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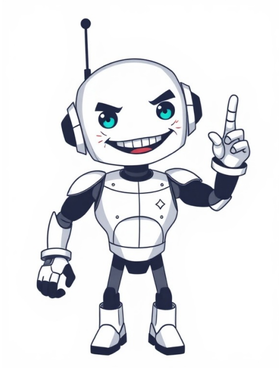

High-Performance Thin-Layer Chromatography (HPTLC): Advanced Technique for Separating Compounds ========== High-performance thin-layer chromatography (HPTLC) is an extension of traditional thin-layer chromatography (TLC), offering several advantages in the quantitative analysis of compounds. By employing higher-quality TLC plates with finer particle sizes, HPTLC enhances compound resolution and provides improved results. One key improvement in HPTLC is the use of repeated plate development using a multiple development device, which further refines the separation process. This approach allows for superior resolution and lower Limit of Detection (LODs) compared to traditional TLC methods. HPTLC offers several benefits, including providing straightforward information about compound effects in complex or natural samples, combining chromatographic separation with effect-directed detection, and aiding in selecting important compounds for further characterization using high-resolution mass spectrometry. The technique comprises three modes: linear, circular, and anticircular mode. The anticircular mode is the fastest in theory and practice, allowing for maximum sample capacity while minimizing time, layer, and mobile phase consumption. This approach also enables automated quantification due to its narrow spot-path. To begin HPTLC, a stationary phase must be determined to separate different compounds within a mixture. Most pharmaceutical separations are performed on normal phase silica gel, but other phases such as alumina can be used for samples with dissociating compounds and cellulose for ionic compounds. The selection of the mobile phase is crucial in HPTLC and follows a 'trial and error' pathway. The 'PRISMA' system provides a guideline for finding the optimal mobile phase, taking into account the absorptivity of the stationary phase and the composition of the compound of interest. Notable HPTLC devices such as the Linomat 5 and Automatic TLC Sampler 4 (ATS 4) by CAMAG feature automated 'spray-on' sample application techniques, which provide high resolution and narrow bands. These devices also offer alternative approaches for automation, including piezoelectric devices and inkjet printers. Upon chromatographic detection, HPTLC plates are usually developed in saturated twin-trough chambers with filter paper for optimal outcomes. A general mechanism for the HPTLC device involves placing a fitted filter paper in the rear trough of the chamber. ========== The mobile phase is poured into the rear trough to absorb all the solvent on the filter paper. The chamber is tilted at an angle of ~45° so that both troughs have equal amounts of solvent and left undisturbed for about 20 mins.[5] Next, the HPTLC plate is placed in the chamber to develop. To get accurate results, the mobile phase and filter paper are swapped between each sample reading.[8] By using two different solvents, the spot capacity can be increased through a technique called two-dimensional chromatography.[8] This technique involves developing the plate with one solvent first, removing it, and then rotating it 90° to develop with another solvent. HPTLC is widely used in various fields such as pharmaceuticals, clinical chemistry, forensic science, biochemistry, cosmetology, food analysis, environmental analysis, and more due to its numerous benefits. It stands out because it's the only chromatographic method that can display results as images, making it simple and cost-effective. HPTLC also offers parallel analysis of samples, high sample capacity, rapid results, and the option for multiple detection methods. Research by Le Roux's team found that HPTLC is suitable for determining salbutamol serum levels in clinical trials.[3] HPTLC has been valuable in lichenology for analyzing and identifying lichen substances. In comparison to standard TLC, it offers several advantages: it allows twice as many samples to be analyzed on one plate, requires less solvent (4 mL per plate compared to 250 mL), completes the chromatographic process in under 10 minutes, and can detect substances at lower concentrations.[9] The technique's increased sensitivity has led to the discovery of previously unidentified lichen compounds and a greater understanding of chemical variation within lichen species. Since the early 1990s, HPTLC has been used as an alternative to standard TLC for routine screening of lichen substances. However, proper plate drying is crucial due to the technique's increased sensitivity to atmospheric humidity.[9] HPTLC has also been successfully used in the separation of various lipid subclasses with consistent and promising results.[7] Several clinical medicine studies have reported positive findings, leading to the strong recommendation of HPTLC for drug analysis in serum and other tissues.