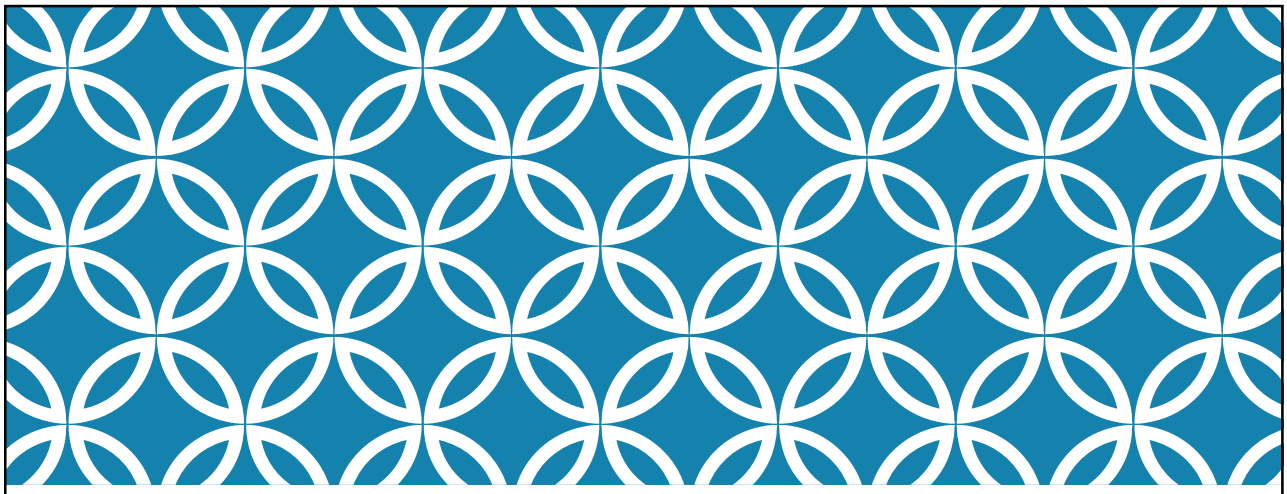


HIGH PERFORMANCE LIQUID CHROMATOGRAPHY - A REFRESHER

Loice Kikwai, PhD

OUTLINE - TODAY

- High performance liquid chromatography(HPLC):
  - Theory and practical implications of HPLC concepts such as
    - Mobile phase,
    - Detection wavelength
    - Injection volume
    - Column chemistry, and ion pairing along with proper
    - System and sample preparation methods
    - Quantification and calculations

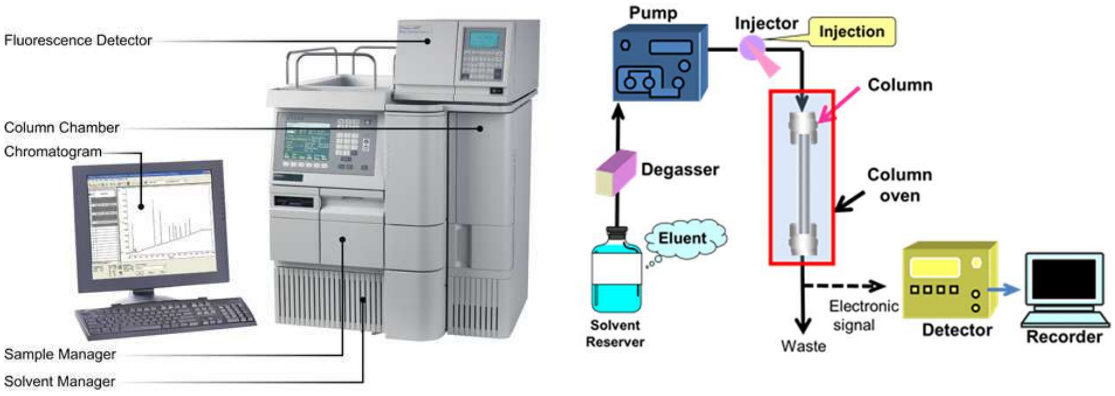
OUTLINE – NEXT PRESENTATIONS

- **Presentation 2**
- HPLC methodology
- Content of HPLC test procedure
- System Suitability Testing (SST)
- Validation of HPLC analytical procedure (validation parameters role).
  - Selectivity
  - Sensitivity (LLOQ)
  - Linearity
  - Accuracy and Precision
  - Ruggedness

- **Presentation 3**
- Small Molecule Quantitation
- Method Development
- Method Validation
- Validation criteria
- Subject Sample Analysis

WHAT IS HPLC?

- HPLC is a technique for separation, identification and quantification of components in a mixture.
- It is especially suitable for compounds which are not easily volatilized, thermally unstable and have high molecular weights.



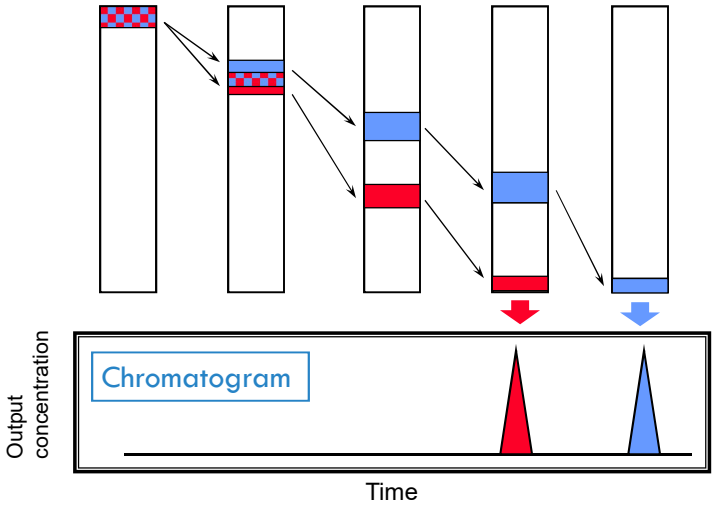
CHROMATO-GRAPHY / -GRAPH / -GRAM / -GRAPHER

- Chromatography: Analytical technique
- Chromatograph: Instrument
- Chromatogram: Obtained "picture"
- Chromatographer: Person

LIQUID CHROMATOGRAPHY

- Chromatography in which the mobile phase is a liquid.
  - The liquid used as the mobile phase is called the "eluent".
- The stationary phase is usually a solid or a liquid.
- In general, it is possible to analyze any substance that can be stably dissolved in the mobile phase.
- Differences in the interactions between the solutes and stationary and mobile phases enable separation.

