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SUDHEER BOBBA

Editor

Research Interest

- HPLC, Preparative HPLC; Cap-LC; LC-MS (Triple Quad, Ion Trap and TOF); QTRAP 4000 & 5500; LTQ-Orbitrap; Q-exactive; Q-TOF; GC; GC-MS; FT-IR; UV-Vis; Spectrofluorometry; NMR; Chiral separation; Chromatography skills; Sample preparation techniques (protein precipitation, liquid and solid phase extraction); Data interpretation; analysis; and management; Technical report preparation; Stability studies; Out of trend; Out of specification and Dissolution studies

Biography

- Sudheer Bobba completed his doctoral studies in May 2013 at University of Missouri-Kansas City. He was awarded the prestigious preparing future faculty fellowship for the academic years 2011-2014. He served as vice president and treasurer of pharmaceutical sciences graduate student association and also served as member of interdisciplinary student council executive board. Dr. Bobba has done summer internships at Johnson and Johnson (J&J), Vertex Pharmaceuticals, and Stowers Institute for Medical Research. He has authored/co-authored several peer reviewed research articles in reputed international journals. He is also a member of American Association of Pharmaceutical Scientists (AAPS) and American Society for Mass Spectrometry (ASMS). Dr. Bobba is also an honorary member of Phi Kappa Phi and Sigma Xi. Dr. Bobba joined Covance Labs in July 2014 as a scientist in drug metabolism group. His responsibilities include HPLC, LC-MS, and LC-MS/MS analytical method development for incoming test articles (drug substance and drug product) and its metabolites along with structural identification and characterization of drug metabolites using advanced LC-MS/MS and radiochromatographic techniques. He is currently serving as reviewer for several journals such as Journal of Drug Metabolism & Toxicology, European Journal of Pharmaceutical Sciences, Journal of Veterinary Pharmacology and Therapeutics, Biomedical Chromatography, Clinical Pharmacology & Biopharmaceutics, Pharmaceutical Sciences, Pharmaceutica Analytica Acta, Pharmaceutical Care & Health Systems, Advances in Pharmacoepidemiology & Drug Safety.

Recent Publication

- Ion-pairing liquid chromatography-tandem mass spectrometry-based quantification of uridine diphosphate-linked intermediates in the Staphylococcus aureus cell wall biosynthesis pathway.

- Quantitative lc-ms/ms analysis of cytoplasmic bacterial cell wall biosynthesis intermediates: application to vancomycin resistance VRE (982.2)

- Ion-pairing LC-MS/MS-based quantification of UDP-linked intermediates in the< i> S. aureus</i> cell wall biosynthesis pathway

Research Interest Topic

- **HPLC**

High-performance liquid chromatography (HPLC; formerly referred to as high-pressure liquid chromatography), is a technique in analytic chemistry used to separate the components in a mixture, to identify each component, and to quantify each component. It relies on pumps to pass a pressurized liquid solvent containing the sample mixture through a column filled with a solid adsorbent material. Each component in the sample interacts slightly differently with the adsorbent material, causing different flow rates for the different components and leading to the separation of the components as they flow out the column.

HPLC has been used for medical (e.g. detecting vitamin D levels in blood serum), legal (e.g. detecting performance enhancement drugs in urine), research (e.g. separating the components of a complex biological sample, or of similar synthetic chemicals from each other), and manufacturing (e.g. during the production process of pharmaceutical and biological products) purposes.

Principle of Chromatography

Chromatography is a technique by which a mixture sample is separated into components. Although originally intended to separate and recover (isolate and purify) the components of a sample, today, complete chromatography systems are often used to both separate and quantify sample components.

- **What does HPLC stand for?**

HPLC is short for the High Performance LC. HPLC is an analysis method that yields high performance and high speed compared with traditional column chromatography because of the forcibly pumped mobile phase. Recently, ultrafast analysis using a high-pressure-resistant apparatus has been attracting attention. UHPLC (Ultra High Performance LC) is becoming established as an abbreviation for this ultrafast LC method.
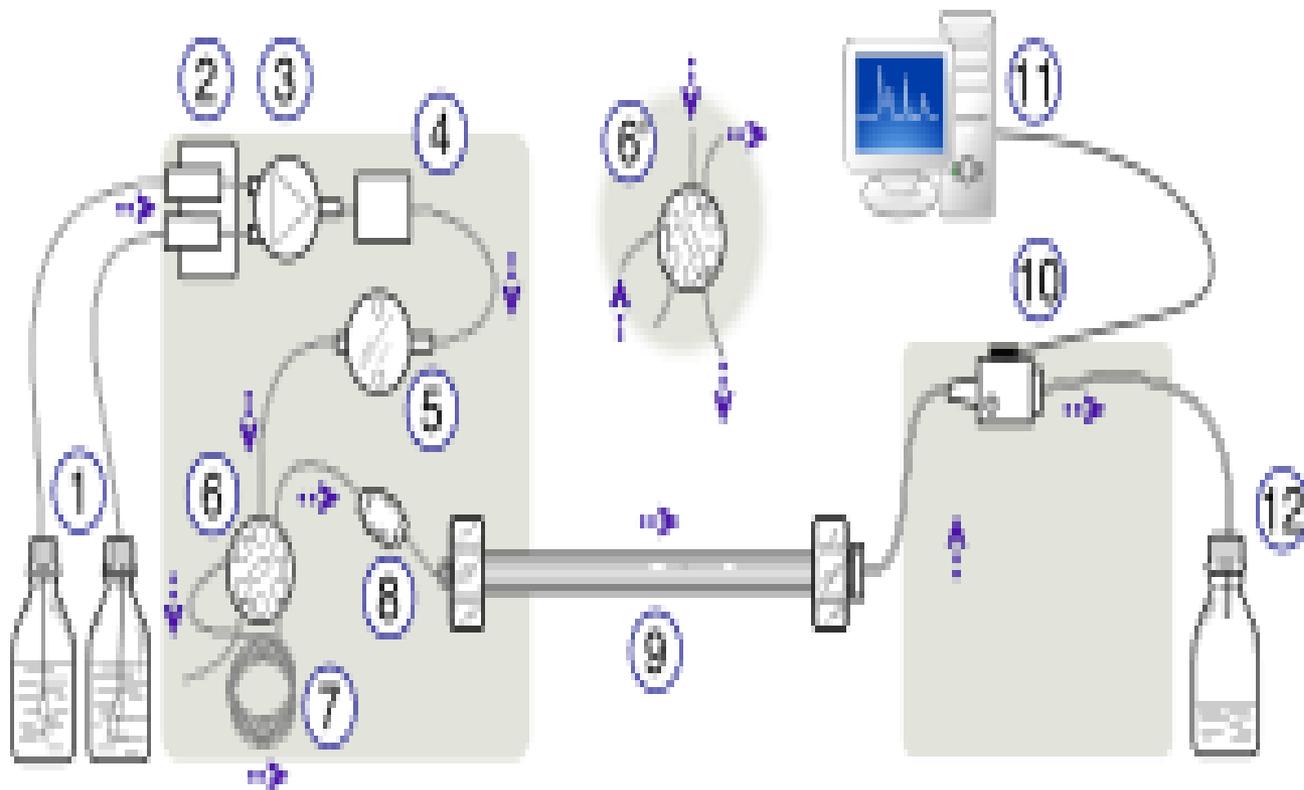
Schematic representation of an HPLC unit:
(1) Solvent reservoirs, (2) Solvent degasser, (3) Gradient valve, (4) Mixing vessel for delivery of the mobile phase, (5) High-pressure pump, (6) Switching valve in "inject position", (6') Switching valve in "load position", (7) Sample injection loop, (8) Pre-column (guard column), (9) Analytical column, (10) Detector (i.e. IR, UV), (11) Data acquisition, (12) Waste or fraction collector.

Types of HPLC

Normal phase HPLC:

- This is essentially just the same as you will already have read about in thin layer chromatography or column chromatography. Although it is described as "normal", it isn't the most commonly used form of HPLC.

- The column is filled with tiny silica particles, and the solvent is non-polar - hexane, for example. A typical column has an internal diameter of 4.6 mm (and may be less than that), and a length of 150 to 250 mm.

- Polar compounds in the mixture being passed through the column will stick longer to the polar silica than non-polar compounds will. The non-polar ones will therefore pass more quickly through the column.

- ## *Reversed phase HPLC*

- In this case, the column size is the same, but the silica is modified to make it non-polar by attaching long hydrocarbon chains to its surface - typically with either 8 or 18 carbon atoms in them. A polar solvent is used - for example, a mixture of water and an alcohol such as methanol.

- In this case, there will be a strong attraction between the polar solvent and polar molecules in the mixture being passed through the column. There won't be as much attraction between the hydrocarbon chains attached to the silica (the stationary phase) and the polar molecules in the solution. Polar molecules in the mixture will therefore spend most of their time moving with the solvent.

- Non-polar compounds in the mixture will tend to form attractions with the hydrocarbon groups because of van der Waals dispersion forces. They will also be less soluble in the solvent because of the need to break hydrogen bonds as they squeeze in between the water or methanol molecules, for example. They therefore spend less time in solution in the solvent and this will slow them down on their way through the column.

- That means that now it is the polar molecules that will travel through the column more quickly.
- Reversed phase HPLC is the most commonly used form of HPLC.

