Fourth Edition

Effective Organic Compound Purification

Guidelines & Tactics for Flash Chromatography

TELEDYNE ISCO
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Effective Organic Compound Purification:
Guidelines and Tactics for Flash Chromatography
Fourth Edition

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Teledyne Isco, Inc.
P.O. Box 82531
Lincoln, NE 68501-2531 USA

Worldwide Web: www.isco.com
E-mail: IscoInfo@teledyne.com
Telephone: (800) 228-4373
(402) 464-0231
Facsimile: (402) 465-3022

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Introduction to Flash Chromatography

Chromatographic Purification in Organic Chemistry

During the course of developing a chemical reaction to produce a desired product, the synthetic organic chemist typically goes through the repeated sequence of reaction set-up, work-up, purification, and final product analysis.

When the chemist reaches the purification step, there are several purification techniques to choose from, including crystallization, filtration, distillation, and column chromatography.

Traditional column chromatography applies a crude reaction mixture on top of a bed of silica gel loaded in a glass column. A gravity-fed solvent mixture (*mobile phase*) passes through the vertical column of silica gel (*stationary phase*), separating the individual products of the crude reaction mixture.

The separation of the compounds in the mixture is based on their different affinity for the mobile and stationary phases, which causes the compounds to migrate through the column at different rates and emerge from the bottom of the column at different times. The stationary phase and mobile phase are chosen to achieve the best possible separation of components, based on the nature of the sample mixture.

The separated products are collected in test tubes positioned below the column outlet. Then, identical fractions are gathered and concentrated.

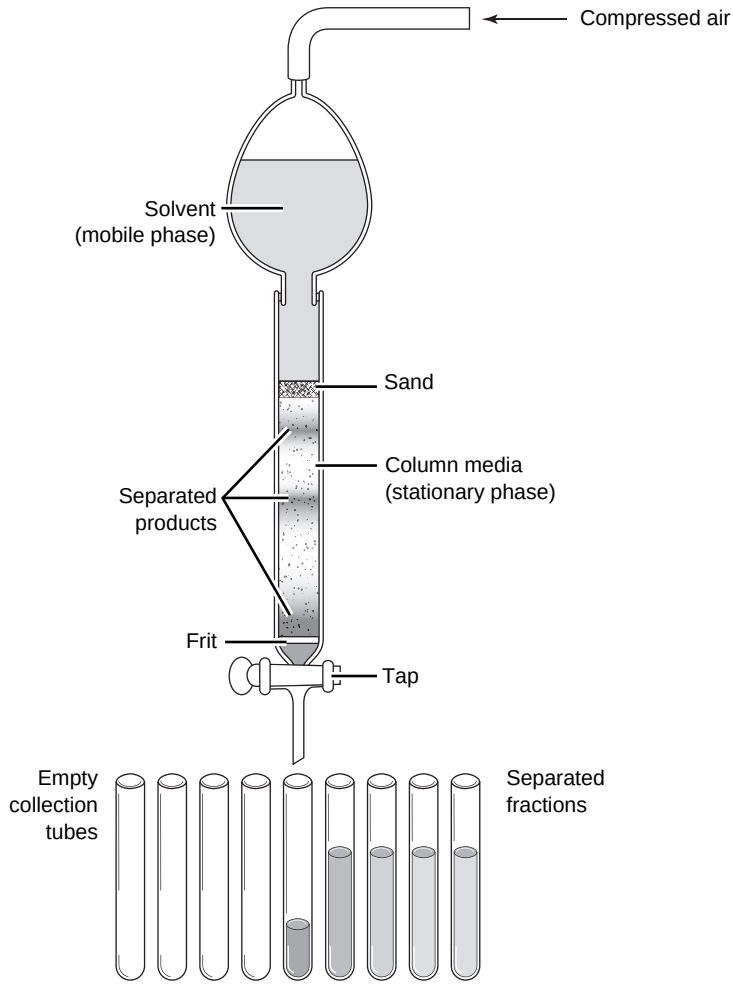


Figure 1: Illustration of basic elements in a traditional Flash column chromatography apparatus

The term *Flash chromatography* was coined in 1978 by W. Clark Still and coworkers at Columbia University to describe separations in which a gas-pressurized solvent reservoir is used to accelerate solvent flow and achieve superior chemical separations in less time than traditional gravity-based column chromatography.

Today, Flash chromatography is a totally automated preparative technique thanks in part to the Combi*Flash* equipment designed by Teledyne Isco. The advantages of using automated Flash chromatography are many. It's easy, fast, inexpensive, requires minimal development time, and has high resolution.

Flash chromatography is currently one of the most popular techniques for purifying pharmaceutical intermediates, as well as final organic products. It is also widely used in natural products research.

Although silica gel was the media first employed in Flash chromatography, the introduction of automated systems by Teledyne Isco has extended the technique to include other media such as reversed phase C18 and other bonded phases, alumina, and ion exchange resins. This has greatly expanded the application base of Flash chromatography.

Chapter 2

Flash Chromatography Essentials

Flash chromatography is an easy and simple purification technique that requires minimal method development. Even though there are only a few factors to consider when preparing for a Flash chromatography purification, they all need to be selected thoughtfully in order to achieve a successful separation. Mobile phase, stationary phase, type of gradient elution, column loading capacity, and sample loading technique are some of these factors. The following paragraphs will describe in detail their influence on the final result and how they ought to be approached and selected.

Compound Solubility

The solubility of the crude products mixture to be separated is a factor the organic chemist should consider when choosing the solvent system mixture, or mobile phase.

A mobile phase with low polarity properties may precipitate oily crude mixture products in the flask during dissolution prior to loading the sample on the column, or after being loaded on top of the column when the low polarity solvent mixture progression starts.

To avoid having the sample precipitate unintentionally (or *crash*), it is important to choose a solvent system polar enough to cover both the solubility issue upon sample loading on column and the maximized separation conditions obtained from thin-layer chromatography (see *Using TLC to Predict Separation*, on page 8).

Should the sample precipitate in the flask prior to column loading or be in an initial solid state, the solid loading technique is recommended (see *Solid sample loading*, on page 28).

In the event the sample precipitates after being loaded onto the column, increasing the polarity of the solvent system through gradient solvent elution (see *Gradient Elution*, on page 17) would eventually reach a solvent system mixture polar enough to solubilize it. However, precipitated samples often raise the system pressure thereby reducing the solvent flow. Higher pressure Flash systems, such as the Combi*Flash* Rf with 200 psi capability, are better able to push the solvent through making it easier to increase the polarity. Once solubilized, the sample moves through the stationary phase.

Mobile Phase

The solvent system or mobile phase choice for Flash chromatography is dependent on the polarity of the product(s) to be isolated and the type of stationary phase to be used.

Typically, the organic chemist will first proceed with a few TLC analytical trials to determine which solvent system will provide the optimal separation conditions with respect to the polarity of the desired product(s) and the selected stationary phase.

The retention distance, R_f, on a TLC plate represents the distance a given compound migrates from the origin with respect to the solvent front on the plate. (See *Method Development Using TLC*, on page 10.)

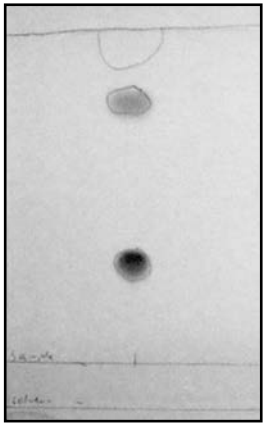


Figure 2: Photo of TLC plate *Annotations include baseline, sample starting point, separated compounds, and final solvent front.*

Figure 3: *Table of common solvents and their characteristics in liquid chromatography* (by increasing polarity)

SOLVENT	POLARITY	VISCOSITY (cp 20°)	BOILING POINT (°C)	UV CUTOFF (nm)
Hexane	0.06	0.33	69	210
n-Heptane	0.20	0.41	98	200
Toluene	2.40	0.59	111	285
Methylene chloride (DCM)	3.40	0.44	40	245
Tetrahydrofuran	4.20	0.55	66	220
Ethanol	4.30	1.20	79	210
Ethyl acetate	4.30	0.45	77	260
i-Propanol	4.30	2.37	82	210
Acetonitrile	6.20	0.37	82	210
Methanol	6.60	0.60	65	210
Water	10.20	1.00	100	—

During the TLC analytical trials, the chemist will seek the solvent system that moves the desired product to $R_f = 0.25 \pm 0.05$ and keeps other undesired products to a distance of at least $\Delta R_f = 0.2$. These TLC parameters constitute the ideal Flash chromatography conditions because of high compound-stationary phase contact time predisposing to high compound resolution during the column separation.

Many organic solvents are available. Figure 3 lists commonly used solvents. Figure 131 on page 135 lists additional solvents that may be more suitable for specialized purifications.

The solvent system strength and selectivity refer respectively to the solvent system's ability to migrate all compounds simultaneously on the column (*i.e.* purification duration) and to migrate one specific compound differently from the others (*i.e.* separation resolution).

Typically, the solvent system is a binary mixture of a higher and a lower strength (polarity) solvent. For instance, organic chemists commonly initiate their solvent system evaluation and selection

with hexane/ethyl acetate 1:1 and/or dichloromethane/methanol 95:5 for a normal phase silica gel stationary phase. The different strength and selectivities of these two solvent mixtures provide information useful in identifying an appropriate solvent system for purification of the reaction mixture.

The mobile phase selection is a function of the stationary phase chosen for the purification. Normal and reversed-phase silica gels are the most common stationary phases used by organic chemists. Typically, the solvent system selected for a normal phase silica gel will have lower protic properties (*e.g.* hexane/ethyl acetate, hexane/ether, or dichloromethane/methanol), whereas reversed-phase silica gel will have higher protic properties (*e.g.* water/acetonitrile, water/isopropanol).

Mobile Phase Modifiers

Acidic and basic organic compounds interact with residual surface silanol groups on a chromatographic support and cause peak tailing. The addition of a *mobile phase modifier* (typically one percent or less concentration) reduces peak tailing and sharpens peaks, improving the resolution in separations of basic or acidic compounds.

Triethylamine, ammonium hydroxide, acetic acid, and trifluoroacetic acid are common mobile phase modifiers.

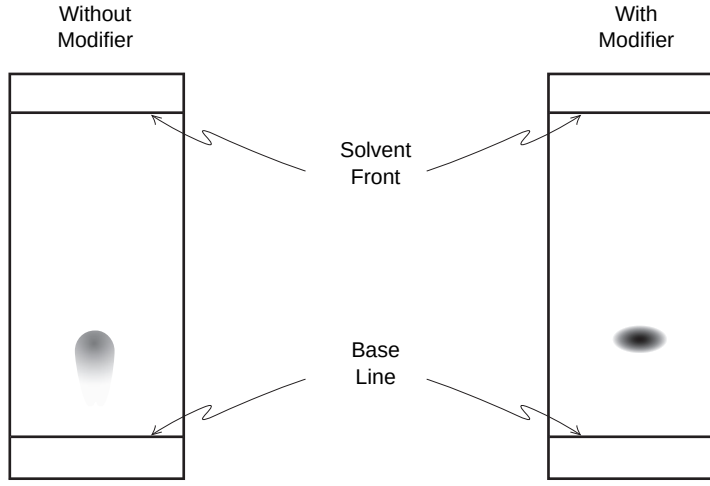


Figure 4: Illustration of mobile phase modifier reducing peak tailing on TLC plates

Stationary Phase

Stationary phase selection is driven by the nature of the products to be separated. Factors such as compound polarity and functionalities greatly influence the media selection.

The majority of reaction products organic chemists need to isolate can be purified using a normal-phase or a reversed-phase silica gel as the stationary phase.

For some specific types of compounds, however, it is difficult to achieve an overall satisfactory degree of separation using these common Flash chromatography stationary phases. The silica gel suppliers have designed and marketed functionalized silica gel to provide chemists additional purification media options. Thus, organic chemists now have a wide range of purification tools available, which facilitates isolation of compounds with very different physico-chemical properties.

Appendix A of this guide provides a stationary phase selection guide and more information on media types.

Using TLC to Predict Separation

Thin-layer chromatography (TLC) is a simple and practical chromatography technique organic chemists use to monitor the evolution of chemical reactions. TLC is also used to optimize Flash chromatography conditions for purification of crude reaction mixtures.

Correlating TLC and Flash

The strength with which a compound binds to the stationary phase is called *retention*. Provided that the stationary phase is identical, a correlation can be made between compound retention in TLC and Flash chromatography.

Retention Factor and Column Volumes

Retention (R_f) of a compound in TLC is measured by the distance it moves relative to the naturally moving solvent front. This differs from Flash chromatography, in which the solvent is pumped through the stationary phase. Instead of relative distances, retention in Flash chromatography is generally defined in term of the volume of solvent necessary to move the components through the column. This volume, expressed in *column volumes* (CV), is the

amount of solvent the column can hold in the interstitial space between the media particles.

Although the measures of retention differ, methods developed using TLC are generally transferable to Flash chromatography because of the relationship between R_f and CV:

$$R_f = \frac{1}{CV}$$

Figure 5 illustrates this relationship between R_f and CV. A compound with low retention that moves easily through TLC, (*e.g.* R_f=0.80), can be expected to elute quickly (1.25 CV). Conversely, a highly retentive compound (*e.g.* R_f=0.10), binds more strongly to the stationary phase media and can be expected to elute much later (10.0 CV).

Figure 5: Table of Rf to CV conversions

R_f	CV
0.90	1.10
0.85	1.17
0.80	1.25
0.75	1.33
0.70	1.40
0.65	1.54
0.60	1.65
0.55	1.81
0.50	2.00
0.45	2.22
0.40	2.50
0.35	2.86
0.30	3.33
0.25	4.00
0.20	5.00
0.15	6.67
0.10	10.00

Method Development Using TLC

Since CV is the measure of compound retention, then ΔCV is the measure of compound resolution, or the degree to which the desired product can be isolated from other components in the mixture.

Chemists perform multiple analytical TLCs to attempt to identify a solvent system that migrates the desired product spot to $R_f = 0.25 \pm 0.05$ (optimal retention), while migrating all other spots as far as possible from the desired product (optimal selectivity).

The following figures illustrate this process. Figure 6 shows progressive attempts to optimize a solvent system to move the desired compound to optimal retention conditions.

Figure 7 shows the sequential solvent selection attempts to reach optimal selectivity for a given mixture.

After identifying a solvent system that performs well under the system conditions, maximum ΔCV for Flash chromatography is achieved, reflected in the column volume chromatogram of Figure 8.

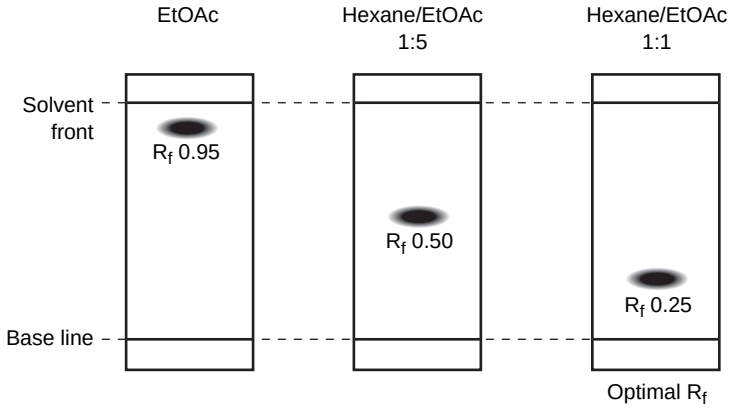


Figure 6: Illustration of solvent strength optimization

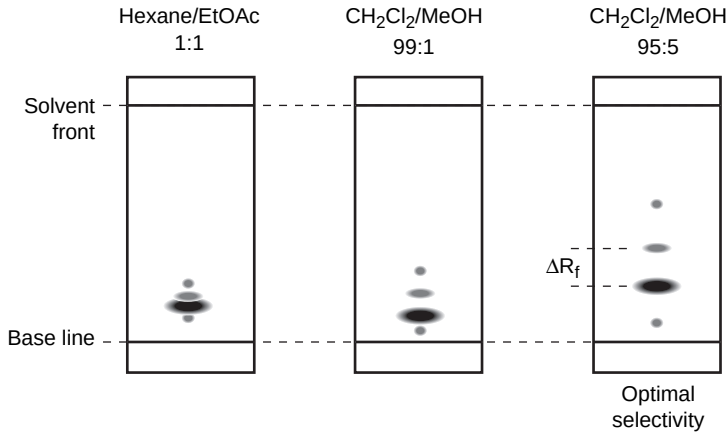


Figure 7: Illustration of solvent system selectivity optimization

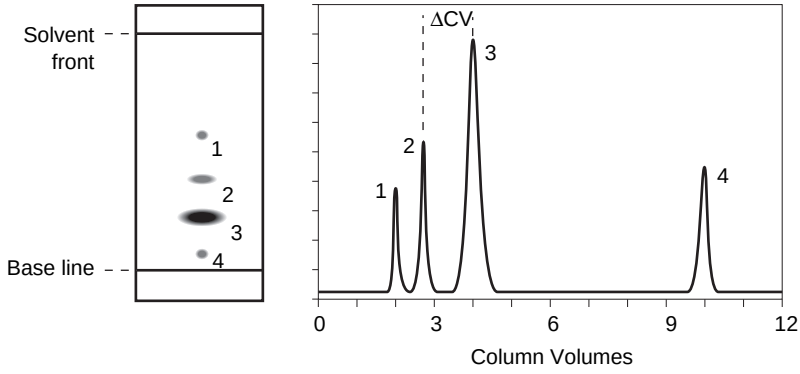


Figure 8: Illustration of a solvent system optimized for compound selectivity and its reflection on the column volume chromatogram

The selectivity obtained will determine the sample loading capacity on the column. The lower the retention time R_f and the higher the selectivity ΔR_f between product spots on the TLC plate, the higher the amount of sample can be loaded.

Figure 9: Table of suggested loading of RediSep Rf silica gel columns based on Rf differences from TLC plates.
The 125 g column is designed for high loads of easily separated compounds.

	Loading			
	Light Loading	**Moderate**	**Significant**	**Heavy**
Column size (g silica)	**ΔRf < 0.2**	**0.2 – 0.4**	**0.4 – 0.6**	**> 0.6**
4 g (69-2203-304)	0.0004 – 0.004	0.004 – 0.16	0.16 – 0.28	0.28 – 0.4
12 g (69-2203-312)	0.0012 – 0.012	0.012 – 0.48	0.48 – 0.84	0.84 – 1.2
24 g (69-2203-324)	0.0024 – 0.024	0.024 – 0.96	0.96 – 1.68	1.68 – 2.4
40 g (69-2203-340)	0.004 – 0.04	0.04 – 1.6	1.6 – 2.8	2.8 – 4
80 g (60-2203-380)	0.008 – 0.08	0.08 – 3.2	3.2 – 5.6	5.6 – 8
120 g (69-2203-320)	0.012 – 0.12	0.12 – 4.8	4.8 – 8.4	8.4 – 12
125 g (69-2203-314)	—	—	5 – 8.75	8.75 – 12.5
220 g (69-2203-422)	0.022 – 0.22	0.22 – 8.8	8.8 – 15.4	15.4 – 22
330 g (69-2203-330)	0.033 – 0.33	0.33 – 13.2	13.2 – 23.1	23.1 – 33
750 g (69-2203-275)	0.075 – 0.75	0.75 – 30	30 – 52.5	52.5 – 75
1500 g (69-2203-277)	0.15 – 1.5	1.5 – 60	60 – 105	105 – 150

Typically, a crude reaction mixture amount corresponding to 1–10% weight of the normal phase silica gel quantity will be loaded on the column for low selectivity conditions. An amount up to 10% weight of the normal phase silica gel quantity will be loaded on the column with high selectivity conditions.

To summarize, when developing a method for Flash chromatography purification with TLC plates, it is recommended to:

- Use identical stationary phase for related TLC experiments and subsequent column runs since the sorbent quality varies from one manufacturer to another.
- Choose a solvent system that moves the desired product to $R_f = 0.25 \pm 0.05$ and keeps other products in the mixture at a distance of at least $\Delta R_f = 0.2$.

TLC and Mobile Phase Techniques

Because TLC separations closely mimic the behavior of compounds in a silica gel column and mobile phase combination, chemists have come to rely upon TLC to scout for optimal separation conditions.

