Combi*Flash*® Gradient Methods

Abstract

Purifying compounds require two processes: the actual purification and drying the sample afterwards. Several gradient methods were evaluated on a reaction mixture to evaluate run time and number of fractions to collect the desired compound. Reducing the volume of fractions is important to reduce the time required to dry the purified material. On large runs, drying time may be equal to, or greater than, the purification time. An optimized step gradient was found to be most efficient for repeated purifications, but required several runs to optimize. The most efficient gradient for general use was programmed into Peak*Trak*®. A comparison was also run between various automated gradients and a manual method: Dry Column Vacuum Chromatography. The Peak*Trak* Gradient Optimizer was found to be efficient when purifying compounds that eluted close to each other on TLC plates.

Introduction

There are several different gradients that can be run on a Combi*Flash* system:

Isocratic: The solvent composition remains the same throughout the run.

Step Gradient: The strong solvent changes abruptly to a higher concentration.

Linear Gradient: The concentration of the strong solvent gradually increases over time. The slope of the linear gradient can be changed to vary the resolution between eluting peaks.

Mixed Gradients: These are a combination of linear gradients with isocratic holds. They can be generated with the Gradient Optimizer on the Combi*Flash* system.

Each gradient type has advantages and disadvantages which will be discussed in the Experimental and Results section.

Experimental and Results

Synthesis of 3-(2-nitrophenyl amino) propionitrile

2-Nitroaniline (304 g) was dissolved in 1500 mL reagent alcohol. Triton B (45 mL) was added and the mixture was heated to reflux. Acrylonitrile (420 mL) was added with stirring. The mixture was stirred overnight at reflux. The alcohol was evaporated to yield ~550 g of a tarry mixture. A portion of this mixture was loaded onto silica by dissolving it in dichloromethane/methanol/water and adsorbing onto silica (1 part reaction mixture to 4 parts silica, 20% load on bulk Redi*Sep*®silica) and the solvents evaporated. This sample was used for all experiments (except where otherwise noted) in this application note; 4.0g was used for each experiment (0.80g reaction mixture, 1.0% load on an 80g Redi*Sep* silica column PN 69-2203-380).

All purifications were run on an Combi*Flash* system using solvent A= hexanes; solvent B= ethyl acetate. Peak collection used 254 nm.

Isocratic

Isocratic is the easiest method to develop and is also the easiest to run because no special equipment is required if the solvent is premixed. Isocratic methods are best run on simple mixtures that are well resolved.
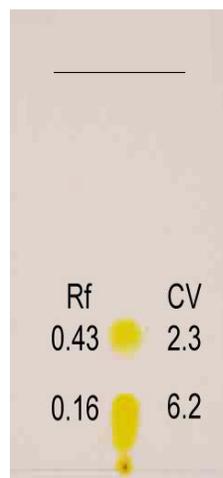

Figure 1: TLC of reaction mixture

The Thin Layer Chromatography experiment (TLC) in *Figure 1* was used to determine the run conditions for this experiment. The plate was developed with 25% ethyl acetate in hexane. The upper spot had a had an R_f of 0.43 while the second spot had an R_f of 0.16 corresponding to elution in 2.3 and 6.2 column volumes respectively[1]. The actual purification showed elution times close to those predicted by the TLC plate. A Redi*Sep* Silica TLC plate (PN 69-2203-400) was used for this experiment.

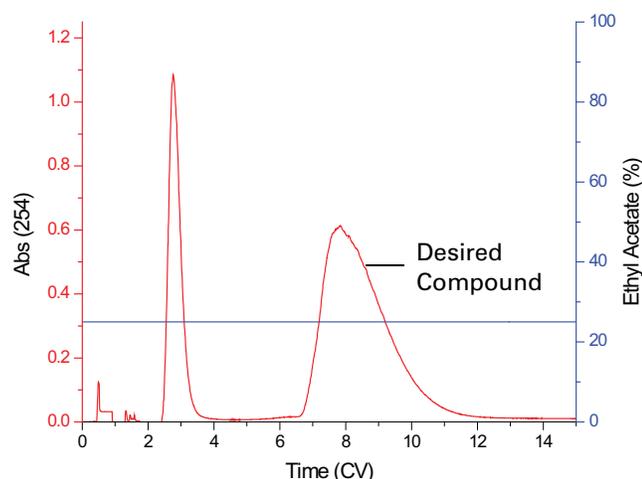1. For an isocratic run, elution time in $CV = 1/R_f$

Figure 2: Isocratic purification of 3-(2-nitrophenyl-amino) propionitrile

The desired compound was completely eluted by 12 column volumes. The first peak was collected in fraction totaling 125 mL solvent and the desired compound was collected in 500 mL solvent.