A flow scheme for HPLC
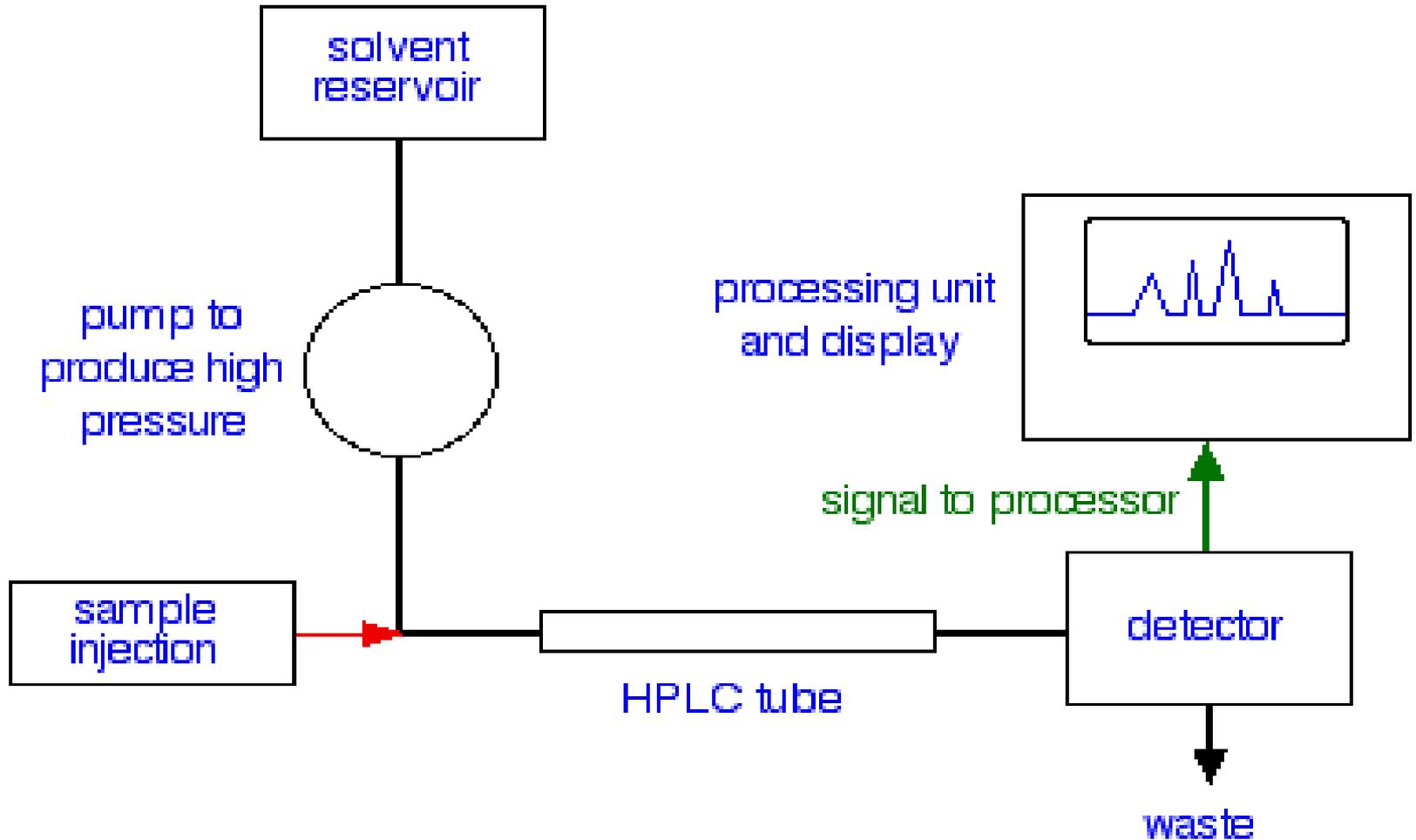
Injection of the sample

Injection of the sample is entirely automated, and you wouldn't be expected to know how this is done at this introductory level. Because of the pressures involved, it is *not* the same as in gas chromatography (if you have already studied that).

Retention time

The time taken for a particular compound to travel through the column to the detector is known as its **retention time**. This time is measured from the time at which the sample is injected to the point at which the display shows a maximum peak height for that compound.

Different compounds have different retention times. For a particular compound, the retention time will vary depending on:
- the pressure used (because that affects the flow rate of the solvent)
- the nature of the stationary phase (not only what material it is made of, but also particle size)
- the exact composition of the solvent
- the temperature of the column

That means that conditions have to be carefully controlled if you are using retention times as a way of identifying compounds.

The detector

There are several ways of detecting when a substance has passed through the column. A common method which is easy to explain uses ultra-violet absorption.

Many organic compounds absorb UV light of various wavelengths. If you have a beam of UV light shining through the stream of liquid coming out of the column, and a UV detector on the opposite side of the stream, you can get a direct reading of how much of the light is absorbed.

The amount of light absorbed will depend on the amount of a particular compound that is passing through the beam at the time.
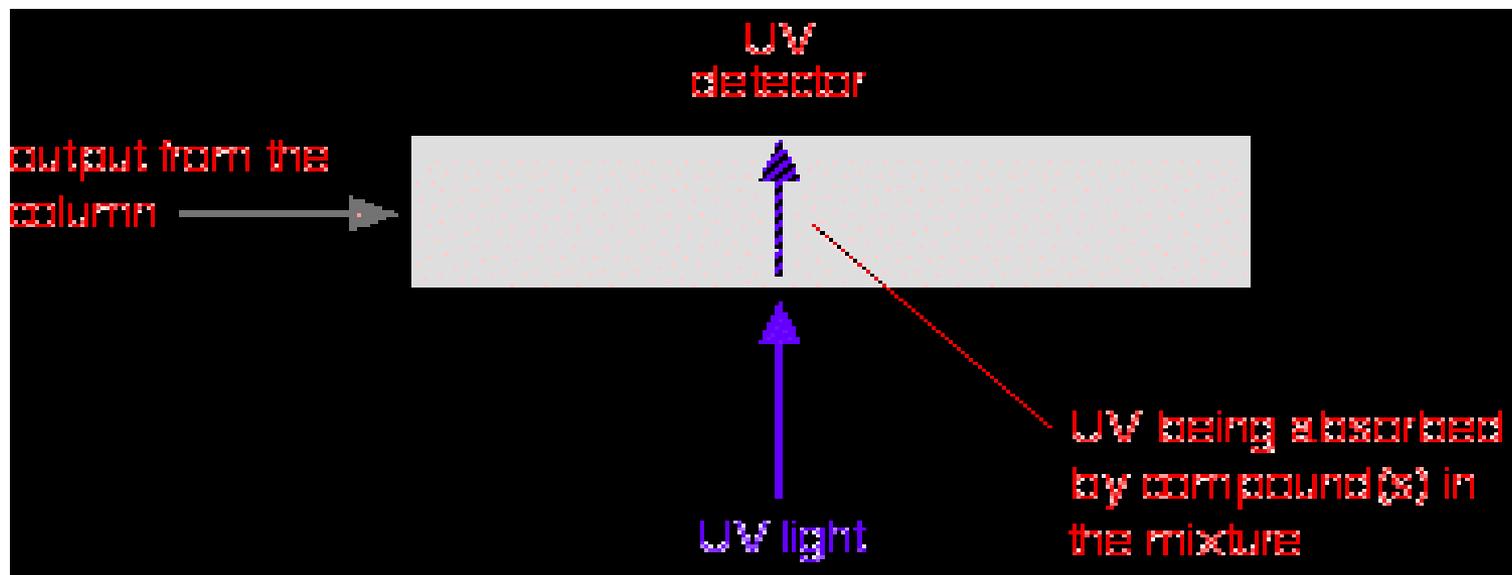
Interpreting the output from the detector

The output will be recorded as a series of peaks - each one representing a compound in the mixture passing through the detector and absorbing UV light. As long as you were careful to control the conditions on the column, you could use the retention times to help to identify the compounds present - provided, of course, that you (or somebody else) had already measured them for pure samples of the various compounds under those identical conditions.

But you can also use the peaks as a way of measuring the quantities of the compounds present. Let's suppose that you are interested in a particular compound, X.

If you injected a solution containing a known amount of pure X into the machine, not only could you record its retention time, but you could also relate the amount of X to the peak that was formed.

The area under the peak is proportional to the amount of X which has passed the detector, and this area can be calculated automatically by the computer linked to the display. The area it would measure is shown in green in the (very simplified) diagram.

If the solution of X was less concentrated, the area under the peak would be less - although the retention time will still be the same. For example:

This means that it is possible to calibrate the machine so that it can be used to find how much of a substance is present - even in very small quantities.

Be careful, though! If you had two different substances in the mixture (X and Y) could you say anything about their relative amounts? Not if you were using UV absorption as your detection method.

In the diagram, the area under the peak for Y is less than that for X. That may be because there is less Y than X, but it could equally well be because Y absorbs UV light at the wavelength you are using less than X does. There might be large quantities of Y present, but if it only absorbed weakly, it would only give a small peak.

Separations

Injector

Mixer

Pumps

Column

Solvents

Detector

Waste

High-Performance Liquid Chromatograph
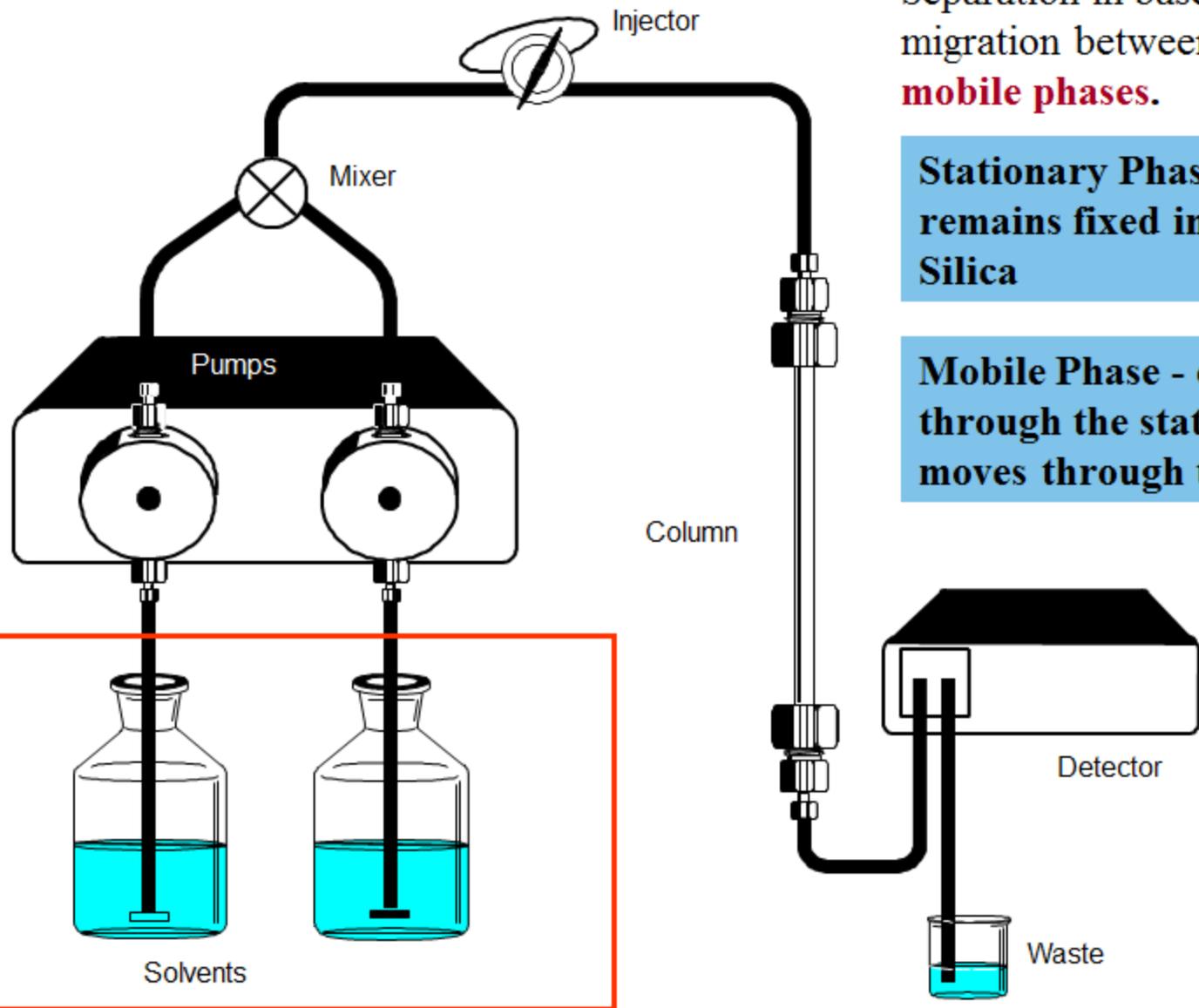
Separation in based upon differential migration between the **stationary** and **mobile phases**.

