Chromatographic Methods

Chapter Overview

Chromatography is one of the most important separation methods in modern analytical chemistry and biochemistry. It enables effective separation, identification, and quantification of components in complex mixtures based on their different interactions with the mobile and stationary phases. In clinical biochemistry and laboratory diagnostics, chromatography has fundamental importance—it is used for the determination of amino acids, vitamins, hormones, drugs, metabolites, and for monitoring disease markers.

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1 Introduction to Chromatographic Methods

1.1 Introduction: Why Chromatography?

Imagine you are handed a glass of fruit juice and someone asks you:

“What exactly is in it?”

At first glance, you only see an orange liquid. But in reality, it contains dozens of different substances—sugars, acids, vitamins, pigments, aromatic compounds. And if it were blood plasma, the number of components would rise to hundreds, even thousands. How can we make sense of such a complex mixture?

This is where chromatography comes in. Its purpose is to transform a single complicated, complex mixture into a clear record of individual components. This way we can find out what is there and how much of it is present.

1.1.1 Where Can We Encounter Chromatography?

Chromatographic methods are used in many fields:

- **Clinical laboratories:** help detect drugs, metabolites, or toxic substances in blood
- **Pharmacy:** used for quality control of medications
- **Food industry and environmental monitoring:** show whether unwanted substances are present in food or water
- **Science:** allow researchers to better understand complex biological and chemical processes

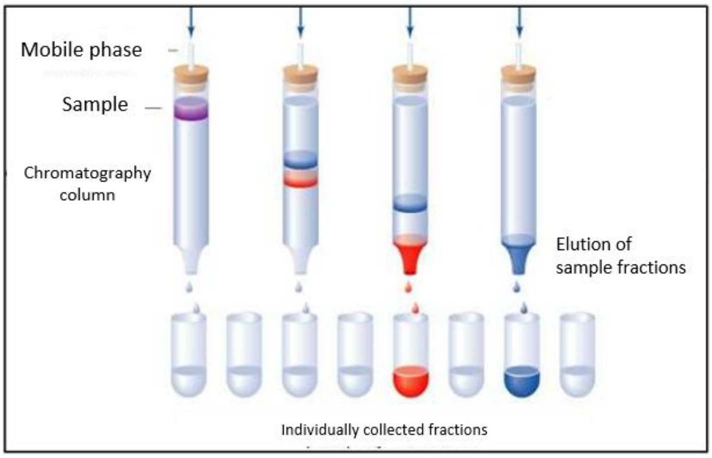
Whether it is fruit juice, a blood sample, or a drug, the principle is always the same: separate the components so that each can be individually identified and quantified.

1.2 How Chromatography Works

The basic idea is surprisingly simple. The sample is distributed between two environments—the **mobile phase** and the **stationary phase**.

- The **mobile phase** flows through the system and carries the sample.
- The **stationary phase** remains fixed, and some substances adhere to it more strongly than others.

Each substance has its own “nature”—some interact more strongly with the stationary phase, others more weakly. Thus, each component of the mixture interacts differently with the stationary and mobile phases and moves through the system at a different speed. Those that bind tightly emerge later; those that move quickly appear earlier. In this way, one mixture becomes a series of separated components, each emerging at a different time. This moment is called the **retention time**.



(Source: https://is.muni.cz/el/med/jaro2018/BLIT0222p/um/Chromatografie_ucebni_text.pdf)

In general, a chromatographic system always contains a **mobile phase** and a **stationary phase**:

- The **mobile phase** is a liquid (or a gas in gas chromatography) that flows through the column and carries the sample. It may consist of pure water or buffer, organic solvents (methanol, acetonitrile), or their mixtures in various ratios. By selecting the right composition of the mobile phase, retention and separation of individual substances can be deliberately controlled.
- The **stationary phase** is a solid support (most commonly silica particles), whose surface can either remain in its original polar form or be chemically modified to carry non-polar or other specific functional groups (e.g., C18 chains, phenyl, fluorinated groups). The type of stationary phase determines the interactions that analytes undergo in the column.

(Source: https://www.chromservis.eu/en/ymc-pack-pro-c18-s-5-m-12nm-analytical-hplc-column-250-x-4-6mm-312947)

What Phenomena Occur During Separation?

As the mixture moves through the chromatographic system, individual molecules "decide" whether to remain in the mobile phase or to temporarily "stick" to the stationary phase. This constant process of repeated binding and release forms the basis of chromatographic separation.

