بسم الله الرحمن الرحيم

High Performance Liquid Chromatography

Fundamentals & Applications

HPLC

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Outline

- **HPLC: A**n **O**verview
- **HPLC A**nalysis & **S**eparation **M**echanisms
- **M**ain **P**hysical **C**omponents
- **HPLC P**arameters & **M**ethod **D**evelopment
- **HPLC A**pplications

HPLC: An Overview

Since its advent in 1974 by Horvath, HPLC has expanded very quickly.

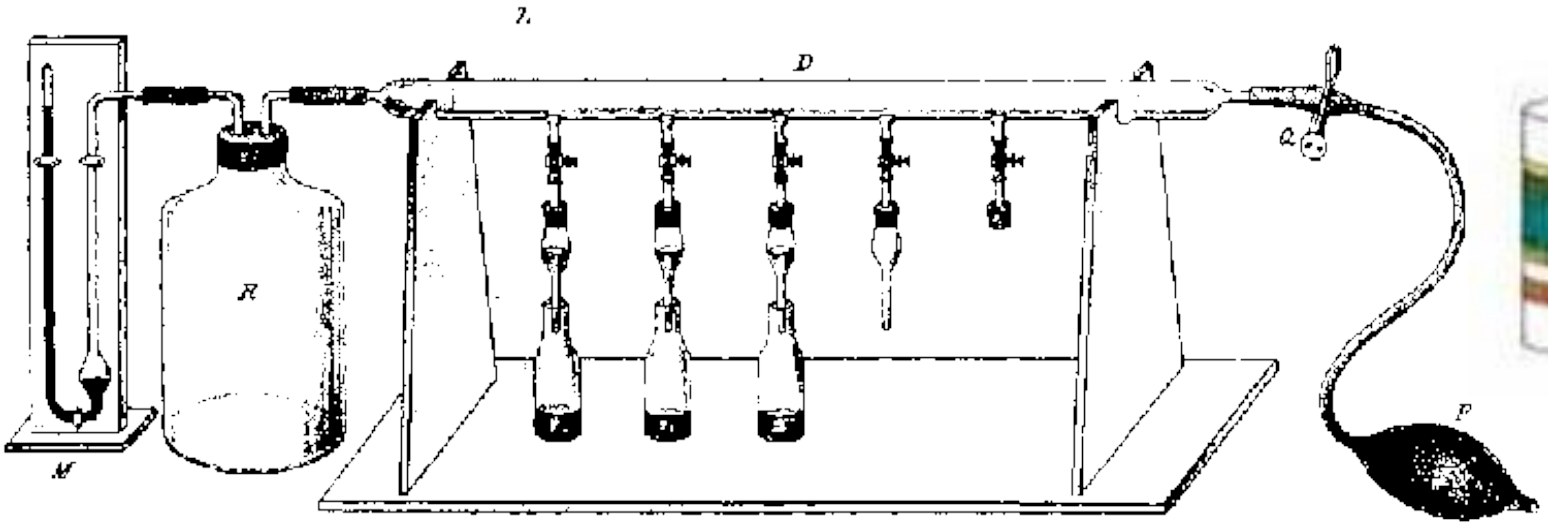
HPLC is an abbreviation for High-Performance Liquid Chromatography (It has also been referred to as High-Pressure Liquid Chromatography).

HPLC is a type of LC in which the mobile phase is liquid that forced through a reusable column by means of a pumping system, use to separate compounds that are dissolved in solution.

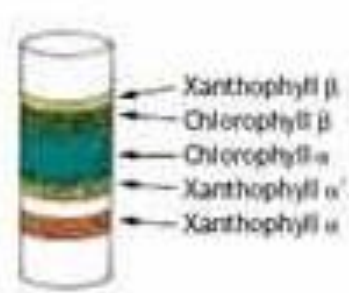
HPLC is very efficient technique and can be applied to about every kind of sample; it yields excellent separations in a short time, and can work with minimal training of the operator. Today, HPLC is one the most widely used techniques for separating and analyzing mixtures of chemical substances, compounds and materials.

Impetus behind high-pressure

Early LC, including the original work by **Tswett**, was carried out in glass columns with diameter of 1–5 cm and lengths of 50–500 cm and 150–200 µm stationary phase particle diameter.

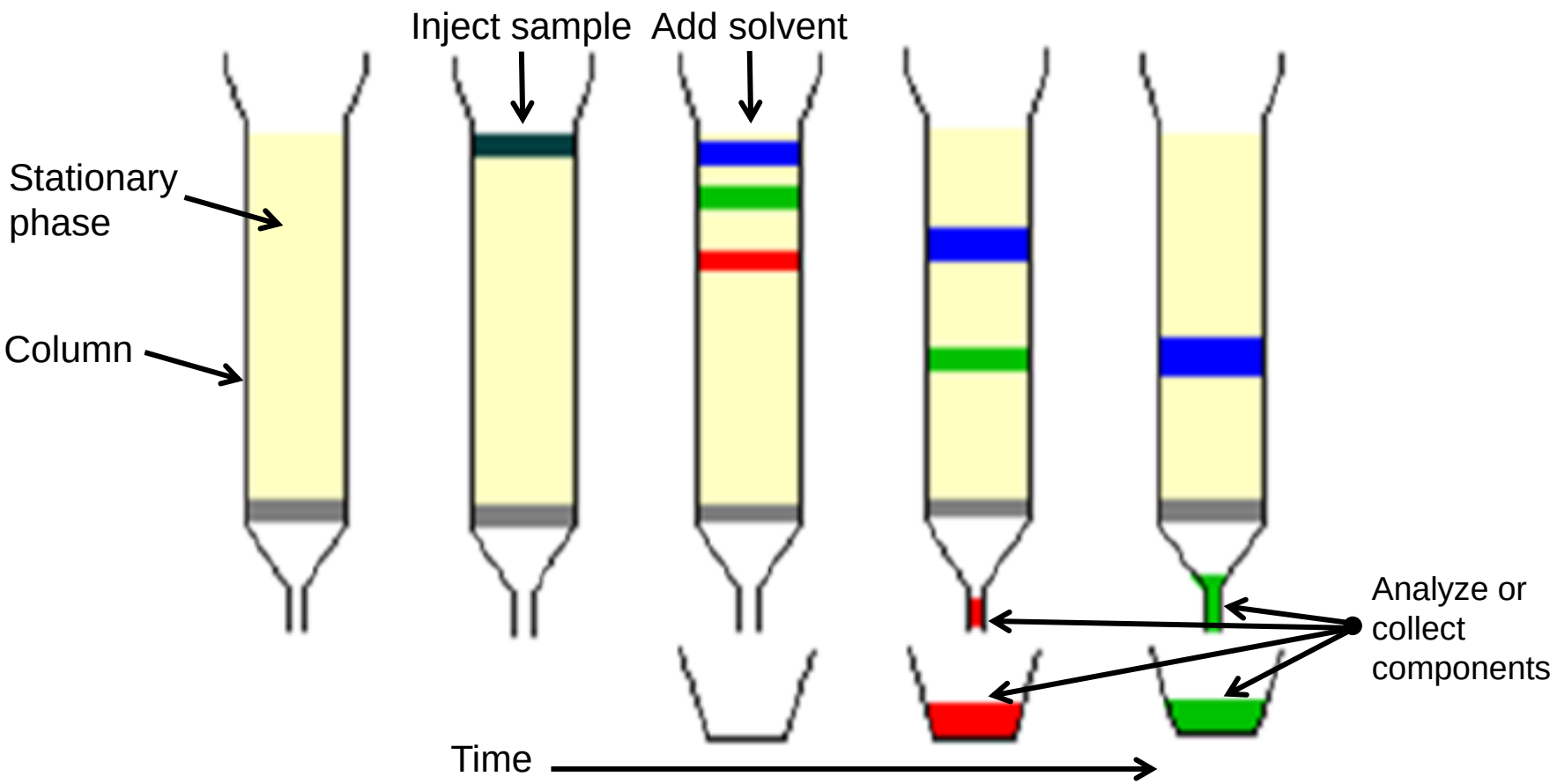


Tswett's apparatus



Scientist realized that the major increases in column efficiency could be brought about by decreasing the particle size of packings to increase the surface area and thus interaction sites and retention, this required sophisticated instruments with high-pressure pumps to assure reasonable flow rate through the very narrow pores.

Principles of liquid chromatography



The components of the sample are separated from one another by the column packing that involves various chemical and/or physical interactions between their molecules and the packing particles. The separated components are analyzed or collected at the exit of this column and identified by an external measurement technique. Separation is achieved by a different (affinity; interaction; retention) for the mobile and stationary phases for the compounds to be separated.

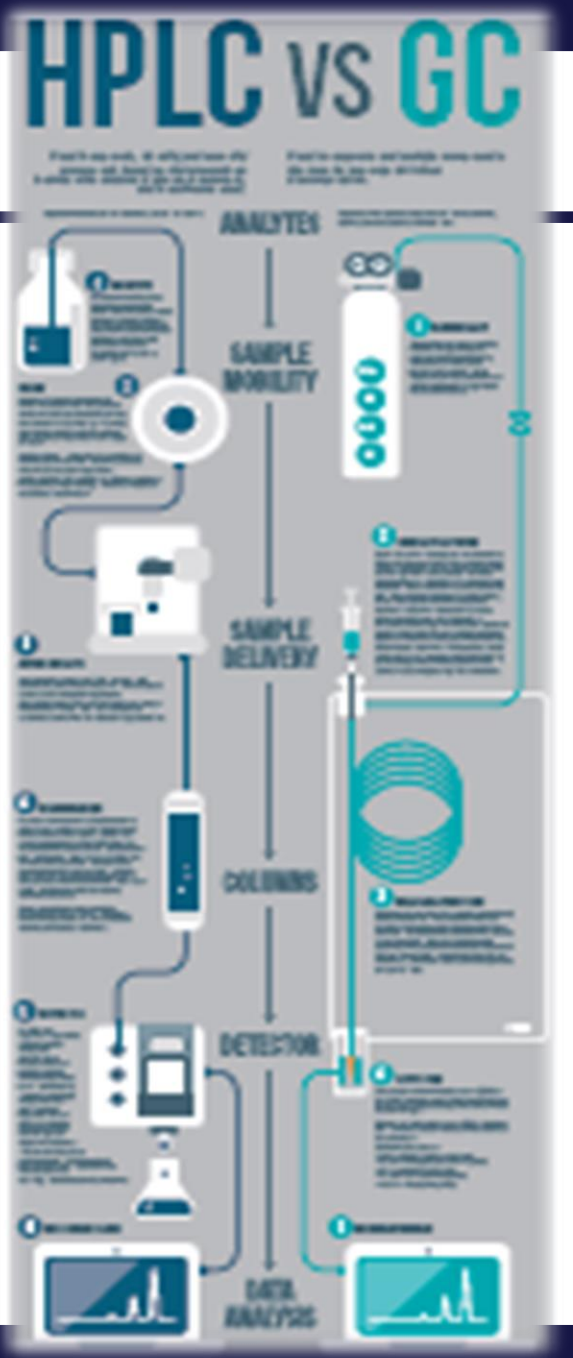
HPLC versus GC

LC is a much older technique than GC, but was overshadowed by the rapid development of GC in the 1950's and 1960's.

-LC: mobile phase is liquid and interact with both solutes and stationary phase in the separation processes.
-GC: mobile phase is inert gas (carrier gas), don't participate in the separation processes.

LC is currently the dominate type of chromatography and is even replacing GC in its more traditional applications.

-GC: commercialized available in the 1950's
-HPLC: commercialized available in the 1970's



Advantages of LC compared to GC:

-LC can be applied to the separation of any compound that is soluble in a liquid phase. LC more useful in the separation of biological compounds, synthetic or natural polymers and inorganic compounds such materials include amino acids, proteins, nucleic acids, drugs, steroids, terpenoids, pesticides, antibiotics and metal-organic species.

-LC is suitable for separating nonvolatile compounds

-Liquid mobile phase allows LC to be used at lower temperatures than required by GC. LC better suited than GC for separating compounds that may be thermally labile.

-Retention of solutes in LC depend on their interaction with both the mobile phase and stationary phase. GC retention based on volatility and interaction with stationary phase. LC is more flexible in optimizing separations, change either stationary or mobile phase.

-Most LC detectors are non-destructive. Most GC detectors are destructive. LC is better suited for preparative or process-scale separations.

Disadvantage of LC compared to GC:

-LC is subject to greater peak or band-broadening. RESOLUTION !!!!
much larger diffusion coefficients of solutes in gases vs. liquids.

Separation Mechanisms and LC Modes

Liquid chromatography is a technique used to separate a sample into its individual parts. This separation occurs based on the chemical or physical interactions of the sample with the mobile and stationary phases. Because there are many stationary/mobile phase combinations that can be employed when separating a mixture, there are several different types of chromatography that are classified based on the physical states of those phases.

A useful classification of the various LC techniques is based on the type of distribution mechanism applied in the separation. Individual HPLC columns may use any one of a number of different phases or processes to induce this resolution.

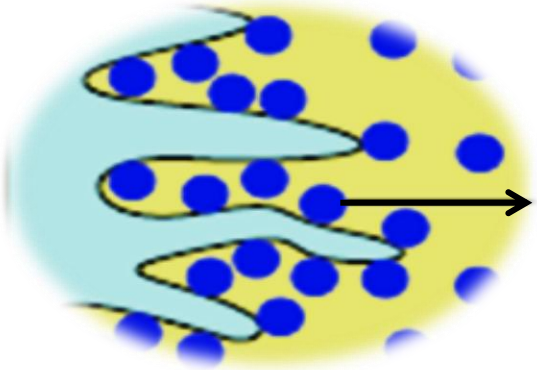
A variety of chromatographic modes have been developed, on the basis of the mechanisms of retention and operation, which consider as the more common classification.

Major Modes of **HPLC** separation

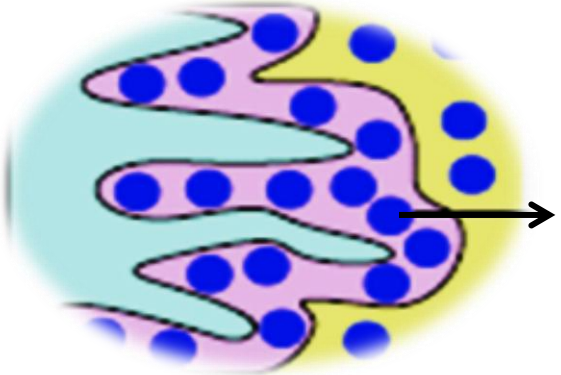
Normal-phase	Reversed-phase	Ion-exchange	Size-exclusion

Sub-modes of **HPLC** separation

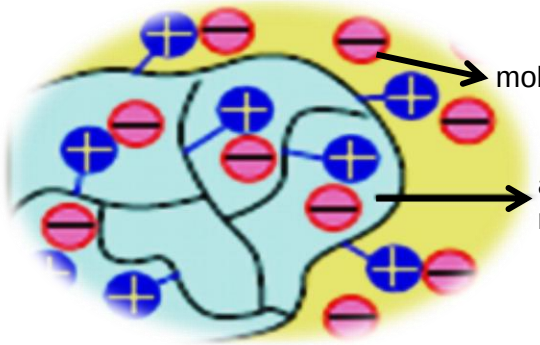
Hydrophobic interaction	Hydrophilic interaction
Affinity chromatography	Chiral separations
Ion suppression	Ion pair



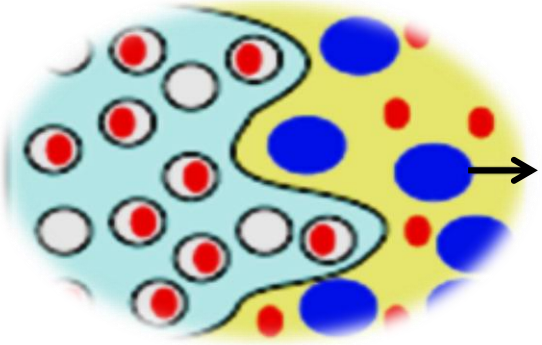
Adsorption



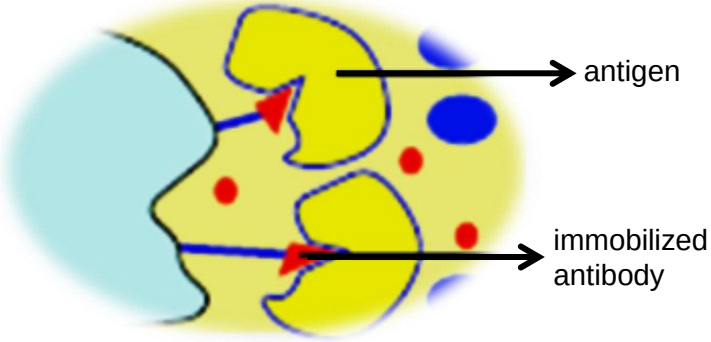
Partition



Ion-exchange



Molecular exclusion



Affinity

Normal-phase chromatography (NPC)

NP-HPLC was the first kind of HPLC setup used (discovered by Tswett in 1903).

Normally bonded phase HPLC columns are composed of a stationary bed, which is strongly polar in nature, and a mobile phase that is nonpolar. Polar samples are thus retained on the polar surface of the column packing longer than less polar materials.

Mobile phases in NPC …

Mobile phases in NP-HPLC are based on nonpolar solvents (such as hexane, heptane, octane, etc.) with the small addition of polar modifier (i.e., methanol, ethanol or isopropanol).

Stationary phases in NPC …

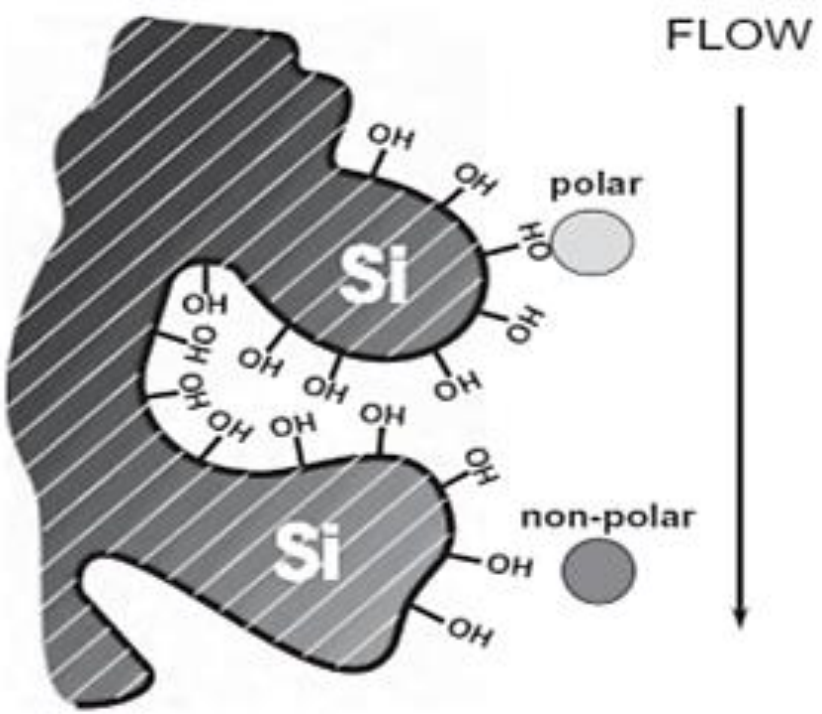
Packing materials traditionally used in NP-HPLC are usually porous oxides such as silica (SiO_2) or alumina (Al_2O_3). Chemically modified stationary phases can also be used in NP-HPLC. Silica modified with trimethoxy glycidoxypropyl silanes (common name: diol phase) is typical packing material with decreased surface polarity.

Suggested retention and interaction mechanisms in NPC …

- Competition model

- Solvent interaction model

The figure shows a schematic diagram of part of a porous silica particle with silanol groups (Si-OH) residing at the surface and inside its pores. Polar analytes migrate slowly through the column due to strong interactions with the silanol groups.



Although NP chromatography can be performed using either **partition** or **adsorption** mechanisms (based on the physical state of the polar stationary phase), the dominant retention mechanism is adsorption (solid stationary phase).

NP-HPLC applications …

NP-HPLC is used when the analyte of interest is neutral species has a polar nature, and the retention on the basis of polarity.

Selection of using NP-HPLC as the chromatographic method of choice is usually related to the sample solubility in specific mobile phases. Since NP uses mainly nonpolar solvents, it is the method of choice for highly hydrophobic compounds (which may show very stronger interaction in RP-HPLC), which are insoluble in polar or aqueous solvents.

NP chromatography is particularly well suited to the separation of isomers and to class separations. It is also possible to separate species using NP chromatography on the basis of the number of electronegative atoms such as oxygen or nitrogen. Fat and water soluble vitamins, hydrocarbons and pesticides have all been separated using hexane as the mobile phase.

Reversed-phase chromatography (RPC)

RP-HPLC columns are the inverse of NPC. The stationary bed is nonpolar (hydrophobic) in nature, while the mobile phase is a polar liquid. RP-HPLC was developed due to the increasing interest in large nonpolar biomolecules.

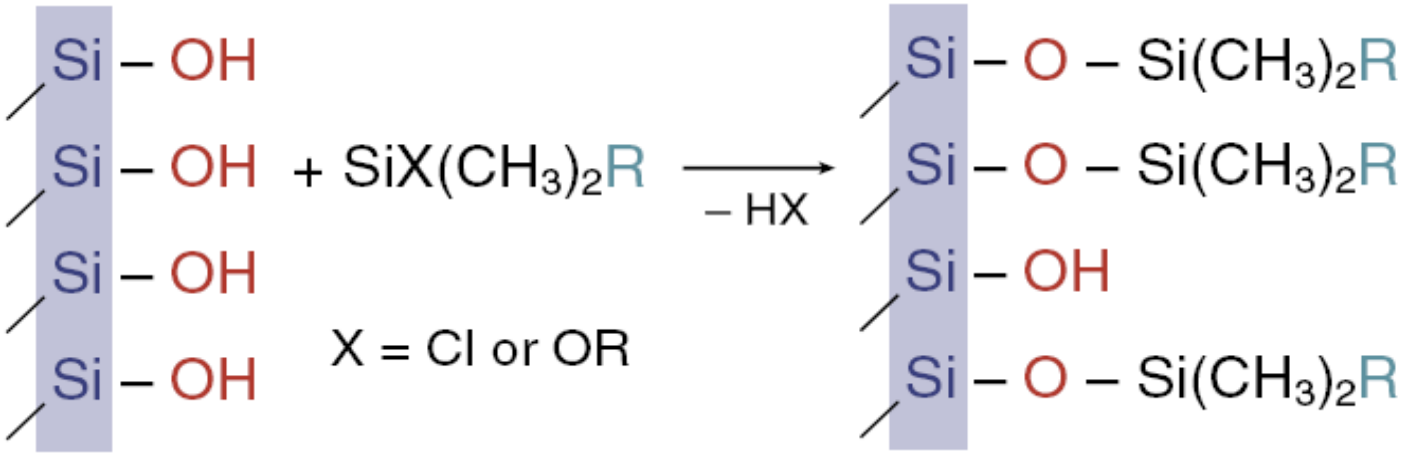
Mobile phases in RPC …

Mobile phase is by far the major tool for the control of analyte retention in RP-HPLC. The mobile phases used in RPC are based on a polar solvent, typically water, to which a less polar solvent such as acetonitrile or methanol is added. The eluting strength of the solvent is inversely related to its polarity. Variations of the eluent composition, type of organic modifier, pH and buffer concentration can have important effects on the analyte retention and selectivity in RPC, which provide the chromatographer with a valuable set of variables for successful development of a separation method.

