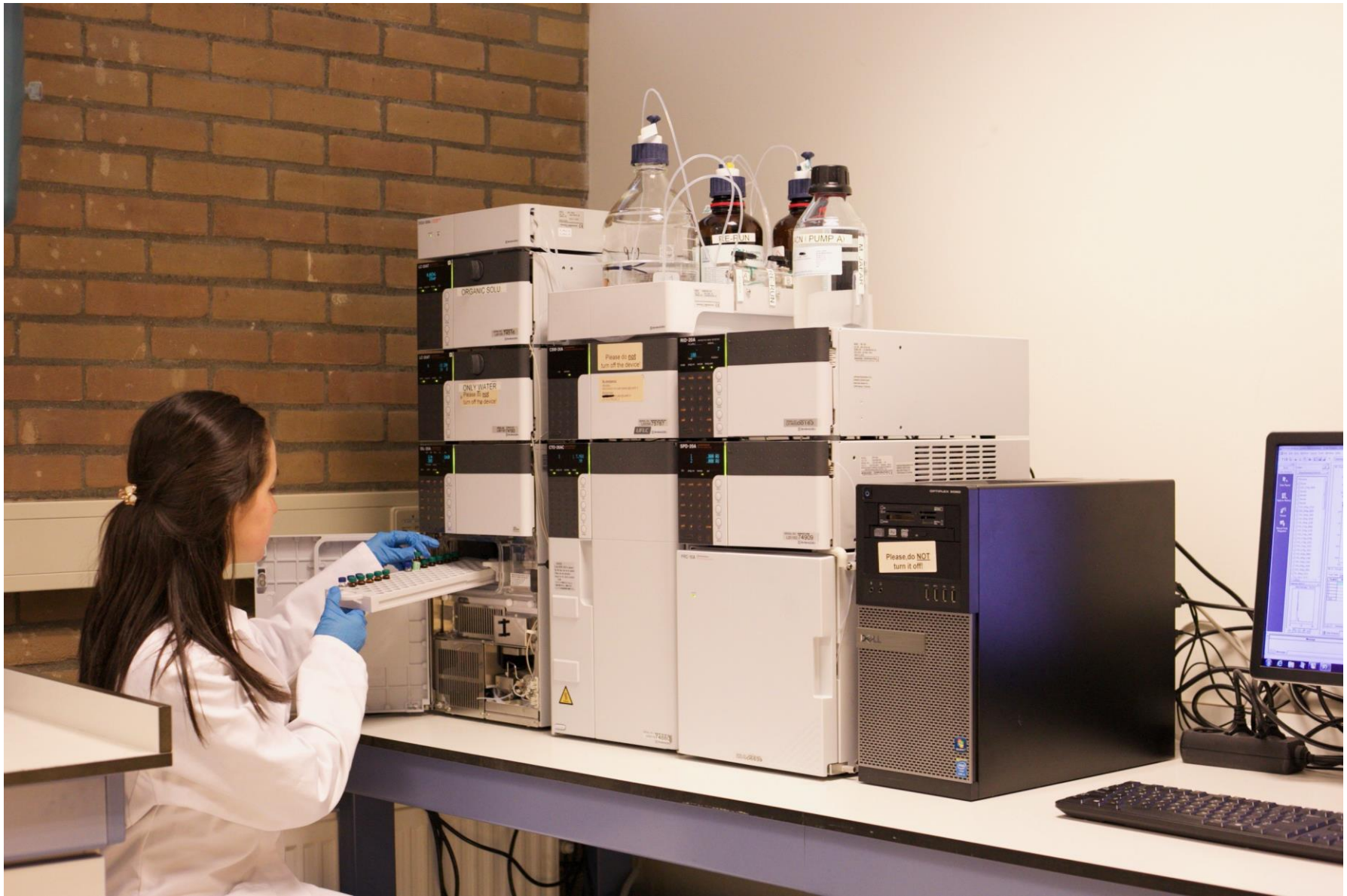
HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

HPLC



Different types of chromatography

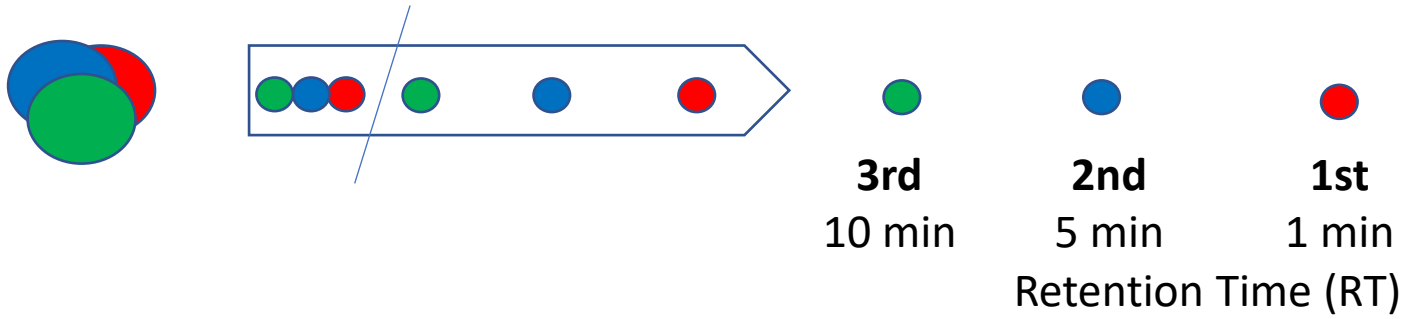
~~(Will be / were dealt with at some other time)~~

Chromatography technique can be explained / **classified** into three fundamental ways:

- Based on the shape of chromatographic bed.
  - ~~Planar chromatography (TLC and paper chromatpography)~~
  - ~~Column chromatography~~
- Based on the physical nature of the stationary and mobile phases.
  - ~~Gas chromatography (GC)~~
  - **Liquid chromatography (LC, HPLC, UHPLC)**
  - ~~Supercritical fluid chromatography (SFC)~~
- Based on the mechanism of the separation.
  - ~~Ion- exchange~~
  - ~~Affinity~~
  - ~~Size exclusion~~
  - ~~Hydrophobic interaction~~

Identifying Components of a Mixture

Chromatography is a process by which compounds within a mixture are separated. Compounds are be separated by properties such as size, and how the compounds interact with the mobile and stationary phases of chromatography.



The sample is mixed into the **mobile phase**, usually a liquid or a gas, which is then passed over the stationary phase, usually a solid or a liquid. If a compound (compound A) within a mixture has a low affinity for the **stationary phase**, then it will not interact much with the stationary phase. However, if another compound (compound B) within a mixture has a high affinity for the stationary phase, then it will bind to the stationary phase. This results in compound A moving through the mobile phase more quickly than compound B, and thus compounds A and B can be separated from the mixture.

Identifying Components of a Mixture

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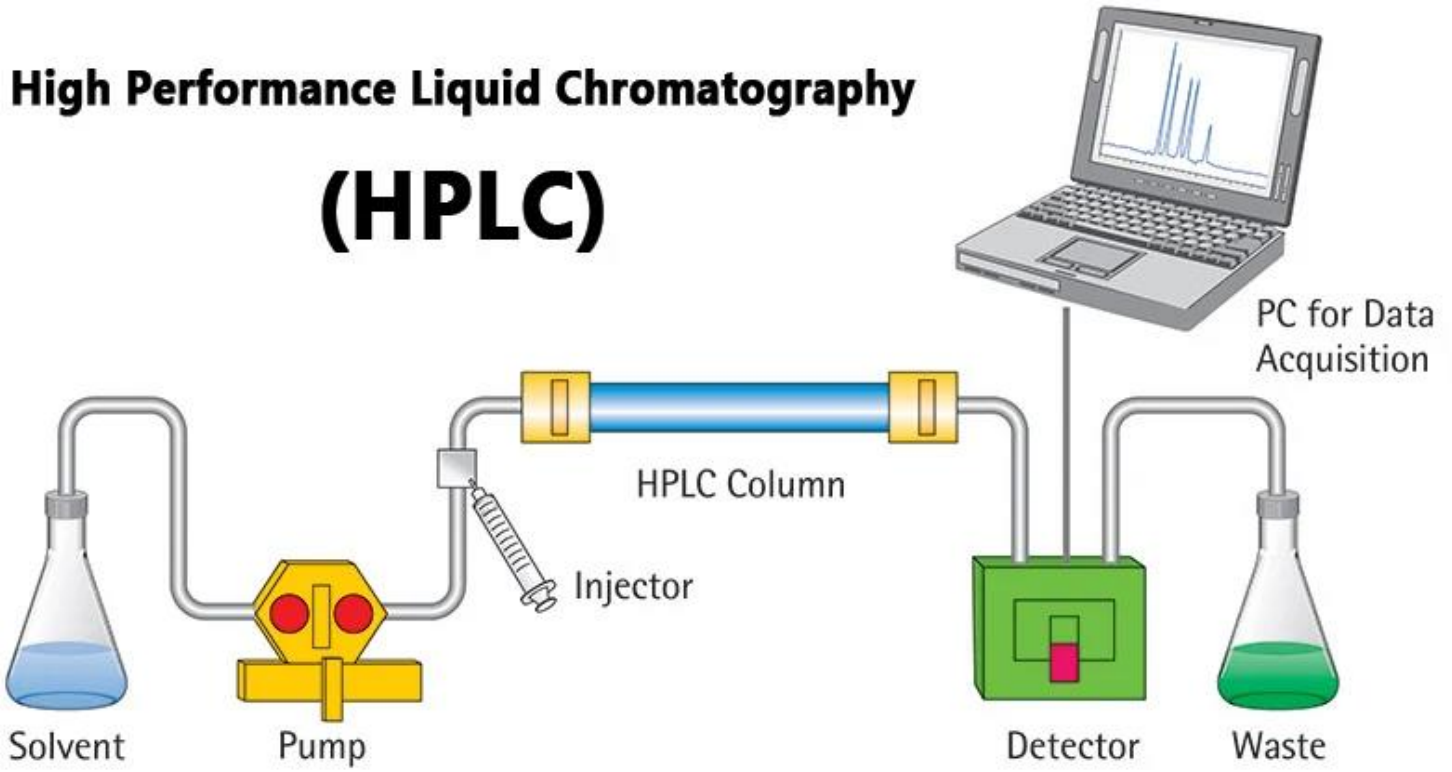


HIGH PERFORMANCE LIQUID CHROMATOGRAPHY
HPLC

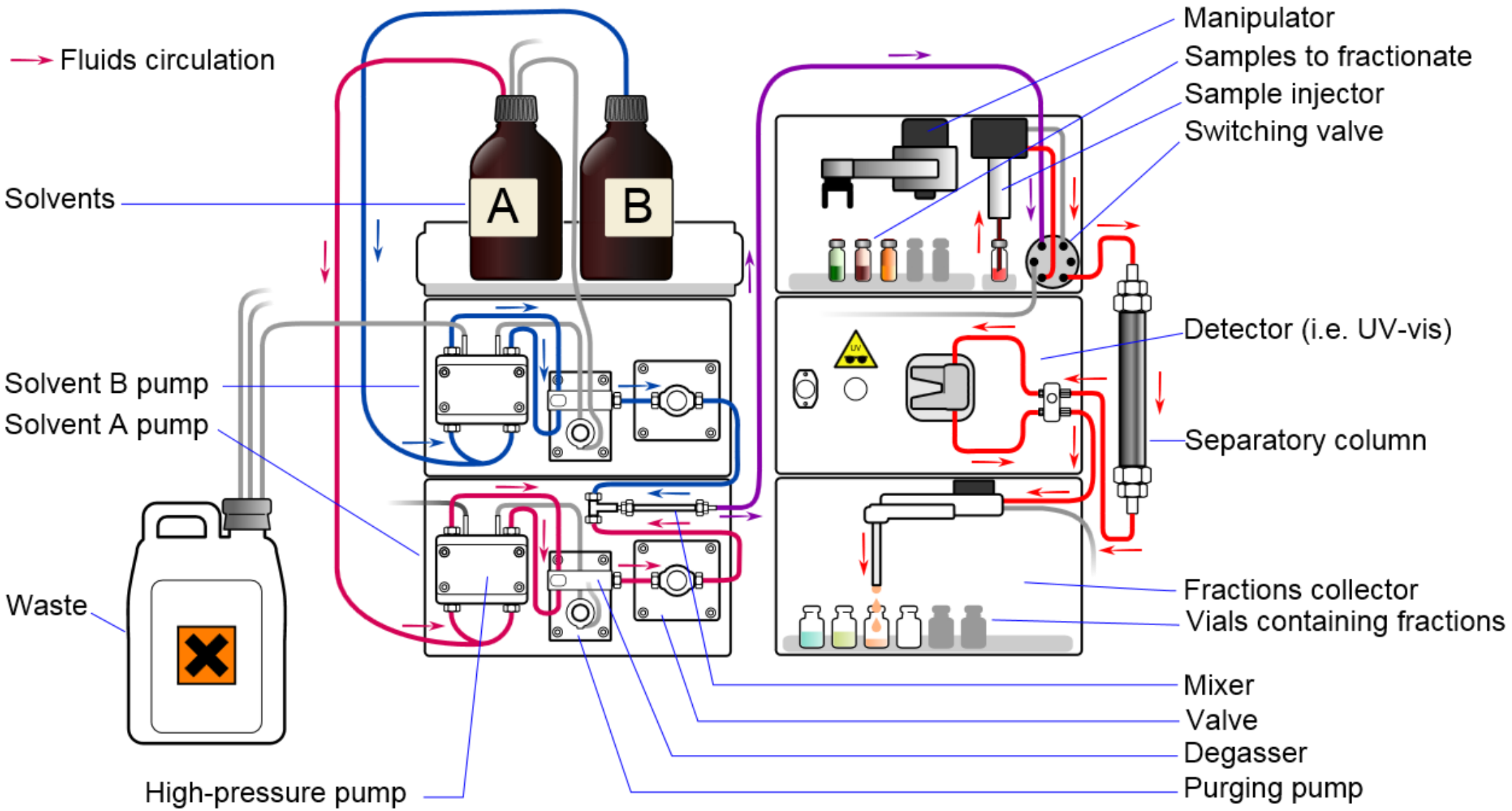
HPLC stands for **High-Performance Liquid Chromatography**. It is an analytical technique used to separate, identify, and quantify the components in a mixture of chemicals or compounds.

In HPLC, a sample is dissolved in a **liquid solvent** and injected into a column packed with a stationary phase. The **stationary phase is typically a solid material that is coated with a liquid phase, and the mobile phase is a liquid that is pumped through the column under high pressure**. As the mobile phase flows through the column, the different components of the sample interact with the stationary phase to different extents, which causes them to separate from one another.

The separated components then exit the column and are detected by a detector, which generates a signal that can be analyzed to identify and quantify the individual components. HPLC is widely used in a variety of industries, including pharmaceuticals, environmental analysis, food and beverage analysis, and forensics, among others.

HIGH PERFORMANCE LIQUID CHROMATOGRAPH

HPLC



HPLC parts (1)

Mobile Phase Reservoir: This is a container that holds the solvent or mobile phase that is used to transport the sample through the system.

Pump: This is a device that generates high-pressure flow of the mobile phase to ensure consistent and reproducible separation of the sample components.

Solvent Degasser: This is a device that removes any dissolved gases from the mobile phase to prevent bubble formation that could interfere with the separation.

Autosampler: This is a device that can automatically introduce multiple samples into the system, increasing throughput and efficiency. Or manual injection

Injector: This is a mechanism that introduces the sample into the mobile phase stream.

Guard column: It is a small chromatography column that is placed before the analytical column in an HPLC system. Its main purpose is to protect the analytical column from sample matrix components that can accumulate over time and cause column fouling or decrease the column's lifetime.

HPLC parts (2)

Column Oven: This is a temperature-controlled compartment that maintains the column at a constant temperature to ensure consistent separation.

Column: This is a narrow tube or column packed with a stationary phase material, typically silica or a polymer. The sample is separated based on the interactions between its components and the stationary phase material.

Detector: This is a device that detects the components as they elute from the column and produces a signal that is used to generate a chromatogram.

Waste disposal vessel: Every remaining liquids from both the sample and the mobile phase should be collected and disposed properly.

Data System: This is a computer-based system that controls the instrument and collects, analyzes, and displays the data generated by the detector.

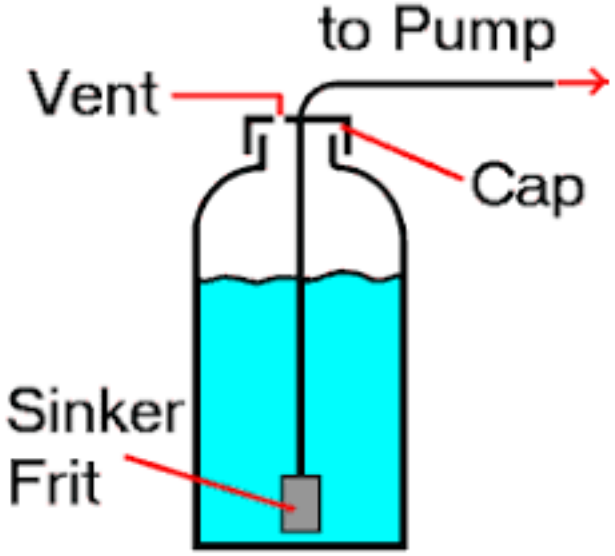
Solvent reservoir

HPLC solvent reservoirs are containers used to store the mobile phase solvents used in High Performance Liquid Chromatography (HPLC) systems.

Solvent reservoirs for HPLC systems come in various sizes, depending on the application and the amount of solvent required for the analysis. They are typically made of glass or plastic and have airtight caps to prevent contamination of the solvent.

It is important to properly handle and maintain the solvent reservoirs to ensure accurate and reliable results in HPLC analysis. The solvent should be filtered before use to remove any impurities or particles that may affect the separation. **The reservoir should also be cleaned regularly to prevent the buildup of contaminants or residual solvents.**

In addition, it is important to use the correct solvent for the specific application and to **properly label the solvent reservoir to avoid any confusion or errors in the analysis.**



Pumps in HPLC

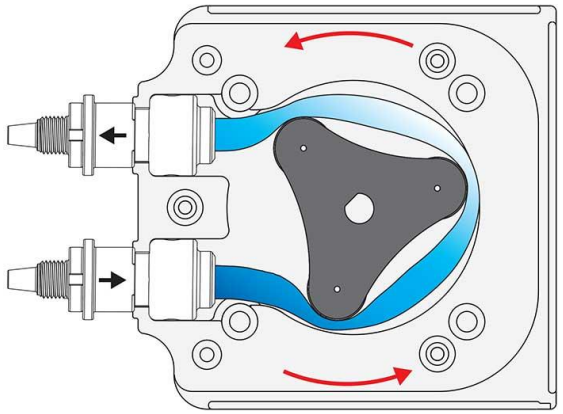
Constant Pressure Pump: This type of pump maintains a constant pressure in the system by adjusting the flow rate of the mobile phase, which can be useful in certain applications.

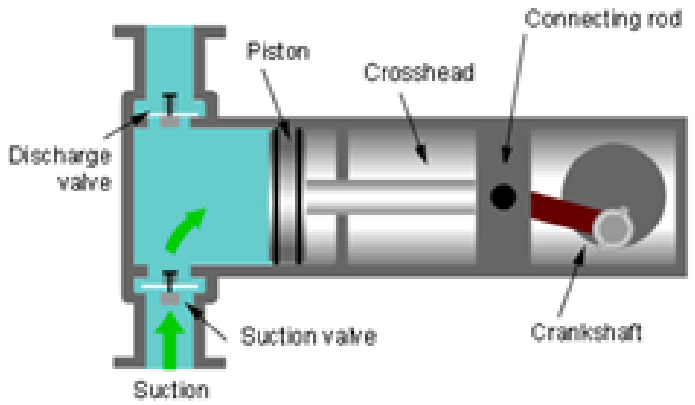
Isocratic Pump: An isocratic pump delivers a constant ratio of solvent components throughout the run, and it is used in isocratic elution mode, where the mobile phase composition is held constant throughout the analysis.