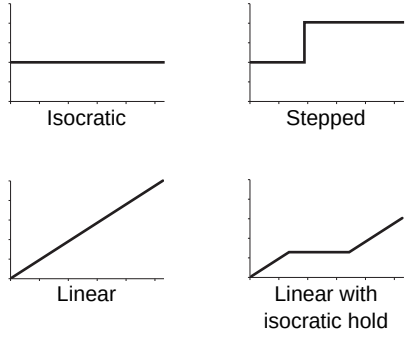


Figure 10: *Illustration of mobile phase techniques* *plotted as solvent strength (Y-axis) over time or column volumes (X-axis)*

The separation conditions found while scouting with TLC easily translate to columns if separations are *isocratic*. Similarly, a chemist can perform a series of TLCs to determine the ideal mobile phase concentrations and translate the conditions to a *stepped gradient* separation on a column.

When using *linear gradient* Flash chromatography to purify organic compounds, TLC data is less useful because the TLC mobile phase cannot be dynamically varied.

Given this limitation, TLC is still a practical starting point for developing effective separation methods using a linear gradient mobile phase. TLC verifies that the selected solvent system has the appropriate solvent strength, and that the selected stationary phase will separate the compounds while ensuring that the compound of interest will not be permanently retained.

What linear gradient Flash chromatography does is provide the ideal solvent blend for the separation. This is because the gradient solvent systems changes infinitesimally from one extreme to another—at some point the ideal solvent blend is provided for purification. Testing one point or even several using TLC does little to help the chemist empirically determine the ideal solvent blend and gradient curve. The need for analytical TLC prior to purification is greatly reduced.

Isocratic Elution

Most classical Flash chromatography uses an isocratic mobile phase to separate compounds. In an isocratic separation, the mobile phase may be a single solvent or a mixture, but the mobile phase composition is the same throughout the separation.

TLC is an isocratic technique. Therefore, it can closely correlate to isocratic separations scaled up to column chromatography.

An isocratic mobile phase can be optimized to purify nearly any compound of interest. To ensure the separation is selective, the chemist must control the isocratic conditions beyond just the right solvent blend. Sample loading and column capacity also must be closely controlled. But in the end, these efforts yield a specialized method that will not separate a wide variety of compounds.

Column capacity is typically limited when using isocratic mobile phases. If the sample size is increased too much, the mixture's compounds will contaminate each other.

Figures 11 through 15 illustrate tests performed to optimize an isocratic mobile phase. In this example, *Sample A*, a blend of acetophenone (1), methyl paraben (2), and 4-aminobenzoic acid (3) is separated using 20, 30, 40, 50, and 70% EtOAc and Hexane.

When reviewing the results of this TLC series, we learn that for the purification of acetophenone 20% EtOAc is best. For methyl paraben 20 to 30% EtOAc is best, and for the purification of 4-aminobenzoic acid, 40 to 50% of EtOAc is best.

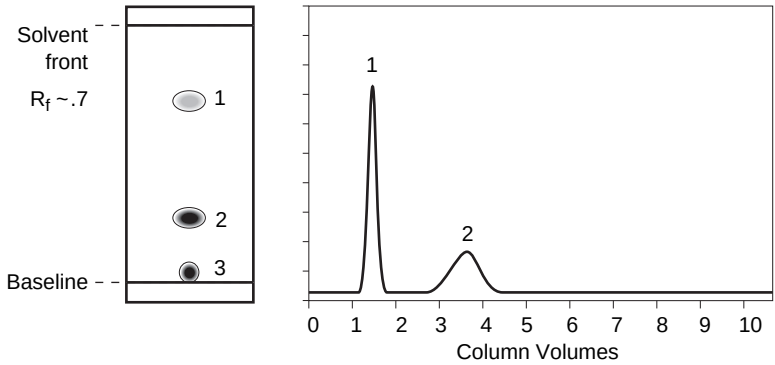


Figure 11: Illustration of isocratic 20% EtOAc in hexane
4-aminobenzoic acid does not move from the TLC baseline, nor does it come off the column.

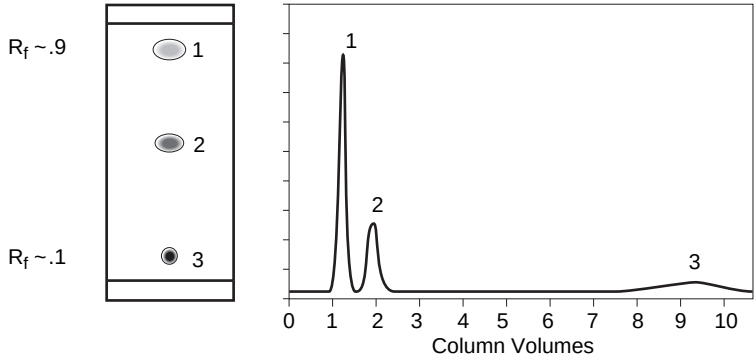


Figure 12: Illustration of isocratic 30% EtOAc in hexane
4-aminobenzoic acid has low R_f. Peak 3 is very spread out.

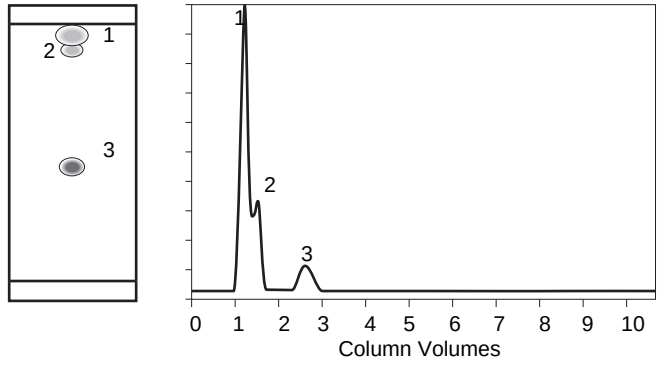


Figure 13: Illustration of isocratic 40% EtOAc in hexane
Acetophenone and methyl paraben are no longer pure. 4-aminobenzoic acid is pure.

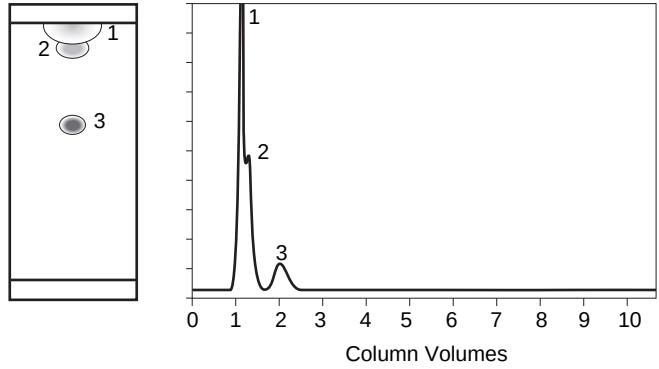


Figure 14: Illustration of isocratic 50% EtOAc in hexane
4-aminobenzoic acid remains pure. It comes off the column sooner while maintaining sufficient resolution.

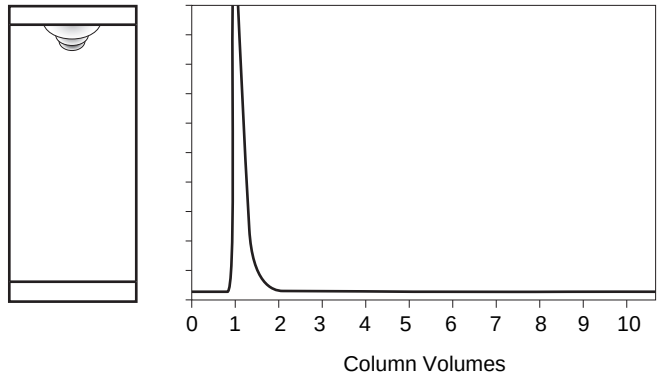


Figure 15: *Illustration of isocratic 70% EtOAc in hexane*
Nothing is resolved at 70%.

Gradient Elution

Gradient elution describes techniques for decreasing overall purification time, increasing resolution, and increasing column capacity by varying the mobile phase composition during the chromatographic separation. Gradient methods include both stepwise and continuous changes in the solvent blend, with linear or straight-line gradients being the most common form of continuous gradients. A *binary gradient* is one in which the ratio of two solvents (or solvent mixtures) is varied during the separation. *Ternary* (3-solvent) and *quaternary* (4-solvent) gradients are also used in some cases.

It is extremely powerful to have fully programmable control over the mobile phase components during the course of a separation. This capability allows you to tailor the resolving power for a particular set of species that need to be separated on a chromatographic column.

Until the recent development of automated Flash chromatography systems, the power of programmable gradients was not readily available to organic chemists. Gradients are a means of controlling resolution. By adroit use of gradients, closely eluting compounds may be separated while compounds with long retention times (they may be thought of having highly excess resolution) can be run with reduced time and solvent.

When gradients are applied to Flash purification of small organic molecules there are several key benefits that a chemist can take advantage of:

- shorter elution times
- less dependence on pre-determining optimal separation conditions
- higher purity
- fewer fractions to deal with
- greater sample loading capacity
- greater repeatability

The benefits of gradient chromatography are apparent when compared to isocratic and stepped solvent systems, and how TLC relates to each method.

Stepped Gradient

Stepped gradients are a classical technique used in Flash chromatography. The solvent system is a blend of solvents. Several different blends are prepared at increasingly polar solvent strengths.

In the same way an optimal solvent is chosen for an isocratic separation, optimal solvent blends for stepped gradients are identified through TLC trials. The goal of the TLC trials is to determine a blend that moves the compound of interest.

These blends are introduced onto the Flash column in turn. The solvent strength is increased only after the previous compound has separated, greatly improving selectivity. As a result, column capacity can be increased.

Referring back to our example separating Sample A, Figure 16 illustrates a stepped gradient developed from the analytical TLC trials. A stepped gradient starting at 20% EtOAc and moving to 40% after 4 column volumes will allow the separation of the three compounds in a single run.

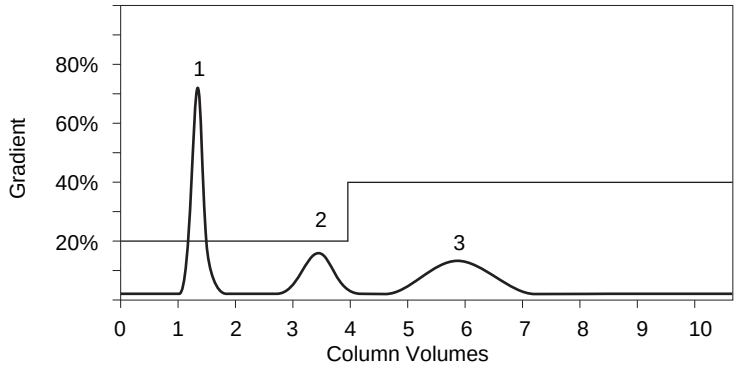


Figure 16: Illustration of a stepped gradient and chromatogram

Linear Gradient

Linear gradients begin with a low-strength solvent blend. The gradient is advanced in infinitesimal steps (limited by the resolution of the solvent pumping system) until the separation ends at a high-strength solvent blend.

With isocratic and stepped gradients, it is very important that a sufficient number of TLCs be performed to determine just the right solvent blend. In the case of linear gradients, this requirement is reduced because the gradient that is best for separation of the compounds is by default provided to the column. This is because at one point along the gradient profile, the best solvent blend for separation of the compounds is delivered to the column.

To continue the example, TLC determined that the ideal concentration of the solvents is between 20 and 40%. To determine this with confidence it required that 20, 30, 40, 50, and possibly 70% blends be prepared and evaluated for separation effectiveness. This is because it is difficult to know at the outset what concentrations will bracket ideal conditions.

However, when a linear gradient is used, since it starts at a concentration that is lower than the optimal and increases to a concentration that exceeds the optimal level, it is not necessary to perform as many TLCs.

Because of this fact, a chemist has only to perform sufficient TLC work to determine that the solvent system and stationary phase combination that is to be applied to the separation will separate the desired compound from the contaminates.

Figure 17 illustrates a linear gradient used to separate the same mixture, Sample A, used in the previous examples. Note that because the 20 and 40% blends are part of the linear gradient profile, it is not necessary to determine the ideal conditions before you begin the separation.

Optimizing resolution of a column is a function of gradient slope and column configuration.

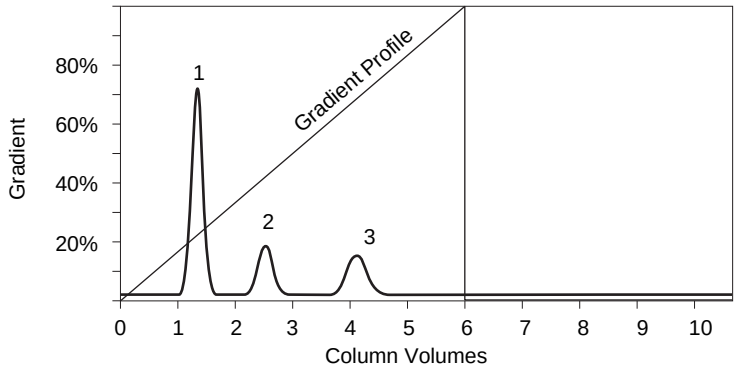


Figure 17: Illustration of a linear gradient and chromatogram

Several adjustments can be made to the slope of the gradient. These include changing the starting and finishing solvent concentrations, and the duration of the separation. Complex gradient curves can also be created. The gradient curve may include points that hold the solvent at a fixed concentration to prevent contamination by a closely eluting compound, or add points to sharply increase the concentration to move highly-retentive compounds.

The slope of the gradient can greatly affect the resolving power of the media/solvent combination.

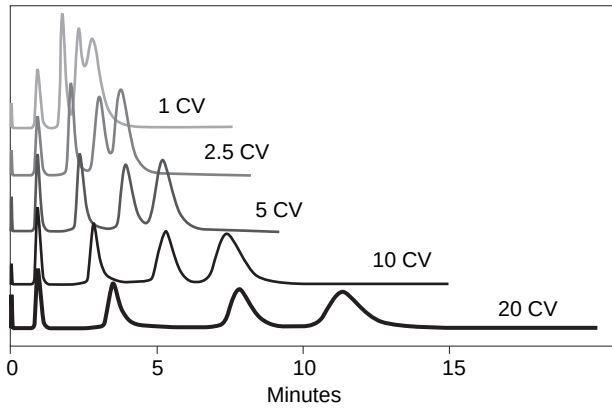


Figure 18: Chromatograms resulting from various gradient slopes *The duration of the gradient can be manipulated to optimize the purity required while minimizing the time needed to complete the procedure.*

By decreasing the slope of the gradient, the separation of the peaks and the broadness of the peaks increase. The trick is to find the ideal gradient slope so that a compromise is struck between purity and time to get the compound off the column. This is most often done by trial and error. A good starting point is to begin with a gradient that extends over ten column volumes.

Mixed Gradients

Mixed gradients are a combination of step and linear gradients. These gradients are used to reduce run time while maintaining a separation between closely eluting components. A linear gradient is started and an isocratic hold is employed during the gradient to maintain the resolution between closely eluting compounds.

Figure 19 shows catechol and resorcinol purified under isocratic conditions and a linear gradient.

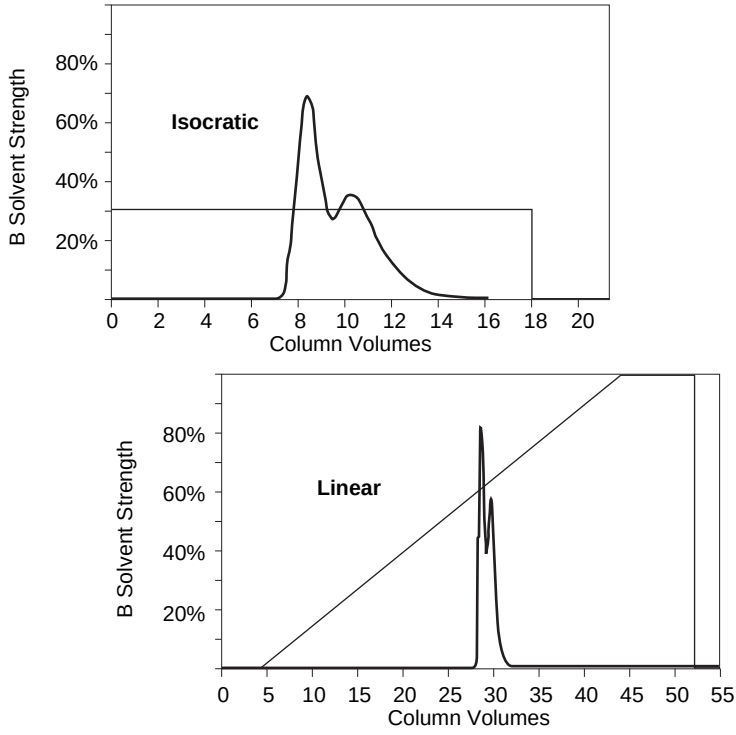


Figure 19: Chromatograms of catechol and resorcinol separations *using isocratic and linear mobile phases*

Under isocratic conditions, the peaks are broad and run together. The linear gradient, while sharpening the peaks, also causes overlap. Reducing the slope of the gradient would separate the peaks but they would also be broadened so there is still overlap between the peaks. Combining a linear gradient with an isocratic hold generates the chromatogram in Figure 20 where nearly complete resolution is achieved between the two diols.

PeakTrak® software on Combi*Flash* systems makes it very easy to create these gradients with just two TLC plates. The retention factors of the compound of interest and the closest impurity are entered into the PeakTrak's Gradient Optimizer window which then calculates the optimal combination of linear gradient and isocratic hold prior to elution of the compounds to give the best separation.

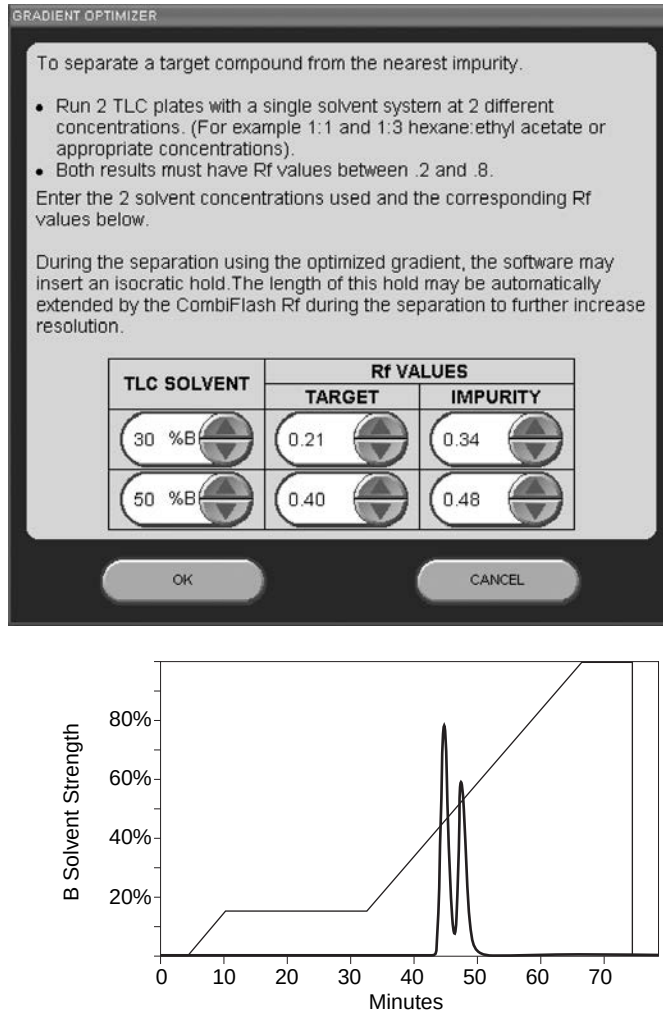


Figure 20: Illustration of CombiFlash Rf Gradient Optimizer and resulting chromatogram *showing nearly complete resolution of catechol and resorcinol.*

Loading Capacity of Column

Loading capacity of a column is dependent on a number of variables. Classical Flash chromatography techniques instruct that for every 1 gram of compound to be purified, 100 grams of silica gel (1% load) are required. With the addition of gradient chromatography and on-line UV-Vis detection, this loading capacity is increased to 1 gram of compound requiring as little as 10 grams of silica (10% load) to purify.

If we refer to the chromatogram presented in Figure 21 you will note that in this isocratic separation at 30% EtOAc the distance between the first two peaks is quite small. An increase in the concentration of either the first or second peak will cause overlap to occur, resulting in loss of purity of both peaks (Figure 22).

In the case where a gradient is used, the capacity of the column is increased because the separation of the peaks is greater and the sharpness of the peaks is greater (Figure 23).