Stationary Phase - the phase which remains fixed in the column, e.g. C18, Silica

Mobile Phase - carries the sample through the stationary phase as it moves through the column.
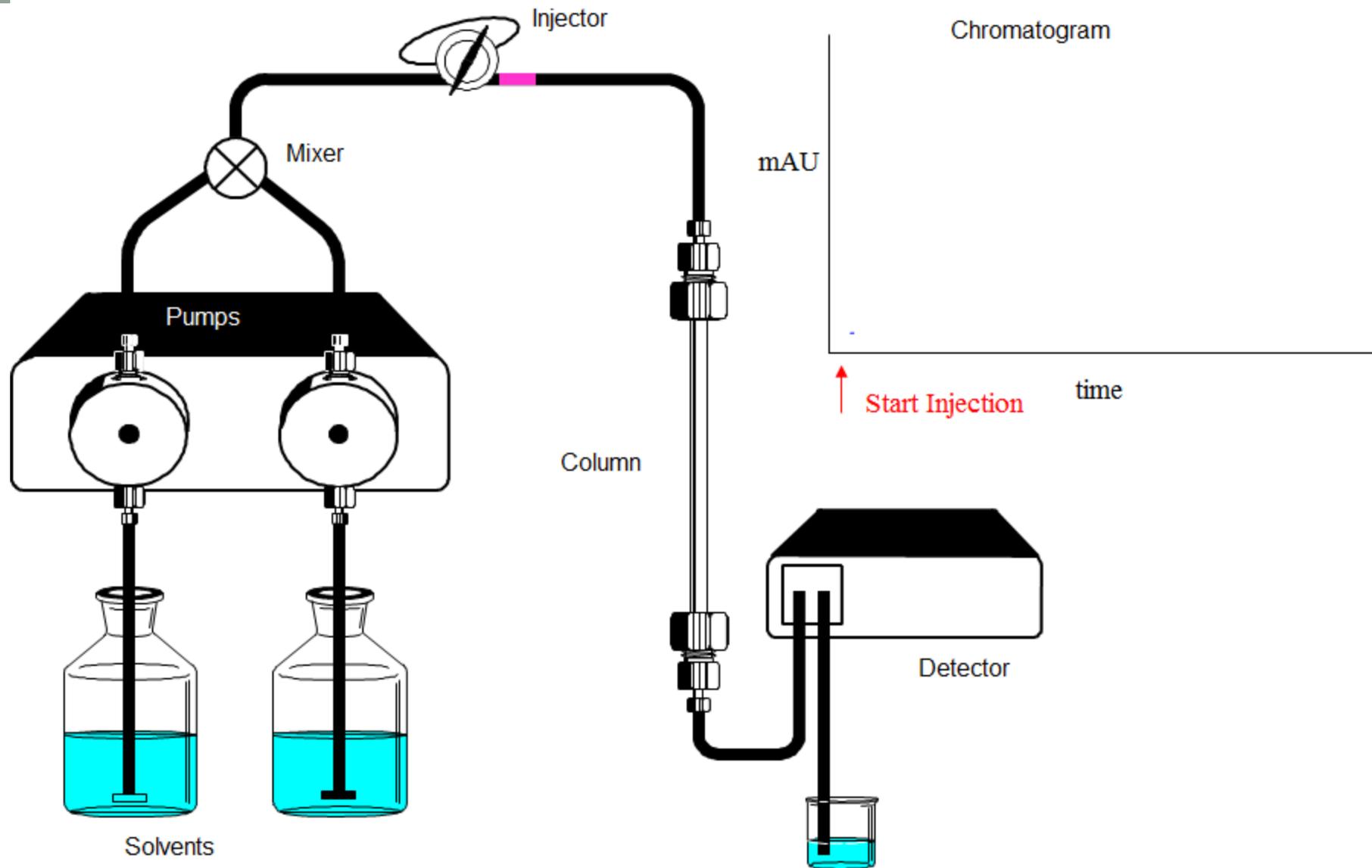
High Performance Liquid Chromatograph
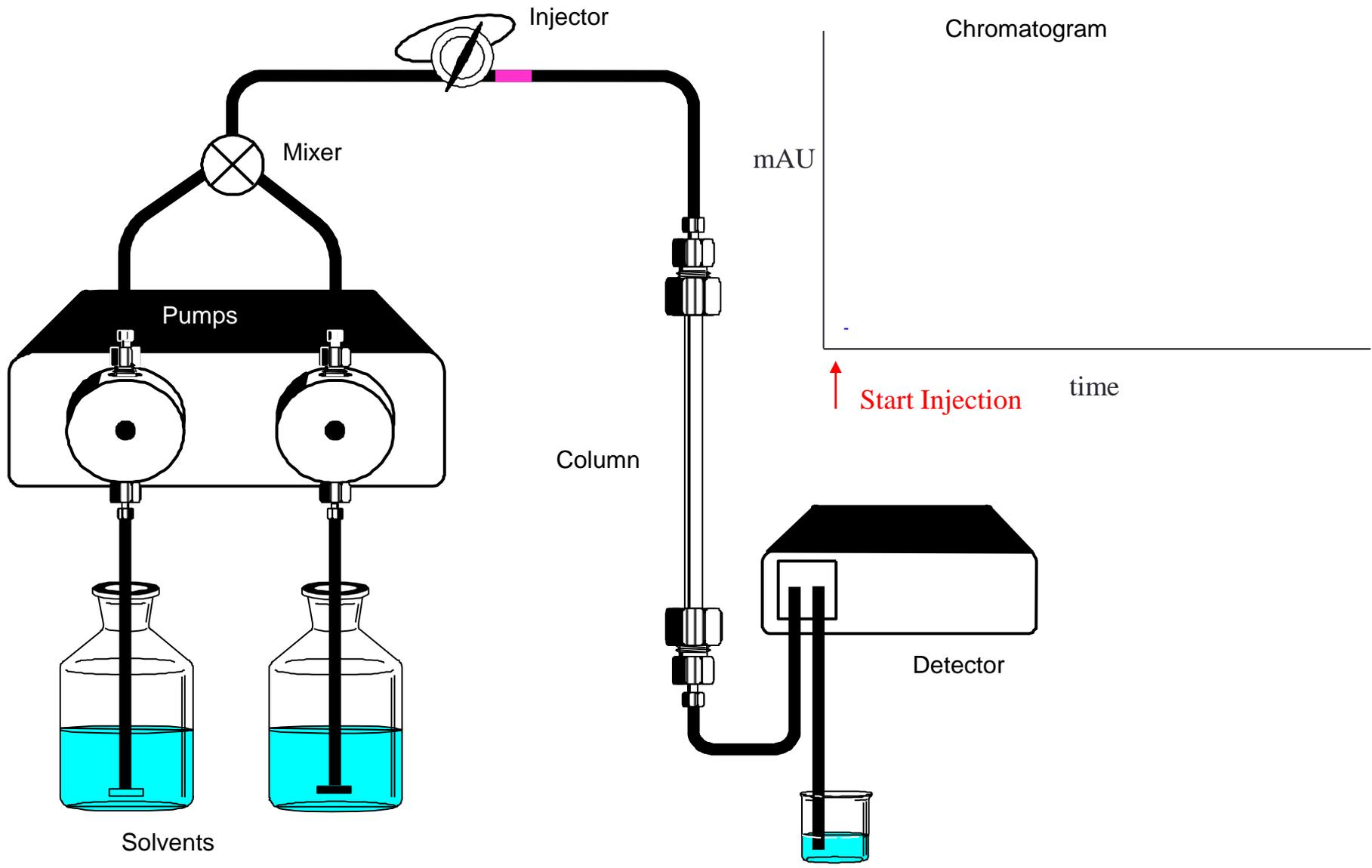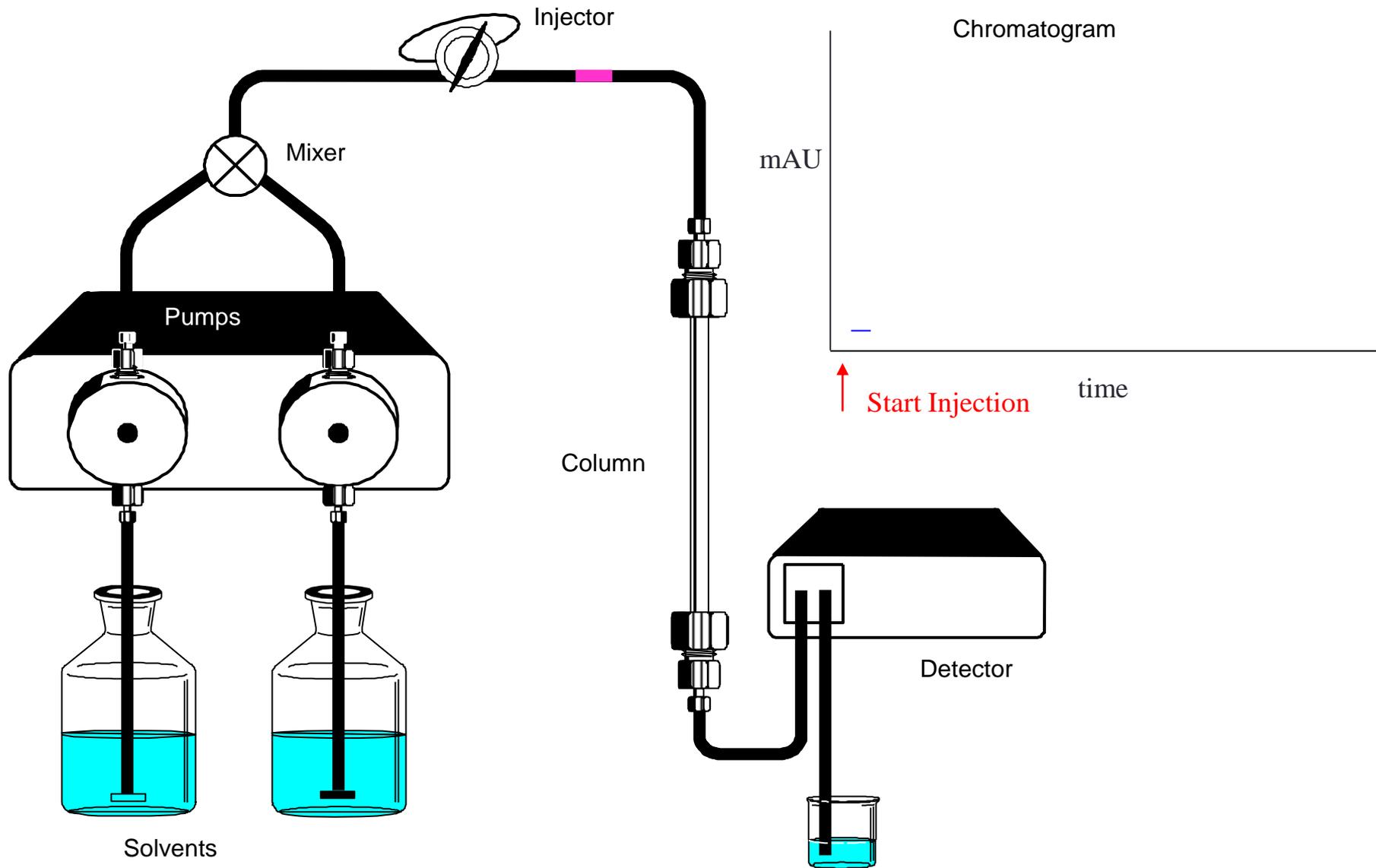