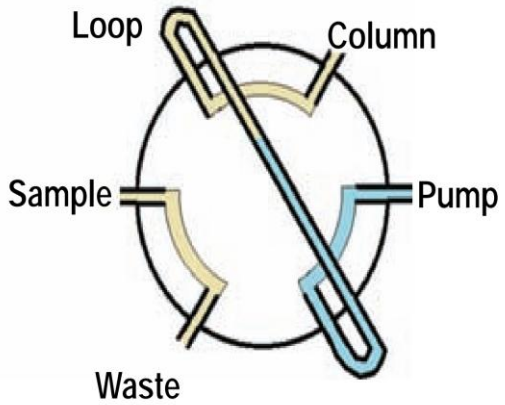
The needle moves down into the vial, metering unit draws the required sample volume into the loop.

Two Mode of loop :

Load Mode– sample is filled in the loop.
Inject Mode – sample is pushed into column along with mobile phase



Load



Inject

3.Column (Heart Of HPLC)

The Agilent 1260 Infinity Thermostated Column Compartment is a stackable temperature- controlled column compartment for LC. It is used for heating and cooling to meet extreme requirements of retention time reproducibility.

LC columns are usually constructed from stainless steel tubing or PEEK and mostly range in **length from 5-25cm** and have inside **diameters of 3-5mm** with most common **particle size of packings 3 or 5µm.**

The particles are composed of silica, alumina or synthetic resin which are porous microparticles.

Working Principle of column

Mobile phase carryover sample from injector to column via injector loop. Here sample is separated in its components and moves to detector.

This HPLC works on the principle of partition chromatography which is distinguishable based on the relative polarities of mobile and stationary phases.

1. Normal Phase Partition Chromatography(NP-HPLC):

Stationary phase: Polar (e.g., water-coated silica).
Mobile phase: Non-polar (e.g., organic solvents like hexane).
Used for: Polar compounds.

2. Reverse Phase Partition Chromatography (RP-HPLC):

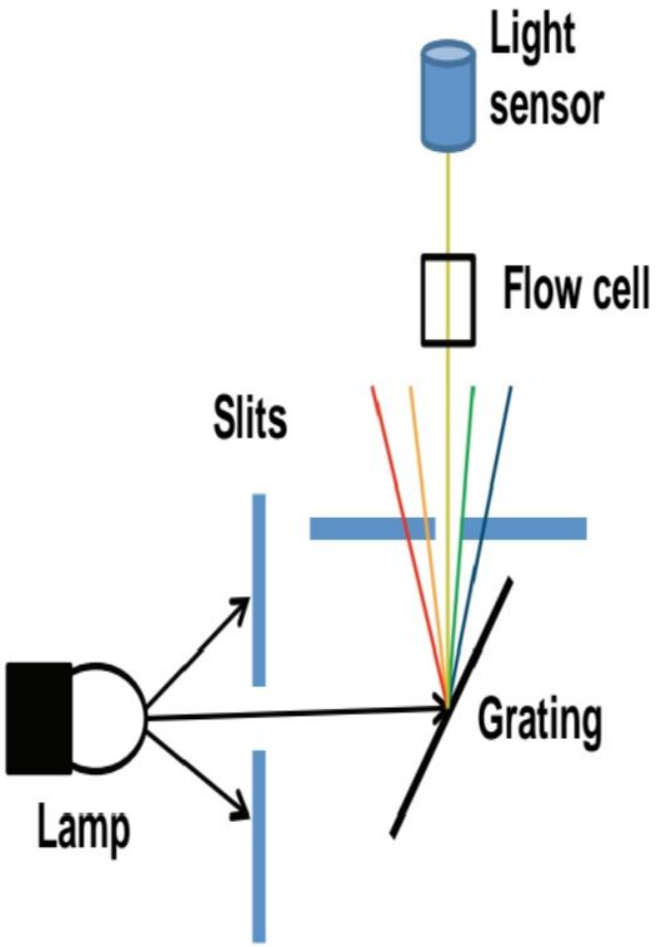
Stationary phase: Non-polar (e.g., C18 silica coated with hydrocarbons).
Mobile phase: Polar (e.g., water-methanol or water-acetonitrile mixtures).
Used for: Non-polar or moderately polar compounds.

The component of the sample having greater affinity with mobile phase will be separated first with low retention time and component having greater affinity with stationary phase will be separated last with high RT.

4. Detectors

UV- visible light absorbance detectors are most commonly employed detectors in HPLC.UV-Vis detector operate in the approx. **wavelength range 190-950nm.**

In UV-vis detectors, light from a source is sent through a slit and split into radiation of different wavelengths in a monochromator. With a filter or a grating, selected wavelengths are passed through the flow cell having mobile phase flows. A signal will be obtained for all compounds that absorb light of appropriate wavelength.



This agilent 1260 infinity II HPLC system is equipped with one more detector i.e. **Evaporative Light Scattering Detector (ELSD).** An **ELSD (Evaporative Light Scattering Detector)** is a type of **universal detector** commonly used in **HPLC** (High Performance Liquid Chromatography), especially for compounds **without UV activity** (e.g., sugars, lipids, polymers, surfactants).

Principle of ELSD

1.Nebulization:

The mobile phase exiting the HPLC column is **nebulized** (converted into an aerosol) using a gas (usually nitrogen).

2.Evaporation:

The aerosol is passed through a heated drift tube where the **mobile phase evaporates**, leaving **non-volatile analyte particles**.

3.Detection:

A laser or light beam is directed through the cloud of analyte particles. These **scatter light**, and the scattered light is detected at a specific angle.

The **intensity of scattered light** is proportional to the **amount of analyte**.

Key Features of ELSD

- **Universal detector**: detects any non-volatile compound.
- **Does not rely on chromophores** (unlike UV detectors).
- **Mass-sensitive**: signal increases with mass of analyte particles.
- **Non-destructive**: sample can be collected after detection (with care).

Applications

- Sugars (mono-, di-, and polysaccharides)
- Lipids, triglycerides
- Surfactants
- Polymers and oligomers
- Pharmaceuticals and nutraceuticals
- Natural products without UV-absorbing groups

Applications

Field	Typical Mixtures separated
Pharmaceuticals	Antibiotic, Sedatives, Steroids, Analgesics
Biochemical	Amino acids, Proteins, Carbohydrates, Lipids
Food Products	Artificial Sweetener, Antioxidants, Aflatoxins, Additives
Industrial Chemicals	Condensed Aromatics, Surfactants, Propellants, Dyes
Pollutants	Pesticides, Herbicides, Phenols, Poly Chlorinated Biphenyls
Forensic Science	Drugs, Poisons, Blood Alcohols, Narcotics
Clinical Chemistry	Bile acids, Drug Metabolites, Urine Extracts, Estrogen