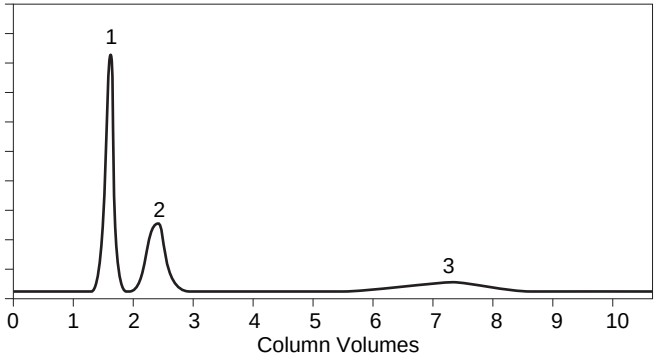


Figure 21: Chromatogram indicating column loading capacity near limit *At an isocratic mobile phase of 30% EtOAc, there is little distance between peaks 1 and 2.*

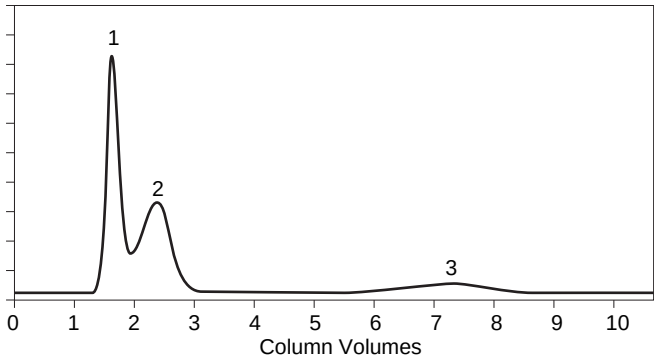


Figure 22: Chromatogram indicating column loading capacity exceeded *Increasing the concentration of methyl paraben (peak 2) causes peaks to overlap under the same conditions.*

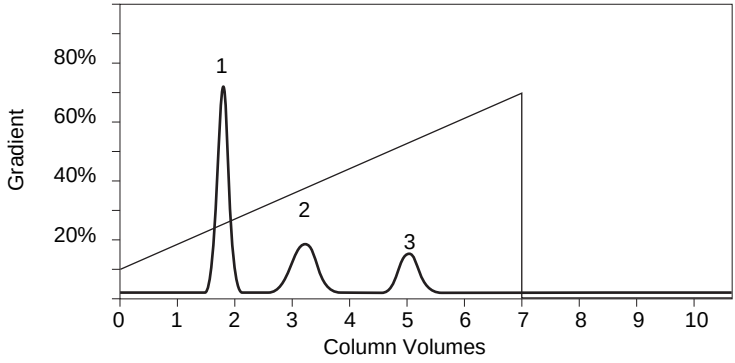


Figure 23: Illustration of gradient mobile phase *The gradient slope can be adjusted to improve selectivity and column loading capacity.*

Column Length Versus Resolution and Purity

Column length is another factor that determines resolution and purity. A longer column increases the distance that each compound must travel through the stationary phase. Given the different rates at which the compounds travel, the resolution increases relative to the increase in distance. However, the separation time and back pressure increase along with resolution and purity. Column stacking (see page 44) is a way to achieve longer column lengths.

Flash Column Packings

Flash chromatography columns typically utilize hard, silica based packing. This silica may be bare or bonded with various functional groups to offer differing binding characteristics for separation of different compounds. The silica particle is also available in different shapes, sizes and porosities which offer unique separation characteristics.

Particle Shape

Silica is manufactured in either irregular or spherical particles (Figure 24).

Irregular silica is produced as a large block of amorphous silica which is ground and then sieved to produce different particle size ranges.

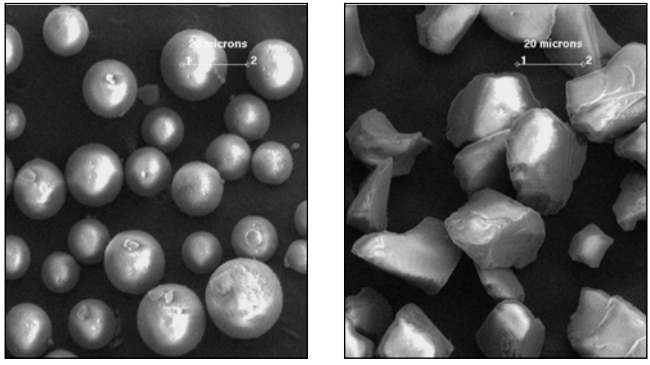


Figure 24: Photos of particle shapes
Microscopic views or spherical (left) and irregular (right) silica

Spherical silica is produced by either spraying a neutral silicate solution and drying the droplets, or by dispersing the solution in an emulsion and allowing the droplets to gel. Conditions are highly controlled under the process to give a well defined size distribution and porosity. This results in a higher manufacturing cost than irregular silica.

Spherical shaped silica packs into a column body more densely and uniformly, resulting in very level and narrow separation bands, thus being the choice for HPLC columns. Irregular shaped particles have edges that catch on each other during the packing process which either break, creating finer particles, or create greater interstitial space and less active surface area in a given column volume. Spherical particles will pack more silica more densely into the same amount of space with less interstitial space and the resulting greater surface area will offer better separation. Teledyne Isco recently added spherical silica in the high performance Redi*Sep* Rf Gold columns for greater resolution.

Particle Size

In liquid chromatography, the smaller the particle size of the column packing leads to greater plate count[1]. However, as particle size decreases the back pressure increases. Typical Flash grade, irregular silica is classified as 40–63 µm or 230 to 400 mesh which refers to the sieves sizes used to produce that particle distribution. This particle size provided adequate resolution while creating low back pressure so gravity and air pressure could produce a separation with glass columns.

Reducing the particle size generates greater back pressure due to the viscosity of the solvent. Reversed phase solvents generally have a higher viscosity, further increasing the back pressure. Irregular particles of the same specified size range typically have more fine particles (>10 µm) in the mixture. Because of the manufacturing and handling processes, spherical media has fewer fine particles than irregular of the same particle distribution, resulting in lower back pressure than irregular particles. Typical Flash columns of 40–63 µm will create back pressure of around 15–20 psi with normal phase solvents and 40–60 psi with reverse phase solvents (without consideration of sample interaction.)

1. Introduction to Modern Liquid Chromatography, Snyder, L.R.; Kirkland, J.J. 2nd edition, John Wiley and Sons,

New, high resolution Flash columns have been introduced in the market with finer packings such as 20–40 µm, irregular, and 15 µm packing. These offer improved resolution, but run at higher pressures. Newer Flash systems, such as the Combi*Flash* Rf, operate at up to 200 psi to accommodate greater back pressures. Teledyne Isco introduced Redi*Sep* Rf Gold high performance columns which combine a smaller 20–40 µm particle size and spherical shape to achieve improved resolution without an increase in back pressure.

Sample Loading Techniques

One of the challenges to producing pure compounds is to load the compound and reaction by-products onto the column. This can be accomplished in a number of ways for both manual and automated chromatography equipment.

Manual Glass Chromatography

Liquid sample loading **—** The sample as an oil or liquid is slowly pipetted inside the glass column preloaded with silica gel topped by a sand layer (Figure 25, left). Then, the solvent mixture selected for optimal resolution is slowly introduced so to preserve the stationary phase packing, causing separation to occur.

Solid sample loading **—** The sample as an oil or solid is dissolved in a minimal amount of polar solvent and powder silica gel is added. The solvent is then removed with a rotary evaporator, leaving the sample coated on the silica gel. This sample on silica is then introduced in the glass column on top of the sand layer (Figure 25, right) covering the packed preloaded silica gel. Then, solvent progression can be initiated to separate the products.

It should be noted that if there is little resolution between compounds, the loading capacity is correspondingly reduced to prevent adjacent peaks mixing with each other.

Automated Chromatography

Syringe injection **—** Syringe injection (Figure 26) is a very common technique as it is very simple and convenient. It also allows equilibration of the column for improved separation.

Syringe injection requires that the compounds are soluble in mobile phase at beginning of gradient.

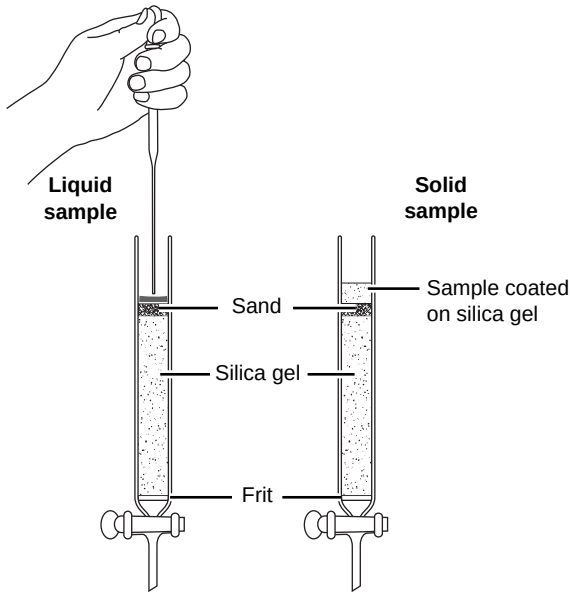


Figure 25: *Illustration of sample injection on glass columns*
A liquid sample is applied evenly to the top of the column with a pipette (left). A solid sample is first adsorbed onto silica gel and then placed on top of the sand (right).

Figure 26: *Photo of syringe injection*
onto a CombiFlash Rf system

Empty solid load cartridges — These cartridges provide a practical method for introducing low-solubility samples onto the column. This method allows equilibration of the column for improved separation.

Solid load cartridges (Figure 27) can be used with a variety of adsorbent materials. The most popular material is silica gel, used by adding powder silica gel onto the neat or dissolved sample to be purified. Then the solvent is removed with a rotary evaporator providing the sample coated on a silica bed. This sample on silica is then poured into an empty cartridge, topped with a frit, and loaded onto the system.

Adsorbents are not limited to silica: Celite®, diatomaceous earth, boiling chips, and even cotton balls and Kimwipes® have been used. Sometimes, chemists insert boiling chips and cotton balls into the rotary evaporator flask to reduce the risk of bumping. These can be removed along with the compound and placed into the solid load cartridge to easily transfer the sample to the purification step. Kimwipes can be used if a flask is inadvertently broken and the contents spill onto the lab bench. This spilled material can be soaked up onto Kimwipes and placed into the solid load cartridge for introduction into the column.

Functionalized media, such as C18, can be used in the solid load cartridge as a guard column or as a scavenger.

Figure 27: Photo of a solid sample load cartridge

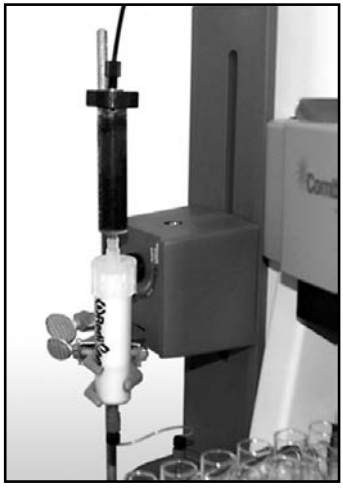
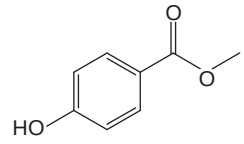


Figure 28: Photo of solid load cartridge connected to column to bypass valves in the instrument

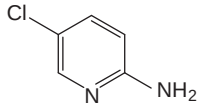
Solid load cartridge techniques do not require that samples be placed on the column bed. However, these solid load cartridges can be directly connected to the columns (Figure 28) when samples that are difficult to keep in solution are being purified. Direct connection to the column eliminates any possibility of contamination of the valves in the automated instrument and also make an easy funnel introduction of the sample onto the column.

Pre-packed solid load cartridges — These cartridges represent a faster solid sample loading technique compared with the use of an empty solid load cartridge. The dissolved sample is introduced into a cartridge pre-packed with an adsorbent. After absorption of dissolved sample by the absorbent, the wet pre-packed cartridge is thoroughly dried with a high vacuum pump and finally loaded onto the system.

Solid load cartridges that have been completely dried often perform better than liquid injections. Figure 29 compares two sample loading techniques in the purification of 260 mg mixtures of a phenolic ester and pyridine derivative on 12 gram Redi*Sep* silica gel columns. The dry solid load cartridge shows better resolution than the liquid injection.

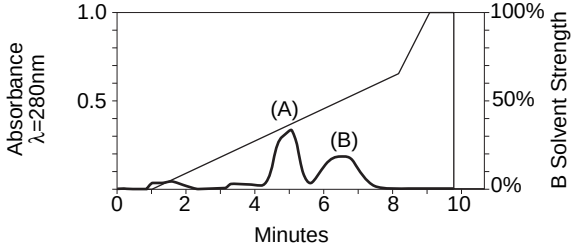


methyl-4-hydroxybenzoate (A)



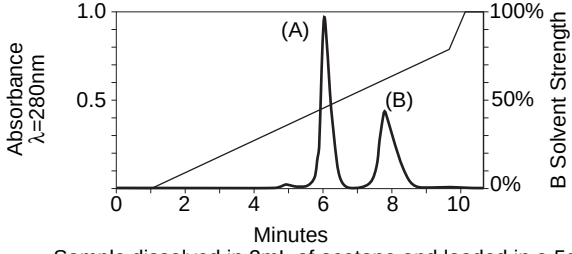
2-amino-5-chloropyridine (B)

Syringe Injection



Sample dissolved in 2mL of acetone loaded injected onto a silica column and followed by a 1mL acetone chase. Acetone used as a strong solvent to completely dissolve the sample. Solvent system hexane/ethyl acetate.

Solid Load Cartridge (dried)



Sample dissolved in 2mL of acetone and loaded in a 5g pre-packed normal phase silica cartridge. Acetone removed with a cartridge dryer.

Figure 29: Chromatograms of syringe injection and dried solid load cartridge techniques. *Acetone used to dissolve, inject, and chase over-dilutes the syringe-injected mixture, causing band broadening and overlapping peaks. The solid load cartridge allows the acetone to be removed, thus retaining the compounds until the increasing solvent strength releases them.*

Figure 30: Photo of pre-packed cartridges on Teledyne Isco's cartridge dryer (Part number 60-2200-010)

Teledyne Isco manufactures a cartridge dryer, part number 60-2200-010, that dries up to four cartridges at a time (Figure 30).

Dry loading column — This technique is ideal for quick cleaning of samples and removing baseline materials. After clean-up, the sample can be moved to the next synthesis step.

When dry loading a column, bypass column equilibration.

Figure 31: Photo of dry loading sample onto the column

Figure 32: Table of RediSep Rf solid load cartridges

Part number	Description
Prepacked Cartridges	
69-3873-238	Sample load prepacked silica gel Rf cartridges, 2.5 gram, pkg. of 20.
69-3873-236	Sample load prepacked silica gel Rf cartridges, 5 gram, pkg. of 20.
69-3873-243	Sample load prepacked silica gel Rf cartridges, 12 gram, pkg. of 15.
69-3873-241	Sample load prepacked silica gel Rf cartridges, 25 gram, pkg. of 15.
69-3873-310	Sample load prepacked silica gel Rf cartridges, 32 gram, pkg. of 12.
69-3873-226	Sample load prepacked silica gel Rf cartridges, 65 gram, pkg. of 12.
69-3873-311	Sample load, prepacked silica gel cartridges 125 grams, pkg. of 4.
68-3873-202	Sample load, prepacked silica gel cartridges 260 grams, pkg. of 4.
69-3873-254	Sample load, prepacked silica gel cartridge. For use on the CombiFlash Torrent and Companion XL systems only.
69-3873-255	Sample load, prepacked silica gel cartridge. For use on the CombiFlash Torrent and Companion XL systems only.
69-3873-247	Sample load, prepacked, C18 Rf cartridges, 2.5 gram, pkg. of 5.
69-3873-237	Sample load, prepacked, C18 Rf cartridges, 5 gram, pkg. of 5.
69-3873-248	Sample load, prepacked, C18 Rf cartridges, 12 gram, pkg. of 4.
69-3873-242	Sample load, prepacked, C18 Rf cartridges, 25 gram, pkg. of 4.
69-3873-249	Sample load, prepacked, C18 Rf cartridges, 32 gram, pkg. of 3.
69-3873-250	Sample load, prepacked, C18 Rf cartridges, 65 gram, pkg. of 3.
69-3873-312	Sample load, prepacked, Celite® Rf cartridges, 2.5 gram, pkg. of 20.
69-3873-313	Sample load, prepacked, Celite® Rf cartridges, 5 gram, pkg. of 20.
69-3873-314	Sample load, prepacked, Celite® Rf cartridges, 12 gram, pkg. of 15.
69-3873-315	Sample load, prepacked, Celite® Rf cartridges, 25 gram, pkg. of 15.
69-3873-318	Sample load, prepacked, Celite® Rf cartridges, 32 gram, pkg. of 12.
69-3873-319	Sample load, prepacked, Celite® Rf cartridges, 65 gram, pkg. of 12.
Empty Cartridges	
69-3873-235	Sample load, empty Rf cartridges (holds up to 5 gram), package of 30.
69-3873-240	Sample load, empty Rf cartridges (holds up to 25 gram) pkg. of 30.
69-3873-225	Sample load, empty Rf cartridges (holds up to 65 gram) pkg. of 12.
69-3873-201	Sample load, empty cartridges (holds up to 260 grams), pkg. of 6.

Chapter 3

From Traditional Glass Columns to Automated Flash Chromatography

Manual Glass Column Chromatography

Since its inception, manual glass-column Flash chromatography has been one of the purification methods most used by several generations of organic chemists.

Figure 33: Photo of a manual Flash system in use

Manual glass-column Flash chromatography is a practical method offering several advantages:

- Low-cost simple material (glass column and silica gel, TLC plates and UV lamp, test tubes, *etc.*).
- Stationary phase introduction and packing modes fully controlled by chemist.
- Either isocratic or step gradient solvent elution can be used.
- Reasonable separation results for simple crude reaction mixtures.
- Familiar technique for organic chemists.

There are however important drawbacks associated with it:

- Time consuming overall process, especially at the collection stage.
- Low resolution.
- Separation of complex crude reaction mixtures is difficult and time consuming.
- Results not reproducible since silica gel column loading and packing parameters are not standardized.
- Only isocratic or step gradient solvent elution are available for use.
- Absence of on-line detection means every tube must be tested by TLC.

Departments with organic compounds preparation as their core activity are demanding increasing efficiency and productivity from synthetic organic chemists. These requirements are conducive to the search of improved working habits in order to maximize their typical reaction sequence. Since the purification step often is the time consuming step in this sequence, chemists are looking for avenues which would significantly improve ease-of-use, reliability, product purity, and overall productivity of this step.

Teledyne Isco has a history of producing automated Flash chromatography instruments meeting these performance criteria.

Benefits of Automation

For instance, the Combi*Flash*® Rf system offered by Teledyne Isco provides automated Flash chromatography that is user-friendly and reliable, without the downsides of manual glass Flash chromatography.

Figure 34: Photo of Teledyne Isco's CombiFlash Rf system with 4x automated sequential purification option

Some of the Combi*Flash* Rf system's clear advantages over glass Flash chromatography columns are:

- Fully automated system from solvent injection to product collection. Purification time is dramatically shortened. Chemists can concentrate on other projects.
- Easy-to-use software.

- Purifies wide range of sample sizes.
- Uses linear gradient elution power for superior peak separation.
- Gradient Optimizer minimizes solvent usage and purification time while preserving resolution.
- Higher productivity. There is no need to pack columns. The pumping system delivers optimal flows and gradients for fast purifications.
- Reliability.
- Safety features such as pressure-limited solvent delivery, solvent vapor sensing to detect spills, and conductive tubing to prevent a static discharge.
- Radio Frequency Identification (RFID) technology speeds setup while minimizing errors.
- Flow rates to 200 mL/minute allow fast purifications and better use of reversed phase columns.
- 200 psi pressure limit allows fast runs and efficient use of C18 and other bonded phases.
- Active solvent sensing prevents columns running dry and resulting loss of resolution. Solvent sensing also protects expensive C18 columns.
- Active waste sensing prevents messy and potentially dangerous solvent spills. Active waste sensing works with most types and sizes of waste containers.
- All-Wavelength Collection triggers collection on any wavelength for improved sample collection.

Column Packing

Manually-packed Columns

Manual column packing can be used to load specialized column media. However, commonly-used media (listed in Appendix A) are readily available as pre-packed columns in a variety of sizes.

