Analytical chemistry

Lecture №33

Liquid chromatography Part II

• **Chromatography** is a technique in which the components of a mixture are separated based on differences in the rates at which they are carried through a fixed or **stationary phase** by a gaseous or liquid **mobile phase.**

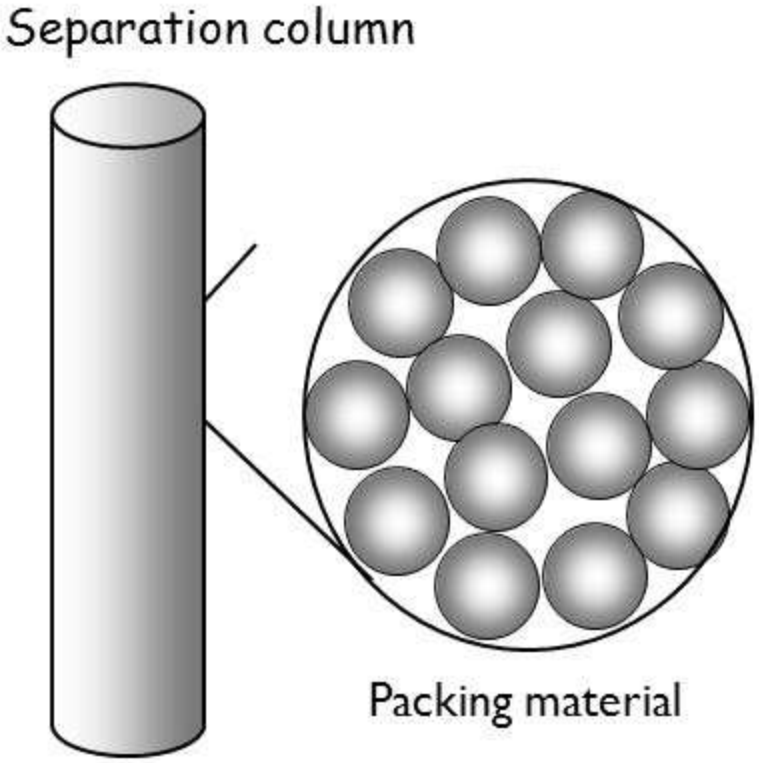
• The **stationary phase** in chromatography is a phase that is fixed in place either in a column or on a planar surface.

• The **mobile phase** in chromatography is a phase that moves over or through the stationary phase carrying with it the analyte mixture. The mobile phase may be a gas, a liquid, or a supercritical fluid.

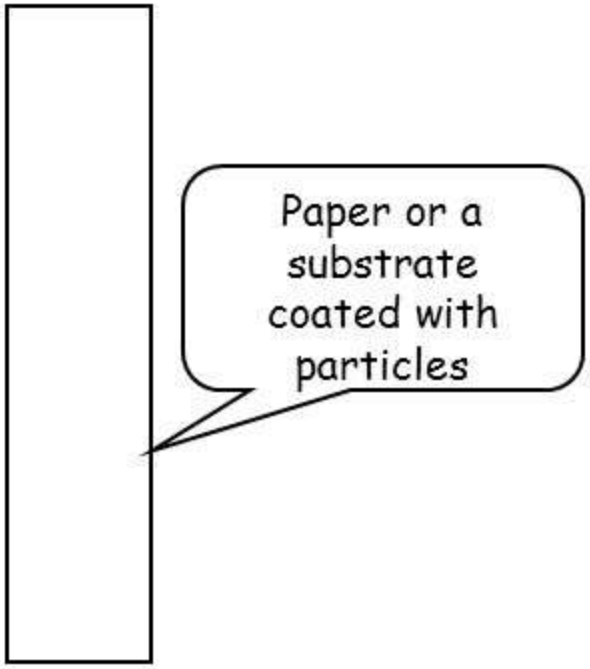
Classification of Column Chromatographic Methods

General Classification	Specific Method	Stationary Phase	Type of Equilibrium
1. Gas chromatography (GC)	a. Gas-liquid (GLC)	Liquid adsorbed or bonded to a solid surface	Partition between gas and liquid
	b. Gas-solid	Solid	Adsorption
2. Liquid Chromatography (LC)	a. Liquid-liquid, or partition	Liquid adsorbed or bonded to a solid surface	Partition between immiscible liquids
	b. Liquid-solid, or adsorption	Solid	Adsorption
	c. Ion exchange	Ion-exchange resin	Ion exchange
	d. Size exclusion	Liquid in interstices of a polymeric solid	Partition/sieving
	e. Affinity	Group specific liquid bonded to a solid surface	Partition between surface liquid and mobile liquid
3. Supercritical fluid chromatography (SFC) (mobile phase: supercritical fluid)		Organic species bonded to a solid surface	Partition between supercritical fluid and bonded surface

Column Chromatography and Planar Chromatography



Column Chromatography



Paper Chromatography
Thin Layer Chromatography (TLC)

Early LC was carried out in large columns with relatively large particles under gravity feed, with manual collection of effluent fractions for off-line measurement; a technique that is still practiced in some synthetic organic chemistry or preparative biochemistry laboratories. In 1964, in a benchmark paper [*Anal. Chem.,* ***36 (1964)***] J. Calvin Giddings (University of Utah) predicted much improved efficiency if small particles are used with the concomitant use of high pressure to overcome the flow resistance. Shortly thereafter, Horvath and Lipsky at Yale University built the first high-pressure liquid chromatograph. The technology of producing small particles that will allow high efficiency and performance came in the 1970s. While HPLC today largely connotes “high-performance liquid chromatography” rather than high pressure, the continued move to smaller particles does necessitate the use of higher and higher pressures.

High-performance liquid chromatography, HPLC, is a type of chromatography that combines a liquid mobile phase and a very finely divided stationary phase. In order to obtain satisfactory flow rates, the liquid must be pressurized to several hundred or more pounds per square inch.

Types of High performance liquid chromatography

Normal phase (NP-HPLC): Polar stationary phase e.g. silica
Non polar mobile phase e.g. Toluene
Polar interaction
Non polar → Polar

Reverse phase (RP-HPLC): Non polar stationary phase e.g. C18
Polar mobile phase such as water
Hydrophobic interaction
Retention time is proportional to the contact surface area around the non-polar segment of the analyte
Polar → Non polar

Some of the serious limitations too often encountered in GC ultimately brought about the development of HPLC, for instance:

• In GC the mixture of components are usually screened in the vapour phase. Hence, either a stable vapour from the mixture is obtained directly or indirectly converting the substance in it to such derivatives that are thermally stable. One 20% of chemical compounds usually come across in analysis are suitable for GC directly i.e., without making their corresponding appropriate derivatives,

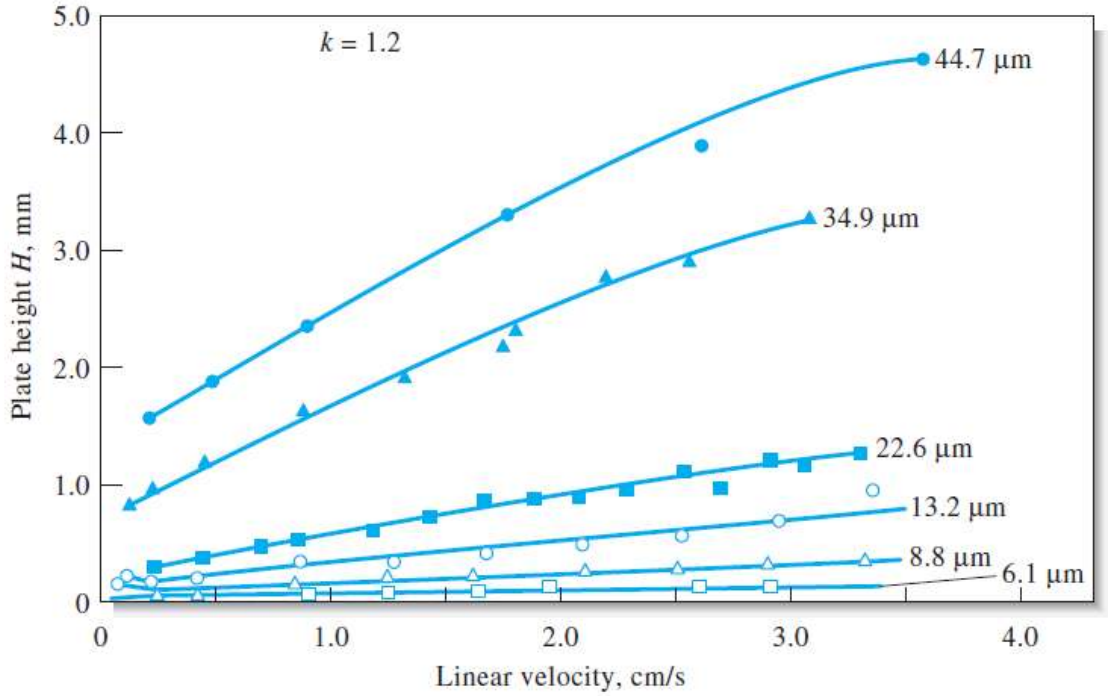
• *The remainder 80% of the chemical compounds are either thermally unstable or involatile in nature,* and

• *Compounds essentially having highly polar or ionizable function groups are very prone to* ***'tailing'*** by GC-analysis. Therefore, HPLC has been evolved as a dire confluence of need, technological supremacy, the emergence of newer theoretical concepts and ideas towards development along rational lines, and above all-**'the human desire to minimise work'.** Interestingly, in HPLC the stationary phase and the mobile-phase is able to interact with the sample selectively. Besides, such interactions as hydrogen bonding or complexation which are absolutely not possible in the GC-mobile phase may be accomplished with much ease in the HPLC-mobile phase. Furthermore, the spectrum of these selective interactions may also be enhanced by an appropriate chemical modification of the silica surface *i.e., the stationary phase. Therefore, HPLC is regarded as a more versatile technique than GC* and capable of achieving more difficult separations.