SEPARATION PROCESS AND CHROMATOGRAM FOR COLUMN CHROMATOGRAPHY



FROM LIQUID CHROMATOGRAPHY TO HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

- Higher degree of separation!
  →Refinement of packing material (3 to 10 µm)
- Reduction of analysis time!
  → Delivery of mobile phase by pump
  → Demand for special equipment that can withstand high pressures

- The arrival of high performance liquid chromatography!

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ADVANTAGES OF HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

- High separation capacity, enabling the batch analysis of multiple components
- Superior quantitative capability and reproducibility
- Moderate analytical conditions
  - Unlike GC, the sample does not need to be vaporized.
- Generally high sensitivity
- Low sample consumption
- Easy preparative separation and purification of samples

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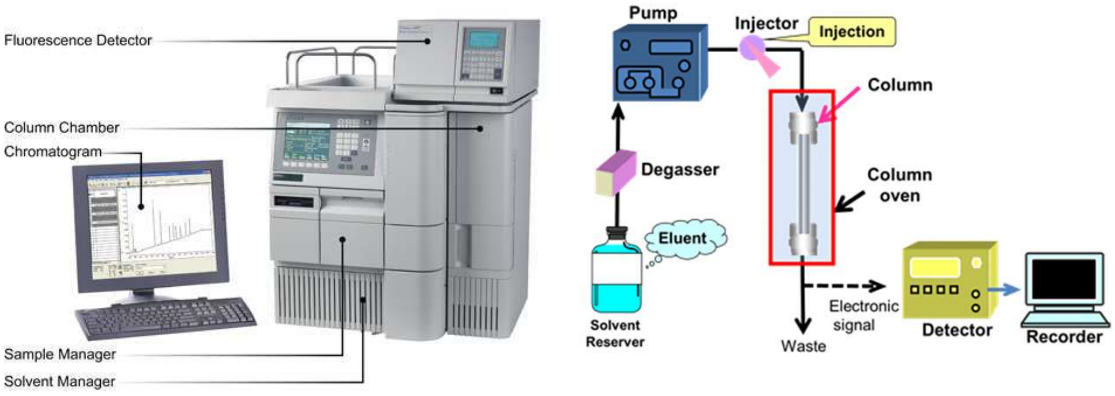
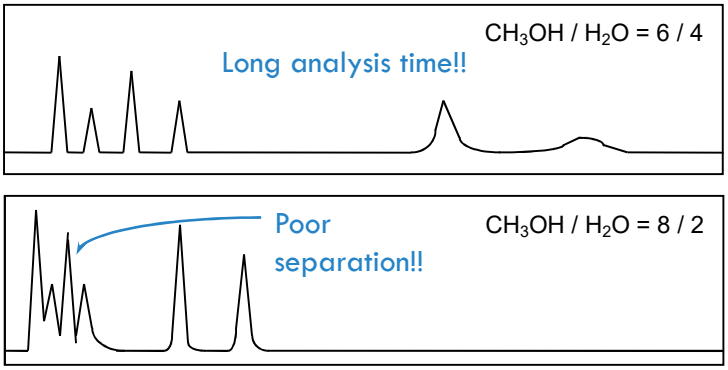


SOLVENT DELIVERY PUMP

- The pump provide a flow of the mobile-phase through the HPLC injector, column, and detector.
- Performance Requirements
  - Capacity to withstand high load pressures.
  - Pulsations that accompany pressure fluctuations are small.
  - Flow rate does not fluctuate.
  - Solvent replacement is easy.
  - The flow rate setting range is wide and the flow rate is accurate.
- Isocratic system
  - Constant eluent composition
- Gradient system
  - Varying eluent composition
    - HPGE (High Pressure Gradient)
    - LPGE (Low Pressure Gradient)

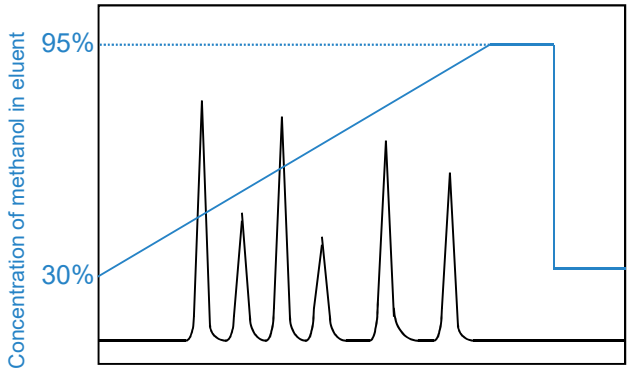
ISOCRATIC SYSTEM



(Column: ODS type)

GRADIENT SYSTEM

- If the eluent composition is changed gradually during analysis...



DEGASSER

- Problems caused by dissolved gas in the mobile phase
  - Unstable delivery of mobile phase by pump
  - More noise and large baseline drift in detector cell

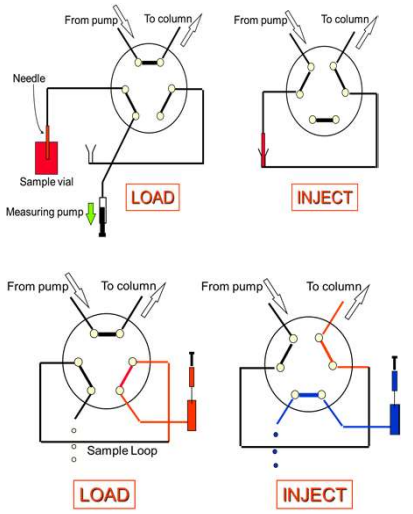
In order to avoid these problems, the eluent must be degassed.