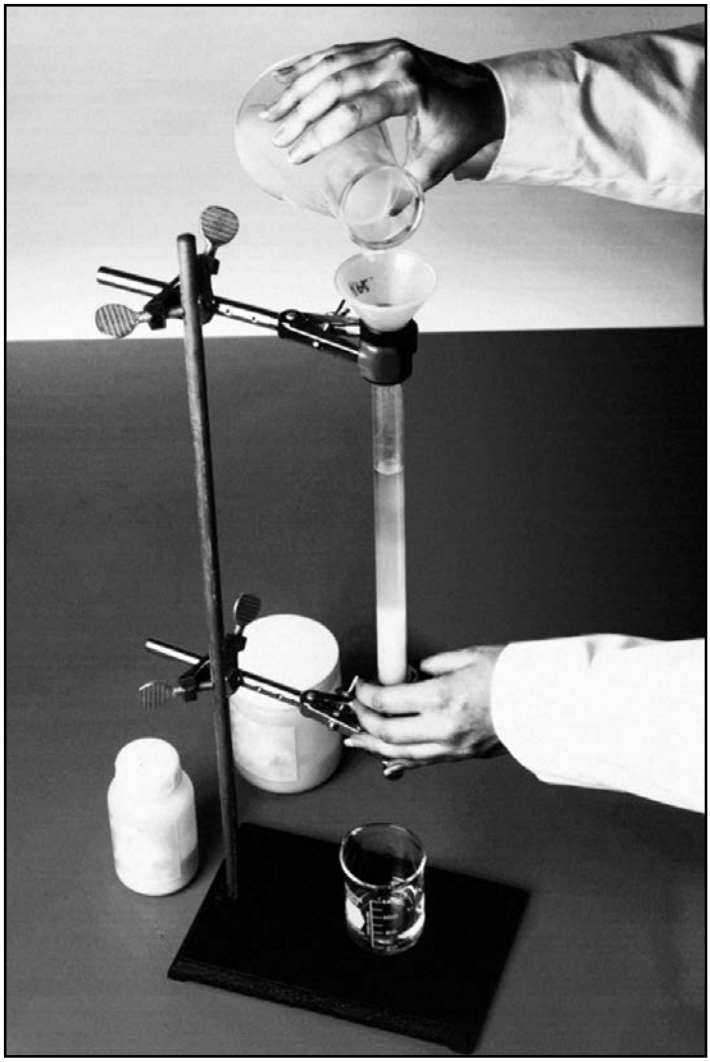


Figure 35: Photo of glass column preparation Manual column packing allows the chemist to load stationary phase media as needed for each purification. Column packing that produces repeatable separation results can be a labor-intensive process.

Pre-packed Columns

Pre-packed columns improve the efficiency of compound purification, offering greater productivity and reproducibility.

By using pre-packed columns, scientists can purify compounds more quickly because they save the time required to pack the column. Pre-packed columns also show higher purification efficiency since the silica is more tightly packed allowing the compounds more interaction with the stationary phase (see Figure 36)

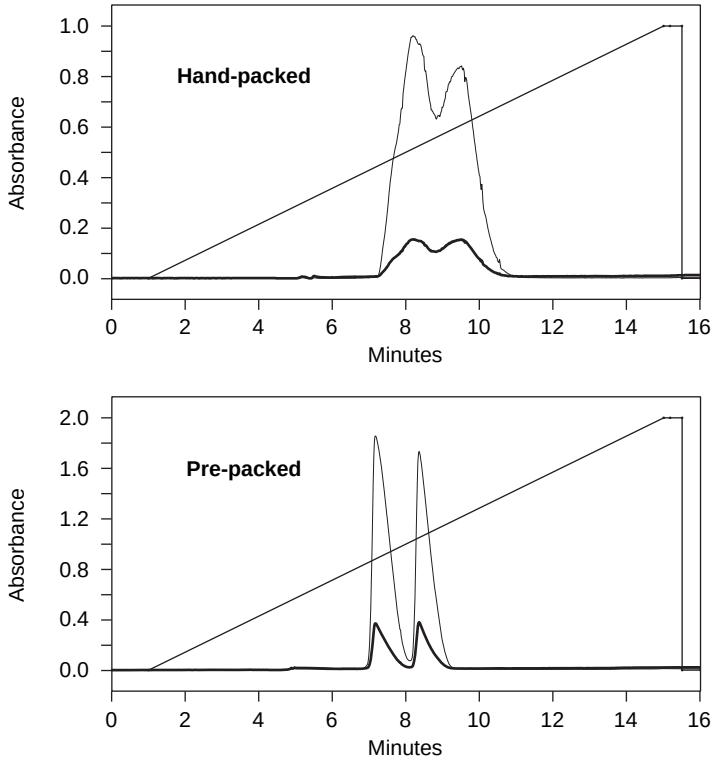


Figure 36: Chromatograms of compounds separated on a hand-packed column *(top) and pre-packed RediSep Rf column (bottom). The prepacked column shows near baseline separation and would result in pure fractions. The hand-packed column gives mixed fractions that would need to be run again at additional time to improve product yield.*

The use of pre-packed columns is safer than hand packed columns. The user is not exposed to silica dust from packing the column or during disposal of the used silica. The Combi*Flash* Rf will blow out the solvent to waste from silica columns at the end of a run allowing easy disposal of the column. The user is not exposed to the silica or any residual compounds left on the column at the end of the run.

Figure 37: Photo of pre-packed columns Reusable and disposable Flash chromatography columns are a cost-effective alternative to manual column packing, nearly eliminating column setup time. Teledyne Isco's RediSep Rf columns are easy to load, allowing fast changeover between purifications.

TLC Plates

Redi*Sep* TLC plates are useful to run compounds to determine optimal purification conditions or to confirm purity at the end of a run. The plates are small enough to run quickly yet long enough to allow accurate measurement of Rf differences for programming the Gradient Optimizer. Redi*Sep* TLC plates are available in a variety of materials to match your purification needs.

Figure 38: Photo of matching TLC media *TLC media can be prepared to match the performance characteristics of a column. Matched media ensures that favorable analytical TLC results can be developed into effective column purification methods.*

Figure 39: Table of RediSep Rf TLC plates

Part Number	TLC Plate description
69-2203-400	TLC plates, Silica Gel, package of 200, 5x10 cm each.
69-2203-401	TLC plates, C18 Reversed Phase, package of 30, 5x10 cm each.
69-2203-403	TLC plates, Alumina Basic, package of 30, 5x10 cm each.
69-2203-569	TLC plates, Alumina Neutral, package of 30, 5x10 cm each.

Figure 40: Table of solvent migration and plate development time for RediSep C18 TLC plates (69-2203-401)

Solvent	Migration
Isopropanol	Full, 45 min
Water	Plate degrades
Methanol	Full, 15 min
Water/Methanol 1:1	Full, 45 min
Acetonitrile (ACN)	Full, 15 min
Water/ACN 1:1	Full, 45 min
Water/ACN 6:1	75%, 45 min
Water/ACN 7:1	60%, 45 min
Water/ACN 8:1	20%, 45 min
Water/ACN 9:1	10%, 45 min

Figure 41: Table of solvent migration and plate development time for RediSep Basic and Neutral Alumina TLC plates (69-2203-403 and 69-2203-569)

Solvent	Migration
Hexane	Full, 15 min
Ethyl acetate	Full, 15 min
Isopropanol	Full, 30 min
Hexane/Ethyl acetate 1:1	Full, 15 min
Hexane/Isopropanol 5:1	Full, 30 min
Dichloromethane	Full, 15 min
Methanol	Full, 15 min
Dichloromethane/Methanol 9:1	Full, 15 min

High Performance Flash Chromatography

Column Stacking

One way to improve resolution is by column stacking, placing one column on top of another of the same size. If needed, more than one column may be stacked. Column stacking essentially creates a long, thin column (Figure 42).

To develop a method using column stacking, the method should be changed to column volumes. Multiply the time for each segment in the gradient table for a single by the number of columns being stacked, and enter this new time for each segment. The flow rate is kept the same as for a single column since the linear velocity of the solvent is optimized for each column in the Combi*Flash* system. After entering the new gradient segment times into the method, the method may be displayed in time, if desired, rather than column volumes. The change to column volumes is required since the solvent and compound needs to run through two (or more) columns worth of media.

Column stacking works best with compounds that are weakly retained on the column as these benefit most from the increased interaction with the media provided by column stacking. Although column stacking can improve resolution, it does lead to increased back pressure due to the increased column length. The run time is also increased by the number of columns stacked. Figure 43 shows increases in resolution from column stacking compared to using a regular column of a similar size, but note that the peaks are slightly wider. The increased peak width is due to diffusion of the compounds in the mobile phase. This diffusion is more noticeable in compounds that have a stronger interaction with the column media. This diffusion leads to diminishing returns from stacking more columns in terms of improving resolution.

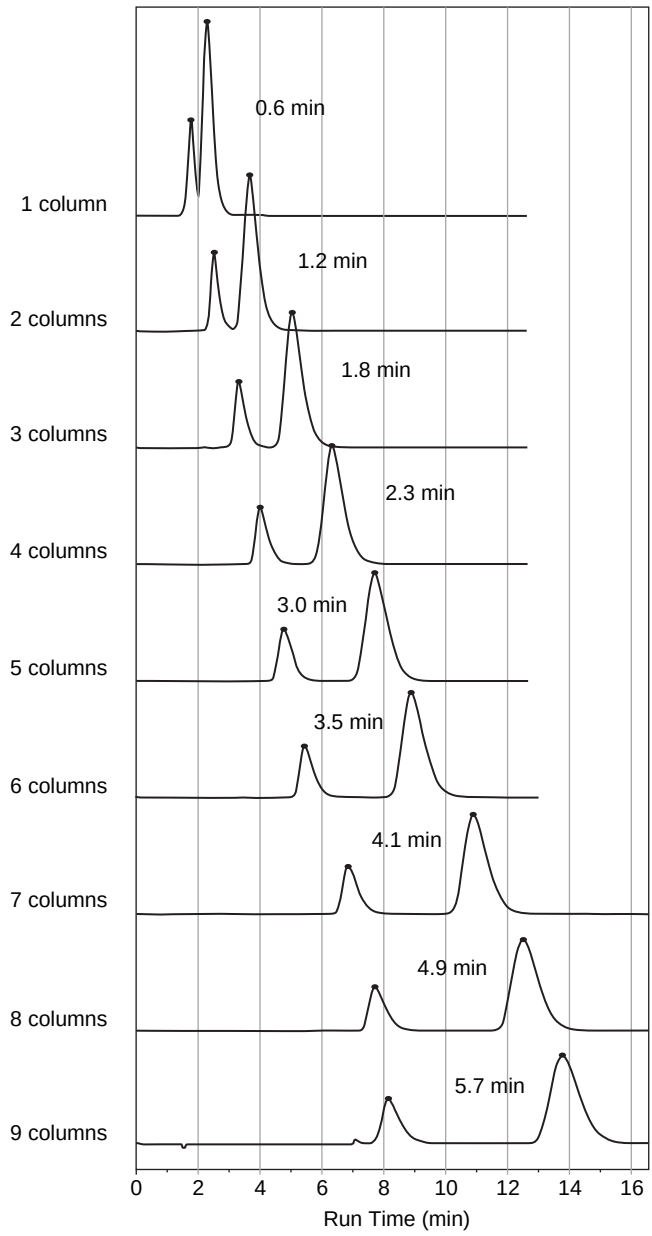


Figure 42: *Chromatograms showing improved resolution* *by stacking columns*

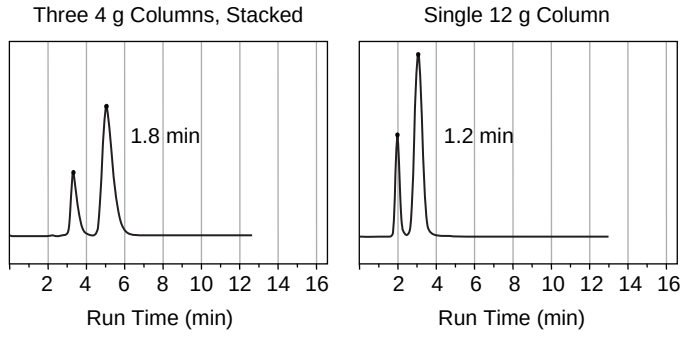


Figure 43: Chromatograms comparing a single column *and three stacked columns containing equivalent amounts of silica.*

An alternative to stacking columns is using a higher resolution column as it will save time and solvent. In Figure 44, the Redi*Sep* Rf Gold column run needed only 19 minutes to purify the compounds compared to 30 minutes for the stacked column. The Gold column used 760 mL solvent compared to 1050 mL for the stacked columns. In addition to the direct time and solvent saved, the peaks from the Gold column were sharper reducing the number of fractions collected resulting in further time savings after the fractions were collected.

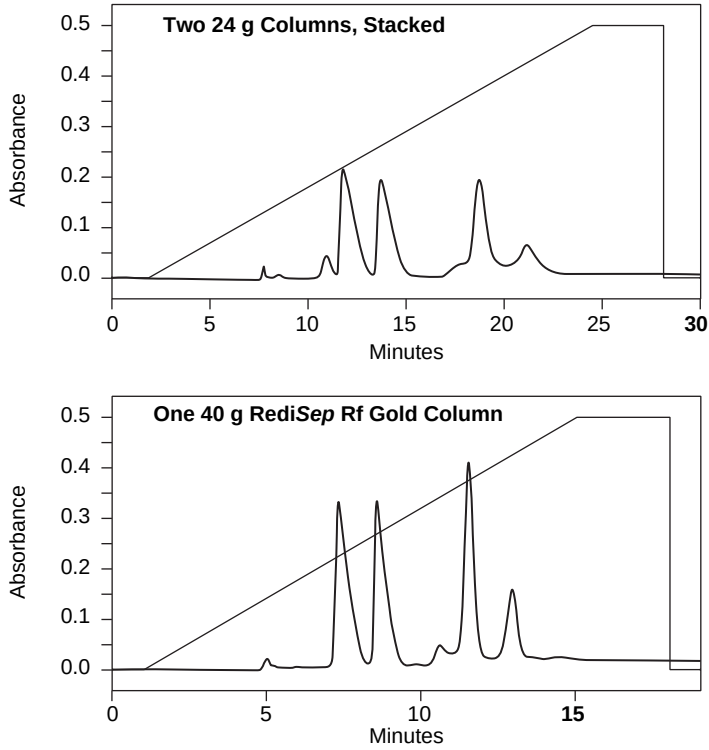
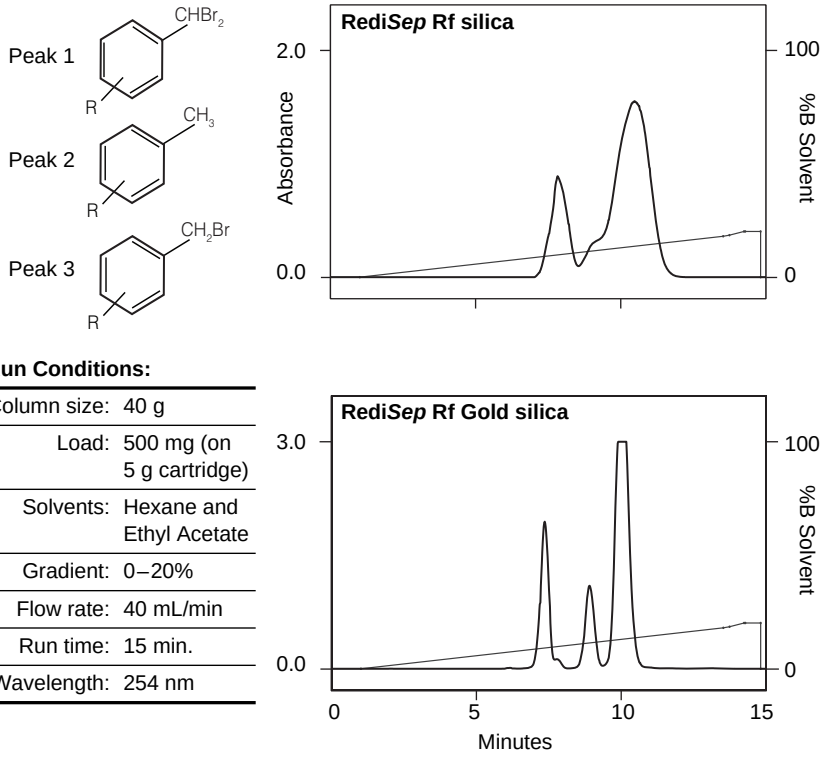


Figure 44: Chromatogram of two 24 g stacked columns compared to a chromatogram of a single 40 g RediSep Rf Gold column



Run Conditions:

Column size:	40 g
Load:	500 mg (on 5 g cartridge)
Solvents:	Hexane and Ethyl Acetate
Gradient:	0–20%
Flow rate:	40 mL/min
Run time:	15 min.
Wavelength:	254 nm

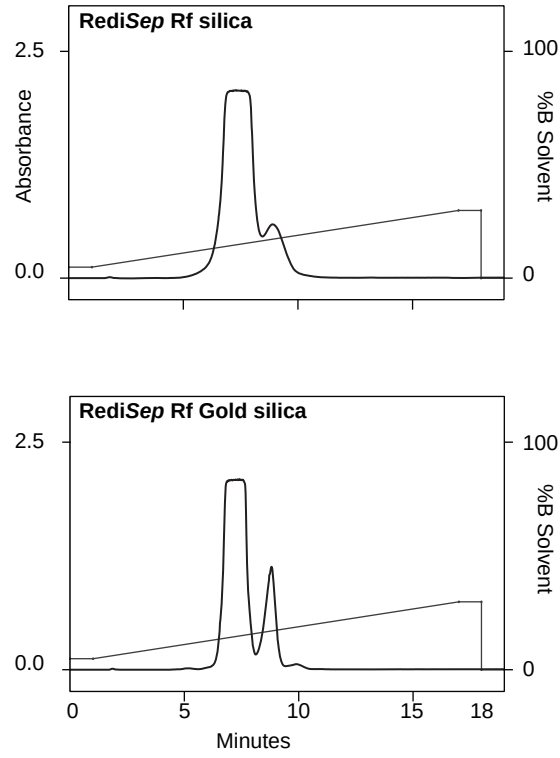
Figure 45: *Chromatogram of bromotoluenes purification* *A 1% sample load of a bromotoluene mixture on an irregular shaped, larger particle media (RediSep column, top) and a spherical shaped, smaller particle media (RediSep Rf Gold, bottom)*

Why spherical media?

Redi*Sep* Rf Gold spherical media has a smaller particle size than standard Flash media so it provides higher resolution. The spherical media reduces back pressure allowing high flow rates (See Chapter 2 for more information).

Higher Resolution with small spherical media

Redi*Sep* Rf Gold silica columns have a particle size range of 20–40 µm compared to 40–60 µm for standard silica. The smaller particle size allows higher resolution so closely eluting peaks can be resolved (Figure 45).



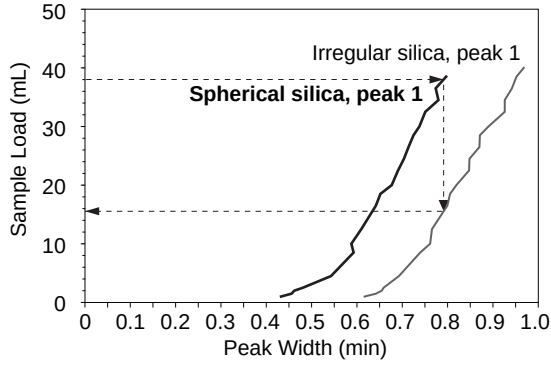
Run Conditions:

Column size:	40 g
Load:	500 mg (1.1% load)
Solvents:	Hexane and Ethyl Acetate
Gradient:	0–20%
Flow rate:	40 mL/min
Run time:	19 min.
Wavelength:	254 nm

Figure 46: Chromatogram of minor compound separation of a proprietary sample using an irregular shaped, larger particle media (RediSep column, top) and a spherical shaped, smaller particle media (RediSep Rf Gold, bottom).

This increased resolution is useful when purifying minor components which are chemically similar to the major compound. These minor compounds are collected for the purposes of screening and patent protection. The minor compounds may show activity similar to the desired material and may be of further interest.

Collection of minor compounds is also important to demonstrate that these materials do not affect the efficacy or toxicity of the pharmaceutical product. Minor compounds are often difficult to resolve from the main compounds since they are generally an isomer of the main compound The sharper peaks allow the minor compounds to be better resolved from the major peak (Figure 46).



