
بسم الله الرحمن الرحيم

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

(HPLC)

CHROMATOGRAPHY

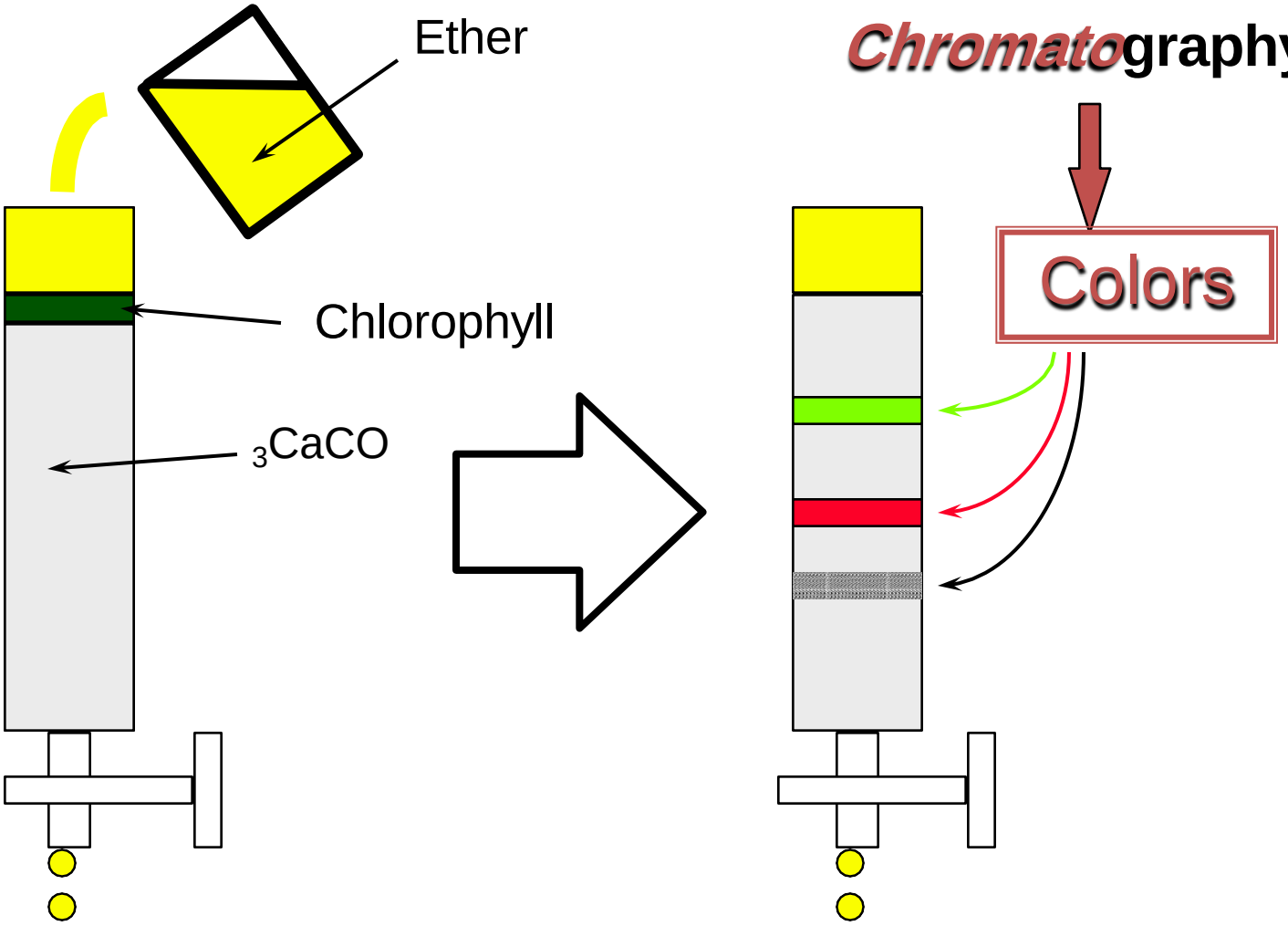
History

Mikhail Tswett, Russian, 1872-1919

- ✓ He was a Botanist
- ✓ In 1906 Tswett used to chromatography to separate plant pigments
- ✓ He called the new technique chromatography because the result of the analysis was 'written in color' along the length of the adsorbent column

Chroma means “**color**” and graphein means to “**write**”

Invention of Chromatography by M. Tswett



Importance

Chromatography has application in every branch of the physical and biological sciences

12

Nobel prizes were awarded between 1937 and 1972 alone for work in which chromatography played a vital role

- **Chromatography is a physical method of separation in which the components to be separated are distributed between two phases.**

- **One of which is stationary (stationary phase) while the other (the mobile phase) moves through it in a definite direction.**

- **The chromatographic process occurs due to differences in the distribution constant of the individual sample components.**

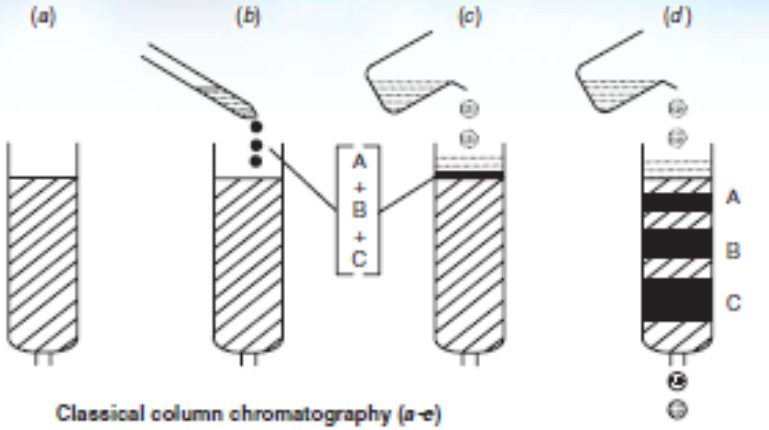
Chromatography

Is a technique used to separate and identify the components of a mixture .

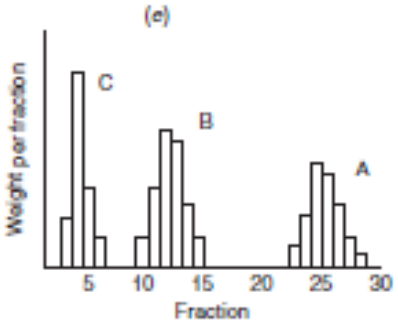
Works by allowing the molecules present in the mixture to distribute themselves between a stationary and a mobile medium.

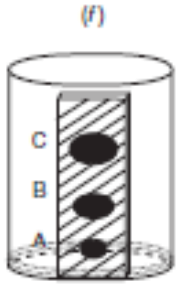
Molecules that spend most of their time in the mobile phase are carried along faster.

Different stage in the development of chromatography

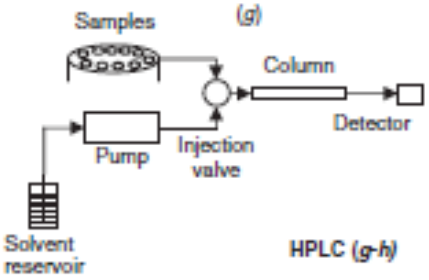


Classical column chromatography (*a-e*)

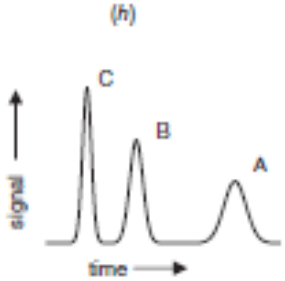




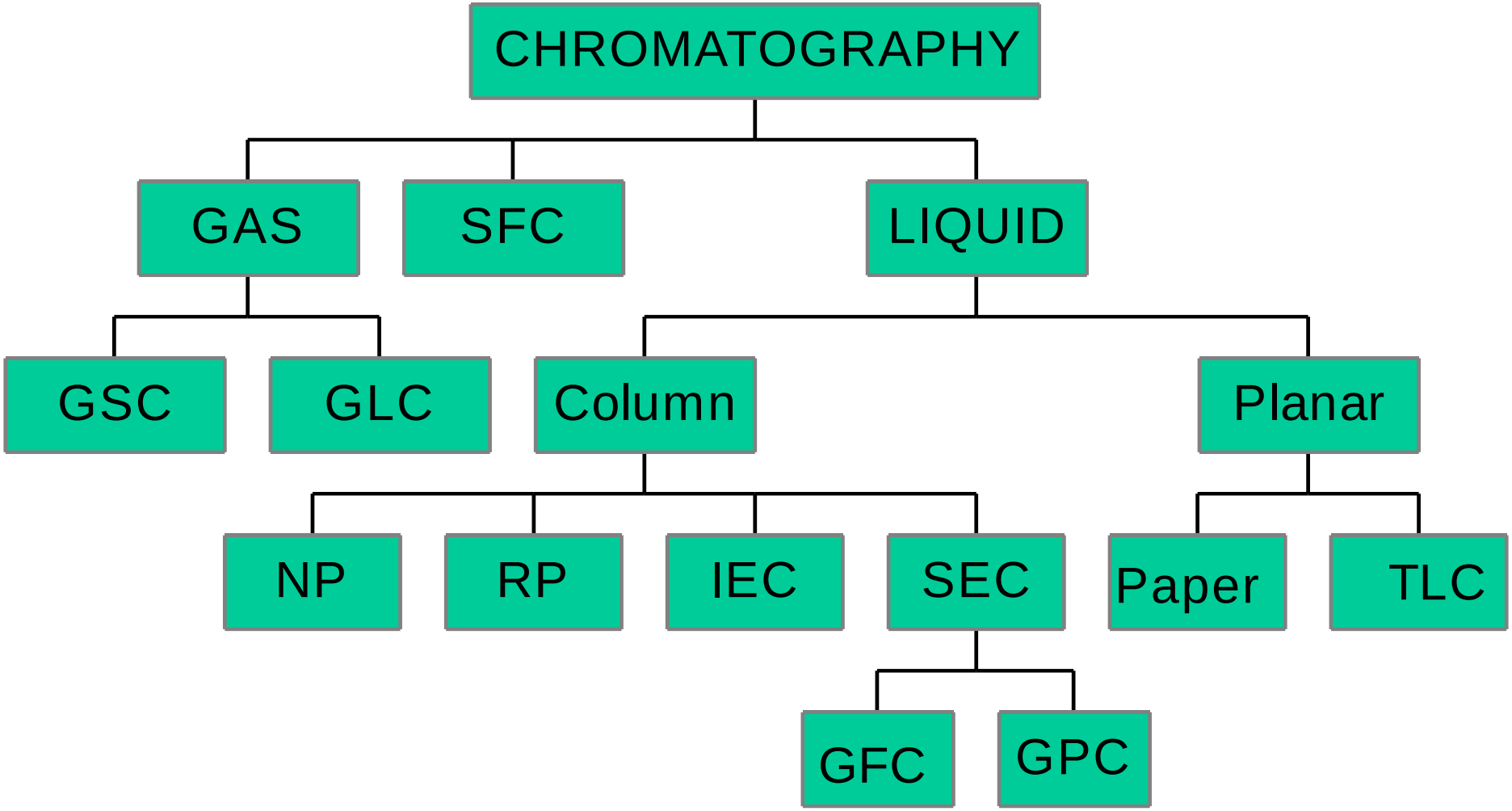
Paper or thin-layer chromatography (*f*)



HPLC (*g-h*)



Chromatography Types



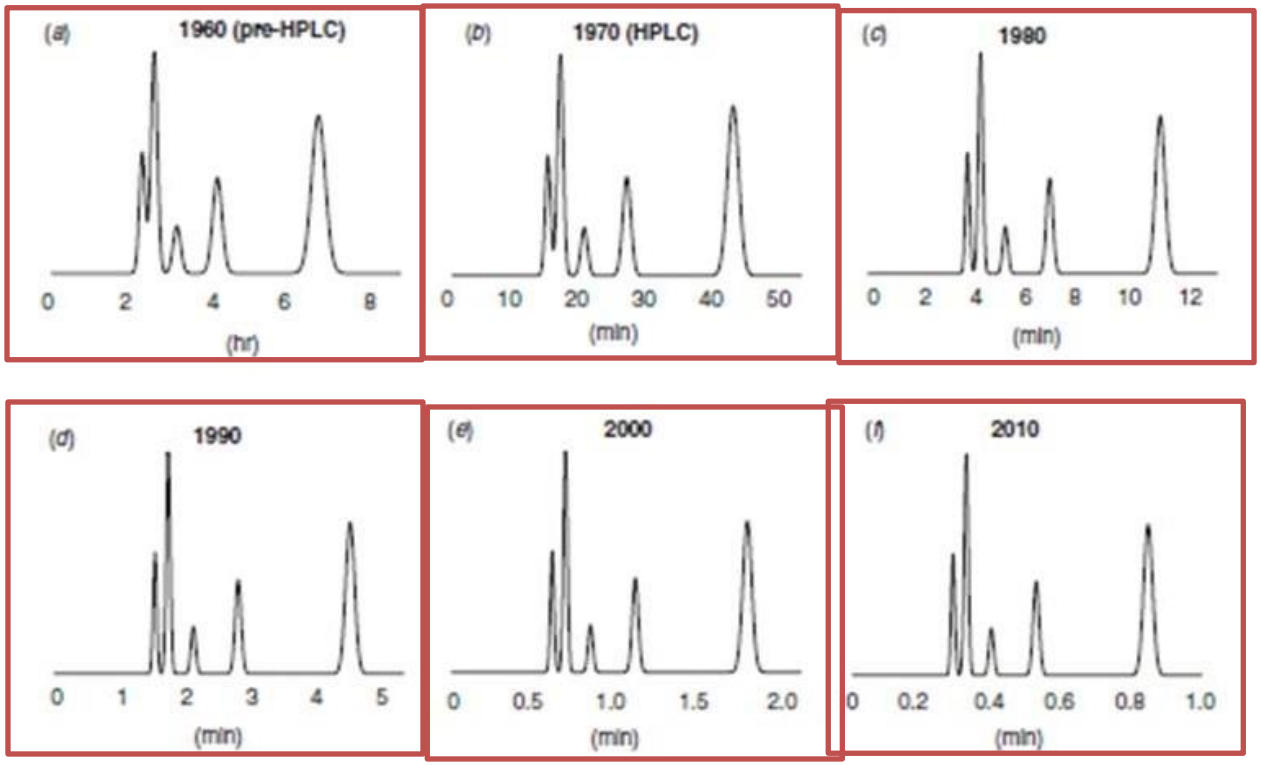
From Liquid Chromatography to High Performance Liquid Chromatography

- Higher degree of separation!
  → Refinement of packing material (3 to 10 µm)

- Reduction of analysis time!
  → Delivery of eluent by pump
  → Demand for special equipment that can withstand high pressures

The arrival of high performance liquid chromatography!

The improvement in HPLC performance overtime



Advantages of HPLC

- Rapid and precise quantitative analysis
  - Typical analysis time of 5-20 min, precision <0.5-1% RSD
- Automated analysis
  - Using autosampler and data system for unattended analysis and report generation
- High sensitivity detection
  - Detection limits of ng to pg
- Quantitative sample recovery
  - Preparative technique from μg to kg quantities
- Amenable to diverse samples
  - Can handle >60% of all existing compounds vs. 15% for GC
  - Can analyze samples with little or minimal preparation

HPLC Advantages vs GC

- Not limited by sample volatility or thermal stability
- Two interacting phases
- Room temperature analysis
- Ease of sample recovery

Comparison of GC and HPLC

- The **diffusion coefficient of the sample** in the mobile phase is much smaller in HPLC than in GC. (this is a drawback because the diffusion coefficient is the most important factor which determines the speed of chromatographic analysis(.
- The **viscosity of the mobile phase** is higher in HPLC than in GC. (this is a drawback because high viscosity result in small diffusion coefficients and in high (.flow resistance of the mobile phase
- The **compressibility of the mobile phase under pressure** is negligibly small in HPLC whereas it is not in GC. (This is an advantage because as a result the flow velocity of the mobile phase is constant over the whole length of the column. Therefore optimum chromatographic conditions exist everywhere if the flow velocity is chosen correctly. Moreover, incompressibility means that a liquid under high pressure is not dangerous(.

