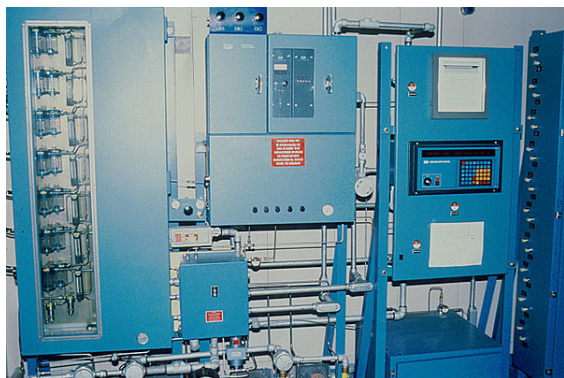


Chromatography



Pictured is a sophisticated gas chromatography system. This instrument records concentrations of acrylonitrile in the air at various points throughout the chemical laboratory.



Automated fraction collector and sampler for chromatographic techniques

Chromatography (/ˌkroʊməˈtɒɡrafi/; from Greek χρῶμα *chroma* “color” and γράφειν *graphein* “to write”^[1]) is the collective term for a set of laboratory techniques for the separation of mixtures. The mixture is dissolved in a fluid called the *mobile phase*, which carries

it through a structure holding another material called the *stationary phase*. The various constituents of the mixture travel at different speeds, causing them to separate. The separation is based on differential partitioning between the mobile and stationary phases. Subtle differences in a compound’s **partition coefficient** result in differential retention on the stationary phase and thus changing the separation.

Chromatography may be preparative or analytical. The purpose of preparative chromatography is to separate the components of a mixture for more advanced use (and is thus a form of **purification**). Analytical chromatography is done normally with smaller amounts of material and is for measuring the relative proportions of analytes in a mixture. The two are not mutually exclusive.

1 History

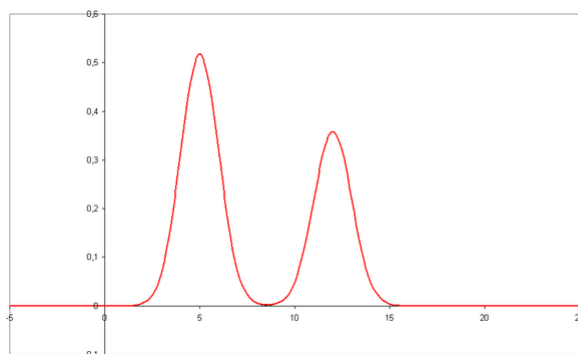
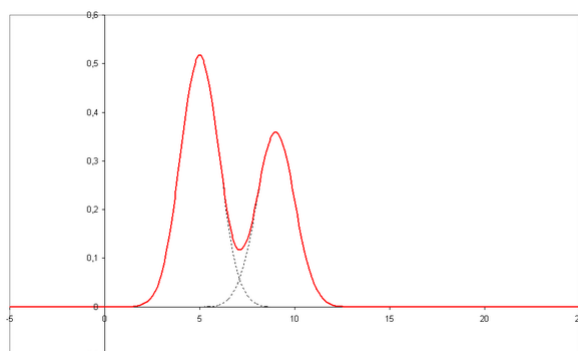
Main article: **History of chromatography**

Chromatography was first employed by the Russian scientist **Mikhail Tsvet** in 1900.^[2] He continued to work with chromatography in the first decade of the 20th century, primarily for the separation of plant **pigments** such as **chlorophyll**, **carotenes**, and **xanthophylls**. Since these components have different colors (green, orange, and yellow, respectively) they gave the technique its name. New types of chromatography developed during the 1930s and 1940s made the technique useful for many **separation processes**.

Chromatography technique developed substantially as a result of the work of **Archer John Porter Martin** and **Richard Laurence Millington Synge** during the 1940s and 1950s. They established the principles and basic techniques of partition chromatography, and their work encouraged the rapid development of several chromatographic methods: **paper chromatography**, **gas chromatography**, and what would become known as **high performance liquid chromatography**. Since then, the technology has advanced rapidly. Researchers found that the main principles of Tsvet’s chromatography could be applied in many different ways, resulting in the different varieties of chromatography described below. Advances are continually improving the technical performance of chromatography, allowing the separation of increasingly similar molecules.