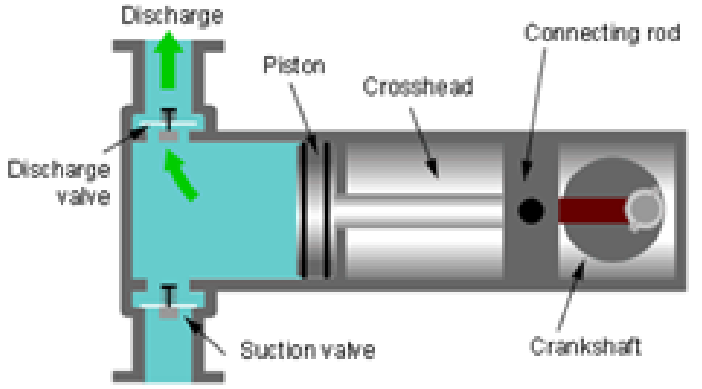
Gradient Pump: Gradient pumps are used in gradient elution mode, where the mobile phase composition changes over time to optimize separation of the analytes. This type of pump is able to deliver two or more solvents at different ratios and can adjust the ratio throughout the analysis.

Syringe Pump: Syringe pumps are used in some HPLC systems to deliver precise amounts of sample or solvent to the column.

Peristaltic Pump: This type of pump uses a flexible tubing to move the solvent, and it is often used in preparative HPLC to transfer large volumes of solvent.







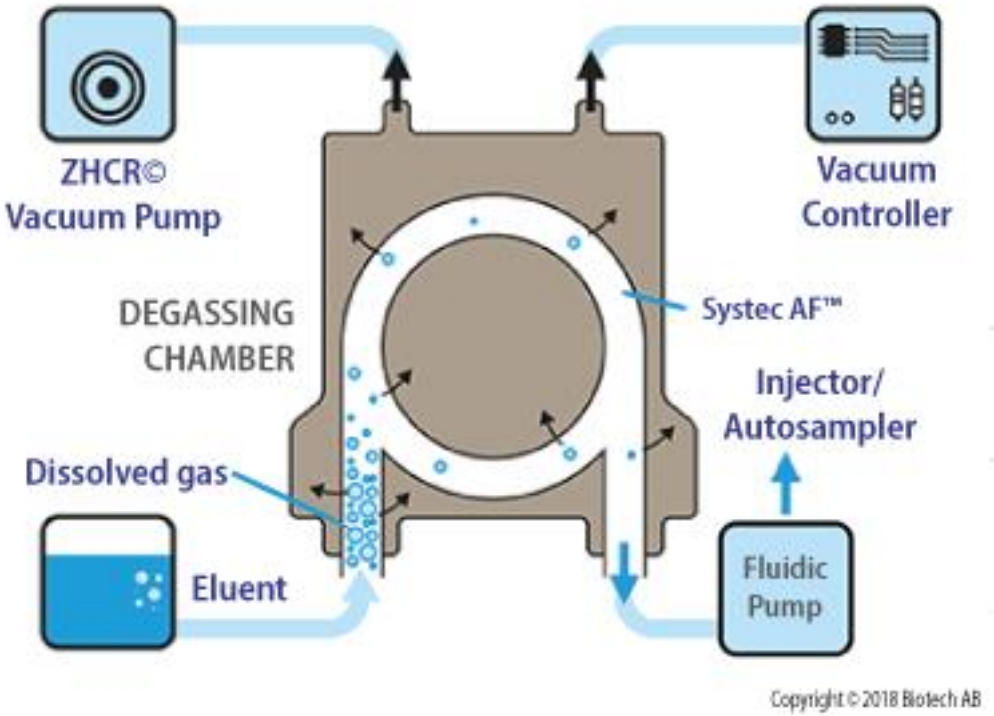
Solvent degasser

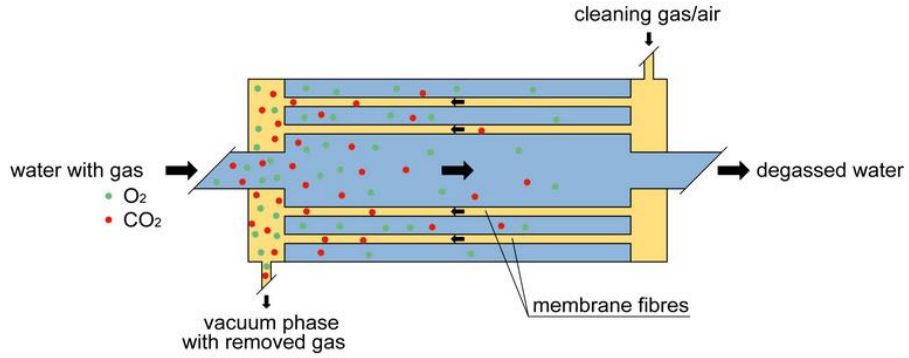
A solvent degasser is a device or component of the system that removes dissolved gases from the solvents used in the chromatographic process. **Dissolved gases in the solvents can cause bubbles and air pockets to form in the system, which can negatively affect the accuracy and reproducibility of the separation.**

A solvent degasser typically consists of a chamber that allows the solvent to flow through a porous material, such as a membrane or a set of tubes, that selectively removes dissolved gases. There are two main types of solvent degassers: vacuum degassers and membrane degassers.

Vacuum degassers work by exposing the solvent to a low-pressure vacuum, which causes the dissolved gases to bubble out of the liquid and collect in a separate chamber. Membrane degassers use a semi-permeable membrane that allows gases to pass through but not the solvent. The solvent flows across the membrane, and the dissolved gases diffuse through the membrane and are removed from the system.

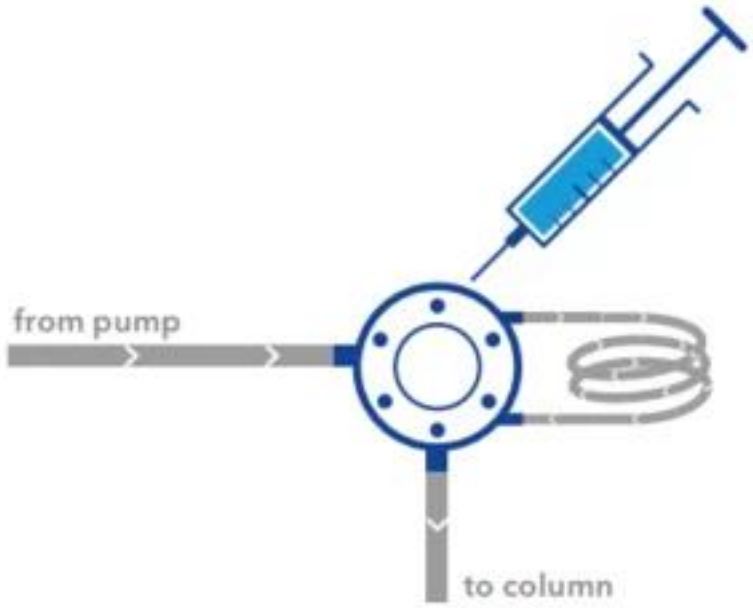
By removing dissolved gases from the solvents, a solvent degasser helps to ensure a stable and consistent flow of solvent through the HPLC system, which is essential for achieving accurate and reproducible chromatographic separations.





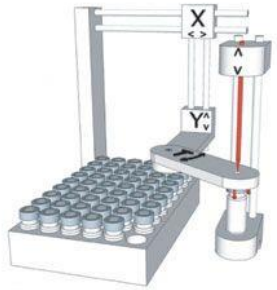
HPLC injection

Fixed loop injection: In this type of injection, a fixed volume of the sample is introduced into the mobile phase by a syringe. The sample is loaded into a loop of known volume, which is then introduced into the mobile phase stream using a valve. This method is commonly used in manual HPLC systems.

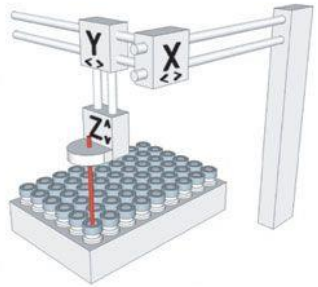


Autosampler injection: In this type of injection, an autosampler is used to automatically inject the sample into the HPLC system. The autosampler is a device that can hold multiple samples in a tray and introduce them into the mobile phase stream in a pre-programmed sequence. Autosamplers can be used for both fixed volume injections and variable volume injections.

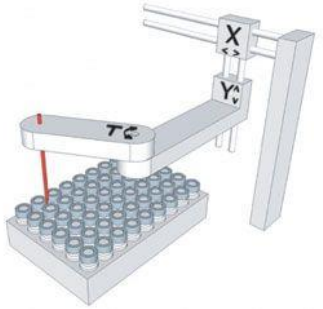
(a) Vial-to-needle configuration



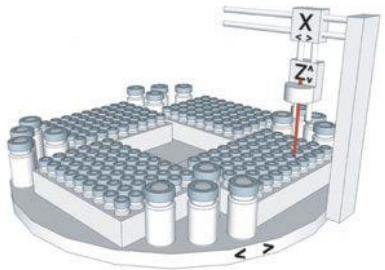
(b) Needle-to-vial configuration
(b1) Static tray with *xyz*



(b2) Static tray with *xy* theta



(b3) Sample carousel with *xz* drive



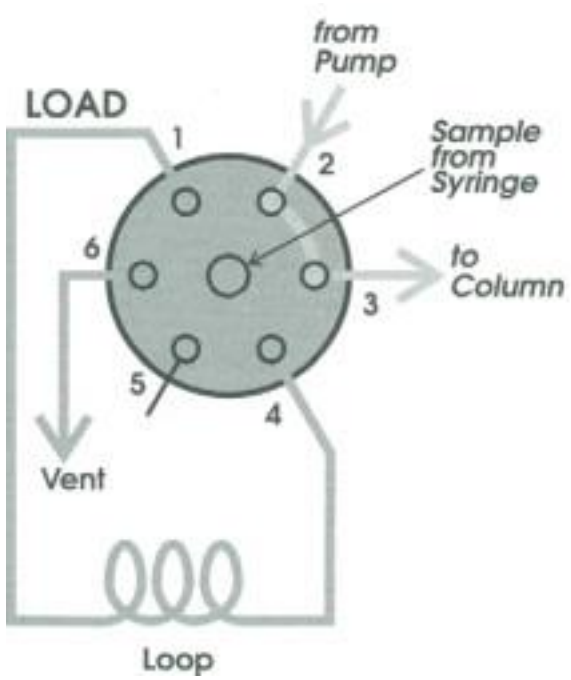
HPLC injection: 6 port valve

A 6-port valve typically consists of a rotor with six positions, each connected to a different port, and a stator that holds the rotor in place and directs the flow of the mobile phase and sample.

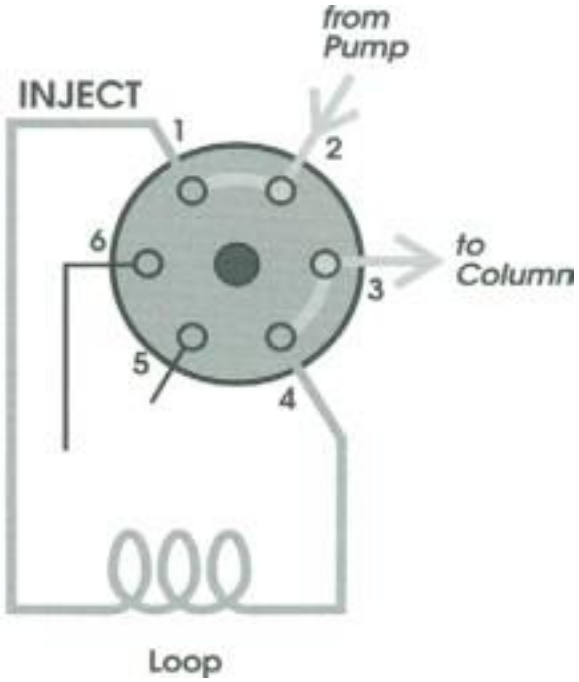
In the sample **loading mode**, the rotor is turned to the "load" position, and a fixed volume of the sample is injected into the valve through a syringe. In **injection mode**, he rotor is then turned to the "inject" position, and the sample is introduced into the mobile phase stream.

Besides for sample injection, a 6-port valve is also used for column switching, and fraction collection, making the HPLC system more versatile and efficient.

Loading mode



Injection mode



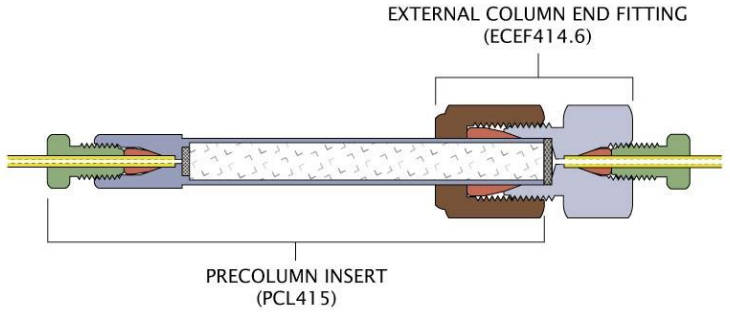
HPLC guard column

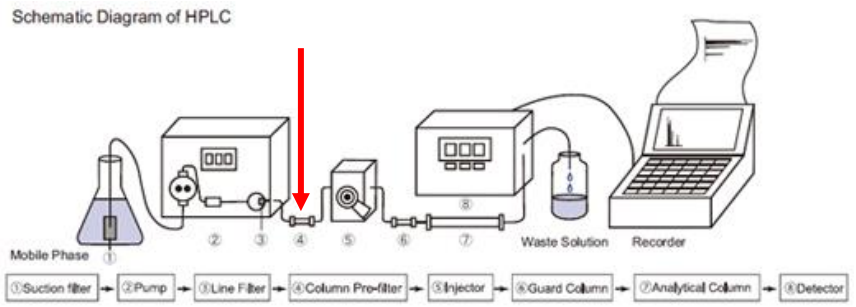
A guard column is a small chromatography column that is placed **before** the analytical column in an HPLC system. Its main purpose is to **protect the analytical column** from sample matrix components that can accumulate over time and cause column fouling or decrease the column's lifetime.

When samples are injected into the HPLC system, they often contain impurities, particulates, or other unwanted components that can stick to the surface of the analytical column. Over time, these accumulated contaminants can cause a reduction in column performance, such as decreased separation efficiency, increased backpressure, or increased baseline noise.

By placing a guard column before the analytical column, the guard column can capture and retain these contaminants, **preventing them from reaching the analytical column**. The guard column can then be replaced or cleaned periodically, prolonging the lifetime of the analytical column.

Guard columns are typically smaller than analytical columns and have the same stationary phase chemistry. They can be purchased separately or as part of a pre-column kit from HPLC column manufacturers. Guard columns can be packed with the same or different stationary phase compared to the analytical column, depending on the type of sample matrix and separation requirements.





HPLC classification according to main separation mechanisms

Reverse-phase chromatography: In reverse-phase chromatography, the stationary phase is a hydrophobic material, typically a C18 or C8 bonded silica or polymer. The mobile phase is typically a polar solvent, such as water or methanol, with a small amount of an organic modifier, such as acetonitrile. This mechanism separates molecules based on their hydrophobicity, with more hydrophobic molecules spending more time interacting with the stationary phase and eluting later.

Normal-phase chromatography: In normal-phase chromatography, the stationary phase is a polar material, such as silica, and the mobile phase is typically a nonpolar solvent, such as hexane or methylene chloride. This mechanism separates molecules based on their polarity, with more polar molecules spending more time interacting with the stationary phase and eluting later.

Ion exchange chromatography: In ion exchange chromatography, the stationary phase is a resin with charged functional groups, typically either positive or negative. The mobile phase contains ions of the opposite charge, which interact with the stationary phase and exchange with the sample ions of the same charge. This mechanism separates molecules based on their charge and ionic properties.

Size exclusion chromatography: In size exclusion chromatography, the stationary phase is a porous material, typically a polymer, with pores of a specific size range. The mobile phase is typically a buffer or salt solution. Molecules of different sizes are excluded from the pores to different extents, resulting in separation based on size.

Affinity chromatography: In affinity chromatography, the stationary phase is a ligand that specifically binds to the target molecule. The mobile phase is typically a buffer or salt solution. This mechanism separates molecules based on their affinity for the ligand.

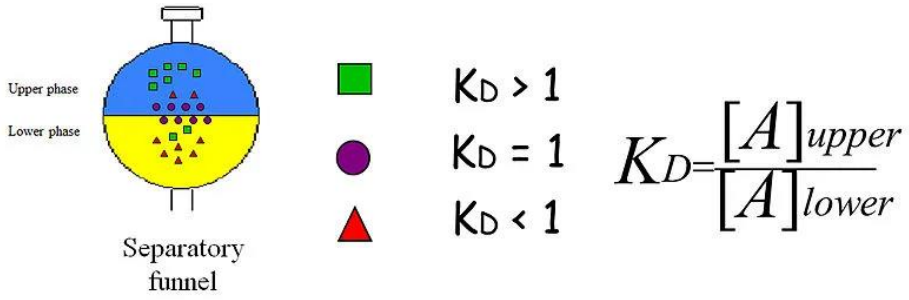
Good ‘ol coefficient of partition

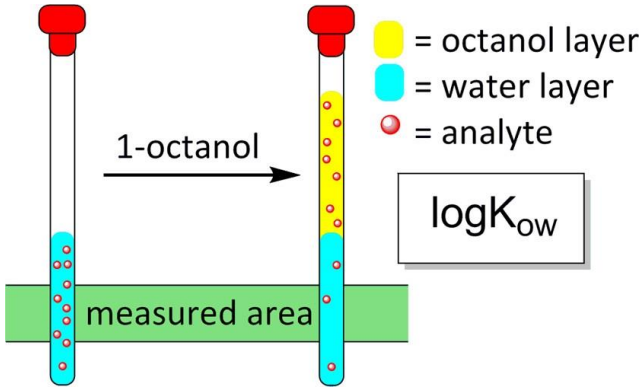
The coefficient of partition, also known as the distribution coefficient or partition coefficient, plays an important role in HPLC. It is a measure of the relative affinity of a molecule for the stationary phase compared to the mobile phase, and is defined as the ratio of the concentration of the solute in the stationary phase to the concentration of the solute in the mobile phase at equilibrium.

In HPLC, the partition coefficient influences the retention time of a solute on the chromatography column, as solutes that have a higher affinity for the stationary phase will be retained on the column for a longer period of time, while solutes with a lower affinity for the stationary phase will elute more quickly.

The partition coefficient is also used to optimize HPLC separations by selecting the appropriate mobile phase composition and stationary phase for a particular sample. By adjusting the mobile phase composition or stationary phase chemistry, the partition coefficient can be manipulated to achieve the desired separation.

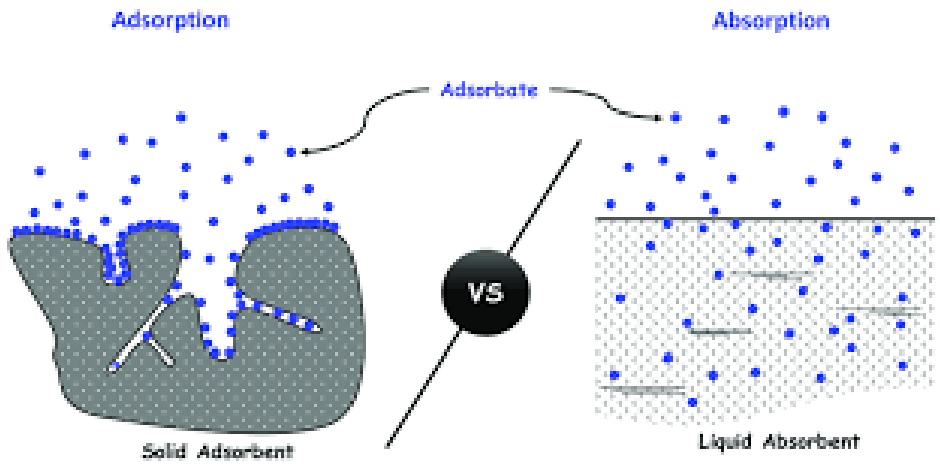
Additionally, the partition coefficient can be used to calculate the capacity factor, which is a measure of the degree of retention of a solute on the column. The capacity factor is defined as the ratio of the retention time of the solute on the column to the time it takes for the mobile phase to pass through the column.