SAMPLE INJECTION UNIT (INJECTOR)

- Introduction of the samples at high pressure into the HPLC system with high precision, without interruption of the mobile phase flow.
- Manual or Automated
  - greater productivity and the highest level of precision
- The valve injection through fixed or variable loop is a common way of introducing the sample.
- The Rheodyne valve is the mostly used devise.
  - The loop can be partially or fully filled.
  - There are both the types of injectors available.
  - The advantage of partial filling is the possibility of using small amount of sample, when there is scarcity of sample.
  - The precision of the injection is 1% RSD

SAMPLE INJECTION UNIT (INJECTOR)

- Performance Requirements
  - No sample remaining in unit
  - Minimal broadening of sample band
  - Free adjustment of injection volume
  - Minimal loss
  - Superior durability and pressure resistance



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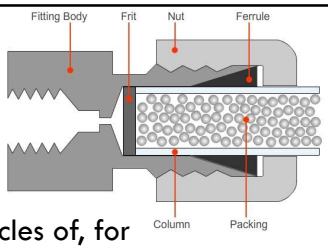
COLUMN



- The most common form of stationary phase consists of fine particles of, for example, silica gel or resin packed into a cylindrical tube.
- These packed particles are called “packing material” or “packing” and the separation tube into which they are packed is called the “separation column” or simply the “column”.
- Packing material:
  - Pellicular (spherical, non-porous, polymer beads with typical diameter of 30 to 40 μm.
  - Porous particles (porous silica particles with typical diameter of 3 to 10 μm.
    - Porous silica particles gives better column efficiency, sample capacity, and speed of analysis
- Stainless steel tube which is sealed with fittings on both ends.
- Guard columns are used to protect the actual separation column from chemical contamination.
- The dimensions: 25-250 mm long, 1-10 mm internal diameter.

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COLUMN OVEN

- This unit maintains the column at a constant temperature. Temperature is an important factor that influences separation, so maintaining the column at a constant temperature makes it possible to improve the quality of separation and the reproducibility. This unit is also called a thermostatic column chamber.
- Air circulation heating type
- Block heating type
  - Aluminum block heater
- Insulated column jacket type
  - Water bath

DETECTOR

- This unit detects the components eluted from the column by monitoring the mobile phase as it emerges from the column
- There are many different types of detectors, based on various operating principles, and the detector used is selected according to the properties of the target compounds and the objective of analysis.
  - UV-VIS absorbance detectors are the most commonly used.
- Performance requirements
  - Adequate sensitivity for the particular task.
  - Good stability and reproducibility.
  - Wide linear dynamic range of response.
  - Short response time that is independent on flow rate.
  - Insensitive to changes in solvent, flow rate, and temperature.
  - Cell design that eliminates remixing of the separated bands.
  - High reliability and ease of use.
  - Non-destructive for the sample

REPRESENTATIVE HPLC DETECTORS

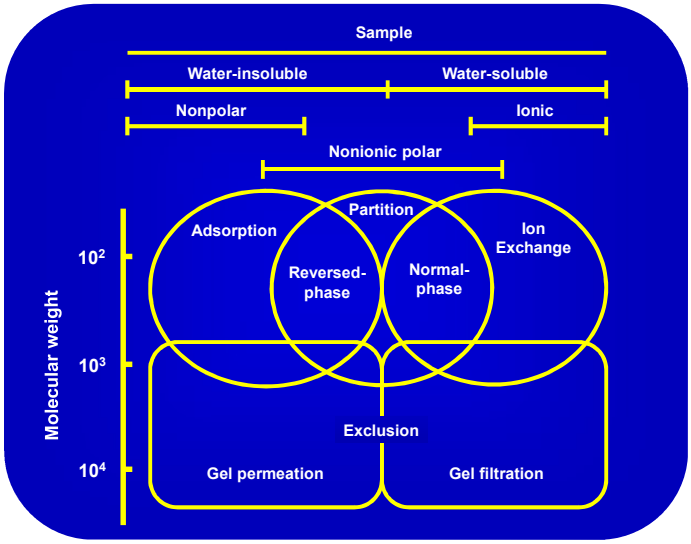
- Photodiode array-type UV-VIS absorbance detector
  - Peak Identification Using Spectra
    - Complementation of identification based on retention time
    - Library searches
  - Evaluation of Peak Purity
    - Purity evaluation performed by comparison of the shape of spectra from the peak detection start point to the peak detection end point
- Mass spectrometer
  - Quantitative analysis with excellent selectivity
  - Peaks can be identified with MS spectra
- UV-VIS absorbance detector
- Fluorescence detector
- Refractive index detector
- Evaporative light scattering detector
- Electrical conductivity detector
- Electrochemical detector

COMPARISON OF DETECTORS

	Selectivity	Sensitivity	Possibility of Gradient System
Absorbance	Light-absorbing substances	ng	Possible
Fluorescence	Fluorescent substances	pg	Possible
Differential refractive index	None	µg	Impossible
Evaporative light scattering	Nonvolatile substances	µg	Possible
Electrical conductivity	Ionic substances	ng	Partially possible
Electrochemical	Oxidizing / reducing substances	pg	Partially possible

Note: The above table indicates general characteristics. There are exceptions.

HOW WE CAN SELECT THE HPLC METHOD ?



