**Chromatographic and Spectrophotometric Separation Techniques applied in the food industry**

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**Course Outline**

* Paper chromatography,
* thin layer chromatography,
* elementary ideas on spectrophotometry

Chromatography and spectrophotometry have grown in popularity, industries such as pharmaceuticals and chemicals began using the method, but one industry that uses chromatography and spectrophotometry extensively is the food industry. These techniques allow the food industry to provide accurate information about the nutrients in a particular food and much more.

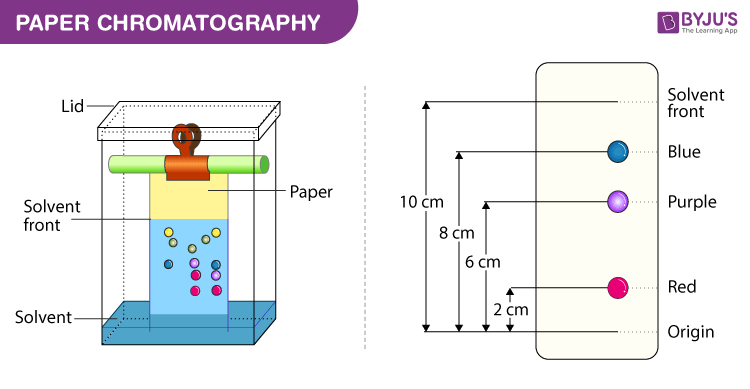
**1. Paper Chromatography**

Chromatography technique that uses paper sheets or strips as the adsorbent being the stationary phase through which a solution is made to pass is called paper chromatography. It is an inexpensive method of separating dissolved chemical substances by their different migration rates across the sheets of paper. It is a powerful analytical tool that uses very small quantities of material. Paper chromatography was discovered by Synge and Martin in the year 1943.

Paper Chromatography Principle

The principle involved can be partition chromatography or adsorption chromatography. Partition chromatography because the substances are partitioned or distributed between liquid phases. The two phases are water held in pores of the filter paper and the other phase is a mobile phase which passes through the paper. When the mobile phase moves, the [separation of the mixture](https://byjus.com/chemistry/separation-of-mixtures/) takes place. The compounds in the mixture separate themselves based on the differences in their affinity towards stationary and mobile phase solvents under the capillary action of pores in the paper. Adsorption chromatography between solid and liquid phases, wherein the solid surface of the paper is the stationary phase and the liquid phase is the mobile phase.

Paper Chromatography Diagram



Paper Chromatography Procedure

Below we have explained the procedure to conduct Paper Chromatography Experiment for easy understanding of students.

1. **Selecting a suitable type of development:**It is decided based on the complexity of the solvent, paper, mixture, etc. Usually ascending type or radial paper chromatography is used as they are easy to perform. Also, it is easy to handle, the chromatogram obtained is faster and the process is less time-consuming.
2. **Selecting a suitable filter paper**: Selection of filter paper is done based on the size of the pores and the sample quality.
3. **Prepare the sample:** Sample preparation includes the dissolution of the sample in a suitable solvent (inert with the sample under analysis) used in making the mobile phase.
4. **Spot the sample on the paper:** Samples should be spotted at a proper position on the paper by using a capillary tube.
5. **Chromatogram development:** Chromatogram development is spotted by immersing the paper in the mobile phase. Due to the capillary action of paper, the mobile phase moves over the sample on the paper.
6. **Paper drying and compound detection**: Once the chromatogram is developed, the paper is dried using an air drier. Also, detecting [solution](https://byjus.com/chemistry/solution-properties-concentration/) can be sprayed on the chromatogram developed paper and dried to identify the sample chromatogram spots.

Paper Chromatography Applications

There are various [applications of paper chromatography](https://byjus.com/chemistry/separation-of-pigments-of-leaves-and-flowers-by-chromatography/). Some of the uses of Paper Chromatography in different fields are discussed below:

* To study the process of fermentation and ripening.
* To check the purity of pharmaceuticals.
* To inspect cosmetics.
* To detect the adulterants.
* To detect the contaminants in drinks and foods.
* To examine the reaction mixtures in biochemical laboratories.
* To determine dopes and drugs in humans and animals.

Types of paper chromatography:

1. Ascending Paper Chromatography – The techniques goes with its name as the solvent moves in an upward direction.
2. Descending Paper Chromatography – The movement of the flow of solvent due to gravitational pull and capillary action is downwards, hence the name descending paper chromatography.
3. Ascending – Descending Paper Chromatography – In this version of paper chromatography, movement of solvent occurs in two directions after a particular point. Initially, the solvent travels upwards on the paper which is folded over a rod and after crossing the rod it continues with its travel in the downward direction.
4. Radial or Circular Paper Chromatography – The sample is deposited at the centre of the circular filter paper. Once the spot is dried, the filter paper is tied horizontally on a Petri dish which contains the solvent.
5. Two Dimensional Paper Chromatography – Substances which have the same rf values can be resolved with the help of two-dimensional paper chromatography.