Run Conditions:

Column size:	12 g
Solvent:	Isocratic EtOC:hexane (10:90)
Flow rate:	30 mL/min
Run time:	9 min.

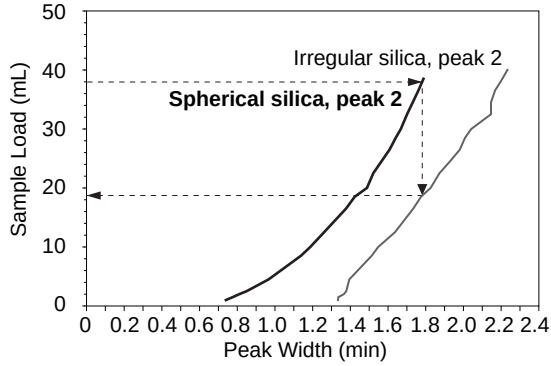
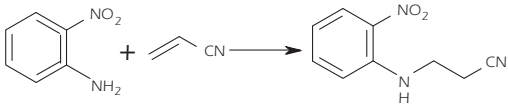


Figure 47: *Diagram of sample load comparison* *by purifying a mixture of acetophenone (Peak 1) and 4'-methoxyacetophenone (Peak 2) in hexane using an irregular silica (non-RediSep) column and a spherical silica RediSep Rf Gold column.*

Improved load capacity

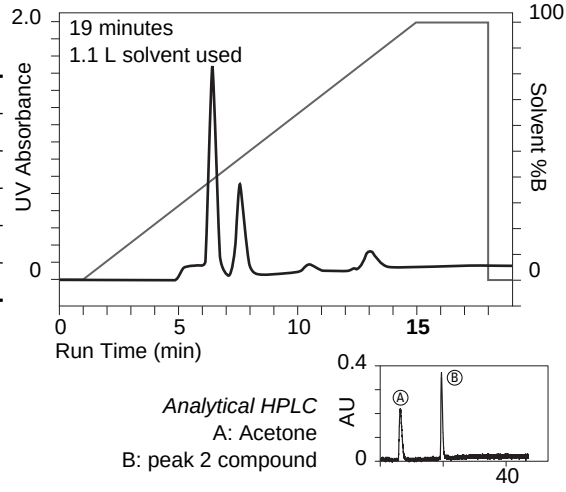
The increased resolution of Redi*Sep* Rf Gold columns can be used for increased loading. Redi*Sep* Rf Gold columns showed a reduced peak width for an equivalent sample load compared to irregular silica of the same particle size.

Figure 47 shows that at a given sample load, the peak width of the spherical silica is the same as that of irregular silica with less than $^1/_2$ the sample load.



Standard Flash Column

Run Conditions:	
Column size:	40 g
Load:	400 mg
Solvent:	Hexane:ACN
Flow rate:	40 mL/min
Run time:	19 min.
Wavelength:	229 nm



RediSep Rf Gold Column

Run Conditions:	
Column size:	40 g
Load:	400 mg
Solvent:	Hexane:ACN
Flow rate:	80 mL/min
Run time:	5 min.
Wavelength:	229 nm

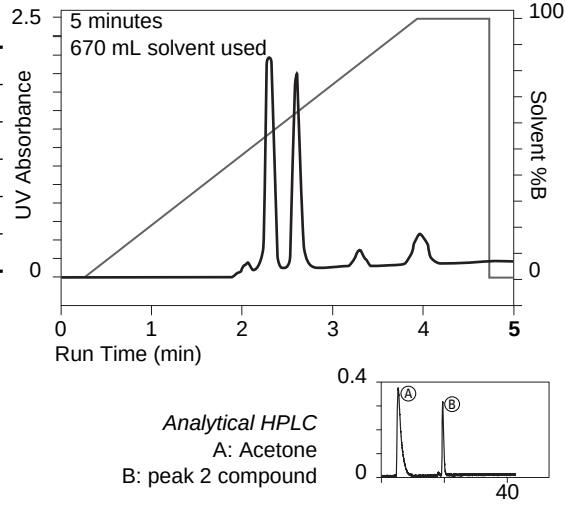


Figure 48: *Chromatograms of 3-(2-nitrophenyl amino) propionitrile purifications* compared to standard Flash grade silica, the RediSep Rf Gold column demonstrated 60% time savings, 30% solvent savings, with no loss in purity.

Faster purifications

The higher resolution of Redi*Sep* Rf gold columns can be used to more quickly purify compounds saving time and solvent. This is accomplished by increasing the gradient; in some cases the flow rate can be increased as well. Using Combi*Flash* Rf systems, the fast parameters are optimized as a "Gold Speed" method.

To use Gold Speed, the retention factor difference between the two compounds of interest should be greater than 0.1.

The resolution of the spherical column under Gold Speed conditions is equivalent to that of a standard Flash column run under standard conditions, but less solvent was used (Figure 48). Since the peaks are much narrower, there is less solvent collected with the peaks reducing the time required to dry the fractions. Gold Speed also allows compounds sensitive to silica gel to be purified since the compound has less contact time on the silica.

Figure 49: Table of RediSep Rf Gold Silica Gel *Disposable Flash Columns, spherical, 20–40 microns*

Sample Size	Column Size (g)	Teledyne Isco Part Number	Description
20 mg - 0.4 g	4	69-2203-344	4 gram RediSep Rf Gold Silica Gel Disposable columns, pkg. of 14.
60 mg - 1.2 g	12	69-2203-345	12 gram RediSep Rf Gold Silica Gel Disposable columns, pkg. of 14.
120 mg - 2.4 g	24	69-2203-346	24 gram RediSep Rf Gold Silica Gel Disposable columns, pkg. of 10.
200 mg - 4 g	40	69-2203-347	40 gram RediSep Rf Gold Silica Gel Disposable columns, pkg. of 10.
400 mg - 8 g	80	69-2203-348	80 gram, RediSep Rf Gold Silica Gel Disposable columns, pkg. of 6.
600 mg - 12 g	120	69-2203-349	120 gram, RediSep Rf Gold Silica Gel Disposable columns, pkg. of 6.
1.1 - 22 g	220	69-2203-359	220 gram, RediSep Rf Gold Silica Gel Disposable columns, pkg. of 4.
1.65 - 33 g	330	69-2203-369	330 gram, RediSep Rf Gold Silica Gel Disposable columns, pkg. of 3.
3.8 - 75 g	750	69-2203-427	750 gram, RediSep Rf Gold Silica Gel Disposable columns, pkg. of 3.
7.5 - 150 g	1500	69-2203-428	1500 gram, RediSep Rf Gold Silica Gel Disposable columns, pkg. of 2.
15 - 300g	3000	69-2203-529	3000 gram, RediSep Rf Gold Silica Gel Disposable column, pkg. of 1.

Chapter 4

C18 Flash Chromatography

Overview of Reversed Phase Chromatography

Normal Phase Silica

"Bare" or unfunctionalized silica gel consists of a tetrahedral cross-linked polymer of [SiO_4] (Figure 50). In aqueous media the free valences of the exposed Si atoms adsorb hydroxyl groups, forming polar silanol groups on the surface. These silanol groups produce the polar adsorption properties that make bare silica such a useful medium for normal-phase Flash chromatography.

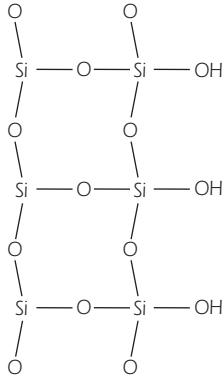


Figure 50: Diagram of normal phase silica *Bare silica allows silanol groups to form on the exposed surfaces.*

Reversed Phase Silica

In reversed phase silica stationary phases, silanol groups are replaced with less polar or non-polar functional groups such as C18 (Figure 51) or phenyl.

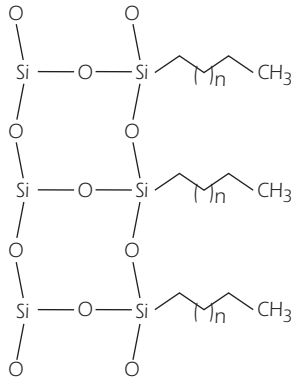


Figure 51: *Diagram of reversed phase silica*

The resulting lipophilic properties obtained are useful for purification of compounds with high polar and high protic characteristics.

The C18 reversed phase usefulness is illustrated in the purification of the following classes of compounds that surveyed organic chemists have pointed out as typically difficult to purify. C18 is the second most common media used for Flash chromatography after silica because it is easy to use and can purify a wide range of compounds. Because the C18 groups protect the silica from hydrolysis, C18 columns are reusable if treated with care. Flash chromatography allows the use of less expensive equipment to purify compounds in reversed phase. The PeakTrak software on Combi*Flash* systems is often easier to use than traditional prep HPLC systems. Acid sensitive compounds can often be run on C18 because the reactive silanol groups are bound to the C18.

C18 columns are generally run at a pH of 8 or less because high pH causes hydrolysis of the siloxane bond causing loss of the C18. Redi*Sep* Rf Gold C18 columns may be run at higher pH for longer periods of time before any performance degradation occurs.

C18 columns are often ranked by “carbon load” which runs between 16 and 22%. This is determined by elemental analysis.

Often a higher carbon load is considered better because it suggests more C18 material bonded onto the silica and a greater interaction between the column and compounds to be purified.

In contrast to silica gel, 100% water can be used on C18 columns after conditioning with organic solvent. If more than 95% water is kept in the column, the long, oil-like, alkyl chains forming the C18 will self-associate; this phenomenon is known as "phase collapse." This also called "dewetting," "chain folding," or "matting." Phase collapse symptoms include retention loss, retention irreproducibility, increased tailing, and long gradient regeneration times although there are some C18 media designed to run under aqueous conditions that exhibit reduced phase collapse.

Figure 52: Table of RediSep Rf C18 Reversed Phase columns
Sample loading capacities

Sample Size	Column Size (g)	Teledyne Isco Part Number	Reverse Phase (C18) RediSep Rf Column Description
4.3 - 86 mg	4.3	69-2203-410	4.3 gram columns, pkg. of 2.
13 - 260 mg	13	69-2203-411	13 gram column, pkg. of 1.
26 - 520 mg	26	69-2203-412	26 gram column, pkg. of 1.
43 - 860 mg	43	69-2203-413	43 gram column, pkg. of 1.
86 mg - 1.72 g	86	69-2203-416	86 gram column, pkg. of 1.
130 mg - 2.6 g	130	69-2203-414	130 gram column, pkg. of 1.
240 mg - 4.8 g	240	69-2203-418	240 gram column, pkg. of 1.
360 mg - 7.2 g	360	69-2203-415	360 gram column, pkg. of 1.

The typical loading on a C18 column is reduced to about 1% (depending on the separation between compounds) compared to loading up to 10% on silica gel.

Other types of reverse phase chromatography include C1, C4, and C8. These are similar to C18 but with shorter chains. These columns have similar characteristics to C18 but show decreased retention for strongly hydrophobic compounds. Peptides and proteins are often purified on reverse phase columns with short chain alkyl groups.

C18 Method Development

Thin Layer Chromatography Plates

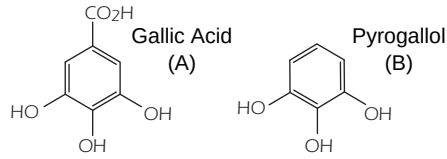
C18 TLC plates can be used for method development if the compound is relatively non-polar. C18 TLC plates generally can't be used in highly aqueous solutions because the stationary phase degrades. Compounds are spotted on C18 TLC plates and developed in a manner similar to silica TLC plates. The PeakTrak software Gradient Optimizer can also be used to develop the gradient. Development times for C18 TLC plates range from 15 to 45 minutes.

Figure 53: *Table of solvent migration and plate development time for RediSep C18 TLC plates* *(69-2203-401)*

Solvent	Migration
Isopropanol	Full, 45 min
Water	Plate degrades
Methanol	Full, 15 min
Water/Methanol 1:1	Full, 45 min
Acetonitrile (ACN)	Full, 15 min
Water/ACN 1:1	Full, 45 min
Water/ACN 6:1	75%, 45 min
Water/ACN 7:1	60%, 45 min
Water/ACN 8:1	20%, 45 min
Water/ACN 9:1	10%, 45 min

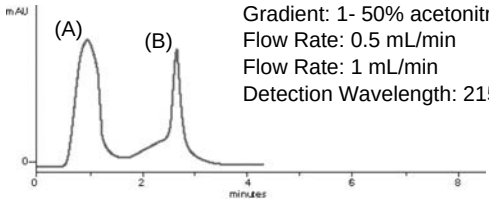
Using HPLC Systems to Generate Flash Methods

Analytical HPLC systems are fairly common in labs. These units produce very accurate gradients and require little sample to develop a method. The steps for creating a method for Flash chromatography are very similar to that for creating an analytical method.



Analytical HPLC

Instrument: Agilent 1100 HPLC
Column: Polaris C18, 3µm, 3x100mm
Gradient: 1- 50% acetonitrile in 10 min
Flow Rate: 0.5 mL/min
Flow Rate: 1 mL/min
Detection Wavelength: 215 nm



Flash System

Instrument: Combi*Flash* Companion
Column: 4.3 g Redi*Sep* C18, 40-63µm
Sample Load: 10 mg
Gradient: 0-50% acetonitrile over 14 min
Flow Rate: 16 mL/min
Detection Wavelength: 215 nm

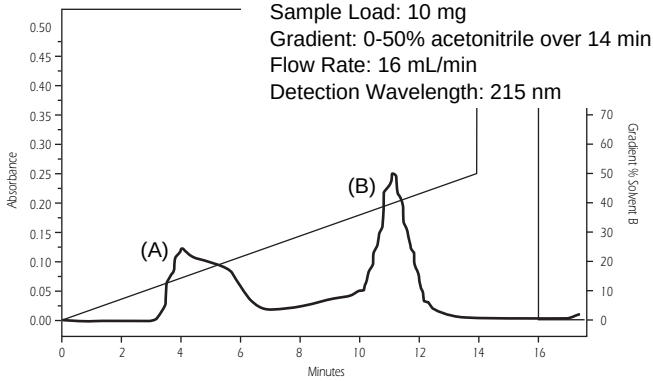


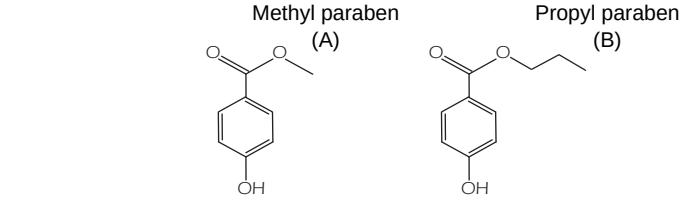
Figure 54: *Illustration of gallic acid and pyrogallol method* *developed on an analytical HPLC and transferred to a Flash reversed phase system*

The first step is to run a gradient and determine where the compound of interest elutes. Adjust the gradient so the desired compound is resolved from contaminants. The next step is to transfer the method to the Flash system. Figure 54 shows gallic acid and pyrogallol developed on an HPLC system and transferred to Flash. The only change was to lengthen the run time, which "flattened" the gradient to allow better resolution.

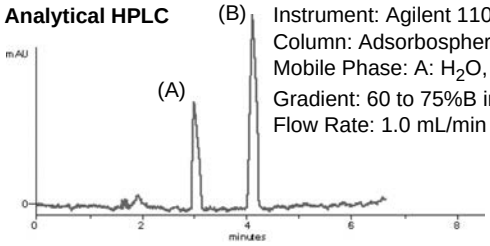
The compounds eluted at three to four minutes in the analytical method. When this method was transferred to the Flash system, the compounds eluted at the same time as the HPLC. Starting the gradient at 0% acetonitrile gave baseline separation while allowing a higher loading of 10 mg. Using an analytical HPLC allows methods to be easily developed with minimal compound use.

Using the Flash Instrument for Method Development

Because C18 columns are reusable, one also can develop the method on the Flash instrument. The use of a 4.3g C18 column reduces run time, solvent, and compound required to develop the method. It is easiest to start with the default method on the instrument and load only a few milligrams to determine where the compounds elute. Increase or reduce the gradient slope to achieve the desired resolution. After the method is developed, save it and change to the column size to be used for your separation. Load the method you saved—the Combi*Flash* system will automatically scale the method for the larger column.



Analytical HPLC



Instrument: Agilent 1100 HPLC
Column: Adsorbosphere C18, 5µm, 4.6x250mm
Mobile Phase: A: H_2O, B: CH_3CN
Gradient: 60 to 75%B in 7 minutes
Flow Rate: 1.0 mL/min

Flash System

Instrument: Combi*Flash* Companion
Column: 4.3 g Redi*Sep* C18, 40-63µm
Sample Load: 10 mg
Mobile Phase: A: H_2O, B: CH_3CN
Gradient: 0 to 100% B in 13 minutes
Flow Rate: 16 mL/min

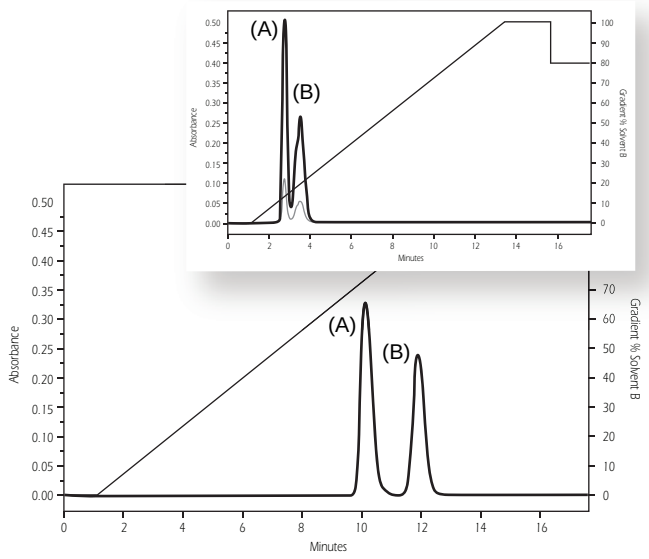


Figure 55: Illustration of methyl- and propyl-paraben method *developed on an analytical HPLC instrument and transferred to a Flash reversed phase system*

Loading Compounds

Loading techniques for C18 are similar to those for silica. The compound can be liquid-loaded onto the column. Alternatively, compounds may be adsorbed onto an inert material such as celite. The compound may also be loaded onto C18 solid load cartridges.

If C18 cartridges are used they should be conditioned by washing them with the "B" solvent to be used in the separation (typically methanol or acetonitrile) followed by the "A" solvent. This double-wash step "raises" the C18 chain from the silica bed so it can interact with the sample. The compound can be loaded in the same fashion as done with a Solid Phase Extraction (SPE) cartridge.

If loose C18 is used, the compound should be dissolved in an organic solvent prior to adding the C18 and drying. This will activate the C18. The compound adsorbed on the C18 can be added to an empty Redi*Sep* Rf solid load cartridge.

Using C18 as an adsorbent (either loose or in a pre-packed cartridge) also acts as a guard column that protects the main column.

Column Care

With proper care, Redi*Sep* Rf C18 columns may be used for over 20 purifications. Redi*Sep* Rf C18 columns are shipped dry-packed; before its first run, the C18 column must be washed with at least 6 column volumes of water:organic solvent prior to use. The organic solvent is typically the "B" solvent that will be used for the separation. The minimum concentration of organic solvent is 1:1; higher concentrations of organic solvent are generally better. Once wetted, a C18 column should never be allowed to dry out or channels will form that will adversely affect future separations.

After initial conditioning, use three column volumes of the initial solvent conditions prior to the run to equilibrate the column. After the run is complete, make sure the system does not purge the solvent from the column (change the method to turn off "Air Purge" prior to the run if needed). If using a Combi*Flash* Rf system with Redi*Sep* Rf columns, the column's RFID tag will tell the system not to purge the solvent.

The C18 methods preprogrammed into Combi*Flash* systems reduce the solvent concentration to 80% B solvent for column storage. Any modifiers (such as TFA) should be washed from the column before storage. Store the column with the end caps in place. The end caps that were shipped with the columns should be retained for this purpose.

For best results, a column run with a particular organic solvent should continue to be run in that solvent.

Solvent Modifiers

The most common solvent modifier used for reversed phase is TFA (TriFluoro acetic Acid). TFA is compatible with the stainless steel fittings used in HPLC and Combi*Flash* systems. Use of a pH above 7.5 to 8 causes the silica gel supporting the C18 to dissolve; TFA keeps the solvent system at a low pH. TFA can also be easily removed by lyophilization. Redi*Sep* Rf Gold columns have demonstrated the ability to perform multiple runs at pH 10 (page 73).