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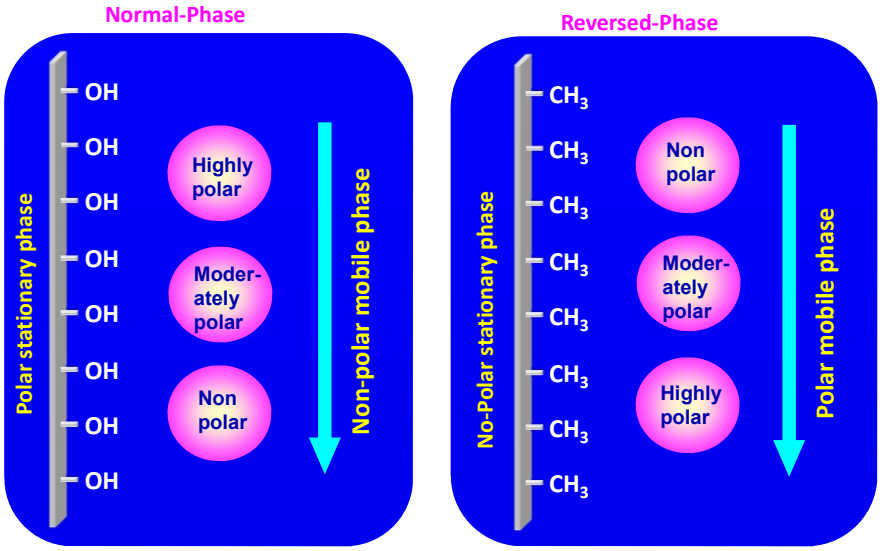
Types of Partition Chromatography



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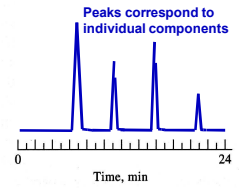
NORMAL PHASE / REVERSED PHASE

	Stationary phase	Mobile phase
Normal phase	High polarity (hydrophilic)	Low polarity (hydrophobic)
Reversed phase	Low polarity (hydrophobic)	High polarity (hydrophilic)

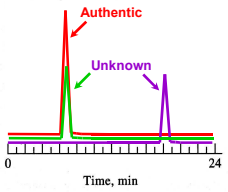
HPLC APPLICATIONS

- Qualitative Analysis
  - **Identification** based on retention time
  - Acquisition of spectra with detector
    - UV spectra
    - MS spectra
  - Transfer to other analytical instruments after preparative separation
  - Purification of compound
- Quantitative Analysis
  - Quantitation performed with peak area or height.
  - Calibration curve created beforehand using a standard.
    - Absolute calibration curve method
    - Internal standard method
      - Not affected by inconsistencies in injection volume
      - Not affected by the pretreatment recovery rate.
    - Standard addition method

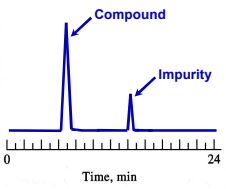
Separation of Mixture Components



Identification of Compounds



Purification of Compounds



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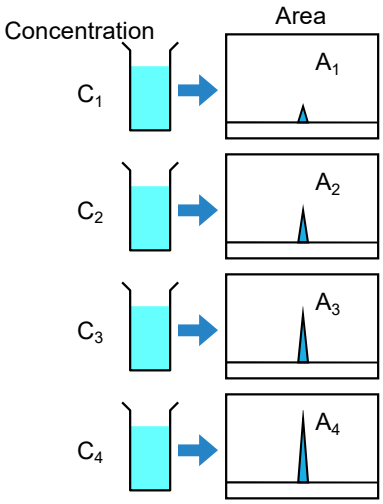
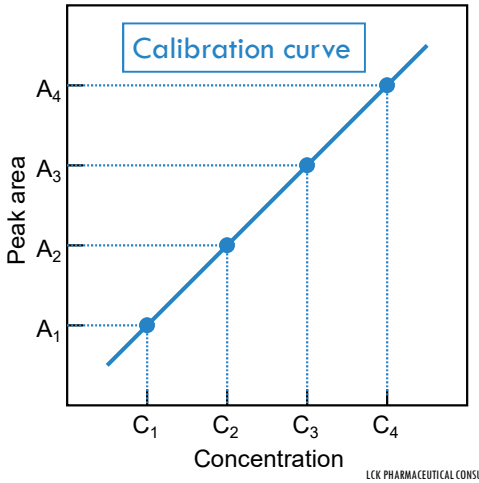
CALIBRATION CURVE: ABSOLUTE STANDARD CURVE METHOD





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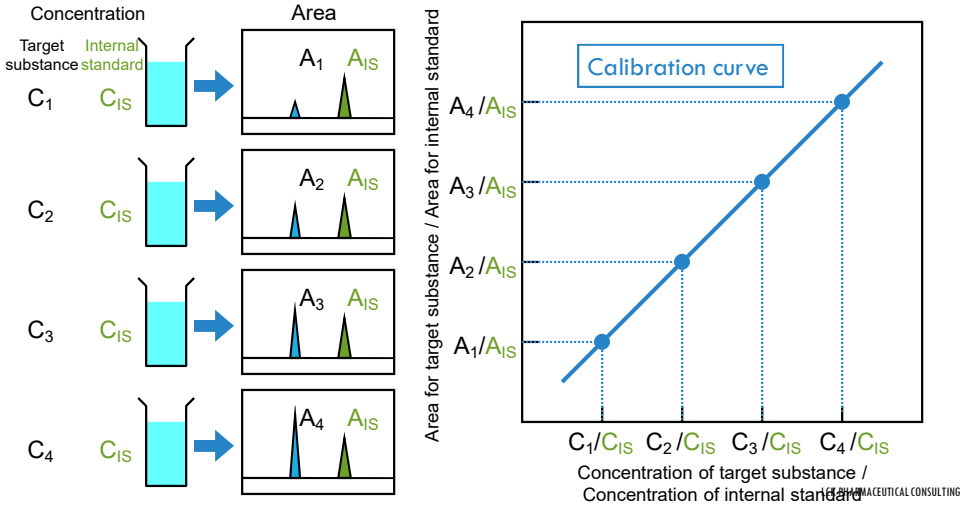
CALIBRATION CURVE: INTERNAL STANDARD METHOD



SELECTION CRITERIA FOR INTERNAL STANDARD

- It must have similar chemical properties to the target substance.
- Its peak must appear relatively near that of the target substance.
- It must not already be contained in the actual samples.
- Its peak must be completely separated from those of other sample components.
- It must be chemically stable.