Stationary phases in RPC …

The majority of packing materials used in RPC are those in which a functional group is chemically attached to a silica support (chemically modified porous silica bonded phases). The most popular bonded phase is silica which has been treated with **RMe_2SiCl**

Where; R is a straight chain alkyl group such as $-CH_3$, $-C_4H_9$, $-C_8H_{17}$ & $-C_{18}H_{37}$, phenyl $-C_6H_5$ groups, cyano $(-CH_2)_3CN$ groups & amino $(-CH_2)_3NH_2$ groups.



Reaction of silica gel with a functional group to produce a RP stationary phase (silylation).

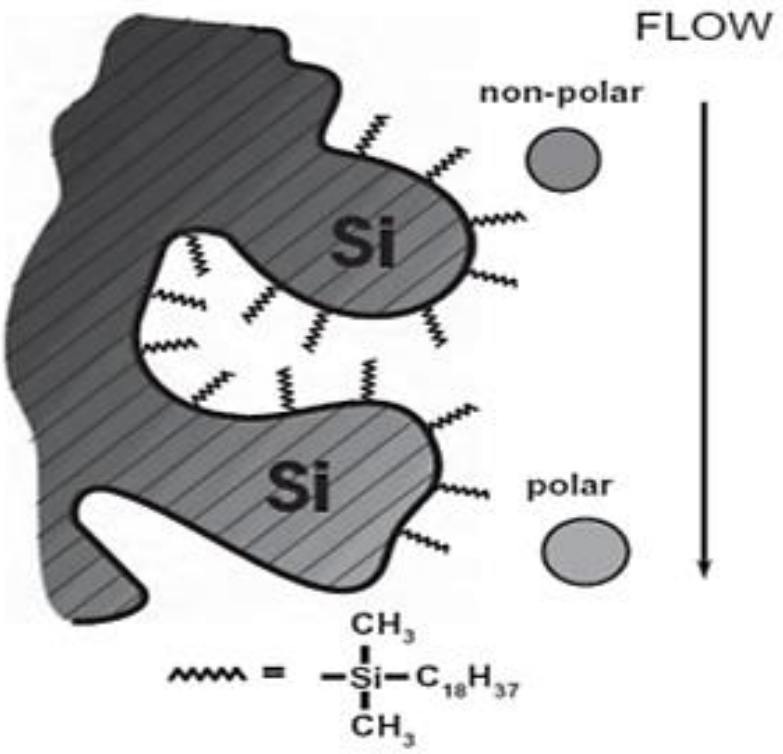
RPC typically refers to the use of chemically bonded stationary phases, where a functional group is bonded to silica. For this reason, RPC is often referred to in the literature as bonded phase chromatography. However, polymeric stationary phases such as polymethacrylate or polystyrene, or solid stationary phases such as porous graphitic carbon are also used as RP stationary phases.

Suggested retention and interaction mechanisms in RPC …

- Solvophobic model

- Partitioning model

A simplified view of reversed-phase is shown in the figure, where polar analytes elute first while nonpolar analytes interact more strongly with the hydrophobic groups such as C_{18} that form a liquid like layer around the solid silica support.

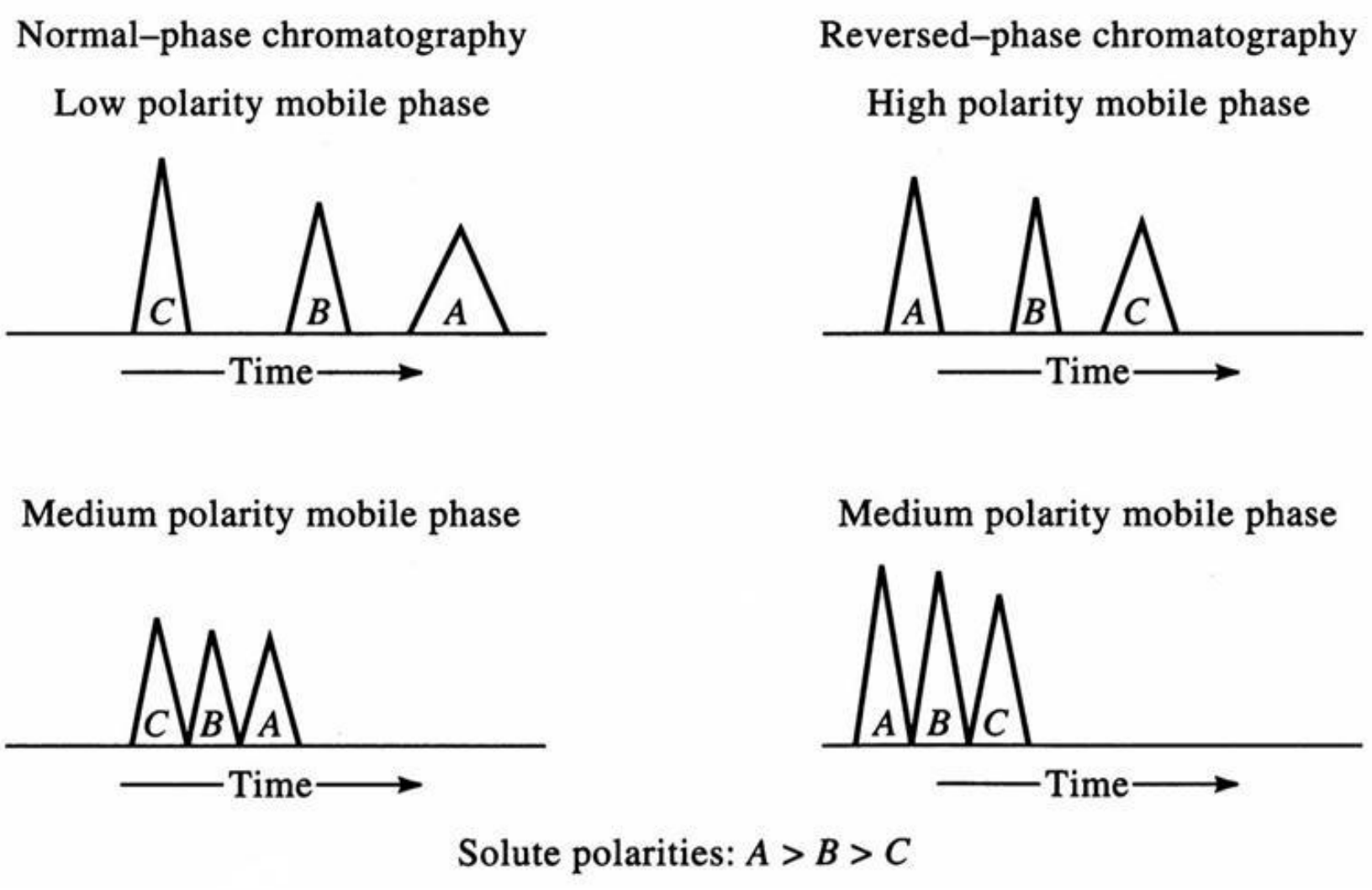


RP-HPLC applications …

RP-HPLC is by far the most popular mode. Almost 80% of all analytical separations are carried out using RP-HPLC.
-Nature and significant of the analysis compounds (pharmaceutical and biological compounds).
-Cheaper mobile phase solvents (water).

RP chromatography is the most popular mode for the separation of low molecular weight (<3000), neutral species that are soluble in water or other polar solvents. It is widely used in the pharmaceutical industry for separation of species such as steroids and vitamins.

It is also used in other areas; for example, in clinical laboratories for analysis of catecholamines, in the chemical industry for analysis of polymer additives, in the environmental arena for analysis of pesticides and herbicides, and in the food and beverage industry for analysis of carbohydrates, sweeteners and food additives.



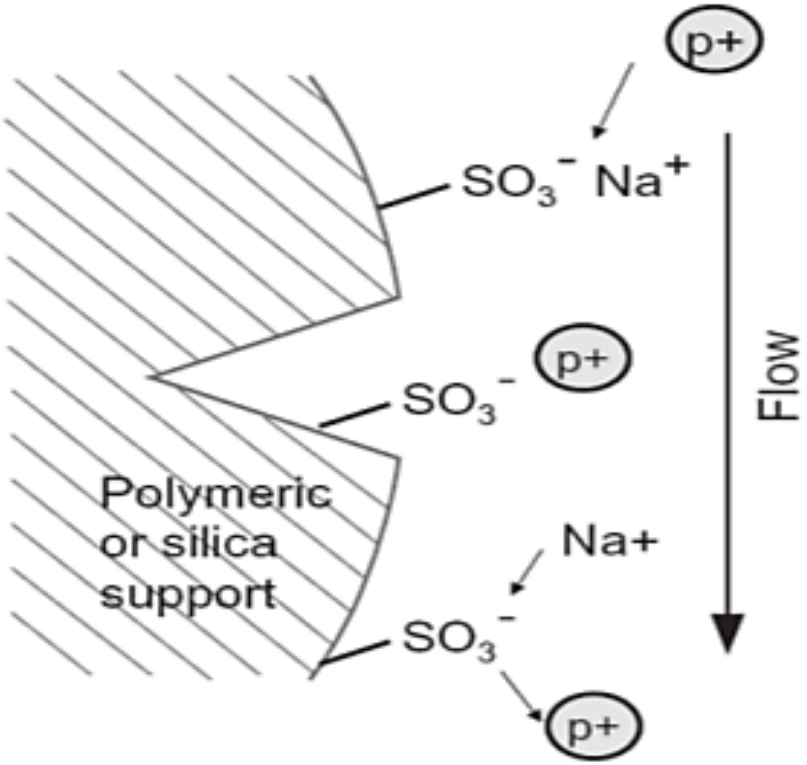
The relationship between polarity and elution times for normal-phase and reversed-phase chromatography.

Ion-exchange chromatography (IEC)

IEC allows the separation of ions and polar molecules based on the charge properties of the molecules. The stationary bed in IE has an ionically +ve or -ve charged surface. This technique is used almost exclusively with ionic or ionizable samples. The stronger the charge on the sample, the stronger it will be attracted to the ionic surface, thus, the longer it will take to elute.

In IEC, species are separated on the basis of differences in electric charge. The primary mechanism of retention is the electrostatic attraction of ionic solutes in solution to fixed ions of opposite charge on the stationary phase.

Separation occurs as a consequence of differences in the size, charge density and structure of the different ionic solutes.



Stationary phases in IEC …

Ion-exchange are characterized by the type of support and by the functional group providing the charge. Functionalized silica and synthetic polymeric (e.g. crosslinked styrene divinylbenzene or methacrylic acid divinylbenzene copolymers) resins are the most common supports. Some inorganic materials such as zeolites are sometimes used.

Depending on the charge of the exchange centers on the surface, the stationary phase or ion-exchanger is classified as:

-**Anion-exchanger** when it carries a +ve charge

-**Cation-exchanger** when it carries a -ve charge.

Ion-exchangers are further sub-divided into strong acid or base and weak acid or base types. The four major types of ion-exchange centers are usually employed:

(1) Sulfonic acid $-SO_3^-H^+$ (**strong cation-exchanger**).

(2) Carboxylic acid $-COO^-H^+$ (**weak cation-exchanger**).

(3) Quaternary amine $-N(CH_3)_3^+OH^-$ (**strong anion-exchanger**).

(4) Tertiary amine $-NH(CH_3)_2^+OH^-$ (**weak anion-exchanger**).

Mobile phases in IEC …

Mobile phases in IEC are aqueous solutions of a salt or mixture of salts, often with a small percentage of an organic solvent added. The salt mixture may be a buffer, or a buffer may be added if required. The main component of the eluent is the competing ion that causes the solute ions to be eluted. Analyte retention and selectivity in IEC are strongly dependent on the pH and ionic strength of the mobile phase.

IEC applications …

Any species that can acquire a charge can be separated by IEC.

Some of the more important applications include analyses of amino acids, nucleotides, carbohydrates and proteins.

IEC found an important application during World War II for the separation of rare earths, as part of the Manhattan project.

The technique also had an important role in the research on transuranium elements. Probably one of the most remarkable achievements of separation science was represented by the separation of element 101 (mendelevium) from elements 99 (einsteinium) and 100 (fermium), involving only 17 atoms.

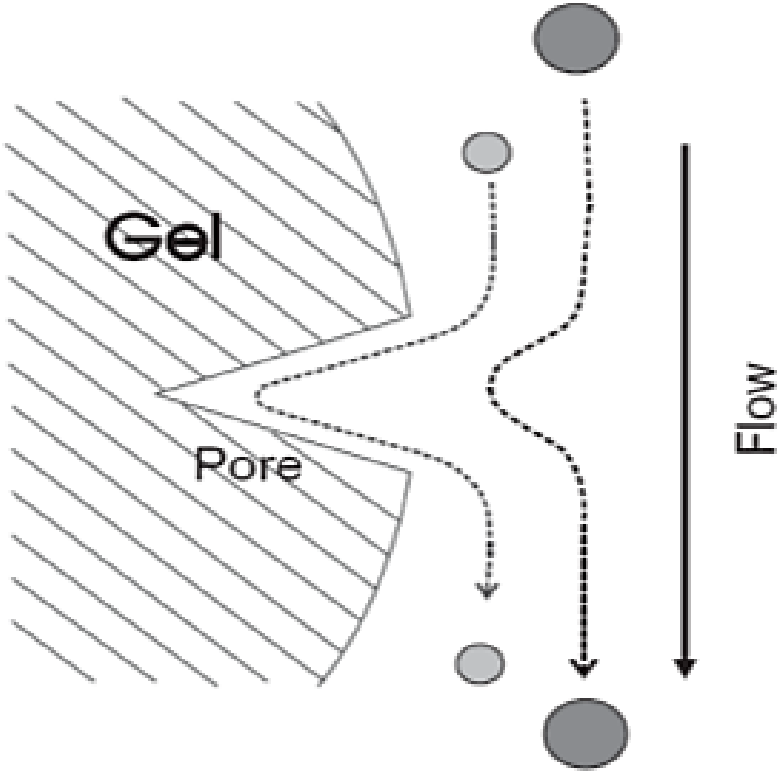
Separation of nucleic acid constituents by IEC is particularly noteworthy.

An important impact of IEC on the overall evolution of chromatography is related to the work of Stanford Moore and William Stein on the analysis of amino acids.

Size-exclusion chromatography (SEC)

SEC is a chromatographic method in which particles are separated by differences in their molecular size (on the absence of any specific analyte interactions with the stationary phase). In SE, the columns are filled with material having precisely controlled pore sizes, and the sample is filtered by the stationary phase according to its solvated molecular size.

The Figure shows that a large molecule is excluded from the pores, migrates quickly and eluted first from the column, whereas a small molecule can penetrate the pores and migrates more slowly down the column. The process depends on the relative size and shape of analyte molecules and the respective pore size of the absorbent. The solvent molecules are usually the smallest, they are normally the last to be eluted.



Two types of SEC are usually distinguished:

-**Gel filtration chromatography** (GFC): use aqueous mobile phases and hydrophilic packings to separate and identify aqueous soluble biological macromolecules. Typical stationary phases for GFC include polydextrans, polyvinyl alcohol gel and silica gel.

-**Gel permeation chromatography** (GPC): using hydrophobic stationary phases and organic mobile phases to obtain molecular weight distribution information and characterization on organic soluble polymers. Stationary phases for GPC are typically crosslinked, rigid polystyrene divinylbenzene gels.

Stationary phases in SEC …

Stationary phases for SEC fall into two broad categories, historically,,

-A **hydrophilic** gel consisting of dextran crosslinked with epichlorohydrin was introduced as a cooperative venture under the trade name Sephadex in 1959 (GFC).

-A **hydrophobic** polystyrene gels led to the development of GPC, enabling the determination of the molecular weight distribution of high molecular weight synthetic polymers.

Mobile phases in SEC …

Mobile phases for SEC fall into two broad categories:

-**Aqueous** buffers for GFC.

-**Organic** solvents for GPC.

In SEC, the mobile phase is selected not to control selectivity but for its ability to dissolve the sample. In addition, the mobile phase should have a low viscosity and be compatible with the detector and column packing.

SEC applications …

SEC is a widely used technique for the purification and analysis of synthetic and biological polymers, such as proteins, polypeptides, macromolecular complexes, polysaccharides, nucleic acids, hydrocarbon polymers, polyamides and polyesters.

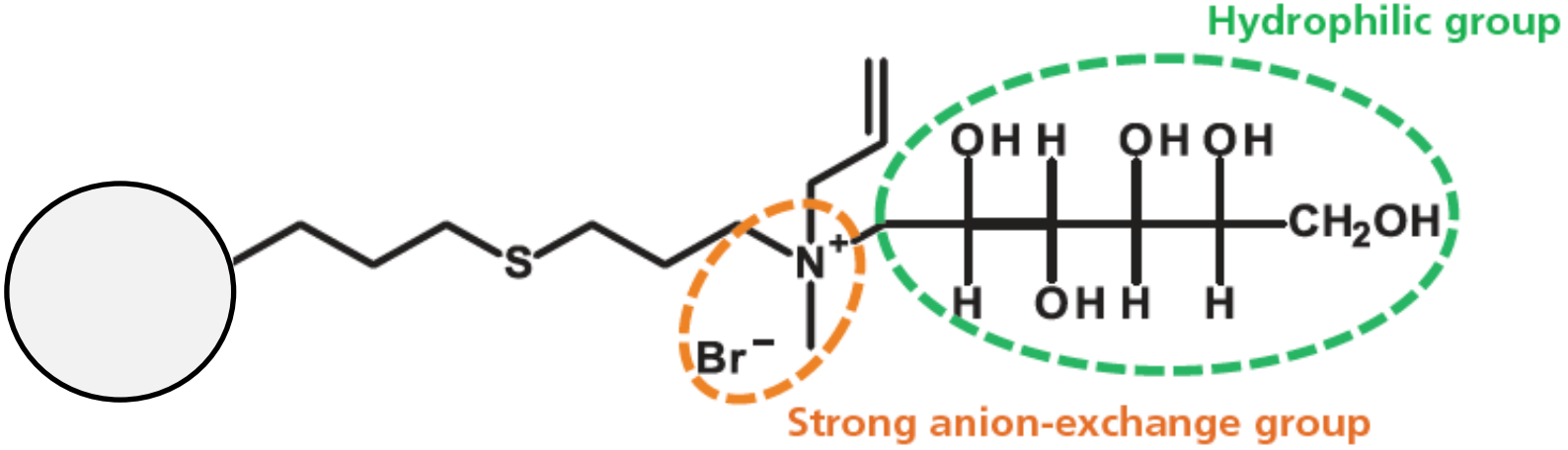
It is also useful for determining the tertiary and quaternary structures of purified proteins, and is the primary technique for determining the average molecular weight of natural and synthetic polymers.

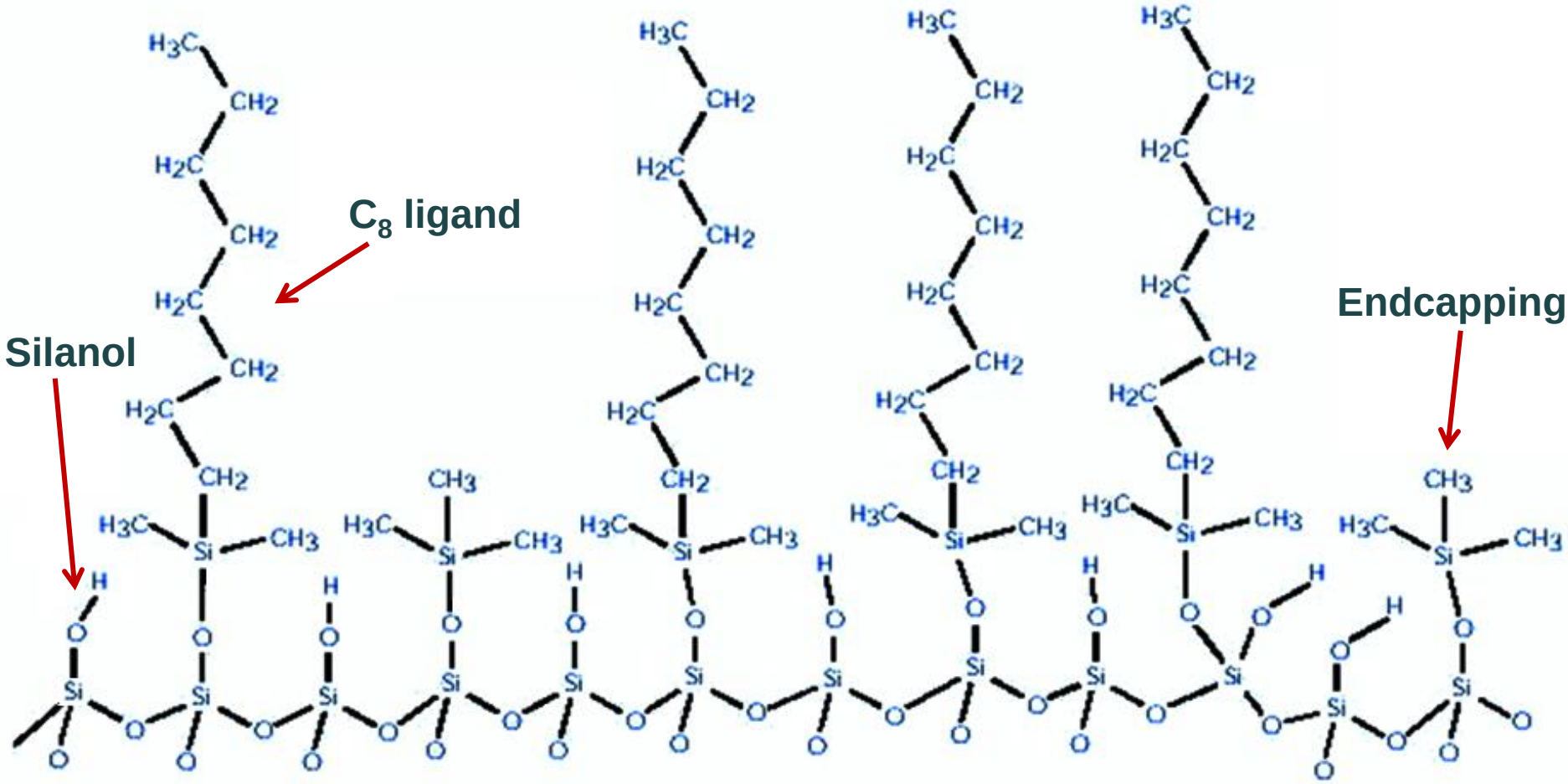
One of the major applications of SEC is polymer characterization. As many of the properties that characterize a polymer, including hardness, brittleness and tensile strength, are related to the molecular weight distribution, GPC can be used to identify subtle differences between polymer materials.

Mixed-mode chromatography

In practice, most LC separations are the result of mixed mechanisms, e.g., in partition chromatography, in most cases contributions due to adsorption/desorption effects are observed.