High Performance Liquid Chromatography (HPLC)

- Most compounds are not sufficiently volatile
- HPLC uses high pressure to force solvent through closed columns containing very fine particles that give high-resolution separations.

In HPLC, a liquid sample, or a solid sample dissolved in a suitable solvent, is carried through a chromatographic column by a liquid mobile phase. Separation is determined by solute/stationary-phase interactions, including liquid–solid adsorption, liquid–liquid partitioning, ion exchange and size exclusion, and by solute/mobile-phase interactions. In each case, however, the basic instrumentation is essentially the same.



Effect of particles size of packing and flow rate on plate height in liquid chromatography.

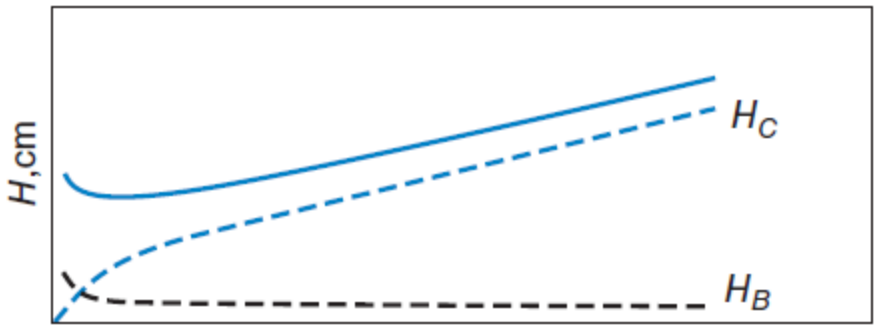
In HPLC, analytes are separated based on their differential affinity between a solid stationary phase and a liquid mobile phase. The kinetics of distribution of solutes between the stationary and the mobile phase is largely diffusion-controlled. Compared to gases, the diffusion coefficient of analytes in liquids is 1000 to 10,000 times slower. To minimize the time required for the interaction of the analytes between the mobile and the stationary phase, two criteria should be met. First, the packing particles should be small and as uniformly and densely packed as possible. This criterion is met by uniformly sized spherical particles and results in a smaller *A value in the van Deemter* equation (smaller eddy diffusion). Second, the stationary phase should be effectively a thin uniform film with no stagnant pools and provide a small *C value (more rapid mass* transport between the phases—necessary for high flow rates). Because molecular diffusion in liquids is small, the *B term in van Deemter Equation is small.*

$$H \approx A + \frac{B}{u} + Cu_x$$

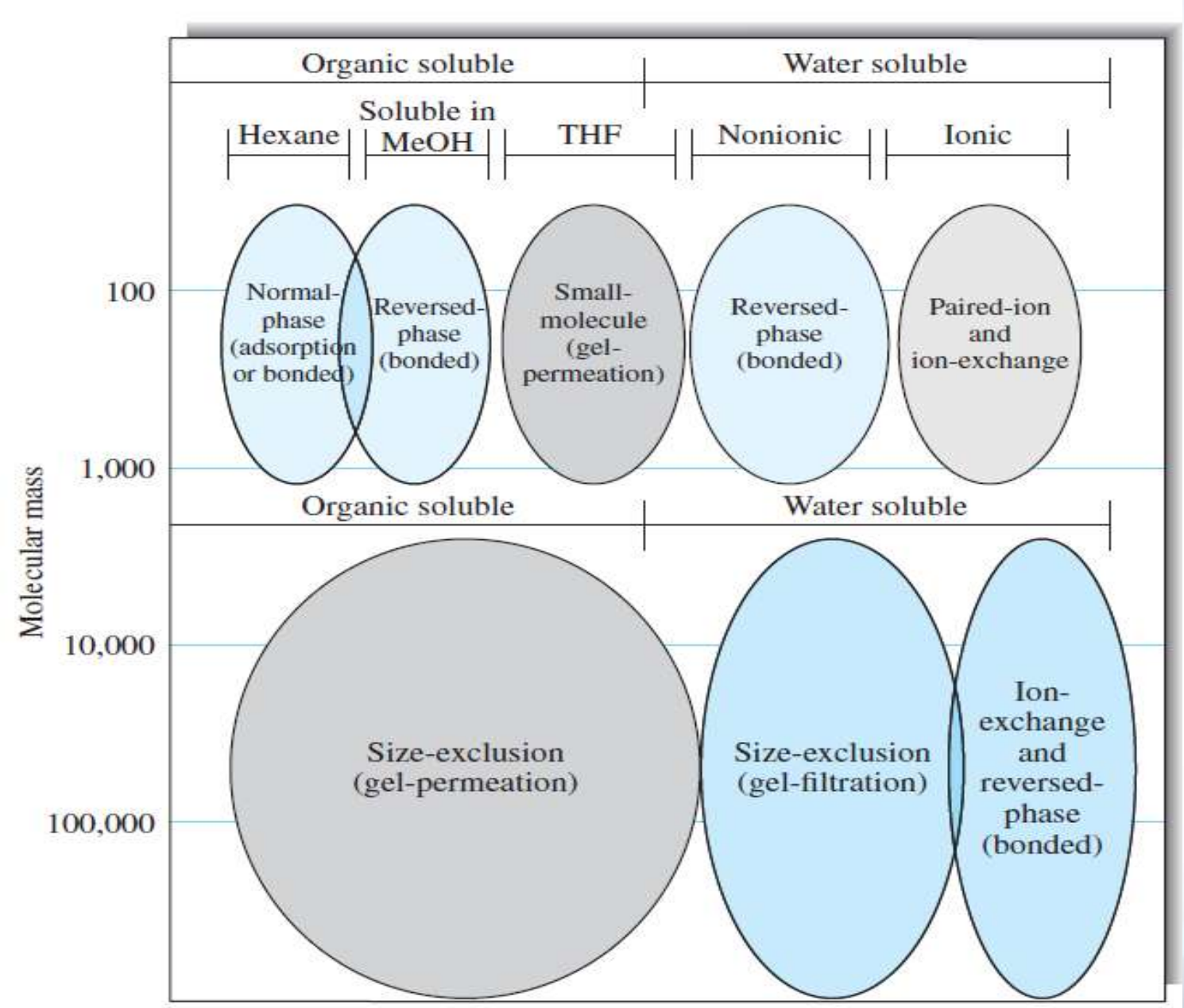
van Deemter plot for HPLC

H-plate height; u-flow rate; A-Multiple paths (Eddy diffusion); B/*u*-Longitudinal diffusion (molecular diffusion); C*u-Equilibration time* (resistance to mass transfer).

- ***Increasing efficiency is equivalent to decreasing H***

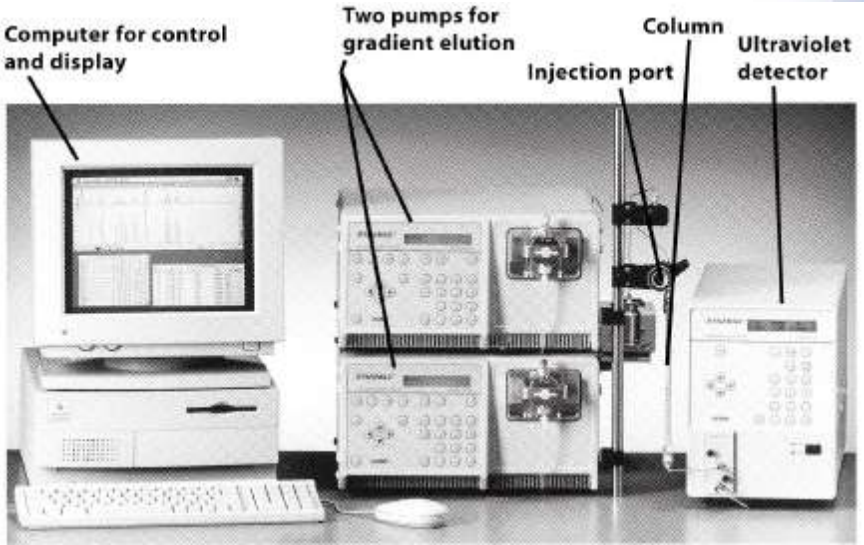


Applications of liquid chromatography. Methods can be chosen based on solubility and molecular mass.