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SAMPLE PREPARATION/PRETREATMENT

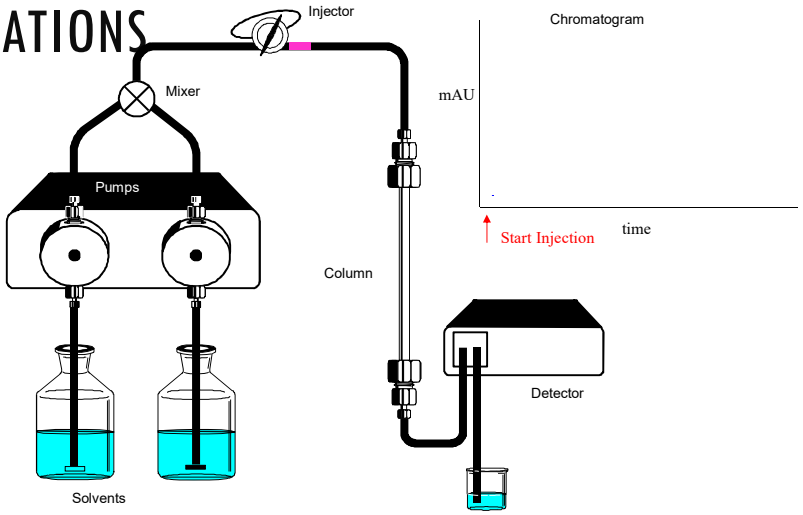
- To improve the **accuracy of quantitative values**
- To improve **sensitivity and selectivity**
- To protect and **prevent the deterioration of columns** and analytical instruments
- To **simplify measurement operations** and procedures
- To **stabilize target substances**

WHY SAMPLE PREPARATION/PRETREATMENT?

- Insoluble substances (e.g., microscopic particles and precipitation)
- Substances that are precipitated in the eluent
- Substances that irreversibly adsorb to the packing material
- Substances that dissolve, or chemically react, with the packing material

- Filtration and Centrifugal Separation
  - In general, filter every sample before injection!
  - It is convenient to use a disposable filter with a pore diameter of approx. 0.45 μm.
  - Centrifugal separation is applicable for samples that are difficult to filter.
- Deproteinization
  - Precipitation
    - Addition of organic solvent (e.g., acetonitrile)
    - Addition of acid (e.g., trichloroacetic acid, perchloric acid)
    - Addition of heavy metal or neutral salt
  - Ultrafiltration
- Solid Phase Extraction
- Pre-Column Derivatization
  - convert the substance to another compound via a chemical reaction before injection

SEPARATIONS



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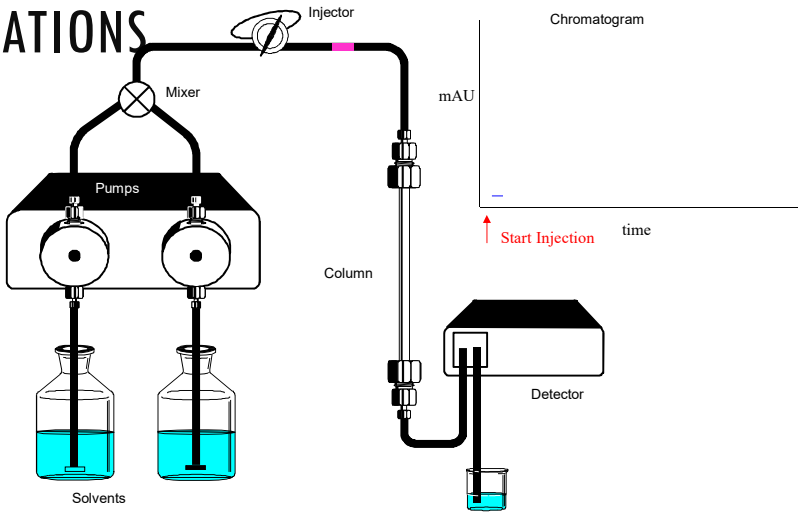

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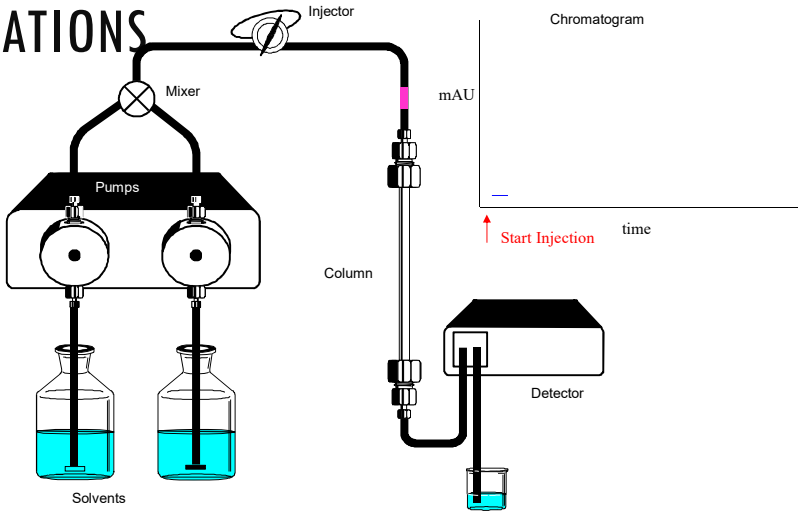