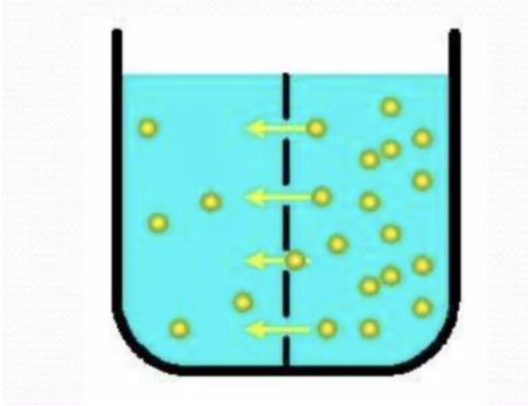


HPLC: Other aspects affecting separation mechanisms

Adsorption principle: The adsorption principle is based on the interaction of analyte molecules with the surface of the stationary phase. The surface of the stationary phase has active sites that can interact with the analyte molecules through weak forces such as van der Waals forces, hydrogen bonding, or dipole-dipole interactions. The degree of interaction of the analyte with the stationary phase is dependent on the strength of the forces and the nature of the surface. The analyte molecules that interact more strongly with the stationary phase will move more slowly through the column and will take longer to elute.



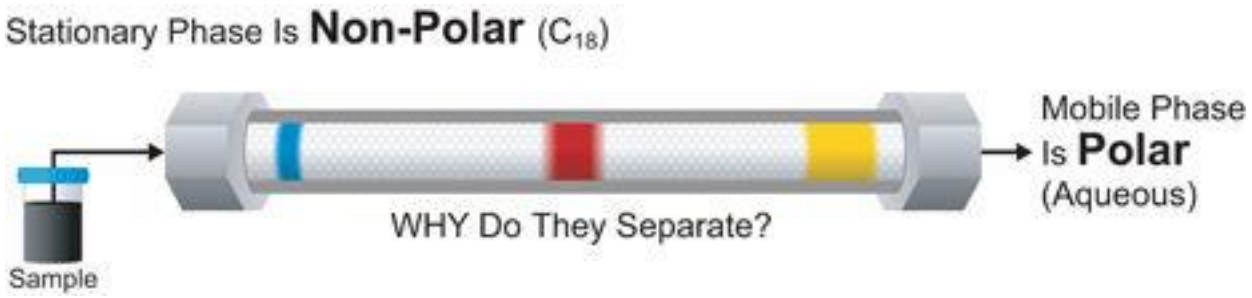
Diffusion principle: The diffusion principle is based on the movement of analyte molecules through the stationary phase. The rate of diffusion of a molecule is dependent on its size and shape, with smaller molecules diffusing more quickly than larger ones. As the analyte moves through the stationary phase, it interacts with the surface of the phase, and its movement is slowed down. This interaction is responsible for the separation of the components in the mixture.



HPLC according to separation mechanisms: Reverse phase

Reverse phase HPLC is a type of chromatography in which **the stationary phase is nonpolar**, and the **mobile phase is polar.** This means that the analytes of interest interact with the stationary phase through **nonpolar interactions, such as hydrophobic interactions and van der Waals forces**. In reverse phase HPLC, the elution order of the analytes is generally determined by their hydrophobicity or lipophilicity, with **more hydrophobic or lipophilic compounds eluting later in the chromatogram.**

Reverse phase HPLC is the most commonly used type of HPLC and is typically used for the separation of polar and moderately polar compounds, such as peptides, proteins, nucleotides, and pharmaceuticals. The stationary phase used in reverse phase HPLC is usually a nonpolar material, such as C18-bonded silica, which is packed into a column. The mobile phase used in reverse phase HPLC is typically a polar solvent, such as **water** or an aqueous buffer, mixed with a nonpolar solvent, such as acetonitrile or methanol. In reverse phase HPLC, the **more polar compounds elute more quickly because they interact less strongly with the nonpolar stationary phase and are less retained in the column. Conversely, the less polar compounds interact more strongly with the nonpolar stationary phase and are retained longer in the column**. Reverse phase HPLC is versatile and can be used for a wide range of applications, including drug discovery and development, quality control, and environmental analysis.

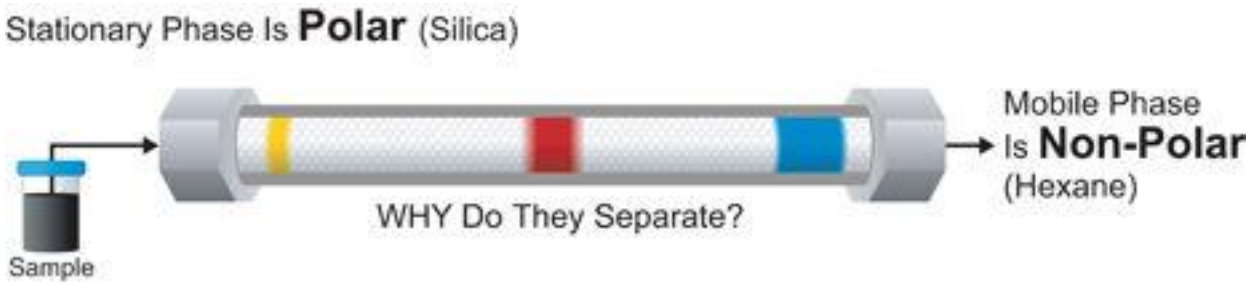


HPLC according to separation mechanisms: Normal phase

Normal phase HPLC is a type of chromatography in which the **stationary phase is polar**, and the **mobile phase is nonpolar**. This means that the analytes of interest interact with the stationary phase through **polar interactions, such as dipole-dipole interactions, hydrogen bonding, and van der Waals forces.** In normal phase HPLC, the elution order of the analytes is generally determined by their polarity and their ability to interact with the stationary phase.

Normal phase HPLC is typically used for the separation of nonpolar or moderately polar compounds, such as lipids, steroids, and some natural products. The stationary phase used in normal phase HPLC is usually a polar adsorbent material, such as silica gel or alumina, which is packed into a column. The mobile phase used in normal phase HPLC is typically a nonpolar solvent, such as hexane or heptane, mixed with a polar solvent, such as ethanol or methanol.

In normal phase HPLC, the more polar compounds interact more strongly with the stationary phase and are retained longer in the column. Conversely, the less polar compounds interact less strongly with the stationary phase and are eluted more quickly. Normal phase HPLC is often used in combination with other types of chromatography, such as reversed-phase chromatography, to provide complementary information about the properties of the analytes.

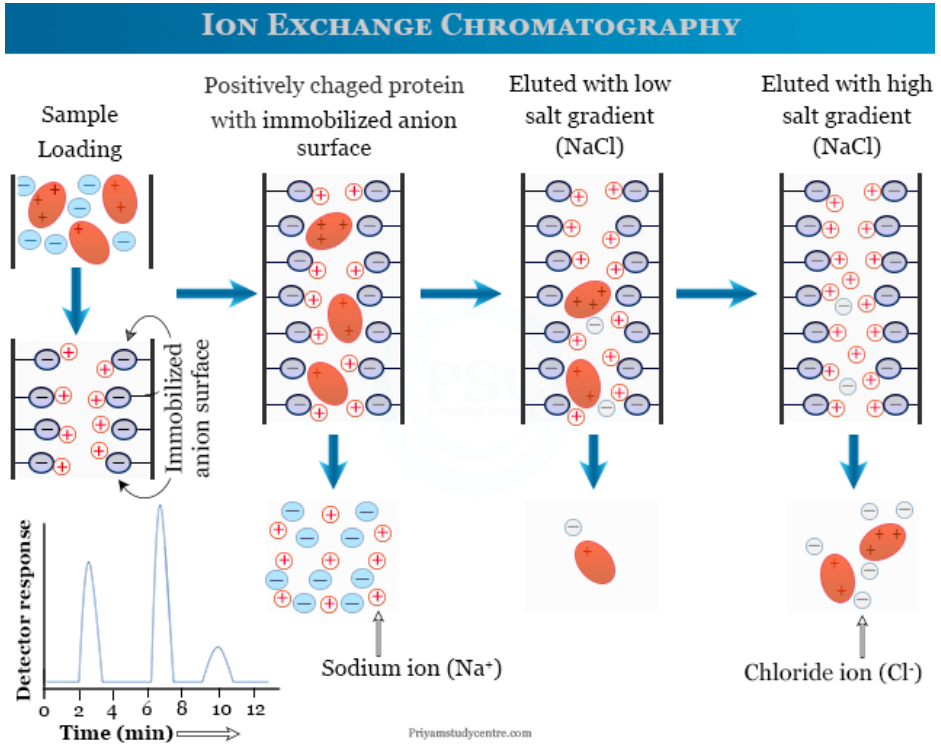


HPLC according to separation mechanisms: Ion exchange

Ion exchange HPLC is a type of chromatography in which the stationary phase contains charged functional groups that **can interact with analytes of opposite charge in the sample. The analytes of interest are separated based on differences in their ionic properties, such as charge, size, and shape. Ion exchange HPLC is particularly useful for the separation of ionic compounds, such as amino acids, proteins, and nucleotides.**

The stationary phase used in ion exchange HPLC is typically a resin containing functional groups that are either positively charged (anion exchange) or negatively charged (cation exchange). In anion exchange HPLC, the stationary phase contains negatively charged functional groups, such as carboxylic acid or sulfonic acid groups. In cation exchange HPLC, the stationary phase contains positively charged functional groups, such as amino or quaternary ammonium groups.

The mobile phase used in ion exchange HPLC is typically an aqueous buffer containing ions of opposite charge to the stationary phase. When the sample is introduced into the column, the charged analytes interact with the stationary phase via electrostatic interactions. Analytes with a strong interaction with the stationary phase are retained longer in the column, while analytes with a weaker interaction are eluted more quickly. The elution order of analytes in ion exchange HPLC depends on several factors, such as the charge, size, and shape of the analytes, as well as the type and concentration of ions in the mobile phase. The selectivity of ion exchange HPLC can be adjusted by changing the pH and/or the concentration of the mobile phase buffer. **Ion exchange HPLC is a powerful tool for the separation and purification of charged compounds in a wide range of applications, such as biopharmaceuticals, food analysis, and environmental monitoring.**

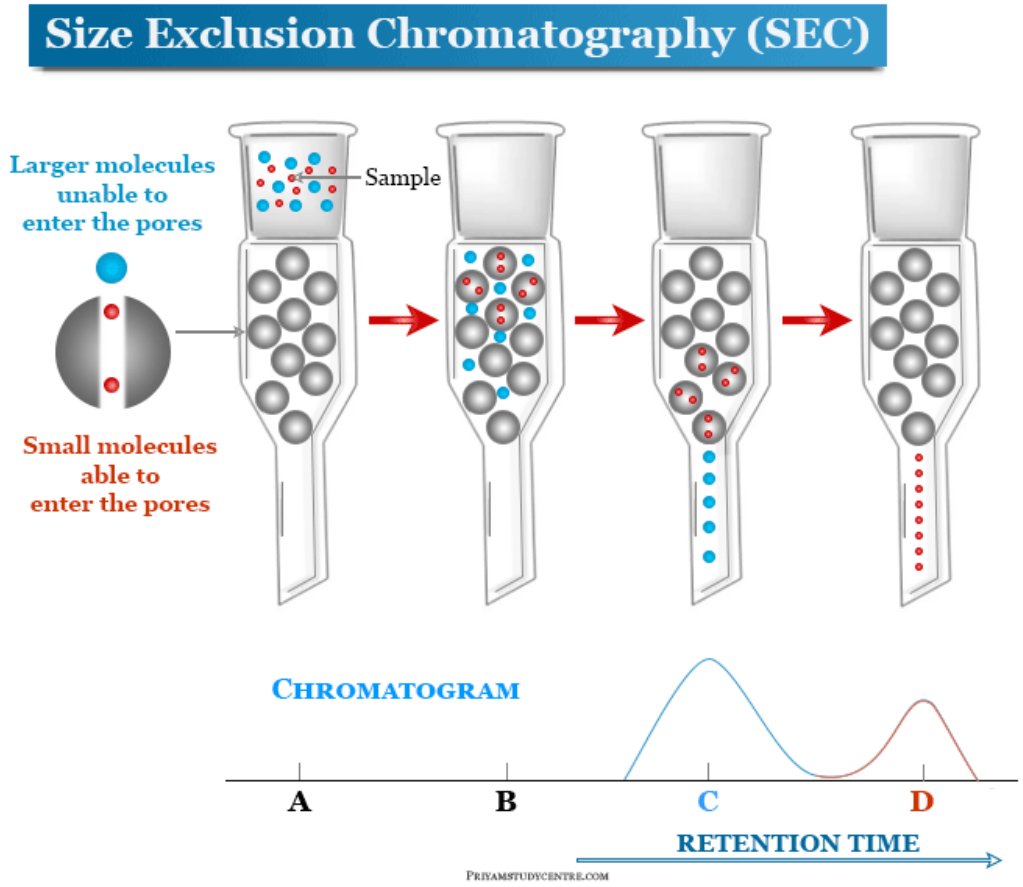


HPLC according to separation mechanisms: Size exclusion

Size exclusion HPLC, also known as gel filtration chromatography, is a type of chromatography that **separates molecules based on their size and shape. In size exclusion HPLC, the stationary phase is a porous gel matrix,** typically made of cross-linked agarose or dextran, which contains a range of pore sizes. The mobile phase is typically an aqueous buffer that flows through the stationary phase and carries the sample molecules with it.

The stationary phase separates molecules based on their size, with larger molecules excluded from the pores and eluting first, while smaller molecules penetrate the pores and elute later. This separation occurs because larger molecules do not penetrate the pores of the stationary phase and are thus not significantly retained, while smaller molecules enter the pores and interact more strongly with the stationary phase, resulting in longer retention times.

Size exclusion HPLC is particularly useful for the separation of biomolecules, such as proteins, nucleic acids, and carbohydrates. The technique can be used to determine the molecular weight of proteins and other molecules by comparing their elution volumes with those of known molecular weight markers. The selectivity of size exclusion HPLC can be adjusted by changing the pore size of the stationary phase or by selecting a different stationary phase with a different pore size distribution. Size exclusion HPLC is a widely used technique in biochemical and biopharmaceutical research, as well as in industrial applications such as the production of therapeutic proteins and monoclonal antibodies.



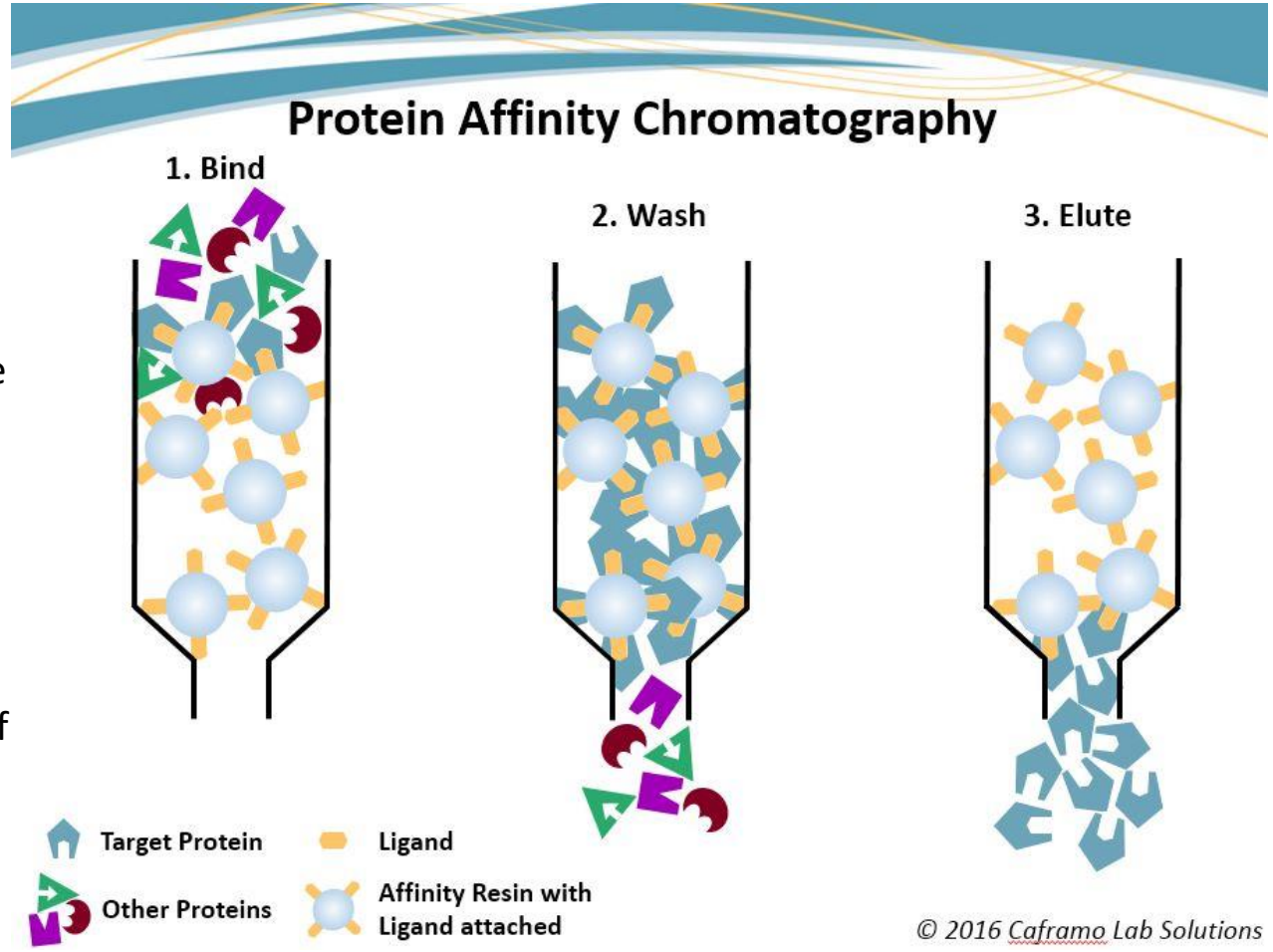
HPLC according to separation mechanisms: Affinity

Affinity HPLC is a type of chromatography that separates molecules based on their **specific binding interactions with a ligand** immobilized on the stationary phase. **The ligand can be an antibody, enzyme, receptor, or other molecule that specifically binds to the analyte of interest.** The stationary phase typically consists of a resin that is chemically modified to covalently attach the ligand.

In affinity HPLC, the sample containing the analyte of interest is introduced into the column and the molecules in the sample interact with the immobilized ligand on the stationary phase. Analytes that have a strong binding affinity for the ligand are retained on the column for a longer period of time, while those with weaker binding affinity elute more quickly.

The technique can be used to purify, identify, and quantify proteins, peptides, nucleic acids, and other biomolecules. Affinity HPLC is also used in drug discovery and development, as well as in the production of biopharmaceuticals.

One of the main advantages of affinity HPLC is its high specificity, which allows for the isolation and purification of a single molecule from a complex mixture.