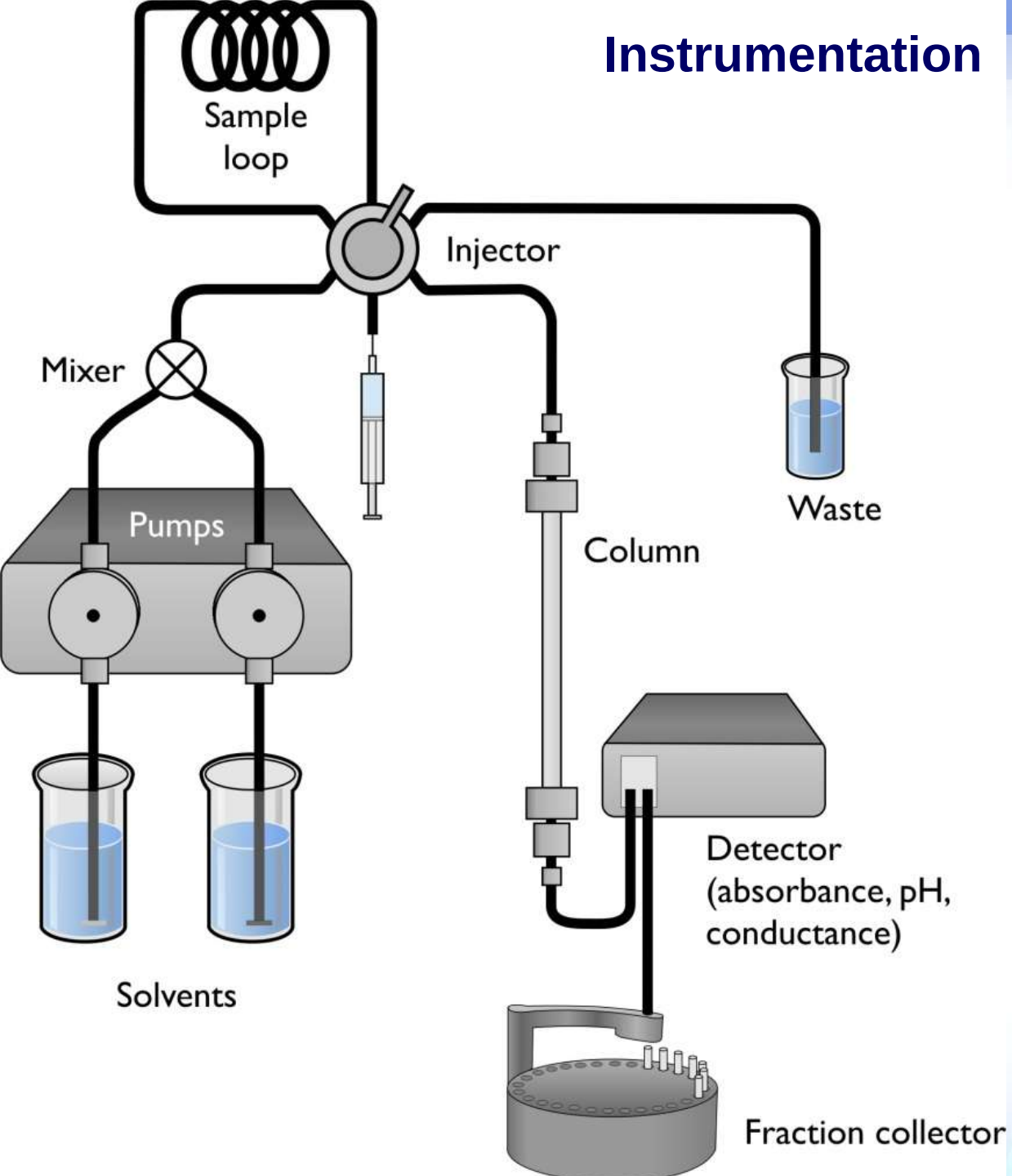


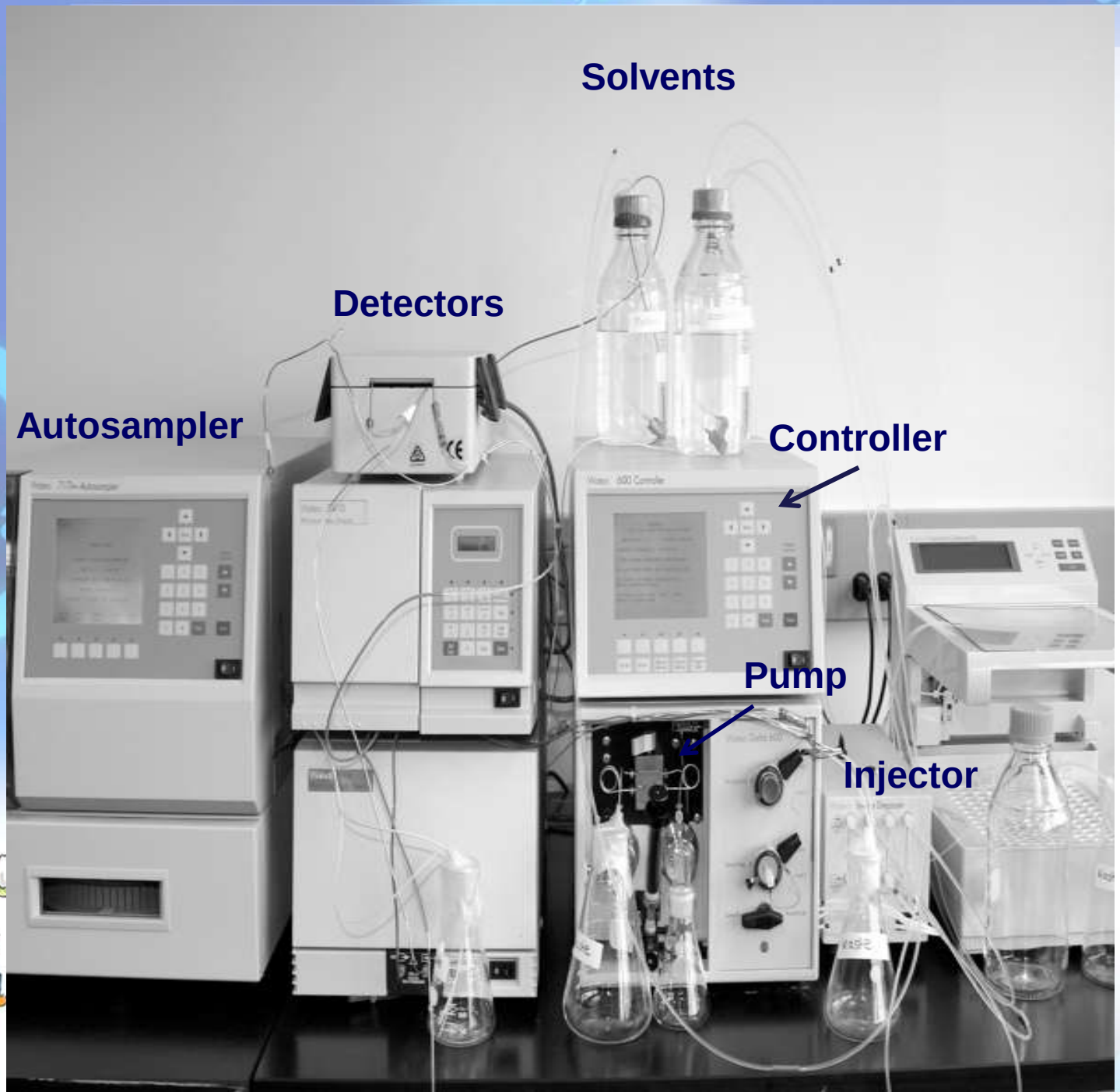
A schematic diagram of a typical HPLC instrument

- *Solvent reservoir and degassing system,*
- *Pressure, flow and temperature,*
- *Pumps and sample injection system,*
- *Columns,*
- *Detectors,*
- *Strip-chart recorder, and*
- *Data handling device and microprocessor control.*