The outcome is determined mainly by physicochemical interactions between the analyte and the stationary phase:

- **Hydrophobic/hydrophilic interactions** – water-soluble (hydrophilic) substances tend to interact with polar phases, while hydrophobic ones prefer nonpolar phases
- **Hydrogen bonding** – certain functional groups can form bonds with the stationary phase, increasing retention time
- **Ionic interactions** – charged molecules may bind to oppositely charged sites on the stationary phase

What Determines the Speed of Elution?

- A substance with **higher affinity** for the stationary phase will be retained **longer** in the column → it elutes later.
- A substance that “prefers” the mobile phase will barely stop and **pass quickly** through the column → it elutes earlier.

It is therefore a constant **“tug of war” between two environments**:

- The mobile phase encourages molecules to move forward
- The stationary phase holds them back according to the strength of their interactions

These differences in interactions are the key to separation.

1.2.1 Chromatography – Separation in Normal and Reversed Phase

Liquid chromatography can be classified according to many criteria. Based on the physicochemical nature of separation, the most common division is into **normal-phase (NP)** and **reversed-phase (RP)** chromatography.

In the early days of chromatography, NP separation was commonly used, where the stationary phase was polar (typically silica gel) and the mobile phase consisted of nonpolar solvents or their mixtures, such as hexane, dichloromethane, chloroform, or ethyl acetate. Today, however, RP chromatography predominates. These columns use nonpolar stationary phases based on silica gel, the surface of which is modified—most commonly with octadecyl (C18), phenylhexyl, pentafluorophenyl, or octyl groups. The mobile phase usually consists of a mixture of polar solvents, such as acetonitrile or methanol, with water or buffers.

2 Examples of Separation Techniques by Interaction of the Analyte with the Mobile and Stationary Phase

2.1 Adsorption Chromatography

This method is based on the fact that some substances tend to adhere more strongly to the surface of the sorbent, which forms the stationary phase. These substances are therefore “held back” longer, since they must repeatedly bind and release, while other substances pass through more quickly.

- **Analogy:** it’s like walking down a road—those who often stop and get distracted by interesting details arrive later than those who walk straight ahead.
- **Application:** thin-layer chromatography (TLC), used, for example, to check drug purity.

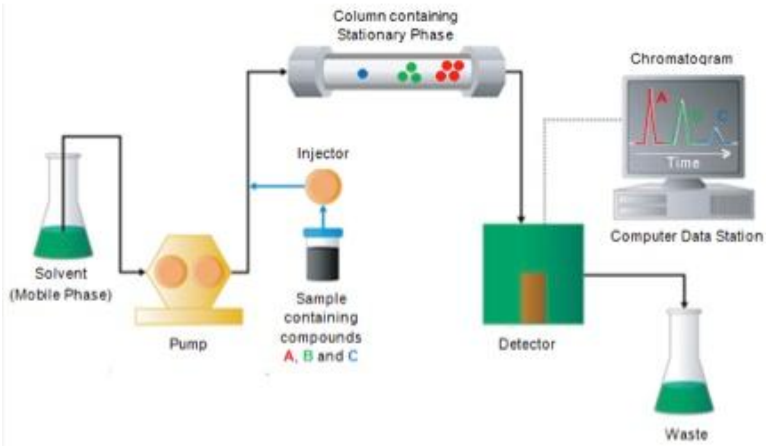
(Source: https://chemistryhall.com/thin-layer-chromatography/)

2.2 Partition Chromatography

Here, separation is based on the distribution of substances between two immiscible liquids. One is the mobile phase (flowing through the column), the other is immobilized as the stationary phase. Individual substances are then separated according to which phase they interact with more strongly.

- If a substance dissolves better in the mobile phase, it passes through the column faster.
- If it dissolves better in the stationary phase, it is retained longer.

Practical example: liquid chromatography (HPLC) used in clinical chemistry for the determination of drugs, hormones, or amino acids.



(Source: https://theory.labster.com/niche_hplc/)

2.3 Ion-Exchange Chromatography

Here, the electrical charge of molecules plays a role. The stationary phase carries a surface charge (positive or negative). Ions of opposite charge are electrostatically attracted and temporarily retained. Only by changing conditions (e.g., eluent composition or pH) can they be “displaced” from the column.

- Analogy: It works similarly to a magnet – the stationary phase attracts only particles of opposite charge, while particles with the same charge are repelled and neutral particles pass through unaffected.
- Medical application: analysis of electrolytes, protein separation by charge, sometimes purification of biomolecules for research.