[7] ========== ========== Optimizing Mobile Phase for Planar Chromatography The mobile phase in planar chromatography plays a crucial role in separating mixture components. A proper choice of mobile phase can significantly enhance the efficiency and effectiveness of the TLC process. The mobile phase, typically a liquid solvent or a solvent mixture, travels up the TLC plate, carrying sample drops at varying speeds. In high-performance thin-layer chromatography (HPTLC), a silica gel stationary phase is used, which has polar hydroxyl groups that participate in dipole-dipole interactions with other polar substances. The Rf value, ratio of solute to solvent distance, quantifies the separation process on a scale of 0 to 1. Choosing an appropriate solvent system for TLC analysis involves considering its solubility in the system and affinity with mobile and stationary phases. Non-polar solvents are commonly used in TLC analyses, allowing non-polar compounds to move further up the plate. A non-polar solvent like hexane is often used in thin layer chromatography (TLC) because it keeps most nonpolar compounds closer to the starting line. ==========___ A polar solvent system, such as ethyl acetate or methanol, will prompt polar components to move further up the plate. The goal of a TLC analysis is to get all components off the starting line. ==========___ The functional groups in the solution determine its behavior on the TLC plate. Polar substances interact strongly with the stationary phase and are absorbed slowly, while nonpolar substances move faster and don't stick to the silica gel. ==========___ The following steps describe how to perform a TLC analysis: First draw a line near the bottom of your TLC plate to mark the starting point. Then spot a small amount of the substance being analyzed onto the plate at that line. ==========___ Next, add a solvent system to the development chamber and insert the spotted TLC plate. Make sure the solutions don't touch the solvent before the TLC begins. Cover the beaker so the solvent doesn't evaporate. ==========___ Allow the TLC plate to develop until the solvent reaches the top of the plate and mark that boundary with another line. Then allow the TLC plate to dry completely. ==========___ To examine the developed plate, use a UV light source or stain it with iodine to make the spots more visible. Assign an Rf value to each dot on the plate by measuring its distance traveled as a fraction of the distance traveled by the solvent system. ==========___ This step allows you to compare the solubility of different dots in the same solvent system. Smaller Rf values indicate less soluble polar solutions, while larger Rf values denote highly soluble nonpolar solutions. ==========___ Chemists use TLC to monitor reactions and determine whether a reactant remains after a specific reaction by calculating Rf values of the reactant and product.Thin Layer Chromatography: A Technique to Maximize Separation ========== === TLC plates are typically coated with a thin layer of stationary phase, usually silica gel, which is around 95% of the time used in organic synthesis. This allows scientists to develop a ratio in which to mix such components, maximizing the distance between two components on their column chromatography plate. The goal of TLC is to isolate and compare different compounds from blood, urine, and other body fluids for clinical applications. In pharmaceutical industries, it fuels quality assurance investigations to identify harmful substances and quantitatively determine drug purity. Additionally, researchers use TLC to detect healthy levels of sweeteners, preservatives, and other additives in foods and food products. As a synthetic organic chemist with extensive experience in the lab, I have run thousands of TLC and flash columns in any solvent combination imaginable. This guide aims to provide a comprehensive tutorial for beginners and advanced chemists alike, covering various aspects of thin layer chromatography. TLC is an essential technique for separating components of a mixture. It involves using a stationary phase, usually silica gel, which is deposited over a glass or aluminum support. Mixtures of compounds are then spotted on the plate, followed by elution with an organic solvent, resulting in the separation of different components based on their affinity to the stationary and mobile phases. The technique is cheap, quick, and easy to perform, making it an ideal choice for synthetic chemists to monitor chemical reactions and purifications. Throughout this guide, I will share my knowledge and expertise, covering various topics such as solvent systems, TLC plates, and tips for optimal results. Whether you are a beginner or an experienced chemist, this tutorial is designed to be accessible and informative, providing a solid foundation for understanding the principles of thin layer chromatography. ========== You "load" the plate. You basically put it vertically inside a closed chamber which contains an amount of an appropriate solvent mixture. The solvent flows slowly up the plate through capillary action.The stationary phase, silica gel contains Si–O–H bonds that bind to the different compounds of the mixtures in a variable manner depending on the polarity of the compounds. Also, depending on the nature of the solvent used (more polar or less polar), it will pull upwards some compounds faster than others.In general, more polar compounds will “climb” slower up through the TLC plate, and less polar ones will fly upwards.Then you just need to check how many and where in the TLC plate each spot is. Each spot corresponds to a different chemical compound on the mixture.Separation of two main components of a mixture (pink spot and red spot). Usually you will need a UV (Ultraviolet) lamp to visualize the different spots, but if the compounds are strongly colored, as in the picture above, you can easily see the different components of the mixture.First you need to cut a piece of TLC plate of the appropriate size. What is the appropriate size? It depends on the purpose of the TLC, and how many spots you need to separate. If you just want to take a look on how many compounds you have in a mixture, one spot is enough.TLC plates are generally made of aluminum coated by the stationary phase, and can be cut with scissors. Sometimes, the supporting material is glass and you will need a glass cutter to do the job.Usually, a thin layer chromatography plate is around 5–7 cm high, and a line is drawn around 0.5–1.0 cm from the bottom. That is the line in which you will spot your mixtures to separate. It is important that you spot the mixtures above the solvent level on your elution chamber!Also, remember to leave some separation between each spot at the bottom spotting line (so they don’t mix to each other!) and also leave a similar separation (of around half a centimeter) from each edge of the TLC plate.Typical TLC size and arrangement.Then is time to prepare the samples of the mixtures to separate, and spot them on the TLC plate.For simplicity, let’s start off with just a single spot, in which we will put a solution of a mixture of several compounds.First we need to prepare a solution of our mixture. The usual average concentration of these solutions is a few miligrams of mixture/compound in around 0.5–1 mL of solvent. Those few miligrams are totally approximate. Just add a spatula or Pasteur pipette tip and dissolve it in a bit of solvent!Once you got the solutions prepared (in this case, just the one!), it’s time to spot it on the bottom line of the TLC. You need to use a capillary tube (see the corresponding section for details). Take up some mixture solution with the capillary tube and press it lightly into the corresponding marked spot (use ALWAYS a pencil to mark in a TLC! Pen ink will elute with organic solvents, pencil graphite will not!) at the line around 0.5–1 cm above the bottom of the TLC.Spot the TLC mixtures at the corresponding mark in the line above the bottom of the plate. Then elute the plate and see how many compounds there is in your mixture, and how polar are they, just by checking out the different spots.Try to spot your mixtures as tightly as possible. Make very small spots of sample. Very wide spots will make the different compounds overlap leading to a not so nice separations. Maybe even some compounds will be hidden since those will be basically co-eluting with other massive spots. Generally speaking, more diluted and smaller spots are they way to go.Then is time to elute the plate. For this you need an elution chamber. There are commercial options, as the one in the picture below, specific for that purpose.But you can use any glass container that you can cap, actually. A beaker works. A a clean jam jar will also do the job!Then you need to fill it with about 0.5 cm height of the desired solvent system.There is no absolute best starting point for selecting a solvent system. However, a extremely quick summary would be:If you are working with absolutely apolar organic molecules (no polar functional groups, only C and H), such as naphthalene, start with pure pentane or hexane.If you want to separate a compound with one or two mildly polar functional groups (ether, ketone, ester…), go for a 4:1 hexane/EtOAc mixture.If your molecule has one or two very polar groups (alcohol, amine, etc), go for 1:1 hexane/EtOAc.If your molecule is much more polar than that (e.g. a sugar, an amino acid…), swap hexane for DCM, and keep EtOAc as polar component. Use a 1:1 ratio for starters.If your compounds are so polar that do not move at all from the baseline with DCM/EtOAc, go for 9:1 DCM/MeOH or even 9:1 EtOAc/MeOH.If none of this works, you can also try different combinations or consult a more detailed guide. ========== Choosing a Solvent System for Thin