TLC, Isocratic, and Gradient

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Chromatography Application Note AN24

Application Overview

This application note explores the considerations and process of taking samples from a simple Thin Layer Chromatography (TLC) separation to a productive, useful, gradient separation. We will look at how a TLC separation, which is inherently isocratic, relates to the same sample separated on an isocratically run instrument at the same concentration.

Gradient separations will also be discussed, paying particular attention to how a gradient solvent system can perform better separations in a shorter amount of time. This application note will provide tips on how to verify proper solvent selection, which will help you separate your sample successfully in a single gradient run.

TLC

TLC in General

> ☑ ***Note***
>
> It may be beneficial to obtain application note #9 from Isco's library. The PDF file contains a calculator that will aid you in determining the fraction tube that contains your compound.

TLC plates are almost always run on the reaction products or samples to be purified. The TLC results reveal a great deal about the sample's likely performance in column chromatography. It is beneficial to obtain TLC plates that have the same media as the columns that will be used. This will give a more accurate correlation between TLC and liquid chromatography. Isco Redi*Sep*® TLC plates and Redi*Sep* columns meet this requirement.

A good TLC with an R_f between 0.1–0.5 gives an indication of whether the sample can be successfully injected. If, with the TLC conditions used, the sample failed to move from the place where it was spotted then it is likely that the sample will stick at the top of the column and fail to move through the column and separate. Or, if the sample moves with or just behind the solvent front then it is likely that the sample will race through the column in an uncontrolled elution and no separation will result. In either case, the TLC solvent conditions have not been optimized.

TLC to Isocratic

A sample's R_f values obtained from TLC tells us if it can be separated by isocratic column chromatography. If the values fall in the range of 0.1 to 0.5 then it is possible to separate them isocratically.

The following are a range of examples using Isco Test Mix A. This is a mixture of methyl paraben, 4-aminobenzoic acid, and acetophenone. The compounds are mixed and then adsorbed onto silica, which is then loaded into a solid sample cartridge. Test Mix A is available from Isco; order part number 60-3877-010.

For the TLC plate, dried sample was simply taken up in ethyl acetate and spotted onto Redi*Sep* Normal Phase plates, Isco part number 69-2203-400. These plates are 5×10 cm, 250 micron, UV254 w/organic binder, silica gel plates and were run as follows.

1. The TLC chamber is equilibrated with the indicated eluant for 10 minutes, allowing for chamber equilibration between vapor and liquid phase.
2. A line was made 4 mm from the bottom of the plate for the solvent level (solvent line).
3. A line was made 4 mm above the solvent line for the samples (sample line).
4. Sample was then spotted onto the plate via a capillary tube.
5. Once the chamber had equilibrated, the plate was inserted into the chamber and allowed to run for 40 minutes.
6. The plate was removed and the solvent front is marked (final solvent front).
7. The plate was allowed to dry and the spots were marked.
8. R_f was calculated for each spot.

If the R_f is in the desirable range of 0.1–0.5 then an isocratic separation can be performed under those same conditions. Separations outside this range may be possible but the resolution may be reduced. Note in the following examples that all three peaks of the standard never fall in the desired range. This data shows that an isocratic separation will not be suitable for this type of sample.

Data follows in order of increasing strength of ethyl acetate.

10%

The TLC plate depicted in Figure 1 (left) was run with 10% ethyl acetate 90% Hexane. This mixture is too weak to move the bottom sample from the sample line. The middle spot R_f is 0.17 and the top spot 0.60.

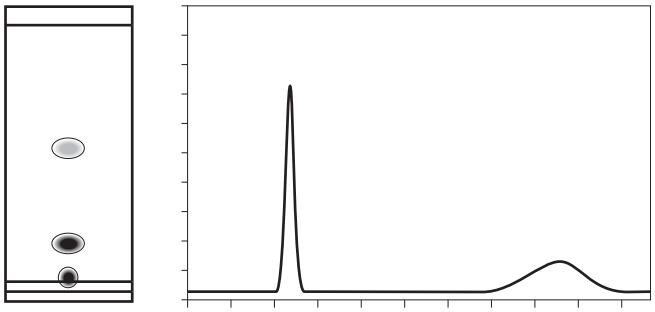

Figure 1: Isocratic 10% EtOAc in Hexane

The same isocratic conditions were run on an Isco Combi*Flash*® Companion™ with a 12 gram Redi*Sep* normal phase column. The chromatogram (Figure 1, right) confirms the TLC trial results.

As evident in the chromatogram, the first peak (acetophenone) is acceptable and corresponds with the top spot on the TLC plate. The second peak (methyl paraben) is spread out so much that it is nearly unusable and the third peak (4-aminobenzoic acid) is not seen.