2 Chromatography terms

- The **analyte** is the substance to be separated during chromatography. It is also normally what is needed from the mixture.
- **Analytical chromatography** is used to determine the existence and possibly also the concentration of analyte(s) in a **sample**.
- A **bonded phase** is a stationary phase that is covalently bonded to the support particles or to the inside wall of the column tubing.
- A **chromatogram** is the visual output of the chromatograph. In the case of an optimal separation, different peaks or patterns on the chromatogram correspond to different components of the separated mixture.



Plotted on the x-axis is the retention time and plotted on the y-axis a signal (for example obtained by a **spectrophotometer**, **mass spectrometer** or a variety of other detectors) corresponding to the response created by the analytes exiting the system. In the case of an optimal system the signal is proportional to the concentration of the specific analyte separated.

- A **chromatograph** is equipment that enables a sophisticated separation, e.g. gas chromatographic or liquid chromatographic separation.
- **Chromatography** is a physical method of separation that distributes components to separate between

two phases, one stationary (stationary phase), the other (the mobile phase) moving in a definite direction.

- The **eluate** is the mobile phase leaving the column.
- The **eluent** is the solvent that carries the analyte.
- An **eluotropic series** is a list of solvents ranked according to their eluting power.
- An **immobilized phase** is a stationary phase that is immobilized on the support particles, or on the inner wall of the column tubing.
- The **mobile phase** is the phase that moves in a definite direction. It may be a liquid (LC and Capillary Electrochromatography (CEC)), a gas (GC), or a supercritical fluid (supercritical-fluid chromatography, SFC). The mobile phase consists of the sample being separated/analyzed and the solvent that moves the sample through the column. In the case of **HPLC** the mobile phase consists of a non-polar solvent(s) such as hexane in normal phase or polar solvents in reverse phase chromatography and the sample being separated. The mobile phase moves through the chromatography column (the stationary phase) where the sample interacts with the stationary phase and is separated.
- **Preparative chromatography** is used to purify sufficient quantities of a substance for further use, rather than analysis.
- The **retention time** is the characteristic time it takes for a particular analyte to pass through the system (from the column inlet to the detector) under set conditions. See also: **Kovats' retention index**
- The **sample** is the matter analyzed in chromatography. It may consist of a single component or it may be a mixture of components. When the sample is treated in the course of an analysis, the phase or the phases containing the analytes of interest is/are referred to as the sample whereas everything out of interest separated from the sample before or in the course of the analysis is referred to as waste.
- The **solute** refers to the sample components in partition chromatography.
- The **solvent** refers to any substance capable of solubilizing another substance, and especially the liquid mobile phase in liquid chromatography.
- The **stationary phase** is the substance fixed in place for the chromatography procedure. Examples include the **silica layer** in **thin layer chromatography**
- The **detector** refers to the instrument used for qualitative and quantitative detection of analytes after separation.

Chromatography is based on the concept of partition coefficient. Any solute partitions between two immiscible solvents. When we make one solvent immobile (by adsorption on a solid support matrix) and another mobile it results in most common applications of chromatography. If matrix support is polar (e.g. paper, silica etc.) it is forward phase chromatography, and if it is non-polar (C-18) it is reverse phase.

3 Techniques by chromatographic bed shape

3.1 Column chromatography

For more details on this topic, see [Column chromatography](#).

Column chromatography is a separation technique in which the stationary bed is within a tube. The particles of the solid stationary phase or the support coated with a liquid stationary phase may fill the whole inside volume of the tube (packed column) or be concentrated on or along the inside tube wall leaving an open, unrestricted path for the mobile phase in the middle part of the tube (open tubular column). Differences in rates of movement through the medium are calculated to different retention times of the sample.^[3]

In 1978, W. Clark Still introduced a modified version of column chromatography called **flash column chromatography** (flash).^{[4][5]} The technique is very similar to the traditional column chromatography, except for that the solvent is driven through the column by applying positive pressure. This allowed most separations to be performed in less than 20 minutes, with improved separations compared to the old method. Modern flash chromatography systems are sold as pre-packed plastic cartridges, and the solvent is pumped through the cartridge. Systems may also be linked with detectors and fraction collectors providing automation. The introduction of gradient pumps resulted in quicker separations and less solvent usage.