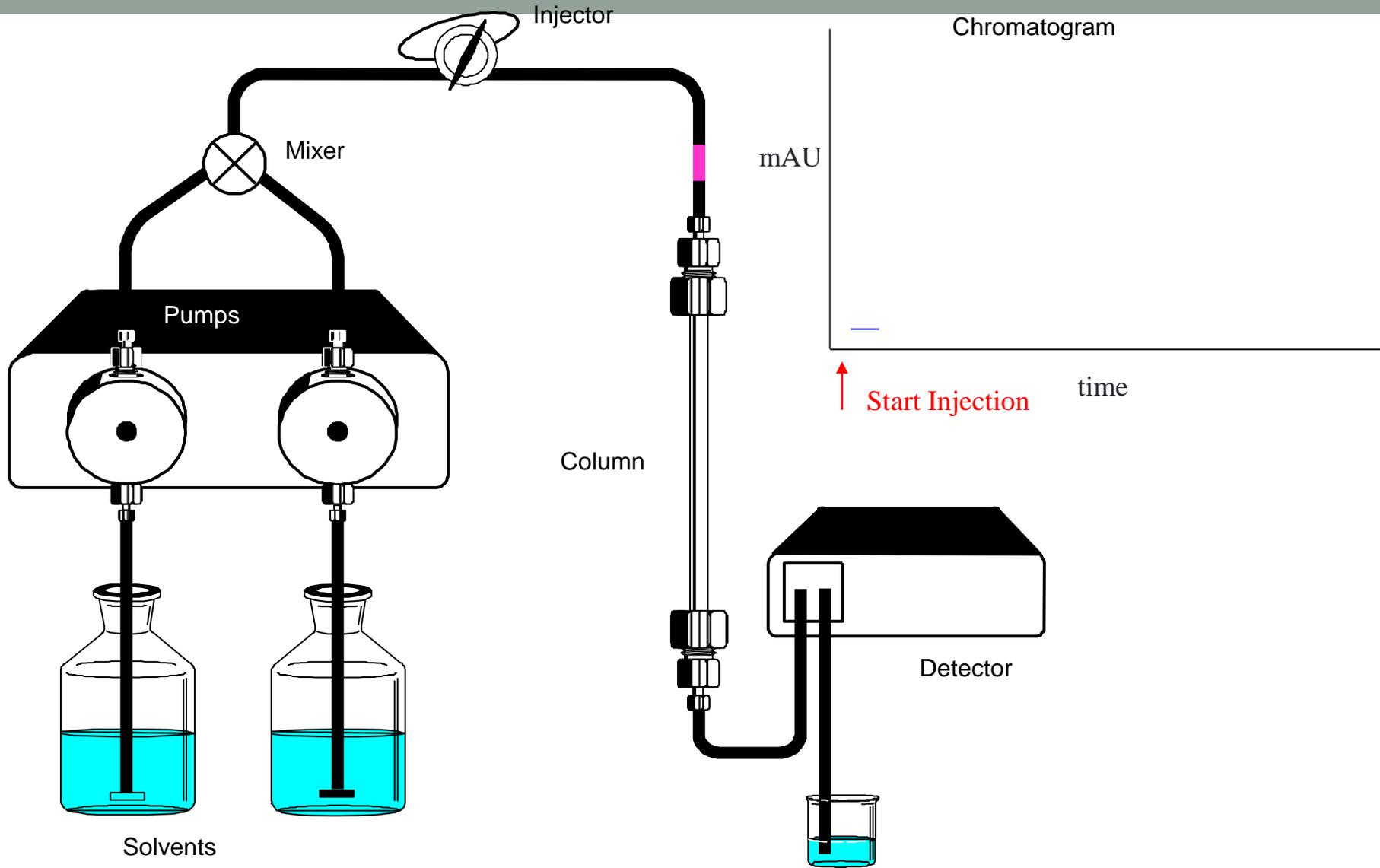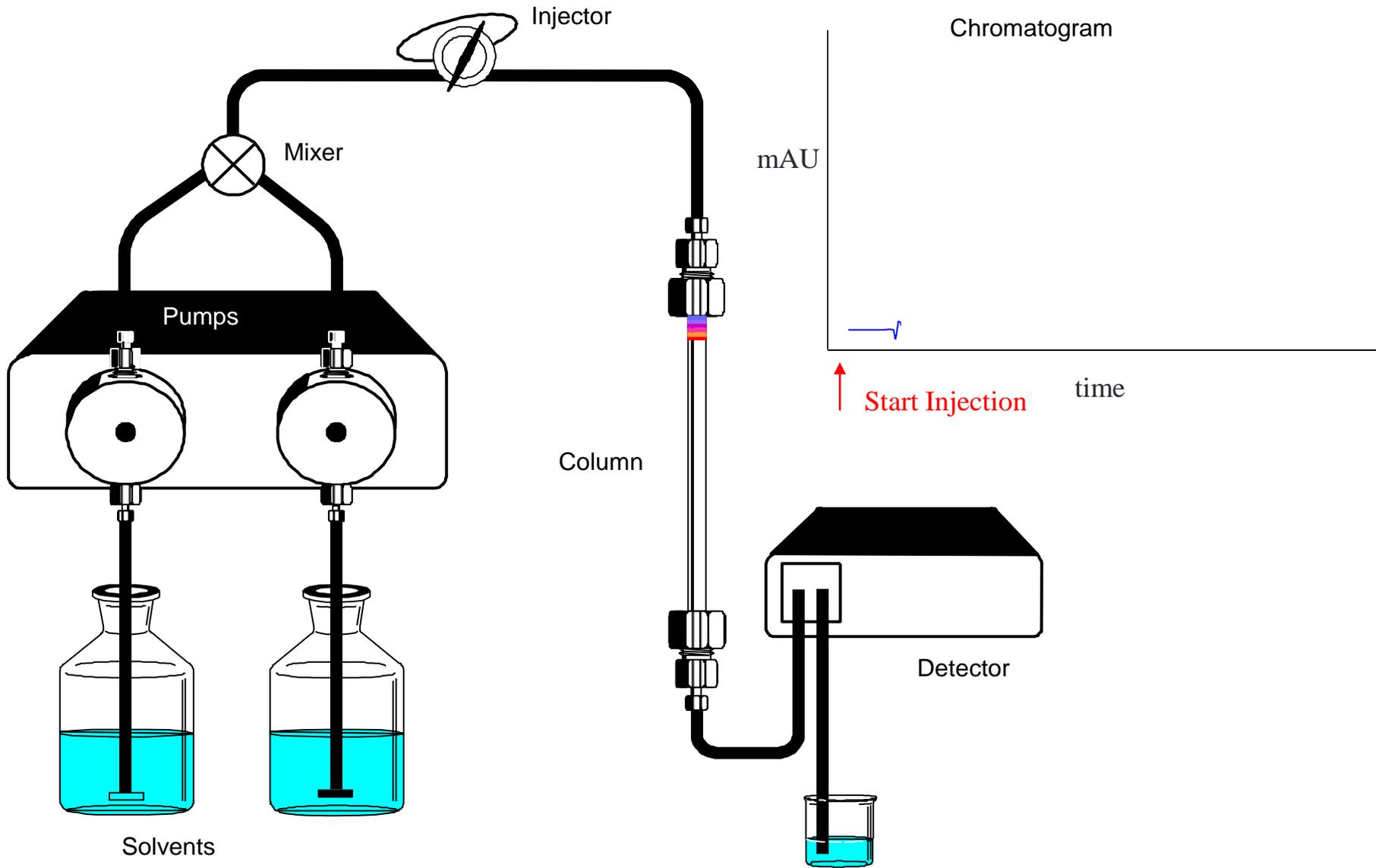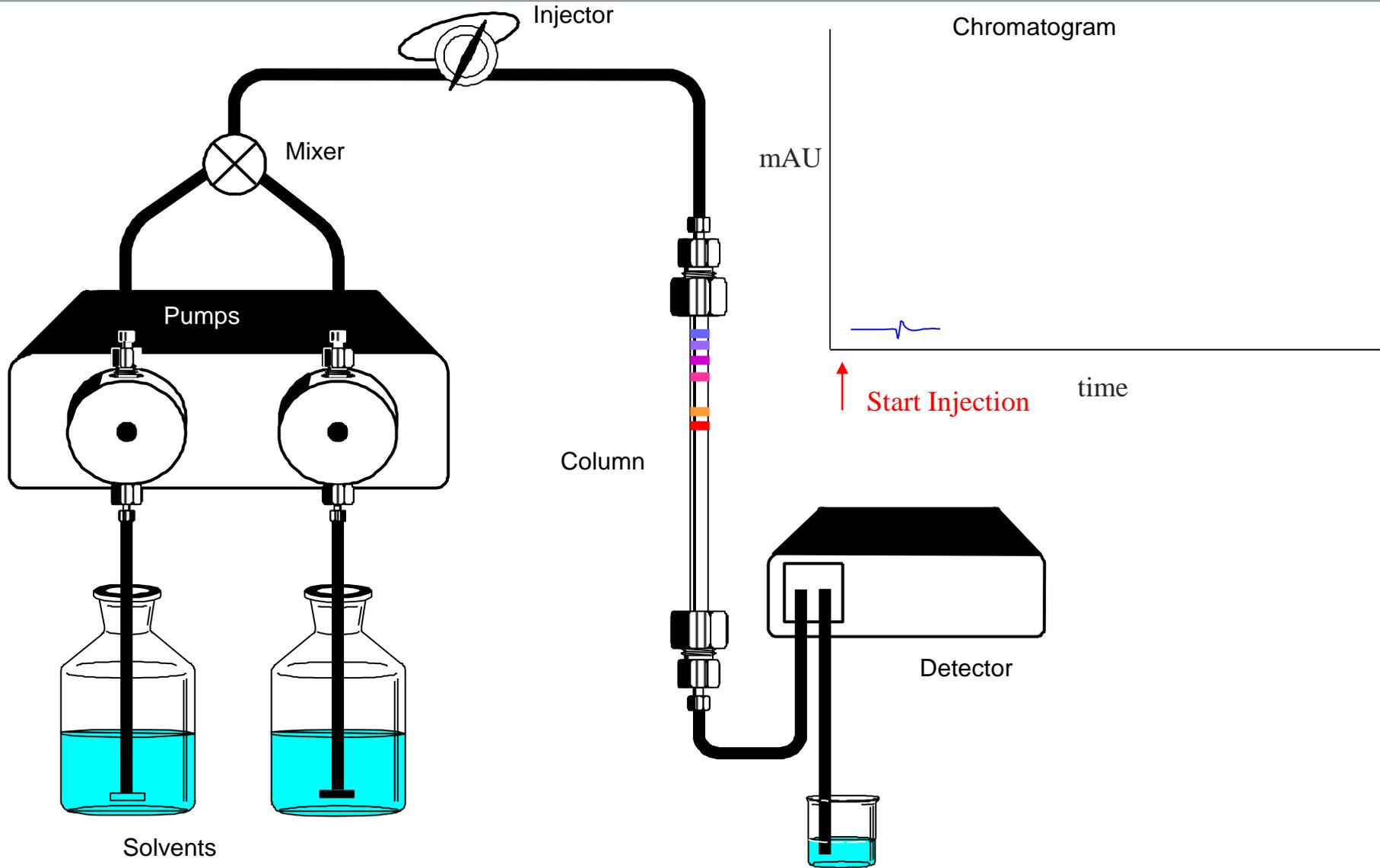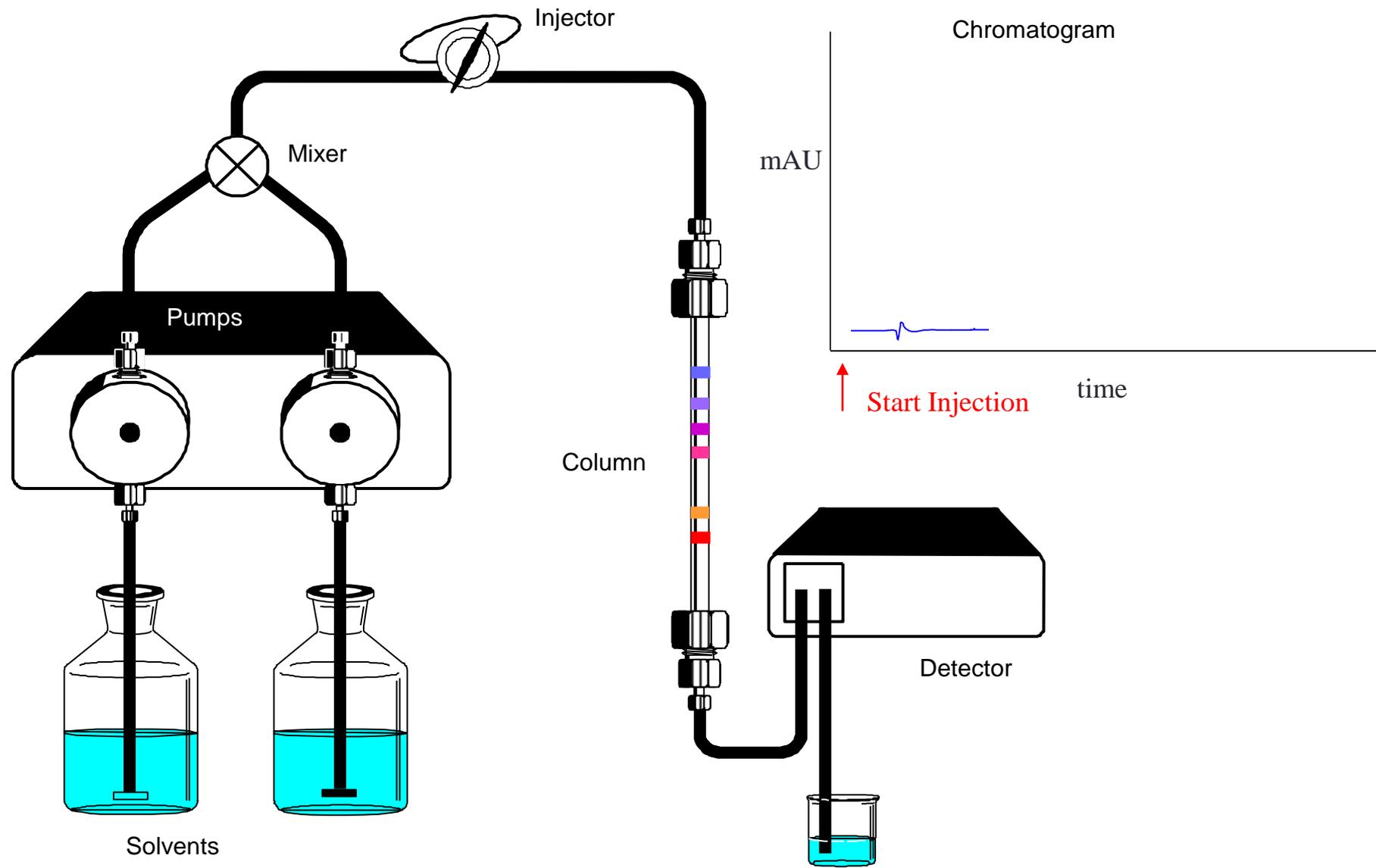