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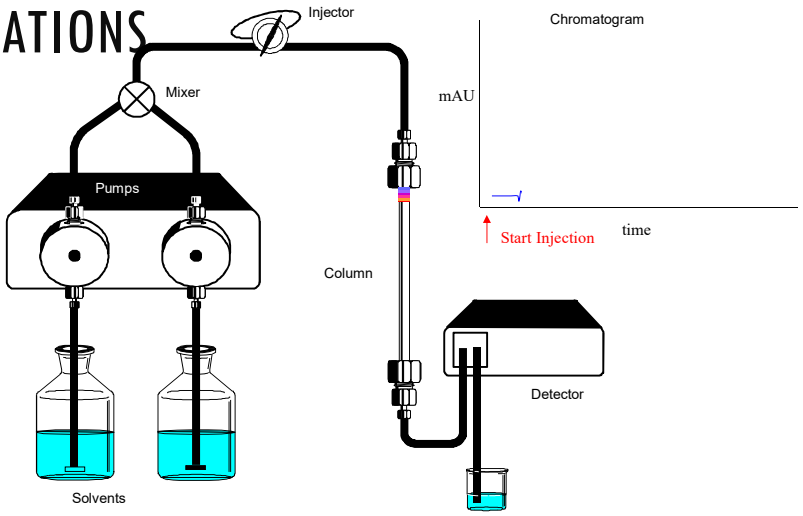

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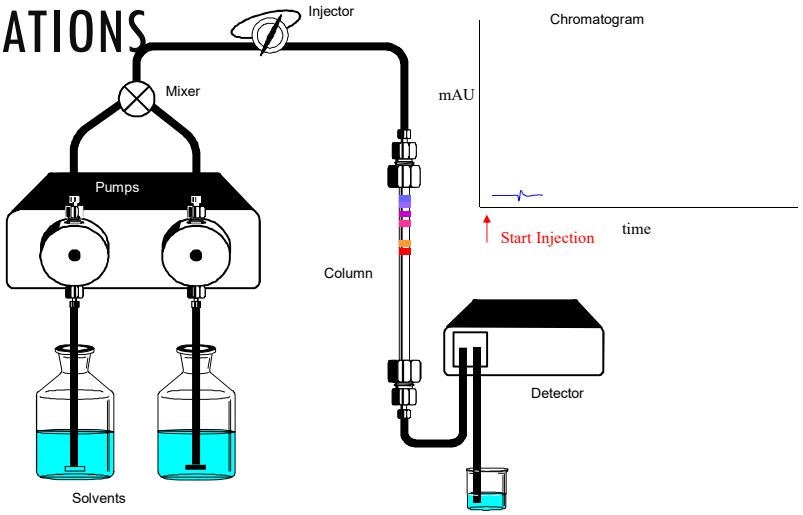

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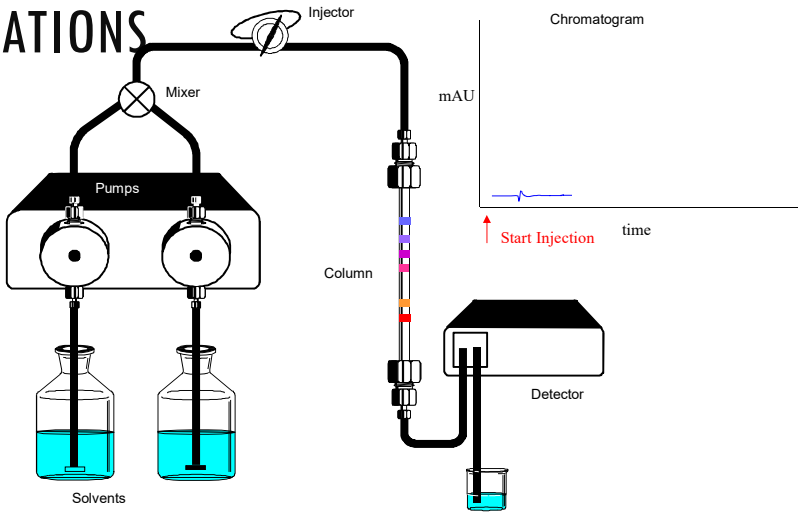

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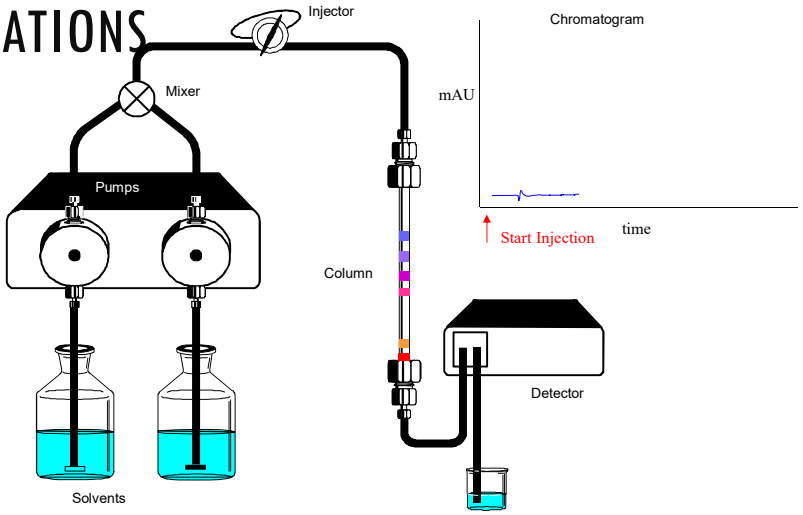