In **expanded bed adsorption**, a fluidized bed is used, rather than a solid phase made by a packed bed. This allows omission of initial clearing steps such as centrifugation and filtration, for culture broths or slurries of broken cells.

Phosphocellulose chromatography utilizes the binding affinity of many DNA-binding proteins for phosphocellulose. The stronger a protein's interaction with DNA, the higher the salt concentration needed to elute that protein.^[6]

3.2 Planar chromatography

Planar chromatography is a separation technique in which the stationary phase is present as or on a plane. The plane can be a paper, serving as such or impregnated by a substance as the stationary bed (**paper chromatography**) or a layer of solid particles spread on a support such as a glass plate (**thin layer chromatography**). Different **compounds** in the sample mixture travel different distances according to how strongly they interact with the stationary phase as compared to the mobile phase. The specific **Retention factor** (R_f) of each chemical can be used to aid in the identification of an unknown substance.

3.2.1 Paper chromatography

For more details on this topic, see [Paper chromatography](#).

Paper chromatography is a technique that involves placing a small dot or line of sample solution onto a strip of **chromatography paper**. The paper is placed in a container with a shallow layer of **solvent** and sealed. As the solvent rises through the paper, it meets the sample mixture, which starts to travel up the paper with the solvent. This paper is made of **cellulose**, a **polar substance**, and the compounds within the mixture travel farther if they are non-polar. More polar substances bond with the cellulose paper more quickly, and therefore do not travel as far.

3.2.2 Thin layer chromatography

For more details on this topic, see [Thin layer chromatography](#).

Thin layer chromatography (TLC) is a widely employed laboratory technique and is similar to **paper chromatography**. However, instead of using a stationary phase of paper, it involves a stationary phase of a thin layer of **adsorbent** like **silica gel**, **alumina**, or **cellulose** on a flat, inert **substrate**. Compared to paper, it has the advantage of faster runs, better separations, and the choice between different adsorbents. For even better resolution and to allow for quantification, **high-performance TLC** can be used. An older popular use had been to differentiate chromosomes by observing distance in gel (separation of was a separate step).

4 Displacement chromatography

The basic principle of **displacement chromatography** is: A molecule with a high affinity for the chromatography matrix (the displacer) competes effectively for binding sites, and thus displace all molecules with lesser

affinities.^[7] There are distinct differences between displacement and elution chromatography. In elution mode, substances typically emerge from a column in narrow, Gaussian peaks. Wide separation of peaks, preferably to baseline, is desired for maximum purification. The speed at which any component of a mixture travels down the column in elution mode depends on many factors. But for two substances to travel at different speeds, and thereby be resolved, there must be substantial differences in some interaction between the biomolecules and the chromatography matrix. Operating parameters are adjusted to maximize the effect of this difference. In many cases, baseline separation of the peaks can be achieved only with gradient elution and low column loadings. Thus, two drawbacks to elution mode chromatography, especially at the preparative scale, are operational complexity, due to gradient solvent pumping, and low throughput, due to low column loadings. Displacement chromatography has advantages over elution chromatography in that components are resolved into consecutive zones of pure substances rather than “peaks”. Because the process takes advantage of the nonlinearity of the isotherms, a larger column feed can be separated on a given column with the purified components recovered at significantly higher concentrations.

5 Techniques by physical state of mobile phase

5.1 Gas chromatography

For more details on this topic, see [Gas chromatography](#).

Gas chromatography (GC), also sometimes known as gas-liquid chromatography, (GLC), is a separation technique in which the mobile phase is a gas. Gas chromatographic separation is always carried out in a column, which is typically “packed” or “capillary”. Packed columns are the routine work horses of gas chromatography, being cheaper and easier to use and often giving adequate performance. Capillary columns generally give far superior resolution and although more expensive are becoming widely used, especially for complex mixtures. Both types of column are made from non-adsorbent and chemically inert materials. Stainless steel and glass are the usual materials for packed columns and quartz or fused silica for capillary columns.