Comparison of GC and HPLC

Problem	GC	HPLC
Difficult separation	Possible	Possible
Speed	Yes	Yes
Automation	Possible	Possible
Adaptation of system to separation problem	By change in stationary phase	By change in stationary and mobile phase
Application restricted by	Lack of volatility, thermal decomposition	Insolubility
Typical number of separation plates	Per column	Per metre
GC with packed columns	2000	1000
GC with capillary columns	50 000	3000
Classical liquid chromatography	100	200
HPLC	5000	50 000

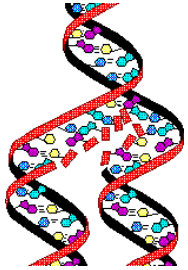
TLC vs. HPLC

	TLC	HPLC
Type of Analysis	qualitative only	qualitative & quantitative
Stationary Phase	-2dimensional thin layer plate	-3dimensional column
Instrumentation	minimal!	much! with many adjustable parameters
Sample Application	spotting (capillary(	injection (Rheodyne injector(
Mobile Phase Movement	capillary action (during development(	high pressure (solvent delivery(
Visualization of Results	UV lightbox	“on-line” detection (variable UV/Vis(
Form of Results	spots, R_f’s (retention factors(	peaks, R_t’s (retention times(

HPLC Applications

Chemical

polystyrenes
dyes
phthalates



Bioscience

proteins
peptides
nucleotides

Pharmaceuticals

tetracyclines
corticosteroids

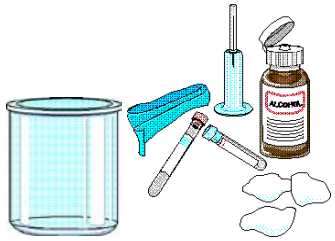
antidepressants
barbiturates

Consumer Products

lipids
antioxidants
sugars

Environmental

polyaromatic hydrocarbons
Inorganic ions
herbicides



Clinical

amino acids
vitamins
homocysteine

Fields in Which High Performance Liquid Chromatography Is Used

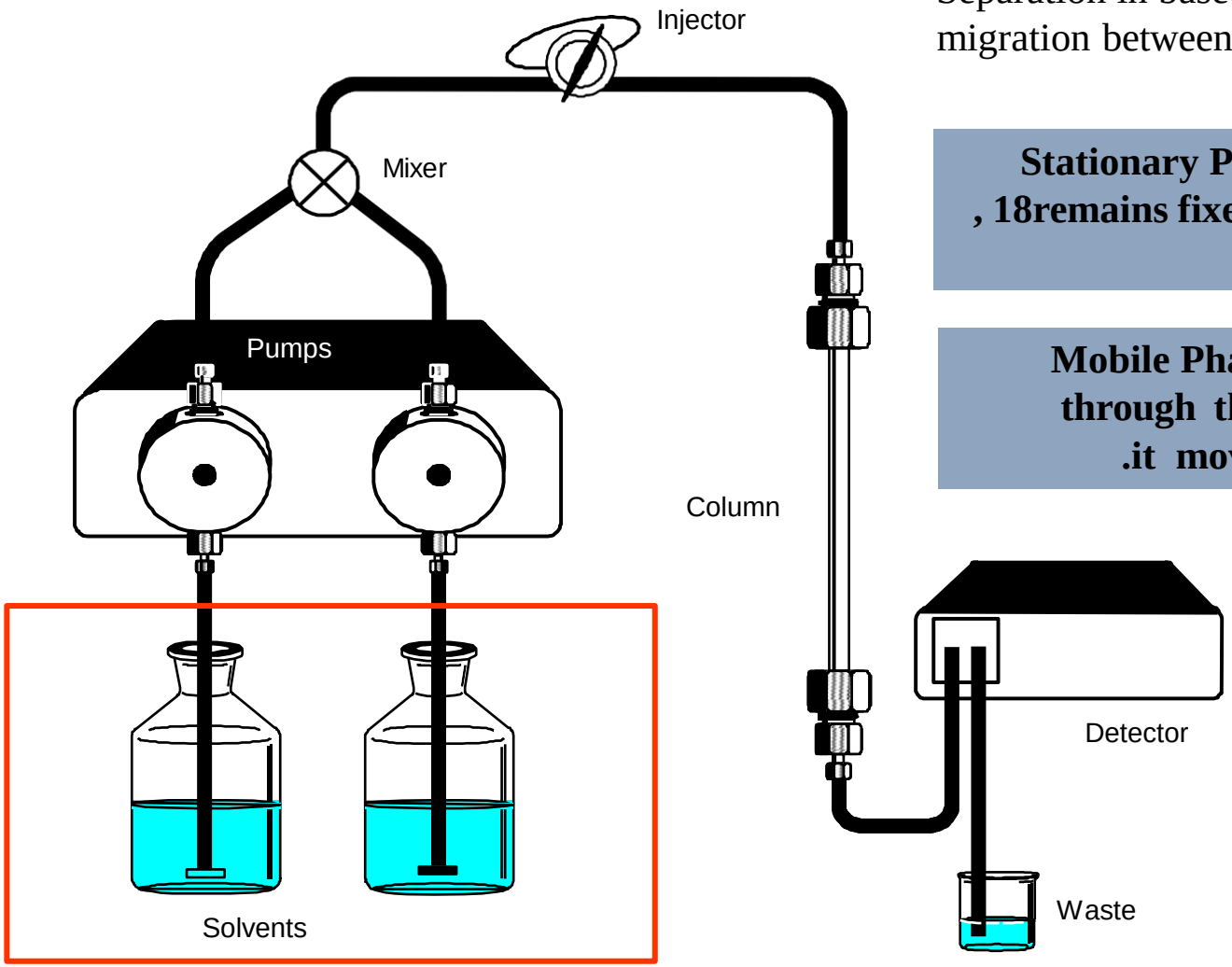
- Biogenic substances
  - Sugars, lipids, nucleic acids, amino acids, proteins, peptides, steroids, amines, etc.
- Medical products
  - Drugs, antibiotics, etc.
- Food products
  - Vitamins, food additives, sugars, organic acids, amino acids, etc.
- Environmental samples
  - Inorganic ions
  - Hazardous organic substances, etc.
- Organic industrial products
  - Synthetic polymers, additives, surfactants, etc.

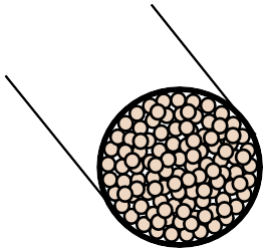
Separation

Separation in based upon differential migration between the **stationary** and **.mobile phases**

Stationary Phase - the phase which , 18remains fixed in the column, e.g. C Silica

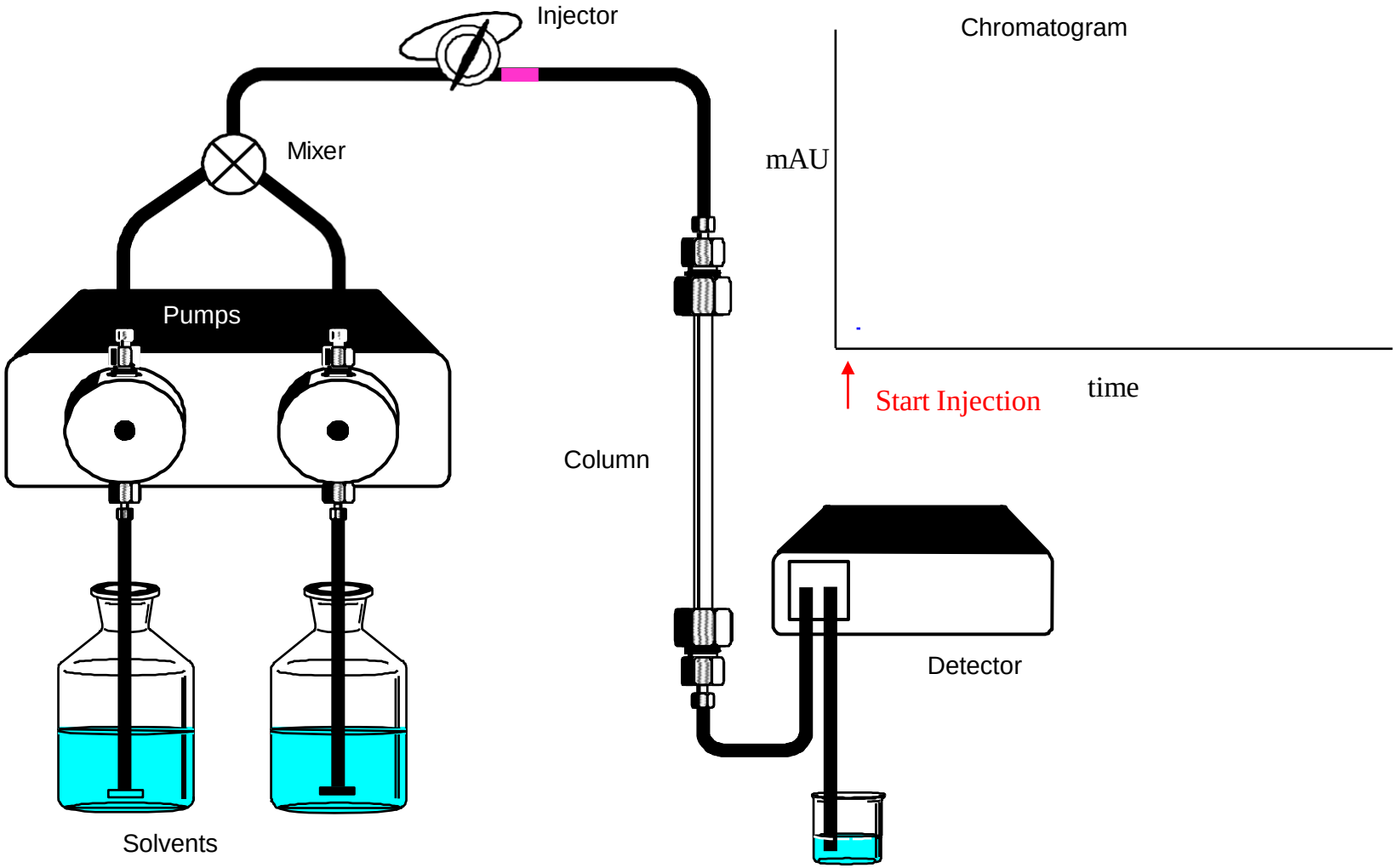
Mobile Phase - carries the sample through the stationary phase as .it moves through the column





High Performance Liquid Chromatograph

Separation


Injector
Chromatogram
Mixer
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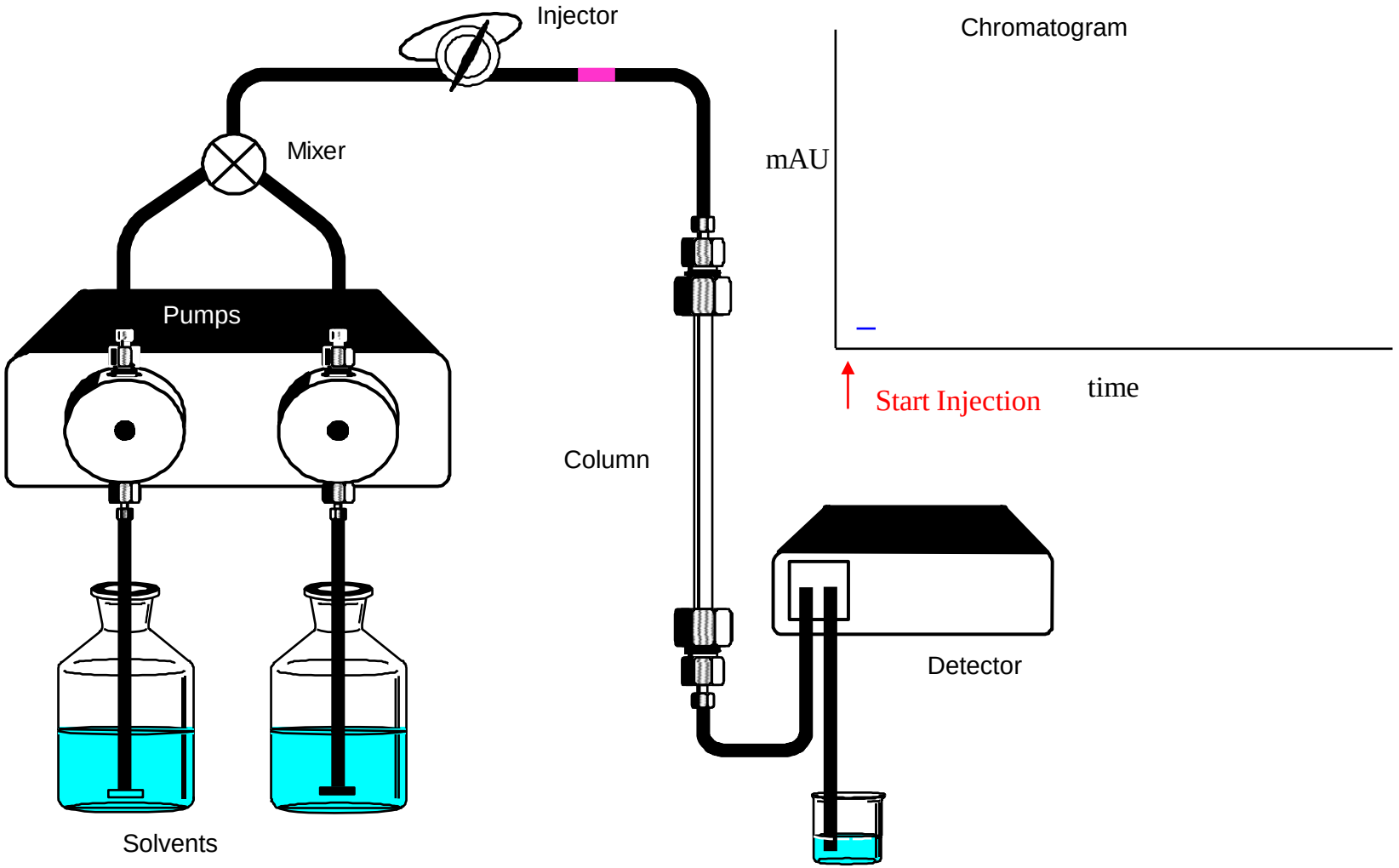
Pumps
Column
Start Injection
time
Detector
Solvents