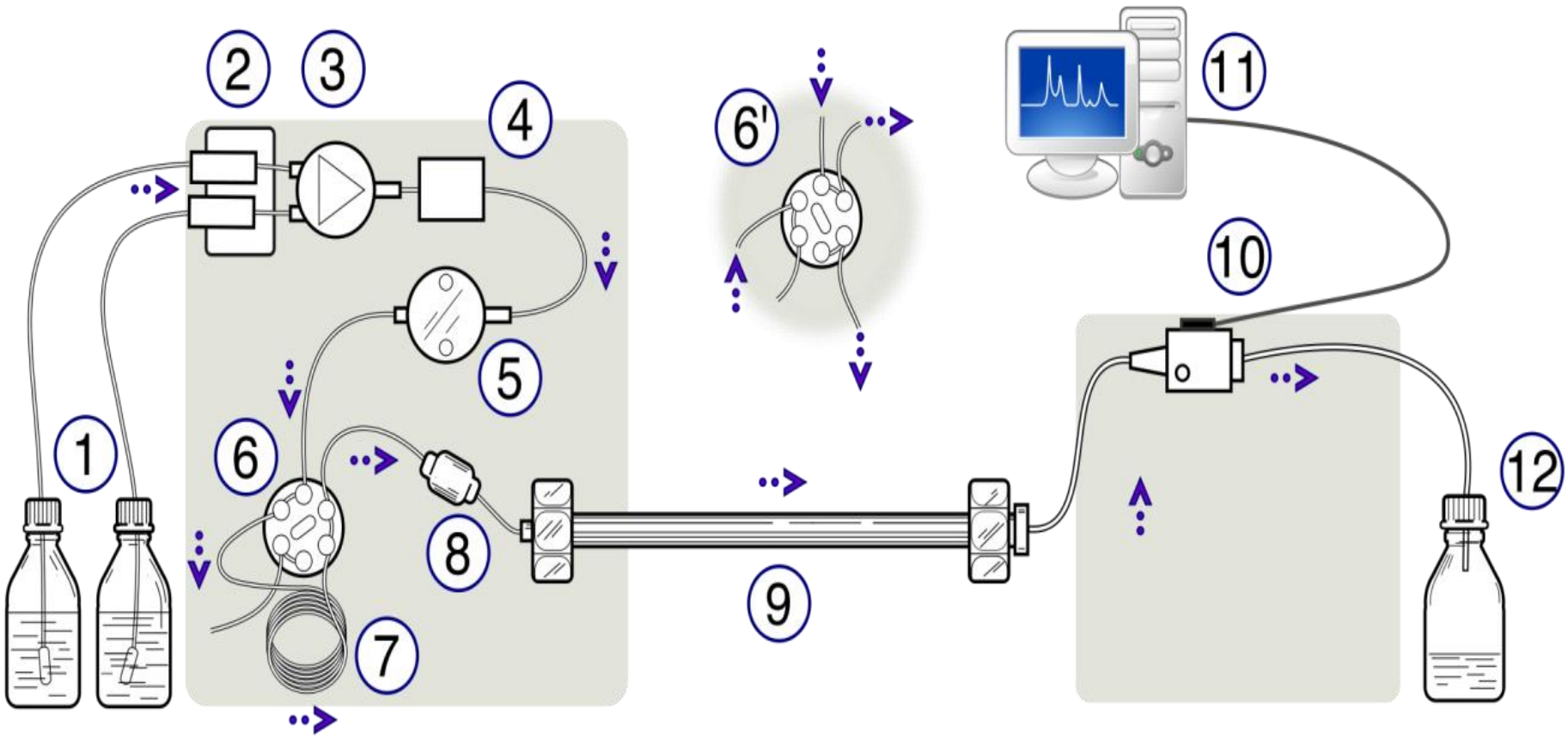
Mixed-mode chromatography, or multimodal chromatography, refers to chromatographic methods that utilize more than one form of interaction between the stationary phase and analytes in order to achieve their separation.





Most **LC** applications (about 80%) are done with **RP-LC**, i.e., a nonpolar stationary phase and a polar mobile phase. **RP-LC** is ideally suited for the analysis of polar and ionic analytes, which are not directly amenable to **GC** analysis.

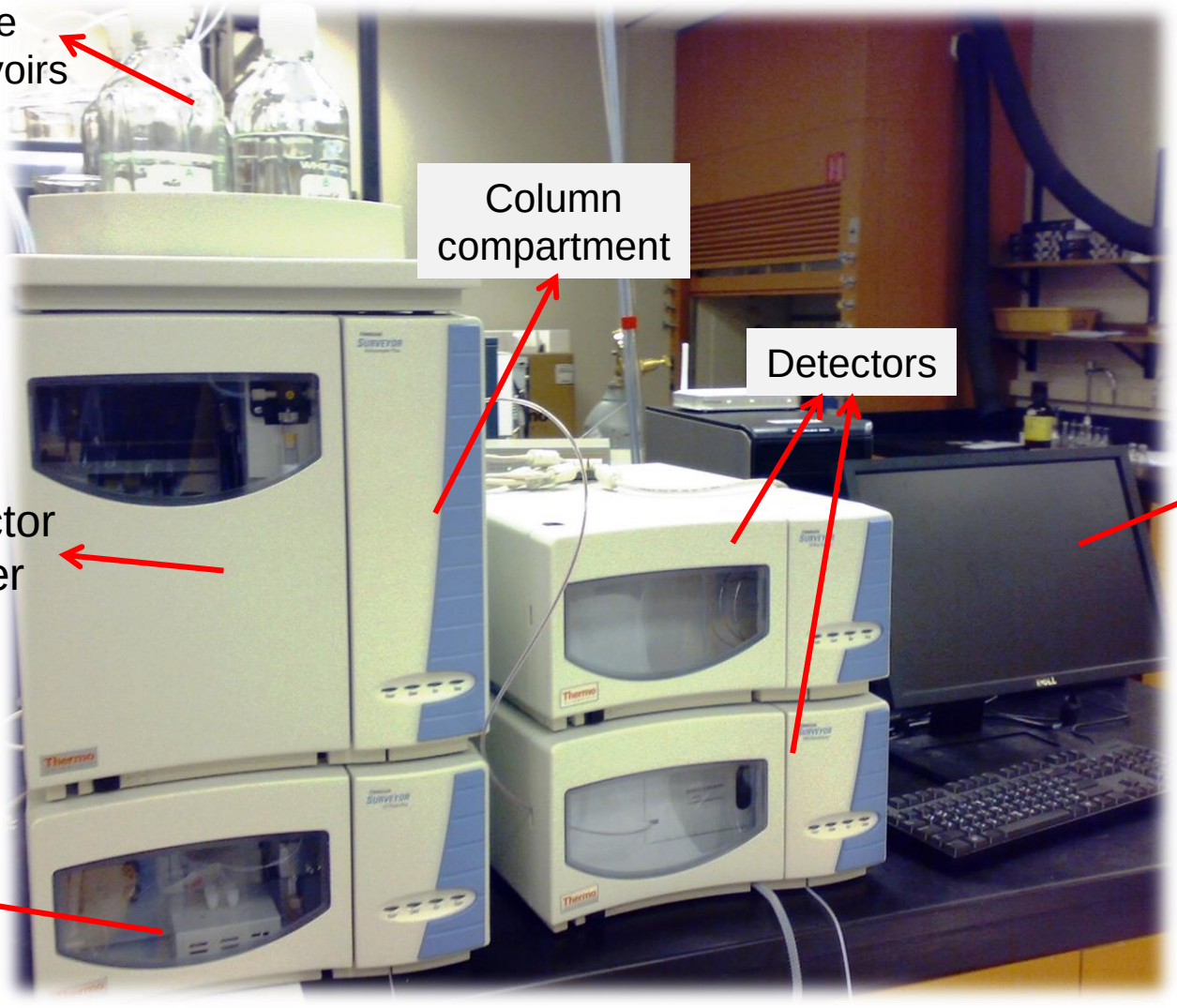
HPLC system (main physical components)



Schematic of an HPLC system

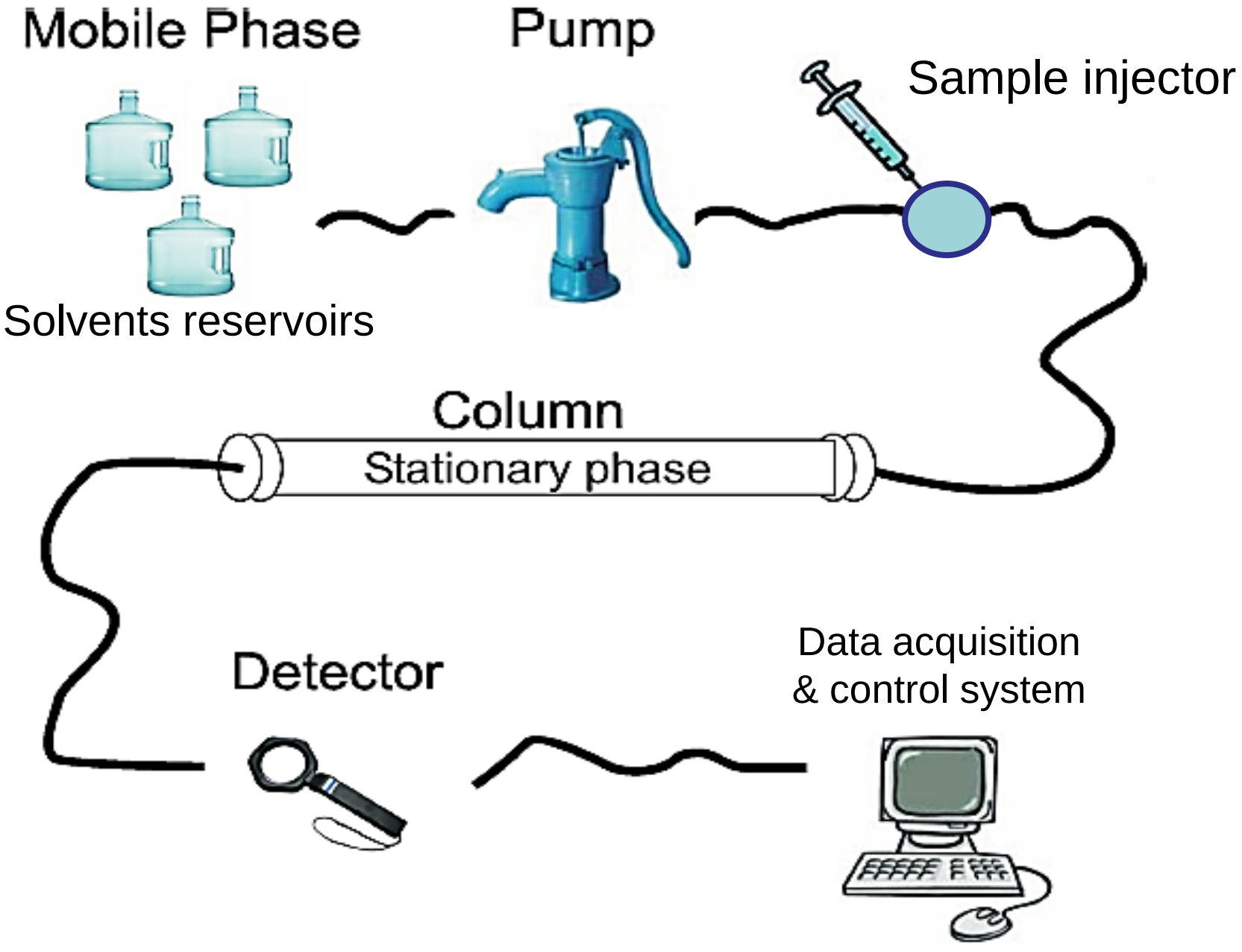
1= Mobile phase reservoir, **2=** Solvent degasser, **3=** Gradient valve, **4=** Mixing vessel, **5=** Solvent delivery system (a pump), **6=** Switching valve in inject position, **6'=** Switching valve in load position, **7=** Sample introduction device (the injector), **8=** Pre-column (guard column), **9=** Analytical column (a separation column that contains the stationary phase on which the separation is to be performed), **10=** Detector (one or more detectors), **11=** Data acquisition or read out, **12=** Waste or fraction collector.

HPLC instrument



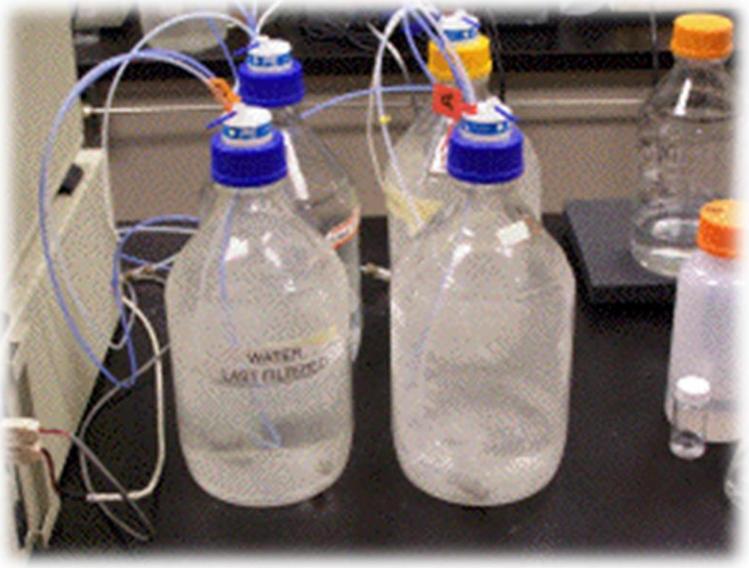

SURVEYOR
SURVEYOR

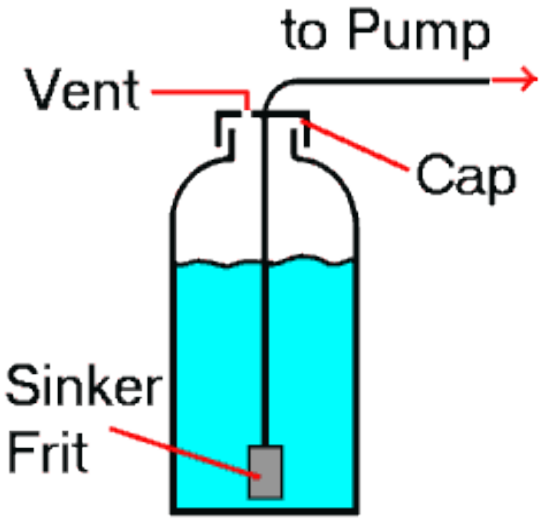

A B C D E
Samples syringe
Samples tray
Column oven
Column
Injector and sample loop
Pump heads
Mixing vessel
Mobile phase tubes


Mobile Phase
Pump
Sample injector
Solvents reservoirs
Column
Stationary phase
Detector
Data acquisition
& control system

Solvent reservoirs (eluent containers)

The mobile phase reservoir can be any clean, inert container. Storage of sufficient amount of **HPLC-grade solvents** for continuous operation of the system. It could be single eluent, but usually multiple solvents are used in HPLC (binary, tertiary and quaternary).





Solvent reservoirs should have a cap that allows for a tubing inlet line, which feeds mobile phase to the solvent delivery system. The cap also serves to keep out dust, reduce solvent evaporation, allow for pressurization of the bottle, offer ports for additional inlet lines, and sparging.

The material of the mobile phase reservoir depends on the type of application. Glass is typically used to prevent leaching of materials from plastics into the eluent, which may interfere with analysis. For RNA, protein, and electrochemical analysis, plastic mobile phase reservoirs are often used, as glass containers can leach trace ions, interfering with analysis.

Mobile phase

The mobile phase is one or more solvent used to **solubilize** the components and move them at different rates across the column and the system.

In contrast to GC, the mobile phase in LC participates in the interaction with both solutes and stationary phase. Therefore, it should be carefully selected.

Selection of the suitable mobile phase mainly depend on the application and the interaction mechanisms (suitability for the solutes and stationary phase).

All mobile phases solvents should be freshly **filtered** and preferably **degassed**. On-line degassers, which are primarily used to remove small gas bubbles and reduce dissolved air, are now popular additions to many HPLC systems, and they eliminate the need to degas mobile phase offline.

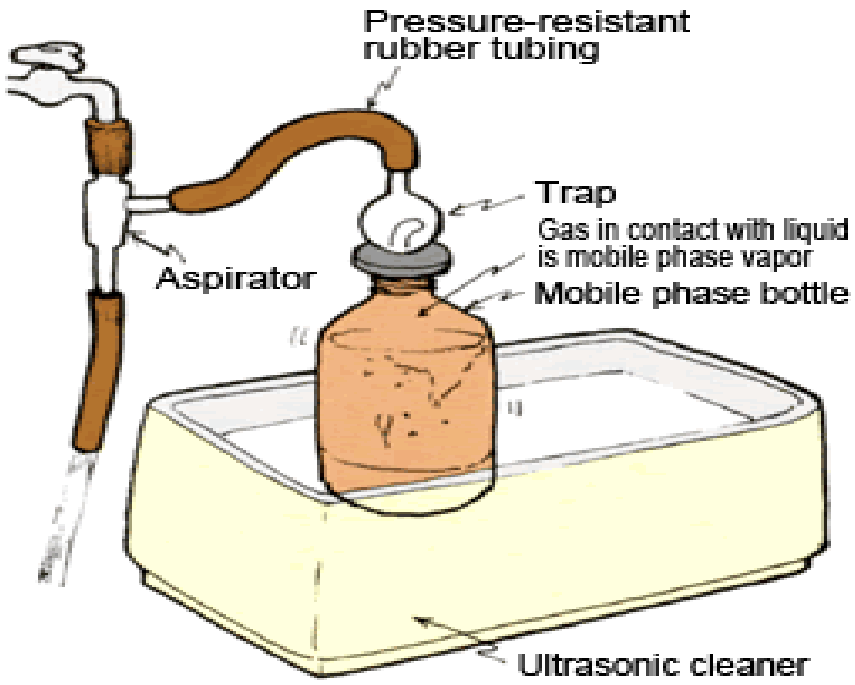
Solvent degassing is important to ensure that **air bubbles** do not outgas and interfere with accurate delivery of flow. Solvents can be degassed by:

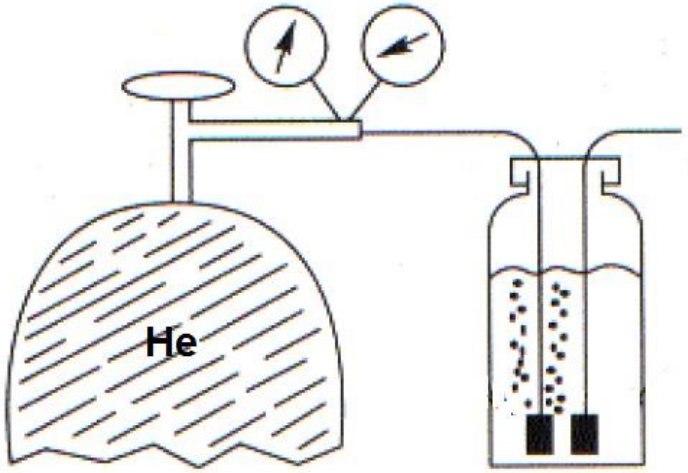
-Ultrasonic degassing.

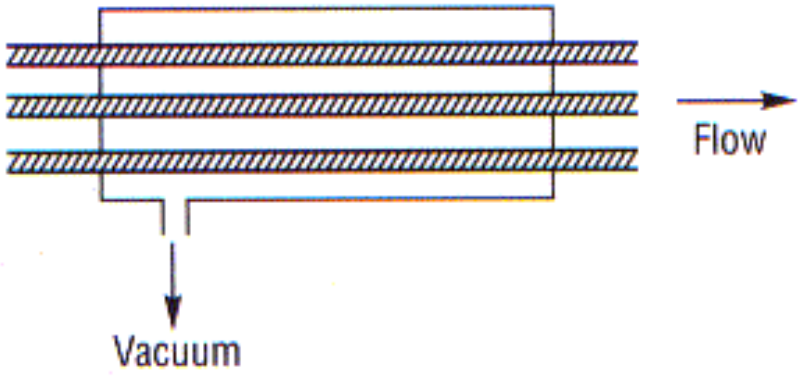
-External vacuum degassing.

-Bubbling helium gas into the eluent.

-On-line degassing methods.







Mobile phase main criteria:

-Highly pure solvents, HPLC-grade, purity > 99%, filtered with at least 0.45 µm membranes. Contaminated solvents can cause baseline inconsistencies, decrease detector signal (UV and mass spectrometric) and destroy the column (blockage). HPLC-grade solvents are highly purified, spectral-grade solvents having low UV absorbance which minimize background noise.

-Dissolves the analytes (solubility).

-Not react to sample and stationary phase.

-Not interfere in the detection (suitable for the detector). e.g., phosphate buffers should be avoided in LC-MS analysis. The phosphate buffers are non-volatile and clog up the MS, and will accumulate around the cone on ESI systems.

-Column back-pressure (viscosity), temperature (boiling point), volatility and flow. The viscosity of the eluent causes changes in back-pressure.

-Free from air bubbles (degassing, sparging).

-Mobile phase composition type, single, binary or more solvent, percentage, concentration, etc. To control the separation process.

-Mobile phase additives; acid, base, buffer, ion pairing reagent, salt, etc. To control pH, ionic strength, and improve the retention of the analytes.