**2. Thin-layer chromatography**

**Thin-layer chromatography** (TLC) is a [chromatography](https://en.wikipedia.org/wiki/Chromatography) technique used to separate non-volatile mixtures.[[1]](https://en.wikipedia.org/wiki/Thin-layer_chromatography#cite_note-HarwoodMoodyEOCPAP-1). Thin-layer chromatography is performed on a sheet of an inert substrate such as glass, plastic, or aluminium foil, which is coated with a thin layer of [adsorbent](https://en.wikipedia.org/wiki/Adsorbent) material, usually [silica gel](https://en.wikipedia.org/wiki/Silica_gel), [aluminium oxide](https://en.wikipedia.org/wiki/Aluminium_oxide" \o "Aluminium oxide) (alumina), or [cellulose](https://en.wikipedia.org/wiki/Cellulose). This layer of adsorbent is known as the [stationary phase](https://en.wikipedia.org/wiki/Stationary_phase_(chemistry)). After the sample has been applied on the plate, a [solvent](https://en.wikipedia.org/wiki/Solvent) or solvent mixture (known as the [mobile phase](https://en.wikipedia.org/wiki/Mobile_phase)) is drawn up the plate via [capillary action](https://en.wikipedia.org/wiki/Capillary_action). Because different [analytes](https://en.wikipedia.org/wiki/Analyte) ascend the TLC plate at different rates, separation is achieved.[[2]](https://en.wikipedia.org/wiki/Thin-layer_chromatography#cite_note-2) It may be performed on the analytical scale as a means of monitoring the progress of a reaction, or on the preparative scale to purify small amounts of a compound. TLC is an analytical tool widely used because of its simplicity, relative low cost, high sensitivity, and speed of separation. TLC functions on the same principle as all chromatography: a compound will have different affinities for the mobile and stationary phases, and this affects the speed at which it migrates. The goal of TLC is to obtain well defined, well separated spots.

The mobile phase has different properties from the stationary phase. For example, with silica gel, a very [polar](https://en.wikipedia.org/wiki/Polarity_(chemistry)) substance, non-polar mobile phases such as [heptane](https://en.wikipedia.org/wiki/Heptane) are used. The mobile phase may be a mixture, allowing chemists to fine-tune the bulk properties of the mobile phase.

After the experiment, the spots are visualized. Often this can be done simply by projecting [ultraviolet](https://en.wikipedia.org/wiki/Ultraviolet) light onto the sheet; the sheets are often treated with a [phosphor](https://en.wikipedia.org/wiki/Phosphor), and dark spots appear on the sheet where compounds absorb the light impinging on a certain area. Chemical processes can also be used to visualize spots; [anisaldehyde](https://en.wikipedia.org/wiki/Anisaldehyde), for example, forms colored adducts with many compounds, and [sulfuric acid](https://en.wikipedia.org/wiki/Sulfuric_acid) will char most organic compounds, leaving a dark spot on the sheet.

To quantify the results, the distance traveled by the substance being considered is divided by the total distance traveled by the mobile phase, this ratio is called the [retardation factor](https://en.wikipedia.org/wiki/Retardation_factor) (*R*f), or sometimes colloquially as *retention factor*. For the result to be quantitative the absorption of solvent must be stopped before the mobile phase reaches the end of the stationary phase. In general, a substance whose structure resembles the stationary phase will have low *R*f, while one that has a similar structure to the mobile phase will have high retardation factor. Retardation factors are characteristic, but will change depending on the exact condition of the mobile and stationary phase. For this reason, chemists usually apply a sample of a known compound to the sheet alongside the unknown samples.