Separation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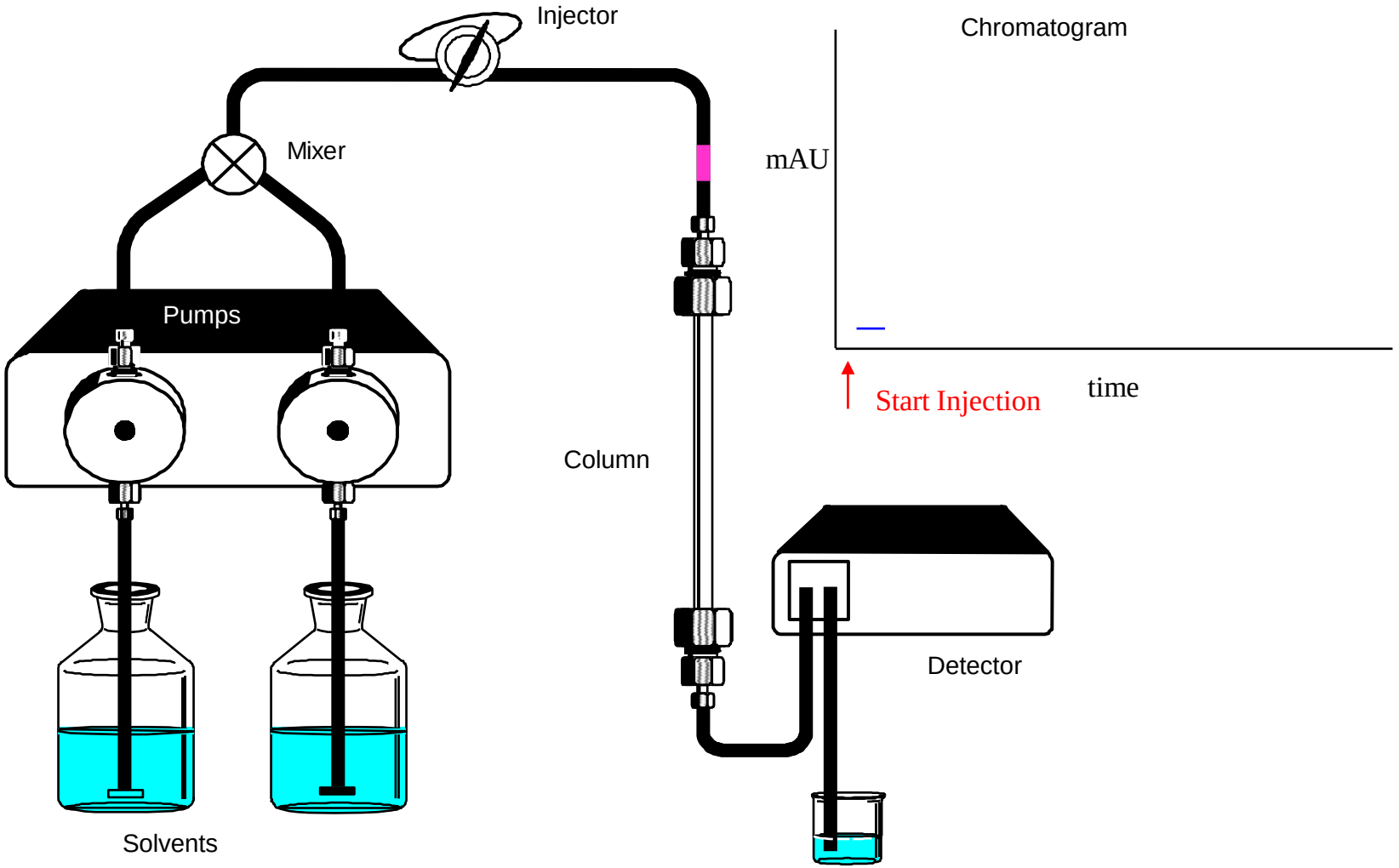
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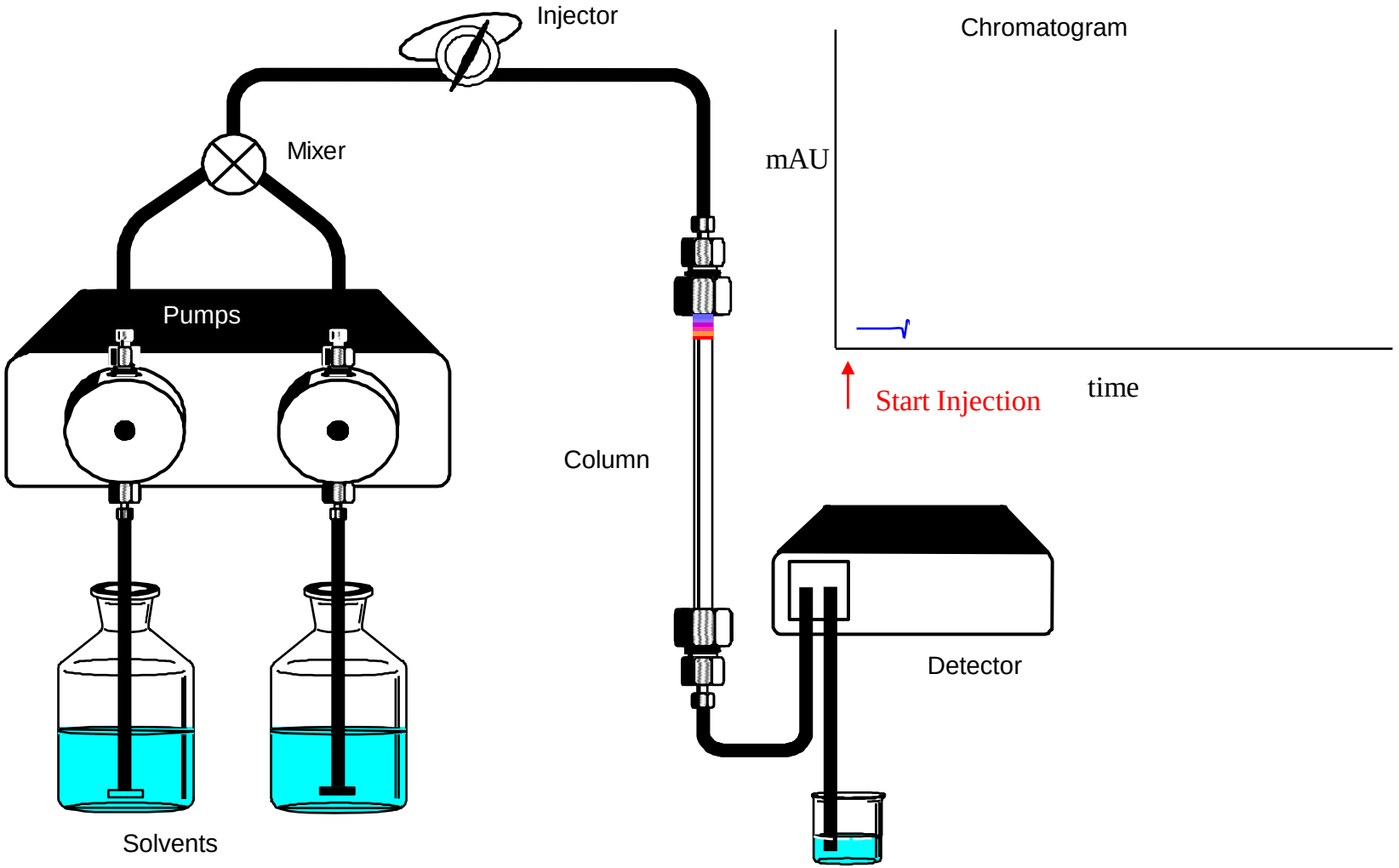
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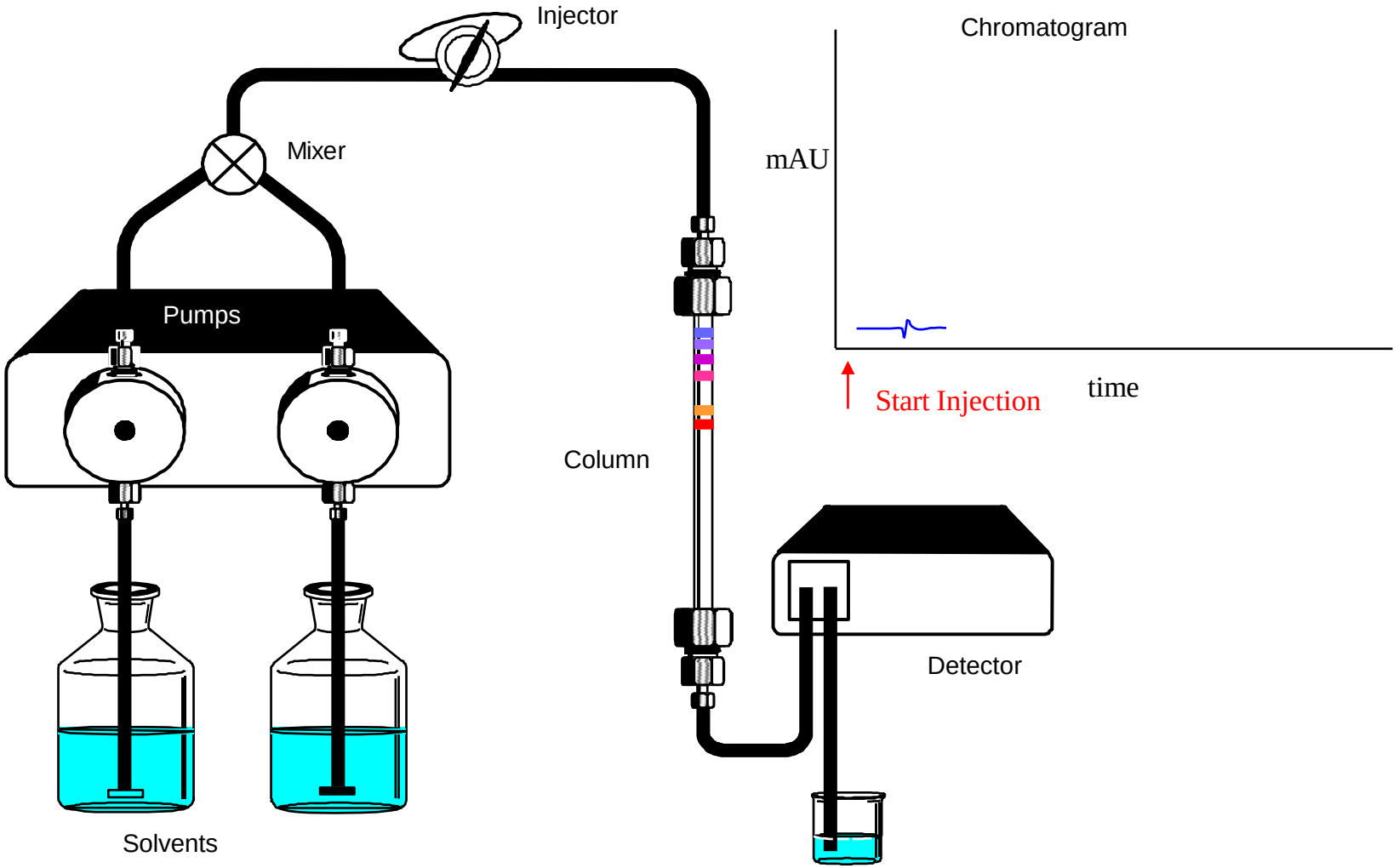
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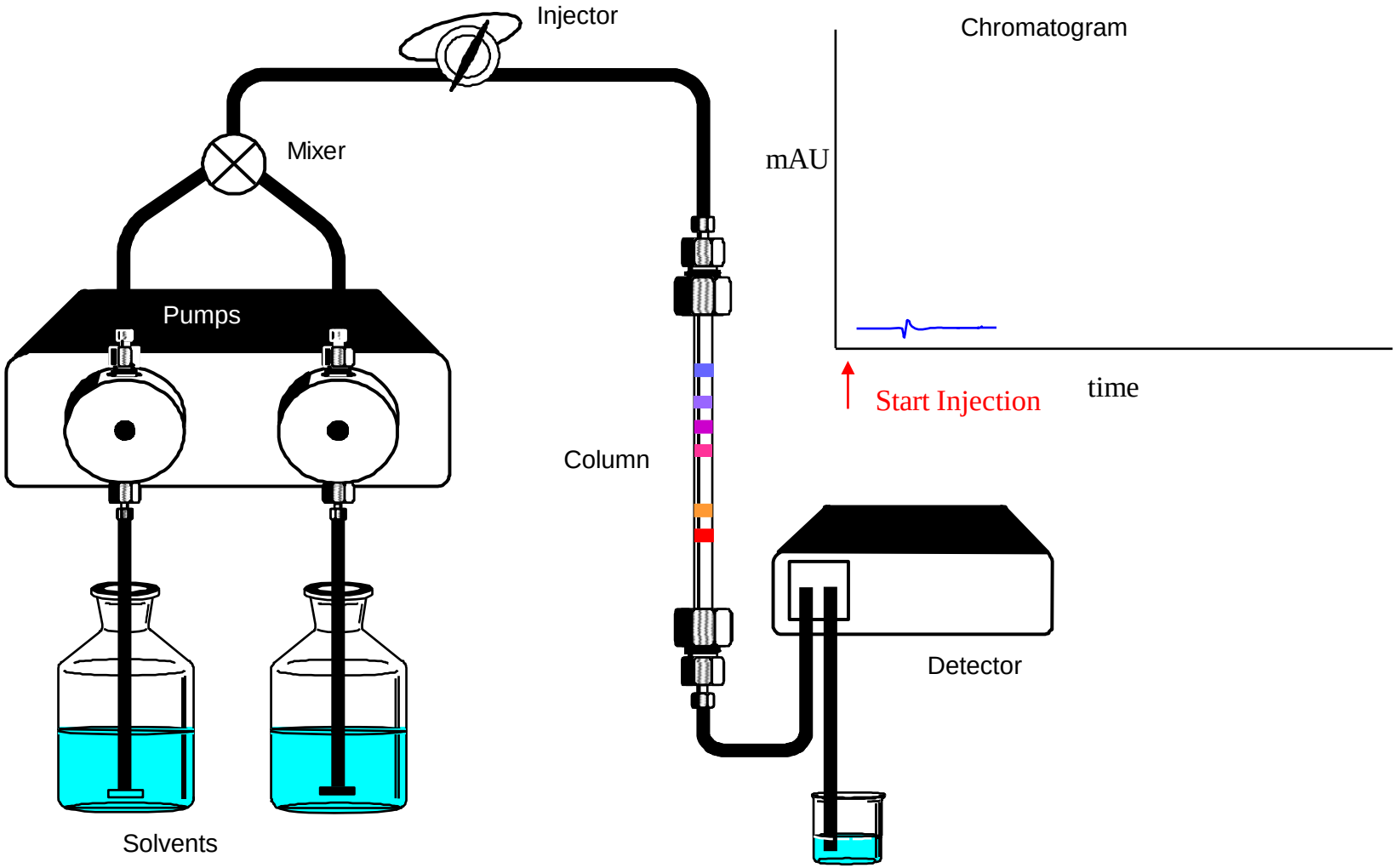
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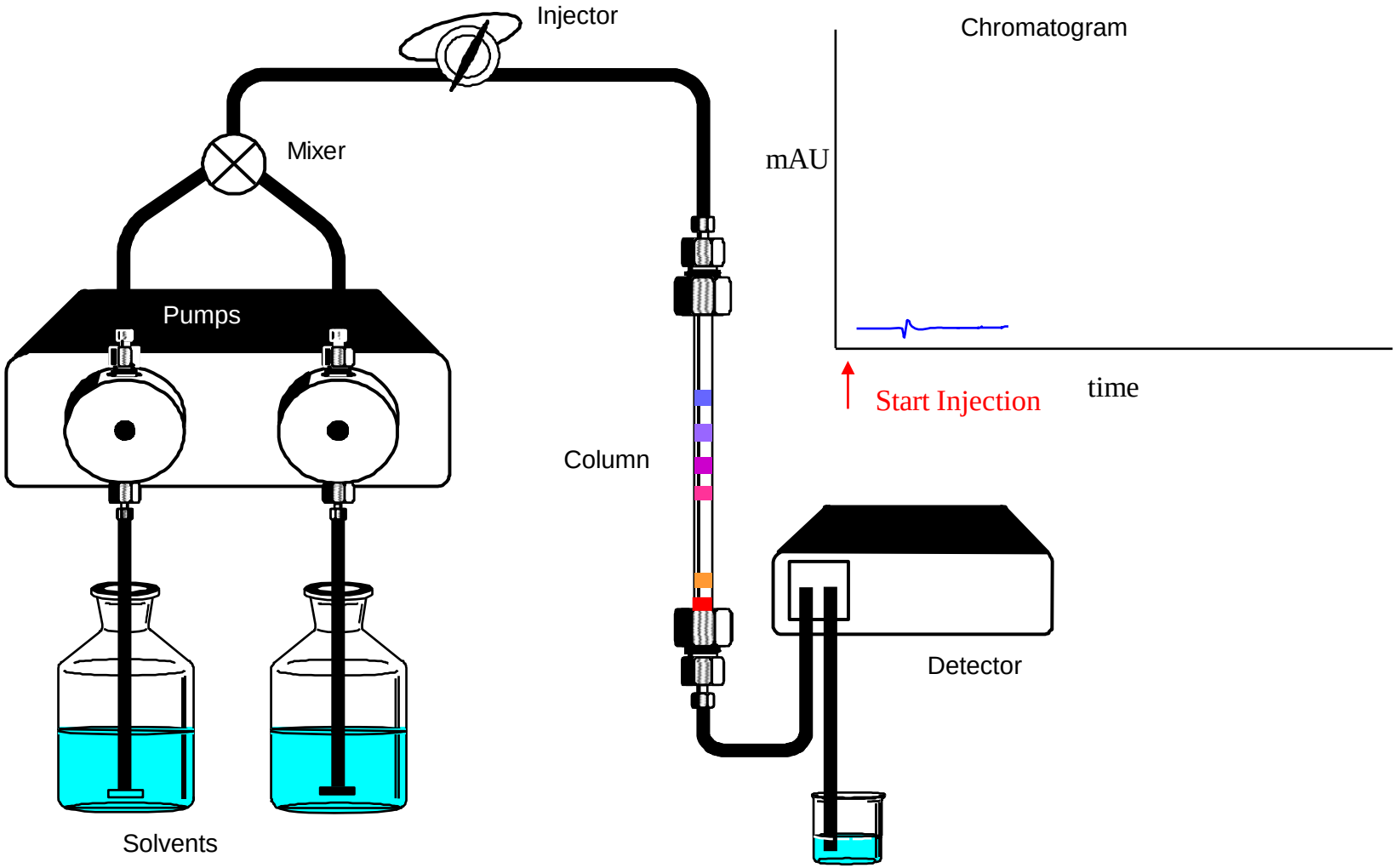