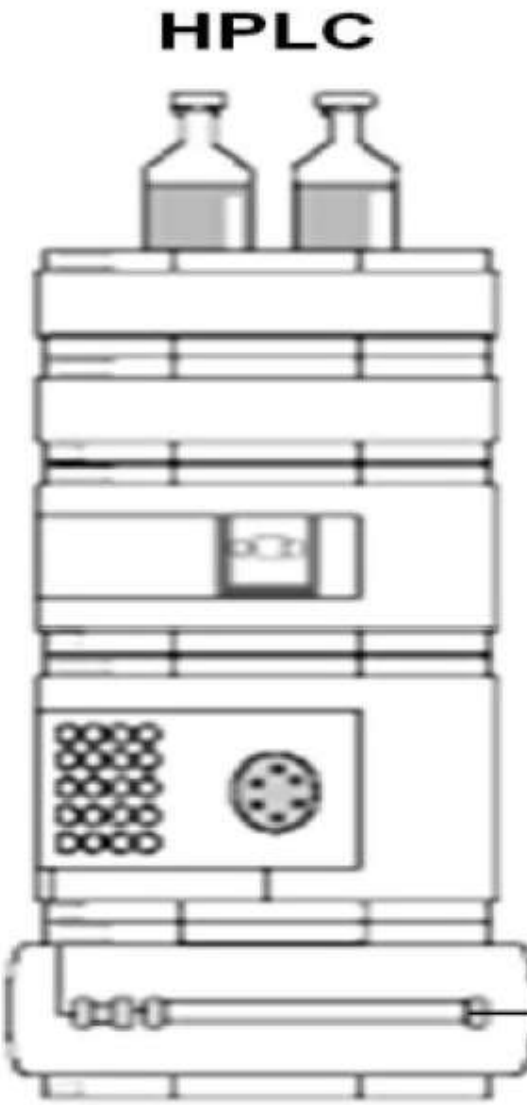


Instrumentation



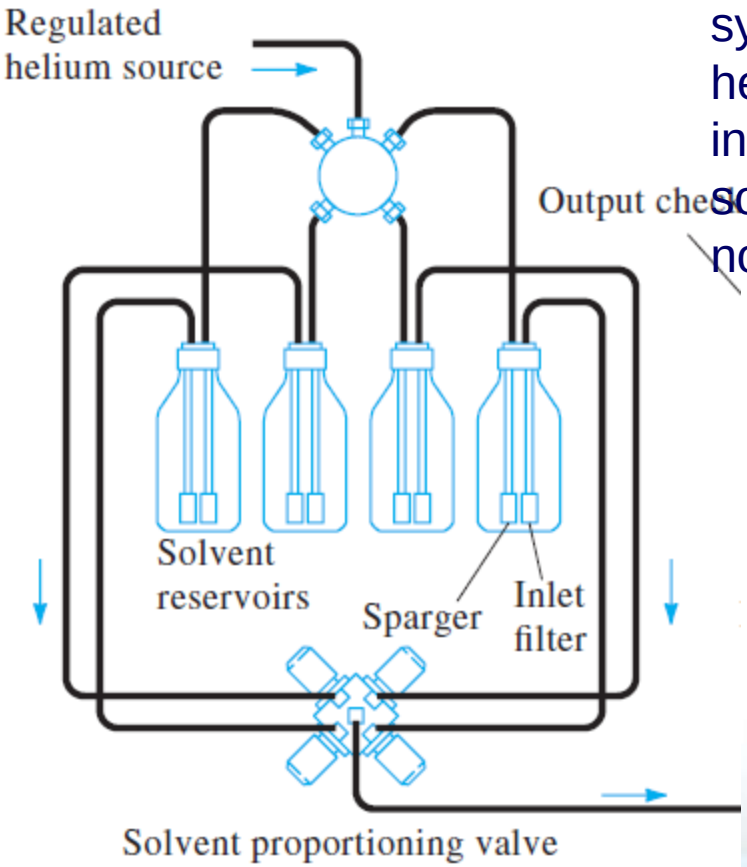

Solvents
Detectors
Autosampler
Controller
Pump
Injector


Eluent bottles
Degasser
Pump
Autosampler /Injector
Column


HPLC

Solvent reservoir and degassing system

A modern HPLC instrument is equipped with one or more glass reservoirs, each of which contains 500 mL or more of a solvent. Provisions are often included to remove dissolved gases and dust from the liquids. Dissolved gases can lead to irreproducible flow rates and band spreading. In addition, both bubbles and dust interfere with the performance of most detectors.



Degassers may consist of a vacuum pumping system, a distillation system, a device for heating and stirring, or, a system for **sparging** in which the dissolved gases are swept out of solution by fine bubbles of an inert gas that is not soluble in the mobile phase.

Sparging is a process in which dissolved gases are swept out of a solvent by bubbles of an inert, insoluble gas.

Pumping Systems

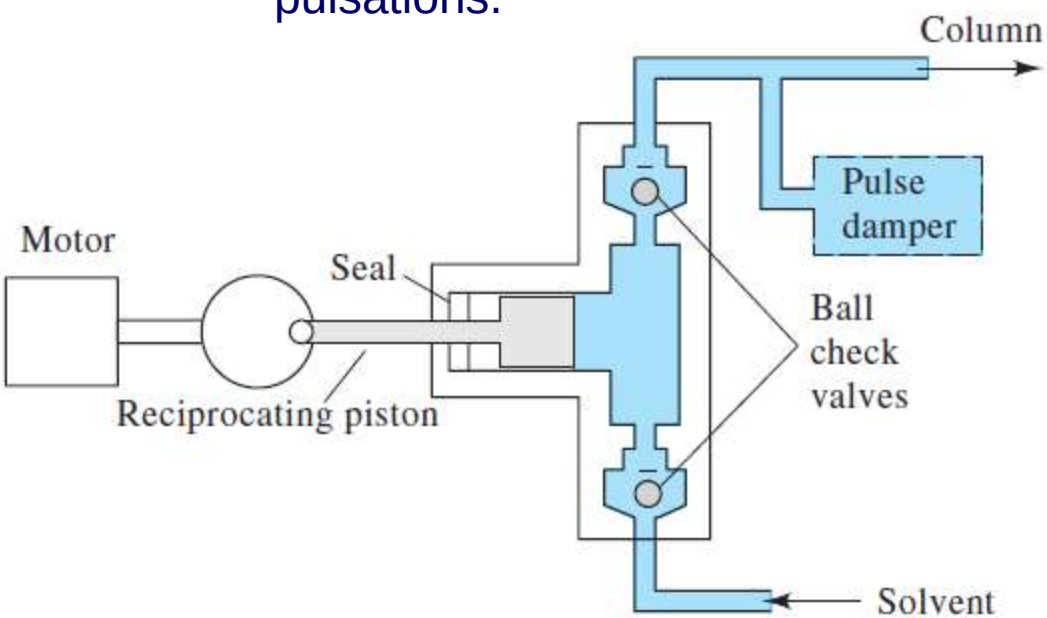
The mobile-phase solvents are pulled from their reservoirs by the action of a pump. Most HPLC instruments use a reciprocating pump consisting of a piston whose back-and-forth movement is capable both of maintaining a constant flow rate of up to several milliliters per minute and of obtaining the high output pressure needed to push the mobile phase through the chromatographic column. A solvent proportioning valve controls the mobile phase's composition, making possible the necessary change in the mobile phase's composition when using a gradient elution. The back and forth movement of a reciprocating pump results in a pulsed flow that contributes noise to the chromatogram. To eliminate this problem a pulse damper is placed at the outlet of the pump.

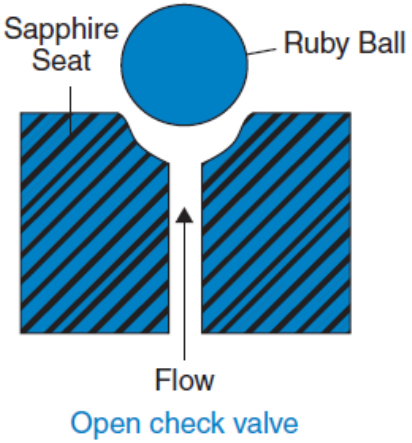
The requirements for liquid chromatographic pumps include
(1) the generation of pressures of up to 6000 psi (lb/in2),
(2) (2) pulse-free output,
(3) (3) flow rates ranging from 0.1 to 10 mL/min,
(4) (4) flow reproducibilities of 0.5% relative or better, and
(5) Resistance to corrosion by a variety of solvents.

Two major types of pumps are used in HPLC instruments: the screw-driven syringe type and the reciprocating pump. Reciprocating types are used in almost all commercial instruments. Syringe-type pumps produce a pulse-free delivery whose flow rate is easily controlled. They suffer, however, from relatively low capacity (250 mL) and are inconvenient when solvents must be changed.

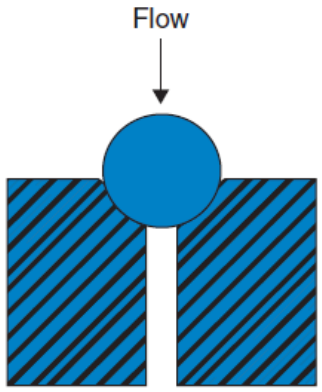
Reciprocating Pump

This device consists of a small cylindrical chamber that is filled and then emptied by the backand- forth motion of a piston. The pumping motion produces a pulsed flow that must be subsequently damped because the pulses appear as baseline noise on the chromatogram. Modern HPLC instruments use dual pump heads or elliptical cams to minimize such pulsations.