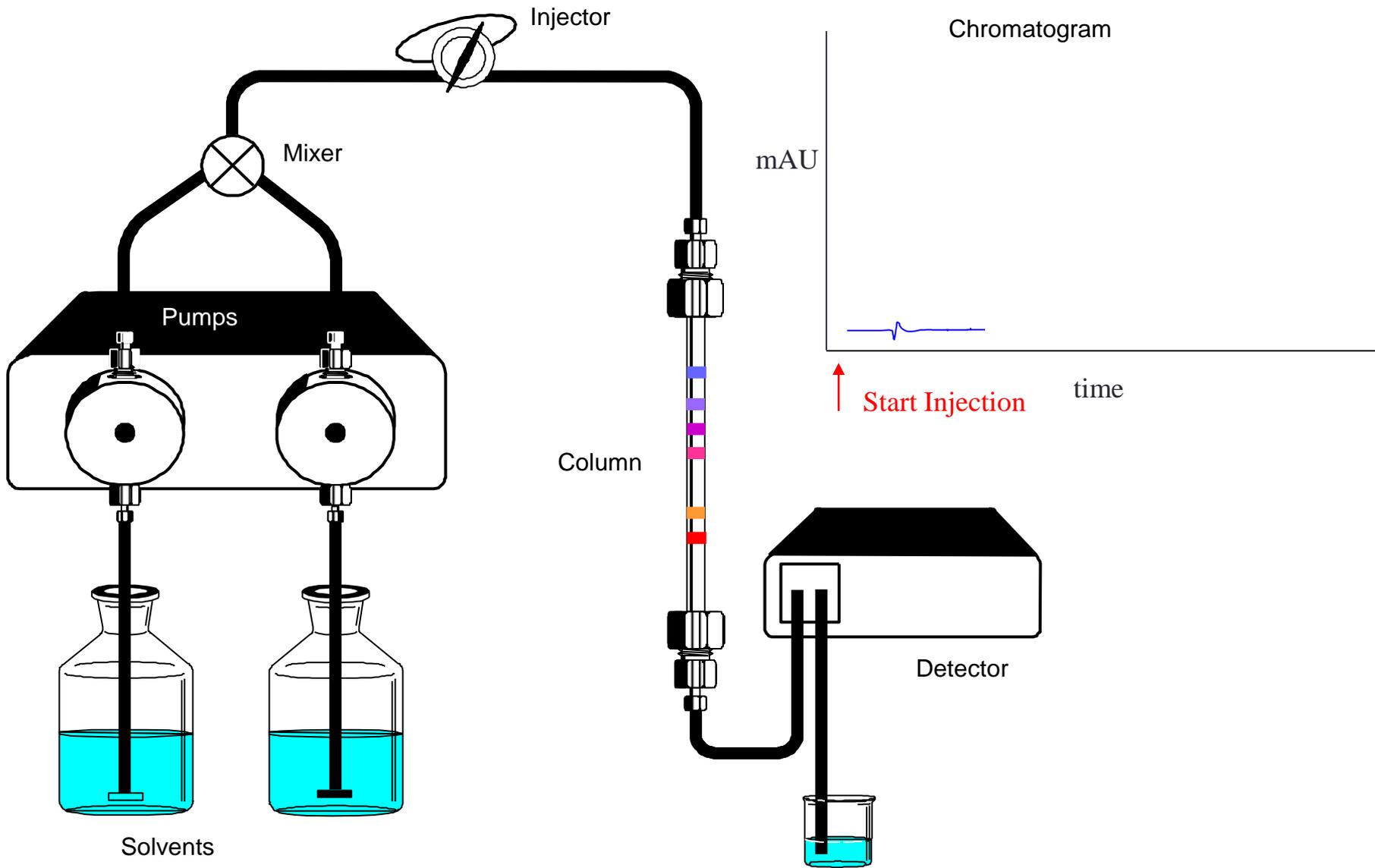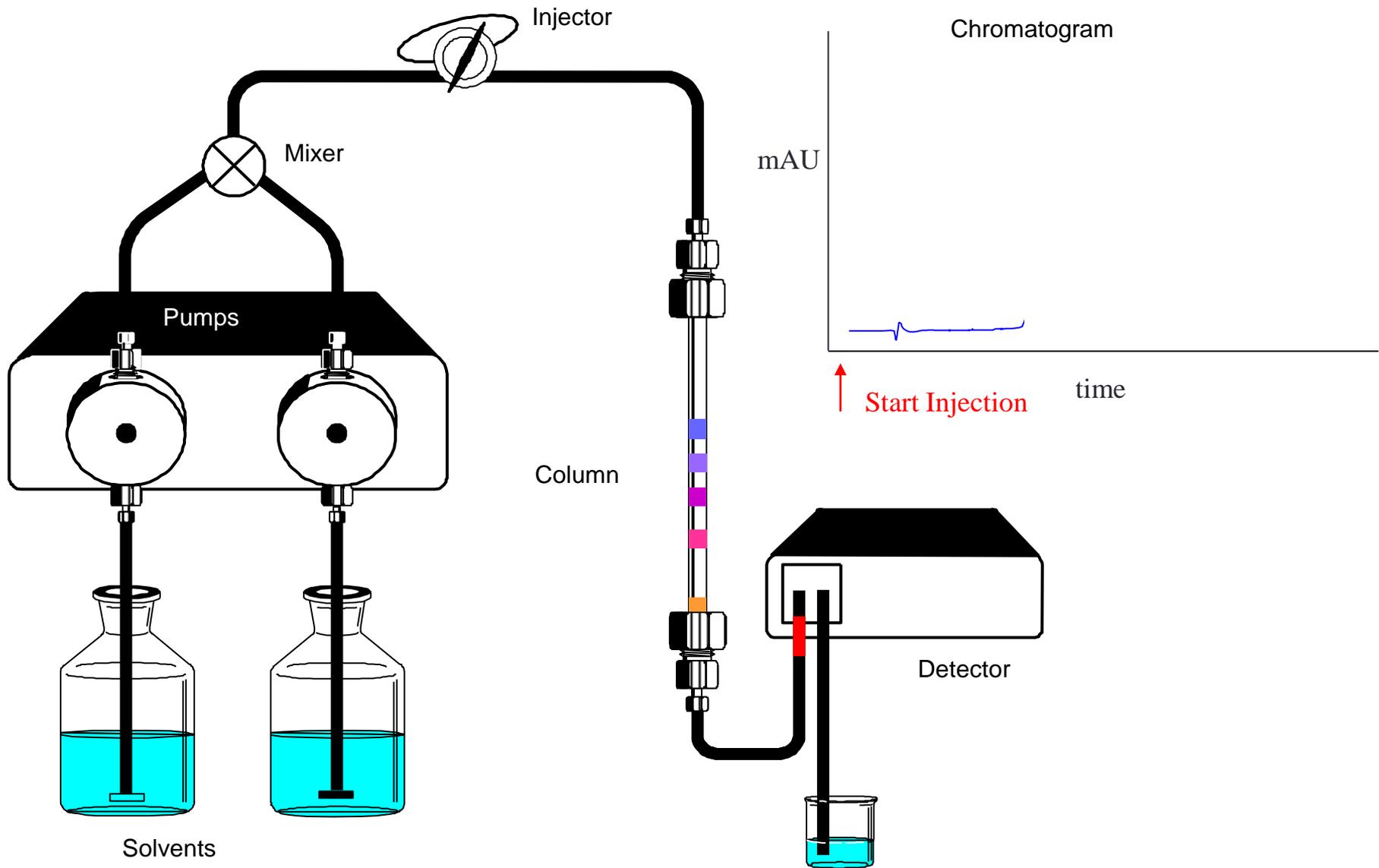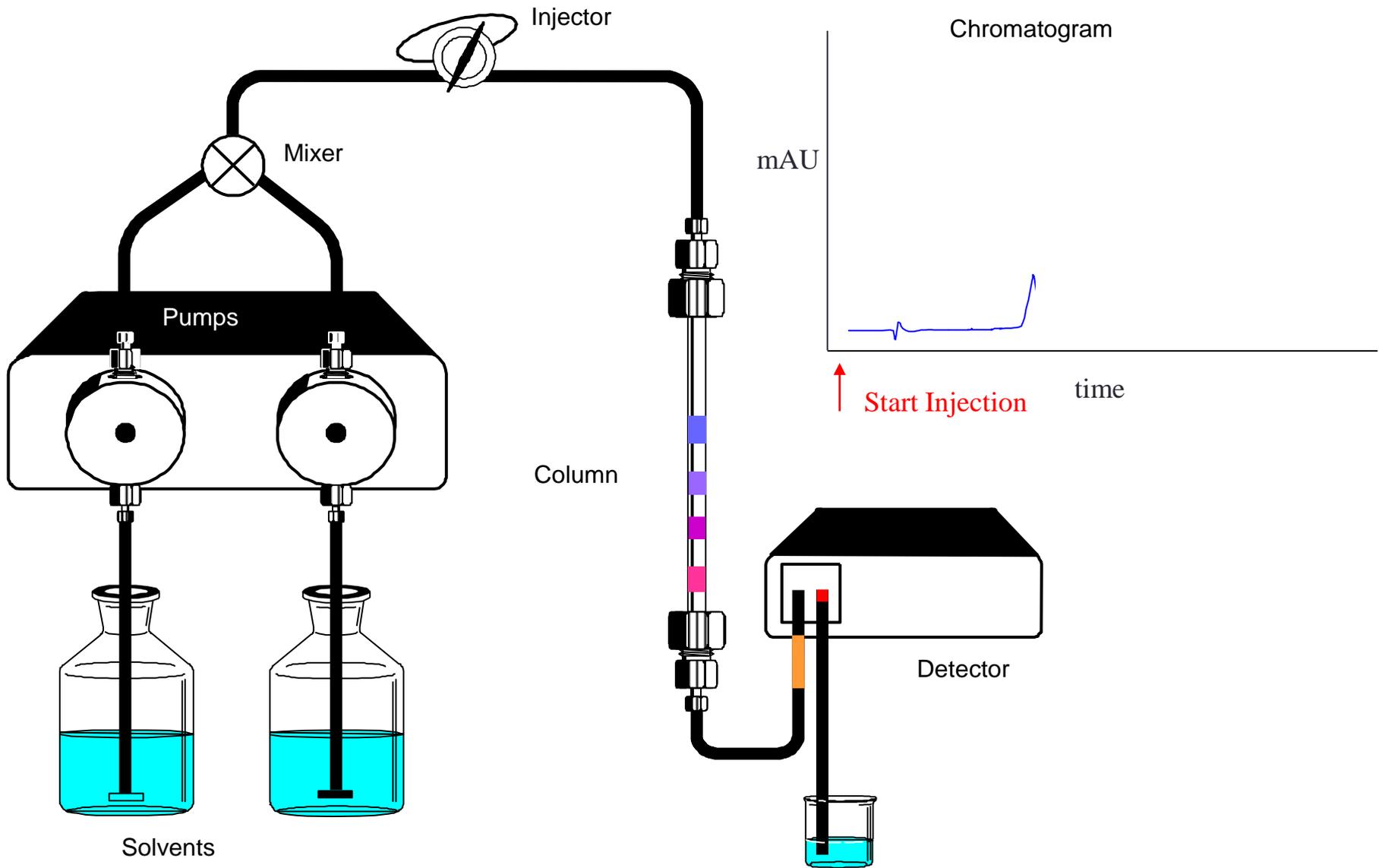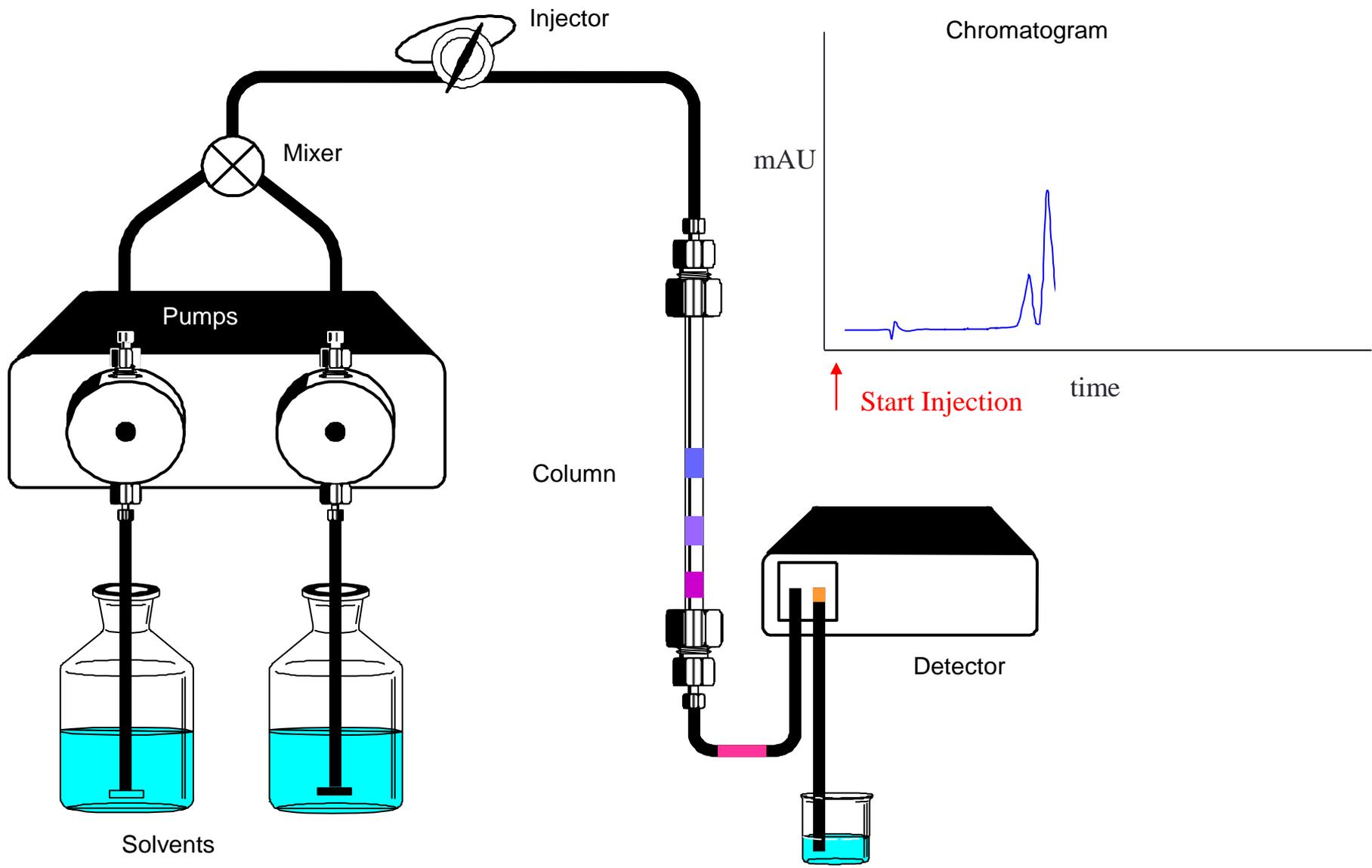
Injector