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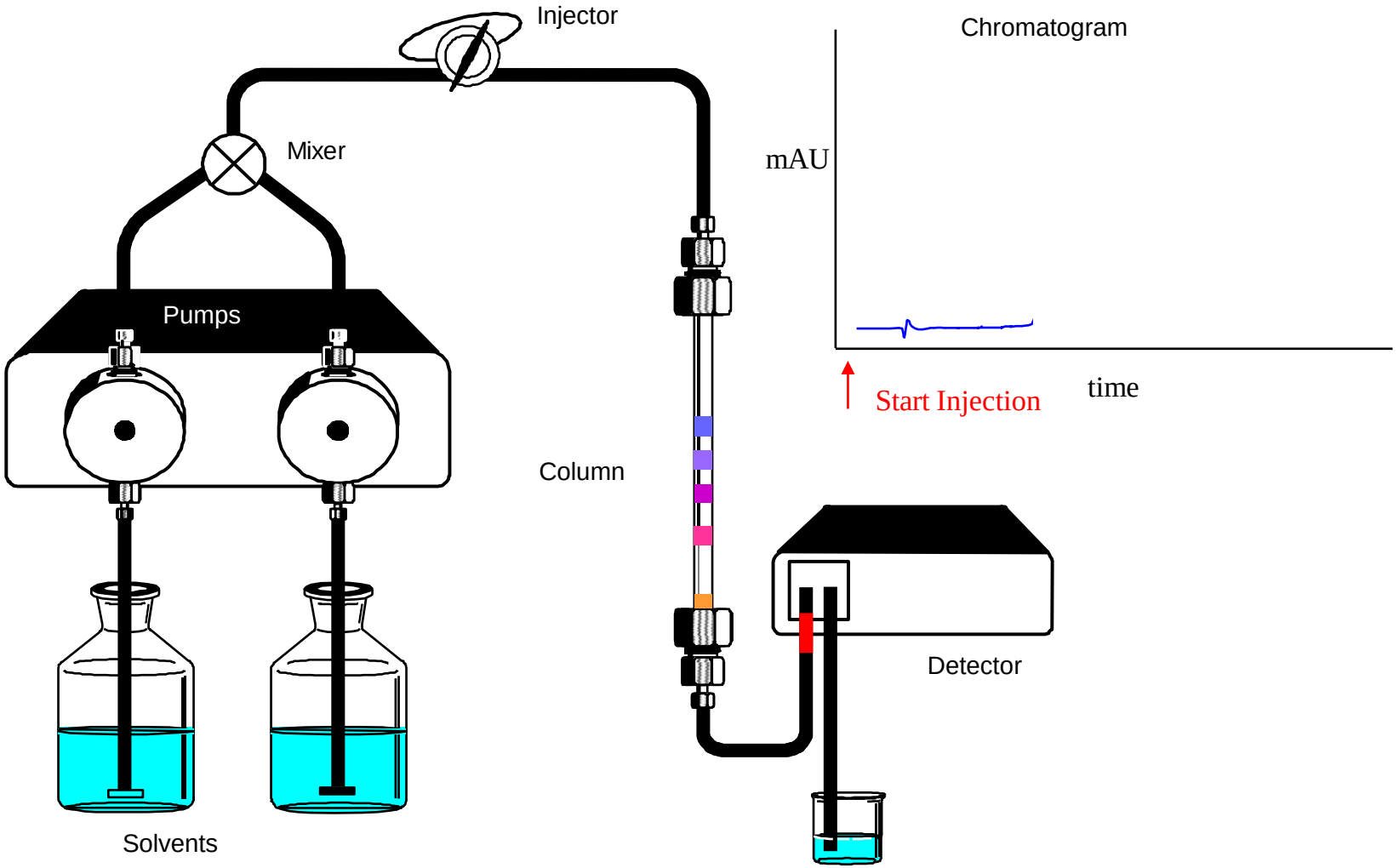
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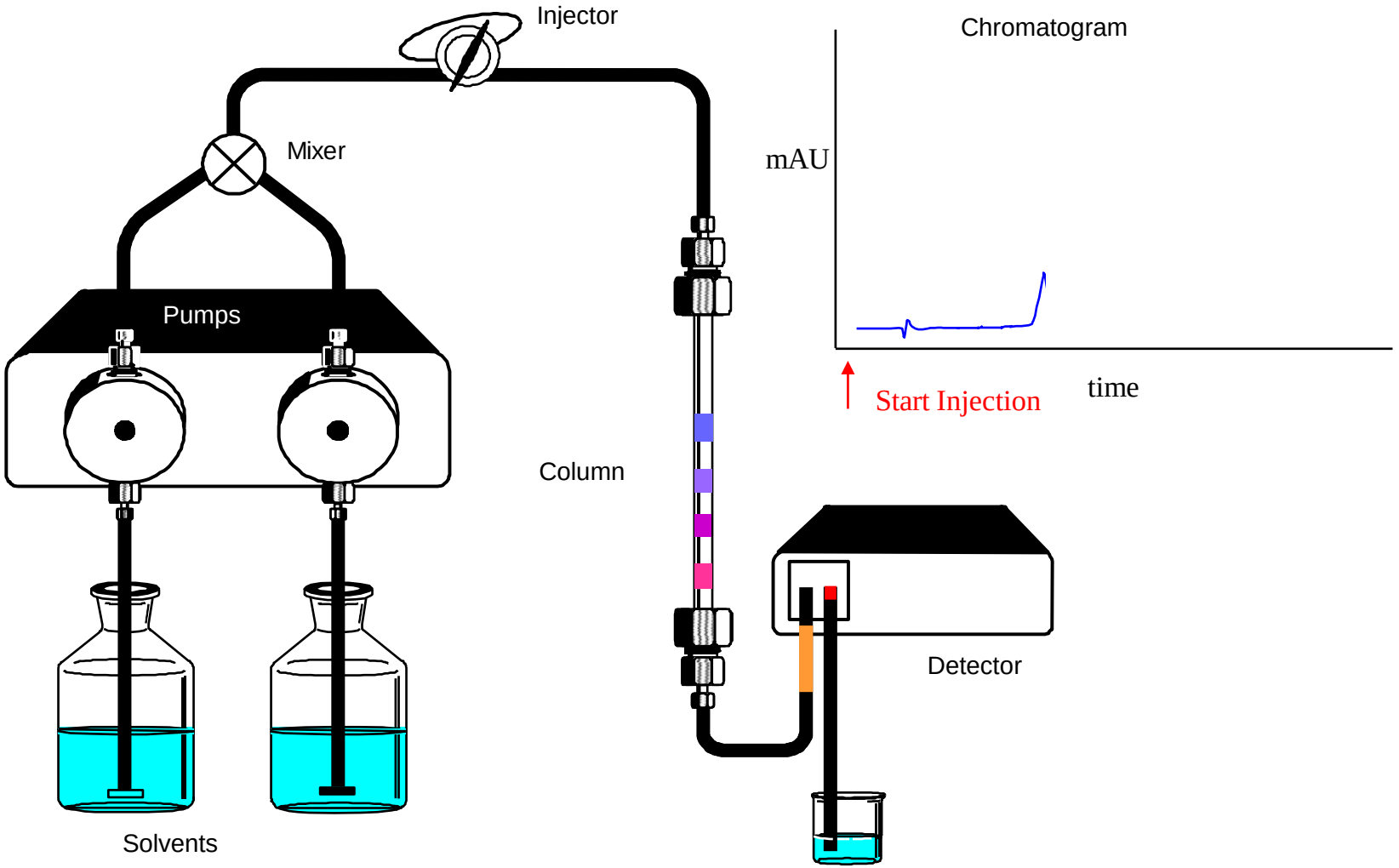
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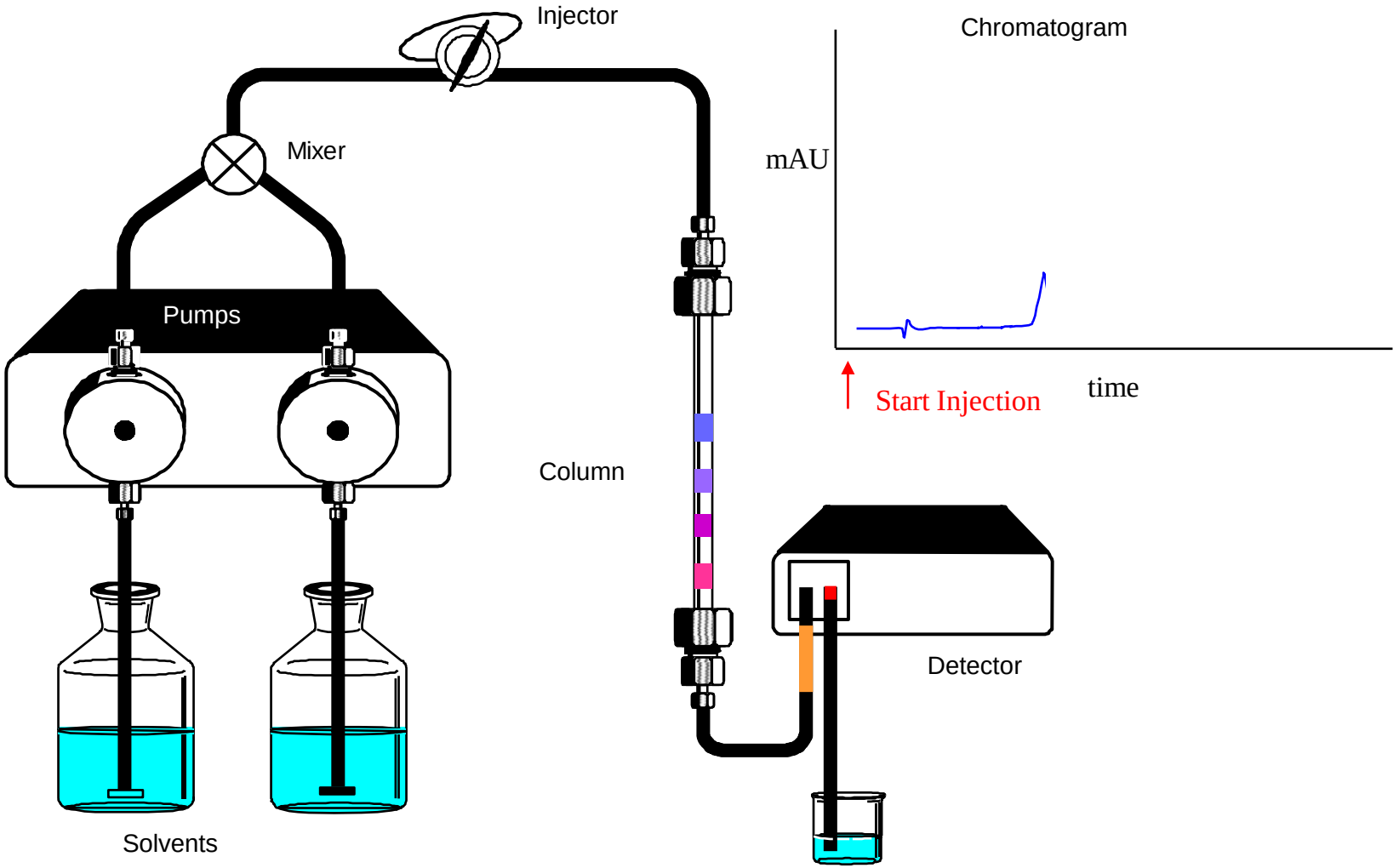
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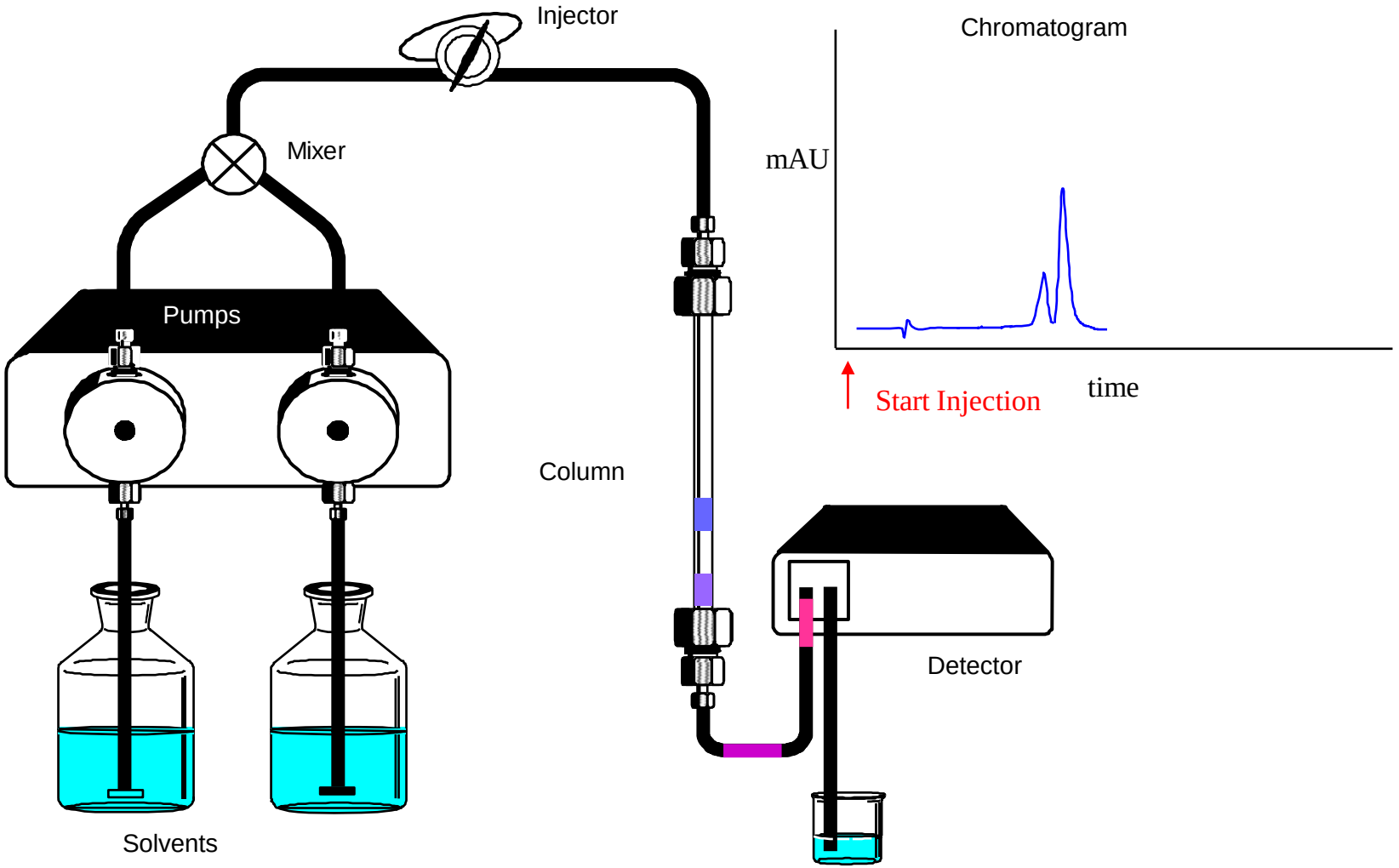
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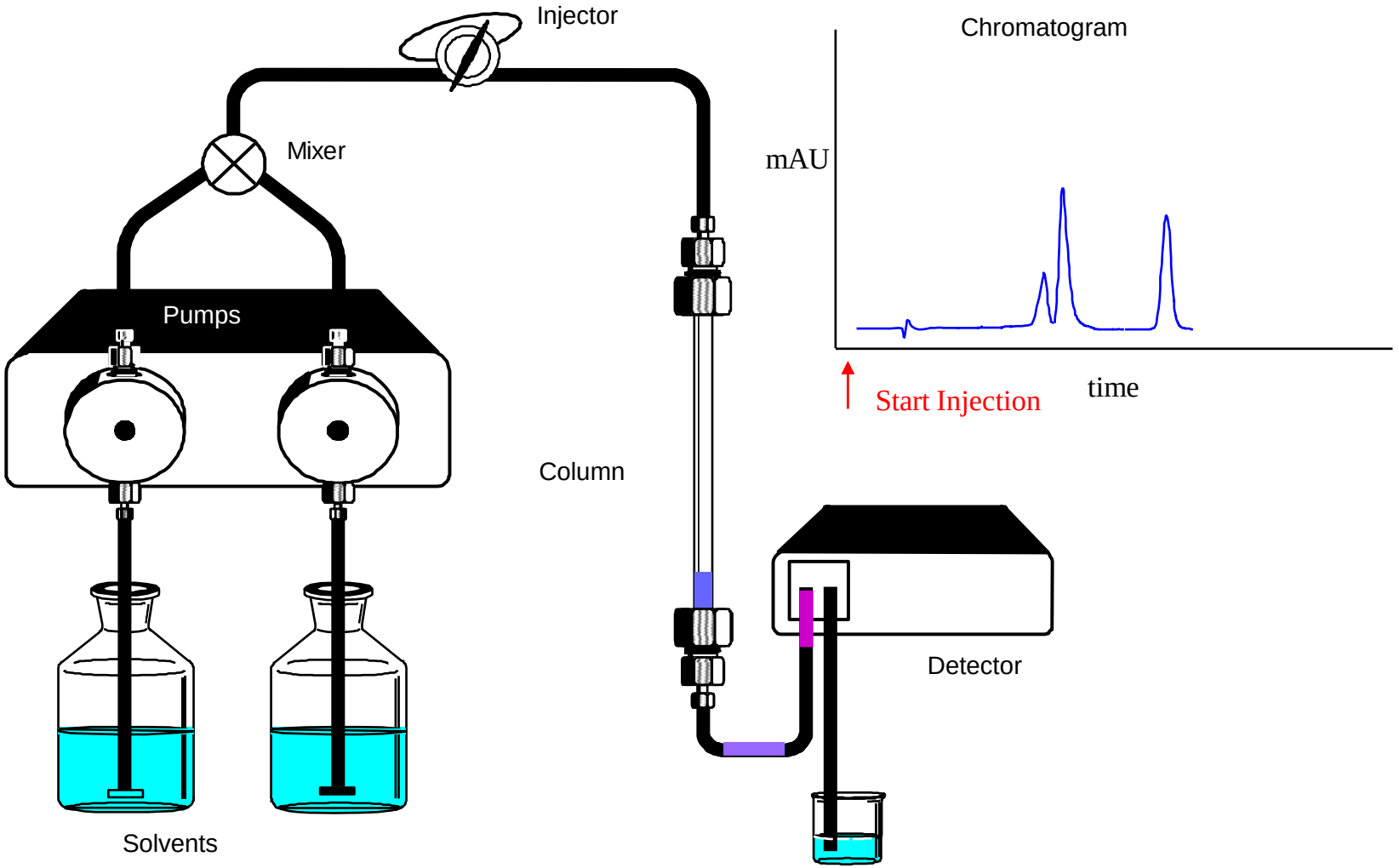