Open check valve



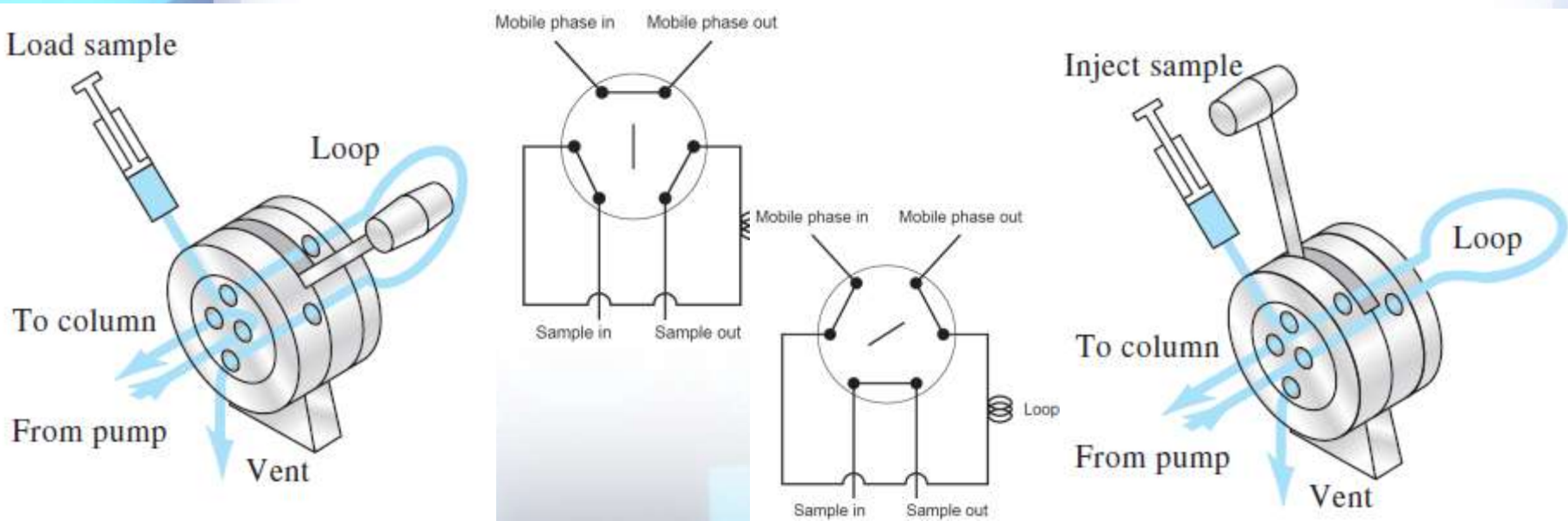
Closed check valve

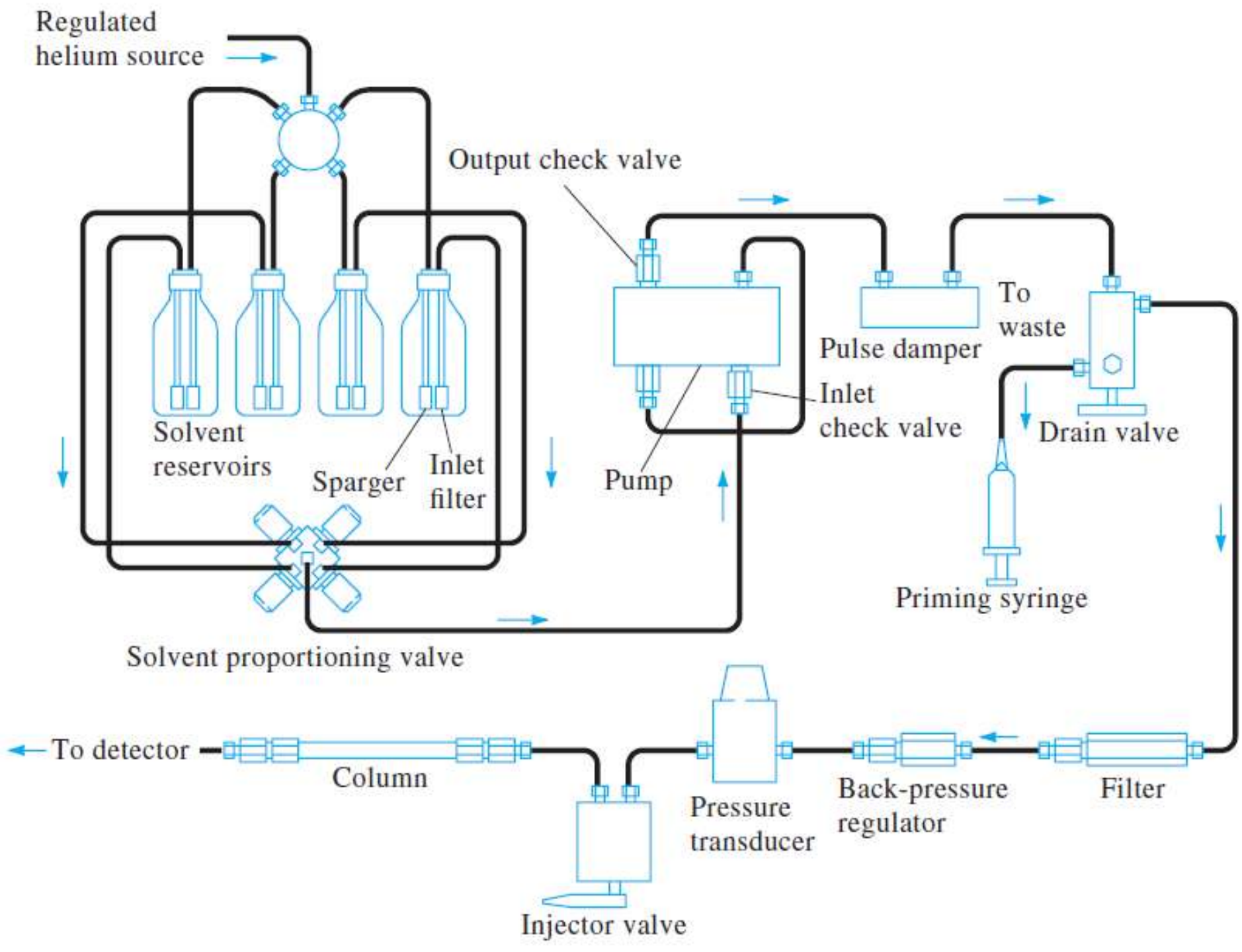
Sample Injection systems

The most widely used method of sample introduction in liquid chromatography is based on a sampling loop. Sampling loops are interchangeable, and available with volumes ranging from 0.5 -2 mL.

loop injector- A means for injecting samples in which the sample is loaded into a short section of tubing and injected onto the column by redirecting the mobile phase through the loop.

In the load position the sampling loop is isolated from the mobile phase and is open to the atmosphere. A syringe with a capacity several times that of the sampling loop is used to place the sample in the loop. Any extra sample beyond that needed to fill the sample loop exits through the waste line. After loading the sample, the injector is turned to the inject position. In this position the mobile phase is directed through the sampling loop, and the sample is swept onto the column.




Regulated
helium source
Output check valve
Solvent
reservoirs
Sparger
Inlet
filter
Pump
Inlet
check valve
Pulse damper
To
waste
Drain valve
Priming syringe
Solvent proportioning valve
To detector
Column
Pressure
transducer
Back-pressure
regulator
Filter
Injector valve

HPLC Columns

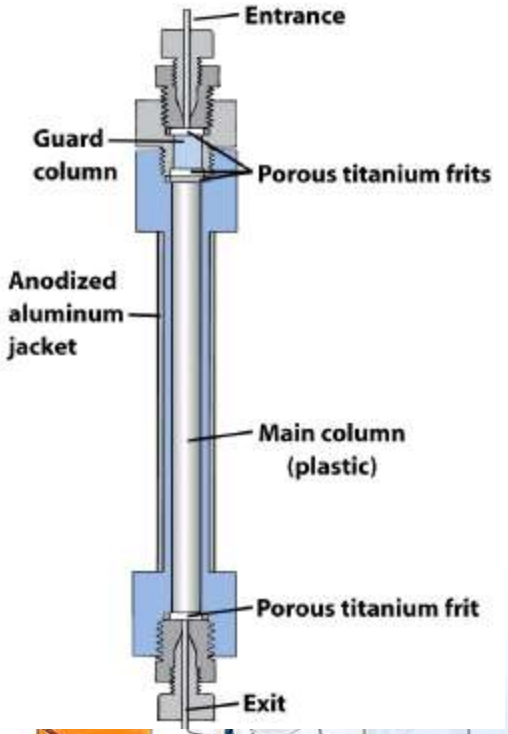
An HPLC typically includes two columns: an analytical column responsible for the separation and a guard column. The guard column is placed before the analytical column, protecting it from contamination.

The most commonly used columns for HPLC are constructed from stainless steel with internal diameters between 2.1 mm and 4.6 mm, and lengths ranging from approximately 30 mm to 300 mm. These columns are packed with 3–10 mm porous silica particles that may have an irregular or spherical shape. Typical column efficiencies are 40,000–60,000 theoretical plates/m.