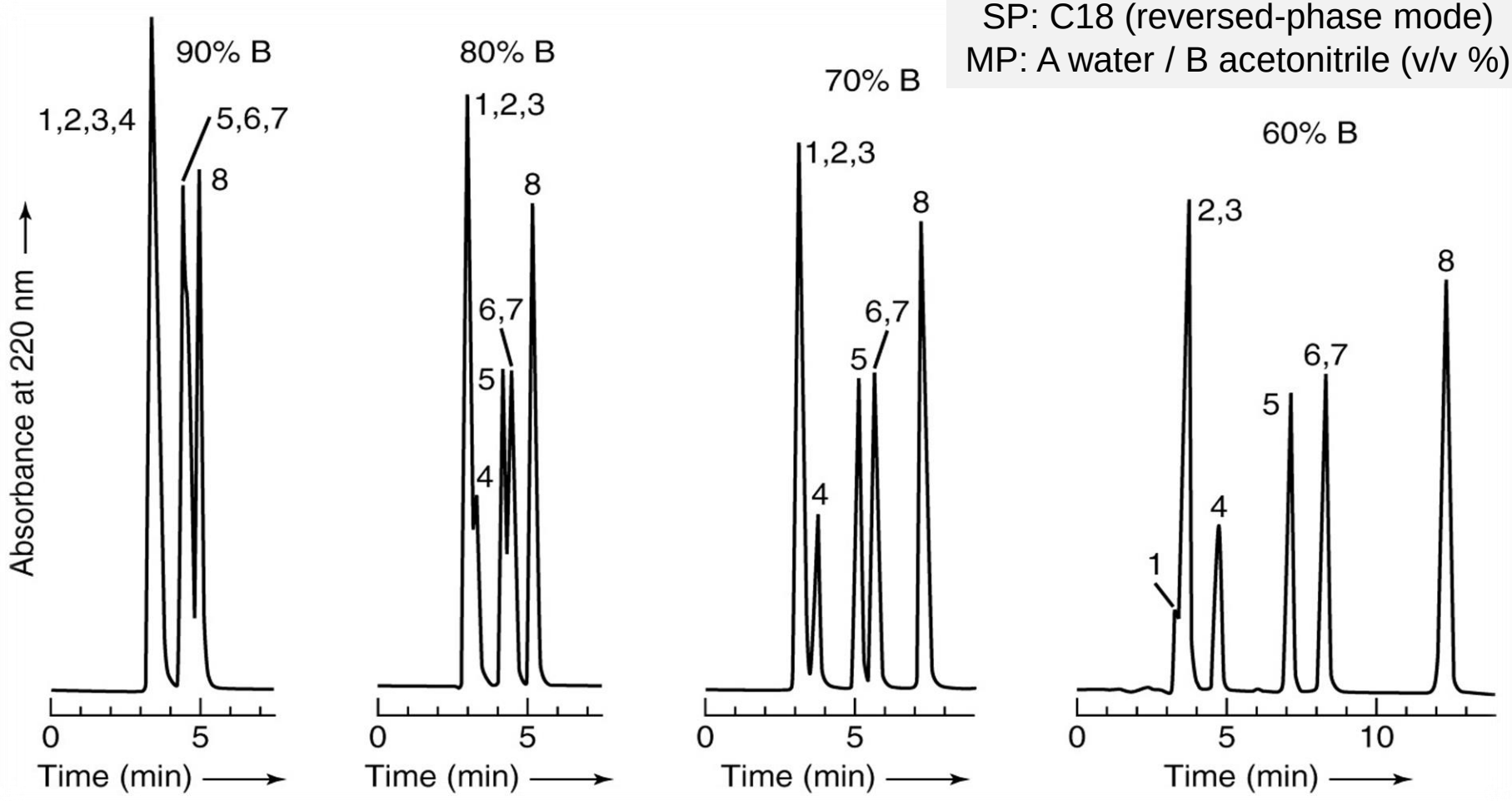
Common chromatographic mobile phases

Solvent	Refractive Index[a]	Viscosity, cP[b]	Boiling Point, °C	Polarity Index, P'	Eluent Strength,[c] ε^0
Fluoroalkanes[d]	1.27–1.29	0.4–2.6	50–174	<–2	–0.25
Cyclohexane	1.423	0.90	81	0.04	–0.2
n-Hexane	1.372	0.30	69	0.1	0.01
l-Chlorobutane	1.400	0.42	78	1.0	0.26
Carbon tetrachloride	1.457	0.90	77	1.6	0.18
i-Propyl ether	1.365	0.38	68	2.4	0.28
Toluene	1.494	0.55	110	2.4	0.29
Diethyl ether	1.350	0.24	35	2.8	0.38
Tetrahydrofuran	1.405	0.46	66	4.0	0.57
Chloroform	1.443	0.53	61	4.1	0.40
Ethanol	1.359	1.08	78	4.3	0.88
Ethyl acetate	1.370	0.43	77	4.4	0.58
Dioxane	1.420	1.2	101	4.8	0.56
Methanol	1.326	0.54	65	5.1	0.95
Acetonitrile	1.341	0.34	82	5.8	0.65
Nitromethane	1.380	0.61	101	6.0	0.64
Ethylene glycol	1.431	16.5	182	6.9	1.11
Water	1.333	0.89	100	10.2	Large

Optimization of mobile phase polarity (type and composition)

-Using binary or more solvents.
-Changing the mobile phase composition alters the separation.
-Weak and strong mobile phase.

SP: C18 (reversed-phase mode)
MP: A water / B acetonitrile (v/v %)



Pump (a solvent delivery system)

The function of the solvent delivery system is to deliver the mobile phase (eluent) through the system, accurately and reproducibly.

The solvent delivery system comprises the pump, check valves, flow controllers, pulse dampeners and pressure transducers. Delivery of the mobile phase must be pulse free to ensure minimal baseline noise from the pump.

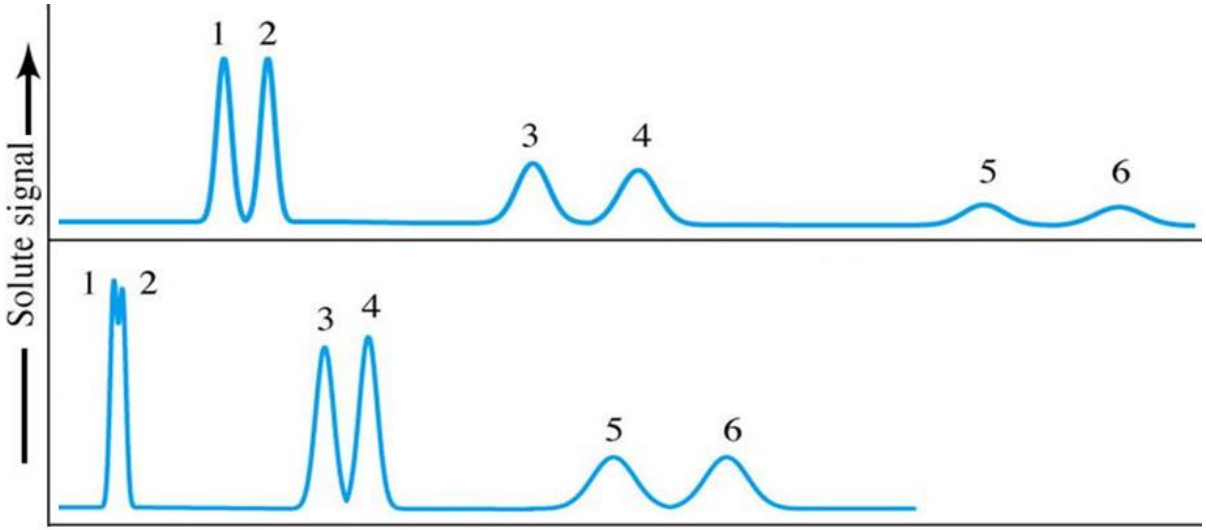
Pumping systems are designed to deliver either a **single eluent** or **multiple** (binary, tertiary and even quaternary) eluents. These are known depending on application which significantly extends the capabilities of the technique.

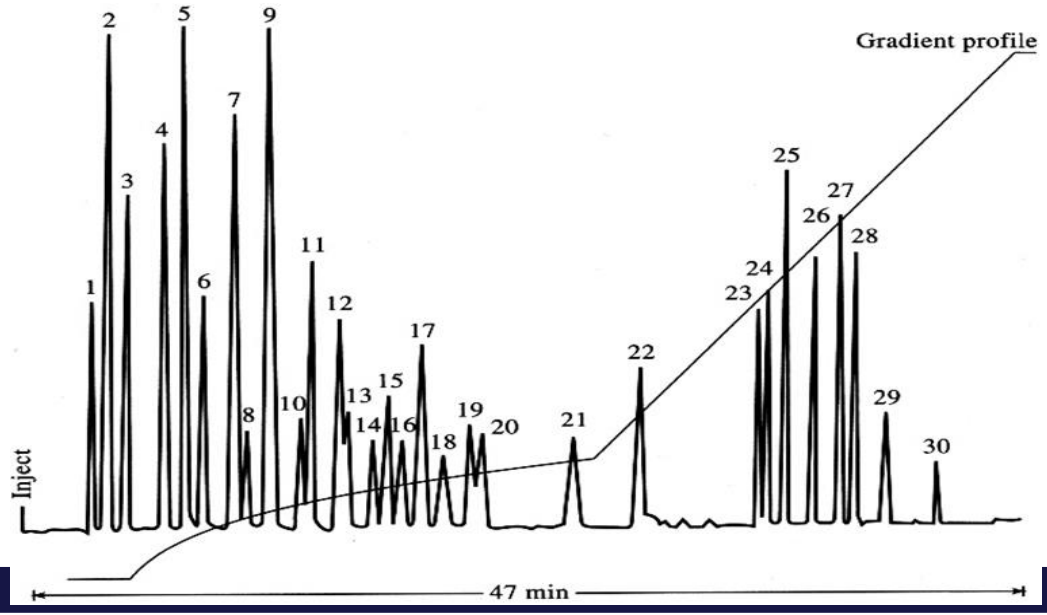
-**Isocratic** (constant mobile phase composition).
-**Gradient** (variable mobile phase composition with the analysis time).

Isocratic vs. Gradient elution …

Similar to GC (isothermal and temperature program), solutes can be eluted from a column by using either a constant column conditions or gradient elution

Isocratic elution:
use of a constant mobile phase composition to elute solutes.
-difficult to elute all solutes with good resolution in a reasonable time; general elution problem





Gradient elution:
changing the composition of the mobile phase with time; solvent programming.
-going from a weak mobile phase to a strong one
-solvent change can be stepwise, linear or non-linear

According to the eluent flow rate that the pump is capable of delivering, pumps may be defined as the following scales;

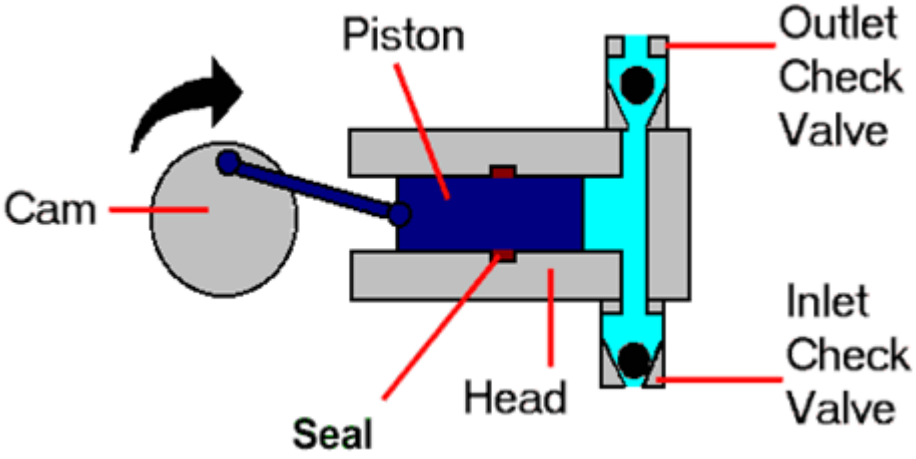
HPLC technique	Flow rate
Preparative	> 10 mL/min
Semi-preparative	5-10 mL/min
Conventional	0.5-2.0 mL/min
Narrowbore	100-500 µL/min
Microbore	10-100 µL/min
Micro LC capillary	1-10 µL/min
Nano LC capillary	10-1000 nL/min

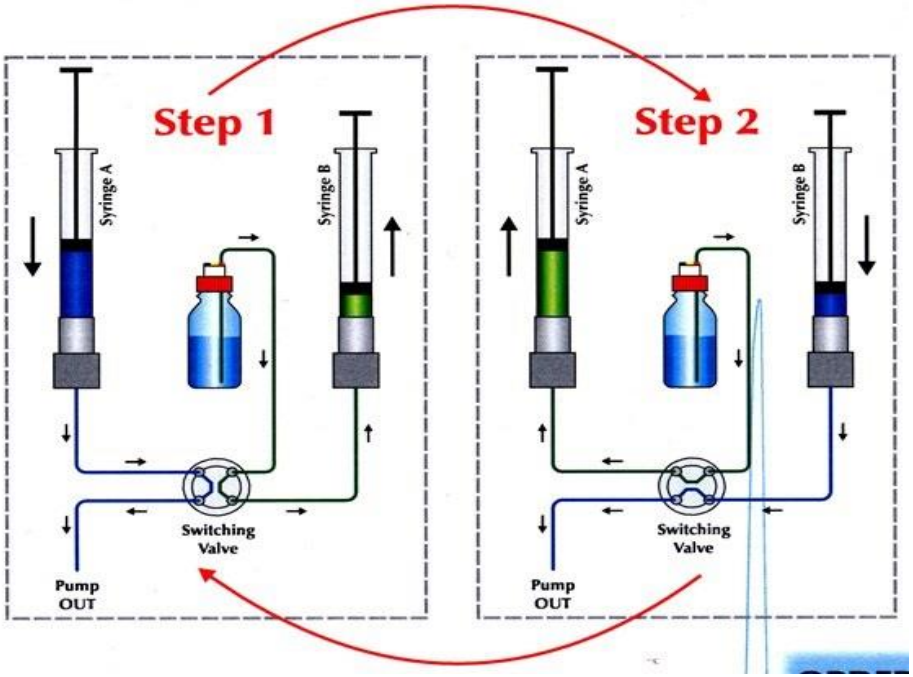
Pumps ant their components may constructed from metallic (steel or titanium) or nonmetallic (PEEK, teflon, or ceramic), depending on the material used for the eluent flow path.

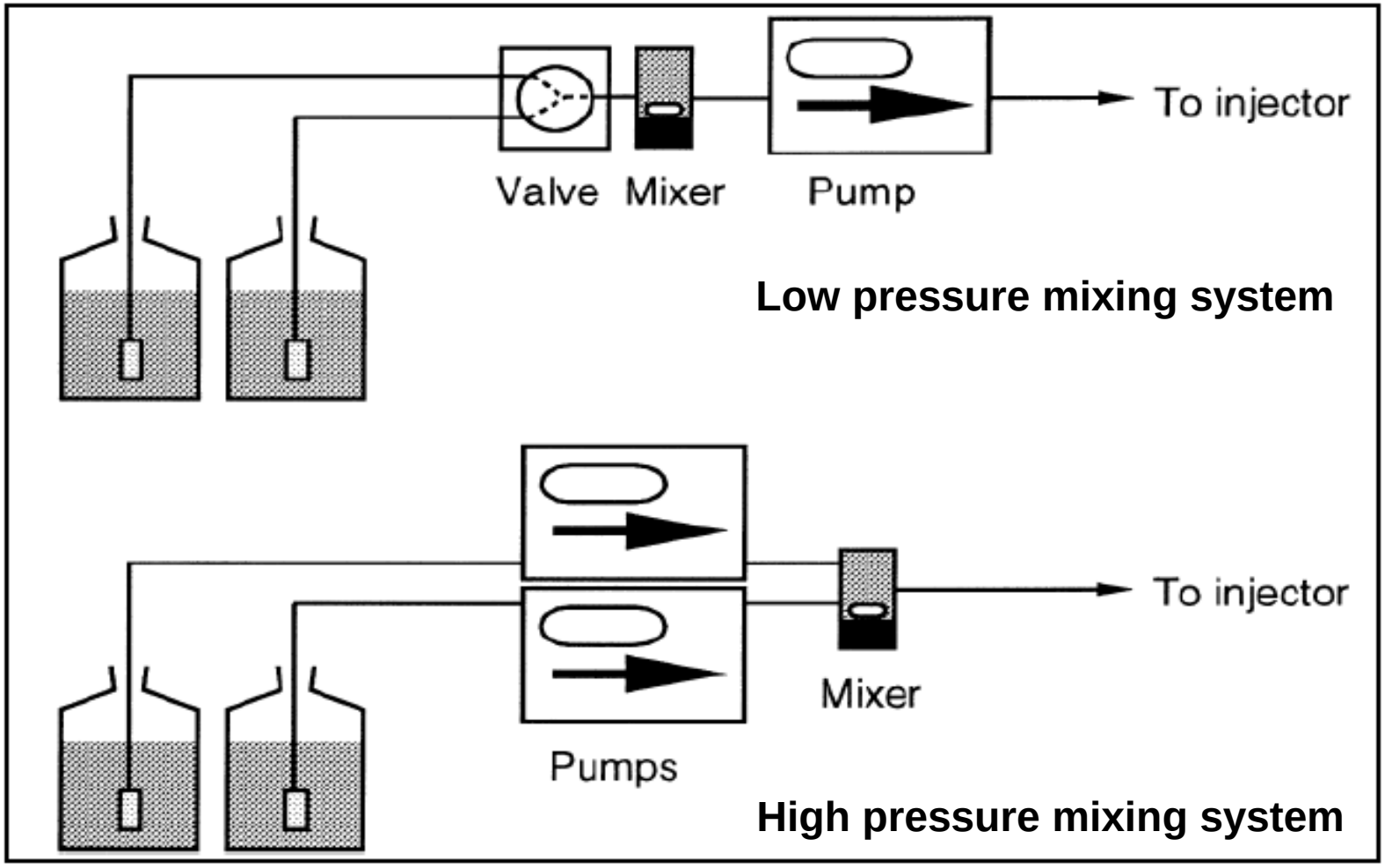
Nearly all LC pumps since the 1980's are based on one of two types according to the mechanism by which the liquid is forced through the chromatograph.

-The reciprocating piston pump is the most common design in modern HPLC, the pump head consists of two sets of moving parts: the check valves and seal piston assembly which may consist of both single or multi-head.

-The syringe pump remains popular for applications requiring pulseless solvent delivery, such as in microscale and capillary LC (where typical flow rates are less than 100 µL/min) or in microbore HPLC connected to an interface to MS.

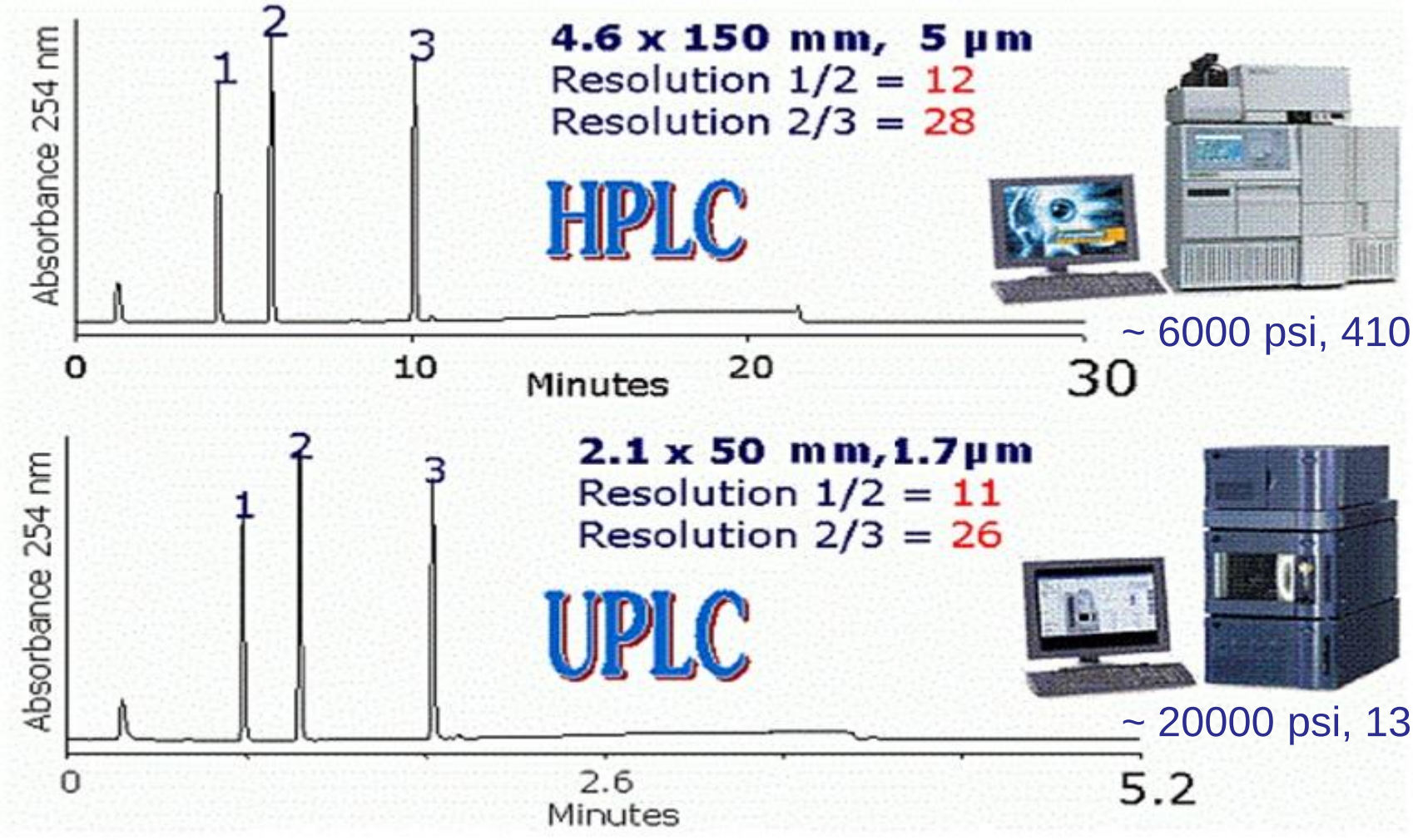






The blending of the solvents can occur in one of two basic ways. In the first, the solvent mixing occurs at high-pressure and in the second the solvents are premixed at low-pressure and then passed to the pump. The high pressure programmer is the simplest but most expensive as each solvent requires its own pump.

Modern **HPLC** systems have been improved to work at much higher pressures, and therefore be able to use much smaller particle sizes in the columns (< 2µm). These are ultra performance liquid chromatography systems or **UPLC's**.



Injector (a sample introduction system)

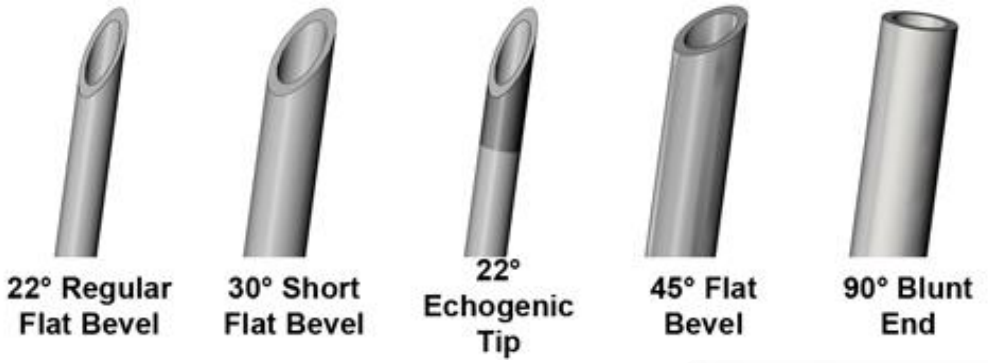
Injection valves are connected between the pump and the column and as close to the top of the column as practically possible, this allows an introduction of the analytes mixture into the stream of the mobile phase before it enters the column.