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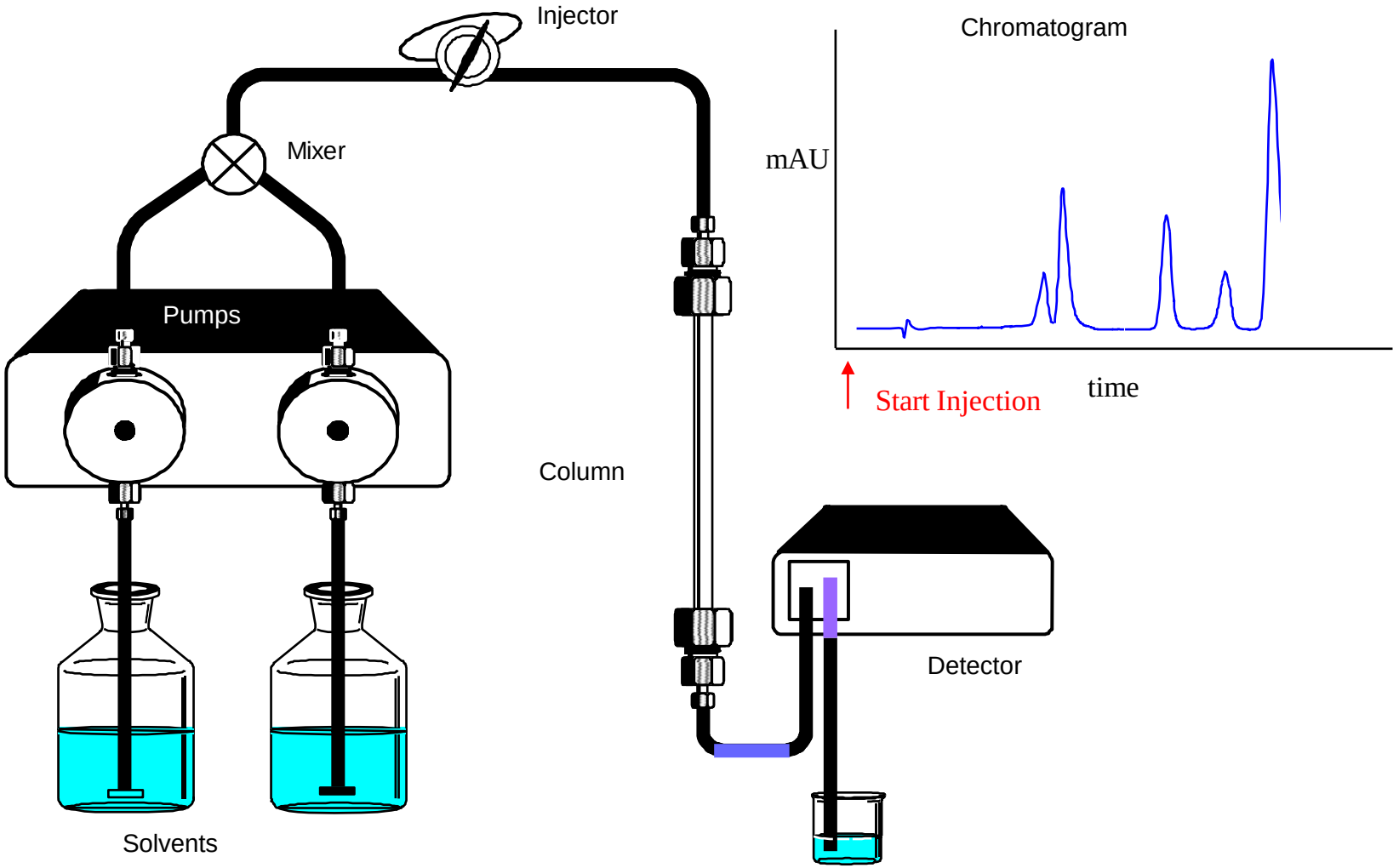
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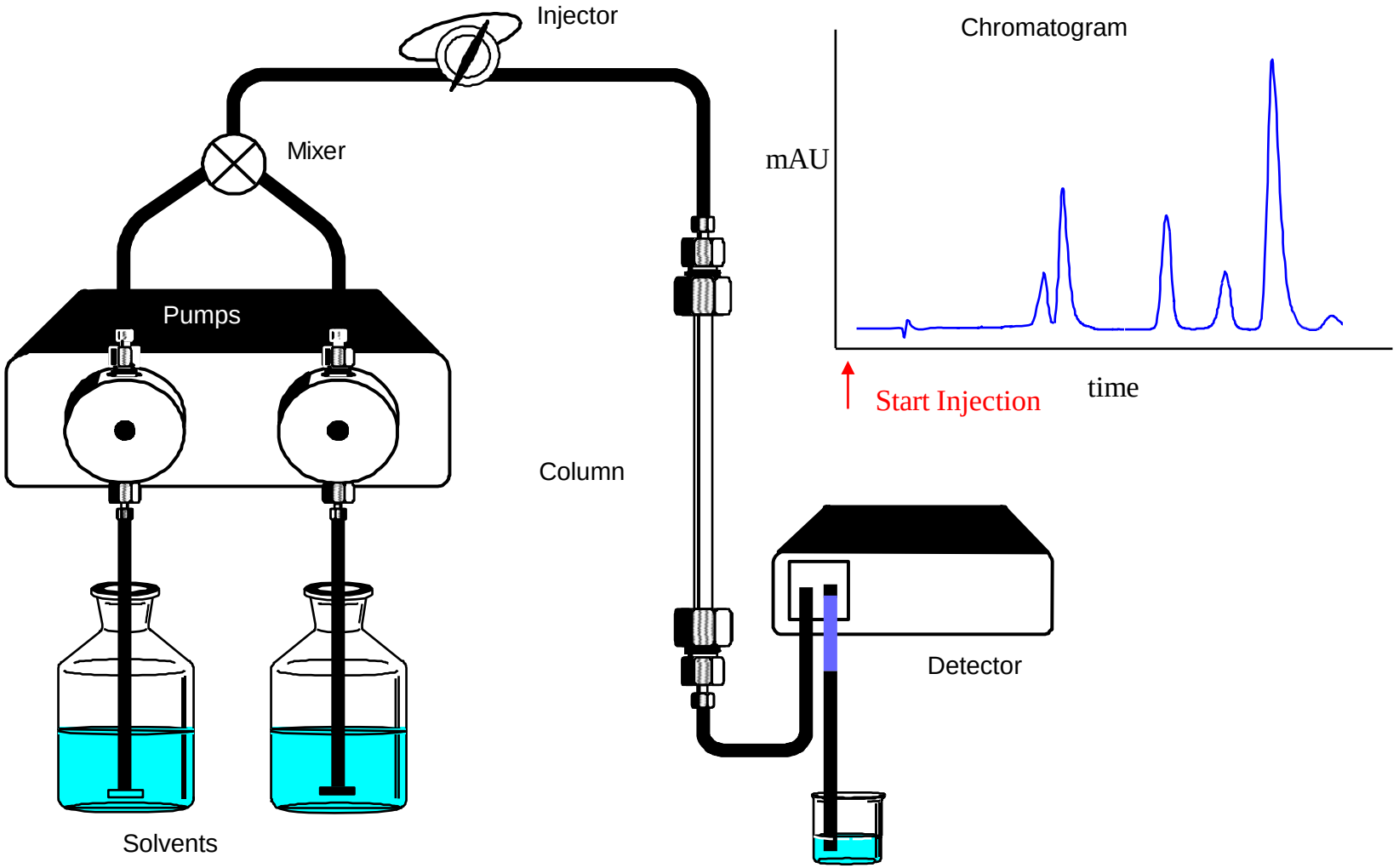
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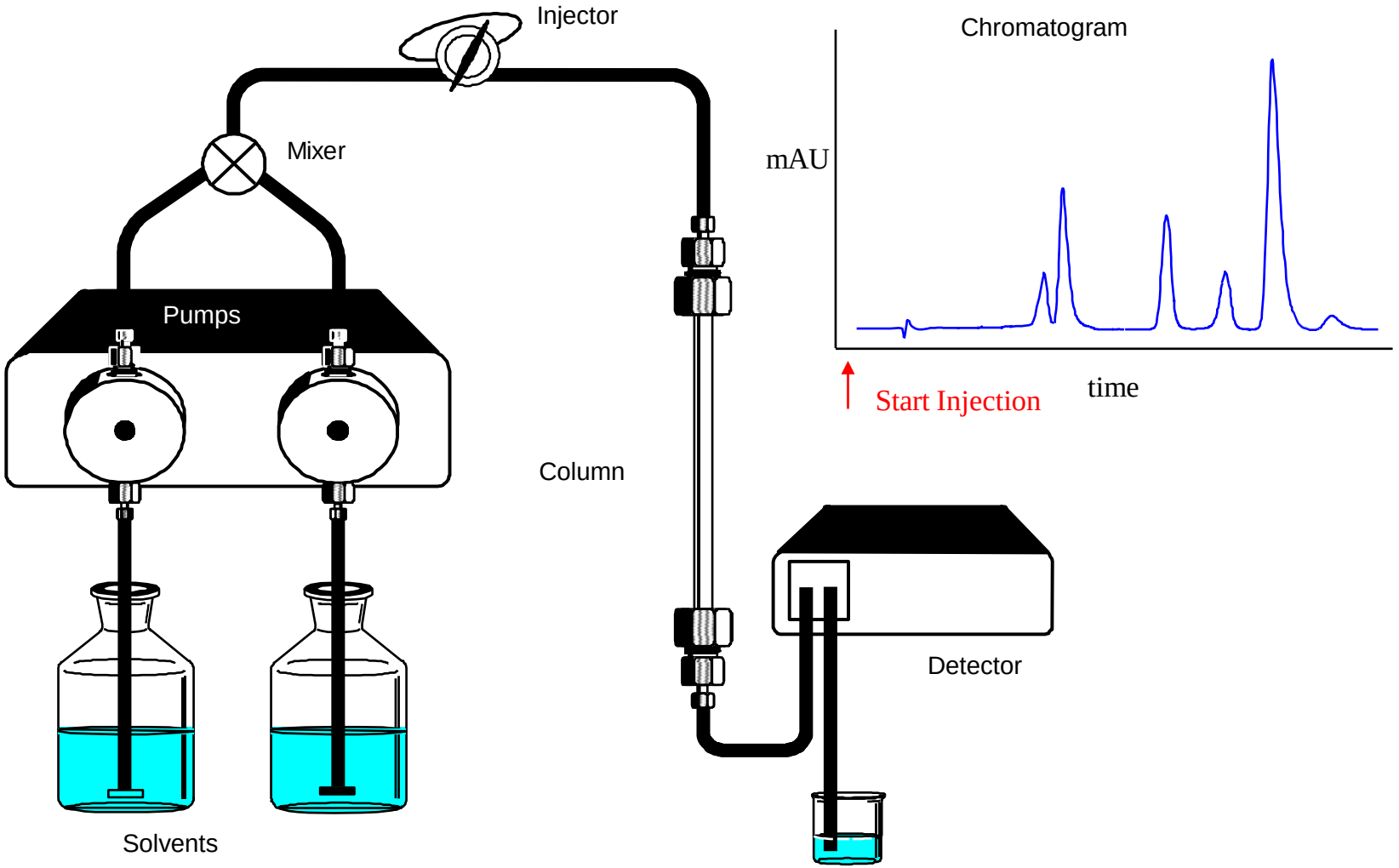
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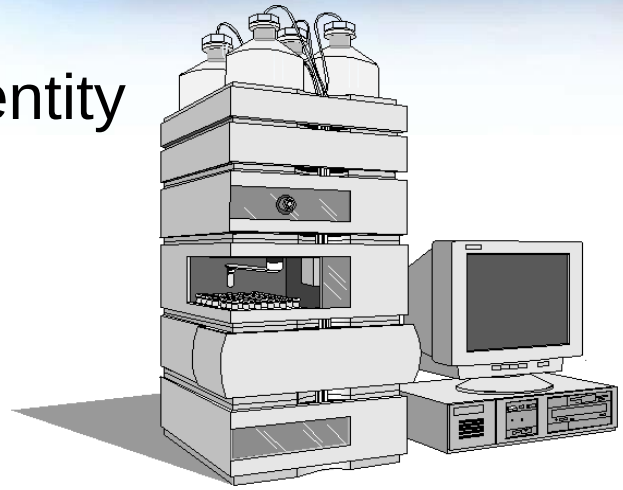
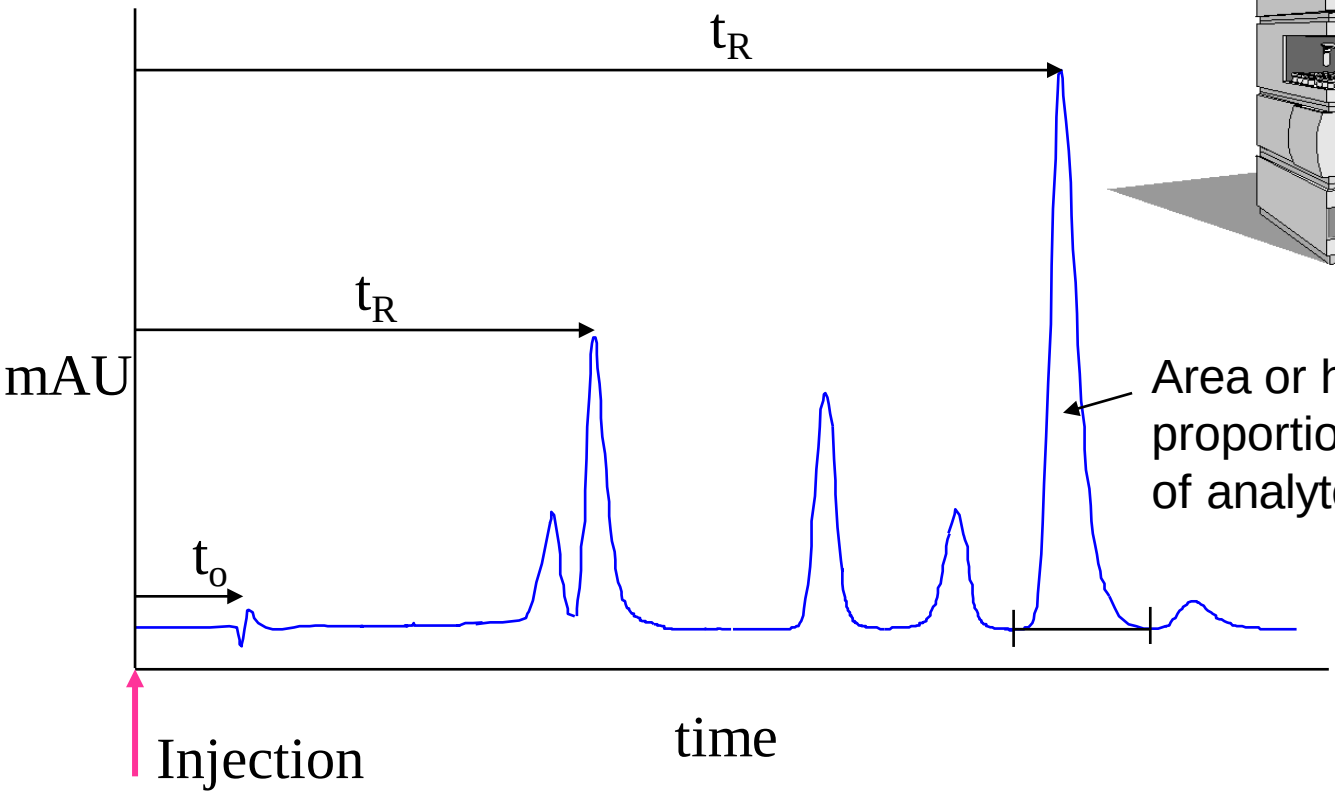
Chromatogram