At neutral pH, acids and bases may form their conjugates and appear as two peaks or as a broad peak. Figures 56 and 57 illustrate this effect and how the use of TFA forces these compounds into a single peak.

When diphenyl acetic acid is purified from esculin (Figure 57) without TFA, the diphenyl acetic acid shows as two peaks at 3.5 and 9 column volumes. The chromatogram appears as if it has contains three compounds with the first tailing into the esculin peak. One hint that the first peak is diphenyl acetic acid is that it tails back while the peak at 6 column volumes tails forward due to the conversion between diphenyl acetic acid and its conjugate base. In more extreme examples, the two peaks will appear to be joined together by the tailing (“bridging”).

Solvent modifiers are not required when purifying neutral compounds.

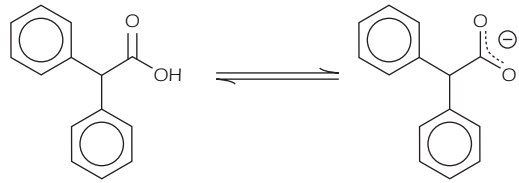


Figure 56: Diagram of interconversion of diphenyl acetic acid *and its conjugate base*

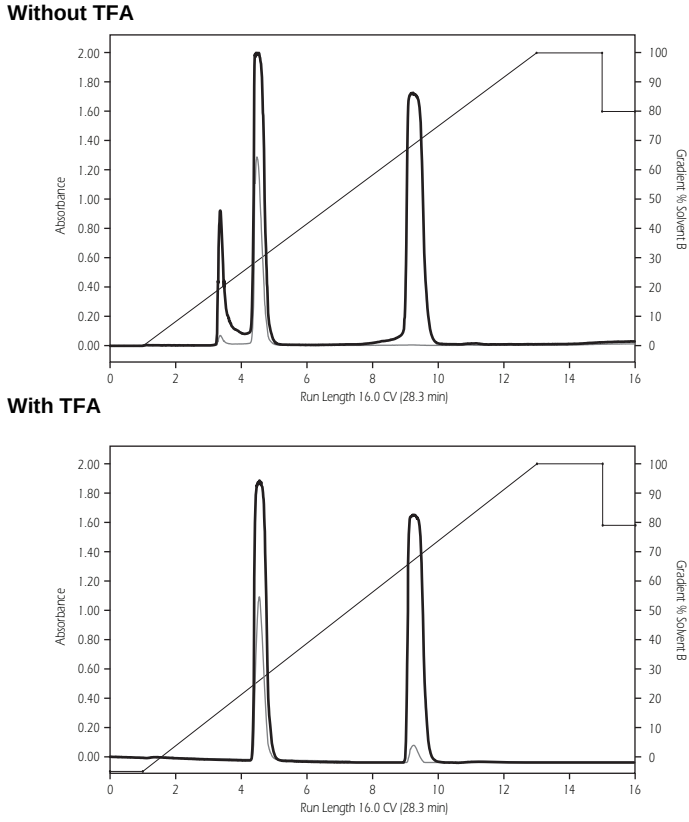
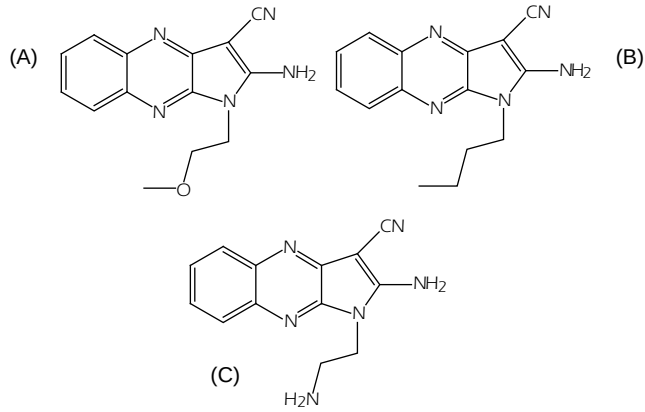


Figure 57: Illustration of esculin/diphenyl acetic acid purifications without and with TFA modifier

Low-solubility Polar Heterocycles

The separation of a mixture of quinoxalines was investigated:



Flash chromatography of the quinoxalines mixture on a Combi-*Flash* Companion system with a C18 reversed phase 4.3g Redi*Sep* column fully separated the products with water + 0.1% TFA/acetonitrile + 0.1% TFA as the mobile phase (Figure 58).

The sample was loaded using the solid sample technique by dissolving the sample in a necessarily high amount of methanol. C18 reversed-phase silica was then added and the solvent was removed *in vacuo*. The resulting pre-coated sample was placed in an empty Redi*Sep* solid load cartridge.

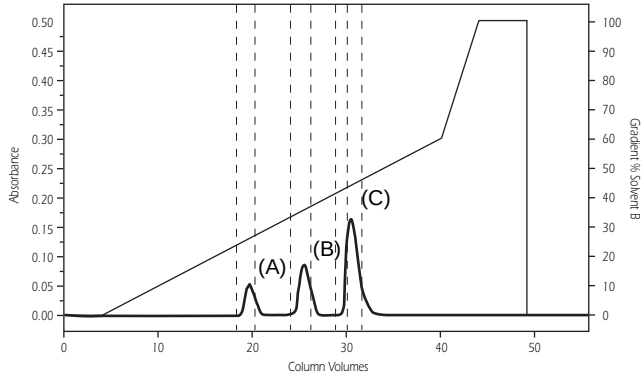
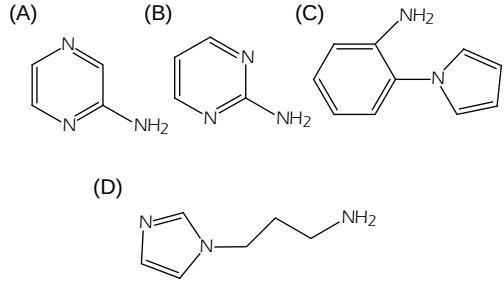


Figure 58: *Chromatogram of quinoxaline mixture purification* on C18 reversed phase RediSep column with water + 0.1% TFA/acetonitrile+ 0.1% TFA

In addition to C18 reversed phase Redi*Sep* Rf columns, amine or basic alumina Redi*Sep* Rf columns are also options to consider for the purification of low-solubility polar heterocycles. Method development would indicate which column and solvents to select.

Primary Amines

The separation of a mixture of primary amines was investigated:



Flash chromatography of the primary amines mixture on a Combi-*Flash* Companion 4x system with C18 reversed phase 4.3g Redi*Sep* column separated the products (Figure 59).

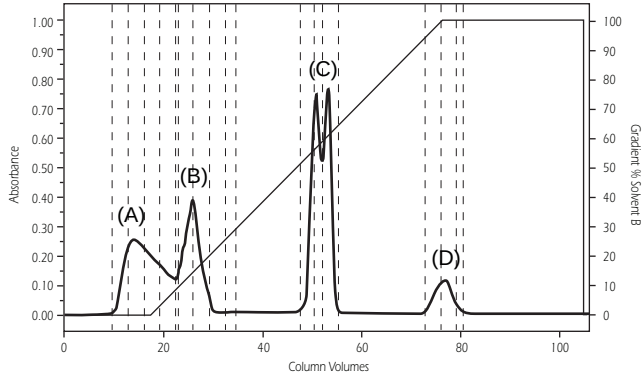
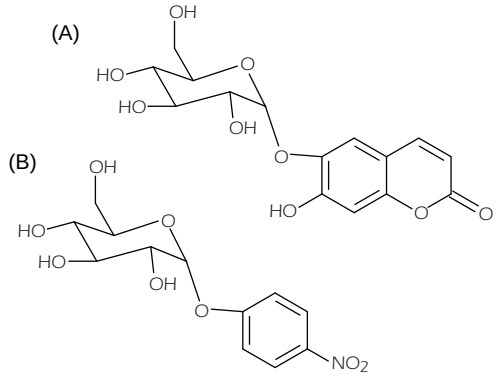


Figure 59: Chromatogram of primary amine mixture purification on C18 reversed phase RediSep column with water/acetonitrile

In addition to C18 reversed phase Redi*Sep* Rf columns, amine or basic alumina Redi*Sep* Rf columns are also options to consider for the purification of primary amines. Method development would indicate which column and solvents to select.

Carbohydrates

The separation of a mixture of carbohydrates was investigated:



Flash chromatography of the carbohydrates mixture on Teledyne Isco Combi*Flash* Companion 4x system with C18 reversed phase 13 g Redi*Sep* column fully separated the products with water/acetonitrile as the mobile phase (Figure 60). The use of a high performance C18 column is discussed later in this chapter.

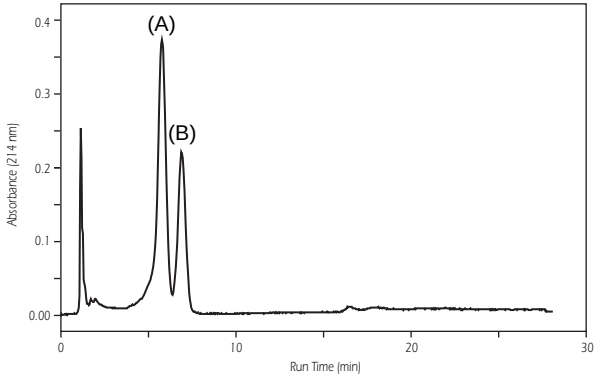


Figure 60: Chromatogram of carbohydrate mixture purification on C18 reversed phase RediSep column with water/acetonitrile

Evaporative Light Scattering Detection (ELSD) is useful when the compound possesses no chromophores. Combi*Flash* Rf and Torrent systems can cut peaks based on a peak signal from an external ELSD detector.

Peptides

The separation of a mixture of peptides was investigated:

Gly-Pro-Ala (A) Val-Tyr-Val (B)

Flash chromatography of the peptides mixture on a Teledyne Isco Combi*Flash* Companion 4x system with C18 reversed phase 13g Redi*Sep* column fully separated the products with water + 0.1% TFA/acetonitrile + 0.1% TFA as the mobile phase (Figure 61).

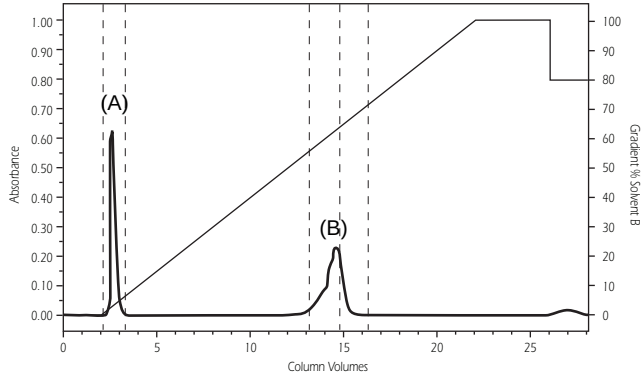
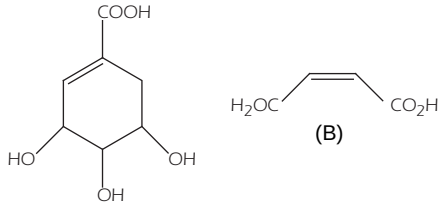


Figure 61: Chromatogram of peptide mixture purification on C18 reversed phase RediSep column with water + 0.1% TFA/acetonitrile + 0.1% TFA

Carboxylic Acids

The separation of a mixture of shikimic acid (A) and maleic acid (B) was investigated:



Flash chromatography of the carboxylic acids mixture on a Teledyne Isco Combi*Flash* Companion 4x with C18 reversed phase 4.3g Redi*Sep* column fully separated the products with water + 0.1% TFA/acetonitrile+ 0.1% TFA as the mobile phase (Figure 62).

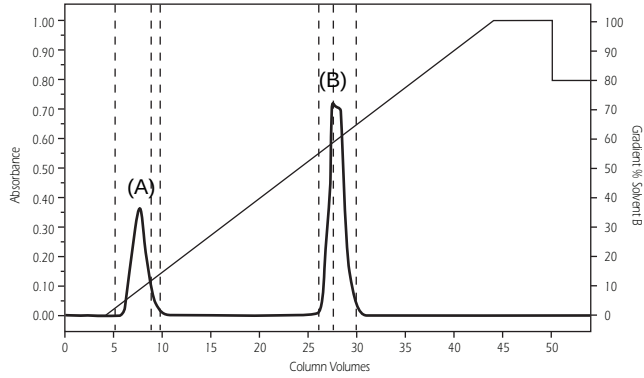
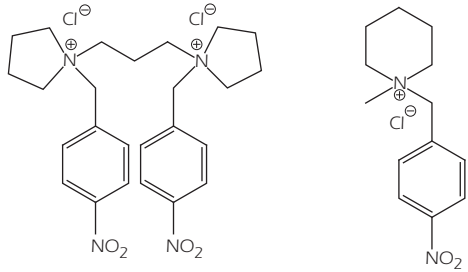


Figure 62: Chromatogram of carboxylic acid mixture purification on C18 reversed phase RediSep column with water + 0.1% TFA/acetonitrile+ 0.1% TFA

Ionic Compounds

The separation of a mixture of pyrrolidinium (A) and piperidinium chlorides (B) was investigated:



Flash chromatography of the ionic mixture on a Teledyne Isco Combi*Flash* Companion 4x system with C18 reversed phase 13g Redi*Sep* column separated the products with water + 0.1% TFA/acetonitrile + 0.1% TFA as the mobile phase (Figure 63).

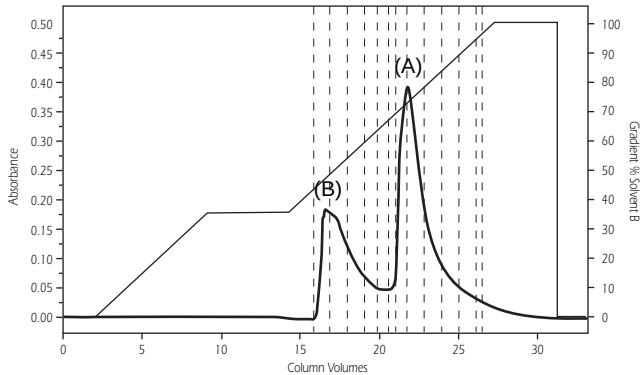
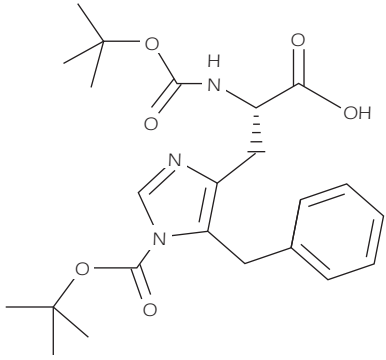


Figure 63: Chromatogram of ionic mixture purification on C18 reversed phase RediSep column with water + 0.1% TFA/acetonitrile + 0.1% TFA

RediSep Rf Gold High Performance C18 Columns

Although the standard Redi*Sep* Rf C18 columns purify most compounds well, there are times when greater resolution is required. Redi*Sep* Rf Gold C18 columns can be used for these purifications. This material provides near-HPLC performance at lower pressure and cost. Redi*Sep* Rf Gold C18 columns utilize the benefits provided by spherical silica in a smaller 30 µm average particle size. Due to their spherical shape, back pressures are 100–150 psi at flow rates providing optimal separation—well within the range of modern Flash equipment.

The purification of a mixture containing 5-benzyl-N-α-N-im-di-*t*-Boc-L-His (Compound A) was investigated[2]:



Compound A was purified on a Combi*Flash* Rf system using Redi*Sep* Rf Gold C18 and standard Redi*Sep* Rf C18 columns (Figure 64). The solvent system was 5–95% ACN:H_2O) both containing 0.1% TFA. The Redi*Sep* Rf Gold C18 column (Figure 65) allowed much higher loading and provided a sharper peak.

For comparison, the same sample was purified using a Waters DeltaPrep 4000 using a Vydac 10×250 mm column, 5µ particle size (Figure 66). Analytical HPLC showed no difference in purity between the compound eluted from the HPLC or that from the Combi*Flash* Rf system using a Redi*Sep* Rf Gold C18 column (Figure 67).

2. The collaboration of Dr. David Smith at Creighton University, School of Pharmacy is gratefully acknowledged.

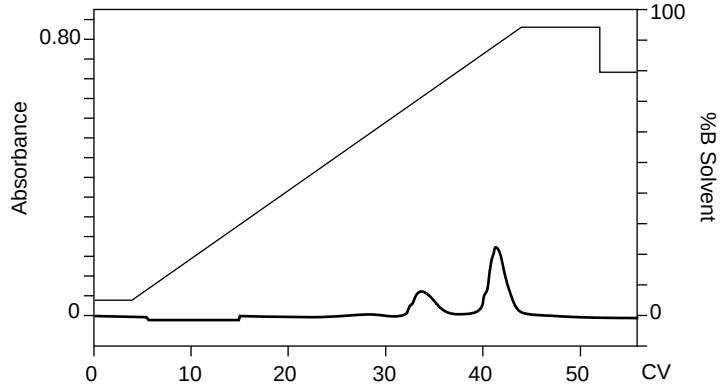


Figure 64: Chromatogram of 10 mg compound A purification on a 13g RediSep Rf C18 column

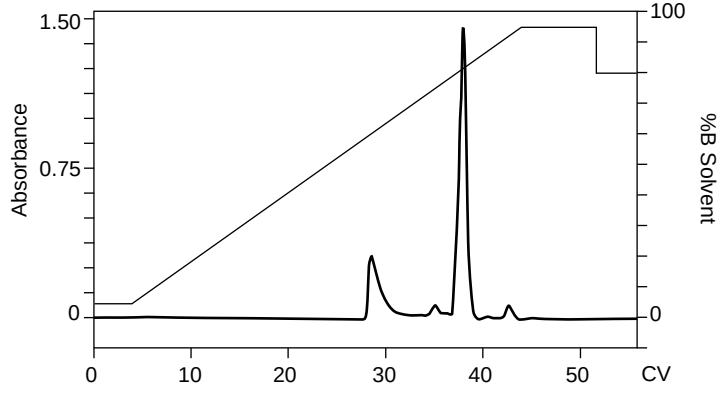


Figure 65: Chromatogram of 46 mg compound A purification on a 15.5g RediSep Rf Gold C18 column

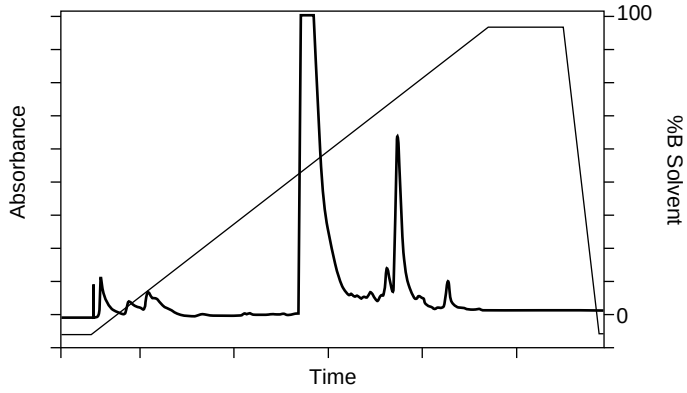


Figure 66: Chromatogram of 10 mg compound A purification on a Waters DeltaPrep 4000 system using a Vydac 10x250 mm column, 5µ particle size

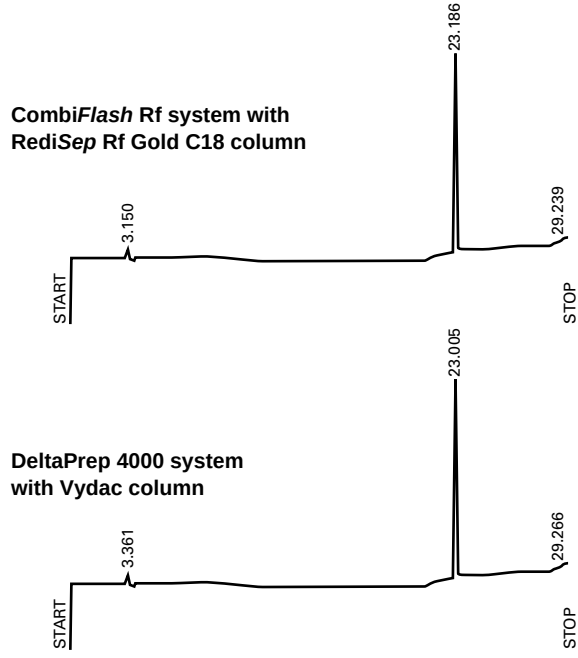


Figure 67: Illustration of analytical HPLC to compare purity of compound A separated on two systems.