Step Gradient

Step Gradients can be very efficient, but require knowledge of the compound elution to be properly optimized. The TLC in *Figure 1* indicated that the compounds are eluted 25% ethyl acetate. The run in *Figure 2* shows there is a great deal of resolution between the two compounds.

> Leaving the starting solvent at 25% B resolves the first peak from the small impurities eluting at 1.5 CV.
>
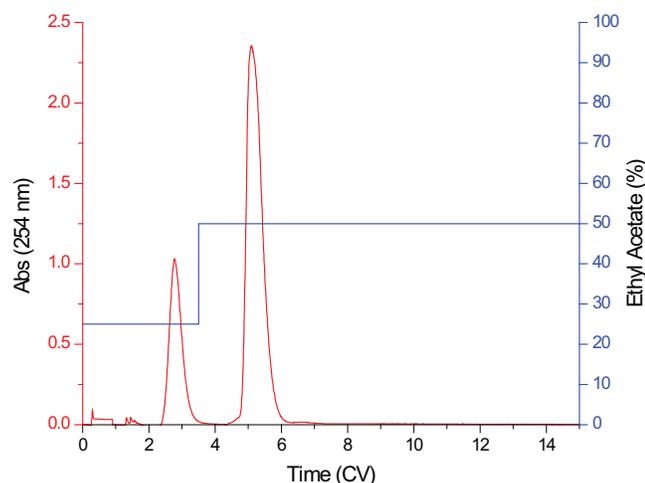> Moving to 50% B allows the second peak to be eluted more quickly.

Figure 3: Step gradient purification

The first peak was collected in 125 mL solvent. The second peak was collected in 225 mL solvent; ½ that for the isocratic experiment.

A further optimization of the step gradient allows further reduction in run time and collected fractions. Changing the gradient from 50% to 100% B causes the second peak to elute within one column volume as the compound will no longer be retained by the column. This knowledge suggests the step be set at 2.6 column volumes, causing the second peak to elute as soon as the first peak finished eluting. With this optimization, the second peak is collected in only 75 mL solvent.

Further optimizations can be made if the only concern is the purity of the second peak. In this case, the starting percentage of B can be increased to cause the first peak to elute more quickly. The step timing would need to be changed so that the elution of the second peak remains separate from the first peak while still resolving the desired compound from the impurities eluting at ~6.7 CV in Figure 4.
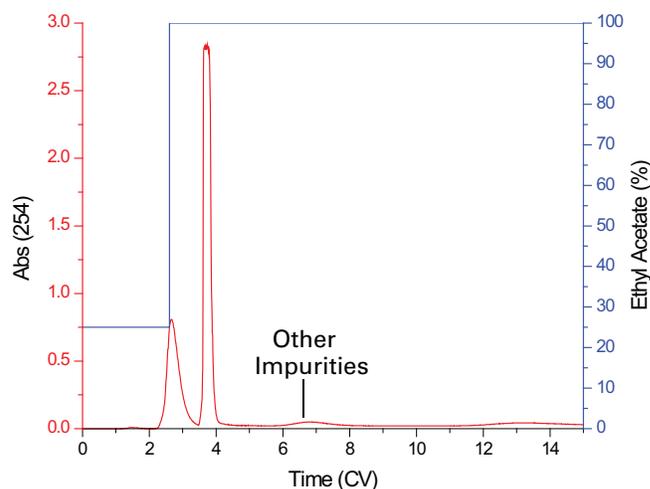

Figure 4: Optimized step gradient

These optimizations are best done in the context of repeated, large, purifications such as those found in a production setting.

Shallow Linear Gradient

Users commonly run a TLC and set the gradient to center around the solvent concentration as run in *Figure 5*.
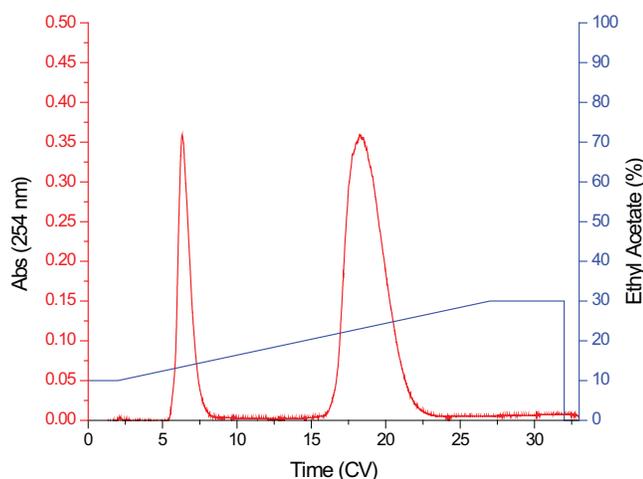
Figure 5: Purification of reaction products using a shallow gradient

This gradient takes longer than using an isocratic elution at the solvent concentration used by the TLC. Compared to the run in *Figure 2*, the second peak takes longer to elute, and is collected in almost 50% more solvent, increasing the evaporation time by that amount.