Thin-layer chromatography can be used to monitor the progress of a reaction, identify compounds present in a given mixture, and determine the purity of a substance. Specific examples of these applications include: analyzing [ceramides](https://en.wikipedia.org/wiki/Ceramide) and [fatty acids](https://en.wikipedia.org/wiki/Fatty_acid), detection of [pesticides](https://en.wikipedia.org/wiki/Pesticide) or [insecticides](https://en.wikipedia.org/wiki/Insecticide) in food and water, analyzing the dye composition of fibers in [forensics](https://en.wikipedia.org/wiki/Forensic), assaying the [radiochemical purity](https://en.wikipedia.org/w/index.php?title=Radiochemical_purity&action=edit&redlink=1) of [radiopharmaceuticals](https://en.wikipedia.org/wiki/Radiopharmaceutical), or identification of [medicinal plants](https://en.wikipedia.org/wiki/Medicinal_plants) and their constituents [[3]](https://en.wikipedia.org/wiki/Thin-layer_chromatography#cite_note-hptlc_med-3)

A number of enhancements can be made to the original method to automate the different steps, to increase the resolution achieved with TLC and to allow more accurate quantitative analysis. This method is referred to as [HPTLC](https://en.wikipedia.org/wiki/HPTLC), or "high-performance TLC". HPTLC typically uses thinner layers of stationary phase and smaller sample volumes, thus reducing the loss of resolution due to [diffusion](https://en.wikipedia.org/wiki/Diffusion).

Plate preparation

TLC plates are usually commercially available, with standard particle size ranges to improve [reproducibility](https://en.wikipedia.org/wiki/Reproducibility). They are prepared by mixing the adsorbent, such as [silica gel](https://en.wikipedia.org/wiki/Silica_gel), with a small amount of [inert](https://en.wikipedia.org/wiki/Chemically_inert) binder like [calcium sulfate](https://en.wikipedia.org/wiki/Calcium_sulfate) (gypsum) and water. This mixture is spread as a thick slurry on an unreactive carrier sheet, usually [glass](https://en.wikipedia.org/wiki/Glass), thick aluminum foil, or plastic. The resultant plate is dried and *activated* by heating in an oven for thirty minutes at 110 °C. The thickness of the absorbent layer is typically around 0.1–0.25 mm for analytical purposes and around 0.5–2.0 mm for preparative TLC.[[4]](https://en.wikipedia.org/wiki/Thin-layer_chromatography#cite_note-4)

Technique

The process is similar to [paper chromatography](https://en.wikipedia.org/wiki/Paper_chromatography) with the advantage of faster runs, better separations, and the choice between different stationary phases. Because of its simplicity and speed, TLC is often used for monitoring [chemical reactions](https://en.wikipedia.org/wiki/Chemical_reaction) and for the qualitative analysis of reaction products. Plates can be labeled before or after the chromatography process using a pencil or other implement that will not interfere or react with the process.

To run a thin layer chromatography plate, the following procedure is carried out:[[5]](https://en.wikipedia.org/wiki/Thin-layer_chromatography#cite_note-5)

* Using a capillary tube, a small spot of [solution](https://en.wikipedia.org/wiki/Solution_(chemistry)) containing the sample is applied to a plate, about 1.5 centimeters from the bottom edge. The [solvent](https://en.wikipedia.org/wiki/Solvent) is allowed to completely evaporate off to prevent it from interfering with sample's interactions with the mobile phase in the next step. If a non-volatile solvent was used to apply the sample, the plate needs to be dried in a [vacuum chamber](https://en.wikipedia.org/wiki/Vacuum_chamber). This step is often repeated to ensure there is enough analyte at the starting spot on the plate to obtain a visible result. Different samples can be placed in a row of spots the same distance from the bottom edge, each of which will move in its own adjacent lane from its own starting point.
* A small amount of an appropriate solvent (eluent) is poured into a glass beaker or any other suitable transparent container (separation chamber) to a depth of less than 1 centimeter. A strip of filter paper (aka "wick") is put into the chamber so that its bottom touches the solvent and the paper lies on the chamber wall and reaches almost to the top of the container. The container is closed with a cover glass or any other lid and is left for a few minutes to let the solvent vapors ascend the filter paper and saturate the air in the chamber. (Failure to saturate the chamber will result in poor separation and non-reproducible results.)
* The TLC plate is then placed in the chamber so that the spot(s) of the sample do not touch the surface of the eluent in the chamber, and the lid is closed. The [solvent](https://en.wikipedia.org/wiki/Solvent) moves up the plate by [capillary action](https://en.wikipedia.org/wiki/Capillary_action), meets the sample mixture and carries it up the plate (elutes the sample). The plate should be removed from the chamber before the solvent front reaches the top of the stationary phase (continuation of the elution will give a misleading result) and dried.
* Without delay, the *solvent front*, the furthest extent of solvent up the plate, is marked.
* The plate is visualized. As some plates are pre-coated with a phosphor such as [zinc sulfide](https://en.wikipedia.org/wiki/Zinc_sulfide), allowing many compounds to be visualized by using [ultraviolet light](https://en.wikipedia.org/wiki/Ultraviolet_light); dark spots appear where the compounds block the UV light from striking the plate. Alternatively, plates can be sprayed or immersed in chemicals after elution. Various visualising agents react with the spots to produce visible results.