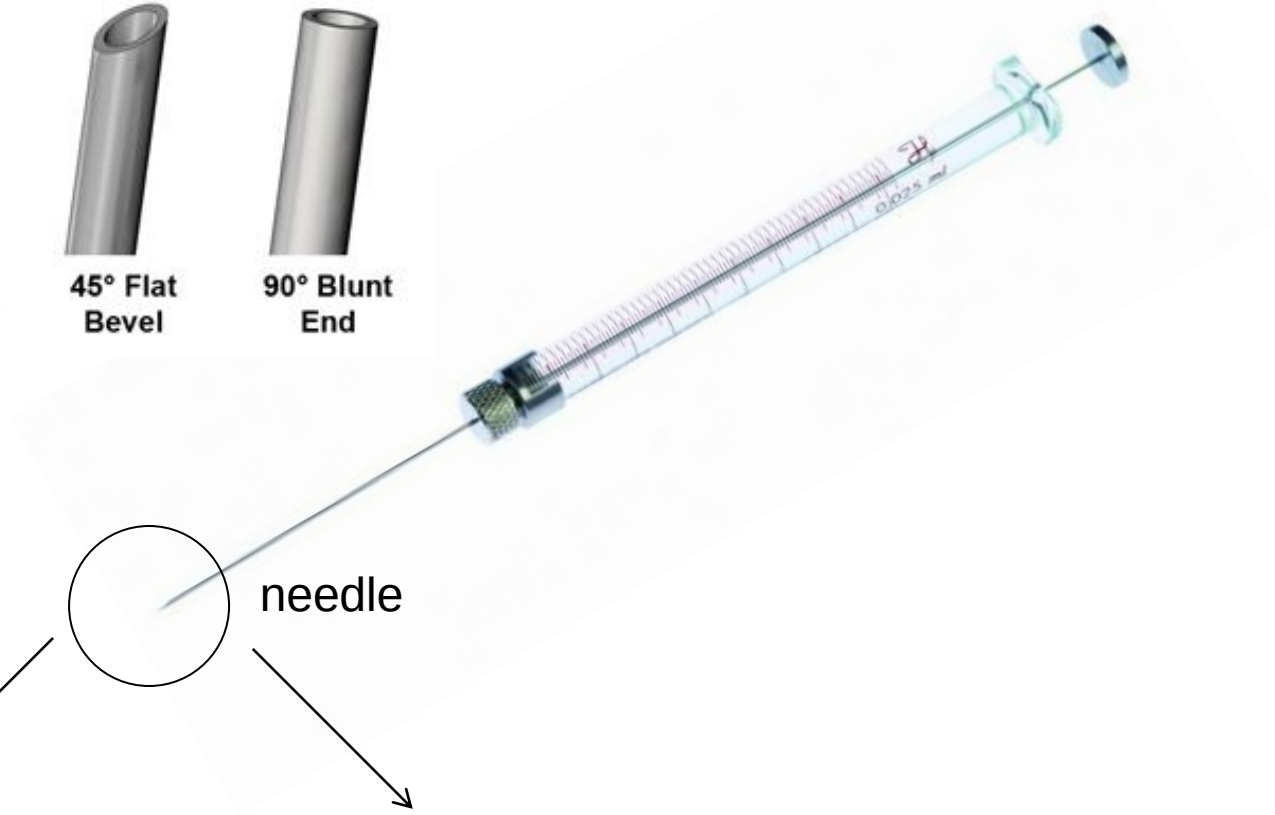
An interchangeable sample loop of discreet volume is connected to the valve and isolated from the flow of mobile phase.

The loop is filled with sample solution and the valve is then turned manually or electronically so that the loop is connected into the flowing mobile phase and the sample is thereby injected onto the column.

Most modern injectors are **autosamplers**, which allow programmed injections (sequence) of different volumes of samples that are withdrawn from the vials in the autosampler tray.

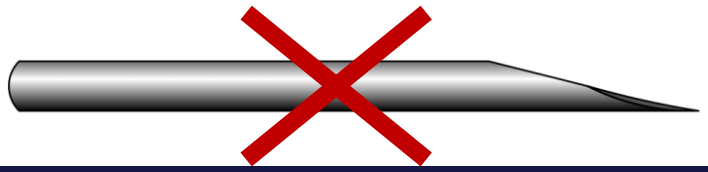
Syringe needle



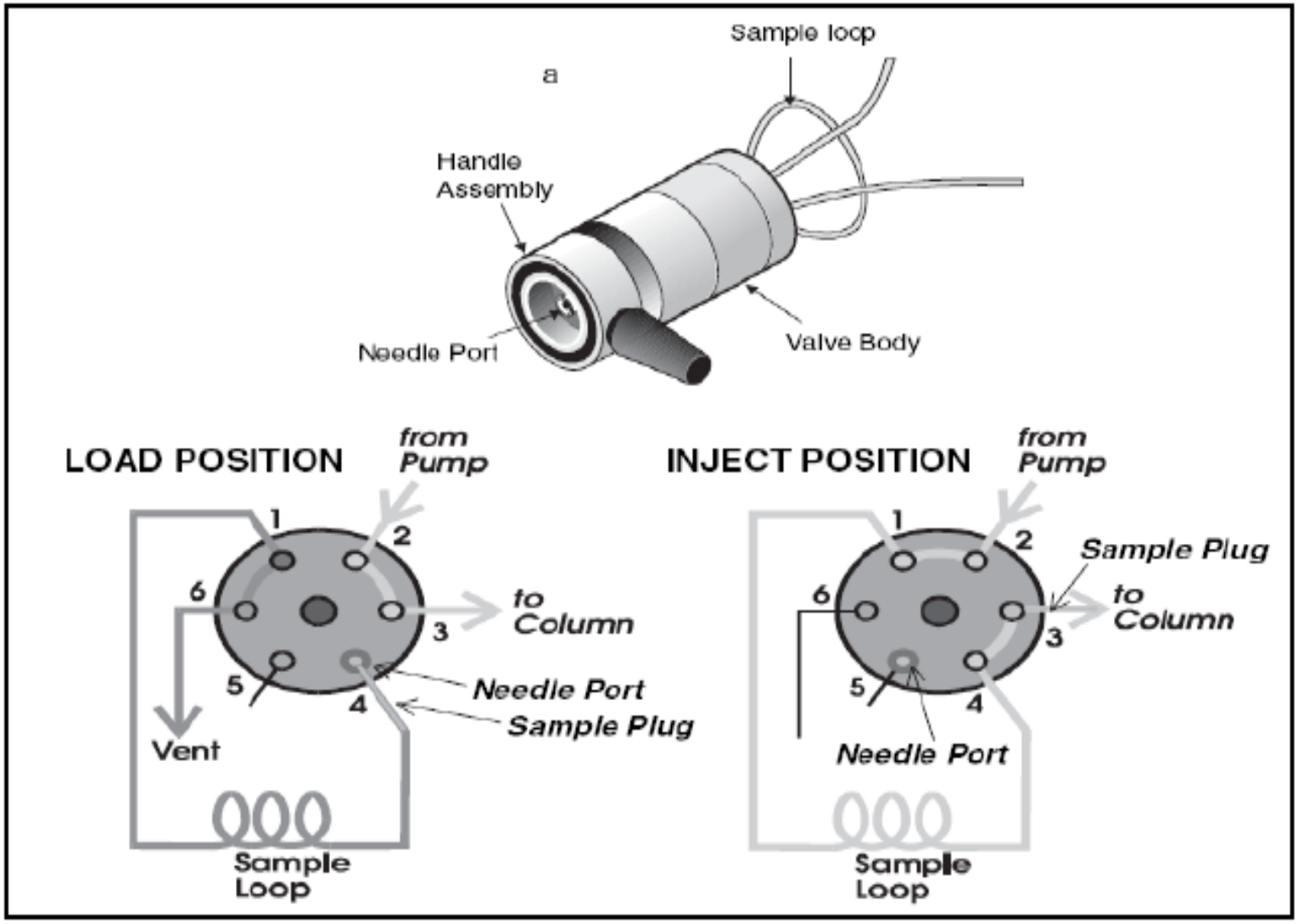


needle

Bevel



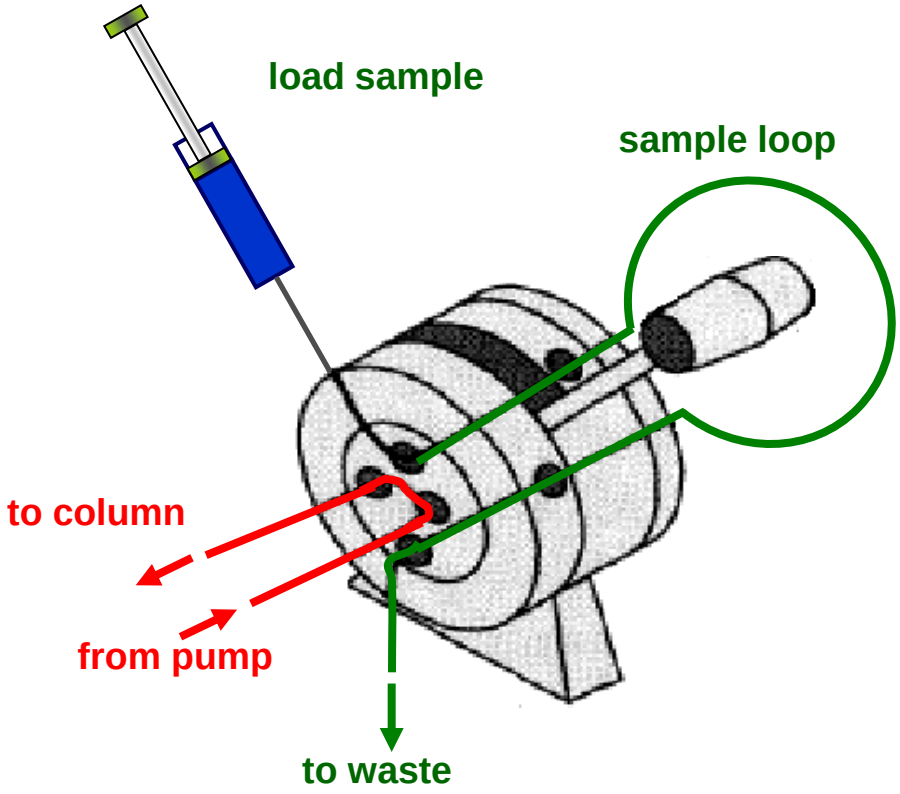
Blunt



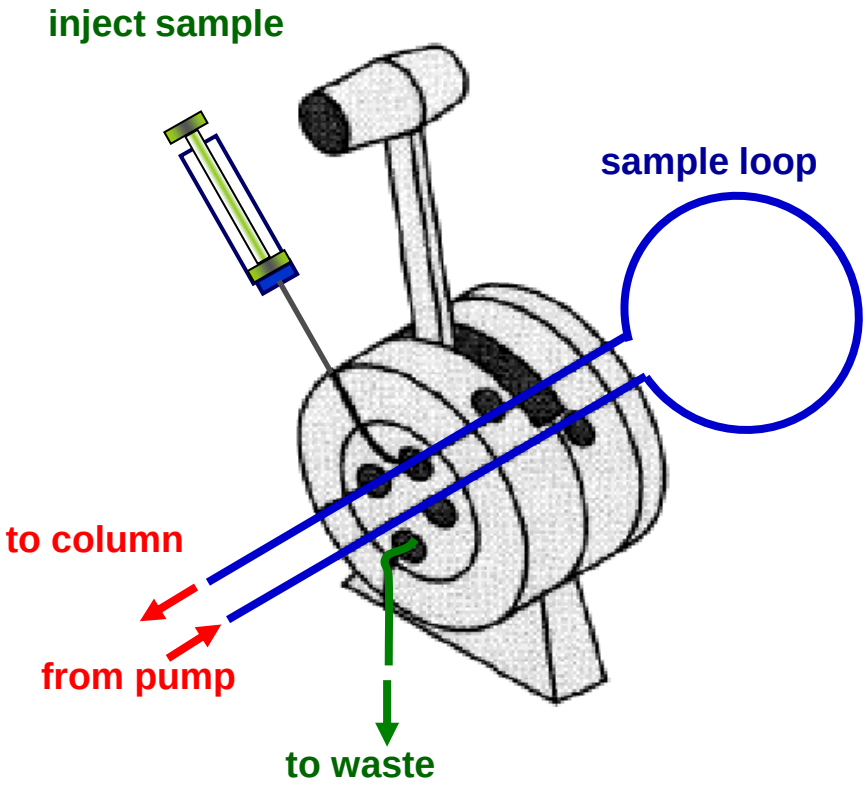
A common injector is the **Rheodyne** model, which consists of a six-port valve with a rotor, a sample loop and a needle port. A syringe with a **22-gauge (0.644 mm) blunt-tip needle** is used to introduce a precise sample aliquot into the sample loop.

6-port valve HPLC injector

« load » position

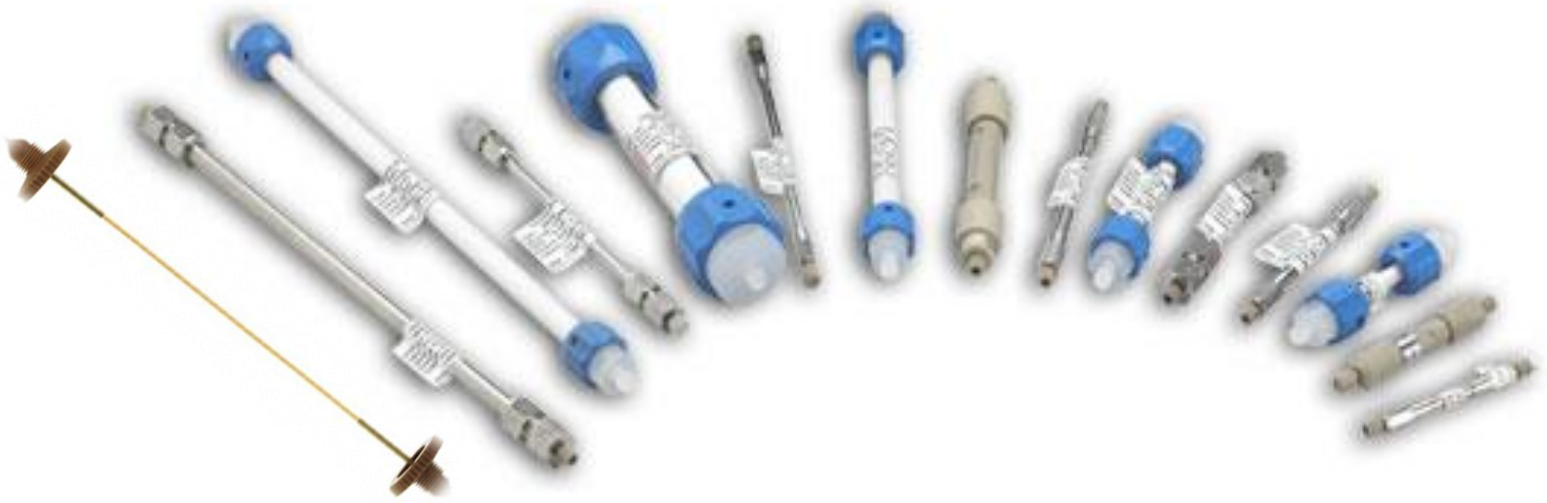


« inject » position



The column

This is the **heart of HPLC system**; it actually produces a separation of the analytes in the mixture. Most of the chromatography development in recent years went toward the design of many different ways to enhance the columns properties and efficiencies.



A modern HPLC column is stainless steel, plastic tubes, glass or fused silica capillaries filled with the stationary phase and arranged with end fittings designed to provide sealed connection with the eluent inlet and outlet lines and to retain packing material inside while allowing liquid to pass through. The length, diameter and construction material of the column affect the lifetime, efficiency and speed of separation. The size and nature of the packing material affect resolution.

Column dimensions - Length

Column length affects both the efficiency and the speed of the separation. Longer columns have higher plate numbers and yield better resolution but with longer analysis times. Note that column pressure drop is also proportional to the column length.

However, the column efficiency tends to increase with length. In general, short columns are used for simple separations. Analytical columns can range from 20 to 500 mm in length.

Column dimensions - Internal diameter

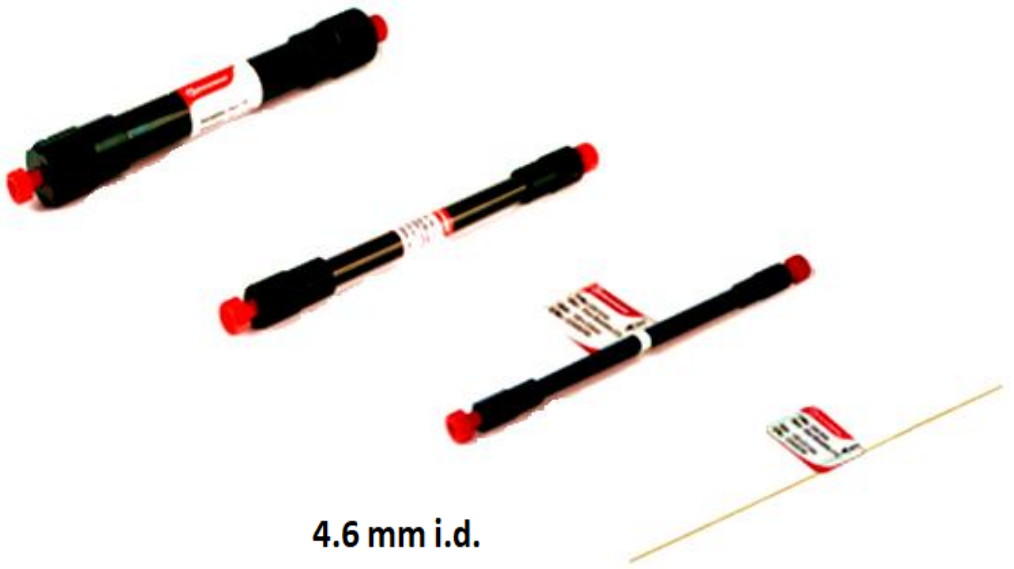
The i.d. of a column is a critical aspect that determines quantity of analyte that can be loaded onto the column, the peak dilution and the flow rate. The larger the i.d., the greater is the loading capacity and the higher is the flow rate. However, peak dilution increases with i.d., and therefore mass sensitivity decreases. In contrast, smaller columns i.d. influences sensitivity and reduce the solvents consumption at the expense of loading capacity.

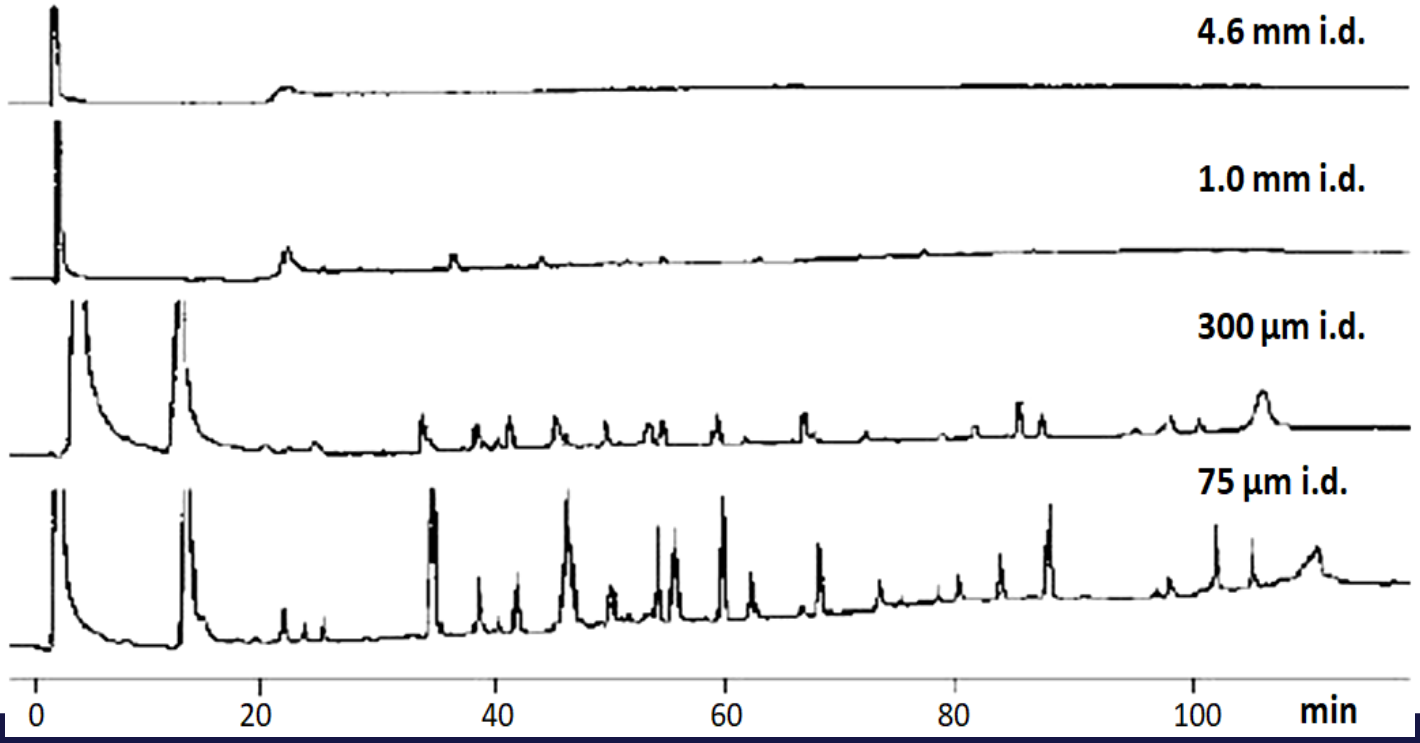
HPLC technique	Column I.D.	Flow rate	Injection volume
Preparative	> 10 mm	> 10 mL/min	---
Semi-preparative	5-10 mm	5-10 mL/min	---
Conventional	3.2-4.6 mm	0.5-2.0 mL/min	10-100 µL
Narrowbore	1.5-3.2 mm	100-500 µL/min	5-10 µL
Microbore	0.5-1.5 mm	10-100 µL/min	1-5 µL
Micro LC capillary	150-500 µm	1-10 µL/min	10-500 nL
Nano LC capillary	10-150 µm	10-1000 nL/min	3-10 nL

Miniaturization in Column Liquid Chromatography

Advantages of using down scales

-Improving detection sensitivity
-Lower chemicals consumption
-Reduce analytical costs & wastes
-Easier coupling to MS interfaces



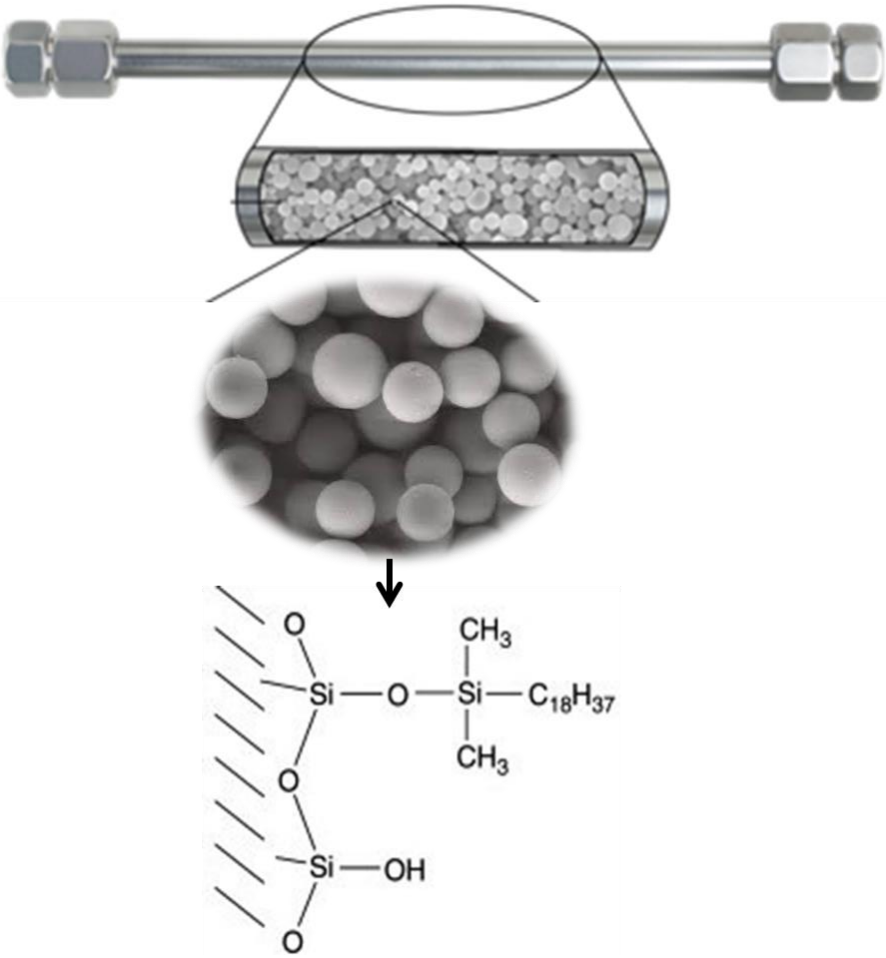


Stationary phase

Although it is usually the smallest part, the column is the most important part in any HPLC system. The column is the only device in the HPLC system which actually separates an injected mixture.