Layer Chromatography (TLC) Before starting a TLC experiment, it is essential to select an appropriate solvent system that can effectively separate the desired compounds from each other. A polar-unsaturated compound and reverse phase are two common considerations when selecting a solvent system. Selecting a Suitable Solvent The primary objective of choosing a solvent system in TLC is to ensure that the solvent level remains below the initial point where you spot your samples, preventing them from getting diluted and losing their distinctiveness. In addition to the above, we recommend you look into more details about choosing a solvent system. Setting up the TLC Chamber Once the chamber is set up, place the TLC plate vertically inside it and wait for the solvent to rise by capillary action. Take out the TLC plate when the solvent level has reached approximately 90% from the top, without allowing it to drown. Marking the Eluent Front Before drying off the plate, mark the eluent front, which is the line on the plate where the solvent level has reached. This marks will be used to determine the retention factor (Rf) of each spot or compound. Drying Off and Visualization Dry off the plate using compressed air, blowing air, or simply waiting for it to dry completely. The next step involves visualization. Depending on the compounds being separated, they may display strongly colored spots that can be easily seen with a UV lamp. Alternatively, certain organic compounds will not produce visible results but will instead absorb UV radiation. Determining Retention Factor (Rf) In TLC, retention factor (Rf) represents the distance traveled by a compound through the stationary phase between the origin spot and the solvent front's position above the origin. The Rf value can be calculated using the simple formula: Rf(spot) = (distance of the spot)/(distance of the solvent front). Calculating Rf for Benzaldehyde A typical example would involve separating benzaldehyde and benzyl alcohol in a TLC plate using 7:3 pentane/diethyl ether as a solvent. By measuring the distance each spot travels, we can determine their retention factors. Conclusion Performing TLC requires knowledge of the suitable solvent system to be used for a given mixture. In some cases, modifying the solvent system may need to be done multiple times until an appropriate separation is achieved. ========== To monitor chemical reactions effectively, it is essential to track the conversion of starting material (SM) into a product. Ideally, the product should have a different polarity than the SM, resulting in distinct retention factors on thin layer chromatography (TLC). A suitable solvent mixture should provide both compounds with retention factors between 0.2 and 0.8, allowing for their separation. A crucial step is to create three spots on TLC: one with the SM, another with the reaction mixture (RM), and a middle spot containing both SM and RM solutions. This enables clear visualization of the reaction progress after elution, especially when the SM and product have similar retention factors. By observing these spots, researchers can confirm whether the SM has been consumed or if only partial conversion has occurred. Monitoring reaction progress using TLC is straightforward. The resulting diagram illustrates three scenarios: (1) no reaction occurs, (2) a product forms but the reaction is not complete, and (3) all SM has been consumed, leaving only products in the RM. By adding spots for the product and co-spot of both product and RM, researchers can verify the desired product's formation. Column chromatography purification builds upon TLC principles. A glass column filled with a stationary phase is used to separate compounds based on their relative polarities. The mixture is eluted from the top to bottom, driven by solvent flow and sometimes pressure. This technique isolates compounds in large quantities, making it an essential tool for chemical synthesis. Before running a flash column chromatography, researchers select an appropriate solvent system using TLC. Ideally, the product should have an Rf of around 0.4 in the chosen eluent to facilitate smooth purification. The resulting TLC diagram demonstrates this ideal separation, where two products are clearly visible and separated. After column chromatography, the resulting fractions are analyzed on TLC to identify and purify individual compounds. By repeating this process, researchers can obtain high-purity products from complex reaction mixtures. ==========eluted in the same solvent system. As you can see, we have two different products that came out of the column pretty close.From fraction 5 to 10, we only have compound one, pure. We can mix these fractions, concentrate them, and we will have pure compound 1.Fractions 11 and 12, have a mixture of the two compounds. Usually we throw away this kind of mixed fractions (unless we don’t actually