Two types of precolumns are used. A precolumn between the mobile phase reservoir and the injector is used for mobile-phase conditioning and is termed a **scavenger column.** The solvent partially dissolves the silica packing and ensures that the mobile phase is saturated with silicic acid prior to entering the analytical column. This saturation minimizes losses of the stationary phase from the analytical column.

A second type of precolumn is a **guard column,** positioned between the injector and the analytical column. A guard column is a short column packed with a similar stationary phase as the analytical column. The purpose of the guard column is to prevent impurities, such as highly retained compounds and particulate matter, from reaching and contaminating the analytical column. The guard column is replaced regularly and serves to increase the lifetime of the analytical column.

Column Temperature Control



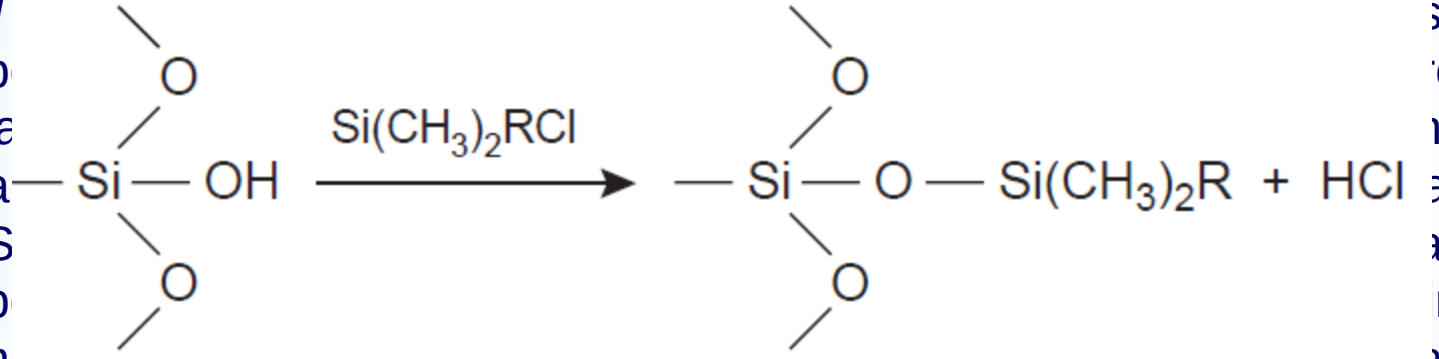
For some applications, close control of column temperature is not necessary, and columns are operated at room temperature. Often, however, better, more reproducible chromatograms are obtained by maintaining constant column temperature.

Most modern commercial instruments are equipped with heaters that control column temperatures to a few tenths of a degree from near room temperature to 150°C. Columns can also be fitted with water jackets fed from a constant-temperature bath to give precise temperature control. Many chromatographers consider temperature control to be essential for reproducible separations.

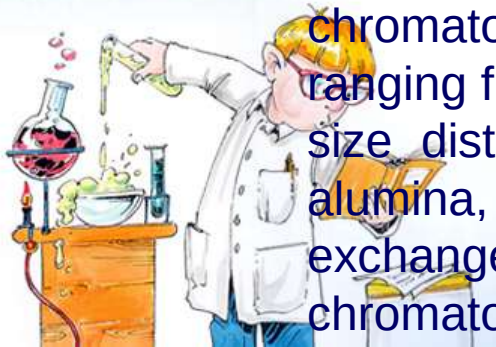
Stationary Phases

In liquid–liquid chromatography the stationary phase is a liquid film coated on a packing material consisting of 3–10 mm porous silica particles. The stationary phase may be partially soluble in the mobile phase, causing it to “bleed” from the column over time. To prevent this loss of stationary phase, it is covalently bound to the silica particles. **Bonded stationary phases** are attached by reacting the silica particles with an organochlorosilane of the general form $Si(CH_3)_2RCl$, where R is an alkyl or substituted alkyl group.

Two types of packings are used in HPLC, *pellicular and porous particle.*



biomolecules. The typical porous particle packing for liquid chromatography consists of porous microparticles having diameters ranging from 3 to 10 mm; for a given size particle, a very narrow particle size distribution is desirable. The particles are composed of silica, alumina, the synthetic resin polystyrene-divinyl benzene, or an ion-exchange resin. Silica is by far the most common packing in liquid chromatography. Silica particles are often coated with thin organic films.



To prevent unwanted interactions between the solutes and any unreacted –SiOH groups, the silica frequently is “capped” by reacting it with $Si(CH_3)_3Cl$; such columns are designated as end-capped.

The properties of a stationary phase are determined by the nature of the organosilane’s alkyl group. If R is a polar functional group, then the stationary phase will be polar. Examples of polar stationary phases include those for which R contains a cyano ($–C_2H_4CN$), diol ($–C_3H_6OCH_2CHOHCH_2OH$), or amino ($–C_3H_6NH_2$) functional group. Since the stationary phase is polar, the mobile phase is a nonpolar or moderately polar solvent. The combination of a polar stationary phase and a nonpolar mobile phase is called **normal-phase chromatography.**

In **reverse-phase chromatography,** which is the more commonly encountered form of HPLC, the stationary phase is nonpolar and the mobile phase is polar. The most common nonpolar stationary phases use an organochlorosilane for which the R group is an *n-octyl (C8) or n-octyldecyl (C18) hydrocarbon chain. Most reversephase* separations are carried out using a buffered aqueous solution as a polar mobile phase. Because the silica substrate is subject to hydrolysis in basic solutions, the pH of the mobile phase must be less than 7.5.

HPLC Detectors

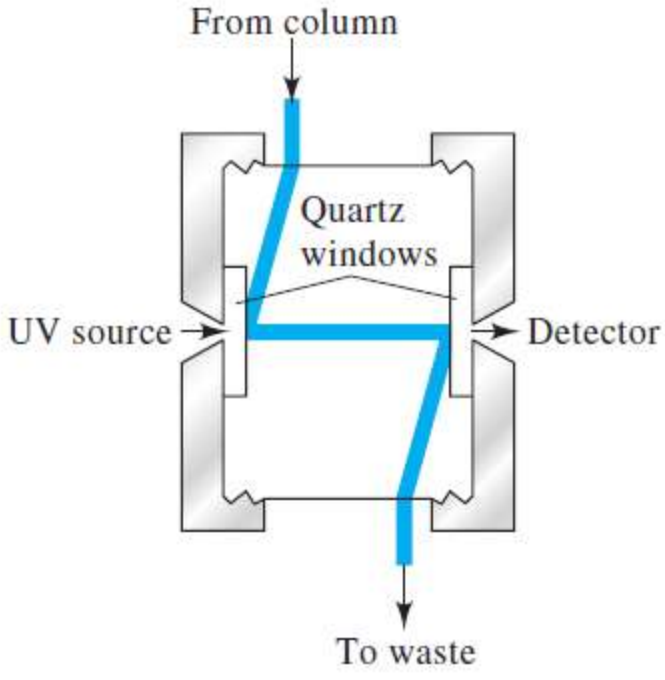
Ideal Detector

- Adequate sensitivity
- Good stability and reproducibility
- A linear response to solutes: over several orders ofmagnitude
- Wide temperature range
- Short response time independent of flow rate
- High reliability and ease of use
- Similarity in response toward all solutes
- Nondestructive

Detector	Approximate limit of detection[a] (ng)
Ultraviolet	0.1–1
Refractive index	100–1 000
Evaporative light-scattering	0.1–1
Electrochemical	0.01–1
Fluorescence	0.001–0.01
Nitrogen ($N \xrightarrow{\text{combustion}} NO \xrightarrow{O_3} NO_2^* \rightarrow h\nu$)	0.3
Conductivity	0.5–1
Mass spectrometry	0.1–1
Fourier transform infrared	1 000

Spectroscopic Detectors

The most widely used detectors for liquid chromatography are based on absorption of ultraviolet or visible radiation. Both photometers and spectrophotometers, specifically designed for use with chromatographic columns, are available from commercial sources. Photometers often make use of the 254- and 280-nm lines from a mercury source because many organic functional groups absorb in the region. Deuterium sources or tungsten-filament sources with interference filters also provide a simple means of detecting absorbing species. Some modern instruments are equipped with filter wheels that contain several interference filters, which can be rapidly switched into place.