Default Linear Gradient

The Default Linear Gradient provided by Combi*Flash* systems provides adequate resolution for most compounds. This gradient is useful for purifying natural products where the desired compound is not known. Medicinal chemists often use the default gradient to capture the minor compounds in addition to the desired reaction product.
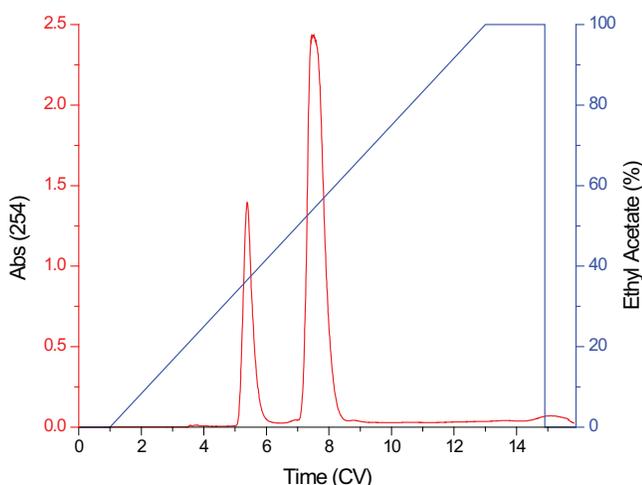

Figure 6: Reaction product eluted with standard Combi*Flash* gradient

For this gradient, the purification is complete before 9 column volumes. The first peak was collected in 125 mL solvent and the second was collected in 175 mL

solvent. For this reaction mixture, these times compare favorably to the step gradient.

Gradient Optimizer

The Gradient Optimizer is a feature in Peak*Trak* that generates a gradient containing an isocratic hold. This method is useful when purifying compounds that elute closely, while minimizing the time required purifying the mixture. The gradient also allows all compounds to elute from the column. The Gradient Optimizer used two TLC runs to generate data to create the method. Compounds that are well resolved will be run with the standard gradient programmed into Peak*Trak*.

For this experiment, a mixture of catechol and resorcinol was used because the reaction mixture used for the other experiments had sufficient resolution between the major compounds that the Peak*Trak* Gradient Optimizer used the default gradient.

Catechol and resorcinol (50 g each) were dissolved in ethyl acetate and mixed with 400 g silica; sufficient ethyl acetate was added to make a slurry. The solvent was evaporated to make a dry, free-flowing powder. The mixture was allowed to "age" so that the purification was similar to that of an actual reaction.

A mixture of catechol and resorcinol was run on TLC plates at 40% and 60% ethyl acetate in hexanes. These concentrations were chosen to elute the compounds of interest between R_f values of 0.2 and 0.8 as per the instructions on the Gradient Optimizer software. The column was loaded with 14 g mixture (2.8g sample, 3.5% sample load).

Figure 7: TLC plates used for Gradient Optimizer data

An 80g silica column was loaded onto the Combi*Flash* system to load the basic parameters for the column such as flow rate. The TLC data was entered into

the Gradient Optimizer (data for the first two eluting spots) and Peak*Trak* altered the method to allow the best purification for this method. The resulting chromatogram is shown in *Figure 8*.
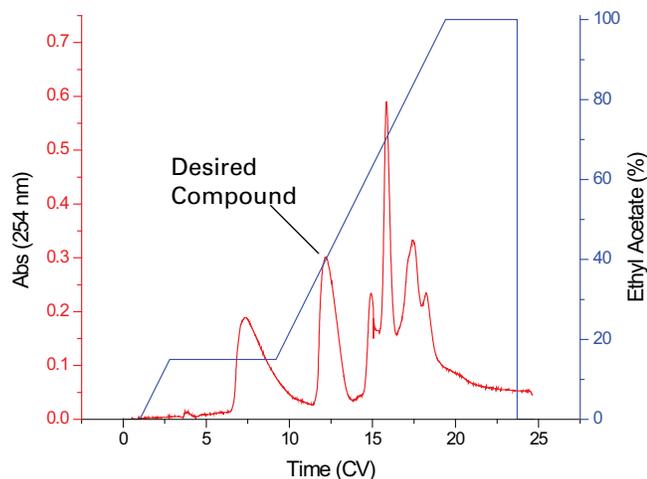