Gas chromatography is based on a [partition equilibrium](#) of analyte between a solid or viscous liquid stationary phase (often a liquid silicone-based material) and a mobile gas (most often helium). The stationary phase is adhered to the inside of a small-diameter (commonly 0.53 - 0.18mm inside diameter) glass or fused-silica tube (a capillary column) or a solid matrix inside a larger metal tube (a packed column). It is widely

used in [analytical chemistry](#); though the high temperatures used in GC make it unsuitable for high molecular weight biopolymers or proteins (heat denatures them), frequently encountered in [biochemistry](#), it is well suited for use in the [petrochemical](#), [environmental monitoring](#) and [remediation](#), and [industrial chemical](#) fields. It is also used extensively in chemistry research.

5.2 Liquid chromatography

Liquid chromatography (LC) is a separation technique in which the mobile phase is a liquid. It can be carried out either in a column or a plane. Present day liquid chromatography that generally utilizes very small packing particles and a relatively high pressure is referred to as [high performance liquid chromatography](#) (HPLC).

In HPLC the sample is forced by a liquid at high pressure (the mobile phase) through a column that is packed with a stationary phase composed of irregularly or spherically shaped particles, a [porous monolithic layer](#), or a porous membrane. HPLC is historically divided into two different sub-classes based on the polarity of the mobile and stationary phases. Methods in which the stationary phase is more polar than the mobile phase (e.g., toluene as the mobile phase, silica as the stationary phase) are termed normal phase liquid chromatography (NPLC) and the opposite (e.g., water-methanol mixture as the mobile phase and C18 = octadecylsilyl as the stationary phase) is termed reversed phase liquid chromatography (RPLC).

Specific techniques under this broad heading are listed below.

6 Affinity chromatography

For more details on this topic, see [Affinity chromatography](#).

Affinity chromatography^[8] is based on selective non-covalent interaction between an analyte and specific molecules. It is very specific, but not very robust. It is often used in biochemistry in the purification of [proteins](#) bound to tags. These [fusion proteins](#) are labeled with compounds such as [His-tags](#), [biotin](#) or [antigens](#), which bind to the stationary phase specifically. After purification, some of these tags are usually removed and the pure protein is obtained.

Affinity chromatography often utilizes a biomolecule's affinity for a metal (Zn, Cu, Fe, etc.). Columns are often manually prepared. Traditional affinity columns are used as a preparative step to flush out unwanted biomolecules.

However, HPLC techniques exist that do utilize affinity chromatography properties. Immobilized Metal Affinity Chromatography (IMAC) is useful to separate aforementioned molecules based on the relative affinity for the

metal (I.e. Dionex IMAC). Often these columns can be loaded with different metals to create a column with a targeted affinity.

6.1 Supercritical fluid chromatography

For more details on this topic, see [Supercritical fluid chromatography](#).

Supercritical fluid chromatography is a separation technique in which the mobile phase is a fluid above and relatively close to its critical temperature and pressure.

7 Techniques by separation mechanism

7.1 Ion exchange chromatography

For more details on this topic, see [Ion exchange chromatography](#).

Ion exchange chromatography (usually referred to as ion chromatography) uses an ion exchange mechanism to separate analytes based on their respective charges. It is usually performed in columns but can also be useful in planar mode. Ion exchange chromatography uses a charged stationary phase to separate charged compounds including [anions](#), [cations](#), [amino acids](#), [peptides](#), and [proteins](#). In conventional methods the stationary phase is an [ion exchange resin](#) that carries charged [functional groups](#) that interact with oppositely charged groups of the compound to retain. Ion exchange chromatography is commonly used to purify proteins using [FPLC](#).

7.2 Size-exclusion chromatography

For more details on this topic, see [Size-exclusion chromatography](#).

Size-exclusion chromatography (SEC) is also known as [gel permeation chromatography](#) (GPC) or [gel filtration chromatography](#) and separates molecules according to their size (or more accurately according to their hydrodynamic diameter or hydrodynamic volume). Smaller molecules are able to enter the pores of the media and, therefore, molecules are trapped and removed from the flow of the mobile phase. The average residence time in the pores depends upon the effective size of the analyte molecules. However, molecules that are larger than the average pore size of the packing are excluded and thus suffer essentially no retention; such species are the first to be eluted. It is generally a low-resolution chromatography technique and thus it is often reserved for the final,

“polishing” step of a purification. It is also useful for determining the [tertiary structure](#) and [quaternary structure](#) of purified proteins, especially since it can be carried out under native [solution](#) conditions.