Column packing materials are the media producing the separation, and properties of this media are of primary importance for successful separations. The selectivity, capacity and efficiency of the column are all affected by the nature of the packing material or the materials of construction.

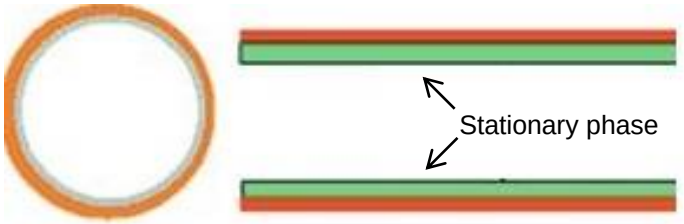
Since materials and polymer science are highly rich in options, reactions and modifications. Therefore, stationary phases faced various developments and can be still improved.



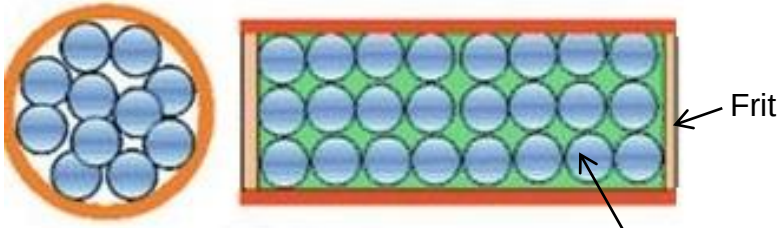
Great varieties of different columns are currently available on the market. Five distinct characteristics could be used for column classification:

(1) Type of packing materials

particulate; non-particulate (interconnected, one-piece or continuous phase); porous; nonporous; core-shell; packed; open-tubular; monolith; etc.

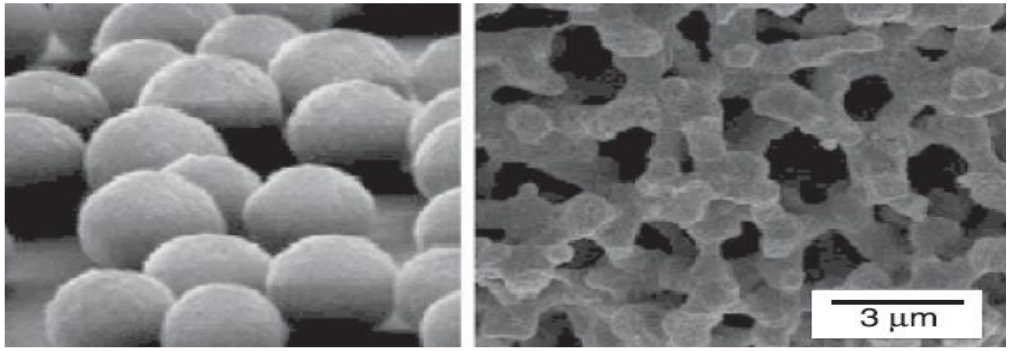


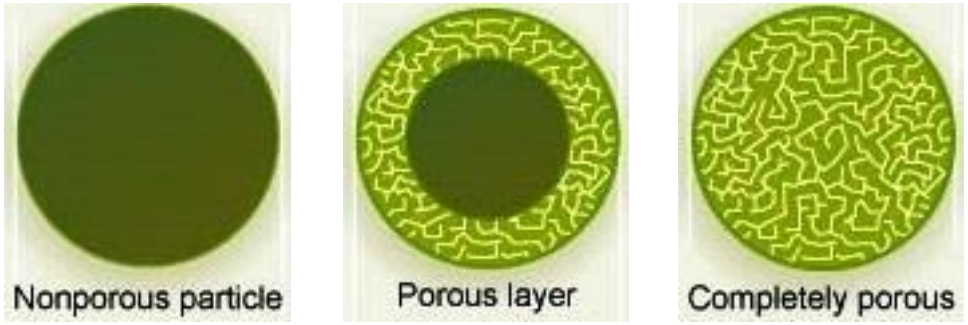
Open tubular column

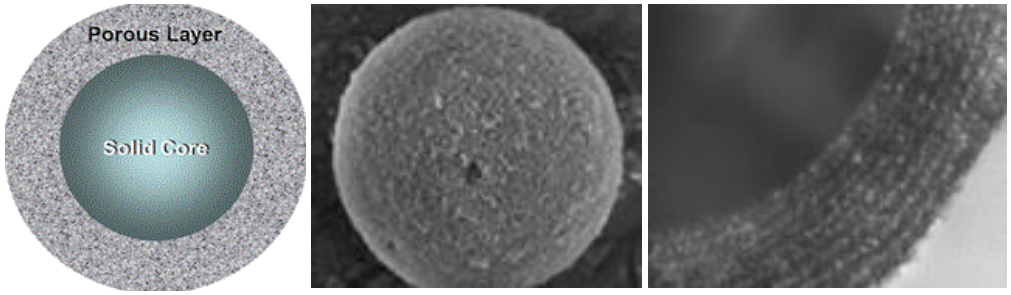


Packed column

Monolithic column







(2) Type of base material

silica SiO_2; polymeric; alumina Al_2O_3; zirconia ZrO_2; etc.

(3) Stationary phase geometry

surface area; interaction sites, pore size or diameter; pore volume; pore size distribution; permeability; particle size; particle shape; particle size distribution; porosity; etc.

(4) Surface chemistry

type of bonded ligands; functional groups; bonding density; carbon content; etc.

(5) Stability and rigidity

surface reactivity; chemical stability, physical stability; mechanical stability; stability under pressure; stability in common LC solvents; pH stability; stability to hydrolysis in acidic and basic media; stability at elevated mobile phase flow rate; column temperature; structural rigidity; re-usability; etc.

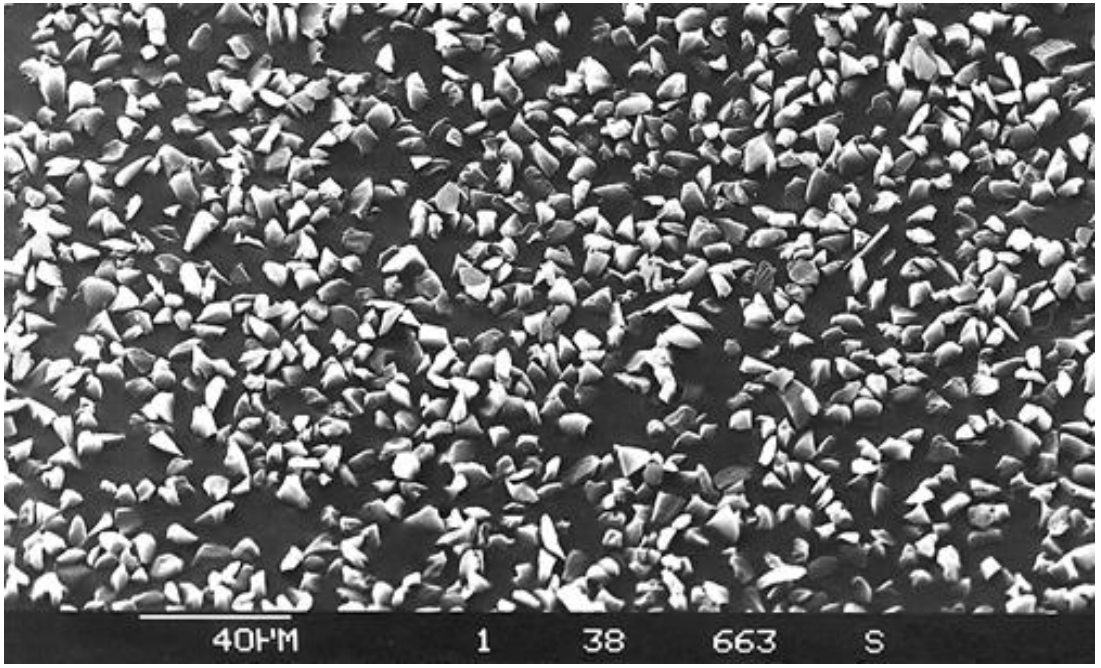
All these parameters are interrelated in their influence on the chromatographic performance of the column. The quality of an HPLC column is a subjective factor, which is dependent on the types of analytes and even on the chromatographic conditions used for the evaluation of the overall quality.

In conclusion, all these characteristics could be classified by either **physical** or **chemical** properties of the stationary phases.

Irregularly shaped silica

1st generation (~ 1970)

-Synthesis via *SIL-GEL* condensation; grinded and sieved.
-Irregular material.
-Contaminated with metal ions ($Fe^{2+/3+}$, Na^{+}, Ca^{2+}, Al^{3+}, ~ 25-75 ppm).
-In use for preparative LC (FLASH, large scale).



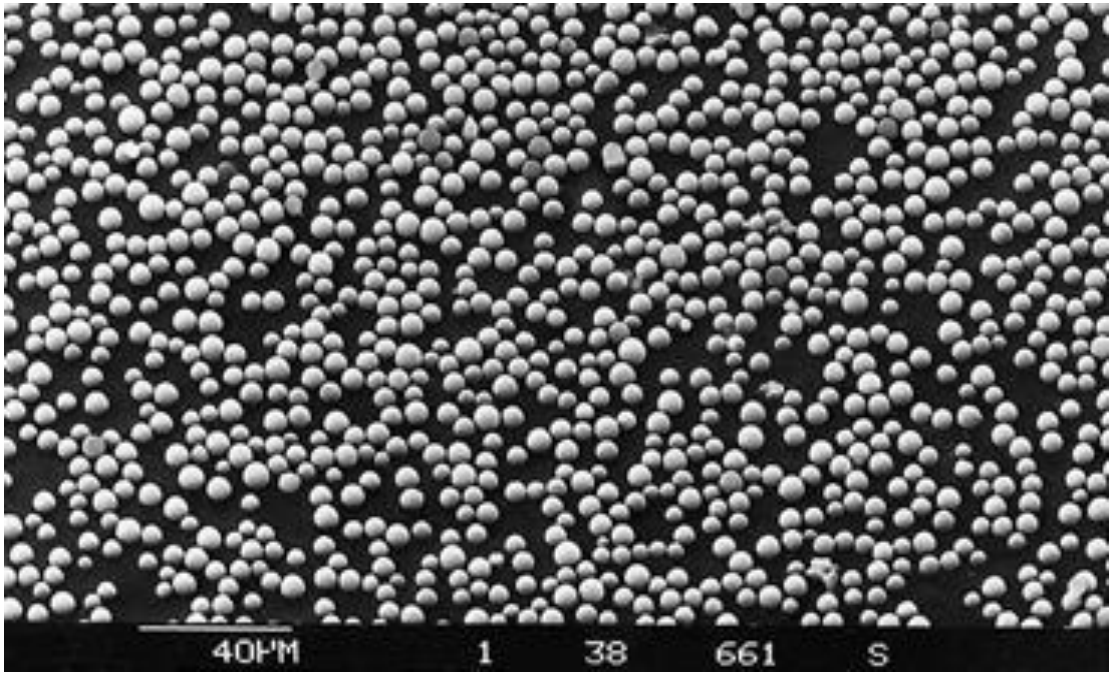
POLYGOSIL® Particle size: 7 µm, SEM micrograph

Spherical silica

2nd generation (~ 1975)

-Synthesis via *SIL-GEL* condensation.
-Spherical material.
-Contaminated with metal ions ($Fe^{2+/3+}$, Na^{+}, Ca^{2+}, Al^{3+}, ~ 25-75 ppm).
-Higher efficiency than irregular silicas in packed HPLC columns.

e.g. colloidal silica solution is sprayed into fine droplets and subsequently dried in a hot air stream.



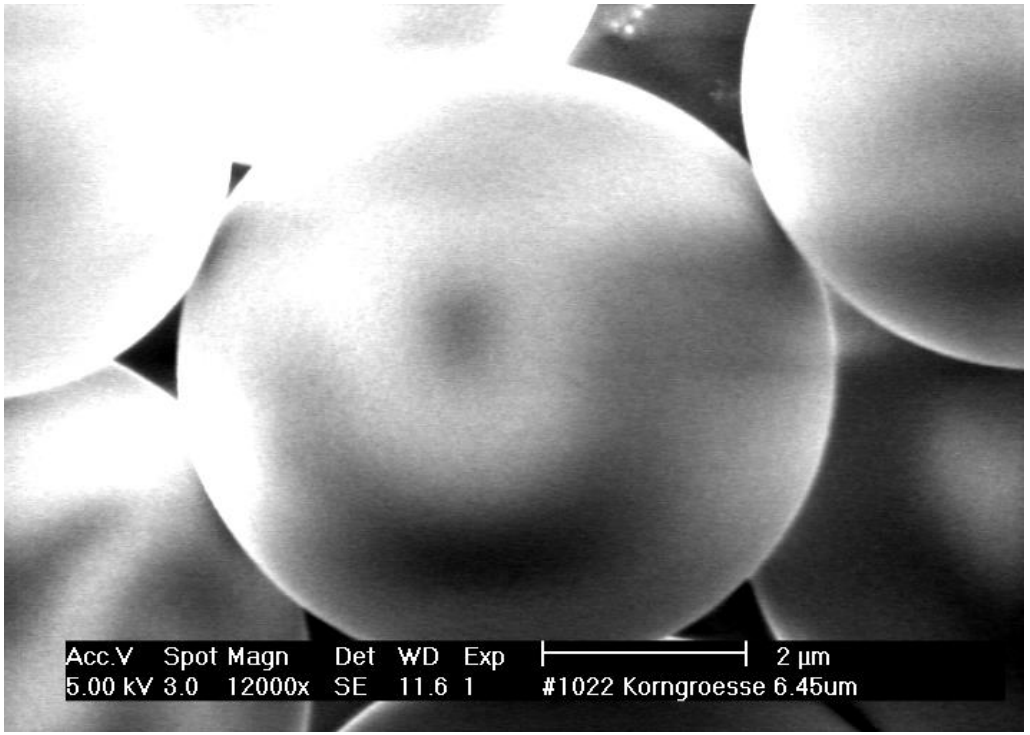
NUCLEOSIL® particle size: 7 µm, SEM micrograph

Spherical silica

3rd generation (~ 1985)

-Synthesis via *Sol-Gel* condensation of alkoxysilanes.
-Spherical material, very homogeneous surface, high mechanical stability.
-Very low concentration of metal ions, ultra-pure (< 10 ppm).

$$Si(OC_2H_5) + 4\,H_2O \rightarrow Si(OH)_4 + 4\,C_2H_5OH \rightarrow n\,Si(OH)_4$$

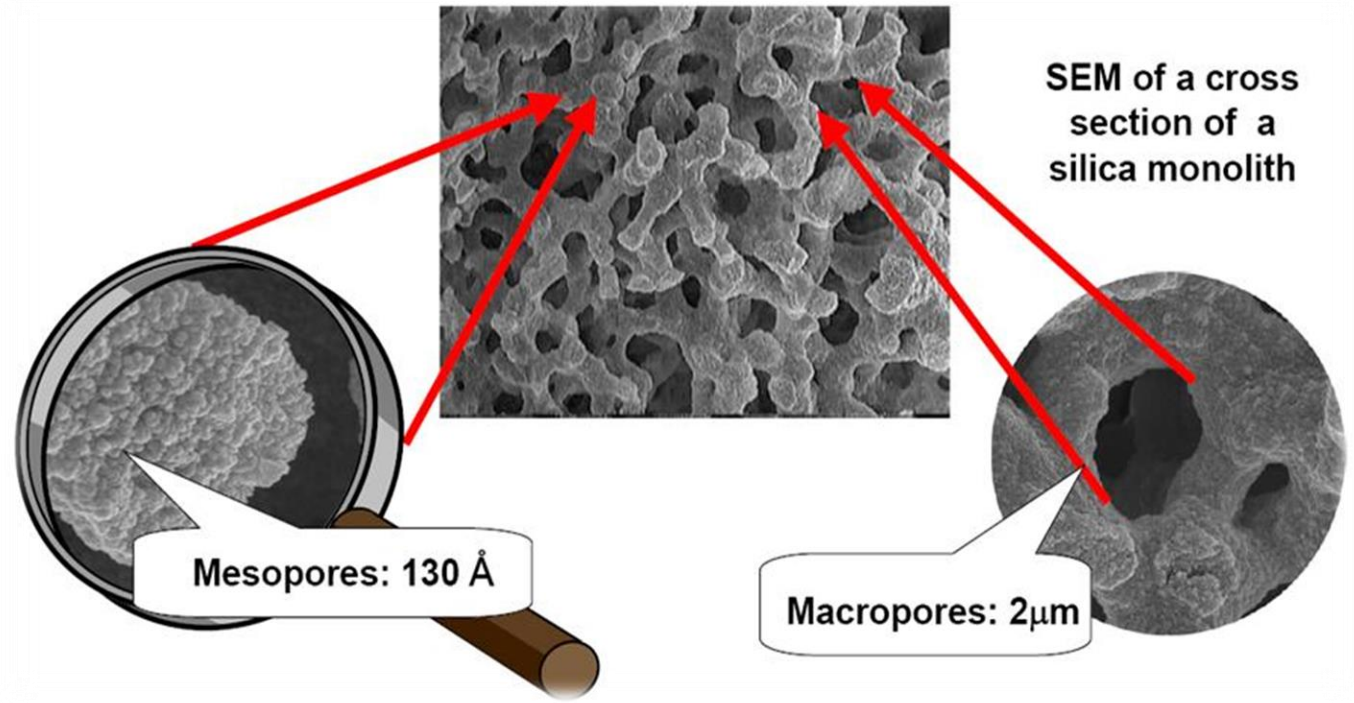


NUCLEODUR® particle size: 5 µm, SEM micrograph

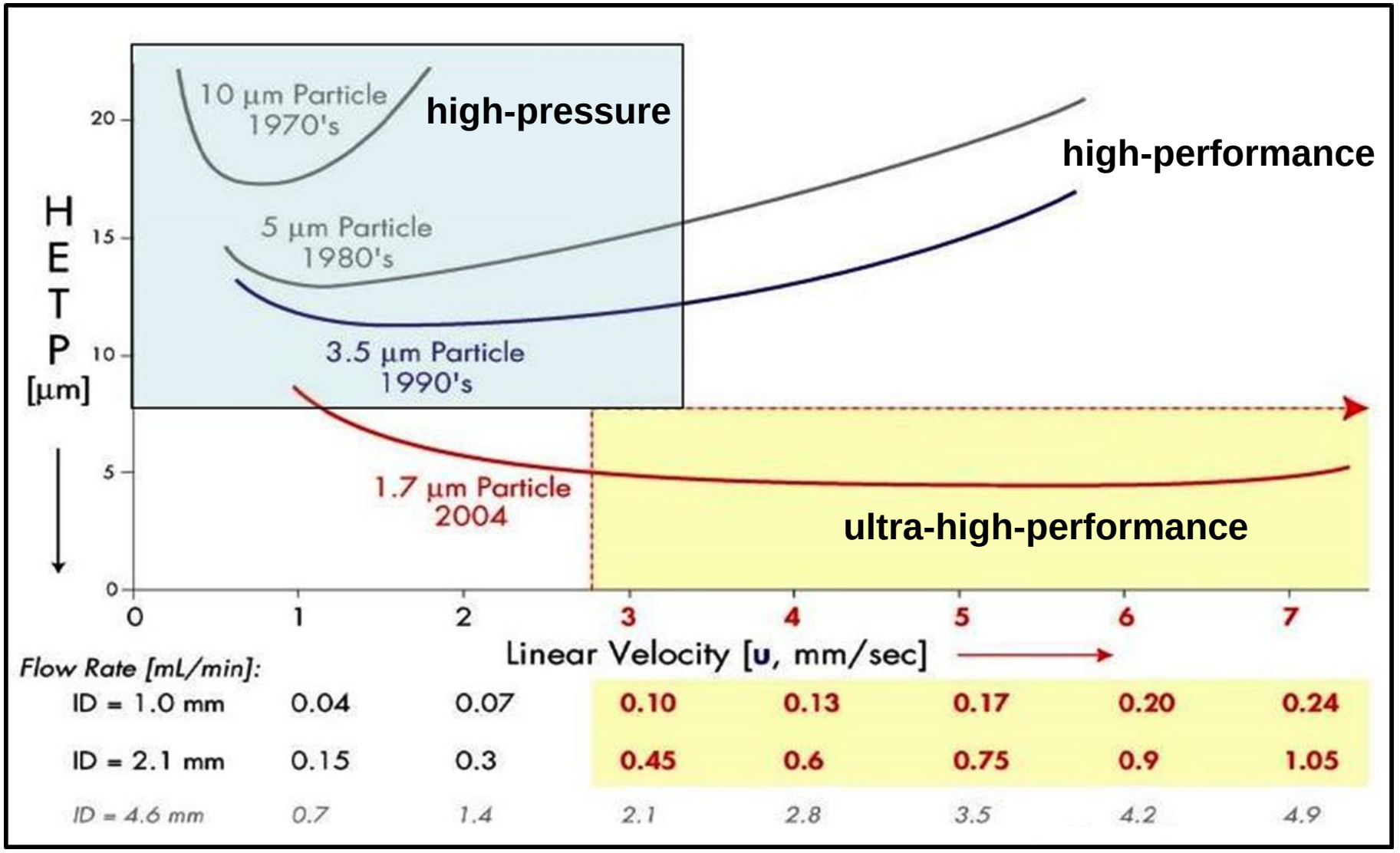
Monolith material

4th generation (~ 1996)

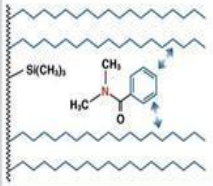
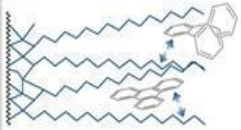
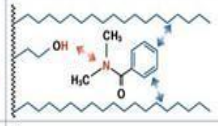
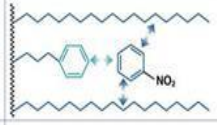
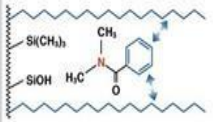
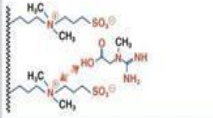
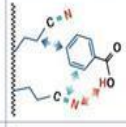
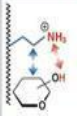
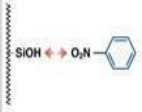
Monoliths are a single block piece of continuous materials made of highly porous rods with two types of bimodal pore structure distribution (macropores and mesopores).