t_o - elution time of unretained peak

t_R- retention time - determines sample identity





Area or height is proportional to the quantity of analyte.

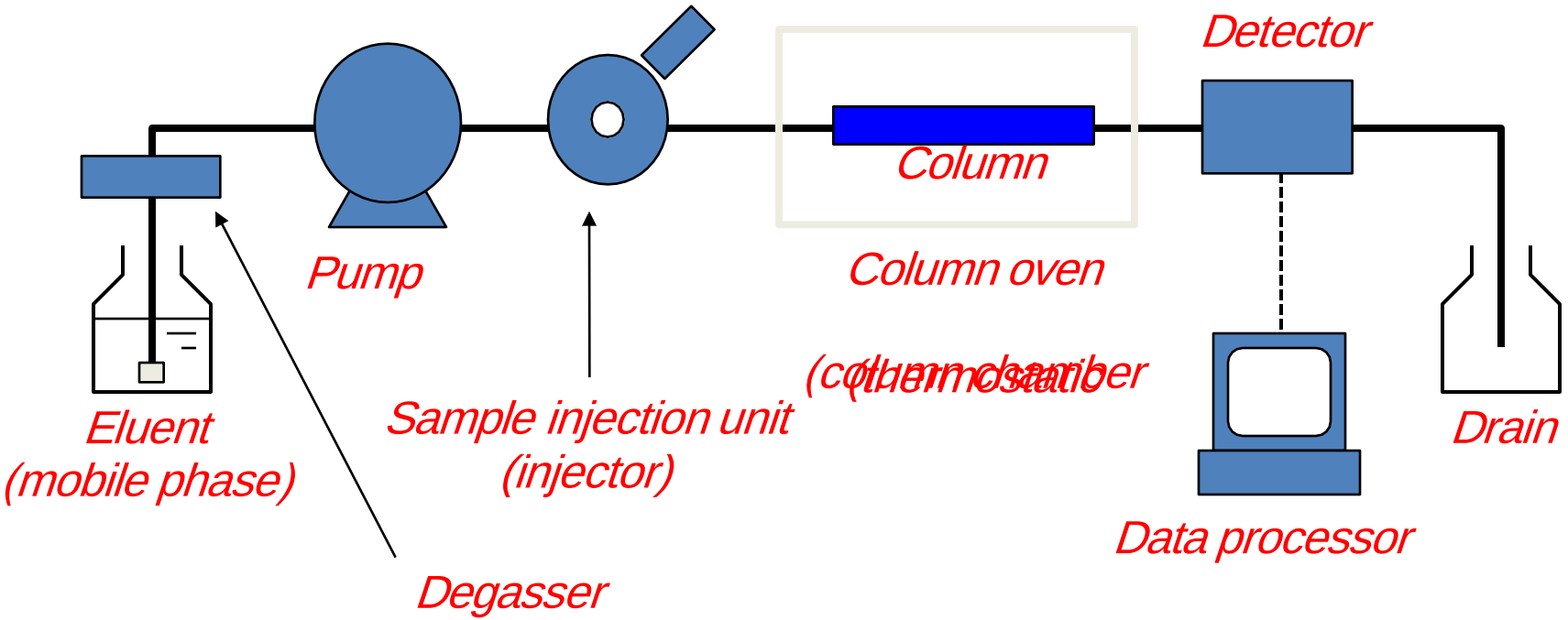
HPLC: Basic Terminology

- Retention Time (t_R)
- Capacity Factor (k)
- Column Efficiency
  - Plate number (n)
  - Height Equivalent of a Theoretical Plate (HETP)
- Selectivity (α)
- Resolution (R_s)

Components

- Mobile phase reservoirs
- HPLC Pump(s(
- Mixing valves
- Sample injector (manual or auto(
- Column
- Detector
- Mobile phase waste container

Flow Channel Diagram for High Performance Liquid Chromatograph



Mobile Phase in HPLC

- Purity of the solvents
- Detector compatibility
- Solubility of the sample
- Low viscosity
- Chemical inertness
- Reasonable price

Common solvents for HPLC mobile phase

Solvent	Solvent strength (E°(	Bp (°C)	Viscosity (cP(	UV cut off (nm(	Refractive index
n-Hexane	0.01	69	0.31	190	1.37
Toluene	0.29	78	0.59	285	1.49
Methylene chloride	0.42	40	0.44	233	1.42
Tetrahydrofuran	0.57	66	0.55	212	1.41
Acetonitrile	0.65	82	0.30	190	1.34
-2propanol	0.82	82	2.30	205	1.38
Methanol	0.95	65	0.54	205	1.33
Water	Large	100	1.00	190>	1.33

Common Mobile phase Modifiers

- Buffers
  - To stabilize pHs of mobile phase under RPC or IEC (phosphate, acetate, citrate(
- Acidifiers
  - To suppress ionization of acidic analytes under RPC (phosphoric acid, acetic acid(
- Ionic strength
  - To control elution of ionic analyte under IEC (i.e., NaCl(
- Ion-pair reagents
  - For separation of ionic compounds under RPC (hexane sulfonate(
- Amine modifiers
  - To reduce tailing of basic analytes under RPC (triethylamine(

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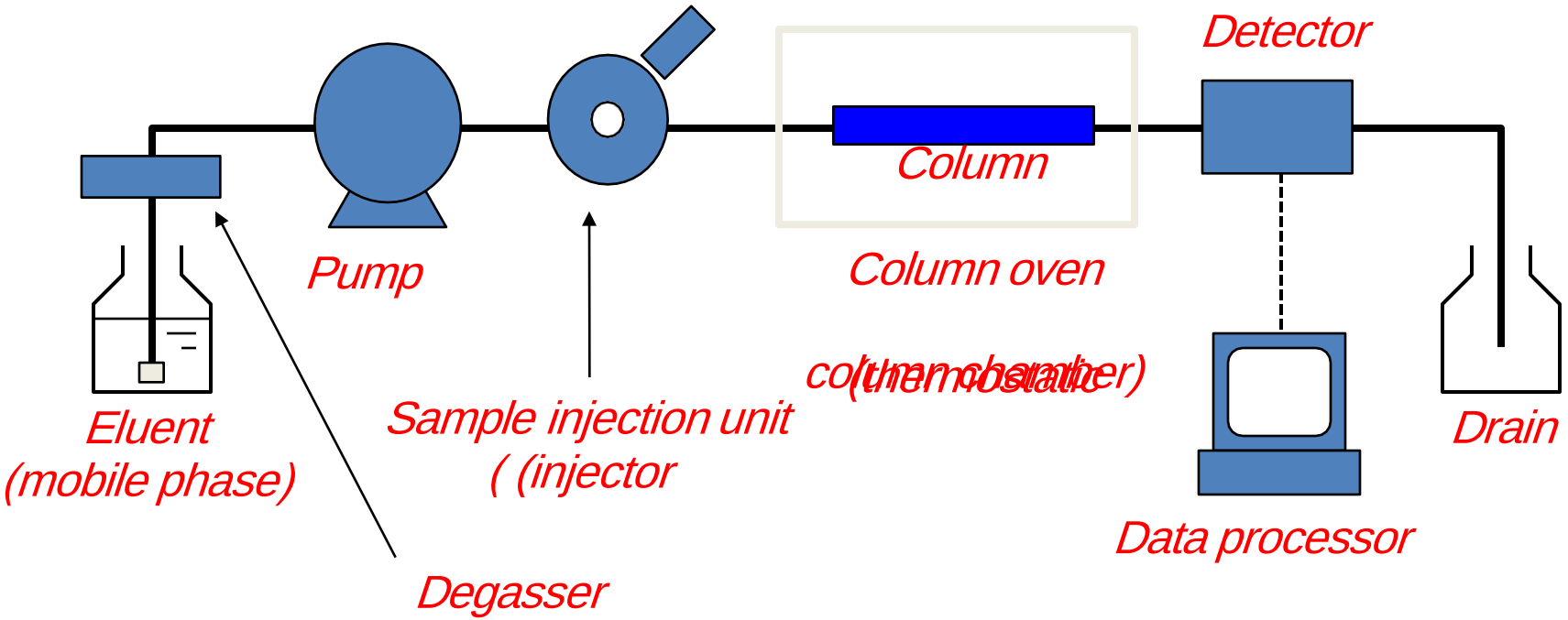
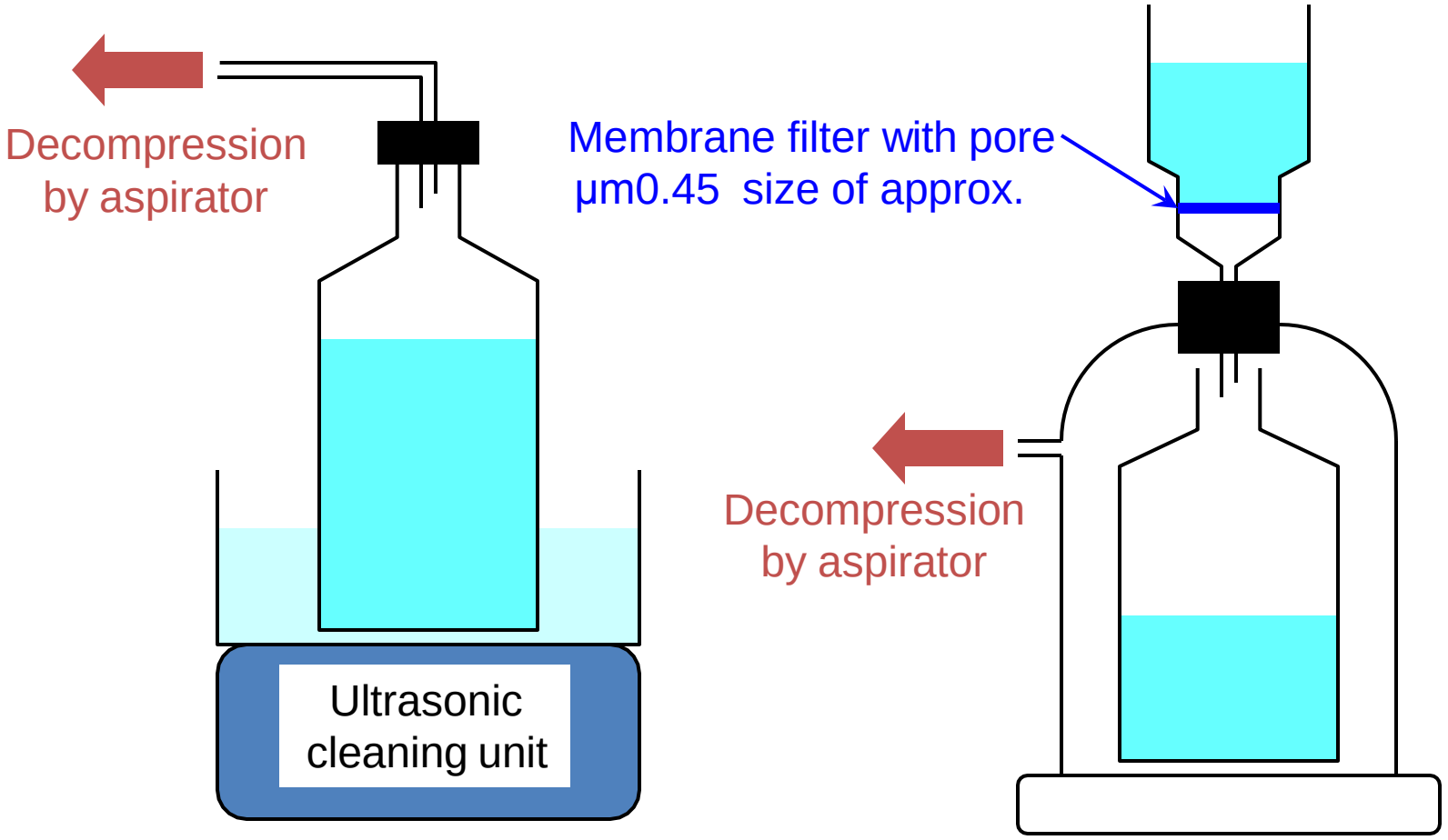