How to drive separation?: Effect of temperature

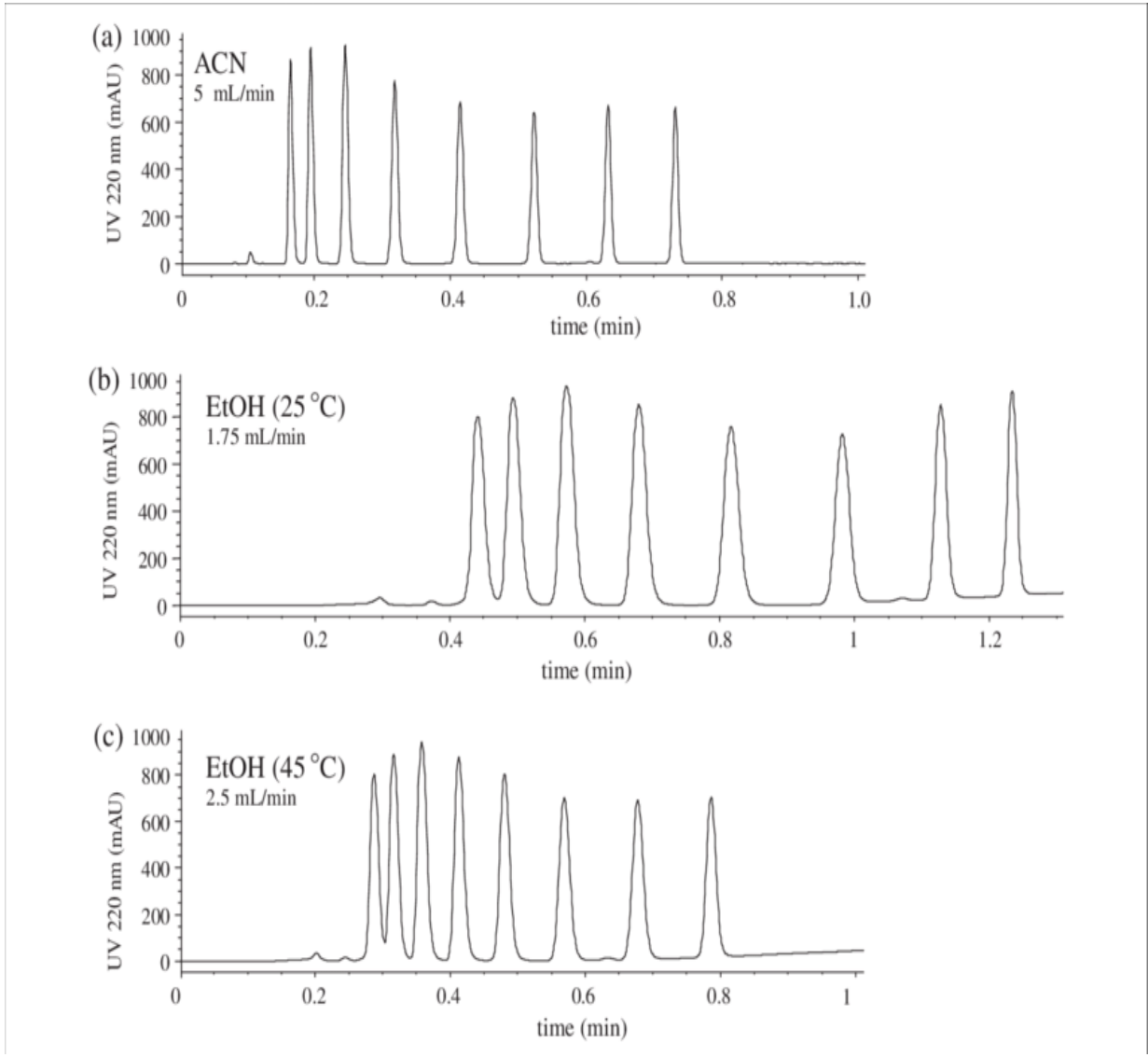
Temperature can have a significant effect on HPLC separations. Specifically, **temperature can affect the retention time, selectivity, resolution, and peak shape of analytes.**

Increasing the temperature in HPLC can lead to decreased retention times of analytes, as the **increased temperature causes the mobile phase to move more rapidly through the column**. This can lead to shorter analysis times and improved productivity.

Changes in temperature can alter the equilibrium between the stationary and mobile phases, leading to changes in the interaction between the analytes and the stationary phase. This can affect the selectivity and resolution of the separation, and can be particularly important for closely related compounds.

Peak shape can also be affected by temperature. Increasing the temperature can lead to peak broadening and tailing, which can negatively impact the resolution of the separation. However, lowering the temperature can lead to peak sharpening, which can improve resolution.

It is important to note that the effect of temperature on HPLC separations can vary depending on the specific column and mobile phase being used. In general, it is recommended to carefully optimize the temperature for each HPLC analysis to achieve the desired separation performance.



How to drive separation?: Effect of pressure/ flow rate

Pressure and flow rate are two important factors that can have a significant effect on the separation process and the quality of the chromatographic results.

Pressure:

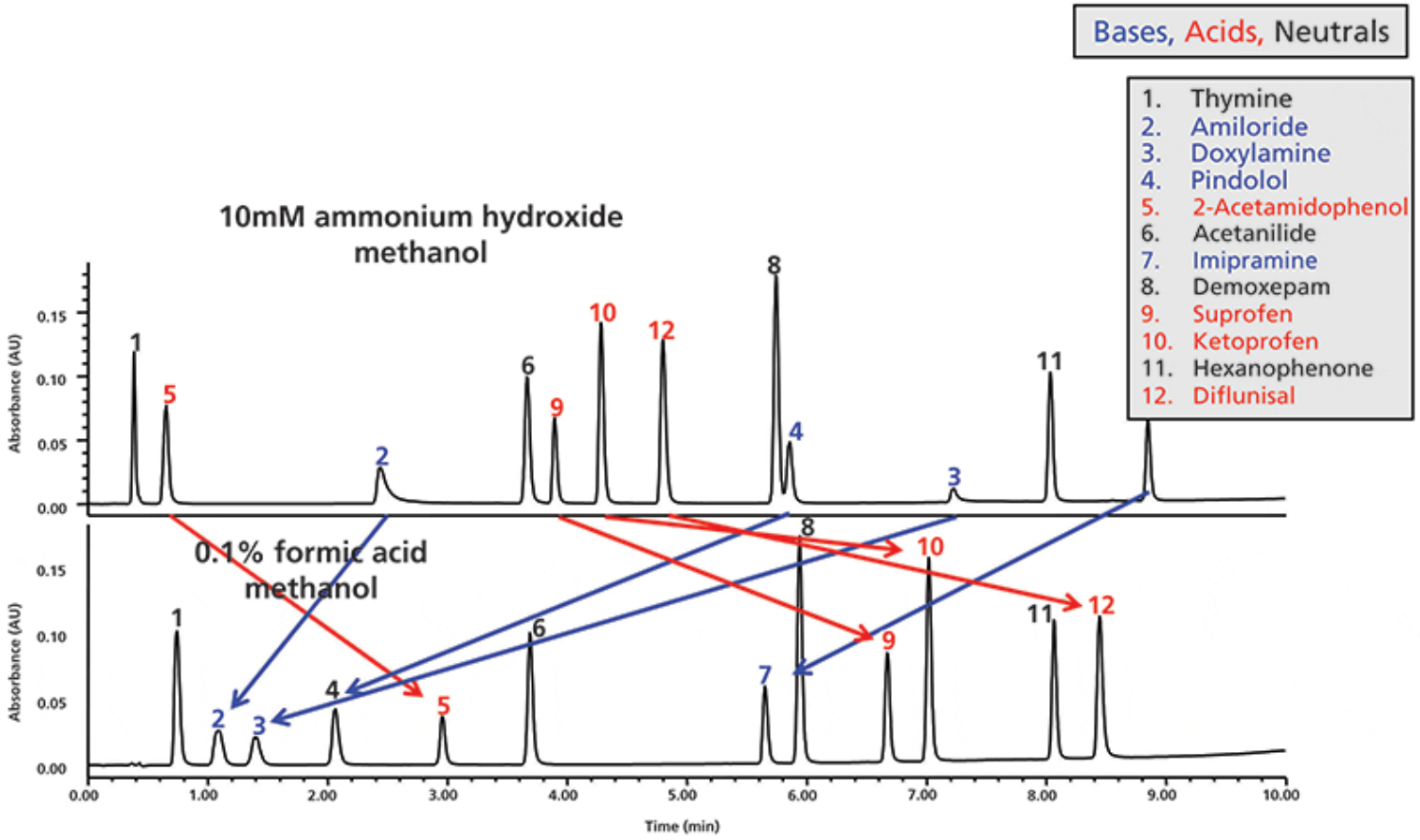
The pressure in HPLC is created by the pump and is used to force the mobile phase (the liquid solvent that carries the sample through the column) through the stationary phase (the packing material inside the column). The pressure in HPLC can range from a few hundred to several thousand psi (pounds per square inch) depending on the instrument and column used. **Higher pressure can improve the efficiency and resolution of the separation, but can also cause problems such as column packing or stationary phase degradation, leaks, and detector saturation.**

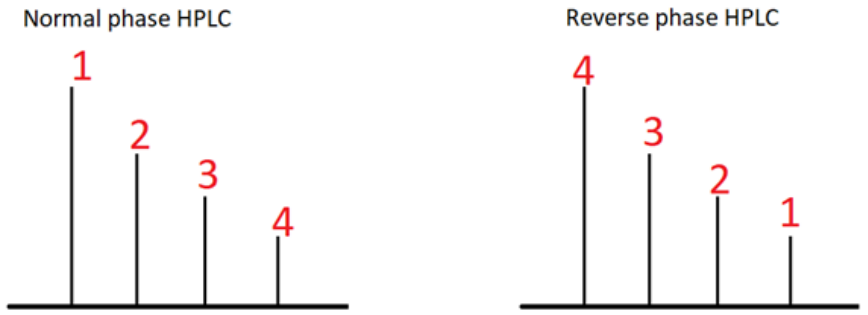
Flow rate:

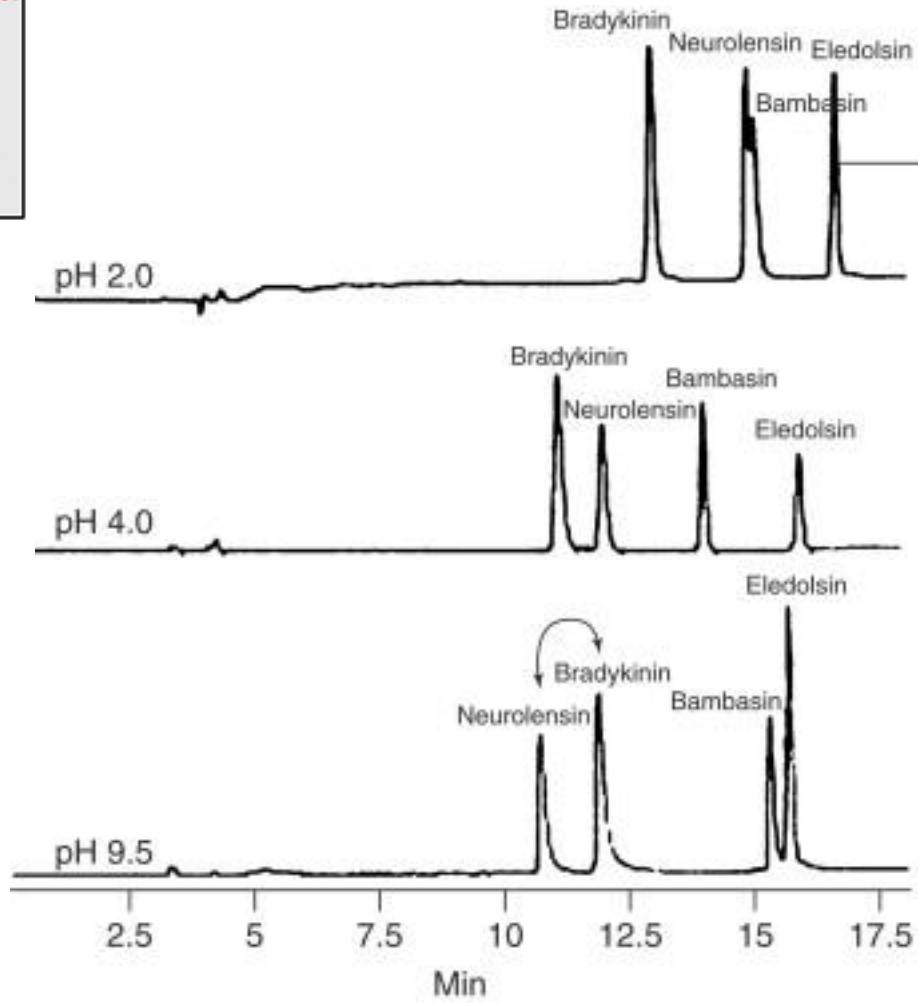
Flow rate in HPLC refers to the rate at which the mobile phase is pumped through the column. It is usually measured in milliliters per minute (mL/min) and can range from a few microliters per minute to several milliliters per minute. The flow rate affects the speed and quality of the separation, as well as the pressure required to achieve the desired flow rate. **A higher flow rate can result in a faster separation, but it can also decrease the resolution and sensitivity of the separation, and may cause peak broadening or loss of efficiency.**

In general, there is a balance between pressure and flow rate in HPLC that must be carefully optimized for each sample and column combination to achieve the best separation and chromatographic results.

How to drive separation?: Mobile phase





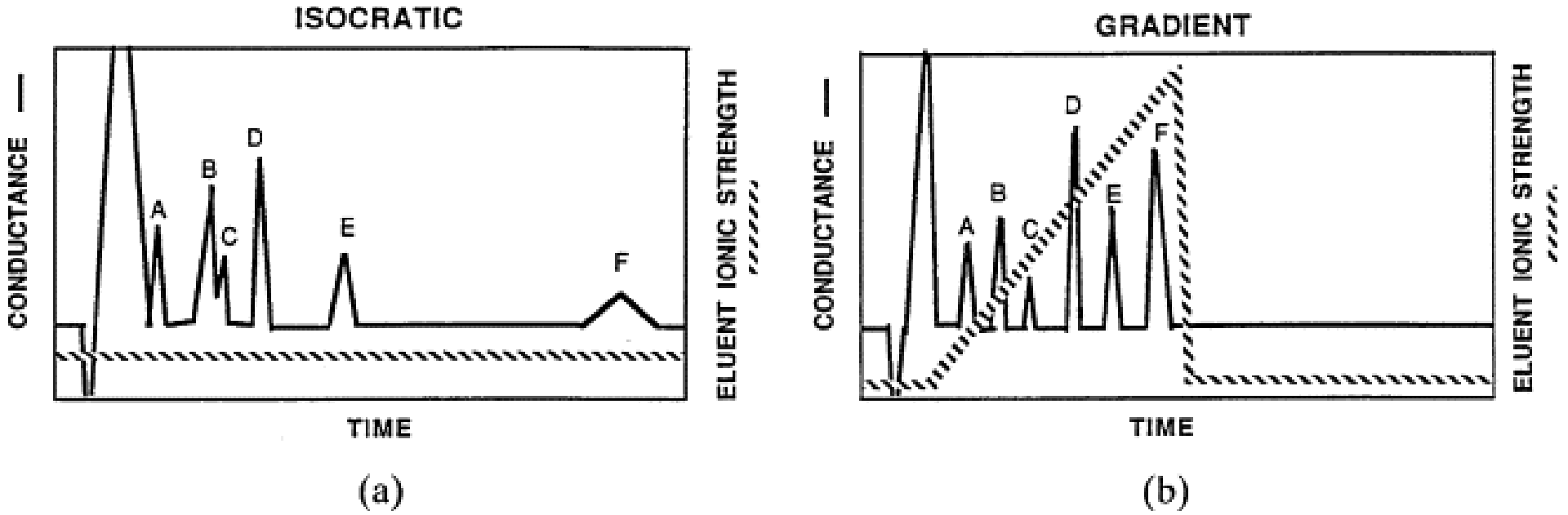


How to drive separation?: Mobile phase

In High-Performance Liquid Chromatography (HPLC), the mobile phase can be delivered to the chromatographic column using two different methods: isocratic elution and gradient elution.

Isocratic HPLC: In isocratic HPLC, the composition of the mobile phase **remains constant** throughout the entire chromatographic run. This means that the same ratio of solvents is used to elute all components of the sample. Isocratic elution is **relatively simple and fast to perform**, and it is commonly used in **routine analyses where separation of a limited number of components is required**. However, it may not be effective for separating complex mixtures or for resolving closely eluting peaks.

Gradient HPLC: In gradient HPLC, the composition of the mobile phase **changes over time**. This is achieved by using a solvent gradient program, where the proportion of one solvent is gradually increased while the proportion of the other solvent is decreased. This gradual change in the composition of the mobile phase results in a change in the eluent strength and provides better resolution of closely eluting peaks in complex samples. Gradient elution can be used to achieve **high selectivity and sensitivity in the analysis of complex mixtures.**



How to drive separation?: Stationary phase. Column

HPLC columns are devices that separate analytes based on their physical and chemical properties in a liquid chromatography system. The specificity of an HPLC column depends on the type and quality of the packing material (stationary phase), the size and shape of the particles, and the chemistry of the mobile phase. Different types of HPLC columns are designed for different applications and analytes, such as small molecules, biomolecules, reversed phase, normal phase, ion exchange, size exclusion, hydrophilic interaction chromatography (HILIC), mixed-mode, and application specific.

Some factors that affect the specificity of an HPLC column are:

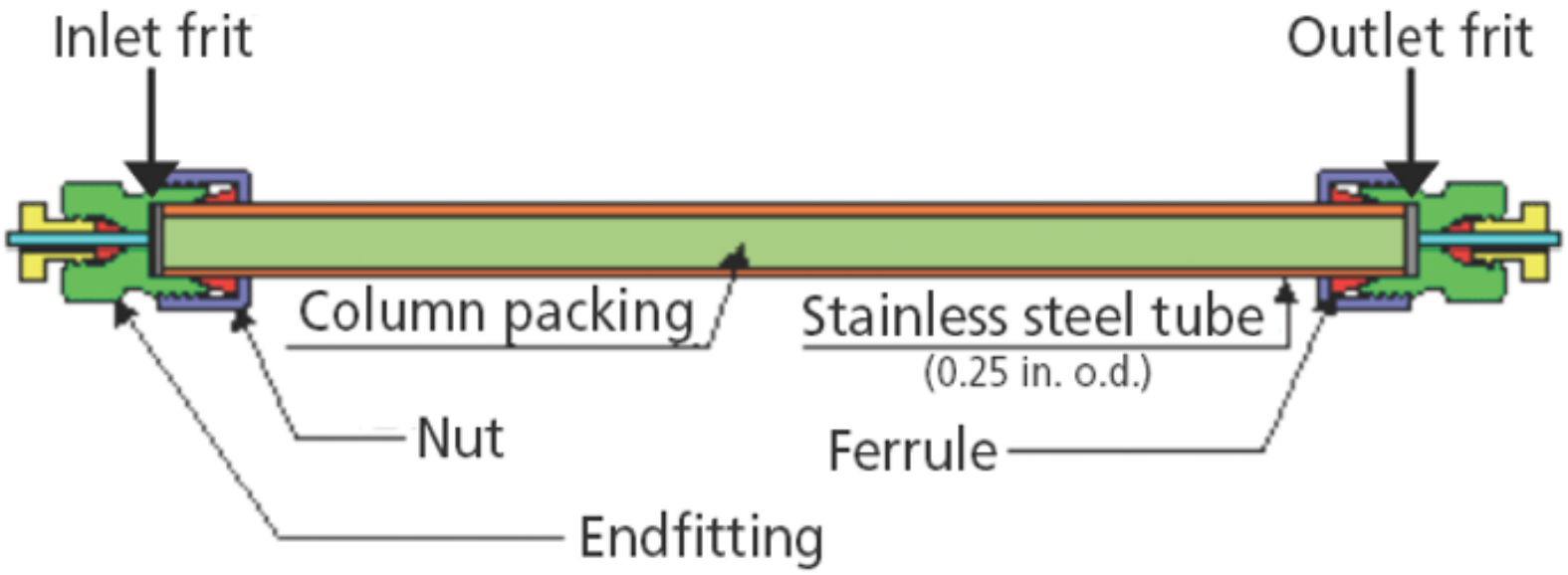
- **The surface area and pore size of the packing material:** Higher surface area and smaller pore size increase the interaction between analytes and stationary phase, resulting in higher resolution and specificity.