RediSep Gold C18 Columns at High pH

Many compounds generate multiple ionic species during the course of C18 purification that cause broadened peaks which reduce resolution. If the interconversion of the compounds is slow, each ionic compound may appear as an individual peak. Trifluoroacetic acid (TFA) often is used for ion pairing (with basic compounds) or ion suppression (acidic compounds) since C18 columns are compatible with low pH.

There is a need to purify compounds on reverse phase column at higher pH. Many compounds are acid sensitive yet form peak-broadening ionic species at neutral pH. Purifying the compounds at basic pH would improve their purity and yield. C18 columns typically are not stable at pH greater than 7.5 because the basic solution dissolves the silica underlying the C18 chains, eventually washing the C18 away and causing a decrease in column performance. These changes take the form of reduced plate counts or changes in retention time. Redi*Sep* Rf Gold C18 columns are end-capped to resist attack of the silica by the mobile phase.

Redi*Sep* Rf Gold C18 columns can be used for purifying compounds up to pH 10 with only minimal change over time. Compared to steel C18 prep HPLC columns, Redi*Sep* Rf Gold C18 columns are inexpensive and useful for milligram to gram scale purifications.

Redi*Sep* Rf Gold columns have been run for several hours exposed to pH 10 mobile phases. Like other silica-based C18 columns, the Redi*Sep* Rf Gold columns will degrade over time. However, the degradation is slow enough that the column is still usable for many runs. The degradation is reduced as the pH is lowered or exposure time is decreased.

Storage of the column after use in high pH

After use, wash the column with 5 column volumes of the organic solvent. Run the column with a standard gradient method from 5 to 95% organic solvent in unbuffered water. The column was stored in 80% organic solvent in water as described earlier (*Column Care*, on page 61). The extended wash is intended to remove as much of the ammonium hydroxide from the column as possible. After washing store the column in 80% methanol or acetonitrile. The column can be stored indefinitely without further degradation as long as it does not dry out.

Figure 68: Table of Reusable RediSep Rf Gold C18 Reversed Phase columns, 20–40 microns *Sample loading capacities*

Sample Size	Column Size (g)	Teledyne Isco Part Number	Description
5.5 - 110 mg	5.5	69-2203-328	5.5 gram RediSep Rf Gold C18 columns, pkg. of 2.
15.5 - 310 mg	15.5	69-2203-334	15.5 gram RediSep Rf Gold C18 column, pkg. of 1.
30 - 600 mg	30	69-2203-335	30 gram RediSep Rf Gold C18 column, pkg. of 1.
50 mg - 1.0 g	50	69-2203-336	50 gram RediSep Rf Gold C18 column, pkg. of 1.
100 mg - 2 g	100	69-2203-337	100 gram RediSep Rf Gold C18 column, pkg. of 1.
150 mg - 3 g	150	69-2203-338	150 gram RediSep Rf Gold C18 column, pkg. of 1.
275 mg - 5.5 g	275	69-2203-339	275 gram RediSep Rf Gold C18 column, pkg. of 1.
415 mg - 8.3 g	415	69-2203-341	415 gram RediSep Rf Gold C18 column, pkg. of 1.
0.95 - 19 g	950	69-2203-492	950 gram RediSep Rf Gold C18 column, pkg. of 1.
1.9 - 38 g	1900	69-2203-493	1.9 kg RediSep Rf Gold C18 column, pkg. of 1.
3.8 - 76 g	3800	69-2203-528	3.8 kg RediSep Rf Gold C18 column, pkg. of 1.

Chapter 5

Advanced Flash Chromatography

Alternative Chromatographic Media

Chemists generally proceed with normal phase silica gel for purification of medium to low polarity products. Reversed phase silica gel is used for high polarity products.

When a mixture cannot be separated on normal or reversed phase silica gel, or when the use of solvent modifiers becomes impractical, the alternative is to use specialty stationary phase media. Specialty media offers different adsorptive properties and selectivities, such as by acidic or basic moiety, or by ionic charge.

Media options include:

- Normal-phase silica
- Amine
- Alumina-basic
- Alumina-neutral
- Alumina-acidic
- C18 reversed phase silica
- Cyano
- Diol
- Strong anion exchange
- Strong cation exchange

Specialty Media

Some specialty media have the carbon tether chain “end-capped” with a functional group conferring the stationary phase specific properties useful for some delicate separations.

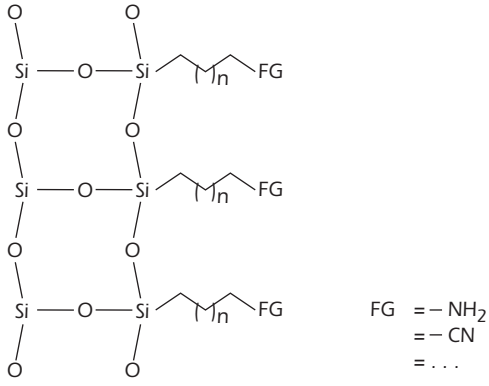


Figure 69: Diagram of functionalized media *Binding chains of functional groups to the tetrahedral silica can create media with an affinity for a specialized characteristic of a compound.*

Some examples of specialty columns and their applications are listed in the following paragraphs.

Amine

Compounds that have an acidic or basic moiety may streak or tail with normal or reversed phase silica. Streaking or tailing will ultimately cause overlapping fractions.

Typically, when using silica, chemists spike their solvents with either triethylamine (TEA) if they have a basic component, or acetic acid (AcOH) if they have an acidic component in their target compound. The problem here is that solvents have to be swapped and primed before compounds can be separated, then purged from the system after the run.

With an acid or base moiety covalently bound to the stationary phase, the need to switch solvents is eliminated. Additionally, TEA or AcOH are not used so they don't need to be removed after the purification is complete.

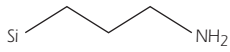
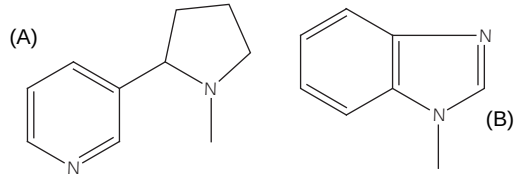


Figure 70: Diagram of amine structure

To illustrate how an amine media can be of assistance, the separation of a mixture of nicotine (A) and 1-methylbenzimidazole (B) was investigated.



Separation of the mixture on normal phase and amine functionalized silica were examined.

Flash chromatography of the heterocycles mixture on a Teledyne Isco Combi*Flash* Companion 4x with an amine functionalized Redi*Sep* column fully separated the products with hexane/ethyl acetate as the mobile phase (Figures 71 and 72). The normal phase column did not separate them correctly.

The amine functionalized silica can be used in either normal or reversed phase conditions. It is useful in purification of compounds holding basic properties. This is particularly true if spiking with TEA is required for purification with normal phase silica. Although amine columns can be used with reversed phase solvents, it is easiest to think of amine as a normal phase column, where a more polar solvent causes faster elution of the compounds.

The use of an amine column can eliminate the need to spike with TEA, and as a result, reduce time required to remove the TEA mixed with the solvent from purified fractions.

Amine columns are reusable because the amine group is bonded to the silica. After the first use, do not allow the column to dry out since drying the column will adversely affect future purifications. Turn off the air purge on your Flash system's method. The Combi*Flash* Rf system will turn off the air purge by reading the column RFID tag. Remove all organic modifiers by flushing with three column volumes of 80% acetonitrile in water or 100% isopropanol and store the column in the wash solvent. If the storage solvents are immiscible with the solvents used for the separation, you may need to wash the column with an intermediate solvent prior to storage.

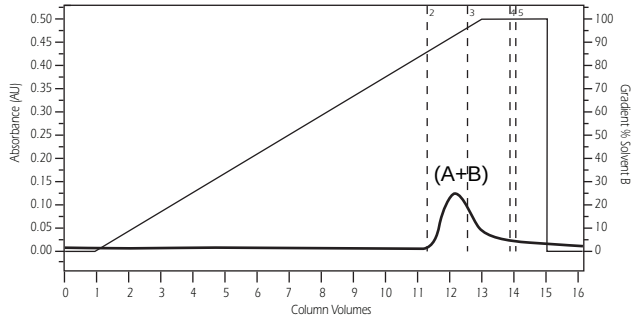


Figure 71: Chromatogram of normal phase column elution with hexane/ethyl acetate Heterocycles mixture did not separated.

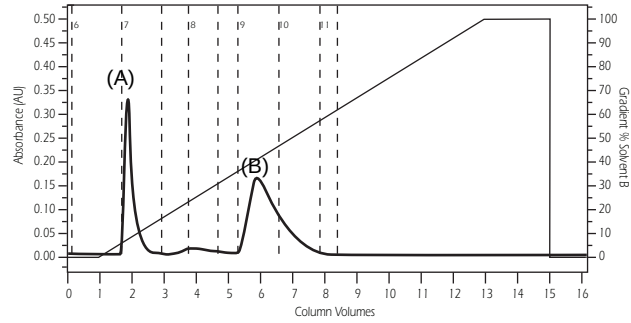


Figure 72: Chromatogram of amine functionalized column with hexane/ethyl acetate Heterocycles mixture successfully separated.

Ketones and aldehydes may react with the aminopropyl stationary phase to form imines so care should be taken when purifying these materials. A small amount should be purified first on a small column, and scaled up if the purification is successful.

Method development for amine columns can be done using a sample with a small column.

Figure 73: Table of Reusable RediSep Rf Amine Columns
40–60 microns

Sample Size	Column Size (g)	Teledyne Isco Part Number	Description
23 - 235 mg	4.7	69-2203-350	4.7 gram Amine RediSep Rf columns, pkg. of 2.
70 mg - 0.7 g	14	69-2203-351	14 gram Amine RediSep Rf column, pkg. of 1.
140 mg - 1.4 g	28	69-2203-352	28 gram Amine RediSep Rf column, pkg. of 1.
235 mg - 2.35 g	47	69-2203-353	47 gram Amine RediSep Rf column, pkg. of 1.
470 mg - 4.7 g	94	69-2203-356	94 gram Amine RediSep Rf column, pkg. of 1.
0.7 - 7 g	140	69-2203-354	140 gram Amine RediSep Rf column, pkg of 1.
1.3 - 12.8 g	260	69-2203-358	260 gram Amine RediSep Rf column, pkg. of 1.
1.92 - 19.25 g	385	69-2203-355	385 gram Amine RediSep Rf column, pkg. of 1.

Figure 74: Table of Reusable RediSep Rf Gold Amine Columns
20–40 microns

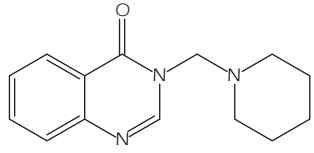
Sample Size	Column Size (g)	Teledyne Isco Part Number	Description
5.5 - 110 mg	5.5	69-2203-504	5.5 gram Amine RediSep Rf Gold columns, pkg. of 2.
15.5 - 310 mg	15.5	69-2203-505	15.5 gram Amine RediSep Rf Gold column, pkg. of 1.
30 - 600 mg	30	69-2203-506	30 gram Amine RediSep Rf Gold column, pkg. of 1.
50 mg - 1.0 g	50	69-2203-507	50 gram Amine RediSep Rf Gold column, pkg. of 1.
100 mg - 2 g	100	69-2203-508	100 gram Amine RediSep Rf Gold column, pkg. of 1.
150 mg - 3 g	150	69-2203-509	150 gram Amine RediSep Rf Gold column, pkg. of 1.
275 mg - 5.5 g	275	69-2203-510	275 gram Amine RediSep Rf Gold column, pkg. of 1.
415 mg - 8.3 g	415	69-2203-511	415 gram Amine RediSep Rf Gold column, pkg. of 1.
0.9 - 19 g	950	69-2203-512	950 gram Amine RediSep Rf Gold column, pkg. of 1.
1.9 - 38 g	1900	69-2203-513	1.9 kg Amine RediSep Rf Gold column, pkg. of 1.
3.8 - 76 g	3800	69-2203-534	3.8 kg Amine RediSep Rf Gold column, pkg. of 1.

Basic Alumina

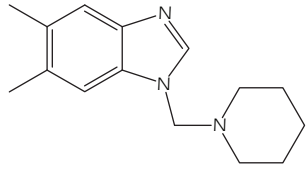
Basic alumina is a mixture of different aluminum oxides that are partially dehydrated. The intrinsic basicity of this media gives it similar applications to the amine media.

To illustrate how a basic alumina media can be of assistance, the separation of a mixture of quinazolinone and benzimidazole derivatives was investigated.

(A) 3-(1-piperdinylmethyl)-4(3H)-quinazolinone



(B) 5,6-dimethyl-1-(piperdinomethyl)benzimidazole



Flash chromatography of the heterocycles mixture on a Teledyne Isco Combi*Flash* Companion 4x with a normal phase silica gel did not separate the two products successfully (Figure 75).

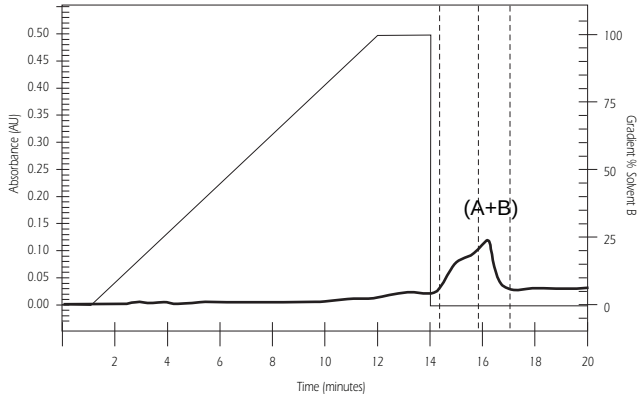


Figure 75: Chromatogram of normal phase silica column
Elution with hexane/ethyl acetate

However, the use of basic alumina Redi*Sep* column successfully separated the two nitrogen-containing heterocycles (Figure 76).

Although the two product peaks on the chromatogram show incomplete baseline resolution, analytical examination of resulting fractions has shown limited cross-contamination.

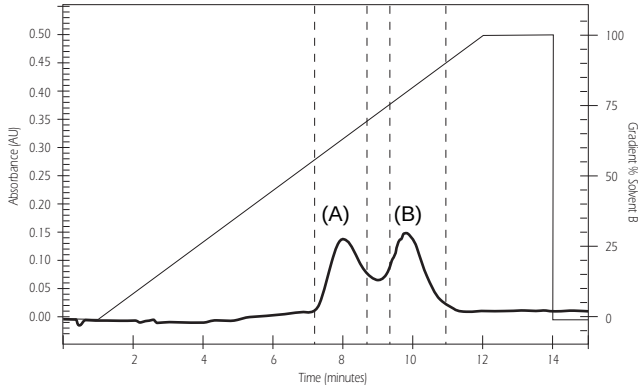


Figure 76: Chromatogram of basic alumina column
Elution with hexane/ethyl acetate

Alumina columns are not multiple use columns, so there is no need for cleaning these columns.

Methods can be developed on basic alumina TLC plates.

Figure 77: Table of solvent migration and plate development time for RediSep Basic Alumina TLC plates *(69-2203-403)*

Solvent	Migration
Hexane	Full, 15 min
Ethyl acetate	Full, 15 min
Isopropanol	Full, 30 min
Hexane/Ethyl acetate 1:1	Full, 15 min
Hexane/Isopropanol 5:1	Full, 30 min
Dichloromethane	Full, 15 min
Methanol	Full, 15 min
Dichloromethane/Methanol 9:1	Full, 15 min

Figure 78: Table of RediSep Rf Alumina Basic Columns

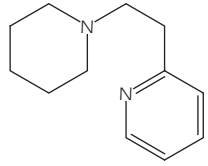
Sample Size	Column Size (g)	Teledyne Isco Part Number	Description
40 - 320 mg	8	69-2203-450	8 gram Alumina Basic RediSep Rf columns, pkg. of 20.
120 - 960 mg	24	69-2203-451	24 gram Alumina Basic RediSep Rf columns, pkg. of 20.
240 mg - 1.92 g	48	69-2203-452	48 gram Alumina Basic RediSep Rf columns, pkg. of 15.
400 mg - 3.2 g	80	69-2203-453	80 gram Alumina Basic RediSep Rf columns, pkg. of 15.
0.8 - 6.4 g	160	69-2203-456	160 gram Alumina Basic RediSep Rf columns, pkg. of 12.
1.2 - 9.6 g	240	69-2203-454	240 gram Alumina Basic RediSep Rf columns, pkg. of 10.
2.2 - 17.6 g	440	69-2203-458	440 gram Alumina Basic RediSep Rf columns, pkg. of 6.
3.3 - 26.4 g	660	69-2203-455	660 gram Alumina Basic RediSep Rf columns, pkg. of 4.

Neutral Alumina

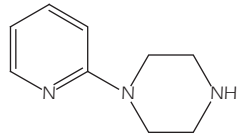
Neutral alumina is a useful media in situations when acid-sensitive products partially or fully degrade during purification due to the intrinsic slight acidity of normal phase silica gel. The neutral properties of this media also allows the purification of substances holding basic properties.

To illustrate this latter possibility, the separation of a mixture of two pyridine derivatives was investigated.

(A) 2-(2-piperdinoethyl)pyridine



(B) 1-(2-pyridiyl) piperazine



Flash chromatography of the heterocycles mixture on a normal phase Redi*Sep* column did not separate the two products successfully (Figure 79).

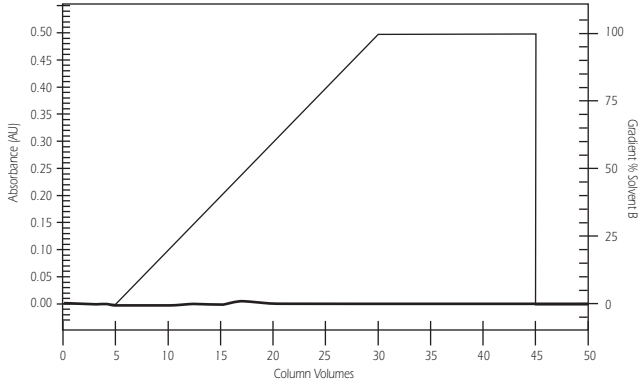


Figure 79: Chromatogram of normal phase silica column
Elution with hexane/ethyl acetate – peaks not visible.

However, the use of neutral alumina Redi*Sep* column successfully separated the two nitrogen-containing heterocycles (Figure 80).

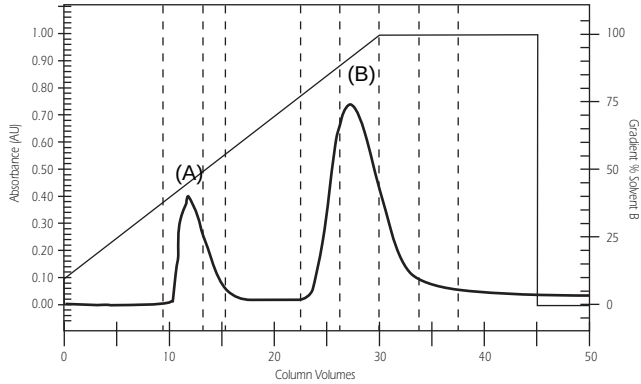


Figure 80: *Chromatogram of neutral alumina column*
Elution with hexane/ethyl acetate

Alumina columns are not multiple use columns, so there is no information for cleaning and storing these columns.

The characteristics that allow Redi*Sep* Alumina to work well for chromatography allow it to be a useful support for catalysts.

Figure 81: Table of RediSep Rf Alumina Neutral Columns

Sample Size	Column Size (g)	Teledyne Isco Part Number	Alumina Neutral RediSep Rf column Description
40 - 320 mg	8	69-2203-440	8 gram columns, pkg. of 20.
120 - 960 mg	24	69-2203-441	24 gram columns, pkg. of 20.
240 mg - 1.92 g	48	69-2203-442	48 gram columns, pkg. of 15.
400 mg - 3.2 g	80	69-2203-443	80 gram columns, pkg. of 15.
800 mg - 6.4 g	160	69-2203-446	160 gram columns, pkg. of 12.
1.2 - 9.6 g	240	69-2203-444	240 gram columns, pkg. of 10.
2.2 - 17.6 g	440	69-2203-448	440 gram columns, pkg. of 6.
3.3 - 26.4 g	660	69-2203-445	660 gram columns, pkg. of 4.