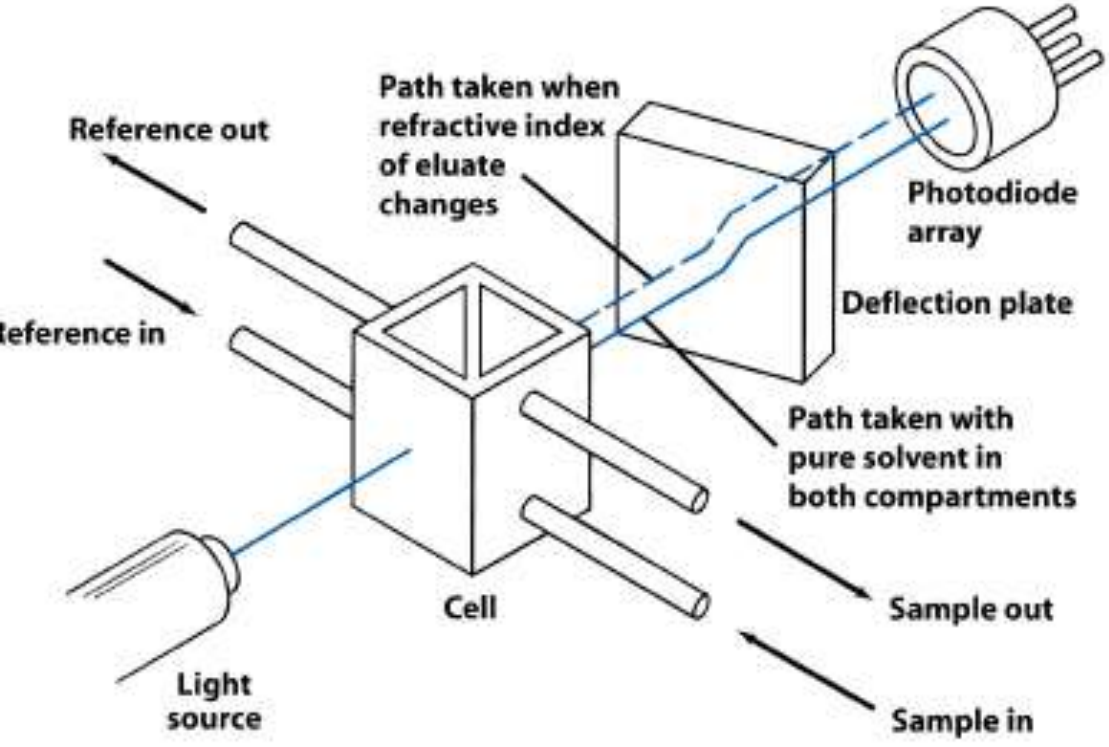
Spectrophotometric detectors are considerably more versatile than photometers and are also widely used in high-performance instruments.

One limitation to using absorbance is that the mobile phase must not absorb strongly at the chosen wavelength.

A refractive index (RI) detector

A refractive index detector is nearly universal, responding to almost all compounds, but has a poorer detection limit of 100 ng–1 µg of injected analyte. Furthermore, a refractive index detector is not useful for a gradient elution unless the mobile-phase components have identical refractive indexes.

The RI detectors measure a bulk property of the mobile phase leaving the column: its ability to refract to bend light (i.e., its refractive index). This property changes as the composition of the mobile phase changes, such as when solutes from the column. By detecting this change, the presence of solutes can be detected.



In this detector, light is created by a source and passed through flowcells containing mobile phase eluting from the column (sample stream) and a reference stream (usually mobile phase with no solute in it). The light passing through these flow0cells is passed through a second time using a mirror and passed to a detector where its intensity is measured.

When the refractive index of liquid in the sample and reference flowcell are the same, little or no bending of light occurs at the interface between the low-cells. This allows the largest amount of light possible to reach the detector.

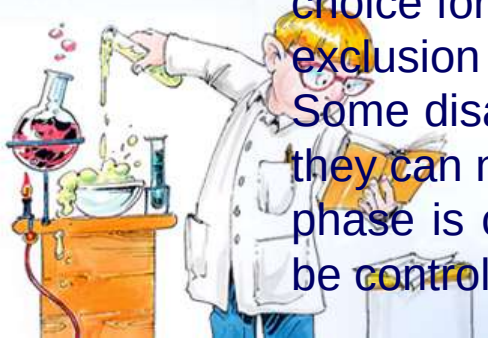
As solute elute from the column, the refractive index of the liquid in the sample flow-cell will be different that that in the reference flow-cell and light will be bent as it passes between them. This changes the amount of light reaching the detector, producing a response.

Applications:

RI detector are universal applicable to the detection of any solute in LC.

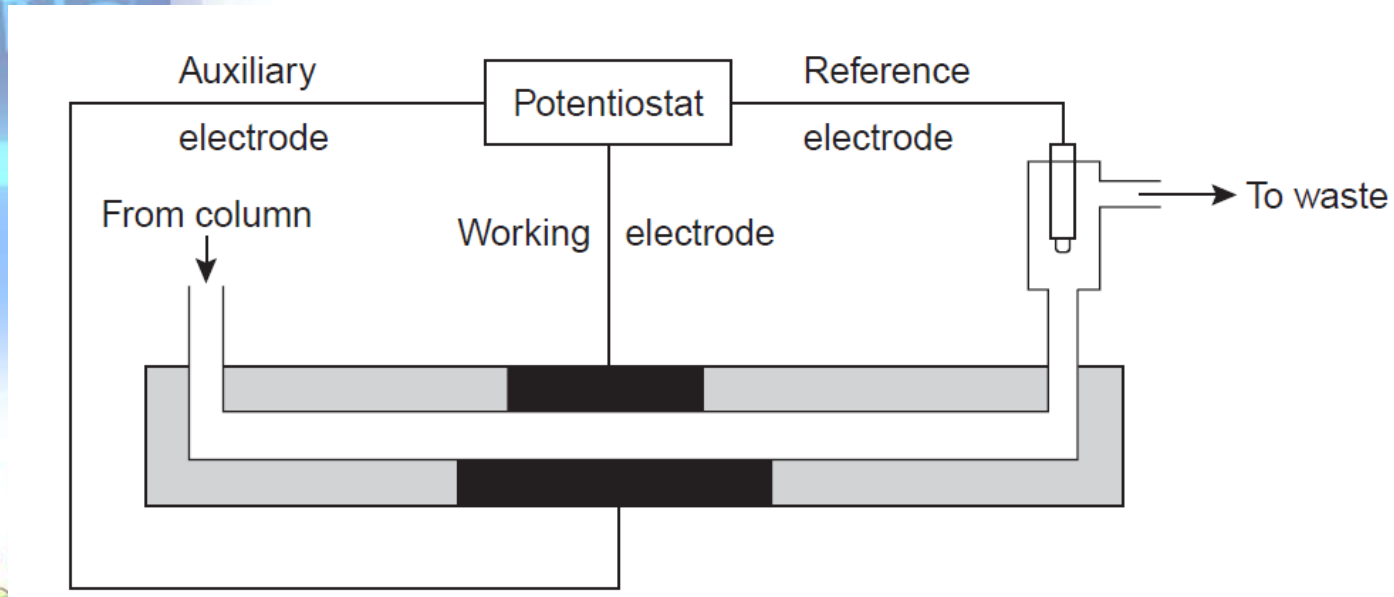
This makes them useful in preliminary work in LC where the nature or properties of a compound may not be known yet. They also the detector of choice for work with carbonhydrates or in the separation of polymer by size-exclusion chromatography.

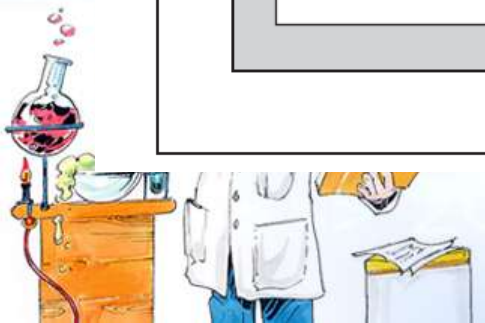
Some disadvantages: (1) they do not have very good limits of detection, (2) they can not used with gradient elution, where the composition of the mobile phase is changing with time. (3) The temperature of the system must also be controlled to avoid baseline fluctuations with these detectors.



Electrochemical Detectors

Another common group of HPLC detectors are those based on electrochemical measurements such as amperometry, voltammetry, coulometry, and conductivity. Figure shows an amperometric flow cell. Effluent from the column passes over the working electrode, which is held at a potential favorable for oxidizing or reducing the analytes. The potential is held constant relative to a downstream reference electrode, and the current flowing between the working and auxiliary electrodes is measured. Detection limits for amperometric electrochemical detection are 10 pg–1 ng of injected analyte.