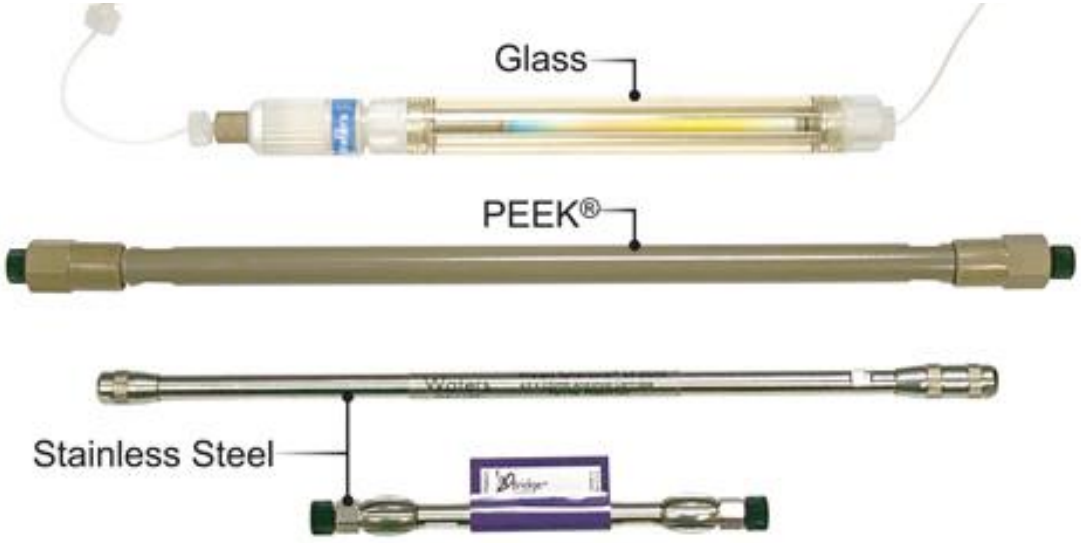
- **The particle size and shape of the packing material:** Smaller and spherical particles reduce diffusion and band broadening effects, leading to higher efficiency and specificity.

- **The chemistry of the packing material:** The functional groups on the surface of the packing material determine its polarity, charge, hydrophobicity/hydrophilicity, selectivity, and stability. Different chemistries can interact with different types of analytes in different ways.

- **The interaction with the mobile phase:** The composition, pH, ionic strength, organic modifier content, buffer type, and additives of the mobile phase affect its polarity, charge, hydrophobicity/hydrophilicity, solubility, and viscosity. These factors influence how analytes partition between mobile and stationary phases, modulating their retention time and specificity.

How to drive separation?: Stationary phase. Column





How to drive separation?: Stationary phase.
Columns (Examples)

There are several types of HPLC columns, each with a different stationary phase chemistry and particle size, designed for specific applications and separations. Here are some of the common types of HPLC columns:

C18 columns: These are the most commonly used HPLC columns, featuring a stationary phase with an octadecylsilane (ODS) functional group. They are widely used for the separation of small molecules, pharmaceuticals, and biomolecules.

C8 columns: Similar to C18 columns, but with a shorter alkyl chain length. They provide less retention than C18 columns and are used for less hydrophobic compounds.

Phenyl columns: These columns feature a stationary phase with a phenyl functional group, which provides aromatic selectivity. They are used for the separation of aromatic compounds and enantiomeric separations.

HILIC columns: HILIC stands for Hydrophilic Interaction Chromatography. These columns have a stationary phase that is polar and hydrophilic, and they are used for the separation of polar compounds such as sugars, peptides, and small polar molecules.

Ion exchange columns: These columns contain a stationary phase that is charged and are used for the separation of charged molecules such as proteins, peptides, and nucleic acids.

How to drive separation?: Stationary phase.
Columns (Examples)

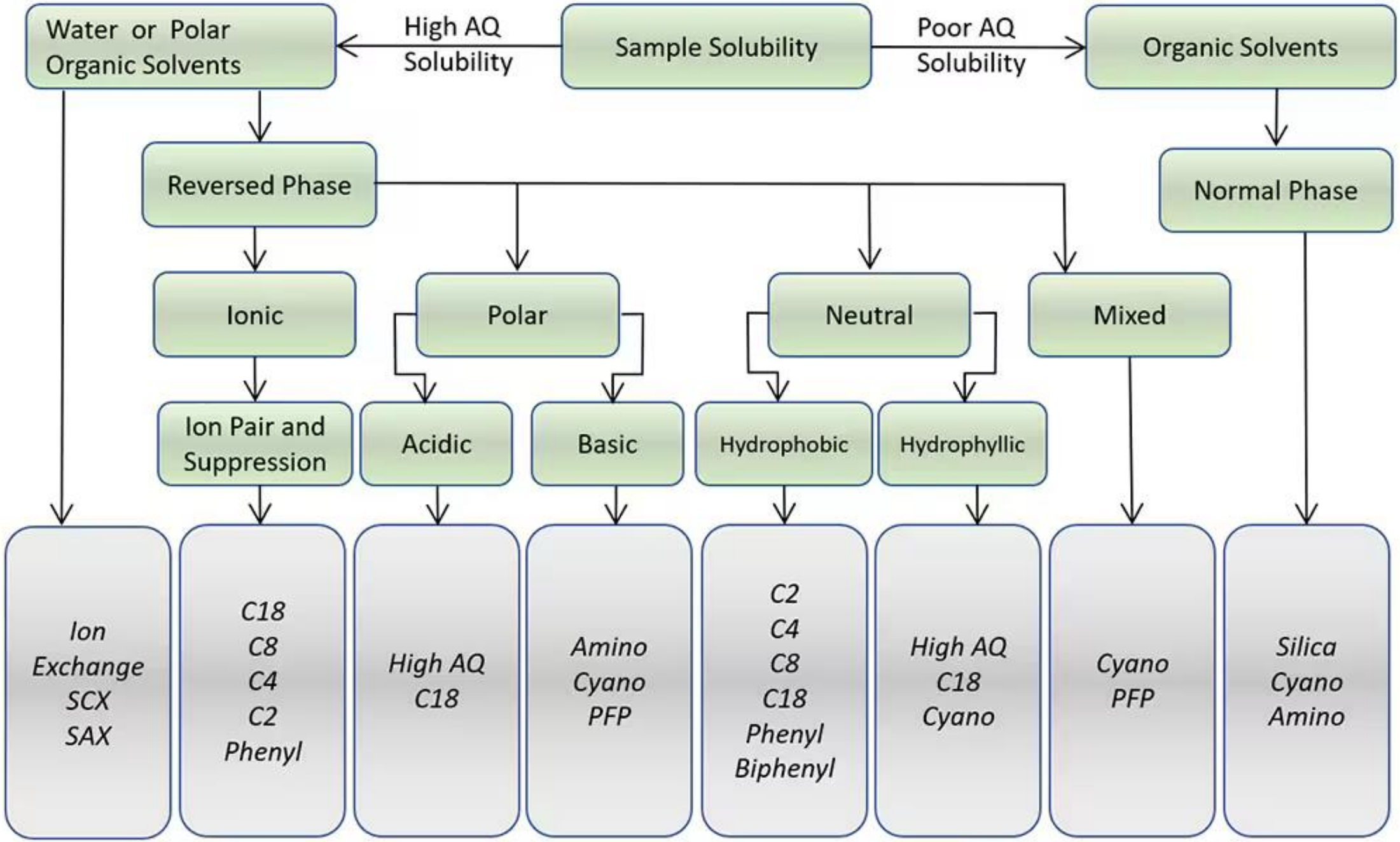
Chiral columns: These columns have a stationary phase that is chiral, which allows for the separation of enantiomers (mirror-image isomers).

Size exclusion columns: These columns contain a stationary phase with porous beads of a specific size range, which separates molecules based on their size and shape. They are often used for the separation of proteins, polymers, and other macromolecules.

Aminex® HPLC Columns for carbohydrate analysis: These columns use polymeric resins with sulfonated cross-linked styrene-divinylbenzene copolymers as stationary phases. They provide high-resolution separation of carbohydrates by ion exclusion mechanism at low pH.

MAbPac™ RP Columns for monoclonal antibody analysis: These columns use silica-based reversed-phase media with a hydrophilic bonded layer that minimizes nonspecific interactions with proteins. They provide high-resolution separation of monoclonal antibodies by hydrophobic interaction mechanism at neutral pH.

ENrich™ Q Columns for peptide analysis: These columns use solid core particles with quaternary amine functional groups as stationary phases. They provide high-resolution separation of peptides by ion exchange mechanism at low pH.


Water or Polar Organic Solvents
High AQ Solubility
Sample Solubility
Poor AQ Solubility
Organic Solvents
Reversed Phase
Normal Phase
Ionic
Polar
Neutral
Mixed
Ion Pair and Suppression
Acidic
Basic
Hydrophobic
Hydrophyllic
Ion Exchange SCX SAX
C18 C8 C4 C2 Phenyl
High AQ C18
Amino Cyano PFP
C2 C4 C8 C18 Phenyl Biphenyl
High AQ C18 Cyano
Cyano PFP
Silica Cyano Amino

HPLC detectors

UV-Vis detectors: These detectors are based on the absorption of light by the sample as it passes through the detector cell. They are commonly used for the detection of small molecules, such as pharmaceuticals and amino acids.

Fluorescence detectors: These detectors are based on the emission of light by the sample as it is excited by a light source. They are commonly used for the detection of fluorescent compounds, such as vitamins, peptides, and nucleic acids.

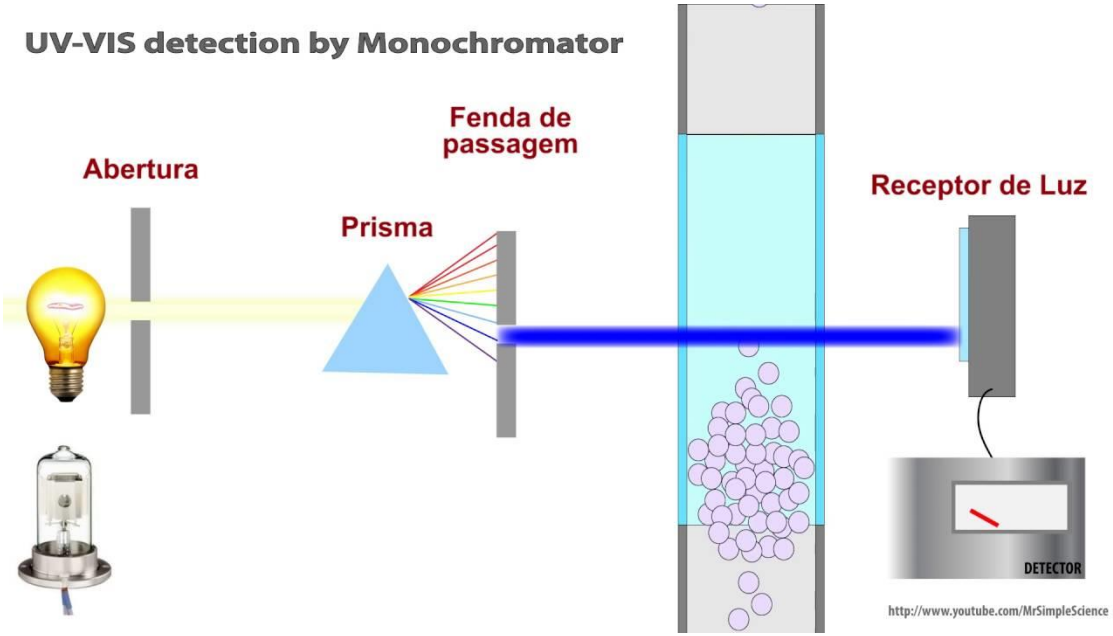
Refractive index detectors: These detectors are based on the refractive index of the sample, which changes as the sample passes through the detector cell. They are commonly used for the detection of non-chromophoric compounds, such as carbohydrates, lipids, and polymers.

Electrochemical detectors: These detectors are based on the electrochemical properties of the sample, such as its ability to conduct electricity. They are commonly used for the detection of compounds with electroactive functional groups, such as catecholamines, neurotransmitters, and amino acids.

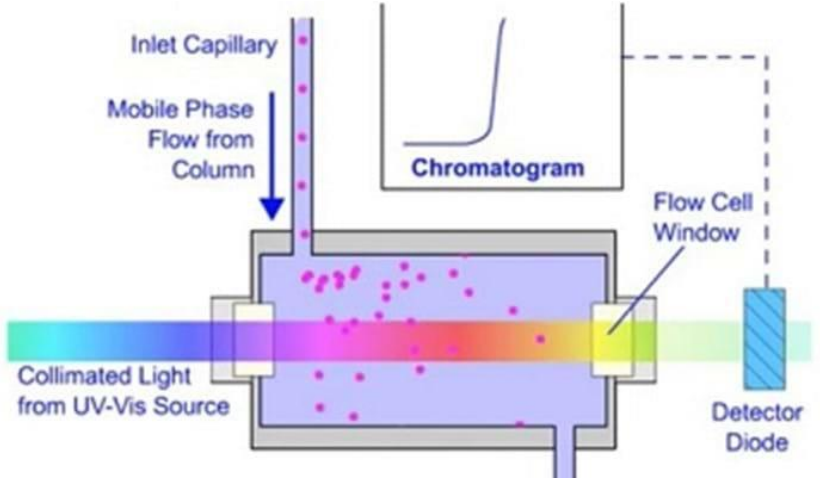
The choice of detector depends on the nature of the sample, the sensitivity and selectivity required, and the budget available for the instrument. Some HPLC systems can be equipped with multiple detectors, which can provide complementary information about the sample and improve the overall detection sensitivity and accuracy.

HPLC detectors: UV-Vis (1)

UV-Vis detectors are one of the most common types of detectors used in HPLC. These detectors operate based on the principle that **some compounds absorb light in the UV and visible regions of the electromagnetic spectrum.** In UV-Vis detection, a sample is passed through a flow cell, where it is exposed to a light beam at a specific wavelength**. The detector measures the amount of light that is absorbed by the sample, and this is used to determine the concentration of the analyte in the sample.**



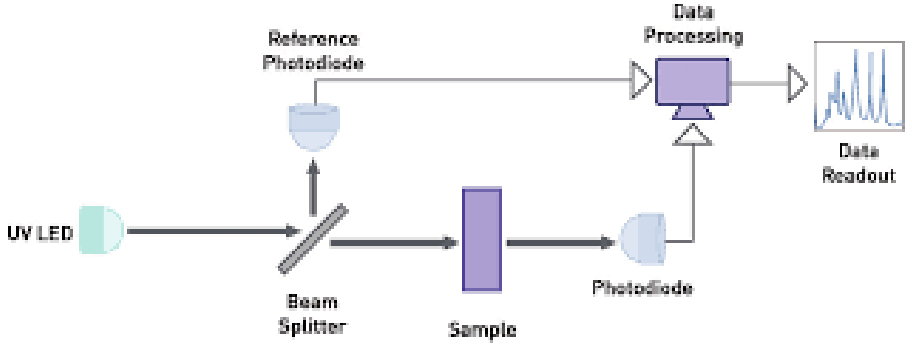
UV-Vis detectors are widely used in HPLC because they are simple, reliable, and provide good sensitivity and selectivity for many types of compounds. They are commonly used for the detection of small molecules, such as drugs, metabolites, and amino acids, as well as for the monitoring of chemical reactions and the purity of synthetic compounds. Some common UV-Vis detector wavelengths used in HPLC include 254 nm, 280 nm, and 360 nm.



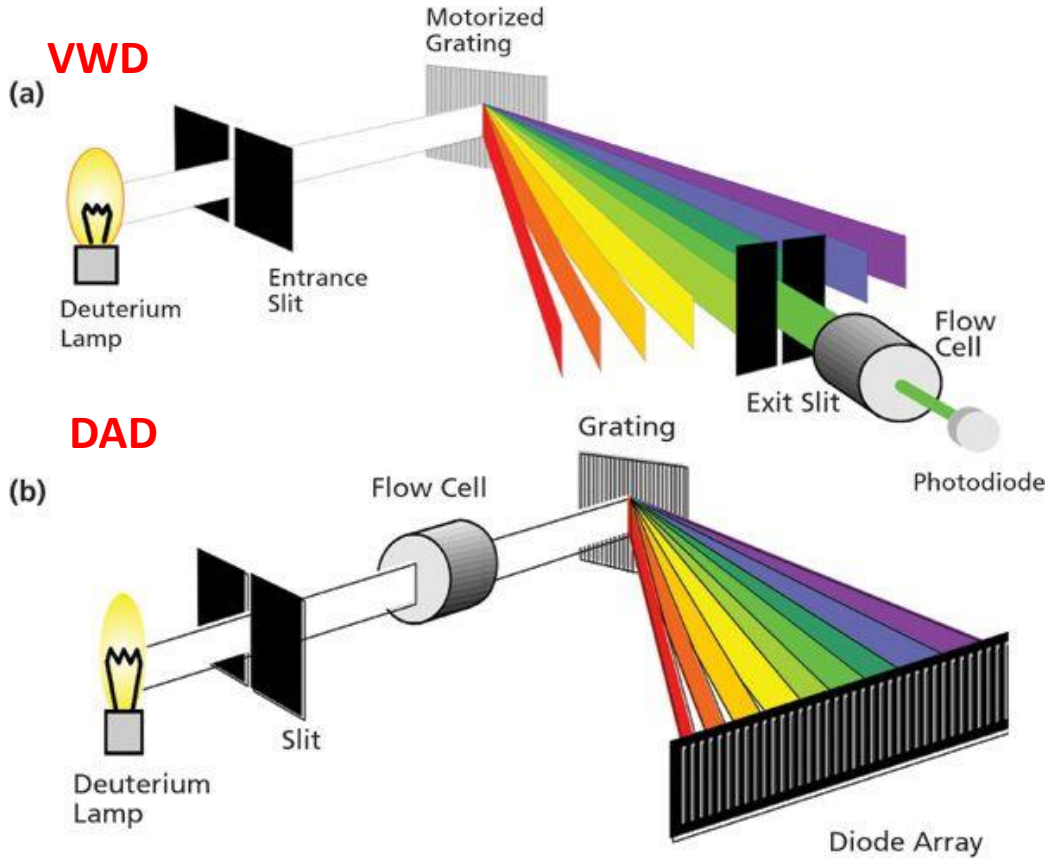
One limitation of UV-Vis detection is that it requires the sample to absorb light in the UV or visible regions, which can limit its usefulness for some types of compounds. Additionally, UV-Vis detectors may not be selective enough to distinguish between closely related compounds, and they may be subject to interference from other compounds in the sample matrix. However, UV-Vis detectors remain a popular and important tool in HPLC analysis.

HPLC detectors: UV-Vis (2)

Fixed-wavelength detectors operate at a specific, pre-determined wavelength (where generally 214, 220, 254, 280, 313, 334 or 365 nm may be used, depending on **the type of lamp**) which is selected based on the absorption properties of the analyte of interest. These detectors are simple and reliable, and they provide good sensitivity for compounds that absorb strongly at the selected wavelength. However, fixed-wavelength detectors are limited in their ability to detect compounds that absorb poorly at the selected wavelength or that have overlapping spectra with other compounds in the sample.

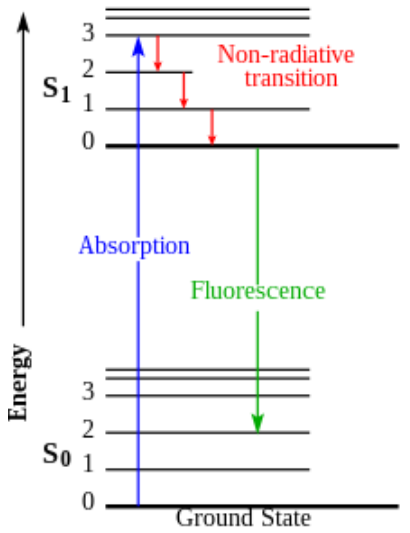


Variable-wavelength detectors, on the other hand, allow the wavelength of the light beam **to be changed** during the analysis. This allows the detector to scan across a range of wavelengths, which can be useful for detecting compounds that absorb poorly at a single wavelength or that have complex spectra. Variable wavelength detectors can have different designs and mechanisms, but they generally consist of a light source, a monochromator or filter, a sample cell and a photodetector. The monochromator or filter allows the selection of a specific wavelength or range of wavelengths to pass through the sample cell, where the absorbance is measured by the photodetector.