**Separation process and principle**[[edit](https://en.wikipedia.org/w/index.php?title=Thin-layer_chromatography&action=edit&section=3)]

Different [compounds](https://en.wikipedia.org/wiki/Chemical_compound) in the sample mixture travel at different rates due to the differences in their attraction to the stationary phase and because of differences in solubility in the solvent.[[6]](https://en.wikipedia.org/wiki/Thin-layer_chromatography#cite_note-mehta-6) By changing the solvent, or perhaps using a mixture, the separation of components (measured by the [*R*f](https://en.wikipedia.org/wiki/Retardation_factor) value) can be adjusted. Also, the separation achieved with a TLC plate can be used to estimate the separation of a [flash chromatography](https://en.wikipedia.org/wiki/Column_chromatography) column. (A compound elutes from a column when the amount of solvent collected is equal to 1/Rf.)[[7]](https://en.wikipedia.org/wiki/Thin-layer_chromatography#cite_note-7) Chemists often use TLC to develop a protocol for separation by chromatography and use TLC to determine which fractions contain the desired compounds.

[Graphical user interface, application

Description automatically generated](https://en.wikipedia.org/wiki/File:Tlc_sequence.svg)

Development of a TLC plate. A purple spot separates into a red and blue spot.

Separation of compounds is based on the competition of the solute and the mobile phase for binding sites on the stationary phase.[[3]](https://en.wikipedia.org/wiki/Thin-layer_chromatography#cite_note-hptlc_med-3) For instance, if normal-phase silica gel is used as the stationary phase, it can be considered polar. Given two compounds that differ in polarity, the more polar compound has a stronger interaction with the silica and is, therefore, better able to displace the mobile phase from the available binding sites. As a consequence, the less polar compound moves higher up the plate (resulting in a higher *R*f value).[[6]](https://en.wikipedia.org/wiki/Thin-layer_chromatography#cite_note-mehta-6) If the mobile phase is changed to a more polar solvent or mixture of solvents, it becomes better at binding to the polar plate and therefore displacing solutes from it, so all compounds on the TLC plate will move higher up the plate. It is commonly said that "strong" solvents (eluents) push the analyzed compounds up the plate, whereas "weak" eluents barely move them. The order of strength/weakness depends on the coating (stationary phase) of the TLC plate. For silica gel-coated TLC plates, the eluent strength increases in the following order: [perfluoroalkane](https://en.wikipedia.org/wiki/Perfluoroalkane" \o "Perfluoroalkane) (weakest), [hexane](https://en.wikipedia.org/wiki/Hexane), [pentane](https://en.wikipedia.org/wiki/Pentane), [carbon tetrachloride](https://en.wikipedia.org/wiki/Carbon_tetrachloride), [benzene](https://en.wikipedia.org/wiki/Benzene)/[toluene](https://en.wikipedia.org/wiki/Toluene), [dichloromethane](https://en.wikipedia.org/wiki/Dichloromethane), [diethyl ether](https://en.wikipedia.org/wiki/Diethyl_ether), [ethyl acetate](https://en.wikipedia.org/wiki/Ethyl_acetate), [acetonitrile](https://en.wikipedia.org/wiki/Acetonitrile), [acetone](https://en.wikipedia.org/wiki/Acetone), [2-propanol](https://en.wikipedia.org/wiki/2-propanol)/[*n*-butanol](https://en.wikipedia.org/wiki/N-butanol), [water](https://en.wikipedia.org/wiki/Water), [methanol](https://en.wikipedia.org/wiki/Methanol), [triethylamine](https://en.wikipedia.org/wiki/Triethylamine), [acetic acid](https://en.wikipedia.org/wiki/Acetic_acid), [formic acid](https://en.wikipedia.org/wiki/Formic_acid) (strongest). For [C18](https://en.wikipedia.org/wiki/Reversed-phase_chromatography)-coated plates the order is reverse. In other words, when the stationary phase is polar and the mobile phase is nonpolar, the method is *normal-phase* as opposed to *reverse-phase*. This means that if a mixture of ethyl acetate and hexane as the mobile phase is used, adding more ethyl acetate results in higher *R*f values for all compounds on the TLC plate. Changing the polarity of the mobile phase will normally not result in reversed order of running of the compounds on the TLC plate. An [eluotropic series](https://en.wikipedia.org/wiki/Eluotropic_series) can be used as a guide in selecting a mobile phase. If a reversed order of running of the compounds is desired, an apolar stationary phase should be used, such as C18-functionalized silica.