care about the impurity, maybe it just doesn’t affect the next step of our synthesis!).Fractions 13 and 14 have pure compound 2. If we also need this compound, we will just concentrate them together as well.As you can see, TLC is extremely important for both reaction monitoring and product purification, the two cornerstones of any synthesis laboratory.Sometimes TLC is just not enough and you don’t know what compound/product is in each of the different fractions that came out of your flash column. Evaporating everything and taking an NMR is really time consuming, so you might want to go for an alternative technique if it’s available to you.If you have access to a GC-MS or LC-MS, you can analyze quickly all the different fractions, and know the molecular mass of the compound(s) present on each of them.Another cool instrument is the TLC-MS. This technique is usually much less available in chemistry labs than GC-MS or LC-MS, but if you can use it is great. Basically this machine automatically scraps off individual spots on an eluted TLC, and makes an MS analysis, so you can check the molecular masses present on of each spot of the TLC in usually less than a minute.Useing an eluent which gives an Rf of 0.4 for your compound is the usual rule of thumb, but it has of course many exceptions. If you have two compounds that are very close together in Rf, this might not be enough. Having two compounds show as two separate spots in TLC doesn’t mean that they will come out separately from flash column.Column bands are like much much wider TLC spots, especially as we scale up the purification. Imagine that typical TLC that you overload with sample and you get two big unresolved overlapping spots. That is a closer picture to what is actually happening in your column chromatography.For this reason, sometimes an Rf of 0.4 will not do the trick. If spots are separated by less than 0.15 Rf, you will usually need to be a bit more conservative and choose an eluent in which they have a retention factor of around 0.3, or even a bit less. Another cool trick to enhance this kind of purification is using thicker columns, this helps a lot with separation. Using longer columns doesn’t usually help, since you are just thickening the bands and making them overlap more!On the flip side of the coin, sometimes your compound of interest just flies on TLC using certain solvent mixture, giving an Rf of 0.7-0.9. This might allow for extremely easy and fast separations in a couple of the first tubes/fractions.You will have to spot reaction mixtures, or reference samples in your TLC using capillary tubes.You can either buy them, or make them yourself. This depends on your lab’s budget, but I don’t think there is much harm in buying some good capillary tubes. The commercial ones I use on a daily basis, usually last for months before breaking, if you are careful enough.But you can make thin capillary tubes out of thicker glass tubes, you just need to heat them up and then pulling. For this, you can either use thicker capillary tubes or glass Pasteur pipettes.It is more complicated than it looks, but making your own capillary tubes for spotting TLC plates can be a nice experiment for undergraduate labs. Just be careful with the flame (or other heating source that you use! Avoid using open flames in the lab if you have alternatives).So, there are actual chambers designed for running TLC, and they are just great, such as these from Fischer:TLC chambers designed for that purposeBut that doesn’t mean you need one of those fancy pieces of glasswares to run a TLC. The beauty and simplicity of this technique is that you can use it in basically any situation!A typical temporary solution, if you are in a rush, is just using a beaker covered with something (like a watch glass, or even aluminum foil), so the solvent doesn’t evaporate and allows for a nice saturated atmosphereIt is very importint to have a good eluent chamber. You need the atmosphere as saturated as possible. This way, the solvent doesn’t evaporate on its way up through the plate, which would cause an uneven movement of the eluent front. This can be detrimental for the separation, so always ensureTLC chambers can be reasonably self-contained systems composed of a beaker, watch glass, and filter paper. The use of a piece of filter paper inside the chamber before elution helps saturate the atmosphere with the solvent, ensuring a more even distribution on the TLC plate. It is essential to be patient when using these homemade TLC chambers, as leaving the eluent in contact with the filter paper for an extended period can aid in better separation. Alternatively, a glass jar with a screw cap can serve as a cost-effective and practical TLC chamber. These "ghetto-chambers" have been instrumental in my undergraduate studies and research experiences. In most cases, silica gel is used as the stationary phase, but specific compounds may