Mixing, Filtration, and Offline Degassing of the Eluent

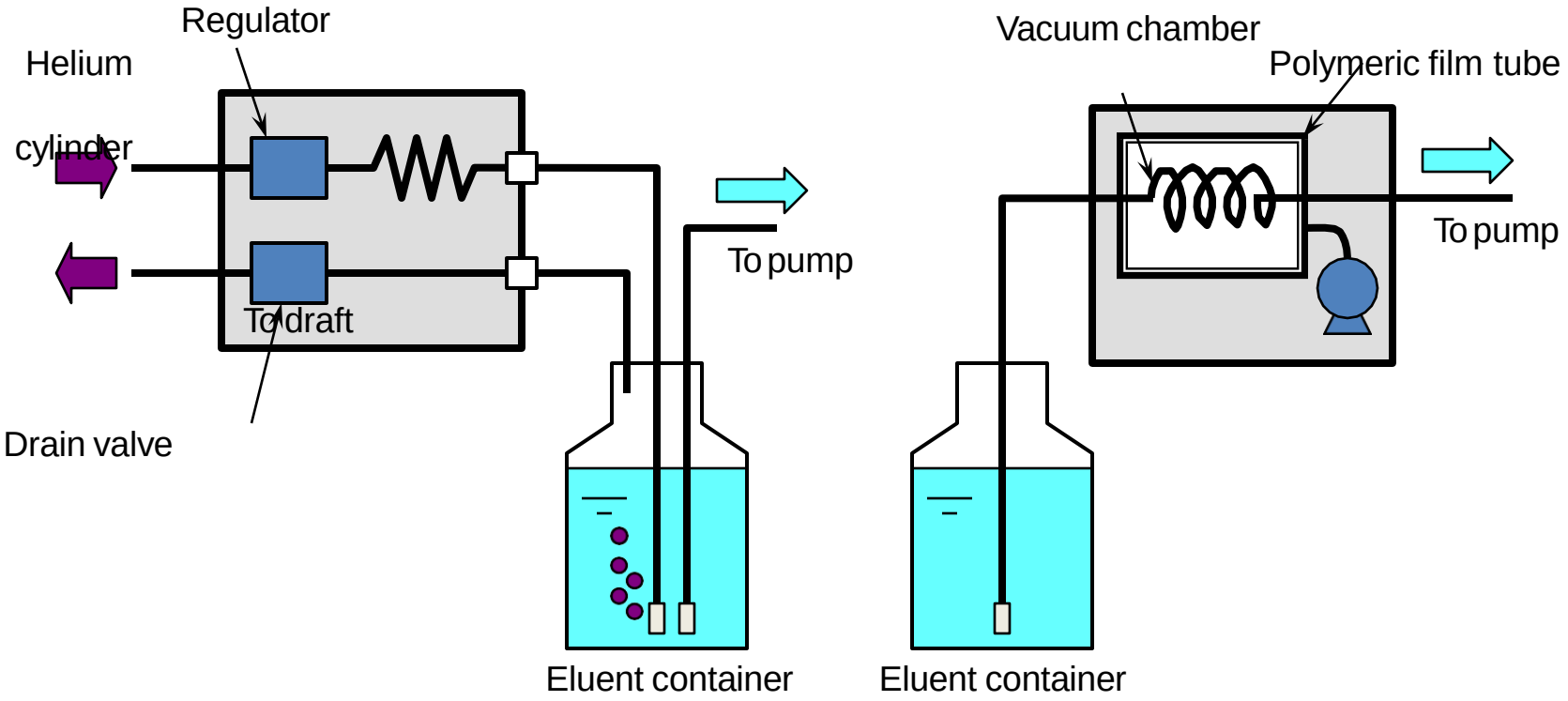


Degasser

- Problems caused by dissolved air in the eluent
  - Unstable delivery by pump
  - More noise and large baseline drift in detector cell

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

Online Degasser

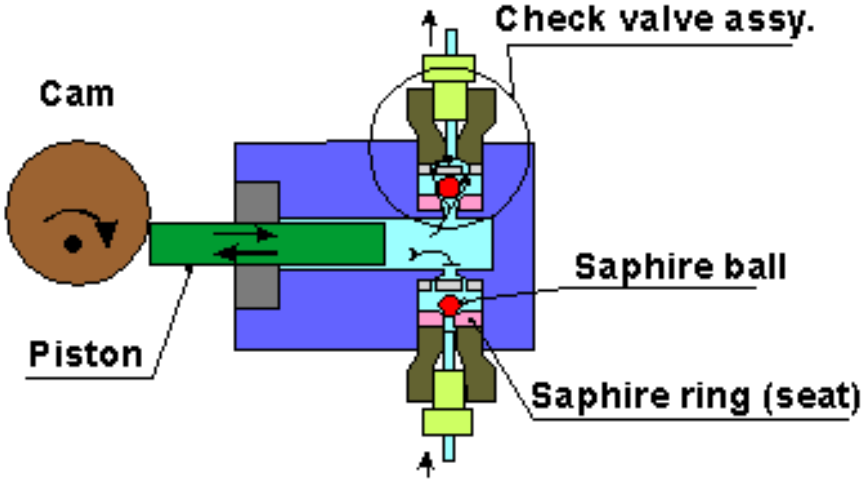


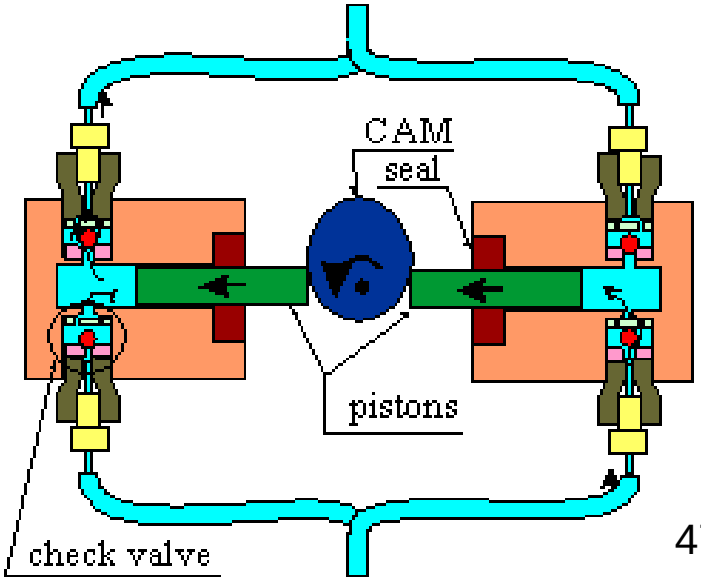
Helium purge method

Gas-liquid separation membrane method

HPLC Pump

- Reciprocating piston pumps are commonly used which have pistons that pull the mobile phase in and push it out into the head of the column





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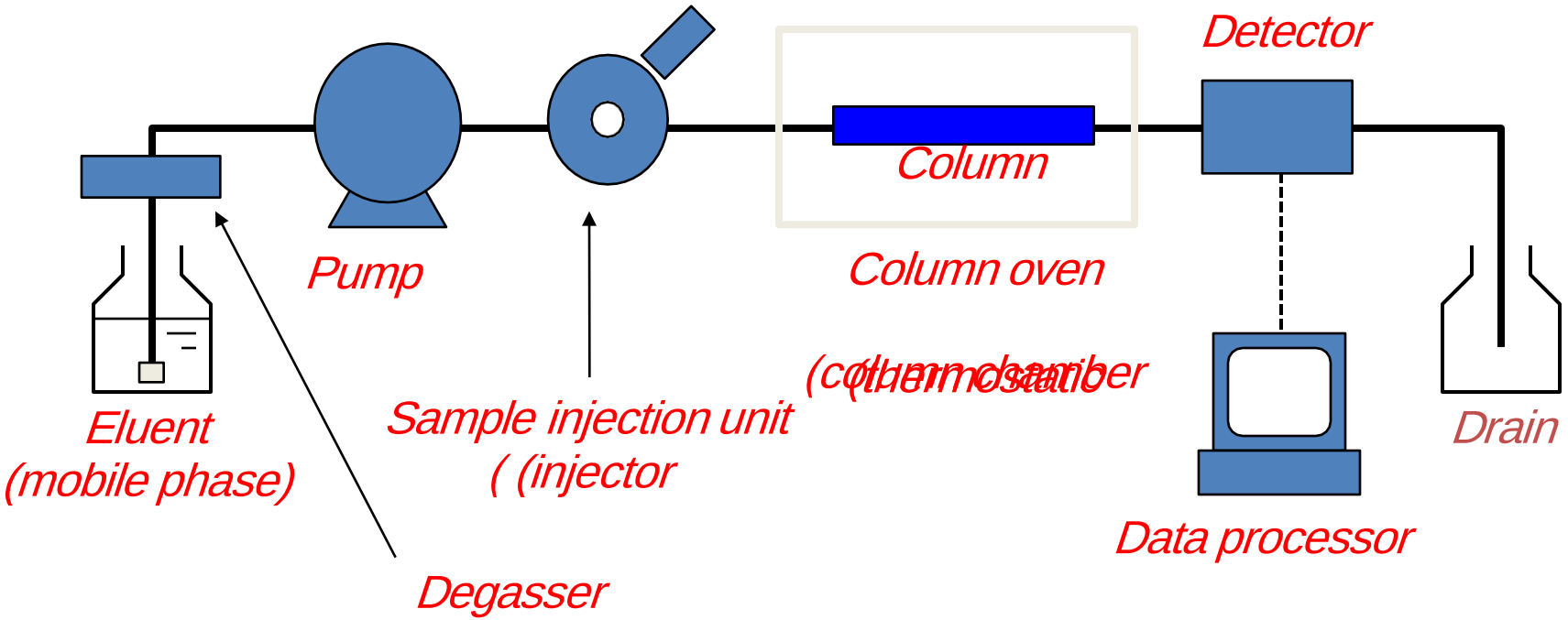
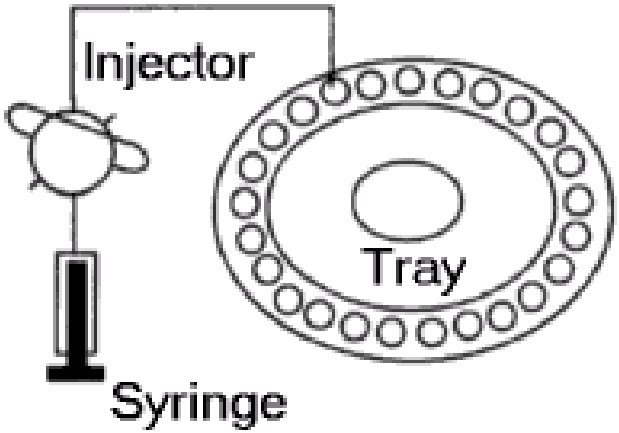
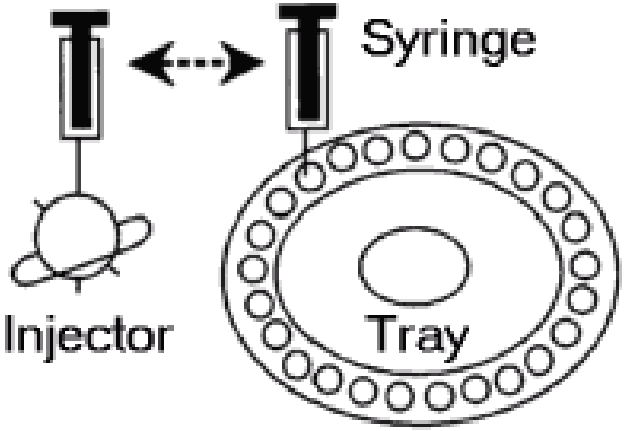


Manual Injector: Injection Method

- Syringe measurement method
  - It is desirable that no more than half the loop volume is injected.
- Loop measurement method
  - It is desirable that at least 3 times the loop volume is injected.

Autosampler





Column

- Types of Columns
  - Microbore
    - Diameter 1-2 mm; Length 7-30 cm; sample size 0.01 mg; flow rate 0.1 mL/min
  - Std. analytical
    - Diameter 3-5 mm; Length 7-30 cm; sample size 0.1 mg; flow rate 1 mL/min
  - Preparative
    - Diameter 5-20 mm; Length 25-50 cm; sample size 10 mg; flow rate 10 mL/min

Column Packing

- Particle size
  - Range from 3-20 µm
  - Smaller the particle, the higher the plate number (N) and need higher pressure to move eluent through the column
  - Analytical application – particle size 10 -3 µm
  - Preparative application – particle size > 10 µm

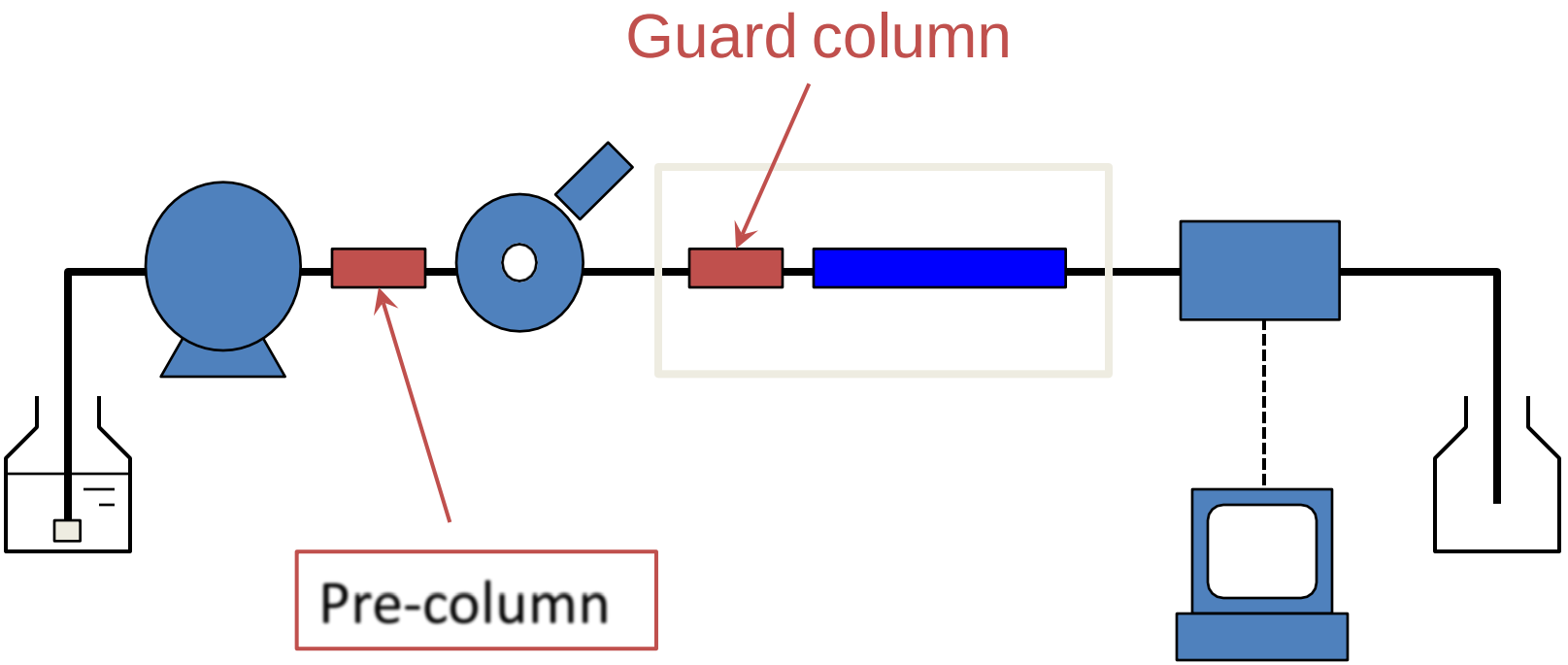
Column Packing Materials

- Support Type
  - Silica (or alumina) or polymer (cross-linked polystyrene(
- Bonded groups
  - C18, C8, C4, amino, cyano, phenyl
  - Diethylaminoethyl (DEAE), sulfonate, quaternary ammonium
- Particle size (d_p): 3-, 5-, 7,- 10- or 20 µm
  - Efficiency is inversely proportional to d_p
  - Column pressure is inversely proportional to $(d_p)^2$
- Pore size (d_{pore}): 60-300A
  - Wide pore materials (300 A) are used for biomolecules or polymers
- Surface area: 90-400 m^2/g
  - High surface area maximizes solute interaction with bonded groups

Column Connection Tubing and Fittings

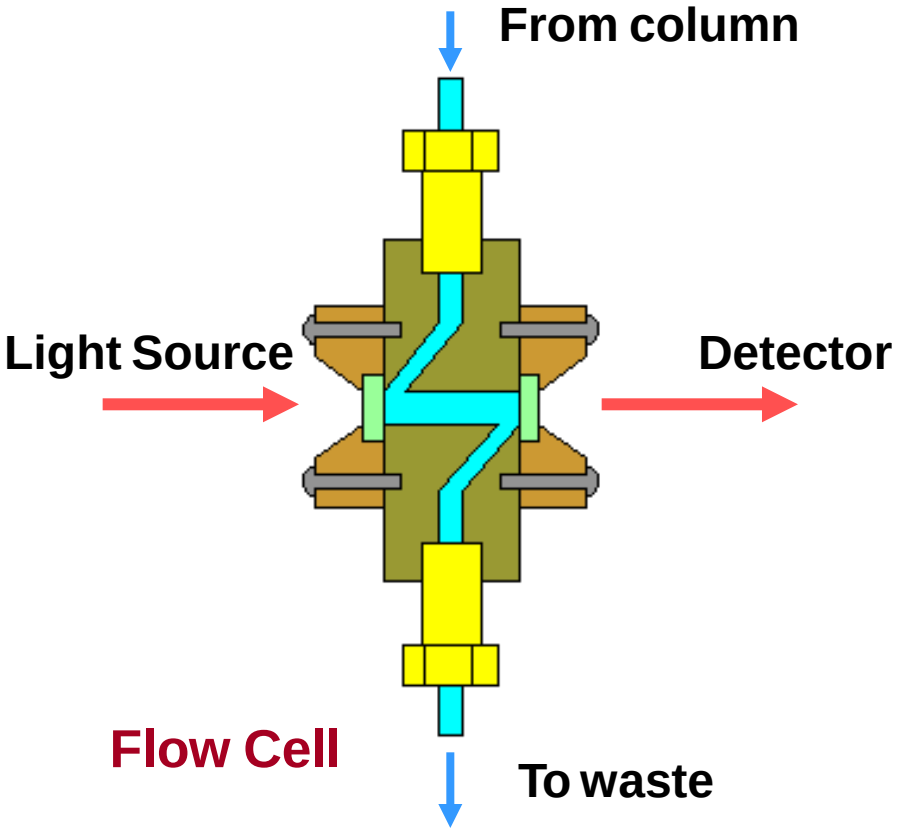
- Connecting Tubing
  - The i.d. and length of capillary tubing between the injector and the detector can affect band spreading
  - This problem of band spreading is pronounced when RT is small e.g. in microbore columns