Figure 8: Gradient Optimizer used to purify a complex mixture
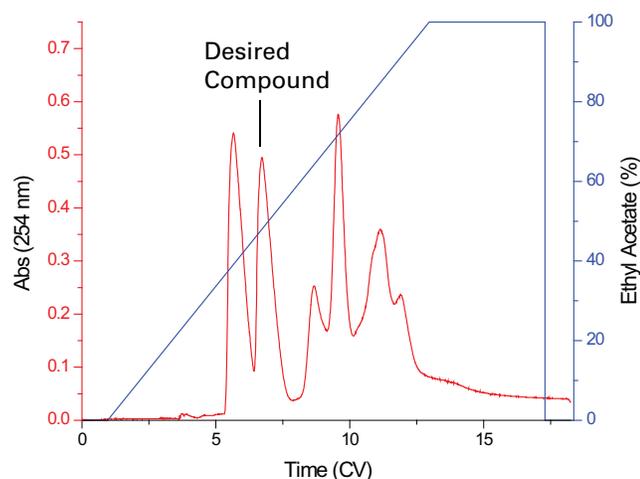

Figure 9: Complex mixture purified with default gradient

The Gradient Optimizer allowed better resolution of the desired compound and minimized run time.

Dry Column Vacuum Chromatography

Dry Column Vacuum Chromatography[2] is a technique considered, by some, to be superior to flash chromatography due to reduced diffusion. A typical experiment is to add ~1 column volume of solvent in increments of 5% or 10% B, drying the column between additions of solvent. The column drying is traditionally done with vacuum. Our modification used the air purge

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2. Pedersen, D.S.; Rosenbohm, C. *Synthesis* 2001, No. 16, 2431-2434

on a Combi*Flash* system to blow the solvent out of the column.

All experiments below used a 40g Redi*Sep* silica column (PN 69-2203-340). The same sample (mixture of acetophenone, methyl paraben, and 4-aminobenzoic acid adsorbed on silica) was run for all experiments. Dry Column Vacuum Chromatography was compared to a run with the standard gradient used in Peak*Trak*, a stepped gradient approximating the steps for a Dry Vacuum Column Chromatography, and a Linear Gradient over the same time. For the Dry Vacuum Column Chromatography experiment, software was written to control the pumps and the air purge through the column.
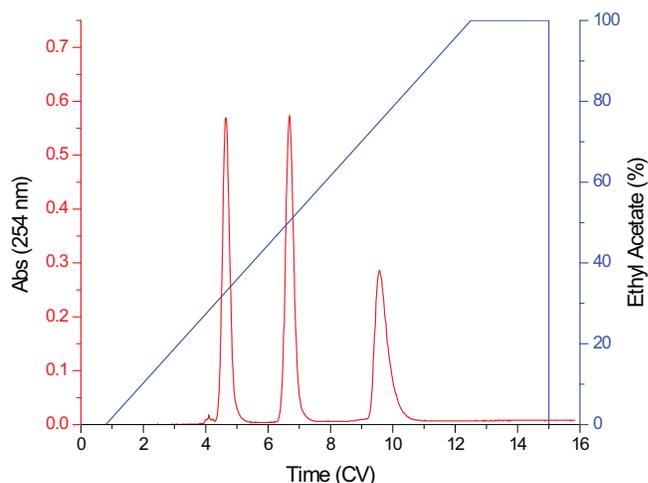

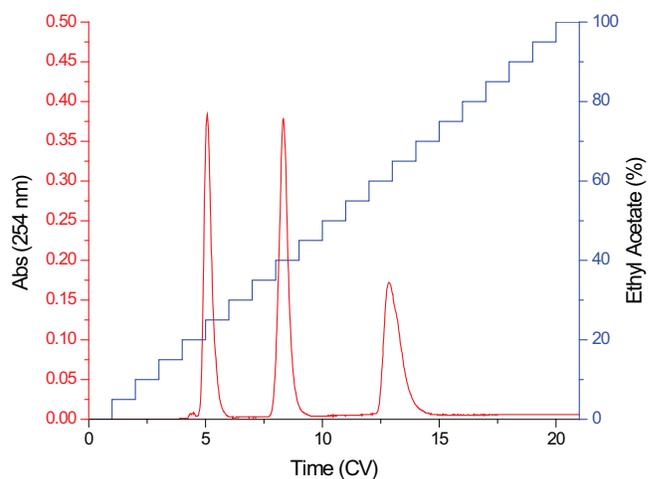**Figure 10: Standard Peak*Trak* gradient**