require neutralization or alternative phases such as alumina or reverse-phase chromatography. Reverse-phase chromatography can be effective for polar molecules that are not easily separated by silica gel-based systems. However, these alternatives come with drawbacks, including higher costs and potentially worse separation and visualization capabilities. The ability to visualize the separated compounds is crucial in TLC; although many organic compounds do not absorb UV light strongly enough, staining agents can aid in this process. ========== It's essential to keep in mind that even if your target compound strongly absorbs UV or visible light, it's still recommended to stain the plate if possible. This is because there might be other impurities present in the mixture that can't be observed correctly under typical UV-Vis conditions. When working with compounds that appear transparent at first glance, make sure to check them under UV light as well. Additionally, even if everything looks fine under UV light, using a general-purpose staining agent will rarely be too much and can always help. The following is a list of the most common staining agents used in TLC, along with their preparation methods. It's worth noting that there are many other options available, some of which are highly specific to certain types of compounds. However, for 95% of organic compounds, a general-purpose stain will work just fine. One popular choice is vanillin solution, which is easy to prepare and reacts well with most functional groups. A small change in the functionality of an organic compound can result in a noticeable color change after vanillin staining and heating. Vanillin stains compounds with polar functional groups more prominently than those without them. To prepare this stain, simply weigh 10-15g of vanillin, dissolve it in 250mL of ethanol, and add 2.5mL of concentrated sulfuric acid. Dip the eluted TLC plate into the solution and heat it using a heating gun. Another general-purpose stain is PMAT (phosphomolybdic acid). It's easy to prepare by dissolving around 5g of phosphomolybdic acid in each 500mL of ethanol. PMAT produces a purple-colored TLC, which can be helpful for identifying compounds that are difficult to distinguish based on Rf values. However, it doesn't always work well with compounds having similar properties. The most classical staining agent is basic KMnO4 (potassium permanganate). The recipe involves dissolving 1.5g of potassium permanganate and 10g of potassium carbonate in 200mL of water, followed by adding 1mL of 10% aqueous NaOH. When handling this mixture, be cautious not to stain yourself or your skin. Another popular stain is Hanessian's Stain (or "blue stain"), which produces a cool pale yellow background after heating. However, if heated too much, the plate can turn blue as well. For those who struggle with finding cerium reagent, there are two alternative recipes available: one involving ammonium molybdate and cerium sulfate, or another using cerium ammonium sulfate. Lastly, there are many other staining agents that cater to specific types of compounds. However, for routine use in the lab, these general-purpose stains will suffice.Choosing the right solvent combination for chromatography can be a daunting task, especially for beginners. With so many different functional groups and potential combinations, it's easy to get overwhelmed. Even experienced researchers may struggle to find reliable guidance. For TLC or column chromatography, selecting the optimal eluent is crucial for achieving accurate results. However, there's no single "best" guide that covers all possible scenarios. This infographic aims to provide a helpful starting point by categorizing organic compounds based on their functional groups and suggesting a corresponding percentage of polar solvent required for effective separation. By grouping compounds according to their functional groups, this chart offers an approximation of the necessary polar solvent ratio for successful TLC. The graphic focuses on classical mixtures of apolar and polar solvents, such as hexane/ethyl acetate or diethyl ether, which are commonly used in organic separations. Keep in mind that while this guide is a valuable resource, it's essential to remember that every compound behaves uniquely. Even small variations can significantly impact separation results. As with any chromatography method, it's crucial to adapt and fine-tune the eluent composition based on individual compounds' properties. ========== ========== Looking at a TLC result as eluent Rf of 0.75, we can conclude that the product is adequately separated from other compounds. By gathering some useful tips and tricks, TLC techniques can be mastered. Two-dimensional TLC was crucial in my grad school days when working with unstable compounds on silica gel. A square TLC plate offers a larger surface area for spotting and elution.