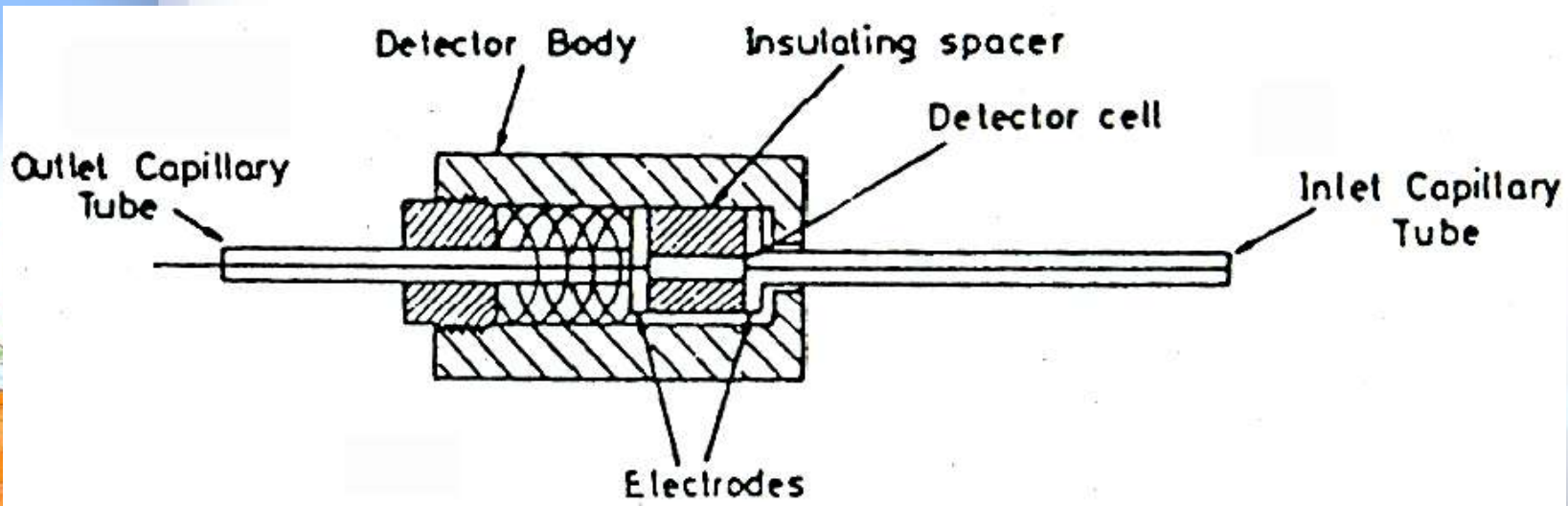


Conductivity Detector

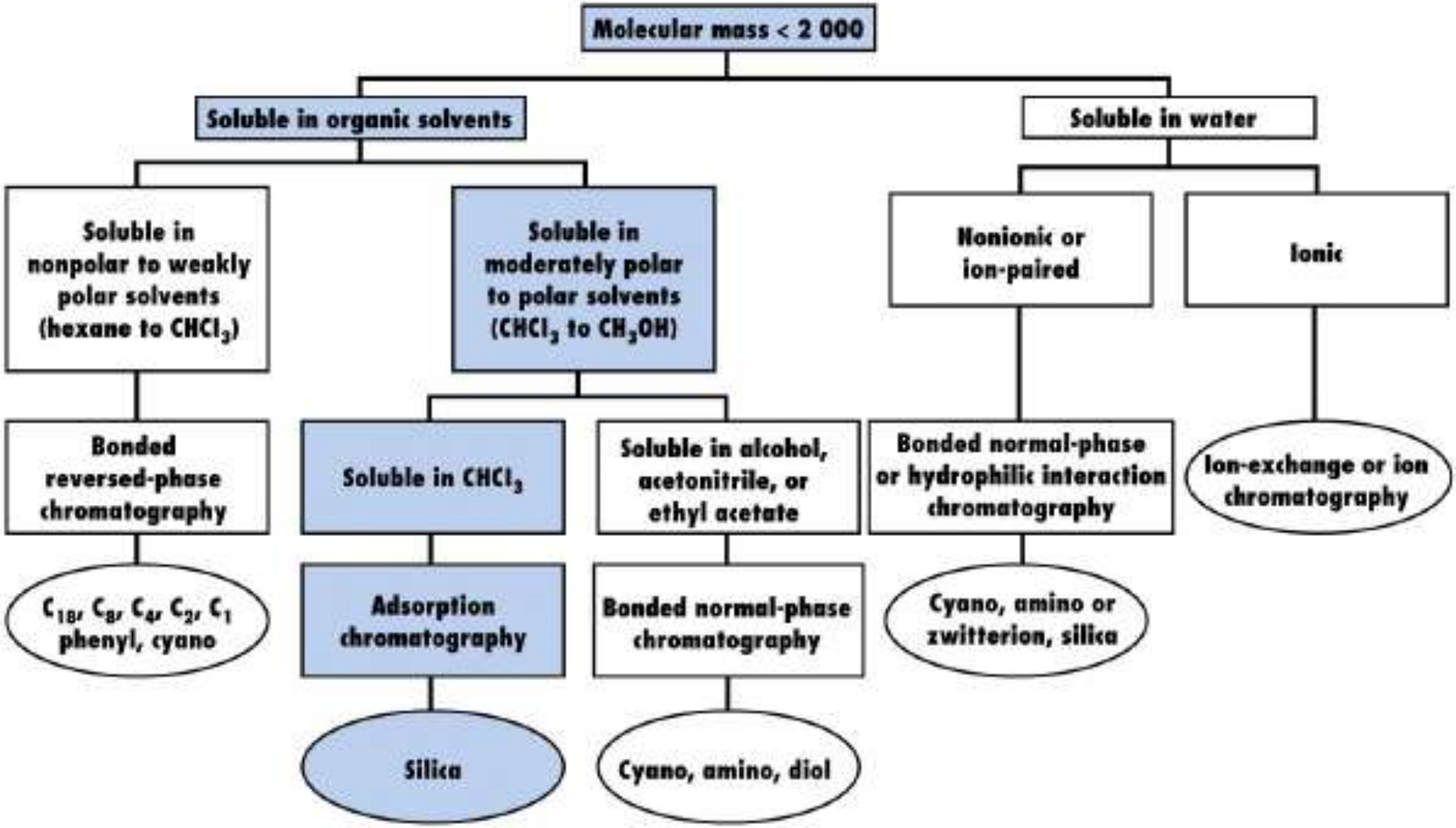
A conductivity detector is an example of a ‘universal’ detector for ionic compound.

This detector measures the ability of a solution to conduct a current when placed in an electrical field. This ability depends on the number of ions or ionic compounds present in the solution.

Applications: for any compound that is ionic or weakly ionic. It is widely used in ion chromatography.



HPLC Mode Selection



Mobile Phases

The elution order of solutes in HPLC is governed by polarity. In a normal-phase separation the least polar solute spends proportionally less time in the polar stationary phase and is the first solute to elute from the column. Retention times are controlled by selecting the mobile phase, with a less polar mobile phase leading to longer retention times. If, for example, a separation is poor because the solutes are eluting too quickly, switching to a less polar mobile phase leads to longer retention times and more opportunity for an acceptable separation. When two solutes are adequately resolved, switching to a more polar mobile phase may provide an acceptable separation with a shorter analysis time. In a reverse-phase separation the order of elution is reversed, with the most polar solute being the first to elute. Increasing the polarity of the mobile phase leads to longer retention times, whereas shorter retention times require a mobile phase of lower polarity.

Often, in choosing a column and mobile phase, the polarity of the stationary phase is matched roughly with that of the analytes; a mobile phase of considerably different polarity is then used for elution.

The polarities of the analyte and the mobile phase are matched but are different from that of the stationary phase.

Quantitative Applications

HPLC is routinely used for both qualitative and quantitative analyses of environmental, pharmaceutical, industrial, forensic, clinical, and consumer product samples.

Preparing Samples for Analysis

Samples in liquid form can be analyzed directly, after a suitable clean-up to remove any particulate materials or after a suitable extraction to remove matrix interferents. In determining polyaromatic hydrocarbons (PAH) in wastewater, for example, an initial extraction with CH_2Cl_2 serves the dual purpose of concentrating the analytes and isolating them from matrix interferents. Solid samples must first be dissolved in a suitable solvent, or the analytes of interest must be brought into solution by extraction. For example, an HPLC analysis for the active ingredients and degradation products in a pharmaceutical tablet often begins by extracting the powdered tablet with a portion of mobile phase. Gases are collected by bubbling through a trap containing a suitable solvent.

Comparison of High Performance Liquid Chromatography and Gas-Liquid Chromatography

Characteristics of Both Methods

- Efficient, highly selective, widely applicable
- Only small sample required
- May be nondestructive of sample
- Readily adapted to quantitative analysis

Advantages of HPLC

- Can accommodate nonvolatile and thermally unstable compounds
- Generally applicable to inorganic ions

Advantages of GC

- Simple and inexpensive equipment
- Rapid
- Unparalleled resolution (with capillary columns)
- Easy to interface with mass spectrometry

When compared with gas chromatography, HPLC has only a few differences in the scale of operation; accuracy; precision; sensitivity; selectivity; and time, cost, and equipment necessary. Injection volumes in HPLC are usually significantly larger than in GC because of the greater capacity of HPLC columns. Precision in HPLC is often better due to the routine use of loop injectors. Because HPLC is not limited to volatile analytes, the range of compounds that can be analyzed is somewhat larger than for GC. Capillary GC columns, on the other hand, have more theoretical plates, providing greater resolving power for complex mixtures.