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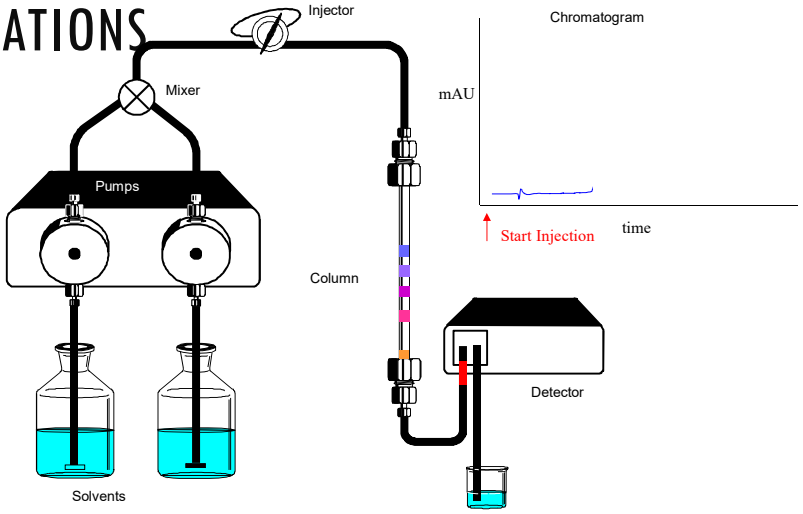

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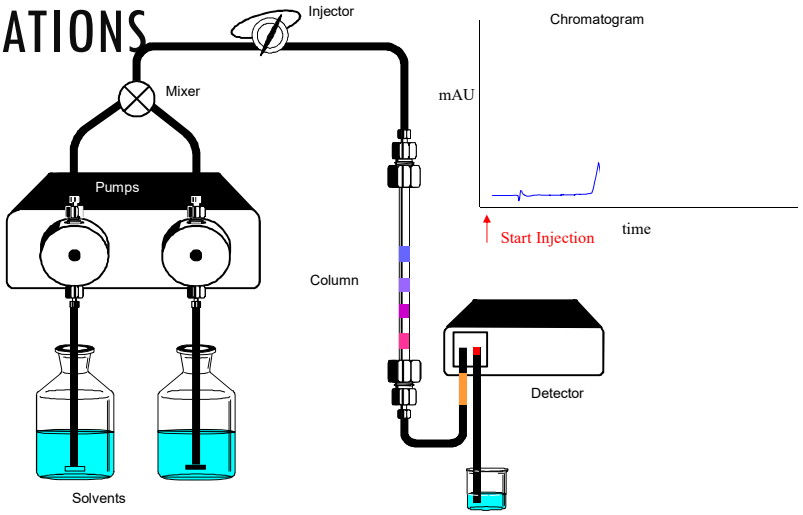

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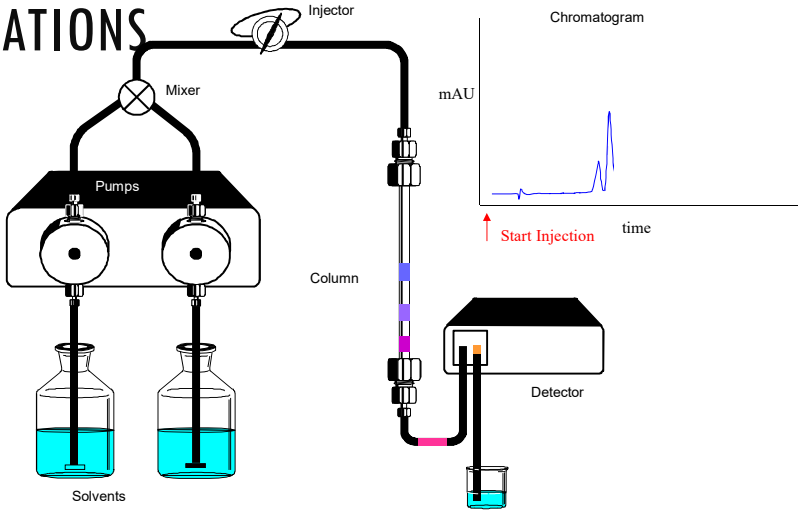

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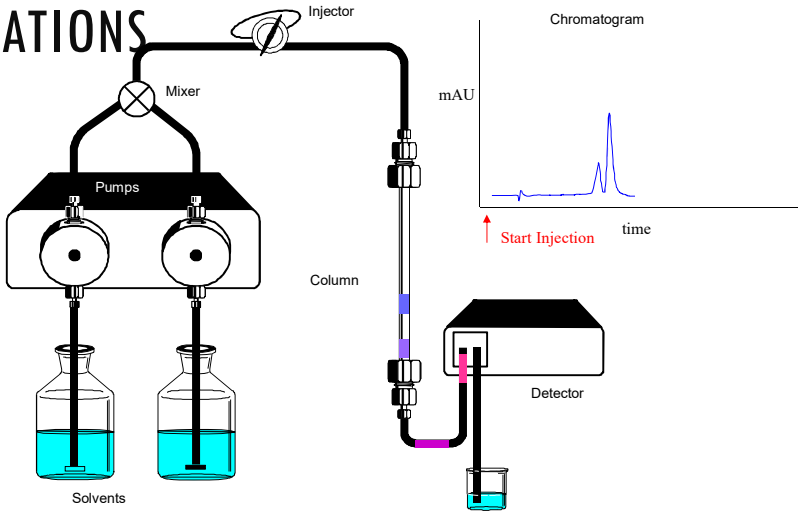

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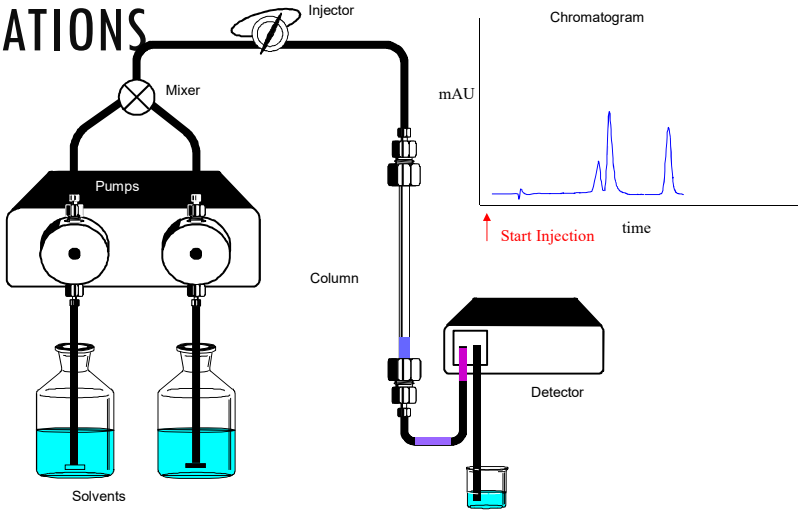