2.4 Bioaffinity Chromatography

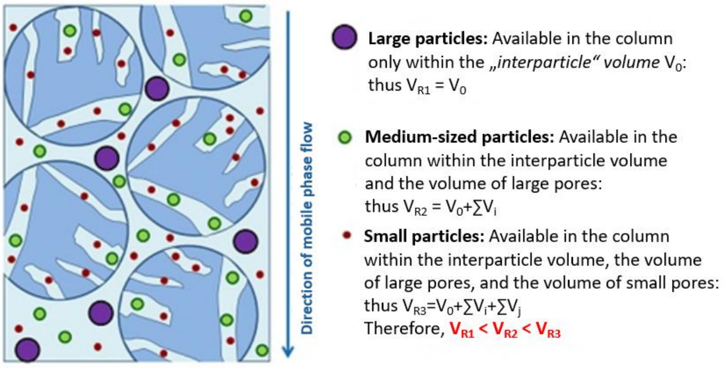
This type of chromatography is unique because it uses specific biological interactions—the natural ability of certain molecules to bind to one another. The stationary phase is linked to a “bait” that recognizes only a specific target molecule—for example, an antibody recognizing an antigen, an enzyme its substrate, or a receptor its hormone. Thus, the target molecule is captured, while others wash through.

- Analogy: like a lock and key—only the correct combination fits.
- Medical application: immunoaffinity chromatography for isolating specific proteins, antibodies, or biomarkers, pregnancy tests, fecal occult blood tests, etc. (see Chapter 5.3)

2.5 Gel Permeation Chromatography (GPC, SEC – Size Exclusion Chromatography)

Unlike the previous methods, separation here is determined not by chemical properties but by size (molecular weight). The stationary phase consists of a special porous gel:

- Smaller molecules enter the pores and must "travel" through them, so they are retained longer.
- Larger molecules cannot enter the pores and pass through the column more quickly.
- Example: separation of proteins, polysaccharides, or determination of the size and molecular weight of biological macromolecules.



3 Selected Chromatographic Techniques

In practice, two main variants are most frequently used: **gas chromatography (GC)** and **liquid chromatography (LC)**. Both methods work on the same principle—the mixture is carried by the mobile phase through a column with a stationary phase, and the components are separated based on their different interactions with the phases. However, they differ in the type of mobile phase and in the kinds of substances for which they are suitable.

3.1 Gas Chromatography

Gas chromatography uses an inert gas (helium, nitrogen, or hydrogen) as the mobile phase. The analyzed mixture is first vaporized in the injector and then carried by the gas stream through a column whose walls are coated with a thin layer of liquid stationary phase. Each component behaves differently—more volatile molecules pass quickly, while less volatile ones or those strongly interacting with the stationary phase are retained longer. The result is a chromatogram, where each compound has its characteristic retention time.

GC is mainly used for volatile and thermally stable substances. These include volatile organic compounds, solvents, toxins, and certain drugs. Non-volatile substances often require chemical modification (derivatization) before GC analysis.

An advantage of **GC** is its high resolution and speed of separation. When coupled with mass spectrometry (**GC-MS**), it becomes a tool with exceptional selectivity and sensitivity. GC-MS is used for example in toxicology (e.g., determination of ethanol in blood), forensic analysis, and doping control, as it enables the reliable identification of even trace amounts of substances in biological samples.

3.2 High-Performance Liquid Chromatography (HPLC)

HPLC uses a liquid mobile phase that is pumped through the column under high pressure. Unlike GC, analytes are not vaporized, making the method suitable for non-volatile or thermally sensitive molecules. Typical analytes include amino acids, peptides, vitamins, hormones, and drug metabolites.

High-performance liquid chromatography (HPLC) is a widely used type of liquid chromatography, applied in areas ranging from clinical biochemistry—where it helps analyze biological samples—to pharmaceutical quality control, where it verifies the purity and composition of drugs. Its versatility lies in the possibility of using different types of stationary phases (reverse phase, ion-exchange, or gel-permeation), allowing HPLC to cover a wide range of analytes.

4 Detection in Chromatography

After a complex sample is separated into individual components, these components exit the chromatographic column at different times. This fact alone would not be very useful—we need to know **which component is eluting (and when) and in what quantity**. For this purpose, a detector is placed after the column in the flow of the mobile phase.

Currently, in liquid chromatography, a variety of detectors are used:

- **UV/VIS**
- **Fluorescence**
- **Electrochemical**
- **Refractive index**
- **ELS (Evaporative Light Scattering)**
- **Conductivity**
- and today, perhaps the most important: the **mass spectrometric detector (MS)**

In the following chapters, we will specify two detection methods in more detail: detection using a UV/VIS detector and mass spectrometry.