Evolution of particle technology



van Deemter plot, illustrating the evolution of particle sizes.

Phase	Specification	Characteristics*	Stability	Structure	Application	Similar phases**	Separation principle · Retention mechanism	
C_{18} / C_8 Gravity	octadecyl phase, high density coating multi-endcapping C_{18} Gravity: 18% C · USP **L1** C_8 Gravity: 11% C · USP **L7**	A C_{18} ⬡⬡⬡⬡⬡ A C_8 ⬡⬡◖ B C_{18} ◖ B C_8 ⬢ C C_{18} ⬡⬡◖ C C_8 ⬡◖	pH stability 1 – 11, suitable for LC/MS	NUCLEODUR® $(Si-O_2)_n$	in general compounds with ionizable functional groups such as basic pharmaceuticals and pesticides for C8 Gravity generally shorter retention times for nonpolar compounds	**NUCLEOSIL® C_{18} HD** Waters Xterra® RP_{18} / MS C_{18}; Phenomenex Luna C18 (2), Synergi™ and Max RP; Zorbax Extend C18; Inertsil ODS III; Purospher RP-18; Star RP-18 **NUCLEOSIL® C_8 HD;** Waters Xterra® RP_8 / MS C_8; Phenomenex Luna C8; Zorbax Eclipse; XDB-C8	only hydrophobic interactions (van der Waals interactions)	
C_{18} Isis	octadecyl phase with specially crosslinked surface modification endcapping 20% C · USP **L1**	A ⬡⬡⬡⬡◖ B ⬢⬢ C ⬡⬡⬡⬡⬡	pH stability 1 – 10, suitable for LC/MS	NUCLEODUR® $(Si-O_2)_n$	high steric selectivity, thus suited for separation of positional and structural isomers, planar/nonplanar molecules	**NUCLEOSIL® C_{18} AB** Inertsil ODS-P; YMC Pro C18RS	steric interactions and hydrophobic interactions	
C_{18} Pyramid	C_{18} modification with polar endcapping 14% C · USP **L1**	A ⬡⬡⬡⬡ B ⬢⬢◖ C ⬡⬡	stable against 100% aqueous eluents, pH stability 1 – 9, suitable for LC/MS	NUCLEODUR® $(Si-O_2)_n$ OH OH	basic pharmaceutical ingredients, very polar compounds, organic acids	Phenomenex Aqua; YMC AQ; Waters Atlantis® dC18	hydrophobic interactions and polar interactions (H bonds)	
Sphinx RP	bifunctional RP phase, propylphenyl and C_{18} ligands; endcapping 15% C; USP **L1** and **L11**	A ⬡⬡⬡ B ⬢⬢◖ C ⬡	pH stability 1 – 10, suitable for LC/MS	NUCLEODUR® $(Si-O_2)_n$	compounds with aromatic and multiple bond systems	no similar phases	π-π interactions and hydrophobic interactions	
C_{18} ec C_8 ec	octadecyl / octyl phase, medium density coating endcapping C_{18} ec: 17.5% C · USP **L1** C_8 ec: 10.5% C · USP **L7**	A C_{18} ⬡⬡⬡⬡ A C_8 ⬡⬡ B C_{18} ⬢ B C_8 ⬢◖ C C_{18} ⬡⬡⬡◖ C C_8 ⬡⬡◖	pH stability 1 – 9	NUCLEODUR® $(Si-O_2)_n$ Si-OH Si-O-Si(CH₃)₃	robust C18 / C8 phase for routine analyses	**NUCLEOSIL® C_{18}** Spherisorb® ODS II; Hypersil ODS; Waters Symmetry® C18; Inertsil ODS II; Kromasil C18; LiChrospher RP 18 **NUCLEOSIL® C_8 ec / C_8** Spherisorb® C8; Hypersil MOS; Waters Symmetry® C8; Kromasil C8; LiChrospher RP 8	only hydrophobic interactions (van der Waals interactions) some residual silanol interactions	
HILIC	zwitterionic ammonium sulfonic acid modification 7% C	A ⬡ B ⬢⬢⬢⬢⬢ C -	pH stability 2 – 8.5, suitable for LC/MS	NUCLEODUR® $(Si-O_2)_n$	hydrophilic compounds such as organic polar acids and bases, polar natural compounds	Merck Sequant ZIC®-HILIC; Sielc Obelisc™	ionic / hydrophilic interactions, electrostatic interactions	
CN / CN-RP	cyano (nitrile) phase for NP and RP separations 7% C · USP **L10**	A ⬡ B ⬢⬢⬢⬢ C -	pH stability 1 – 8, stable towards highly aqueous mobile phases	NUCLEODUR® $(Si-O_2)_n$ C≡N Si-OH C≡N Si-O-Si(CH₃)₃	polar organic compounds (basic drugs), molecules containing π electron systems	**NUCLEOSIL® CN / CN-RP**	π-π interactions, polar interactions (H bonds), hydrophobic interactions	
NH_2 / NH_2-RP	amino phase for NP and RP separations 2.5% C · USP **L8**	A ⬡ B ⬢⬢⬢ C -	pH stability 1 – 8, stable towards highly aqueous mobile phases	NUCLEODUR® $(Si-O_2)_n$ NH_2 Si-OH NH_2 Si-OH	sugars, sugar alcohols and other hydroxy compounds, DNA bases, polar compounds in general	**NUCLEOSIL® NH_2 / NH_2-RP**	polar /ionic interactions, hydrophobic interactions	
SiOH	unmodified high purity silica USP **L3**	A - B n.a. C -	pH stability 2 – 8	NUCLEODUR® $(Si-O_2)_n$ Si-OH Si-OH	polar compounds in general	**unmodified NUCLEOSIL®**	polar /ionic interactions	

* A = ⬡ hydrophobic selectivity, B = ⬢ polar/ionic selectivity, C = ◖ steric selectivity

** phases which provide a similar selectivity based on chemical and physical properties

Common packings and bonded phases in chromatography

Stationary phase	Typical modes and applications
Silica (unmodified)	Polar compounds in general (NP)
Alumina (unmodified)	Similar to silica; can be adjusted for acidic, basic, or neutral analytes (NP)
Polymeric	Used at very high or very low pH, where the silica-based phases might degrade (RP)
Amino: Si-(R)-NH_2	Can be used as a weak ion-exchange phase; ionizable compounds; phenols, petroleum fractionation, sugar, saccharides, drugs, aromatics (NP or RP; depending on R)
Cyano: Si-(R)-CN	Low hydrophobicity, alternative to silica, broad spectrum of mixtures with different polarities; polar organics, peptides, protiens, drugs, metabolites and pesticides (NP or RP; depending on R)
Diol: Si-(R)-CHOH-CH_2-OH	Less acidic than silica, complex mixtures, antibiotics, proteins, peptides (NP or RP; depending on R)
Phenyl: Si-(R)-C_6H_5	Aromatic and moderately polar compounds (RP)
C_{18} (or RP-18): -Si-$(CH_2)_{17}$-CH_3	General purpose; hydrocarbons, drugs, metabolites, pesticides, peptides, organics specially acids (RP)
C_8 (or RP-8): -Si-$(CH_2)_7$-CH_3	Similar to C_{18}; generally less hydrophobic (RP)
C_2 (or RP-2): -Si-CH_2-CH_3	Less retention than C_8 or C_{18}; applications are similar, also used for purification and preparative (RP)

A longer carbon chain means a less polar stationary phase and a higher retention of non-polar solutes

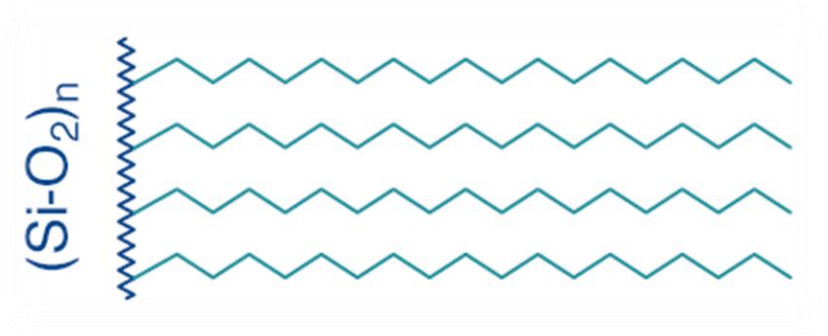
Commercial columns certificate

Example..

NUCLEODUR® C18 Gravity
Macherey-Nagel MN

Description:
-Octadecyl phase; C18
-High density coating, multi-endcapping, 18% C
-Similar phases: NUCLEOSIL® C18 HD, Waters Xterra® RP18/MS C18, Phenomenex Luna® C18 (2), Synergi™, Max RP; Zorbax® Extend C18, Inertsil® ODS III, Purospher® RP-18, Star RP-18

Specifications …	
Catalog number	760102.40
Stationary Phase	Octadecyl phase; C18
Length	250 mm
Internal Diameter	4.0 mm
Particle size	5 µm
pH stability	1-11
Pore size	100 Å
USP type	L1



Recommended applications:
General compounds with ionizable functional groups such as basic pharmaceuticals and pesticides

column specifications (one statement)

C18, 250 x 4 mm, 5 µm, 100 Å

- C18 — bonded phase
- 250 x 4 mm — column dimension (length & i.d.)
- 5 µm — particle size
- 100 Å — pore size

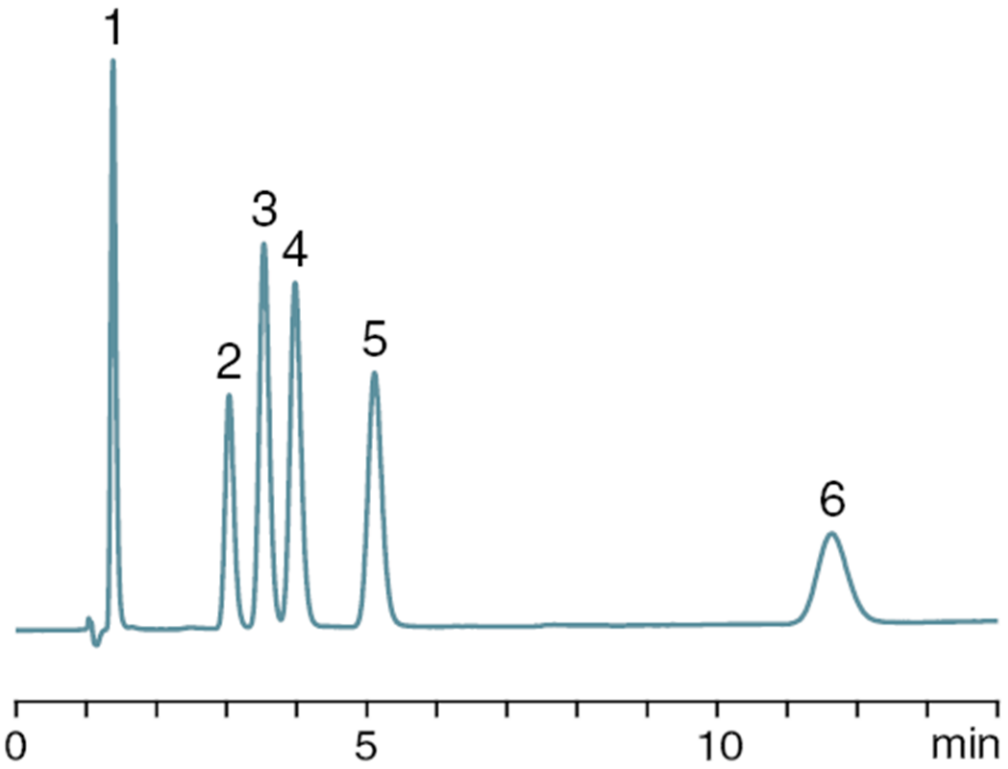
MN application note no. 119860

HPLC Analysis of Sulfonamides Drugs

Separation Conditions

Column	125 x 4 mm NUCLEODUR® Sphinx RP, 5 µm
Eluent	MeOH – 0.1% TFA (20:80, v/v), isocratic mode
Flow rate	1 mL/min
Temperature	22 °C
Detection	UV, 230 nm
Injection volume	3 µL

Peak no.	Sulfonamides	t_R (min)
1	Sulfanilamide	1.42
2	Sulfadiazine	3.16
3	Sulfathiazole	3.74
4	Sulfamerazine	4.18
5	Sulfadimidine	5.24
6	Succinylsulfathiazole	11.63



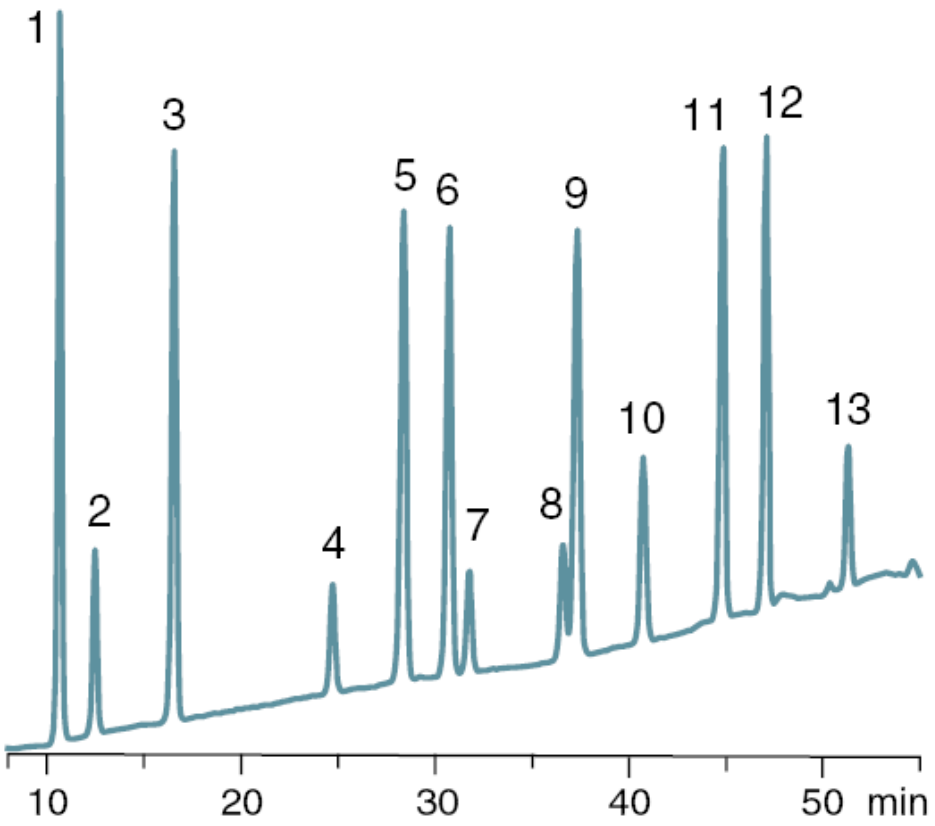
MN application note no. 116170

HPLC Analysis of some Pesticides

Separation Conditions

Column	250 x 4 mm NUCLEOSIL® 100-5 C18 Nautilus
Eluent	A) acetonitrile, B) water
Gradient	90–66% B in 33 min, 66–40% B in 22 min, 10 min at 40% B, in 3 min to 20% B
Flow rate	1 mL/min
Temperature	30 °C
Detection	UV, 218 nm
Injection volume	5 µL

#	Pesticide	#	Pesticide
1	Desisopropylatrazine	8	Metalaxyl
2	2,6-Dichlorobenzamide	9	Atrazine
3	Desethylatrazine	10	Metazachlor
4	Hexazinone	11	Propazine
5	Simazine	12	Terbuthylazine
6	Desethylterbuthylazine	13	Metolachlor
7	Bromacil		

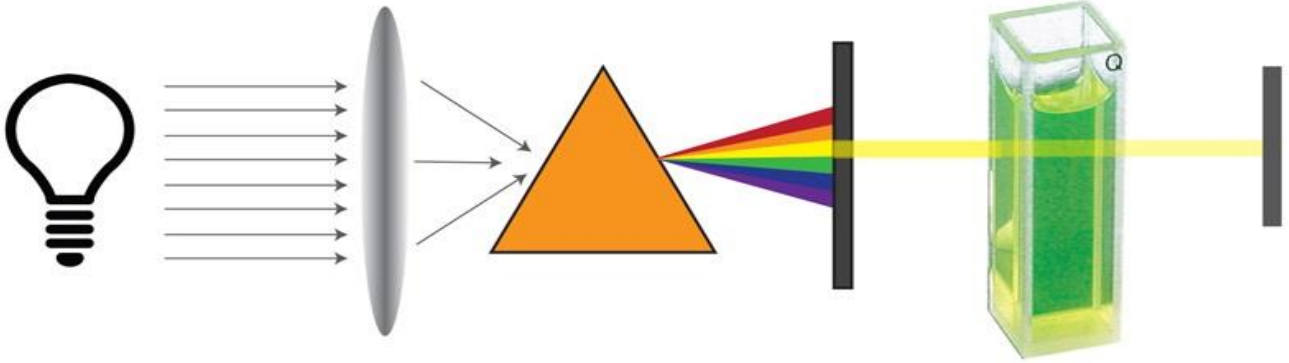


Detector

Although chromatography was discovered late in the 1890's, its development was almost negligible until the 1940's and this was largely due to the lack of an in-line sensitive detector.

The detector measures a **physical parameter** of the column effluent or of components in the column effluent and transform it to an **electrical signal**.

The detector must respond sufficiently fast to the changes in concentration or mass flow in the effluent, otherwise the peaks are distorted.



Detectors can be classified into two types;

-**Universal** (bulk property) detectors
measures some bulk physical property of the **eluent** (such as light scattering or refractive index).

-**Selective** (solute property) detectors
measures some physical or chemical property that is unique to the **solute** (such as UV/Vis or fluorescence).

Detectors may also be classified according to whether they are **destructive** or **nondestructive**. A nondestructive detector is one in which the sample is unaltered by the detection process. Nondestructive detectors are often used in series to obtain extra qualitative information.

Detector may be also be classified for solvent or solute property, and mass or concentration detection.

The choice of detector is often crucial to the success of a particular HPLC method.

The choice of detector is based on intrinsic **properties of the solute**.

Often more than one detector can be used to maximize sample information and confirm peak identities.

For example, an absorbance detector could be placed in series with a conductivity detector for the visualization of a charged, chromophoric solute.

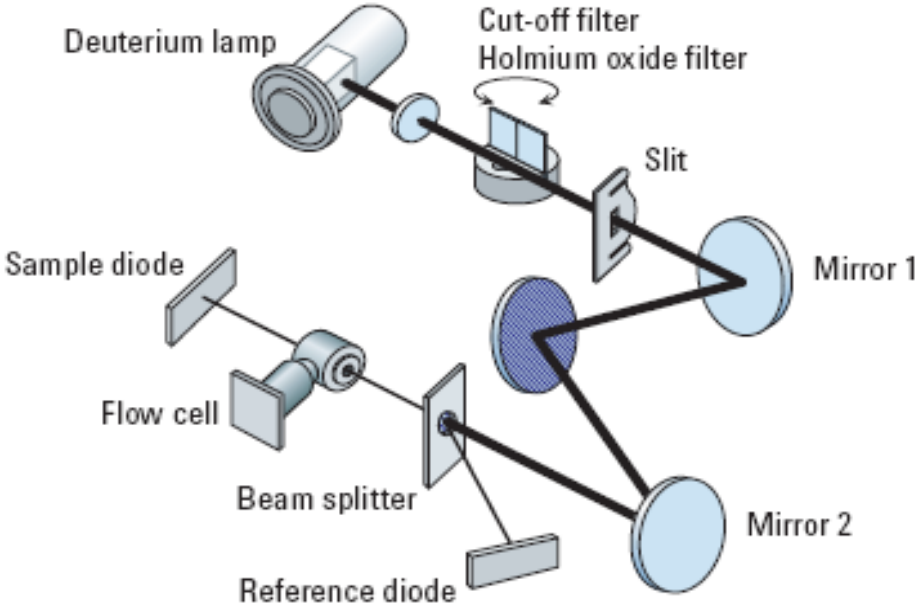
HPLC most common detectors:

Detector in the same instrument:
-Ultraviolet/Visible (UV/Vis) absorption detector
-Reflective index (RI) detector
-Fluorescence detector
-Electrochemical detector
-Electrical conductivity detector
-Light scattering detector

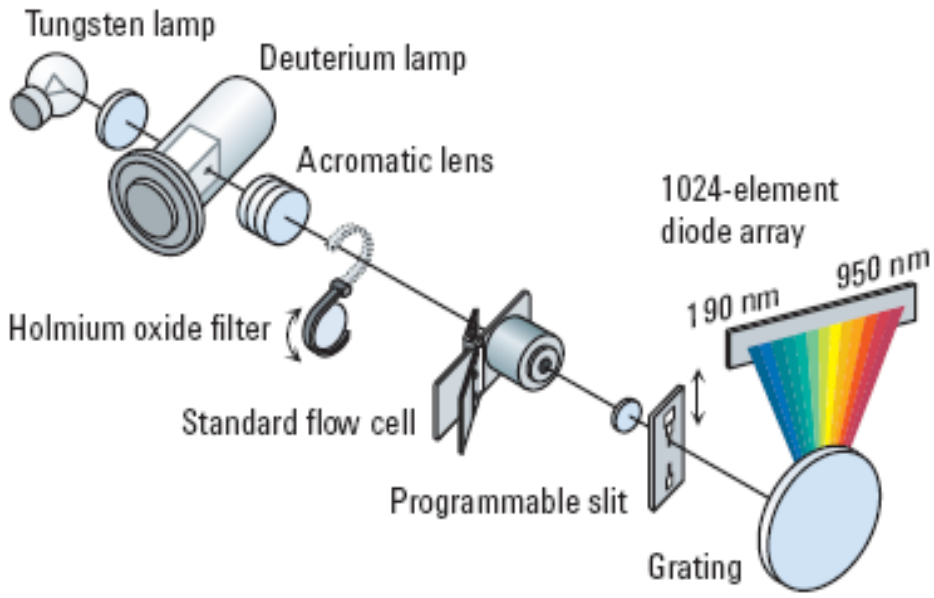
Hyphenated HPLC methods:
-Mass spectrometer detector
-FT-IR detector
-NMR detector

Absorbance ultraviolet/visible (UV/Vis) detector

-An ultraviolet light beam is directed through a flow cell and a sensor measures the light passing through the cell.
-If a compound elutes from the column that absorbs this light energy, it will change the amount of light energy falling on the sensor.
-The resulting change in this electrical signal is amplified and directed to a recorder or data system.
-A UV spectrum is sometimes also obtained which may aid in the identification of a compound or series of compounds.