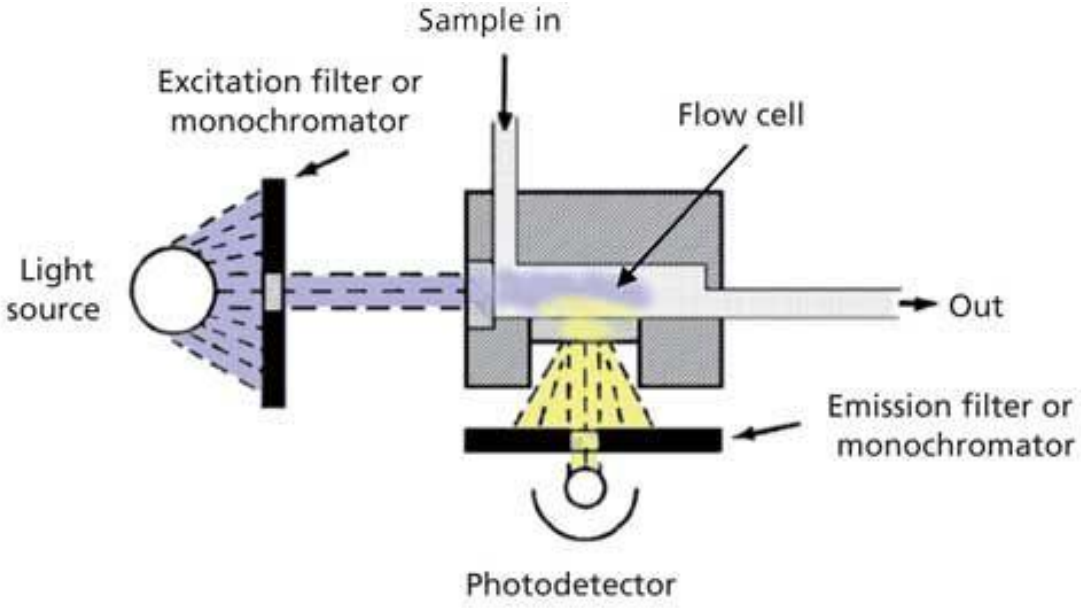


HPLC detectors: Fluorescence

Fluorescence detectors are another type of detector commonly used in HPLC analysis. These detectors are based on the principle that **certain compounds can absorb light at one wavelength and emit light at a longer wavelength, a phenomenon known as fluorescence. Fluorescence detectors measure the intensity of the emitted light, which is proportional to the concentration of the analyte in the sample.**



Fluorescence detection is often more sensitive than UV-Vis detection, particularly for compounds that have low UV absorbance or that are present at low concentrations in the sample. Additionally, fluorescence detectors can be more selective than UV-Vis detectors, as they can distinguish between compounds that have similar UV spectra but different fluorescence properties.



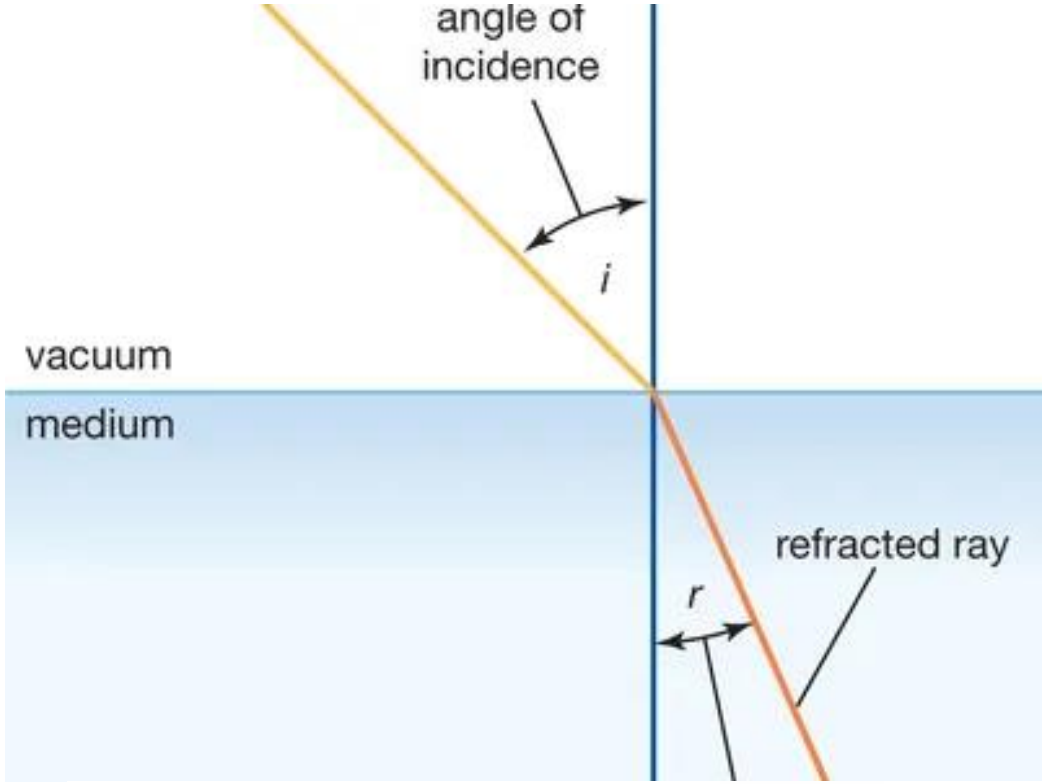
One limitation of fluorescence detection is that not all compounds are fluorescent, so this technique may not be suitable for all samples, although samples may be treated (with reagents like dansyl chloride) if analytes do not have fluorescence absorbance. Additionally, some compounds may exhibit background fluorescence, which can interfere with the measurement of the analyte of interest. Fluorescence detection also requires the use of a fluorophore, which can add an additional step to the sample preparation process.

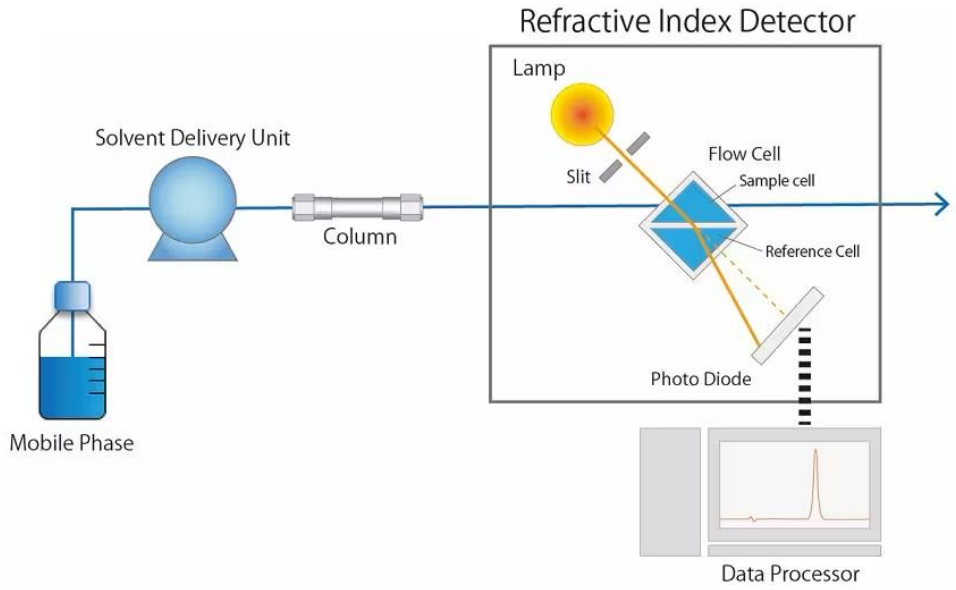
HPLC detectors: RI

A **refractive index (RI) detector** is another type of detector that is commonly used in HPLC analysis. **This type of detector is based on the principle that the refractive index of a material changes with its composition. RI detectors measure the difference in refractive index between the sample and the mobile phase, which is proportional to the concentration of the analyte in the sample.**

RI detectors are particularly useful for the analysis of compounds that do not absorb UV or visible light, such as sugars, alcohols, and polymers. They are also useful for the analysis of non-volatile or thermally unstable compounds that cannot be analyzed by gas chromatography. Additionally, RI detectors are non-destructive, so the sample can be collected after detection for further analysis.

One limitation of RI detection is that it is less sensitive than UV or fluorescence detection, and it is not as selective and it should not be used when there is a solvent gradient as a mobile phase. RI detectors also have a relatively high baseline drift, which can make them less suitable for the analysis of low-concentration samples. RI detectors are commonly used in the analysis of food and beverage products, pharmaceuticals, and polymers. They can be used in combination with other detectors to provide complementary information about the sample.





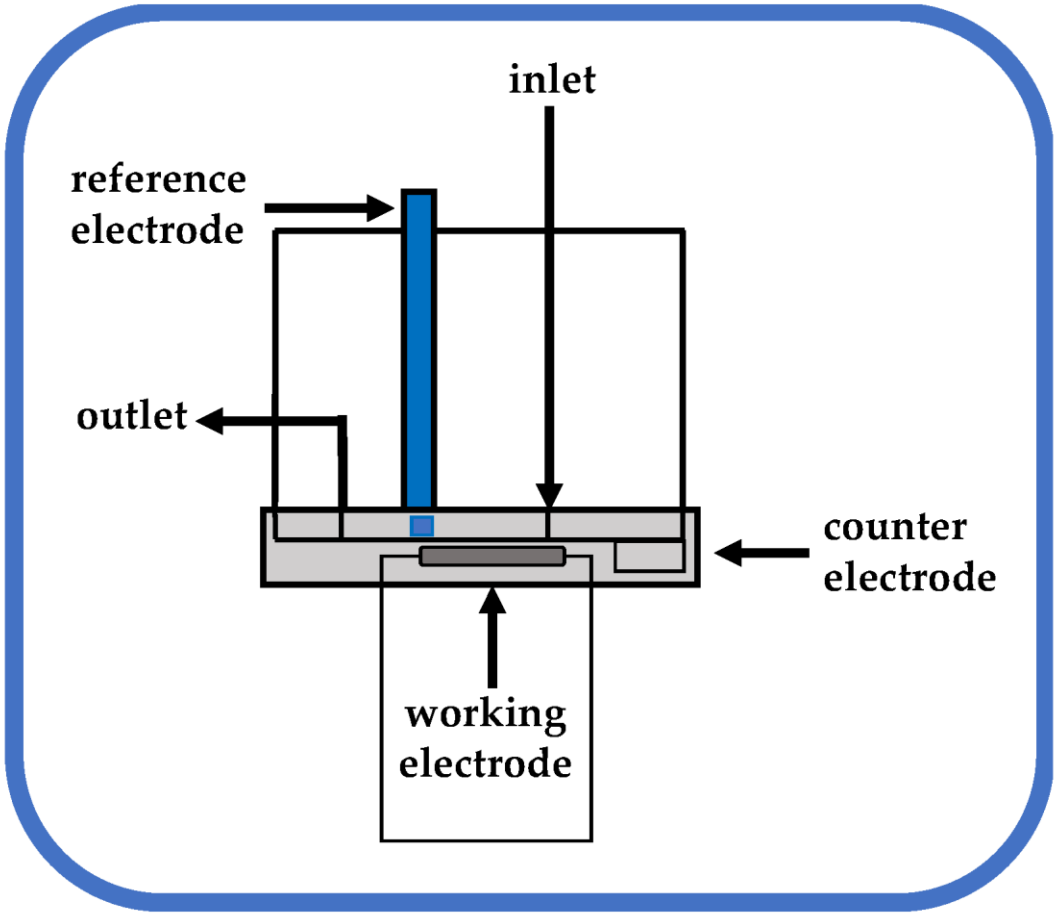
HPLC injection: ECD

An **electrochemical detector (ECD)** is a type of detector used in HPLC to detect and quantify analytes with **electroactive functional groups**. The ECD works by converting the chemical signal from the analyte into an electrical signal, which is then amplified and recorded.

The ECD contains an electrode, typically made of a noble metal such as platinum or gold, which is polarized to generate an electrochemical potential difference. **When the analyte enters the ECD, it interacts with the electrode and undergoes an electrochemical reaction, generating an electrical signal that is proportional to the concentration of the analyte.**

There are different types of ECDs, including amperometric and coulometric detectors. Amperometric detectors measure the current generated by the electrochemical reaction, while coulometric detectors measure the amount of charge transferred during the reaction.

ECD is suitable only for analytes with electrochemically active groups such as hydroxyl, amine, methoxyl, thioles, carbohydrates, etc. The ECD is also highly sensitive and selective, making it a popular choice for a wide range of applications in the fields of pharmaceuticals, environmental monitoring, and biochemistry.

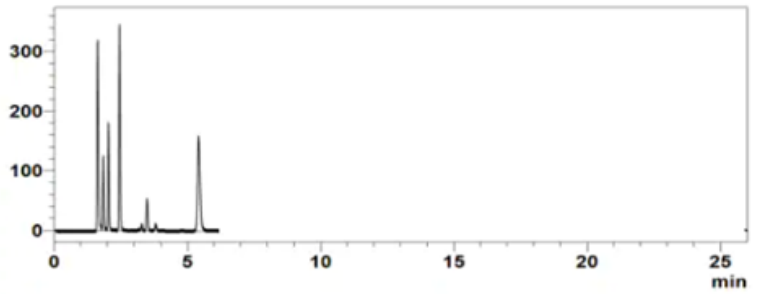
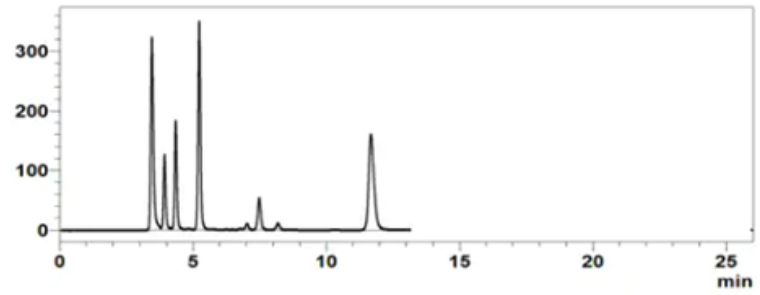
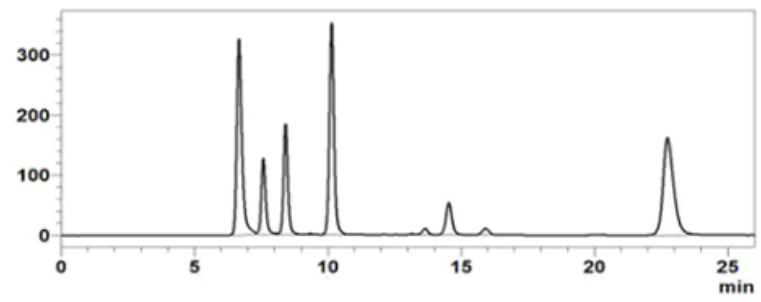


HPLC in steroids: UHPLC

Ultra high-performance liquid chromatography (UHPLC) is a type of HPLC that uses columns packed with **smaller particle sizes and operates at higher pressures** than traditional HPLC. UHPLC typically uses columns with particle sizes of 1.7-2.5 µm, compared to 3-5 µm for traditional HPLC, and operates at pressures up to 15,000 psi, compared to 4000-5000 psi for traditional HPLC.

The smaller particle sizes used in UHPLC columns allow for **faster separations and higher resolution than traditional** HPLC, due to the increased surface area available for interaction between the sample and stationary phase. The higher pressures used in UHPLC also help to increase efficiency and speed of separations.

UHPLC is used in a wide range of applications, including pharmaceuticals, biotechnology, environmental monitoring, and food and beverage analysis. It is particularly useful for the analysis of complex mixtures and for the separation of compounds that have similar properties.

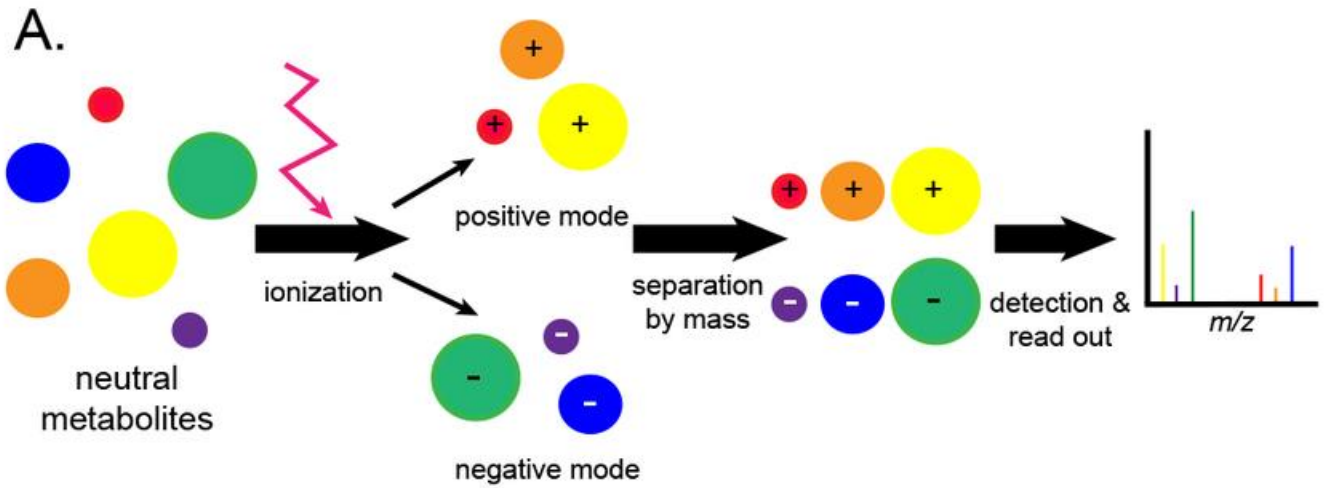
UHPLC — Highest resolution — Highest throughput — 90 to 120 runs per 8 hr. day — Highest Performance	1.7 - 5 µm	XS 105 MPa 15,000 PSI X3 130 MPa 19,000 PSI	
UHPLC — High resolution — High throughput — 40 to 60 runs per 8 hr. day — Performance	3- 5 µm	66 MPa 10,000 PSI	
Conventional HPLC — Forgiving for new users — 16 to 24 runs per 8 hr. day — Economy	5 µm	42 MPa 6,000 PSI	

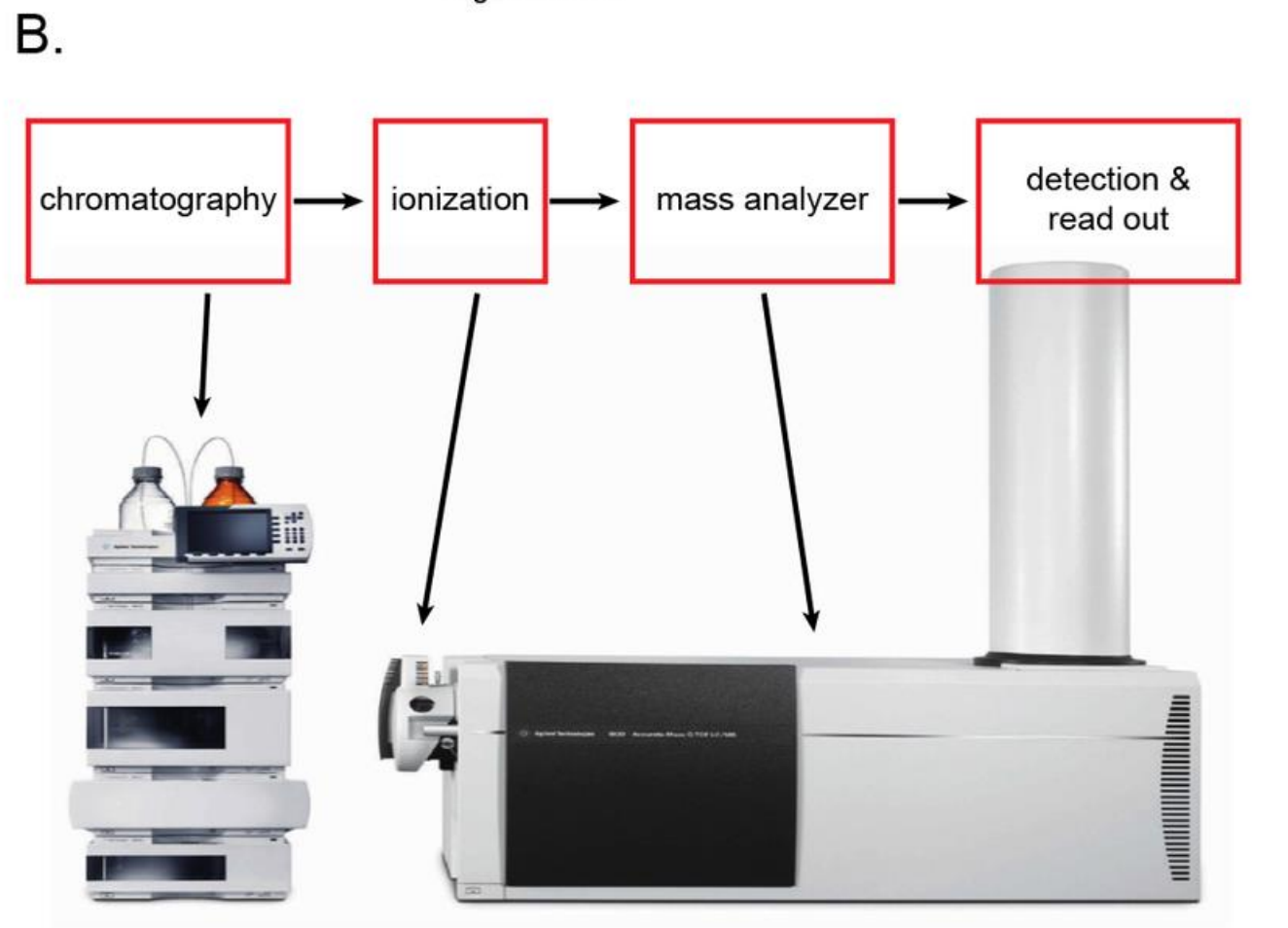
HPLC in steroids: HPLC-MS

HPLC-MS (high-performance liquid chromatography-mass spectrometry) is a hybrid analytical technique that combines the separation capabilities of HPLC with the detection and identification capabilities of mass spectrometry.