7.3 Expanded Bed Adsorption (EBA) Chromatographic Separation

Expanded Bed Adsorption (EBA) Chromatographic Separation captures a target protein from a crude feed stream when it passes through a chromatography column system containing adsorbent beads. With this technique the crude feedstock can be treated directly in the chromatographic column, avoiding the traditional clarification and pre-treatment steps. EBA Chromatographic Separation is highly scalable, from laboratory-based 1 cm diameter columns to large production columns up to 2 meter in diameter. These columns can typically handle feed stock throughput of more than 1,000,000 liter per day with a production capacity of 1000 MT protein per year.

8 Special techniques

8.1 Reversed-phase chromatography

For more details on this topic, see [Reversed-phase chromatography](#).

Reversed-phase chromatography (RPC) is any liquid chromatography procedure in which the mobile phase is significantly more polar than the stationary phase. It is so named because in normal-phase liquid chromatography, the mobile phase is significantly less polar than the stationary phase. Hydrophobic molecules in the mobile phase tend to adsorb to the relatively hydrophobic stationary phase. Hydrophilic molecules in the mobile phase will tend to elute first. Separating columns typically comprise a C8 or C18 carbon-chain bonded to a silica particle substrate.

Hydrophobic interactions between proteins and the chromatographic matrix can be exploited to purify the proteins. In hydrophobic interaction chromatography, the matrix material is lightly substituted with octyl or phenyl groups. At high salt concentrations, nonpolar groups on the surface on proteins “interact” with the hydrophobic groups; that is, both types of groups are excluded by the polar solvent (hydrophobic effects are augmented by increased ionic strength). The eluant is typically an aqueous buffer with decreasing salt concentrations, increasing concentrations of detergent (which disrupts hydrophobic interactions), or changes in pH.

8.2 Two-dimensional chromatography

In some cases, the chemistry within a given column can be insufficient to separate some analytes. It is possible to direct a series of unresolved peaks onto a second column with different physico-chemical (**Chemical classification**) properties. Since the mechanism of retention on this new solid support is different from the first dimensional separation, it can be possible to separate compounds that are indistinguishable by one-dimensional chromatography. The sample is spotted at one corner of a square plate, developed, air-dried, then rotated by 90° and usually redeveloped in a second solvent system.

8.3 Simulated moving-bed chromatography

For more details on this topic, see **Simulated moving bed**.

The simulated moving bed (SMB) technique is a variant of high performance liquid chromatography; it is used to separate particles and/or chemical compounds that would be difficult or impossible to resolve otherwise. This increased separation is brought about by a valve-and-column arrangement that is used to lengthen the stationary phase indefinitely. In the moving bed technique of preparative chromatography the feed entry and the analyte recovery are simultaneous and continuous, but because of practical difficulties with a continuously moving bed, simulated moving bed technique was proposed. In the simulated moving bed technique instead of moving the bed, the sample inlet and the analyte exit positions are moved continuously, giving the impression of a moving bed. True moving bed chromatography (TMBC) is only a theoretical concept. Its simulation, SMBC is achieved by the use of a multiplicity of columns in series and a complex valve arrangement, which provides for sample and solvent feed, and also analyte and waste takeoff at appropriate locations of any column, whereby it allows switching at regular intervals the sample entry in one direction, the solvent entry in the opposite direction, whilst changing the analyte and waste takeoff positions appropriately as well.

8.4 Pyrolysis gas chromatography

Pyrolysis gas chromatography mass spectrometry is a method of chemical analysis in which the sample is heated to decomposition to produce smaller molecules that are separated by gas chromatography and detected using mass spectrometry.