Single wavelength detector

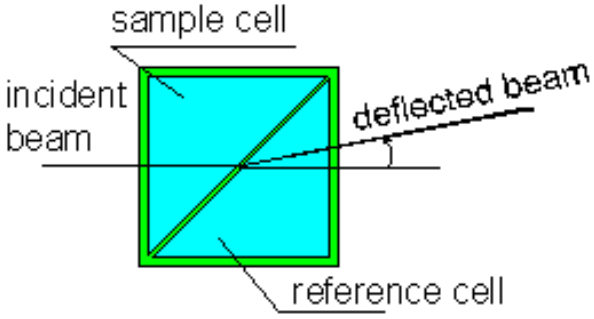


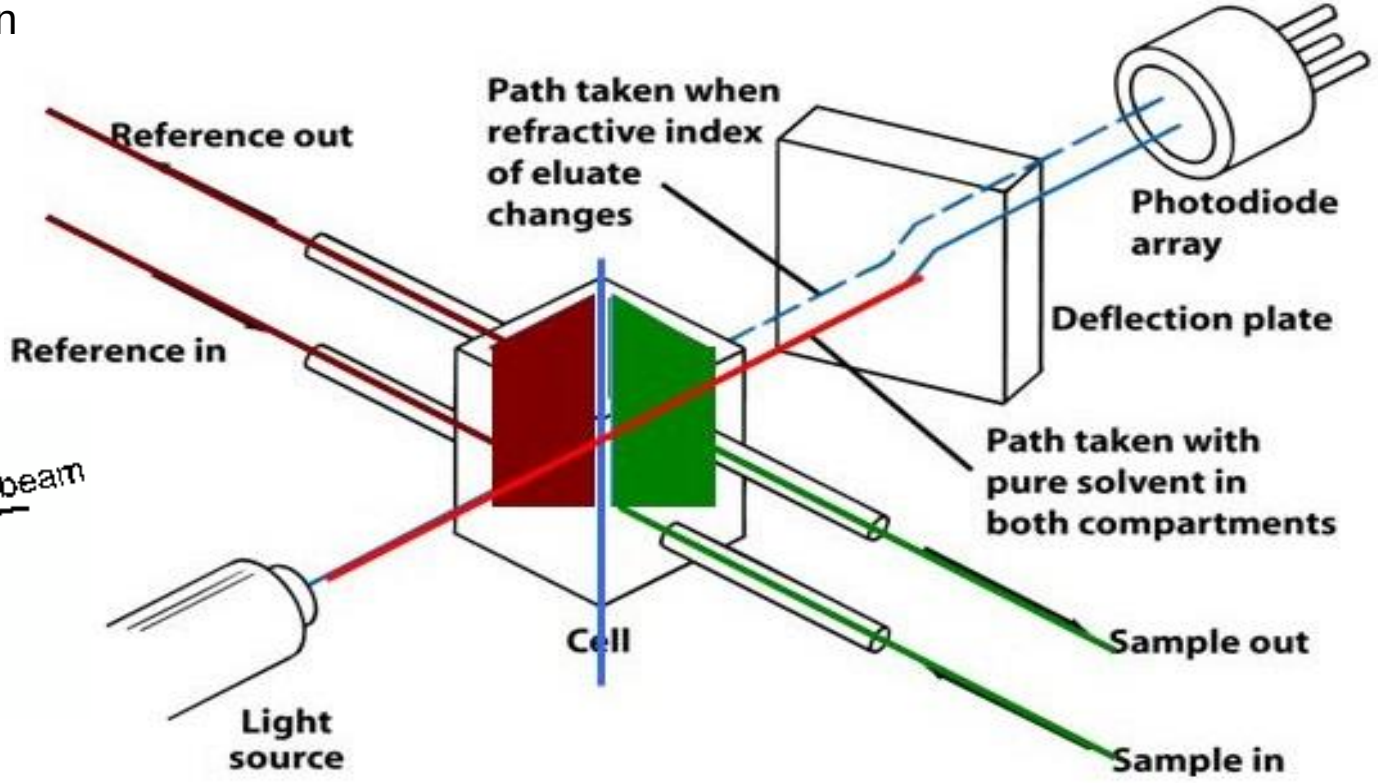
Diode array detector

Refractive index detector (RID)

-The ability of a compound or solvent to deflect or refract light provides a way to detect it.
-The RI is a measure of molecule's ability to deflect or refract light in a flowing mobile phase in a flow cell relative to a static mobile phase contained in a reference flow cell.
-The amount of deflection or bending of the light is proportional to concentration.
-The RI detector is considered to be a universal detector but it is not very sensitive.

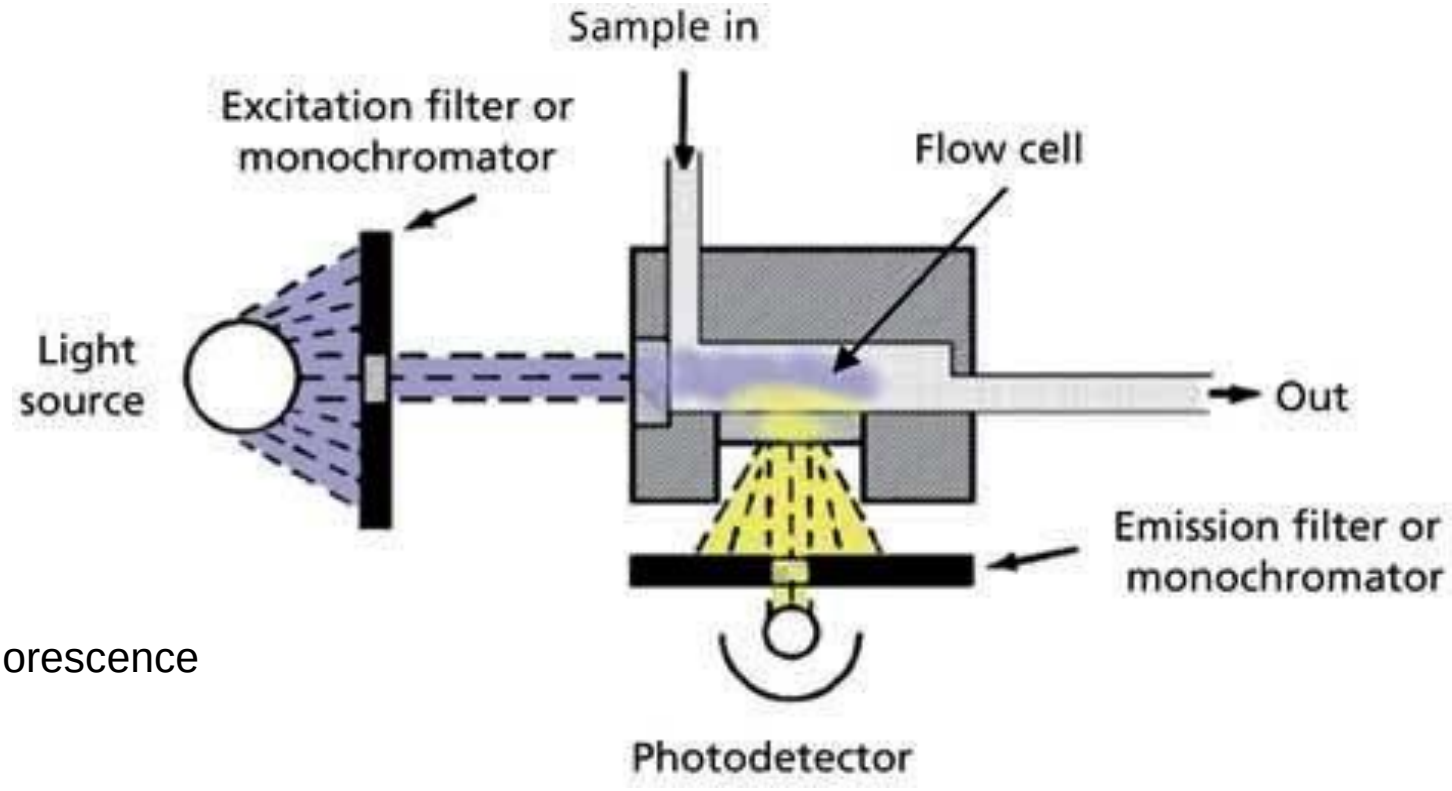
Schematic of a deflection type of RI detector





Fluorescence detector

-Compared to UV/Vis detectors fluorescence detectors offer a higher sensitivity and selectivity that allows to quantify and identify compounds and impurities in complex matrices at extremely low concentration levels (trace level analysis).
-Fluorescence detectors sense only those substances that fluoresce.



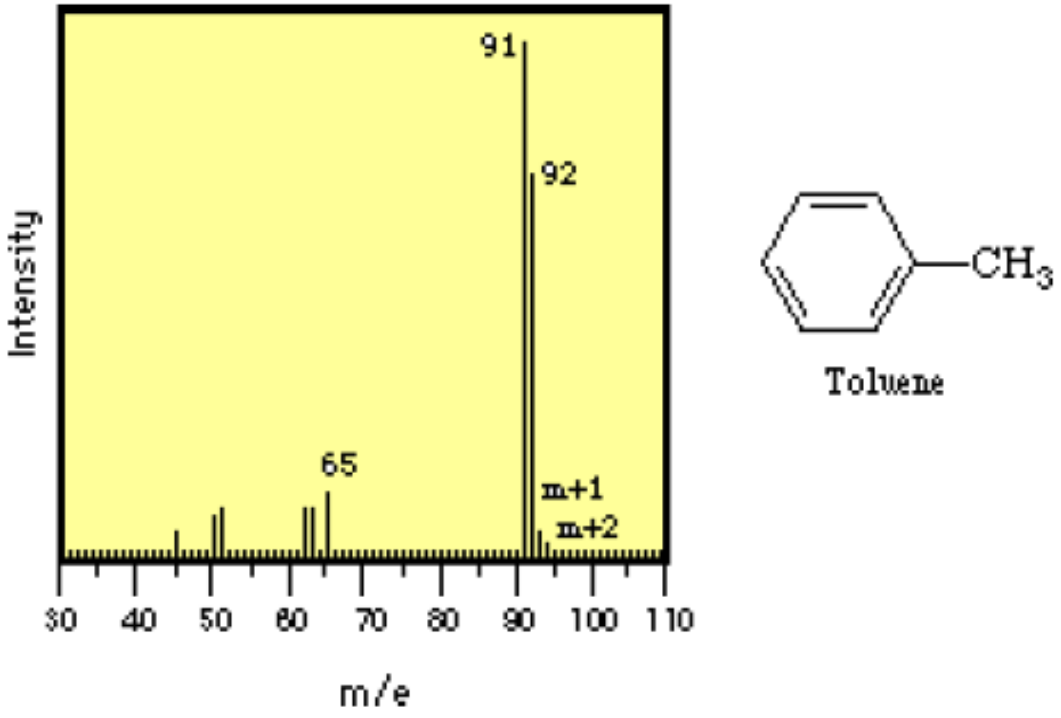
Schematic of a fluorescence detector

Mass spectrometer detector (MS)

-An MS detector senses a compound eluting from the HPLC column first by ionizing it then by measuring it's mass and/or fragmenting the molecule into smaller pieces that are unique to the compound.
-The MS detector can sometimes identify the compound directly since its mass spectrum is like a fingerprint and is quite unique to that compound.

Here is a mass spectrum of a simple chemical compound, **toluene**.

The pattern of lines is very unique to this compound. The largest peak in the spectrum occurs at a mass of 91, which is a fragment ion generated by loss of a hydrogen atom.

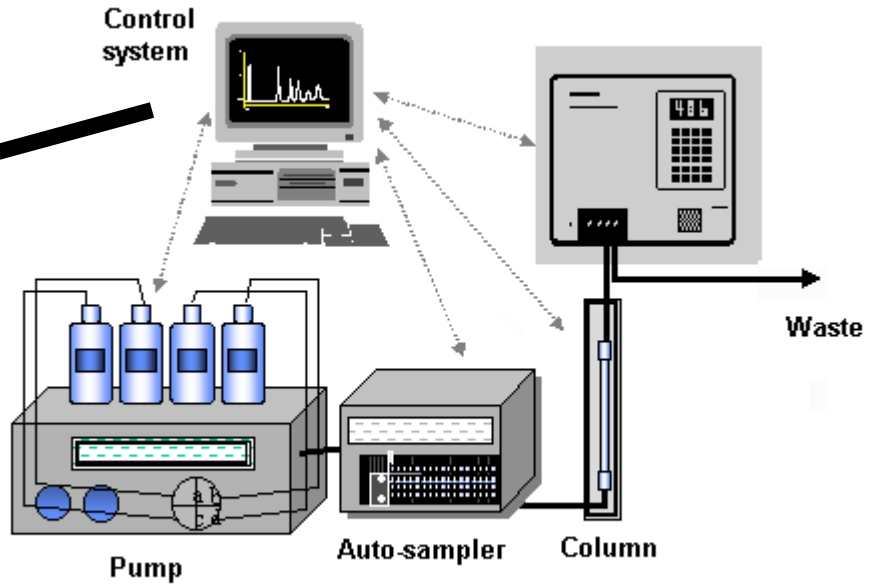


Properties of HPLC detectors

HPLC detector	Analyte/attributes	Mass LOD (typical)
Absorbance (UV/Vis)	Specific: compounds with UV chromophores	ng-pg
Photo diode array (PDA)	Specific: same as UV/Vis detectors, also provides UV spectra	ng-pg
Fluorescence	Very specific: compounds with native fluorescence or with fluorescent tag	fg-pg
Refractive index (RI)	Universal: polymers, sugars, triglycerides, organic acids, excipients; not compatible with gradient analysis	0.1-10 µg
Evaporative light scattering (ELSD)	Universal: nonvalatile or semivolatile compounds	10 ng
Electrochemical	Very specific: electro-active compounds (Redox)	pg
Conductivity	Specific: anions and cations, organic acids and surfactants	ng
Mass spectrometry (MS & MS/MS)	Both universal and specific: structural identification, very sensitive and specific	ng-pg (MS) pg-fg (MS/MS)
Infrared (FT-IR)	Universal: organic compounds	---
Nuclear magnetic resonance (NMR)	Universal: structure elucidation and confirmation	mg-ng
Corona-charged aerosol (CAD)	Universal: use nebulizer and detection of charges induced by a high-voltage corona wire	Low ng
Chemiluminescence nitrogen (CLND)	Specific: N-containing compounds based on pyro-chemiluminescence	<0.1 ng of nitrogen
Radioactivity	Specific: radioactive-labeled compounds	Low levels

Data acquisition and control system

Computer based system are used in every instrumental module and at every stage of analysis. Computers control the flow rate, eluent composition, temperature, injection volume and injection process. Detector output signal is converted from analog form into the digital representation to recognize the presence of peaks, and then at higher level of computer analysis a chromatogram is obtained.

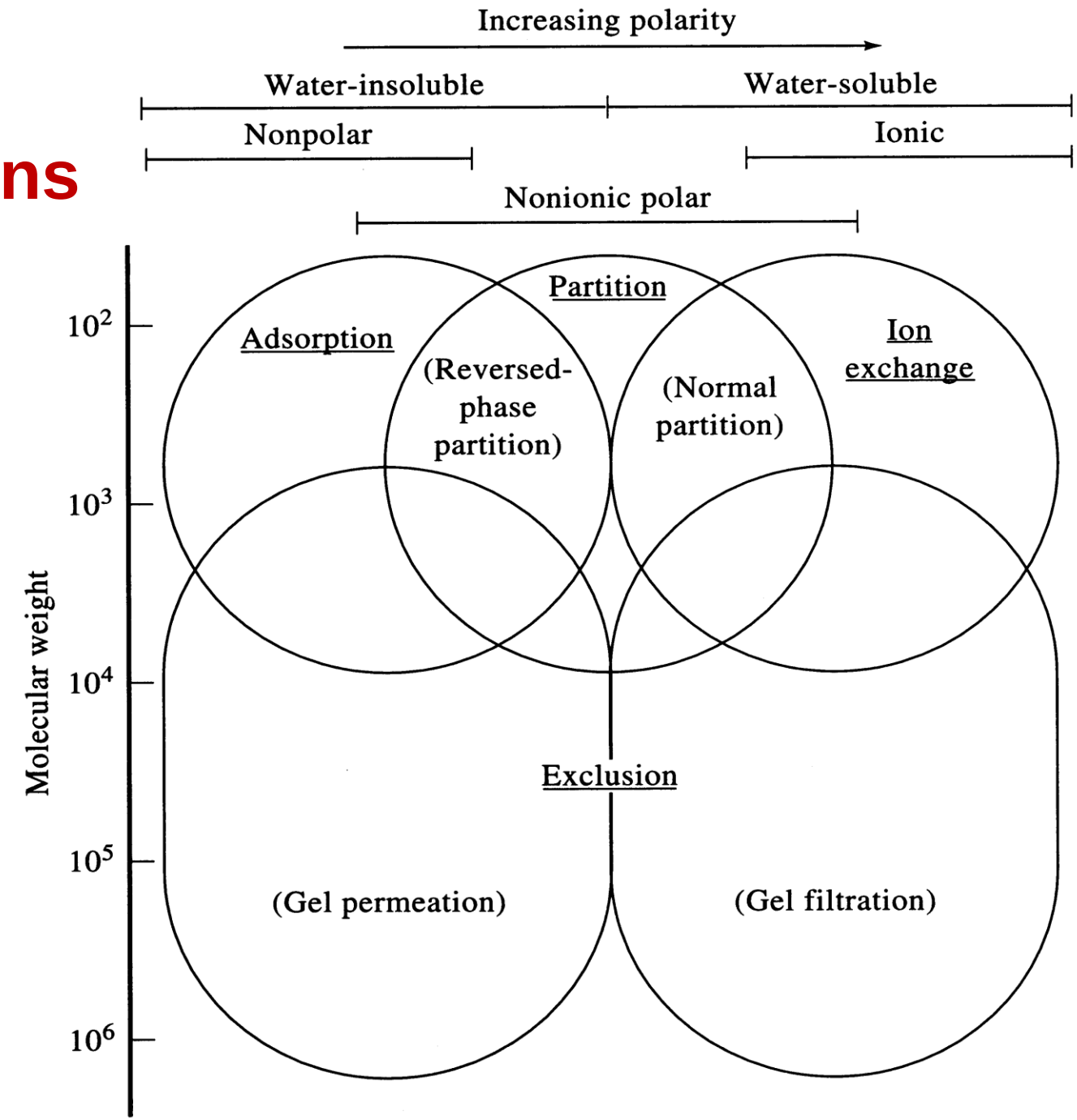


Factors Influencing the HPLC Separation

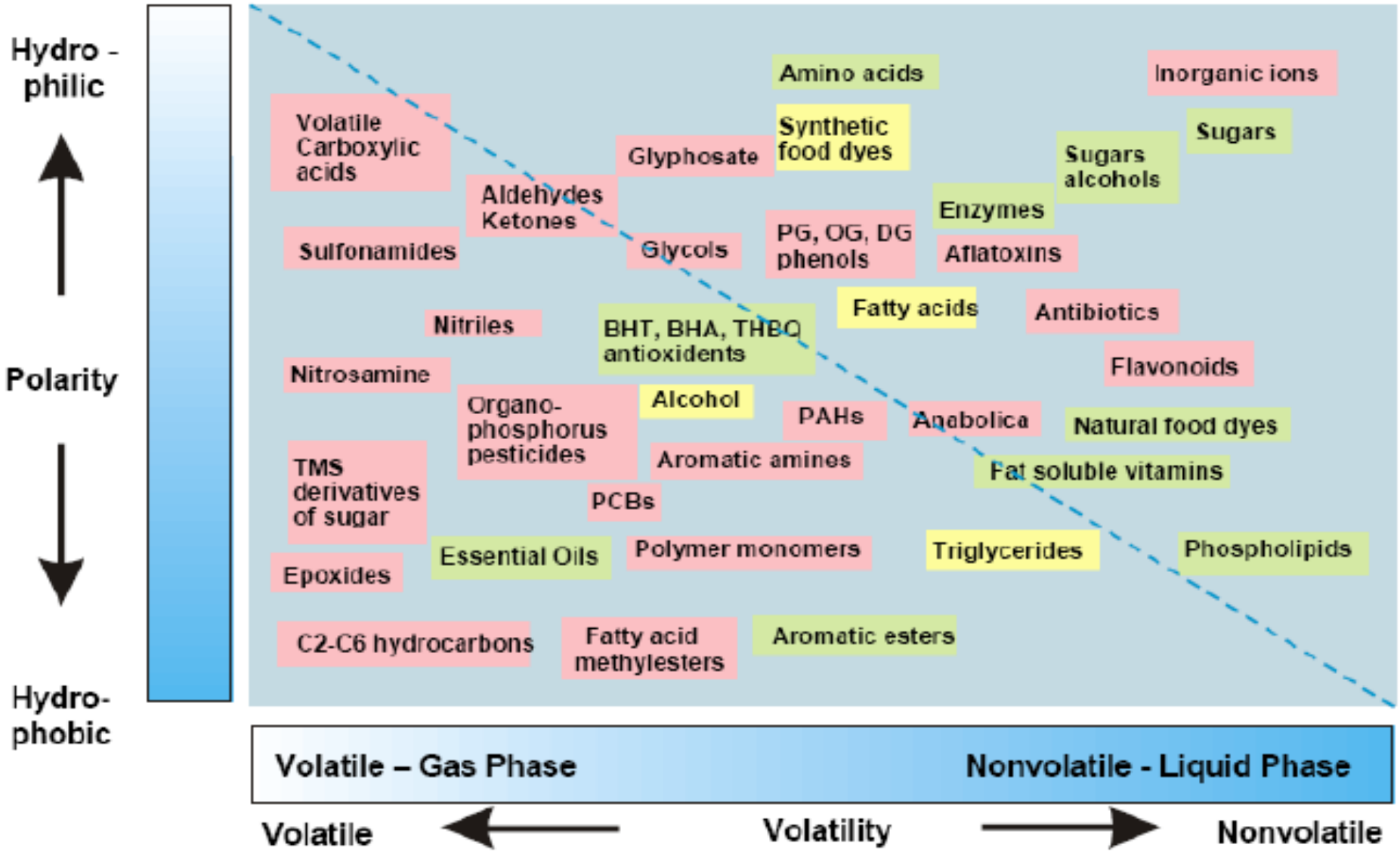
The major interrelated factors to consider

-Mobile phase type and purity
-Mobile phase composition and additives (if any)
-Mobile phase flow rate
-Mobile phase elution (isocratic or gradient mode)
-Injection method and accuracy
-Injection volume and reproducibility
-Column length
-Column internal diameter
-Column temperature
-Stationary phase chemical properties (chemical structure, functional groups, chemical stability, reactivity, purity, etc.)
-Stationary phase physical properties (size, shape, pores, density, uniformity, distribution, etc.)
-Type of detector
-Detector conditions and sensitivity

HPLC Applications



Which separation technique for which compound ?



Typical applications of HPLC chromatography

Field of application	Interesting separation compounds & media
Pharmaceuticals	antibiotics, sedatives, steroids, analgesics, drugs, metabolites
Biochemical	amino acids, peptides, proteins, carbohydrates, lipids
Food Products	artificial sweeteners, antioxidants, preservatives, vitamins
Industrial & Petrochemicals	condensed aromatics, surfactants, propellants, dyes, crude oil, characterization of petroleum products, hydrocarbons, PAHs
Forensic Chemistry	drugs, poisons, blood alcohol, narcotics
Clinical Medicine	bile acids, drug metabolites, urine extracts, estrogens, stimulants
Toxicological	drugs screening, systematic toxicological analysis, drugs of abuse, barbiturates
Environmental & Pollutants	air, water, and soil pollutants, hydrocarbons, PAHs, pesticides, herbicides, phenols, PCBs


Thank You!