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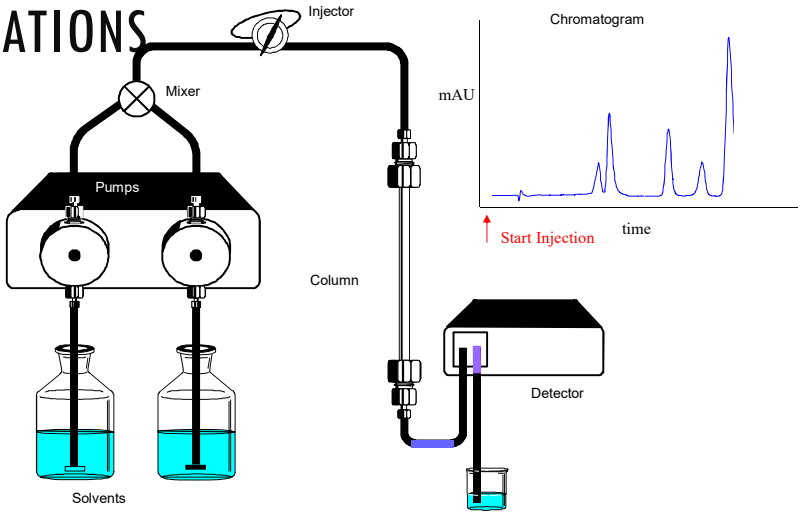

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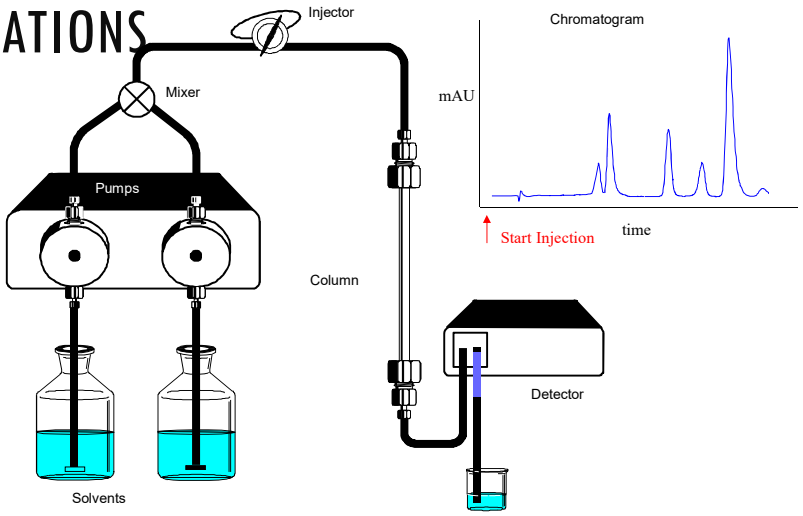

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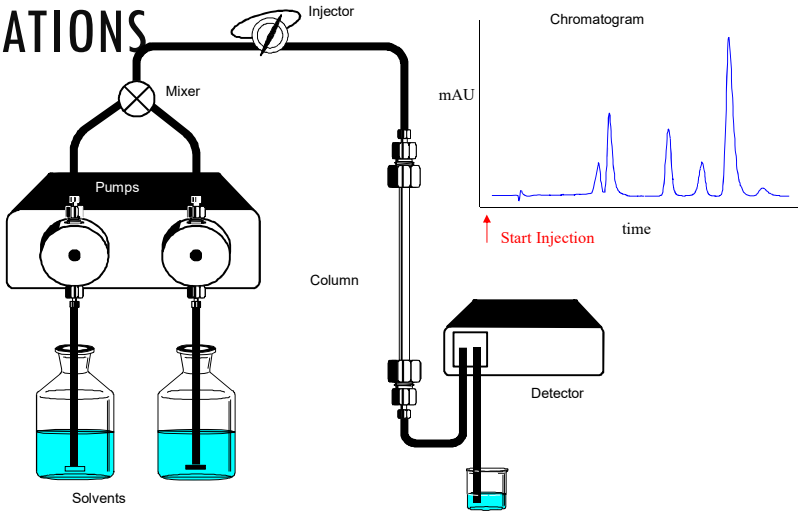

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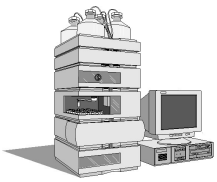
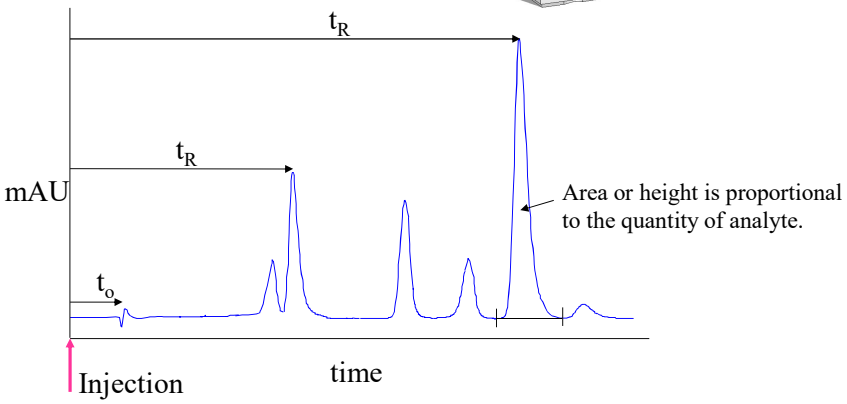
THE CHROMATOGRAM

t_o - elution time of unretained peak

t_R- retention time - determines sample identity





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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

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