Guard Column and Pre-column

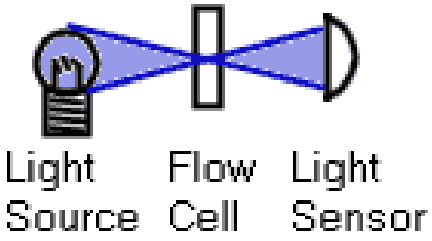


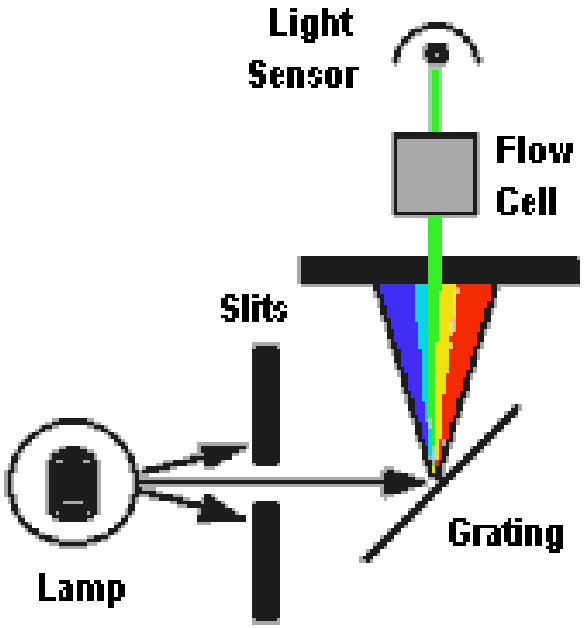
Detector

- Low dead volume: minimize extra column band broadening
  - UV-Vis
  - Fluorescence
  - Electrochemical
  - Refractive index
  - Conductivity
  - Mass Spectrometry
  - FT-IR

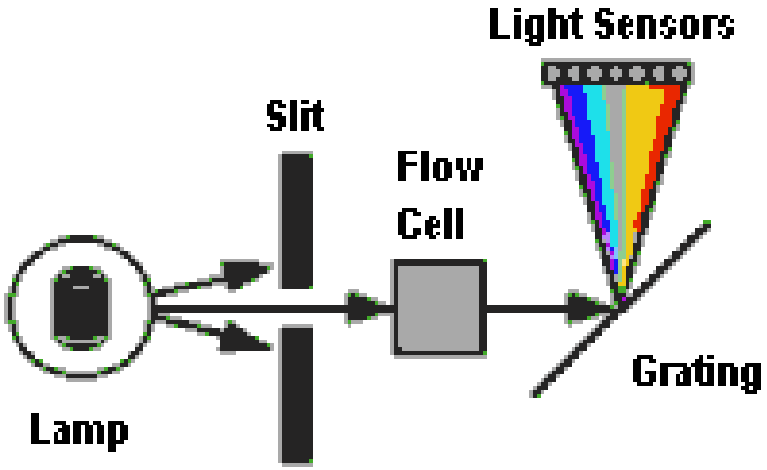


UV-Vis Detectors





UV-Vis



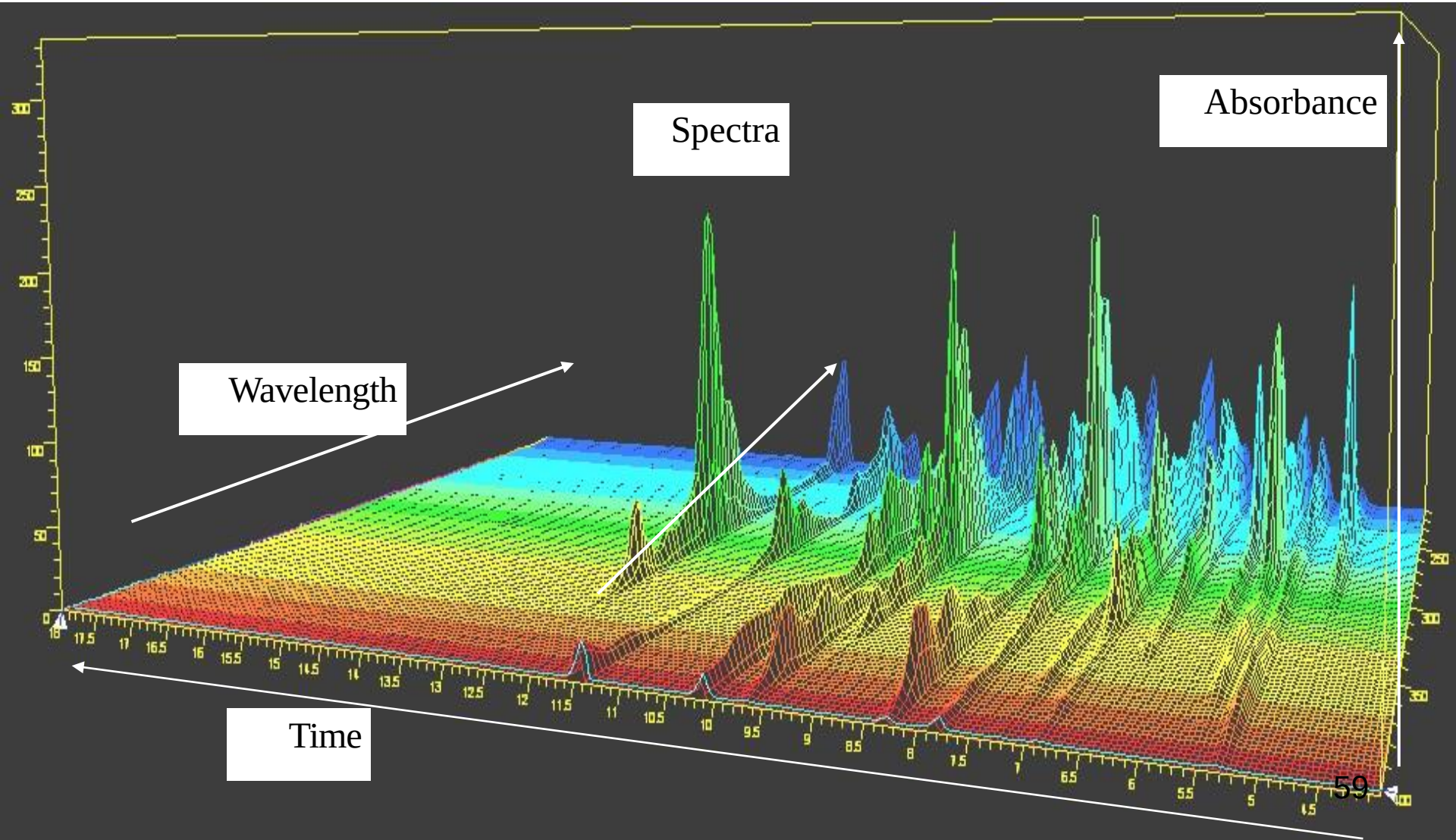
Diode-Array UV-Vis

Advantages of photodiode array detectors

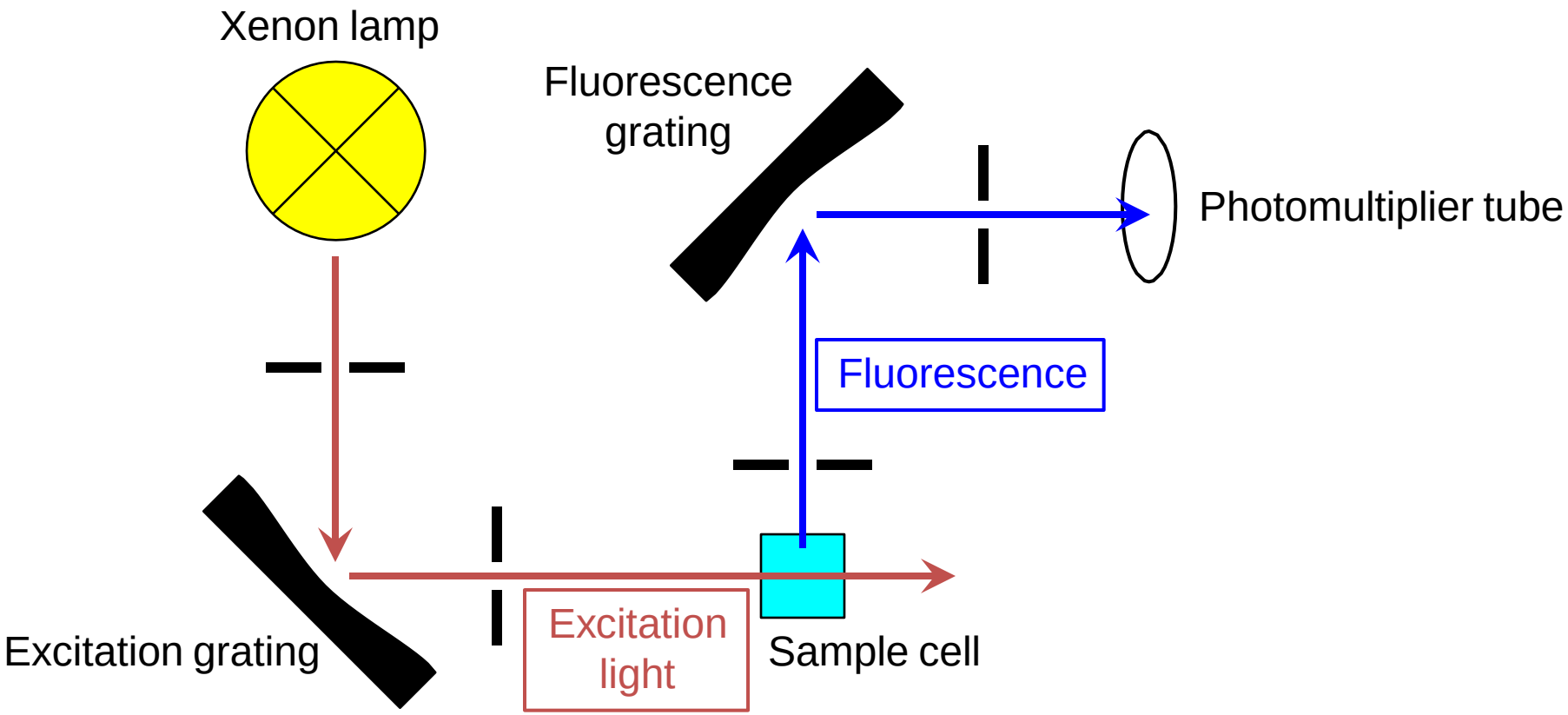
- Peak Identification Using Spectra
  - Complementation of identification based on retention time
  - Library searches

- 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

Online spectra - uv-vis detector

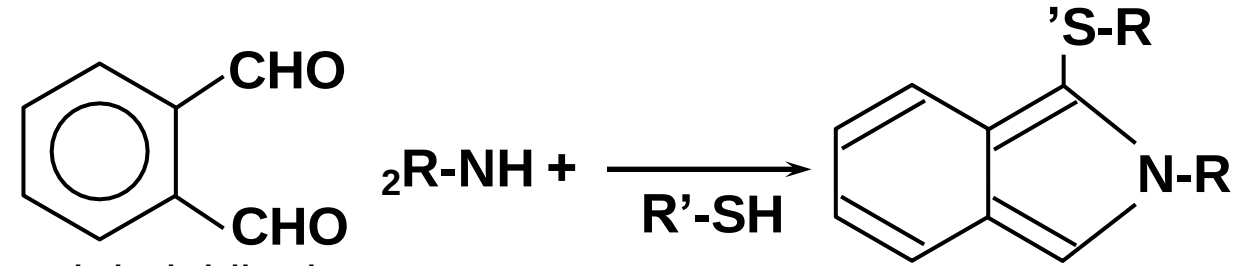


Optical System of Fluorescence Detector



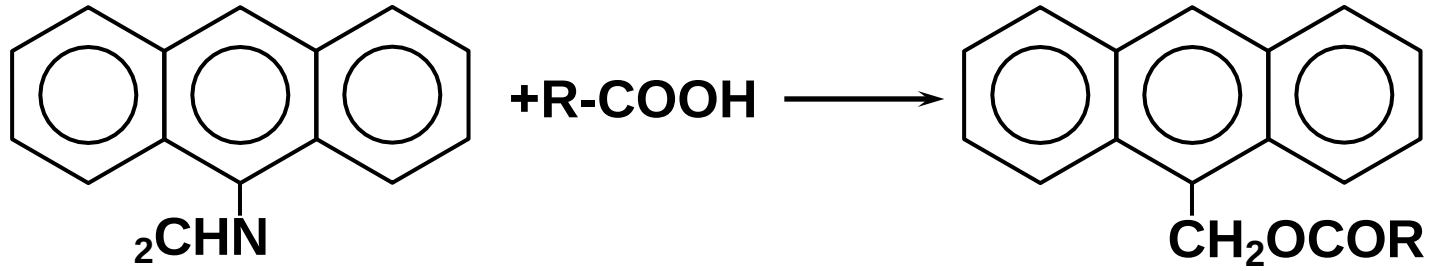
Fluorescence Derivatization Reagents

- OPA Reagent (Reacts with Primary Amines(



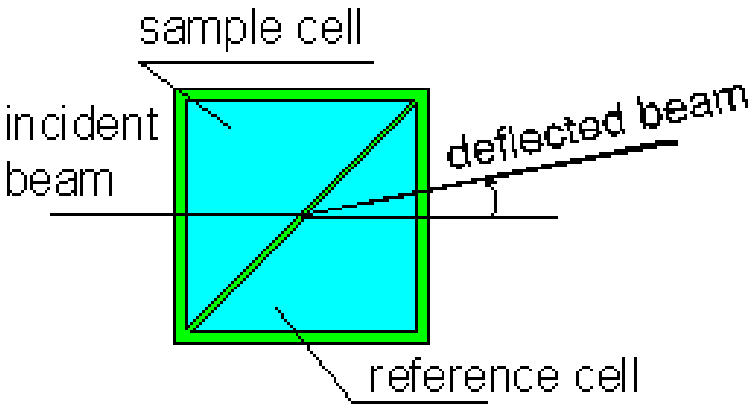
o-phthalaldhyde
(OPA)

- ADAM Reagent (Reacts with Fatty Acids(

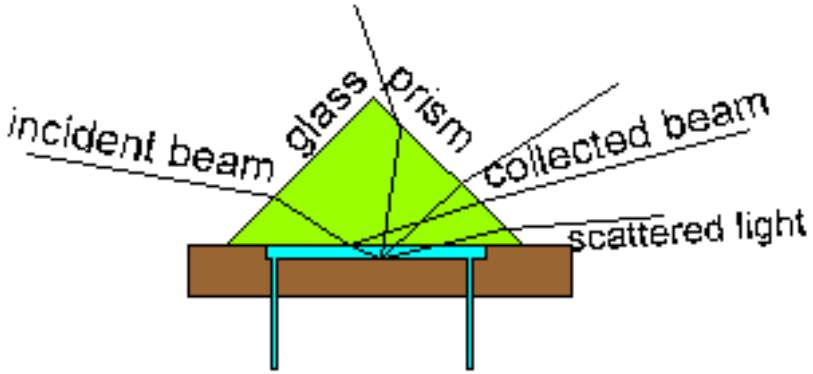


anthryldiazomethane-9
((ADAM

Refractive Index



Deflection detector



Reflective 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


Thanks for your attention
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