Figure 11: Step Gradient approximating those for a Dry Vacuum Column Chromatography

The peaks in *Figure 11* are slightly wider, due to the gradient being longer. The peaks also show slightly later

elution for the same reason. This is confirmed by the linear gradient over the same time range in *Figure 12*.
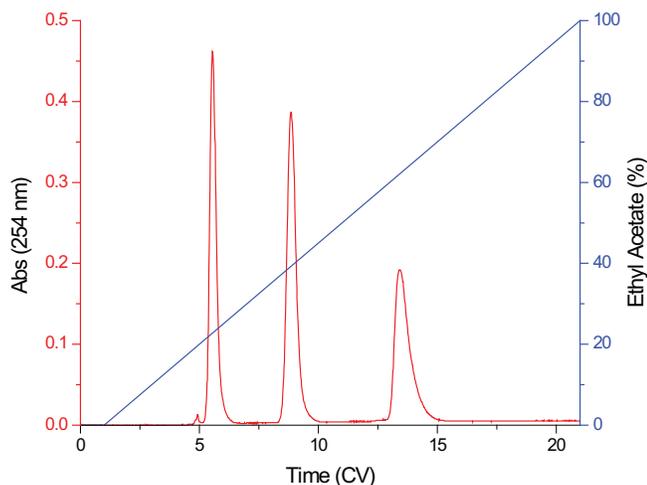

Figure 12: Linear Gradient over similar time range as Dry Vacuum Column Chromatography

The Dry Column Vacuum Chromatography experiment required slightly longer than the others to elute all three peaks due to the time required to dry the column between steps. Peak widths are similar to those of the other runs, with the exception of the standard gradient which still shows narrower peaks.
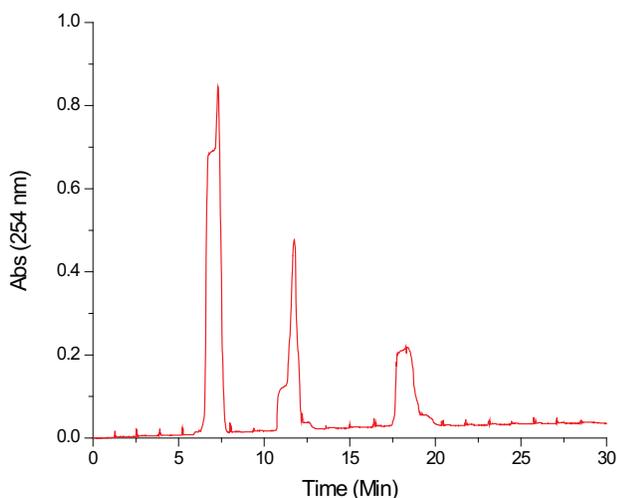

Figure 13: Dry Column Vacuum Chromatography sample run

Table 1 summarizes Dry Column Vacuum Chromatography; this experiment requires longer to run because of

the size of the steps and the air purge between each step.

Table 1: Comparisons of various gradients to Dry Column Vacuum Chromatography (DCVC)

Method	Run Time	A Solvent Used	B Solvent Used	Total Solvent Used
Standard	25	400	400	800
5% Step Gradient	30	500	500	1000
Extended Linear Gradient	30	500	500	1000
DCVC	30	500	500	1000

Conclusions

Table 2: Comparison of run times and collection volumes of various methods used to purify a reaction mixture

Gradient Method	Peak 1 Collected Volume (mL)	Peak 2 Collected Volume (mL)	Elution Time (end of Peak 2, Column Volumes)
Isocratic	125	500	12
Step Gradient (optimized)	125	75	4.5
Shallow Gradient	300	725	23
Standard Gradient	125	175	9

For compounds with good resolution, the optimized Step Gradient results in the fastest purification time and smallest collection volume. However, this required two runs to fully optimize the method making this method more suitable for repeated purifications of the same compounds. The next most efficient purification used the standard gradient programmed into Peak*Trak*. The standard gradient is useful for most research purposes. The Gradient Optimizer is best used for compounds that show reduced resolution on TLC plates. In all cases, TLC plates were used as a starting point to determine the correct method.

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