This solvent mixture is far too weak for a successful isocratic separation of anything other than the first peak.

20%

The next TLC plate run (Figure 2) increases ethyl acetate strength to 20%, with 80% Hexane.

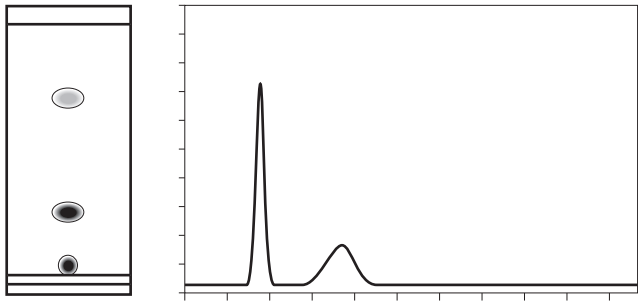

Figure 2: Isocratic 20% EtOAc in Hexane

The bottom sample R_f is .07, the middle sample R_f is .31, and the top sample is .73. This mixture is still too weak to move the bottom compound significantly from the sample line. Only the middle sample has a good R_f at this mixture.

For comparison, the sample is run isocratically on the Companion under 20% B conditions. The first peak and second peak are good. And, just like the 10% mixture, the third peak is not seen. This solvent mixture is too weak for a successful isocratic separation of all three peaks.

30%

Increasing the ethyl acetate to 30% in the next TLC plate run produces the results shown in Figure 3.

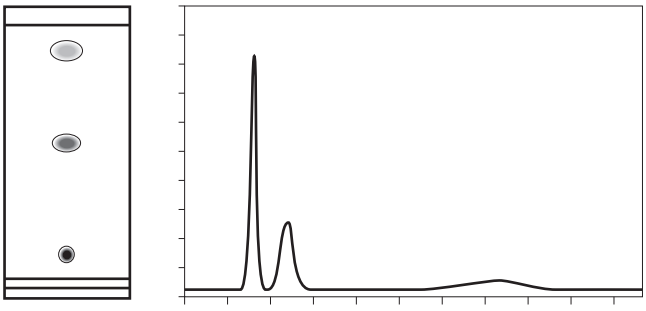

Figure 3: Isocratic 30% in EtOAc in Hexane

This mixture is now strong enough to move the bottom sample significantly from the sample line. The bottom sample R_f is .10, the middle sample R_f is .52, and the top sample .90. The bottom and middle sample have a good R_f at this mixture. This TLC indicates that all three components will elute from the column.

Running the same conditions isocratically on the Companion confirm this assumption. The first and second peaks look good now and the third peak comes off the column but is not usable. This mixture works well for the first two peaks but does not work well for the third. It is likely that the third peak will not be salvageable in a concentration this low.

50%

Figure 4 illustrates the next TLC plate run with 50% Ethyl acetate, 50% Hexane.

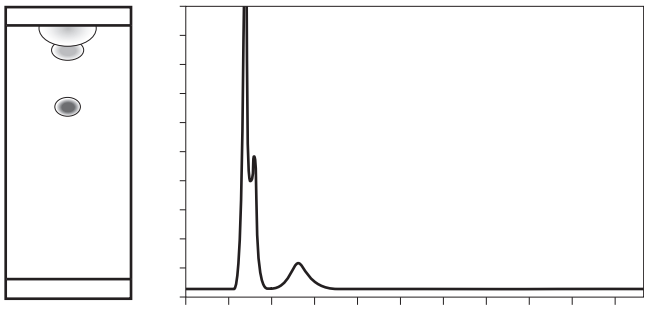

Figure 4: Isocratic 50% EtOAc in Hexane

This mixture is definitely strong enough to move the bottom sample significantly from the sample line. However, the top sample runs with the same velocity as the solvent front. This unretained compound allows for very little separation since it is not interacting with the column. The strength of the solvent is forcing the second spot to be crowded against the first. The bottom sample R_f is .42, the middle sample R_f is .89, and the top sample .92, so the bottom spot has a good R_f at this mixture, but the other two are running too fast.

On the chromatogram, the first and second overlap, as was predicted by the TLC, but the third peak is probably usable. This mixture works well for the third peak but the first two are not sufficiently separated.

TLC to Isocratic summary

The TLC trials reveal that with an isocratic ethyl acetate and hexane solvent system, there is no ideal solvent mixture that will purify all three Test Mix A compounds.

However, to purify the compounds individually, 20% ethyl acetate will purify acetophenone (peak 1); 30 to 40% is best to purify methyl paraben (peak 2); 40 to 50% will purify 4-aminobenzoic acid (peak 3).