Analysis

As the chemicals being separated may be colorless, several methods exist to visualize the spots:

* Fluorescent analytes, like [quinine](https://en.wikipedia.org/wiki/Quinine), may be detected under [blacklight](https://en.wikipedia.org/wiki/Blacklight) (366 nm)
* Often a small amount of a [fluorescent](https://en.wikipedia.org/wiki/Fluorescent) compound, usually [manganese](https://en.wikipedia.org/wiki/Manganese)-activated [zinc silicate](https://en.wikipedia.org/wiki/Willemite), is added to the adsorbent that allows the visualization of spots under UV-C light (254 nm). The adsorbent layer will thus fluoresce light-green by itself, but spots of analyte quench this fluorescence.
* [Iodine](https://en.wikipedia.org/wiki/Iodine) vapors are a general unspecific color [reagent](https://en.wikipedia.org/wiki/Reagent)
* Specific color reagents into which the TLC plate is dipped or which are sprayed onto the plate exist.[[8]](https://en.wikipedia.org/wiki/Thin-layer_chromatography#cite_note-8)[[9]](https://en.wikipedia.org/wiki/Thin-layer_chromatography#cite_note-9)[[10]](https://en.wikipedia.org/wiki/Thin-layer_chromatography#cite_note-10)
  + [Potassium permanganate](https://en.wikipedia.org/wiki/Potassium_permanganate) – oxidation
  + [Bromine](https://en.wikipedia.org/wiki/Bromine)
  + Acidic [vanillin](https://en.wikipedia.org/wiki/Vanillin)
  + [Phosphomolybdic acid](https://en.wikipedia.org/wiki/Phosphomolybdic_acid)
* In the case of lipids, the chromatogram may be transferred to a [polyvinylidene fluoride](https://en.wikipedia.org/wiki/Polyvinylidene_fluoride) membrane and then subjected to further analysis, for example [mass spectrometry](https://en.wikipedia.org/wiki/Mass_spectrometry), a technique known as [far-eastern blot](https://en.wikipedia.org/wiki/Far-eastern_blot).

Once visible, the *Rf* value, or [retardation factor](https://en.wikipedia.org/wiki/Retardation_factor), of each spot can be determined by dividing the distance the product traveled by the distance the solvent front traveled using the initial spotting site as reference. These values depend on the solvent used and the type of TLC plate and are not physical constants.

Applications

**Characterization**

In [organic chemistry](https://en.wikipedia.org/wiki/Organic_chemistry), reactions are qualitatively monitored with TLC. Spots sampled with a capillary tube are placed on the plate: a spot of starting material, a spot from the reaction mixture, and a cross-spot with both. A small (3 by 7 cm) TLC plate takes a couple of minutes to run. The analysis is qualitative, and it will show if the starting material has disappeared, i.e. the reaction is complete, if any product has appeared, and how many products are generated (although this might be underestimated due to co-elution). Unfortunately, TLCs from low-temperature reactions may give misleading results, because the sample is warmed to room temperature in the capillary, which can alter the reaction—the warmed sample analyzed by TLC is not the same as what is in the low-temperature flask. One such reaction is the [DIBALH](https://en.wikipedia.org/wiki/DIBALH) reduction of ester to aldehyde.

In one study TLC has been applied in the screening of [organic reactions](https://en.wikipedia.org/wiki/Organic_reaction),[[11]](https://en.wikipedia.org/wiki/Thin-layer_chromatography#cite_note-11) for example in the fine-tuning of [BINAP](https://en.wikipedia.org/wiki/BINAP) synthesis from [2-naphthol](https://en.wikipedia.org/wiki/2-naphthol). In this method, the alcohol and catalyst solution (for instance [iron(III) chloride](https://en.wikipedia.org/wiki/Iron(III)_chloride)) are placed separately on the baseline, then reacted, and then instantly analyzed.

A special application of TLC is in the characterization of radiolabeled compounds, where it is used to determine [radiochemical purity](https://en.wikipedia.org/w/index.php?title=Radiochemical_purity&action=edit&redlink=1). The TLC sheet is visualized using a sheet of photographic film or an instrument capable of measuring [radioactivity](https://en.wikipedia.org/wiki/Radioactivity). It may be visualized using other means as well. This method is much more sensitive than the others and can be used to detect an extremely small amount of a compound, provided that it carries a radioactive atom.