Pyrolysis is the thermal decomposition of materials in an inert atmosphere or a vacuum. The sample is put into direct contact with a platinum wire, or placed in a quartz sample tube, and rapidly heated to 600–1000 °C. Depending on the application even higher temperatures

are used. Three different heating techniques are used in actual pyrolyzers: Isothermal furnace, inductive heating (Curie Point filament), and resistive heating using platinum filaments. Large molecules cleave at their weakest points and produce smaller, more volatile fragments. These fragments can be separated by gas chromatography. Pyrolysis GC chromatograms are typically complex because a wide range of different decomposition products is formed. The data can either be used as fingerprint to prove material identity or the GC/MS data is used to identify individual fragments to obtain structural information. To increase the volatility of polar fragments, various methylating reagents can be added to a sample before pyrolysis.

Besides the usage of dedicated pyrolyzers, pyrolysis GC of solid and liquid samples can be performed directly inside Programmable Temperature Vaporizer (PTV) injectors that provide quick heating (up to 30 °C/s) and high maximum temperatures of 600–650 °C. This is sufficient for some pyrolysis applications. The main advantage is that no dedicated instrument has to be purchased and pyrolysis can be performed as part of routine GC analysis. In this case quartz GC inlet liners have to be used. Quantitative data can be acquired, and good results of derivatization inside the PTV injector are published as well.

8.5 Fast protein liquid chromatography

For more details on this topic, see **Fast protein liquid chromatography**.

Fast protein liquid chromatography (FPLC), is a form of liquid chromatography that is often used to analyze or purify mixtures of proteins. As in other forms of chromatography, separation is possible because the different components of a mixture have different affinities for two materials, a moving fluid (the “mobile phase”) and a porous solid (the stationary phase). In FPLC the mobile phase is an aqueous solution, or “buffer”. The buffer flow rate is controlled by a positive-displacement pump and is normally kept constant, while the composition of the buffer can be varied by drawing fluids in different proportions from two or more external reservoirs. The stationary phase is a resin composed of beads, usually of cross-linked agarose, packed into a cylindrical glass or plastic column. FPLC resins are available in a wide range of bead sizes and surface ligands depending on the application.

8.6 Countercurrent chromatography

For more details on this topic, see **Countercurrent chromatography**.

Countercurrent chromatography (CCC) is a type of liquid-liquid chromatography, where both the stationary and mobile phases are liquids. The operating principle

of CCC equipment requires a column consisting of an open tube coiled around a bobbin. The bobbin is rotated in a double-axis gyratory motion (a cardioid), which causes a variable gravity (G) field to act on the column during each rotation. This motion causes the column to see one partitioning step per revolution and components of the sample separate in the column due to their partitioning coefficient between the two immiscible liquid phases used. There are many types of CCC available today. These include HSCCC (High Speed CCC) and HPCCC (High Performance CCC). HPCCC is the latest and best performing version of the instrumentation available currently.

8.7 Chiral chromatography

Chiral chromatography involves the separation of stereoisomers. In the case of enantiomers, these have no chemical or physical differences apart from being three-dimensional mirror images. Conventional chromatography or other separation processes are incapable of separating them. To enable chiral separations to take place, either the mobile phase or the stationary phase must themselves be made chiral, giving differing affinities between the analytes. **Chiral chromatography HPLC columns** (with a chiral stationary phase) in both normal and reversed phase are commercially available.

9 See also

10 References

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- [2] The first report of Tsvet's discovery was published in 1905:
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The word "chromatogram" first appeared in print in 1906:

- Mikhail Tswett (1906) "Physikalisch-Chemische Studien über das Chlorophyll. Die Adsorption." (Physical-chemical studies of chlorophyll. Adsorption.) *Berichte der Deutschen botanischen*

Gesellschaft, vol. 24, pp. 316–326. On page 322, Tsvet coins the term "chromatography":

Original : " Wie die Lichtstrahlen im Spektrum, so it werden in der Calciumkarbonatsäule die verschiedenen Komponenten eines Farbstoffgemisches gesetzmässig auseinandergelegt, und lassen sich darin qualitativ und auch quantitativ bestimmen. Ein solches Präparat nenne ich ein Chromatogramm und die entsprechende Methode, die chromatographische Methode."

Translation : Like light rays in a spectrum, so the different components of a mixture of pigments are dispersed in the calcium carbonate column following a set pattern, and [they] can be determined in there qualitatively as well as quantitatively. I call such a preparation a "chromatogram" and the corresponding method, the "chromatographic method".