4.1 UV/VIS Detector – Principle and Use

UV/VIS spectrophotometry is historically one of the most commonly used detectors in chromatography. It is popular due to its simplicity, reliability, and wide applicability. The principle is based on measuring the absorbance of light in the ultraviolet and visible regions of the spectrum (typically 200–700 nm). Any substance containing a so-called chromophore (e.g., an aromatic ring or double bond) can absorb this light and thus be detected.

In a chromatographic system, the eluate from the column passes through the detector, where it is continuously irradiated with light of a specific wavelength. If the analyte absorbs the radiation, the intensity of light reaching the detector decreases. This drop is recorded and appears as a chromatographic peak, the height of which corresponds to the concentration of the substance. The relationship between absorbance and concentration is described by the Lambert–Beer law, which allows quantitative evaluation.

The major advantage of the UV/VIS detector is its versatility—it can be used to measure a wide range of organic compounds, the method is fast, and it does not require complex sample preparation. On the other hand, the technique has its limitations: selectivity is relatively low, since if other substances in the sample absorb at the same wavelength, their signals may overlap. Moreover, the method is only applicable to molecules that actually contain a chromophore—substances without such structures remain “invisible.”

In practice, UV/VIS detectors are used especially for the determination of drugs with aromatic structures, for monitoring the purity of pharmaceutical preparations, or for routine quantification of compounds using HPLC—for example, vitamins, dyes, or certain metabolites.

4.2 Mass Spectrometric Detector – Principle and Use

Mass spectrometry (MS) is currently one of the most important detection systems used in combination with separation methods, particularly chromatography. Its strength lies not only in its high **sensitivity** but also in its exceptional **selectivity**—the ability to reliably distinguish the target analyte from other components of a complex mixture.

Unlike other detectors, such as UV/VIS or fluorescence detectors, which record only signal intensity, a mass spectrometric detector provides additional information—it measures the **mass-to-charge ratio (m/z)** of generated ions. From this value, the molecular weight of a compound can be determined, and in some cases even its structure. Simply put, MS is capable of “weighing” molecules.

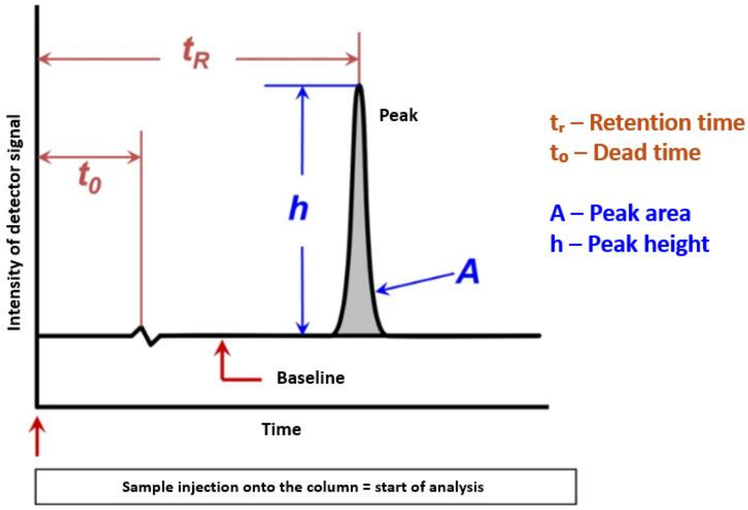
The greatest advantage is its high selectivity. It enables analysis of individual components even in complex biological samples, where signals would overlap and be lost in a standard UV/VIS detector. Another advantage is the **ability to identify compounds**. Modern instruments not only determine the exact m/z value but can also fragment the molecule, thereby revealing its structure. The accuracy of m/z measurement is now so high that even the molecular formula of a compound can be deduced. A third major advantage is extraordinary sensitivity—MS allows detection of substances at trace levels. For example, with the drug fluticasone, concentrations as low as 5 pg/mL can be determined, which is a thousand times less than dissolving a teaspoon of salt in a swimming pool of 100 m^3.

Thanks to these properties, MS detectors are used for determining extremely low concentrations of drugs or metabolites in plasma, as well as in research on metabolic processes and the identification of unknown compounds.

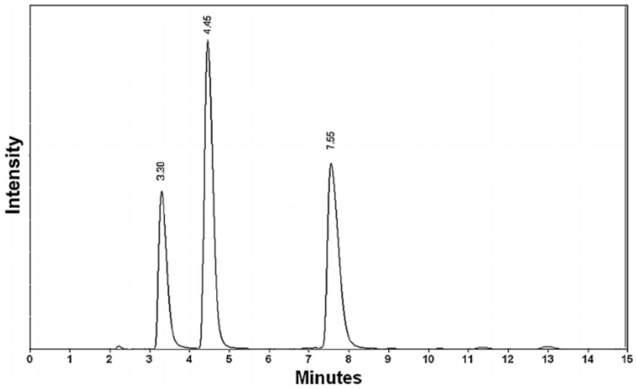
4.3 Chromatogram

The final output is the so-called **chromatogram**—a signal record in the form of peaks. Each peak represents one compound.