Mass spectrometry (MS) is an analytical technique that measures the mass-to-charge ratio of ions to identify and quantify compounds. In MS, a sample is ionized to create charged particles, which are then separated based on their mass-to-charge ratio using an electric or magnetic field. **The separated ions are then detected by a mass analyzer, which generates a spectrum that can be used to identify the compounds in the sample.**

HPLC-MS combines HPLC and MS techniques by using HPLC to separate the components of a mixture and then introducing them into the mass spectrometer for detection and identification based on their mass-to-charge ratio. HPLC-MS is a powerful analytical tool that can be used to identify and quantify a wide range of compounds, including small molecules, peptides, proteins, and nucleic acids.

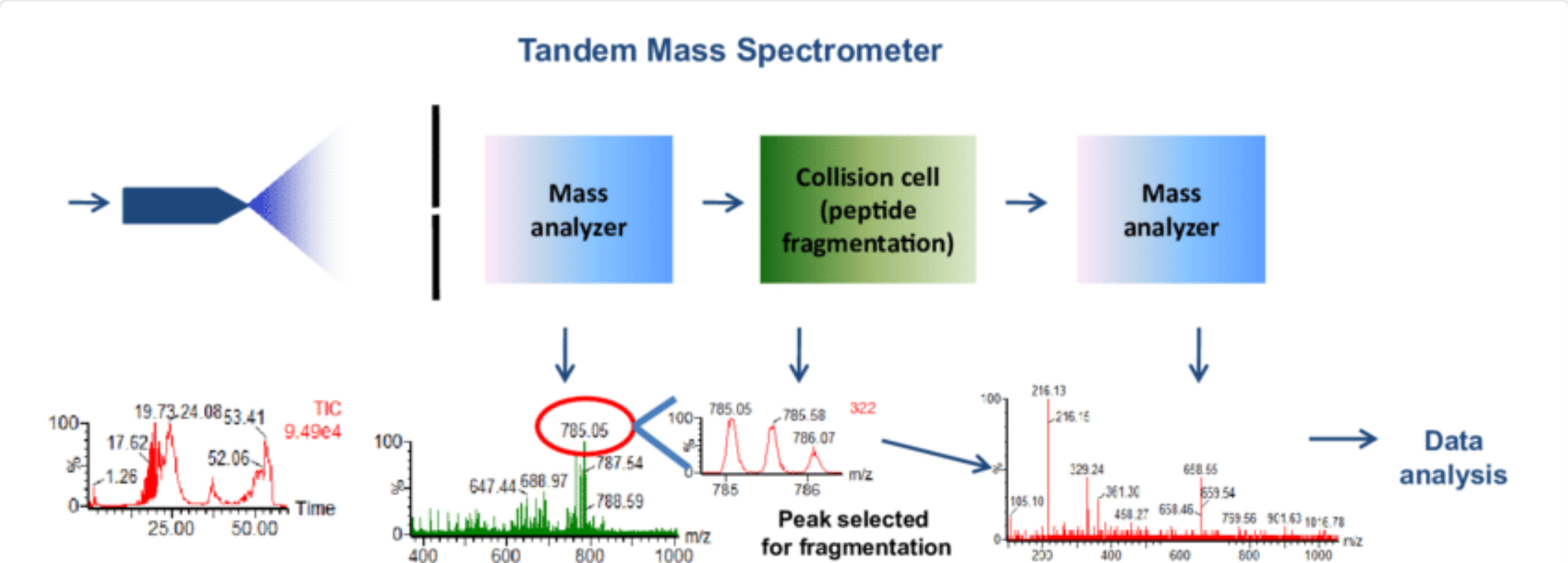




HPLC in lots of steroids: HPLC-MS/MS

HPLC-MS/MS is a combination of High Performance Liquid Chromatography (HPLC) and Tandem Mass Spectrometry (MS/MS). MS/MS is a powerful analytical technique used to identify and quantify individual components in a mixture. In MS/MS, the sample is ionized, and the resulting ions are separated based on their mass-to-charge ratio (m/z) in a mass spectrometer. **The ions are then fragmented into smaller ions, and the resulting fragment ions are analyzed to determine the identity and quantity of the original compounds.**

By combining HPLC and MS/MS, it is possible to separate and detect individual components in complex mixtures with high sensitivity and specificity. HPLC-MS/MS is commonly used in fields such as pharmaceuticals, biochemistry, and environmental science for qualitative and quantitative analysis of small molecules.



HPLC Applications

Chemical

polystyrenes
dyes
phthalates

Bioscience

proteins
peptides
nucleotides

Pharmaceuticals

tetracyclines
corticosteroids
antidepressants
barbiturates

Consumer Products

lipids
antioxidants
sugars

Environmental

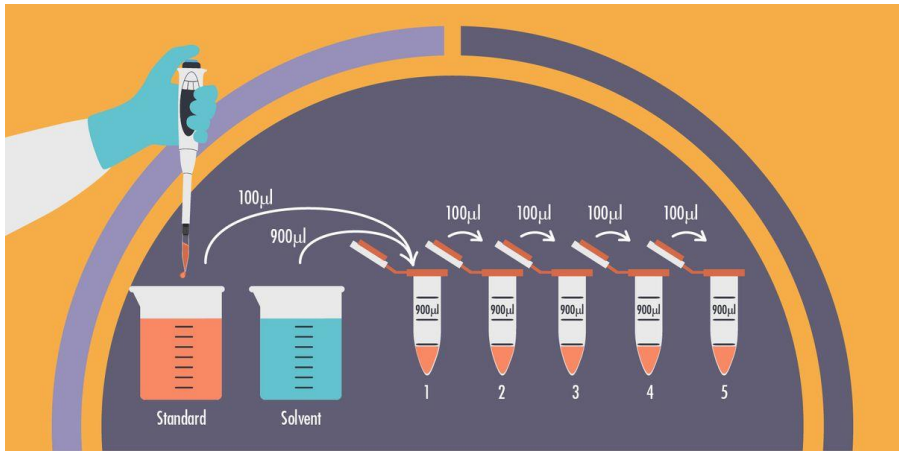
polyaromatic hydrocarbons
Inorganic ions
herbicides

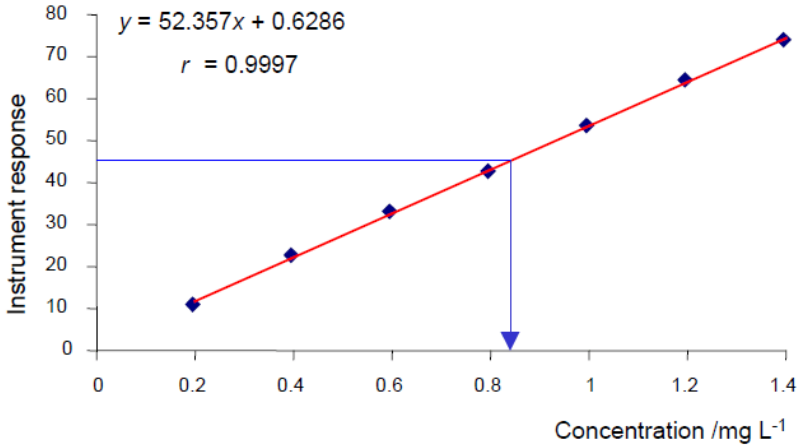
Clinical

amino acids
vitamins
homocysteine

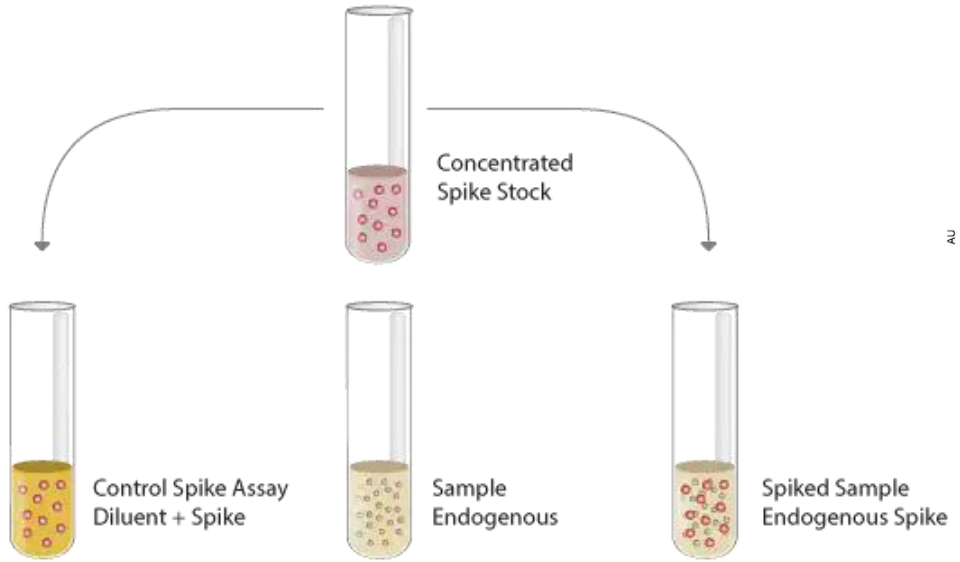
Need for a QA/QC System

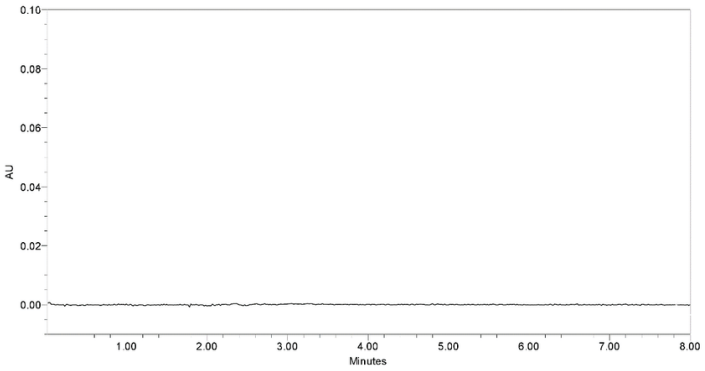
HPLC analyses should be performed within a framework where the quality of the produced data is guaranteed. Analytes must be analyzed within the **range of linearity** already established, where both separation and detection respond in a manner proportional to the concentration. **Calibration curves** must also meet the requirements of linearity, and be prepared with standards of known concentration and traceable to reliable sources. Within each batch, **quality control samples** must also be run and must comply with the specifications for each case (**method, instrument, and solvent blanks, spiked samples and percentages of recovery**, etc.). **Uncertainty** must be calculated and traceability of the sample during the entire process must be registered.

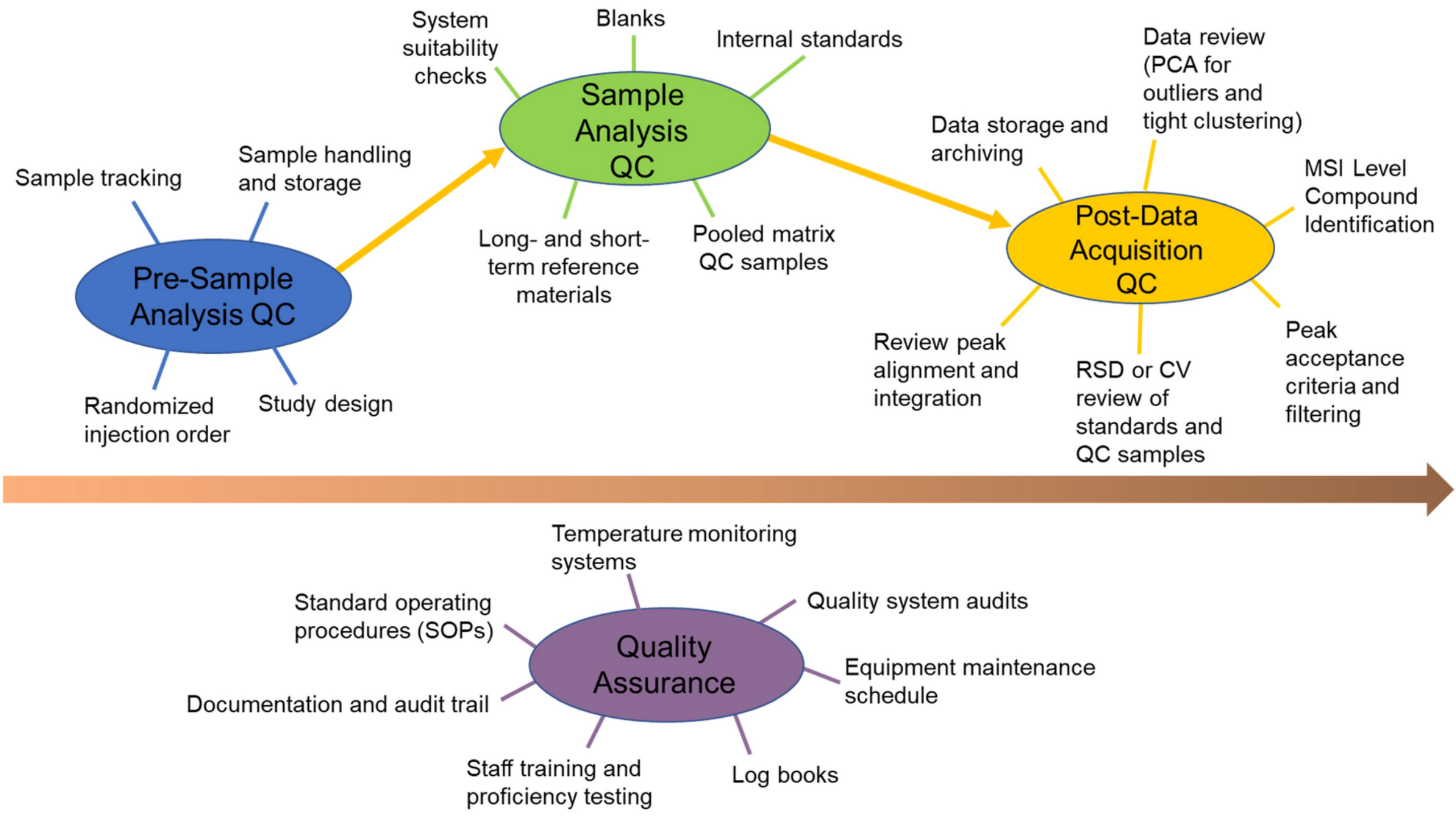




$$\% \text{ Recovery} = \left(\frac{\text{Concentration}_{\text{SPIKED ENDOGENOUS}} - \text{Concentration}_{\text{ENDOGENOUS}}}{\text{Concentration}_{\text{CONTROL SPIKE}}} \right) \text{x100}$$

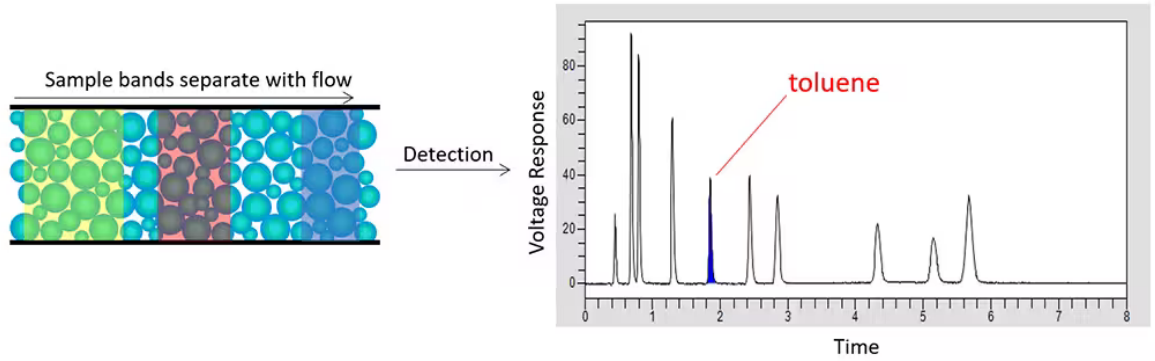


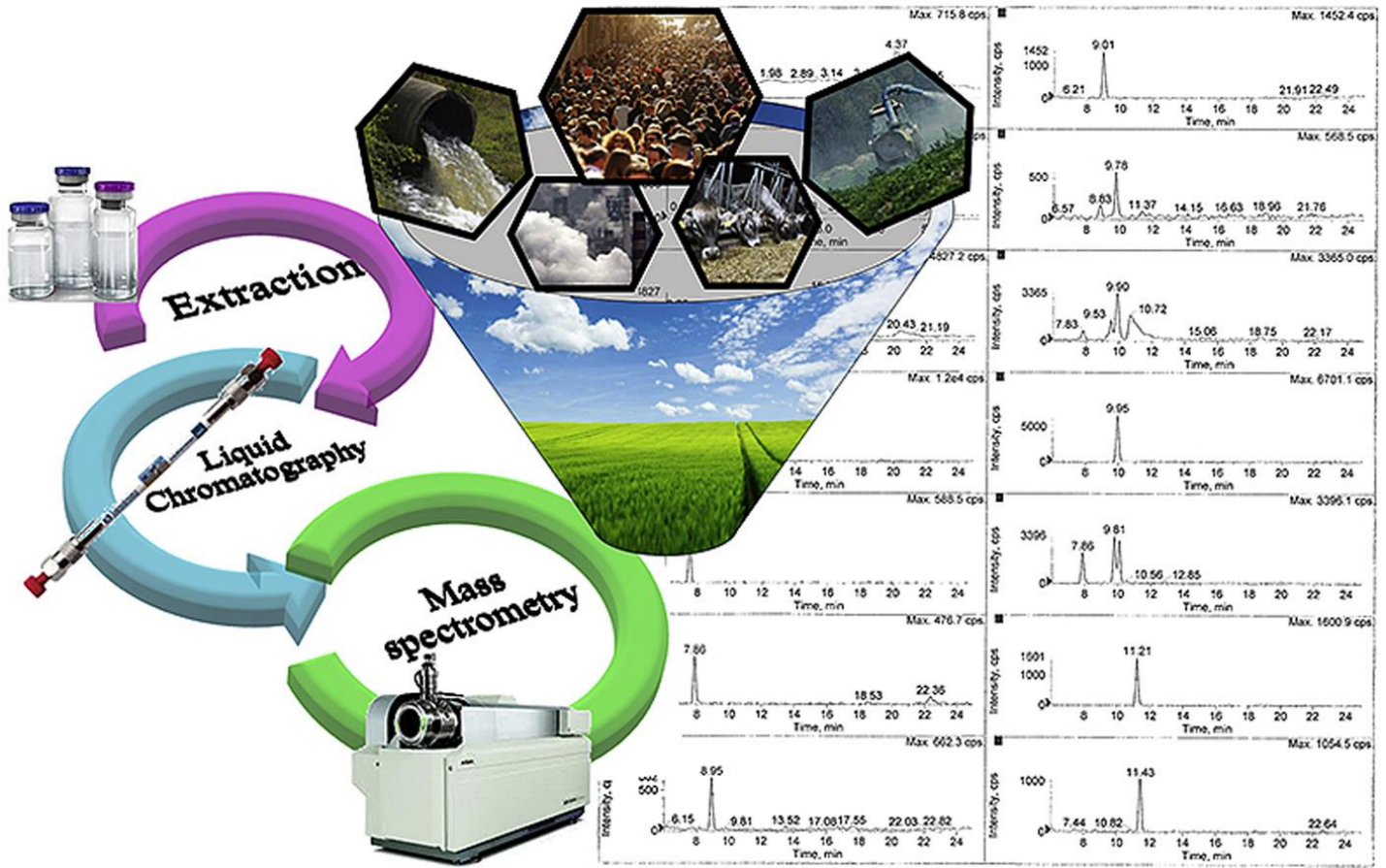



Pre-Sample Analysis QC
Sample tracking
Sample handling and storage
Randomized injection order
Study design
Sample Analysis QC
System suitability checks
Blanks
Internal standards
Long- and short-term reference materials
Pooled matrix QC samples
Post-Data Acquisition QC
Data storage and archiving
Data review (PCA for outliers and tight clustering)
MSI Level Compound Identification
Review peak alignment and integration
RSD or CV review of standards and QC samples
Peak acceptance criteria and filtering
Quality Assurance
Temperature monitoring systems
Standard operating procedures (SOPs)
Quality system audits
Documentation and audit trail
Equipment maintenance schedule
Staff training and proficiency testing
Log books