**Isolation**

Since different compounds will travel a different distance in the stationary phase, chromatography can be used to isolate components of a mixture for further analysis. The separated compounds each occupying a specific area on the plate, they can be scraped off (along with the stationary phase particles) and dissolved into an appropriate solvent. As an example, in the chromatography of an extract of green plant material (for example [spinach](https://en.wikipedia.org/wiki/Spinach)) shown in 7 stages of development, [carotene](https://en.wikipedia.org/wiki/Carotene) elutes quickly and is only visible until step 2. [Chlorophyll A](https://en.wikipedia.org/wiki/Chlorophyll_a) and [B](https://en.wikipedia.org/wiki/Chlorophyll_b) are halfway in the final step and [lutein](https://en.wikipedia.org/wiki/Lutein) the first compound staining yellow. Once the chromatography is over, the carotene can be removed from the plate, extracted into a solvent and placed into a [spectrophotometer](https://en.wikipedia.org/wiki/Spectrophotometer) to determine its spectrum. The quantities extracted are small and a technique such as column chromatography is preferred to separate larger amounts. However, big preparative TLC plates with thick silica gel coatings can be used to separate more than 100 mg of material.

**Examining the progress of reactions and purity of compounds**[[edit](https://en.wikipedia.org/w/index.php?title=Thin-layer_chromatography&action=edit&section=8)]

TLC is also used for the identification of the completion of any chemical reaction. To determine this it is observed that at the beginning of a reaction the entire spot is occupied by the starting chemicals or materials on the plate. As the reaction starts taking place the spot formed by the initial chemicals starts reducing and eventually replaces the whole spot of starting chemicals with a new product present on the plate. The formation of an entirely new spot determines the completion of a reaction.[[12]](https://en.wikipedia.org/wiki/Thin-layer_chromatography#cite_note-12)

Furthermore, two-dimensional TLC is frequently used as a method to check if a compound is stable in the stationary phase (such as silica gel, which is usually slightly acidic). For this purpose, the tested compound mixture is eluted twice in a square-shaped TLC plate, first in one direction and then rotated 90º. If the target compound appears on the diagonal of the square, it is stable in silica gel and safe to purify. If it appears below the diagonal, it is decomposing on silica gel. If this is the case, purification can be attempted using neutralized silica gel (with [triethylamine](https://en.wikipedia.org/wiki/Triethylamine), for example), or an alternative stationary phase such as [neutral alumina](https://en.wikipedia.org/wiki/Aluminium_oxide).[[13]](https://en.wikipedia.org/wiki/Thin-layer_chromatography#cite_note-13)

TLC is also used as an analytical method for the direct separation of [enantiomers](https://en.wikipedia.org/wiki/Enantiomer) and the control of enantiomeric purity, e.g. active pharmaceutical ingredients ([APIs](https://en.wikipedia.org/wiki/Active_Pharmaceutical_Ingredient)) that are chiral.

**3. Spectrophotometry**

**Spectrophotometry** is a branch of [electromagnetic spectroscopy](https://en.wikipedia.org/wiki/Electromagnetic_spectroscopy) concerned with the quantitative measurement of the reflection or transmission properties of a material as a function of wavelength.[[2]](https://en.wikipedia.org/wiki/Spectrophotometry#cite_note-:1-2) Spectrophotometry uses [photometers](https://en.wikipedia.org/wiki/Photometer), known as spectrophotometers, that can measure the intensity of a light beam at different wavelengths. Although spectrophotometry is most commonly applied to ultraviolet, [visible](https://en.wikipedia.org/wiki/Visible_spectrum), and [infrared](https://en.wikipedia.org/wiki/Infrared) radiation, modern spectrophotometers can interrogate wide swaths of the [electromagnetic spectrum](https://en.wikipedia.org/wiki/Electromagnetic_spectrum), including [x-ray](https://en.wikipedia.org/wiki/X-ray), [ultraviolet](https://en.wikipedia.org/wiki/Ultraviolet), [visible](https://en.wikipedia.org/wiki/Visible_spectrum), [infrared](https://en.wikipedia.org/wiki/Infrared), and/or [microwave](https://en.wikipedia.org/wiki/Microwave) wavelengths.



Typical spectrophotometer

Spectrophotometry is a tool that hinges on the quantitative analysis of molecules depending on how much light is absorbed by colored compounds. Important features of spectrophotometers are spectral bandwidth (the range of colors it can transmit through the test sample), the percentage of sample-transmission, the logarithmic range of sample-absorption, and sometimes a percentage of reflectance measurement.