Mixer

Pumps

Solvents

Column

Detector

Chromatogram

mAU

Start Injection

time

Injector

Chromatogram

Mixer

mAU

Pumps

Start Injection

time

Column

Detector

Solvents

Injector

Mixer

Pumps

Solvents

Column

Detector

Chromatogram

mAU

time

Start Injection

Injector

Mixer

Pumps

Solvents

Column

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time

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time

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Detector

Solvents

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Mixer

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Solvents

Column

Chromatogram

mAU

Start Injection

time

Detector

Injector

Mixer

Pumps

Solvents

Column

Detector

Chromatogram

mAU

time

Start Injection

Injector

Mixer

Pumps

Solvents

Column

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mAU

time

Start Injection

Detector
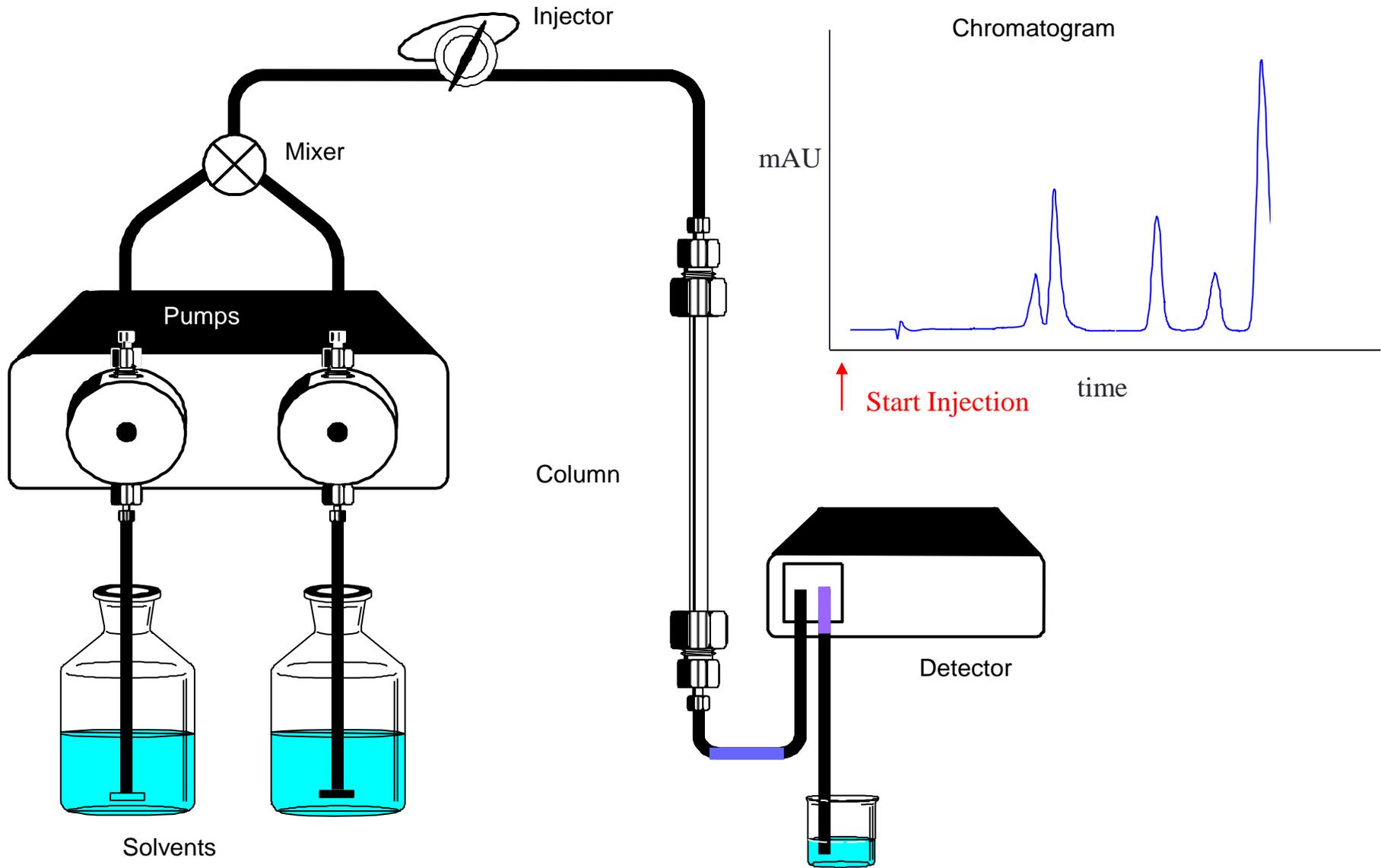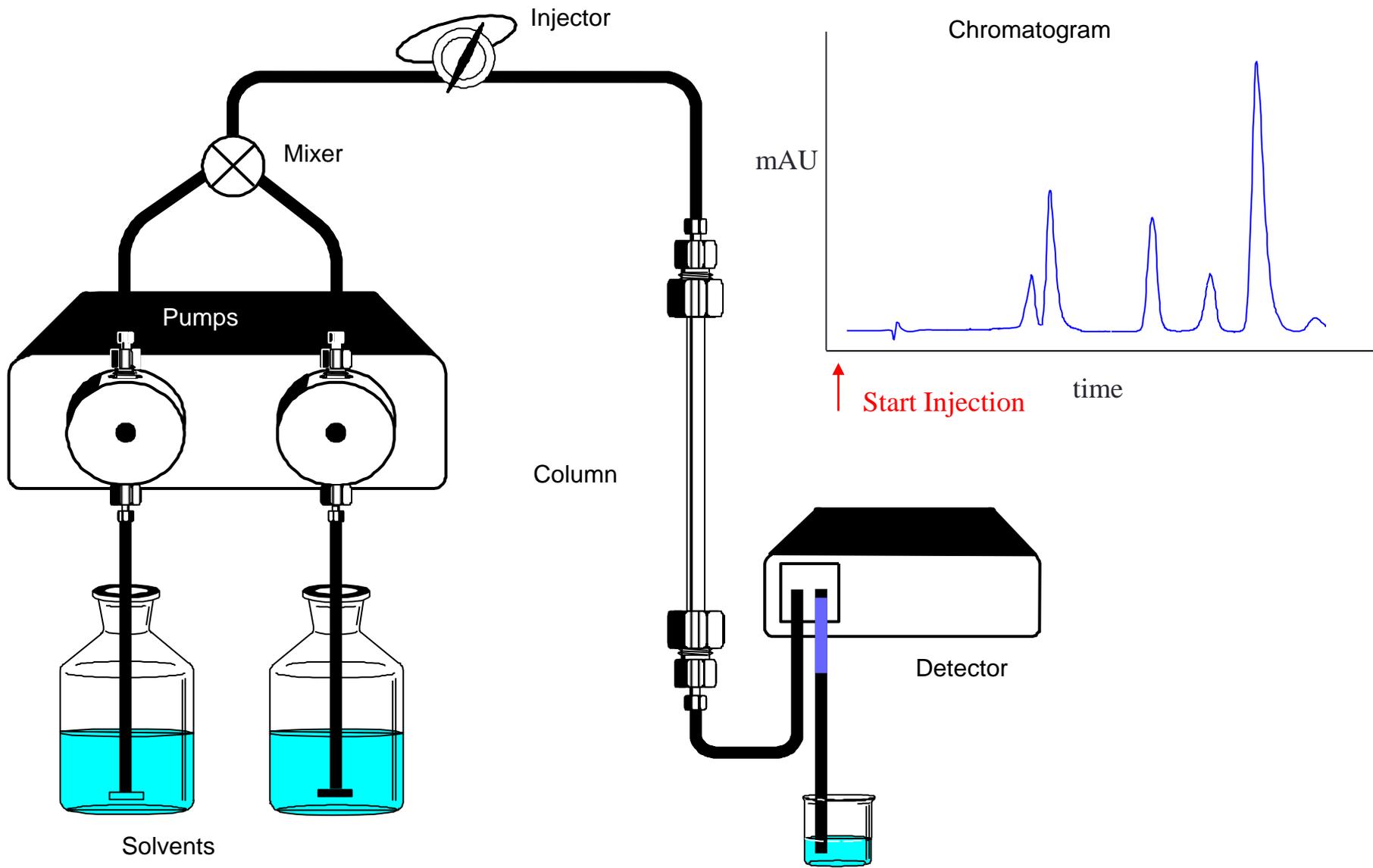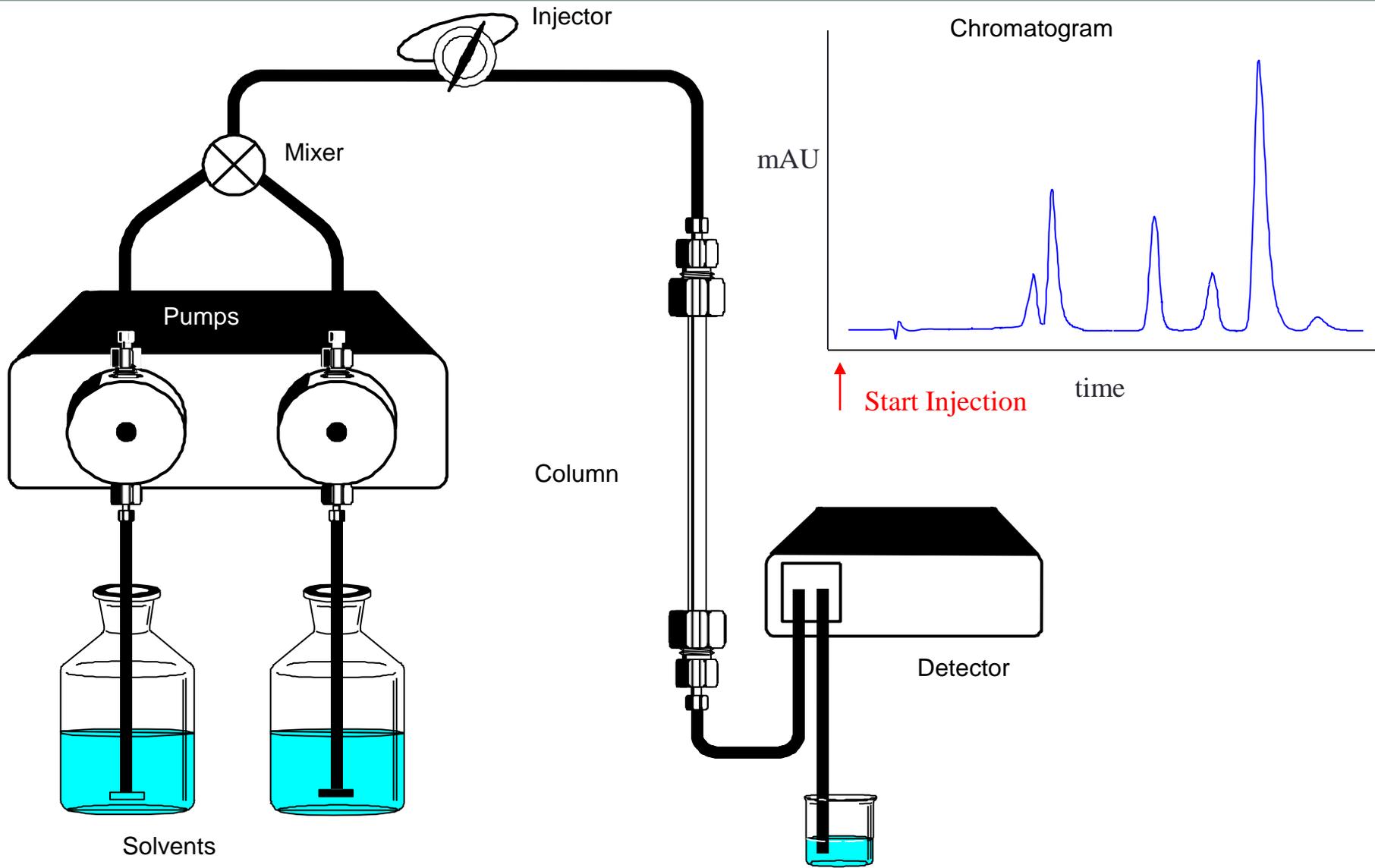
The Chromatogram