To purify all three in a single run, a gradient solvent system may be developed.

Gradient Solvent Systems

The fastest and best solution to purify Test Mix A is to use a gradient separation. Figure 5 illustrates this.

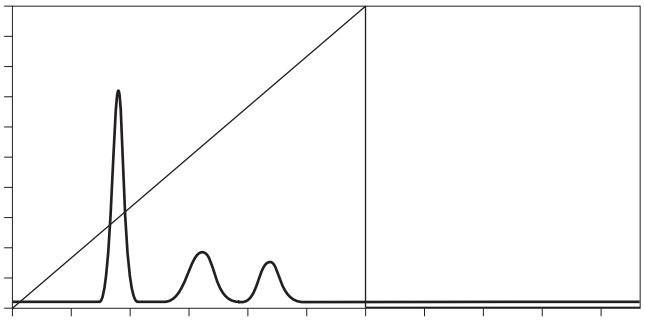

Figure 5: Linear Gradient from 0 to 100% EtOAc in Hexane

Linear gradients offer several advantages:

- The gradient did not have to be optimized; it is simply a 0–100% linear gradient.
- The entire run is complete in 12 column volumes. This is 25% less time than the isocratic runs, using 25% less solvent.
- Far fewer tubes are used, which in turn means disposing of fewer tubes, and less work in drying down sample.

Ideally, initial solvent should be weak enough that the samples are not moved. Normally the starting condition is 0% B. The final solvent strength should be sufficiently strong. A default setting is 100% B but this isn't always necessary.

Solvent Selection for a Gradient run

TLC remains a practical starting point for developing gradient solvent elutions. A couple of TLC runs will establish the suitability of the solvents. For this purpose, these TLCs will be much quicker and easier because they do not require that the spots separate. Instead, the TLC must only move the spots.

Solvent selection — These instructions assume that the flash chromatography system can deliver a two solvent system. Also, the solvents two solvents must be compatible with the system. If so, TLC may be used to bracket the solvent mixture.

Steps:

1. Spot your TLC sample onto two separate plates and allow spots to adequately dry.
2. Choose two compatible, miscible, solvents, *e.g.* hexane/ethyl acetate.
3. Make up two separate TLC chambers. Beakers and watch glasses are generally sufficient.
4. In chamber one, place the weaker of your two solvents, *e.g.* hexane.
5. In chamber two, place the stronger of you two solvents, *e.g.* ethyl acetate.
6. Run the plates as you would normally until the solvent nears the top, mark the solvent final line (Z_f), and allow the plates to dry.
7. Use the appropriate detection lamp and mark the spots.

Ideally, the plate from chamber one should show a grouping of desired species having moved very little, if at all from the sample line.

Conversely, the plate from chamber two should show a grouping near the top, or Z_f. There should not be any of the desired species near the sample line. It is okay, if not common, that undesirable impurities do not move.

For example the same three peak standard from Test Mixture A, when run on a TLC in 100% Hexane, then 100% EtOAc gives the result shown in Figure 6.

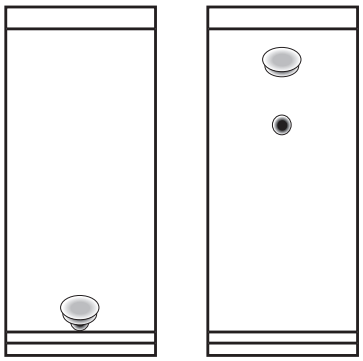

Figure 6: Bracketing solvent performance with TLC

The TLC plates clearly show that neither solvent alone is ideal, but a mixture between the two extremes should give a reasonable result. A gradient does its best work under these conditions.

From these two TLC plates, we learn that under gradient solvent conditions:

- The desired species in the sample are completely soluble in solvent B, if not solvent A. This will prevent loss of sample due to insolubility.
- The weaker solvent is not too strong and will not cause samples to race off of the column unretained and unseparated.
- The stronger solvent is not too weak and is capable of eluting all desired species.

- And finally, two solvents have been identified that should lead to a successful gradient separation.

Summary

Understanding the relationship of TLC, isocratic, and gradient separations provides a background necessary to developing effective gradient elution methods. This application note demonstrates this relationship and how to move to a gradient solvent system.

Gradient solvents systems have many advantages, which include:

- fewer and easier TLC trials,
- better separation resolution,
- faster separation runs,
- less solvent used,
- higher compound purity with less drying time.

Teledyne ISCO
P.O. Box 82531, Lincoln, Nebraska, 68501 USA
Toll-free: (800) 228-4373 • Phone: (402) 464-0231 • Fax: (402) 465-3091
www.teledyneisco.com