A spectrophotometer is commonly used for the measurement of transmittance or reflectance of solutions, transparent or opaque solids, such as polished glass, or gases. Although many biochemicals are colored, as in, they absorb visible light and therefore can be measured by colorimetric procedures, even colorless biochemicals can often be converted to colored compounds suitable for chromogenic color-forming reactions to yield compounds suitable for colorimetric analysis.[[3]](https://en.wikipedia.org/wiki/Spectrophotometry#cite_note-:0-3): 65 However, they can also be designed to measure the [diffusivity](https://en.wikipedia.org/wiki/Reflectivity) on any of the listed light ranges that usually cover around 200–2500 nm using different controls and [calibrations](https://en.wikipedia.org/wiki/Calibrations).[[2]](https://en.wikipedia.org/wiki/Spectrophotometry#cite_note-:1-2) Within these ranges of light, calibrations are needed on the machine using standards that vary in type depending on the [wavelength](https://en.wikipedia.org/wiki/Wavelength) of the *photometric determination*.[[4]](https://en.wikipedia.org/wiki/Spectrophotometry#cite_note-4)

An example of an experiment in which spectrophotometry is used is the determination of the equilibrium constant of a solution. A certain chemical reaction within a solution may occur in a forward and reverse direction, where reactants form products and products break down into reactants. At some point, this chemical reaction will reach a point of balance called an equilibrium point. In order to determine the respective concentrations of reactants and products at this point, the light transmittance of the solution can be tested using spectrophotometry. The amount of light that passes through the solution is indicative of the concentration of certain chemicals that do not allow light to pass through.

The absorption of light is due to the interaction of light with the electronic and vibrational modes of molecules. Each type of molecule has an individual set of energy levels associated with the makeup of its chemical bonds and nuclei and thus will absorb light of specific wavelengths, or energies, resulting in unique spectral properties.[[5]](https://en.wikipedia.org/wiki/Spectrophotometry#cite_note-:2-5) This is based upon its specific and distinct makeup.

The use of spectrophotometers spans various scientific fields, such as [physics](https://en.wikipedia.org/wiki/Physics), [materials science](https://en.wikipedia.org/wiki/Materials_science), [chemistry](https://en.wikipedia.org/wiki/Chemistry), [biochemistry](https://en.wikipedia.org/wiki/Biochemistry), [Chemical Engineering](https://en.wikipedia.org/wiki/Chemical_Engineering), and [molecular biology](https://en.wikipedia.org/wiki/Molecular_biology).[[6]](https://en.wikipedia.org/wiki/Spectrophotometry#cite_note-:3-6) They are widely used in many industries including semiconductors, laser and optical manufacturing, printing and forensic examination, as well as in laboratories for the study of chemical substances. Spectrophotometry is often used in measurements of enzyme activities, determinations of protein concentrations, determinations of enzymatic kinetic constants, and measurements of ligand binding reactions.[[3]](https://en.wikipedia.org/wiki/Spectrophotometry#cite_note-:0-3): 65 Ultimately, a spectrophotometer is able to determine, depending on the control or calibration, what substances are present in a target and exactly how much through calculations of observed wavelengths.

UV-visible spectrophotometry

Most spectrophotometers are used in the [UV](https://en.wikipedia.org/wiki/UV) and [visible](https://en.wikipedia.org/wiki/Visible_spectrum) regions of the spectrum, and some of these instruments also operate into the near-[infrared](https://en.wikipedia.org/wiki/Infrared) region as well. The concentration of a protein can be estimated by measuring the OD at 280 nm due to the presence of tryptophan, tyrosine and phenylalanine. This method is not very accurate since the composition of proteins varies greatly and proteins with none of these amino acids do not have maximum absorption at 280 nm. Nucleic acid contamination can also interfere. This method requires a spectrophotometer capable of measuring in the UV region with quartz cuvettes.[[3]](https://en.wikipedia.org/wiki/Spectrophotometry#cite_note-:0-3): 135

Ultraviolet-visible (UV-vis) spectroscopy involves energy levels that excite electronic transitions. Absorption of UV-vis light excites molecules that are in ground-states to their excited-states.[[5]](https://en.wikipedia.org/wiki/Spectrophotometry#cite_note-:2-5)

Visible region 400–700 nm spectrophotometry is used extensively in [colorimetry](https://en.wikipedia.org/wiki/Colorimetry) science. It is a known fact that it operates best at the range of 0.2–0.8 O.D. Ink manufacturers, printing companies, textiles vendors, and many more, need the data provided through colorimetry. They take readings in the region of every 5–20 nanometers along the visible region, and produce a [spectral reflectance](https://en.wikipedia.org/wiki/Spectral_reflectance) curve or a data stream for alternative presentations. These curves can be used to test a new batch of colorant to check if it makes a match to specifications, e.g., ISO printing standards.