HPLC Applications (Examples)

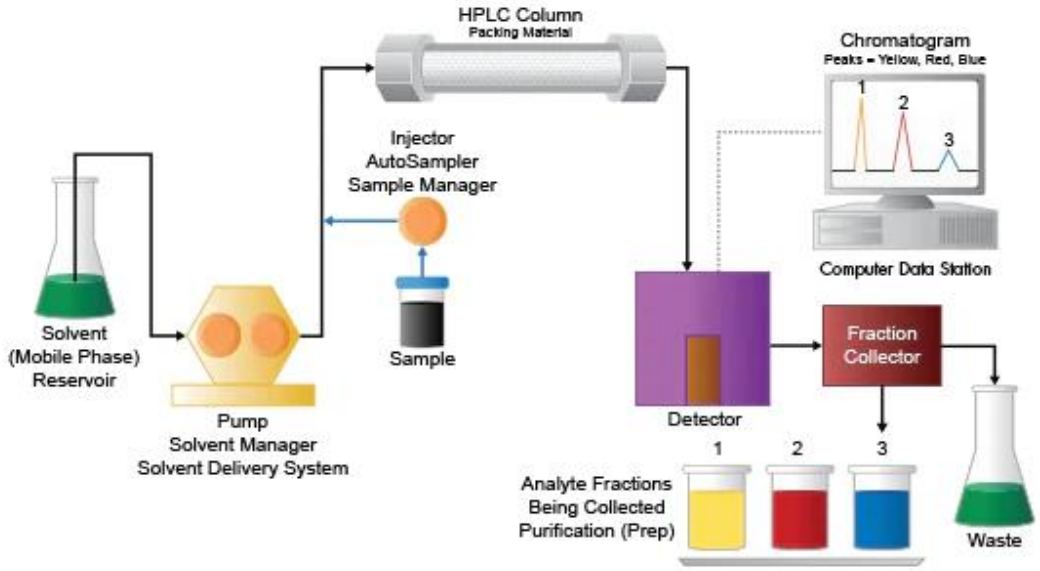
Chemical industry (for polystyrenes, dyes, phtalates, etc.); bioscience (proteins, peptides, nucleotides, etc.); **Pharmaceuticals** (several APIs including tetracyclines, corticosteroids, antidepresants, barbiturates, etc.); **Consumer products** (lipids, antioxidants, sugars, etc.); **Environmental sciences** (polyaromatic hydrocarbons, inorganic ions, herbicides, etc.); or **Clinical applications**, among several others (agricultural products, food technology, etc.). HPLC is included in several international regulations and technical procedures (such as USP, BP, JP, AOAC, ICH, etc.)

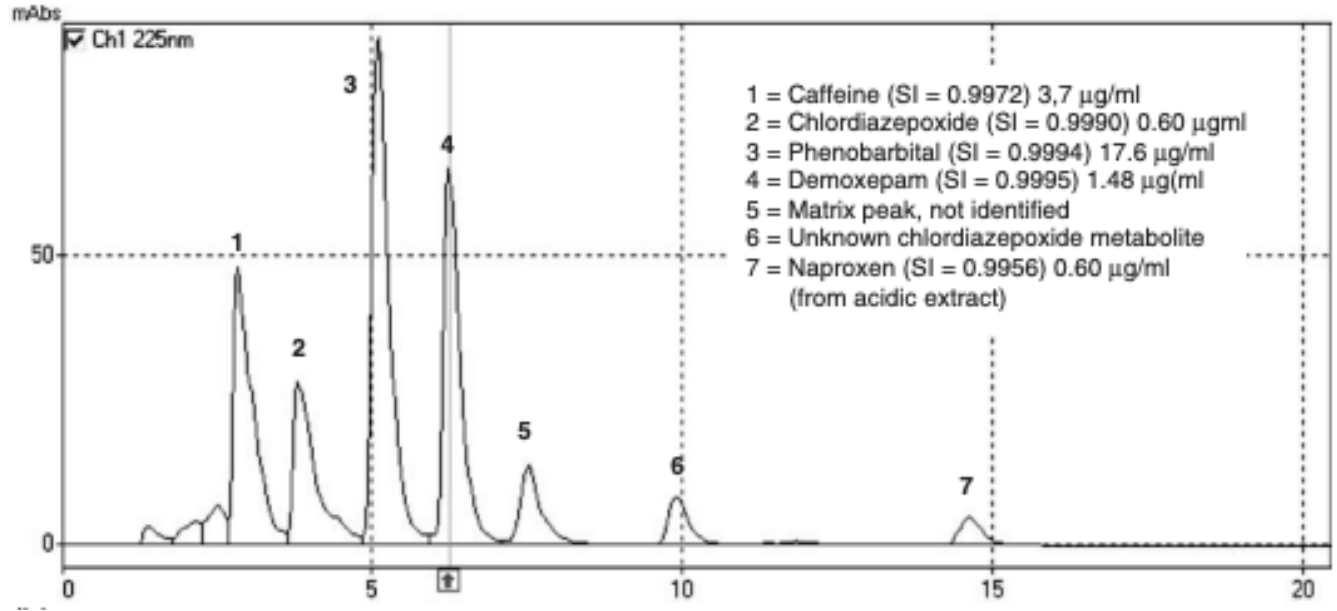




HPLC Applications. Examples (2)

HPLC may also be used for **preparative** purposes. If a non-destructive detector (such as UV-Vis) is used, fractions may be collected thus purification may be achieved. Although this method is expensive and the quantities are very small, thus it is not practical to use it for large manufacturing processes, it still can be used during the R&D stage. In pharmaceutical development, HPLC may also be a reliable and precise way to **check the quality and purity of a product**. In medical diagnosis or legal procedures, HPLC may be used for **determining substances in blood** (either nutrients or vitamins, or drugs such as opioids, LSD, cannabis, cocaine, and/or ketamine) (or their metabolites). Police may then either present or exempt charges based on evidence obtained through this method. In medical research, HPLC may be used to trace **specific metabolites which may be biomarkers of diseases**.





HPLC Applications. Food Industry 1

ADDITIVES

ACIDULANTS

Function: Render foods more palatable and stimulating; intensify certain tastes and mask undesirable aftertastes; buffer pH during and after processing; prevent growth of microorganisms; antioxidant synergist to prevent rancidity and browning; viscosity modifiers in baked goods; melting modifiers in cheese spreads and hard candy; meat curing agents to enhance color or flavor. ***Examples:*** Sorbic, citric, acetic, succinic, lactic, tartaric and phosphoric acids.

ANTIOXIDANTS

Function: Natural vitamins may be present in food or formed by processes such as smoking. Synthetic antioxidants retard onset of rancidity by preventing the oxidative degradation of lipids. ***Examples:*** *Natural:* vitamins C (ascorbic acid) and E (tocopherols). *Synthetic:* butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), mono-tert-butylhydroquinone (TBHQ).

PRESERVATIVES

Function: Inhibit microbial growth in foods and beverages. ***Examples:*** Benzoic, sorbic, and propionic acids; methyl, ethyl and propyl esters of p-hydroxy benzoic acid (PHB).

FLAVORS

Function: Three major classes of flavoring agents: essential oils, bitter compounds and pungency compounds. ***Examples:*** Vanillin, lupulon and humulon (hop bittering compounds).

Other Important Subclasses of Additives

ARTIFICIAL SWEETENERS (*Examples:* Acesulfam, aspartame, saccharin)
COLORANTS (*Examples:* E104 Quinolin yellow, E131 Patent blue)

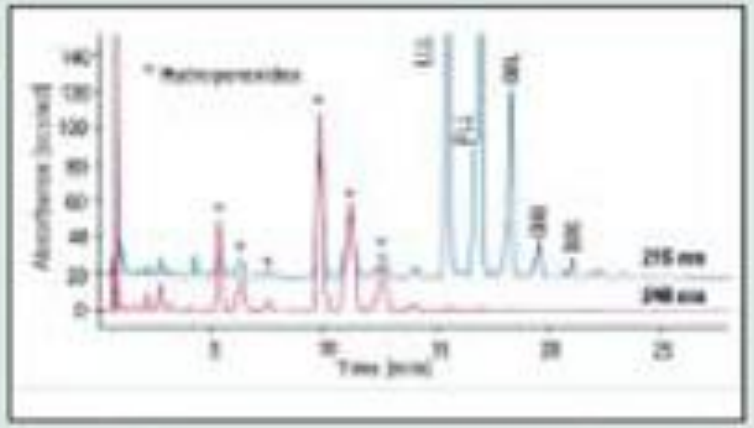
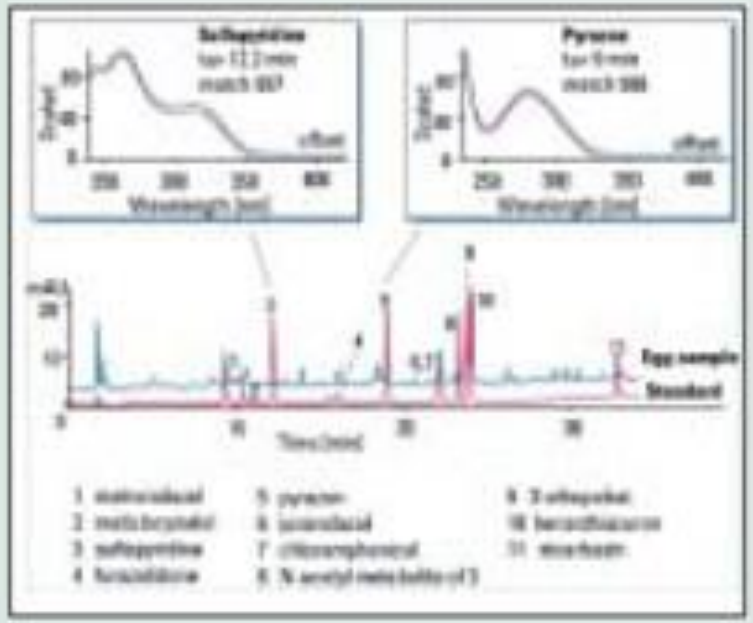


Figure 8. Triglyceride pattern of aged sunflower oil.



HPLC Applications. Food Industry 2

NATURAL COMPONENTS

Inorganic Ions

Function: Nitrites act as preservatives in cured and smoked meats. ***Examples:*** Halogen-, nitrogen-, and sulfur-containing foods and water.

Lipids

Function: Triglycerides containing unsaturated fatty acids react with light and oxygen to form hydroperoxides, which strongly influence the taste and quality of fats and oils. Adulteration with foreign fats and the use of triglycerides modified by a hardening process also can be detected. (Figure 8) ***Examples:*** Saturated and unsaturated triglycerides; fats and oils; complex mixtures of triglycerides, sterols and vitamins.

Biogenic Amines

Function: Present in various food products and beverages, including fish, cheese, wine, and beer. ***Examples:*** Ammonia, histamine, iso-butylamine, methylamine, 3-methyl-butylamine.

Other Important Subclasses of Natural Components

Amino Acids (*Examples:* 23 naturally occurring amino acids.)
Carbohydrates (*Examples:* Glucose, fructose, sorbitol, lactose, maltose, sucrose.)
Fatty Acids (*Examples:* Saturated and unsaturated fatty acids from C4 through C22.)
Vitamins (*Examples:* Fat-soluble: E, D, A. Water-soluble: C, B6, B2, B1, B12.)

RESIDUES AND CONTAMINANTS

Chemotherapeutics and Antiparasitic Drugs

Function: Used in intensive animal breeding for suppression of diseases caused by viruses, bacteria, protozoa, and/or fungi. Residues of these drugs can be found in foods of animal origin such as milk, eggs and meat. (Figure 15) ***Examples:*** Nitrofurans and sulfonamides such as sulfapyridine, chloramphenicol, furazolidone, nicarbazin; tetracyclines; fumonisins.

Mycotoxins

Function: Highly toxic compounds that can contaminate food products when storage conditions are favorable to fungal growth. (Figure 16) ***Examples:*** Aflatoxins G2, G1, B2, B1, M2, M1; ochratoxin A; zearalenone; patulin; more than 100 are known.

Reactive Intermediates

Function: Present in the three most common coatings (epoxy lacquer, organosol lacquer and polyester lacquer) used to protect the inside surfaces of cans used for food packaging. Tends to migrate into and remain stable in the fatty phase of high-fat food; hydrolyzed in water. (Figure 17) ***Example:*** Bisphenol A.

Other Important Subclasses of Residues and Contaminants

Pesticides (*Examples:* Triazines, phenylurea-herbicides, carbamates.(Figure 18)

Figure 15. Analysis of drug residues in an egg sample. Identification through spectra comparison.

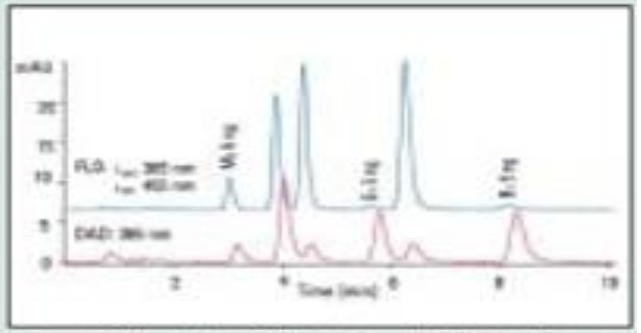


Figure 16. Analysis of aflatoxins with UV and fluorescence detection.

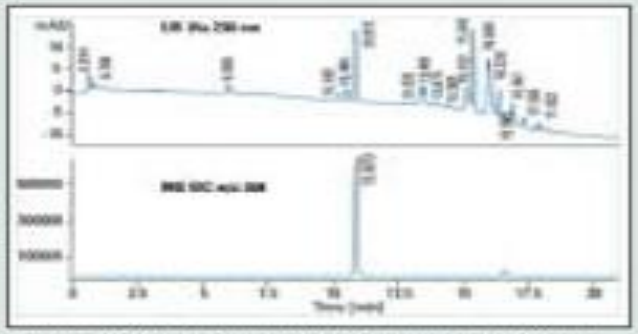


Figure 17. Analysis of extract from sardine 20 ppm.

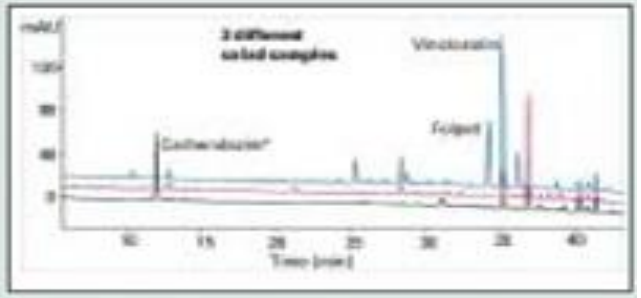


Figure 18. Analysis of pesticide residues in three different salad samples.

Example of parameters used for HPLC Analysis

API	Column	Mobile phase composition	Detection wavelength	Retention time (min)	Flow rate (mL min^{-1})
Amoxicillin Sodium	Waters X-Bridge C18	MeOH: Phosphate buffer pH 3.0 (10:90 v/v)	227 nm	6.00	1.0
Antipyrine	Waters X-Bridge C18	ACN:Water (25:75 v/v)	242 nm	4.12	0.7
Atenolol	ACE 5 C18	MeOH: Phosphate buffer pH 6.0 (20:80 v/v)	226 nm	3.96	0.7
Caffeine	Waters X-Bridge C18	MeOH : Water (30:70 v/v)	270 nm	4.63	0.7
Carbamazepine	Waters X-Bridge C18	ACN : Phosphate buffer pH 6.0 (40 :60 v/v)	285 nm	3.50	1.0
Cimetidine	Waters X-Bridge C18	MeOH : Phosphate buffer pH 3.0 (30:70 v/v)	214 nm	4.67	0.7
Enalapril	Waters X-Bridge C18	MeOH : Phosphate buffer pH 3.0 (5:95 v/v)	208 nm	3.26	0.7
Furosemide	ACE 5 C18	ACN: Phosphate buffer pH 3.0 (40 :60 v/v)	272 nm	4.04	1.0
Hydrochlorothiazide	Waters X-Bridge C18	ACN : Water (30:70 v/v)	271 nm	3.22	0.7
Ibuprofen	ACE 5 C18	ACN: Phosphate buffer pH 3.0 (65 :35 v/v)	224 nm	4.49	1.0
Ketoprofen	ACE 5 C18	ACN: Phosphate buffer pH 3.0 (50 :50 v/v)	259 nm	4.66	1.0
Metoprolol Tartrate	Waters X-Bridge C18	ACN: Phosphate buffer pH 3.0 (25 :75 v/v)	221 nm	3.20	1.0
Methyldopa	ACE 5 C18	MeOH: Phosphate buffer pH 6.0 (20:80 v/v)	280 nm	2.67	0.7
Naproxen Sodium	Waters X-Bridge C18	ACN : Phosphate buffer pH 3.0 (25 :75 v/v)	332 nm	4.09	1.0
Pindolol	Waters X-Bridge C18	ACN: Phosphate buffer pH 3.0 (20 :80 v/v)	264 nm	3.06	1.0
Piroxicam	Waters X-Bridge C18	ACN : Phosphate buffer pH 3.0 (50 :50 v/v)	349 nm	3.20	1.0
Propranolol HCl	Waters X-Bridge C18	ACN : Phosphate buffer pH 6.0 (35 :65 v/v)	228 nm	3.22	1.0
Ranitidine	Waters X-Bridge C18	MeOH : Phosphate buffer pH 6.0 (20 :80 v/v)	321 nm	5.45	0.7
Theophylline	Waters X-Bridge C18	MeOH : Water (30:70 v/v)	271 nm	3.78	0.7
Verapamil HCl	ACE 5 C18	ACN : Phosphate buffer pH 3.0 (40 :60 v/v)	229 nm	4.99	1.0

* 150 x 4.6 mm, 5 µm HPLC columns were used for analyses.

Kaynak *et al.*, 2017

Method selection/ development

1. Use an existing method as a reference

- Pharmacopeia
- Manufacturer’s application libraries –Thermo scientific AppsLab-
- Column catalogues
- Published articles

2. Check analyte / matrix for selection of column /mobile phase (considering efficiency, selectivity, affinity), and detector type. Perform a scouting run.

3. Optimize separation conditions: Column temperature / Mobile phase flow rate / Gradient program /

4. Evaluate: Speed / Resolution / Peak shape

5. **If required:** Test robustness

6. **If required:** Validate in compliance with regulations