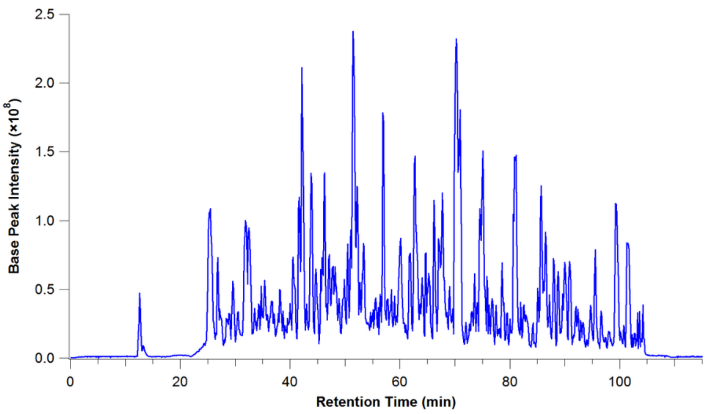
- The time of appearance indicates what the compound is (Retention time – t_R).
- The Peak area (height) shows how much of the compound was present in the sample.



Thus, instead of a complex mixture, we see a clear “fingerprint” of the sample, composed of its individual components.



Or sometimes not: 😊



5 Applications of Chromatographic Separation Techniques in Medicine

Chromatographic methods hold an irreplaceable role in clinical laboratories. They are not primarily intended for routine determination of common biochemical markers (e.g., glucose, ions), which are performed on automated analyzers. Their importance lies rather in the determination of specific compounds that require **high sensitivity and selectivity**.

5.1 Therapeutic Drug Monitoring (TDM)

The main area of application is so-called therapeutic drug monitoring (TDM)—the monitoring of drug levels in blood. This applies especially to substances with a narrow therapeutic window, where plasma concentrations must be kept within an optimal range. Exceeding this range risks toxic effects, while underdosing leads to loss of efficacy.

- Examples: cytostatics, immunosuppressants, but also antihypertensives, antipsychotics, sedatives, and antidepressants.

5.2 Toxicological Screening

Another key area is toxicological analysis, where liquid chromatography with mass detection (LC-MS) is often used. This method can simultaneously quantify a vast number of compounds.

- Example: in University hospital in Hradec Králové, more than 1,200 different drugs, narcotics, and their metabolites are routinely monitored using this technique.
- Frequently tested substances include barbiturates, benzodiazepines, salicylates, amphetamines, cannabinoids, and opiates.

5.3 Chromatography in Everyday Life

We also encounter chromatography outside the laboratory—for example, in simple disposable tests:

- pregnancy tests for detecting hCG in urine,
- fecal occult blood tests.

These tests use the principle of thin-layer immunoaffinity chromatography, where the sorbent is coated with an antibody specific for the analyte of interest.

5.4 Gas Chromatography in Clinical Practice

Gas chromatography is less significant in medicine compared to LC-MS, but it is still used, especially in toxicology laboratories. It is ideal for determining volatile organic compounds—such as methanol, ethanol, acetone, isopropanol, or toluene.

Summary

Chromatography is a universal separation method that enables effective separation of components in complex mixtures based on their different interactions with the stationary and mobile phases. There are many types of chromatographic techniques, differing in their separation principle (adsorption, ion-exchange, gel, affinity) and technical execution (PC, TLC, column, GC, HPLC). Detection of compounds is carried out using various detectors, often coupled with mass spectrometry in modern methods. Clinical applications of chromatography cover a wide spectrum—from the diagnosis of metabolic disorders, through therapeutic drug monitoring, to the detection of drugs. Chromatography, when combined with an appropriate detector, is a sensitive, universal, and precise method, whose limitation lies in its technical complexity and financial demands.

Control Questions

1. What is the principle of chromatography?
2. What is the difference between retention time and retention factor?
3. How do ion-exchange chromatography and gel chromatography differ?
4. What is the advantage of HPLC compared to classical column chromatography?
5. What types of detectors are used in chromatography, and when is it appropriate to use a fluorescence detector?
6. Which clinical test uses HPLC to monitor diabetes?
7. What errors can occur in chromatographic analysis?