t_o - elution time of unretained peak

t_R - retention time - determines sample identity

t_R

t_R

mAU

Area or height is proportional to the quantity of analyte.

t_o

time

Injection
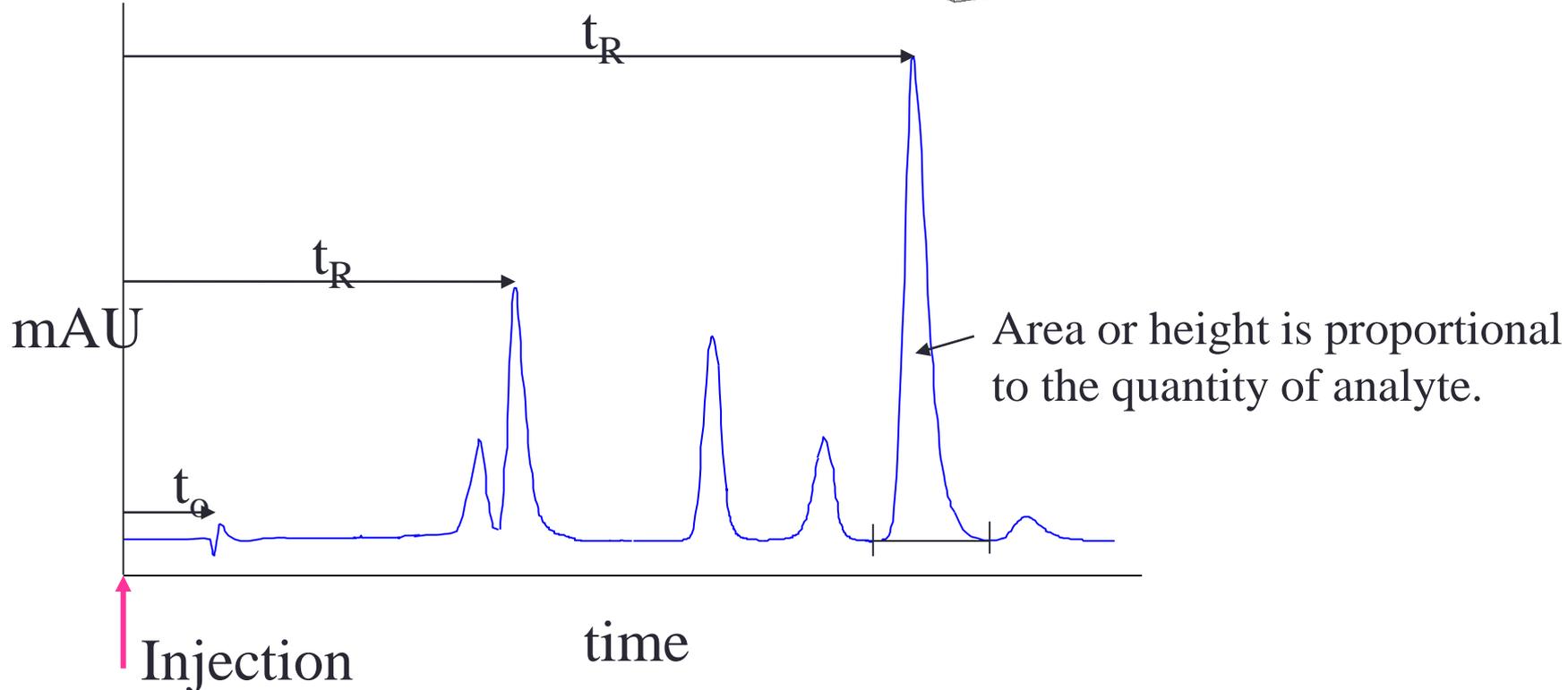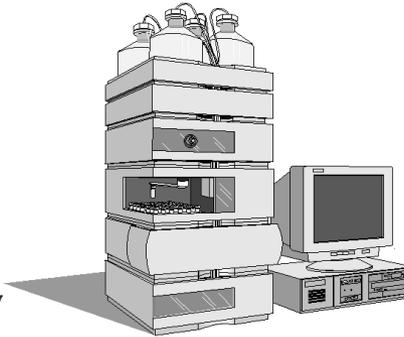
Modes of High Performance Liquid Chromatography

Types of Compounds	Mode	Stationary Phase	Mobile Phase
Neutrals Weak Acids Weak Bases	Reversed Phase	C18, C8, C4 cyano, amino	Water/Organic Modifiers
Ionics, Bases, Acids	Ion Pair	C-18, C-8	Water/Organic Ion-Pair Reagent
Compounds not soluble in water	Normal Phase	Silica, Amino, Cyano, Diol	Organics
Ionics Inorganic Ions	Ion Exchange	Anion or Cation Exchange Resin	Aqueous/Buffer Counter Ion
High Molecular Weight Compounds Polymers	Size Exclusion	Polystyrene Silica	Gel Filtration-Aqueous Gel Permeation-Organic

HPLC Applications
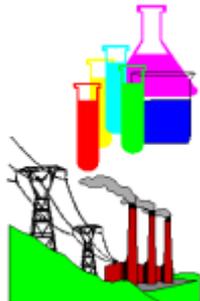
Chemical

polystyrenes
dyes
phthalates
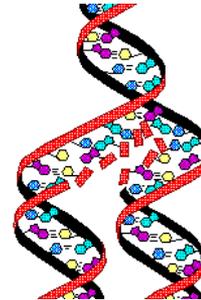
Bioscience

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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