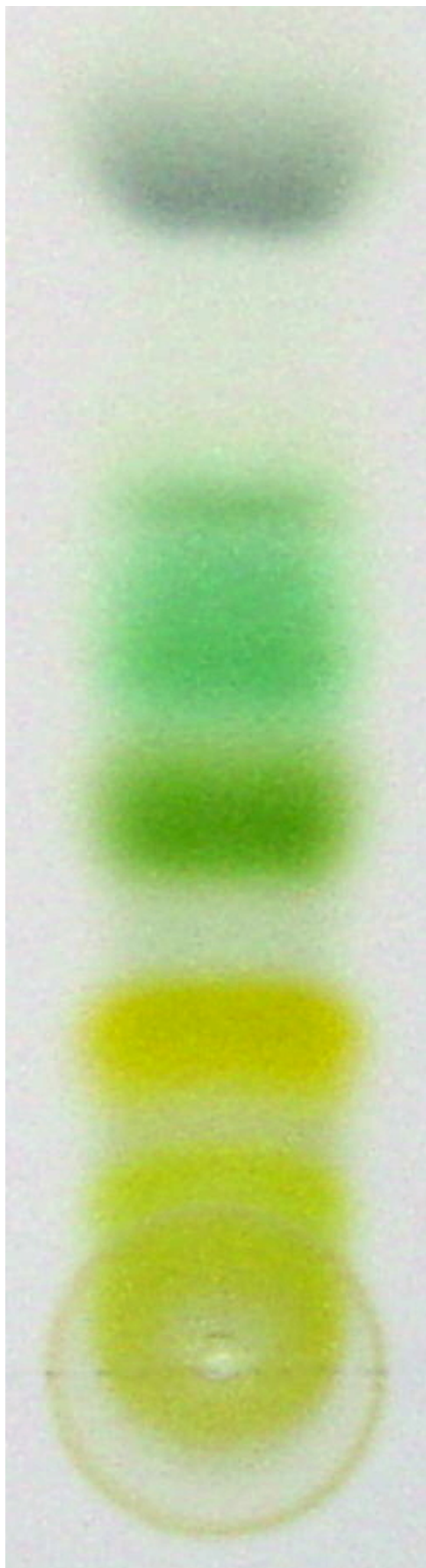
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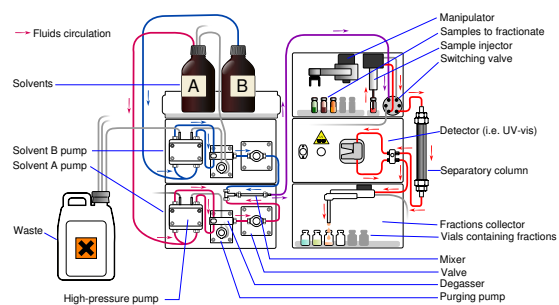
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11 External links

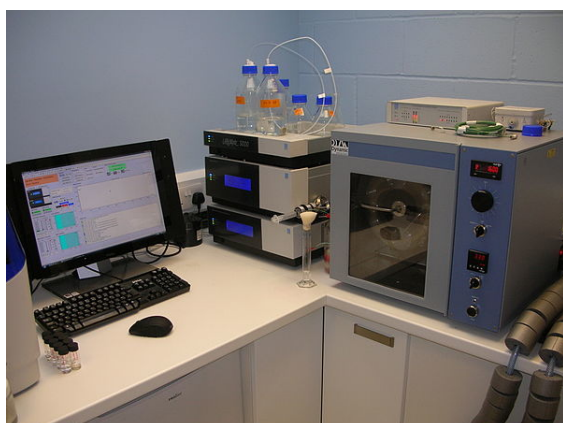
- **IUPAC Nomenclature for Chromatography**
- **Chromedia** On line database and community for chromatography practitioners (paid subscription required)
- **Library 4 Science: Chrom-Ed Series**
- **Hydrophobic Interaction & Reversed Phase Chromatography Handbook**
- **Overlapping Peaks Program – Learning by Simulations**

- [Chromatography Videos – MIT OCW – Digital Lab Techniques Manual](#)
- [Chromatography Equations Calculators – Micro-Solv Technology Corporation](#)





Preparative HPLC apparatus



An example of a HPCCC system

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12.1 Text

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