Traditional visible region spectrophotometers cannot detect if a colorant or the base material has fluorescence. This can make it difficult to manage color issues if for example one or more of the printing inks is fluorescent. Where a colorant contains fluorescence, a [bi-spectral fluorescent spectrophotometer](https://en.wikipedia.org/w/index.php?title=Bi-spectral_fluorescent_spectrophotometer&action=edit&redlink=1) is used. There are two major setups for visual spectrum spectrophotometers, d/8 (spherical) and 0/45. The names are due to the geometry of the light source, observer and interior of the measurement chamber. Scientists use this instrument to measure the amount of compounds in a sample. If the compound is more concentrated more light will be absorbed by the sample; within small ranges, the [Beer–Lambert law](https://en.wikipedia.org/wiki/Beer%E2%80%93Lambert_law) holds and the absorbance between samples vary with concentration linearly. In the case of printing measurements two alternative settings are commonly used- without/with uv filter to control better the effect of uv brighteners within the paper stock.

[A picture containing text, indoor, projector

Description automatically generated](https://en.wikipedia.org/wiki/File:Mettler_Toledo_UV5Nano_micro-volume.jpg)

METTLER TOLEDO UV5Nano Micro-Volume Spectrophotometer

Samples are usually prepared in [cuvettes](https://en.wikipedia.org/wiki/Cuvette); depending on the region of interest, they may be constructed of [glass](https://en.wikipedia.org/wiki/Glass), [plastic](https://en.wikipedia.org/wiki/Plastic) (visible spectrum region of interest), or [quartz](https://en.wikipedia.org/wiki/Quartz_glass) (Far UV spectrum region of interest). Some applications require small volume measurements which can be performed with micro-volume platforms.

**Applications**[[edit](https://en.wikipedia.org/w/index.php?title=Spectrophotometry&action=edit&section=6)]

* Estimating [dissolved organic carbon](https://en.wikipedia.org/wiki/Dissolved_organic_carbon) concentration
* [Specific ultraviolet absorbance](https://en.wikipedia.org/wiki/Specific_ultraviolet_absorbance) for metric of aromaticity
* [Bial's test](https://en.wikipedia.org/wiki/Bial%27s_test) for concentration of pentoses

**Experimental application**

As described in the applications section, spectrophotometry can be used in both qualitative and quantitative analysis of DNA, RNA, and proteins. Qualitative analysis can be used and spectrophotometers are used to record spectra of compounds by scanning broad wavelength regions to determine the absorbance properties (the intensity of the color) of the compound at each wavelength.[[5]](https://en.wikipedia.org/wiki/Spectrophotometry#cite_note-:2-5) One experiment that can demonstrate the various uses that visible spectrophotometry can have is the separation of β-galactosidase from a mixture of various proteins. Largely, spectrophotometry is best used to help quantify the amount of purification your sample has undergone relative to total protein concentration. By running an affinity chromatography, B-Galactosidase can be isolated and tested by reacting collected samples with ONPG and determining if the sample turns yellow.[[3]](https://en.wikipedia.org/wiki/Spectrophotometry#cite_note-:0-3): 21–119 Following this testing the sample at 420 nm for specific interaction with ONPG and at 595 for a Bradford Assay the amount of purification can be assessed quantitatively.[[3]](https://en.wikipedia.org/wiki/Spectrophotometry#cite_note-:0-3): 21–119 In addition to this spectrophotometry can be used in tandem with other techniques such as SDS-Page electrophoresis in order to purify and isolate various protein samples.

**Questions and Answers**

What are the advantages of Paper Chromatography?

Paper Chromatography Has Many Benefits  
Simple and rapid  
Paper chromatography necessitates a minimal amount of quantitative material.  
Paper chromatography is less expensive than other chromatography methods.  
The paper chromatography method can identify both unknown inorganic and organic compounds.  
Paper chromatography takes up little space when compared to other analytical methods or equipment.  
Outstanding resolving power

Why water is not used in paper chromatography?

It is preferable to use a less polar solvent, such as ethanol, so that the non-polar compounds will travel up the paper while the polar compounds will stick to the paper, separating them.

What are the limitations of Paper Chromatography?

Limitations of Paper Chromatography are as follows-  
Paper chromatography cannot handle large amounts of sample.  
Paper chromatography is ineffective in quantitative analysis.  
Paper chromatography cannot separate complex mixtures.  
Less Accurate than HPLC or HPTLC

What is the importance of paper chromatography?

Paper chromatography has traditionally been used to analyse food colours in ice creams, sweets, drinks and beverages, jams and jellies. Only edible colours are permitted for use to ensure that no non-permitted colouring agents are added to the foods. This is where quantification and identification come into play.