Figure 82: Table of RediSep Rf Alumina Acidic Columns

Sample Size	Column Size (g)	Teledyne Isco Part Number	Alumina Acidic RediSep Rf column Description
5.5 - 110 mg	8	69-2203-430	8 gram columns, pkg of 20.
15.5 - 310 mg	24	69-2203-431	24 gram columns, pkg. of 20.
30 - 600 mg	48	69-2203-432	48 gram columns, pkg. of 15.
50 mg - 1.0g	80	69-2203-433	80 gram columns, pkg. of 15.
100 mg - 2 g	160	69-2203-436	160 gram columns, pkg. of 12.
150 mg - 3 g	240	69-2203-434	240 gram columns, pkg. of 10.
415 mg - 8.3 g	440	69-2203-438	440 gram columns, pkg. of 6.
0.9 - 19 g	660	69-2203-435	660 gram columns, pkg. of 4.

Cyano

Cyano functionalized silica acts very similar to normal phase silica when using similar solvents.

In reversed phase conditions, it is similar to C4 reversed phase columns, although the elution order and selectivity of compounds may be different. This allows chemists to purify compounds that may not be well resolved on C18.

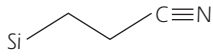


Figure 83: Diagram of cyano structure

Cyano columns are reusable. After the first use, do not allow the column to dry out since drying the column will adversely affect future purifications. Turn off the air purge on your Flash system's method. The Combi*Flash* Rf system will turn off the air purge by reading the column RFID tag. Remove all organic modifiers by flushing with three column volumes of 80% acetonitrile in water or 100% isopropanol and store the column in the wash solvent. If the storage solvents are immiscible with the solvents used for the separation, you may need to wash the column with an intermediate solvent prior to storage.

Figure 84: Table of Reusable RediSep Rf Gold Cyano Columns
20–40 microns

Sample Size	Column Size (g)	Teledyne Isco Part Number	Description
5.5 - 110 mg	5.5	69-2203-494	5.5 gram Cyano RediSep Rf Gold columns, pkg. of 2.
15.5 - 310 mg	15.5	69-2203-495	15.5 gram Cyano RediSep Rf Gold column, pkg. of 1.
30 - 600 mg	30	69-2203-496	30 gram Cyano RediSep Rf Gold column, pkg. of 1.
50 mg - 1.0 g	50	69-2203-497	50 gram Cyano RediSep Rf Gold column, pkg. of 1.
100 mg - 2 g	100	69-2203-498	100 gram Cyano RediSep Rf Gold column, pkg. of 1.
150 mg - 3 g	150	69-2203-499	150 gram Cyano RediSep Rf Gold column, pkg. of 1.
275 mg - 5.5 g	275	69-2203-500	275 gram Cyano RediSep Rf Gold column, pkg. of 1.
415 mg - 8.3 g	415	69-2203-501	415 gram Cyano RediSep Rf Gold column, pkg. of 1.
0.9 - 19 g	950	69-2203-502	950 gram Cyano RediSep Rf Gold column, pkg. of 1.
1.9 - 38 g	1900	69-2203-503	1.9 kg Cyano RediSep Rf Gold column, pkg. of 1.
3.8 - 76 g	3800	69-2203-533	3.8 kg Cyano RediSep Rf Gold column, pkg. of 1.

Diol

Diol functionalized silica is less polar and has higher retention time than normal phase bare silica. Diol functionalized silica offers an interesting alternative to normal phase bare silica for difficult separations. The OH moieties on diol are less active than those on silica making this media useful for compounds that decompose or are difficult to elute with silica. Being normal phase, diol uses solvents that are easy-to-evaporate compared to solvents used in reverse phase.

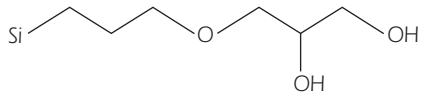


Figure 85: Diagram of diol structure

Diol columns are reusable. After the first use, do not allow the column to dry out since drying the column will adversely affect future purifications. Turn off the air purge on your Flash system's method. The Combi*Flash* Rf system will turn off the air purge by reading the column RFID tag. Remove all organic modifiers by flushing with three column volumes of 80% acetonitrile in water or 100% isopropanol and store the column in the wash solvent. If the storage solvents are immiscible with the solvents used for the separation, you may need to wash the column with an intermediate solvent prior to storage.

Diol columns work well with long-chain compounds. Diol columns are useful for compounds that are unstable on silica or irreversibly bind to silica.

Method development with diol columns is similar to that of silica gel. TLC plates can be used for non-aqueous solvent systems. If aqueous systems are required, it is faster to develop the method with a small diol column using a small amount of sample. Although diol columns are compatible with reverse phase solvents, it is easiest to think of the column as always working in normal phase with the ability to run aqueous solvent systems. The combination of a less active surface and the ability to elute with a strong water solvent allows diol to be used with a wide range of compounds.

Long hydrocarbon chain compounds on diol — Compounds containing long organic chains are easily purified on diol compared to silica. Diol is not as polar as silica so compounds with a strong hydrocarbon character interact better with the stationary phase and do not elute all at once when a slightly more polar solvent is run on the column.

Oleyl Glycerate — Oleyl glycerate is made from oleic acid[3], which is available at only 85% purity. The desired compound can be easily purified with a diol column. The compounds all eluted together using silica (Figure 86).

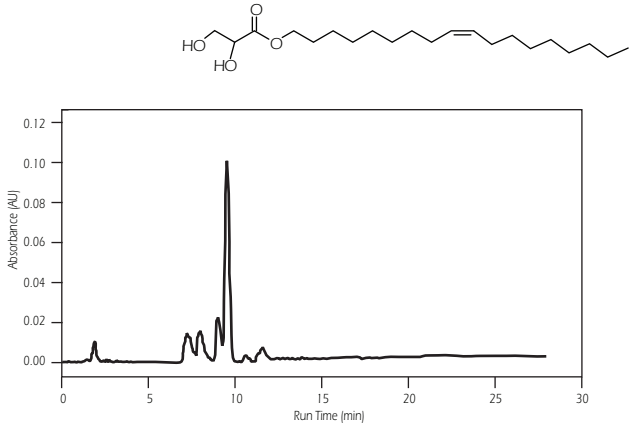


Figure 86: *Purification of oleyl glycerate* with RediSep Rf Gold diol column

Tocopherols — Tocopherols represent another example of compounds with a long hydrocarbon chain. Diol columns were used to isolate the tocopherols from a variety of vegetable oils. The purification was attempted on silica gel but the tocopherols eluted immediately upon addition of any amount of "B" solvent. The diol column provided greater control (Figure 87).

3. Fong, C.; Wells, D.; Krodkiewska, I.; Booth, J.; Hartley, P.G. Synthesis and Mesophases of Glycerate Surfactants *J. Phys Chem B* **2007**, *111*, 1384

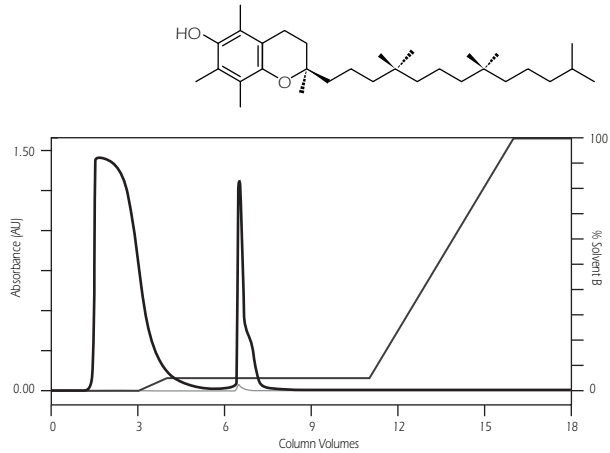


Figure 87: Purification of tocopherols from corn oil *with RediSep Rf Gold diol column*

Diol Column Versatility — As an example of the versatility of the diol column, a methanolic extract of green tea was eluted from a diol column with a gradient starting a hexane/isopropanol gradient and ending with an isopropanol/water gradient in a single run (Figure 88). The sample was adsorbed onto celite. Compounds eluted include chlorophylls (A), xanthine alkaloids (B), catechins (B), and tannins (C). The tannins would bind to silica.

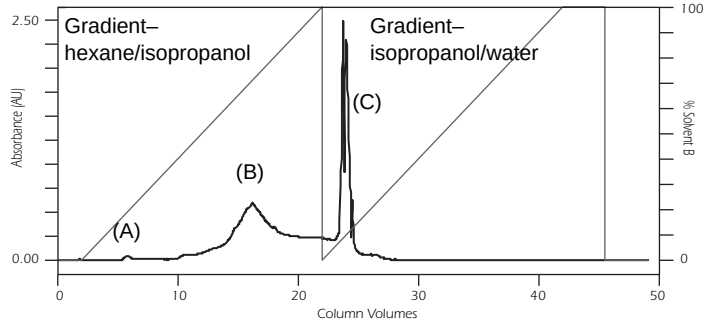


Figure 88: Purification of green tea extract *using a RediSep Rf Gold diol column*

Care should be taken when purifying ketones, 1°, or 2° amines as these may react with the diol stationary phase. Care should also be taken when using acetone (or other ketones) as a solvent as they may react with the diol side chain, especially if trace amounts of acids are present. If in doubt, try the purification on a small column and scale up to a larger column if the purification is successful.

Figure 89: Table of Reusable RediSep Rf Gold Diol Columns
20–40 microns

Sample Size	Column Size (g)	Teledyne Isco Part Number	Description
5.5 - 110 mg	5.5	69-2203-514	5.5 gram Diol RediSep Rf Gold columns, pkg. of 2.
15.5 - 310 mg	15.5	69-2203-515	15.5 gram Diol RediSep Rf Gold column, pkg. of 1.
30 - 600 mg	30	69-2203-516	30 gram Diol RediSep Rf Gold column, pkg. of 1.
50 mg - 1.0 g	50	69-2203-517	50 gram Diol RediSep Rf Gold column, pkg. of 1.
100 mg - 2 g	100	69-2203-518	100 gram Diol RediSep Rf Gold column, pkg. of 1.
150 mg - 3 g	150	69-2203-519	150 gram Diol RediSep Rf Gold column, pkg. of 1.
275 mg - 5.5 g	275	69-2203-520	275 gram Diol RediSep Rf Gold column, pkg. of 1.
415 mg - 8.3 g	415	69-2203-521	415 gram Diol RediSep Rf Gold column, pkg. of 1.
0.9 - 19 g	950	69-2203-522	950 gram Diol RediSep Rf Gold column, pkg. of 1.
1.9 - 38 g	1900	69-2203-523	1900 gram Diol RediSep Rf Gold column, pkg. of 1.
3.8 - 76 g	3800	69-2203-535	3.8 kg Diol RediSep Rf Gold column, pkg. of 1.

Ion Exchange Media

Various strong and weak anion or cation exchange bonded silica gel are available and can be used for the separation of almost any type of charged molecule, from large proteins to small nucleotides and amino acids.

SCX

The SCX (Strong Cation Exchange) media is a silica-bound tosic acid.

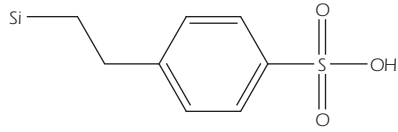


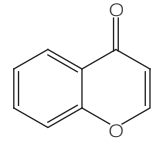
Figure 90: Diagram of SCX structure

The strong acidity of this media induces the full retention of any compounds with basic properties subjected through it. This intrinsic media property can be exploited several ways.

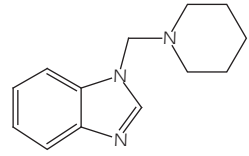
SCX media can be used as a practical and efficient tool for the selective isolation of either basic or non-basic compounds from a crude reaction mixture containing both.

To illustrate how SCX media can be of assistance, the separation of a mixture of chromone and a benzimidazole derivative was investigated.

(A) Chromone



(B) 1-(1-piperidinylmethyl)-1H-benzimidazole



Flash chromatography of the mixture on a normal phase silica Redi*Sep* column showed release of the two products (Figure 91).

The use of a Redi*Sep* SCX column showed total retention of the benzimidazole derivative onto the column and release of the chromone (Figure 92).

This isolation of the basic compound, while allowing one or more organic compounds holding neutral properties to migrate freely through the column, demonstrates that the column can be effective as a clean up tool.

Conversely, the SCX media also represents a practical tool for the isolation of desired compounds holding basic properties. In this case, the contaminants would be the neutral compounds which would be immediately released then discarded by the SCX column run. The compounds holding basic properties retained by the SCX column are liberated by injecting a solution of ammonia in methanol. In this case, the SCX media works as a catch and release process.

SCX columns may be reused. After the first use, do not allow the column to dry out since drying the column will adversely affect future purifications. Turn off the air purge on your Flash system's method. The Combi*Flash* Rf system will turn off the air purge by reading the column RFID tag. Regenerate the column with 10 column volumes of 1M acetic acid and store in 80% methanol in water or 100% isopropanol.

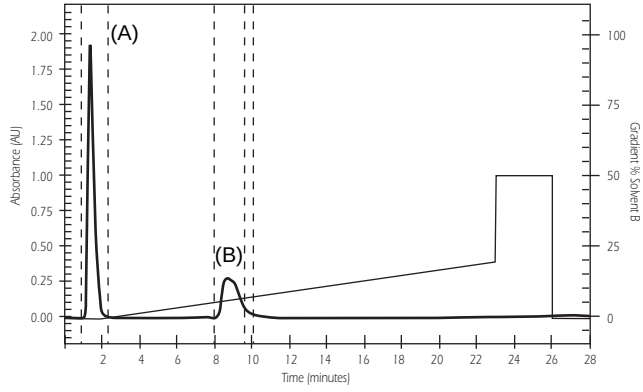


Figure 91: Chromatogram of normal phase column Elution with dichloromethane/methanol

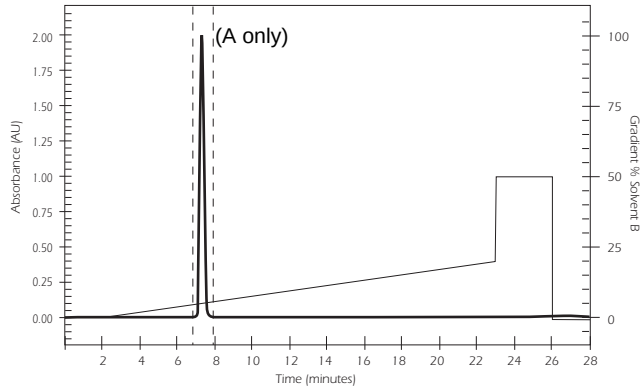


Figure 92: Chromatogram of SCX column Elution with dichloromethane/methanol

Figure 93: Table of Reusable RediSep Rf SCX Columns

Sample Load[a]	Column Size (g)	Teledyne Isco Part Number	Description
≤3.5 mMol	5	69-2203-390	5 gram Strong Cation Exchange RediSep Rf columns, pkg. of 2.
≤10.5 mMol	15	69-2203-391	15 gram Strong Cation Exchange RediSep Rf column, pkg. of 1.
≤21 mMol	30	69-2203-392	30 gram Strong Cation Exchange RediSep Rf column, pkg. of 1.
≤35 mMol	50	69-2203-393	50 gram Strong Cation Exchange RediSep Rf column, pkg. of 1.
≤70 mMol	100	69-2203-396	100 gram Strong Cation Exchange RediSep Rf column, pkg. of 1.
≤105 mMol	150	69-2203-394	150 gram Strong Cation Exchange RediSep Rf column, pkg. of 1.
≤192 mMol	275	69-2203-398	275 gram Strong Cation Exchange RediSep Rf column, pkg. of 1.
≤287 mMol	410	69-2203-395	410 gram Strong Cation Exchange RediSep Rf column, pkg. of 1.

a. Sample load = mMol × compound molecular weight/1000

SAX

The SAX (Strong Anion Exchange) media is a silica-bound quaternary amine.

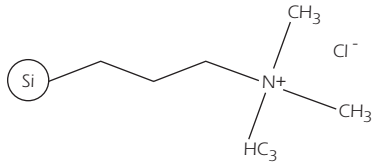
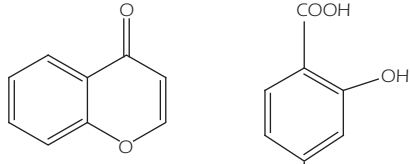


Figure 94: Diagram of SAX structure

Any compounds with acidic properties including weakly acidic compounds subjected through SAX media will be fully retained.

SAX media can be used as a practical and efficient tool for the selective isolation of either acidic or non-acidic compounds from a crude reaction mixture containing both.

To illustrate how SAX media can be of assistance, the separation of a mixture of chromone and 2,4-dihydroxybenzoic acid was investigated.



(A)
Chromone

(B)
2,4-Dihydroxybenzoic acid

Flash chromatography of the mixture on a normal phase Redi*Sep* column showed release of the two products (Figure 95).

The use of a Redi*Sep* SAX column showed total retention of the aromatic carboxylic acid compound onto the column and release of the chromone (Figure 96).

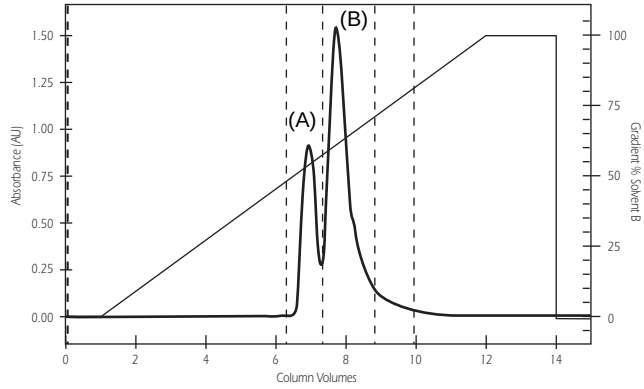


Figure 95: Chromatogram of normal phase column
Elution with hexane/ethyl acetate

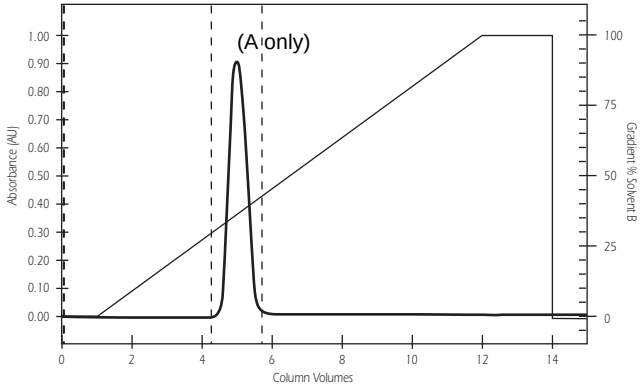


Figure 96: Chromatogram of SAX column
Elution with hexane/ethyl acetate

This isolation of the acidic compound, while allowing one or more organic compounds holding neutral properties to migrate freely through the column, demonstrates that the SAX media can be effective as a clean up tool.

Conversely, the SAX media also represents a practical tool for the isolation of desired compounds holding acidic properties. In this case, the contaminants would be the neutral compounds which would be immediately released then discarded by the SAX column run. The compounds holding acidic properties retained by the SAX column are liberated by injecting a solution of acetic acid in methanol. This solution can be directly injected through the column with a syringe flush. In this case, the SAX column works as a catch and release process.

SAX columns may be reused. After the first use, do not allow the column to dry out since drying the column will adversely affect future purifications. Turn off the air purge on your Flash system's method. The Combi*Flash* Rf system will turn off the air purge by reading the column RFID tag. Regenerate the column with 10 column volumes of 5% NH_4OH in methanol and store in 80% methanol in water, or 100% isopropanol.

Figure 97: Table of Reusable RediSep Rf SAX Columns

Sample Size[a]	Column Size (g)	Teledyne Isco Part Number	Description
≤6.27 mMol	5.7	69-2203-381	5.7 gram Strong Anion Exchange RediSep Rf columns, pkg. of 2.
≤18.7 mMol	17	69-2203-382	17 gram Strong Anion Exchange RediSep Rf column, pkg. of 1.
≤37.4 mMol	34	69-2203-383	34 gram Strong Anion Exchange RediSep Rf column, pkg. of 1.
≤62.7 mMol	57	69-2203-384	57 gram Strong Anion Exchange RediSep Rf column, pkg. of 1.
≤125.4 mMol	114	69-2203-387	114 gram Strong Anion Exchange RediSep Rf column, pkg. of 1.
≤187 mMol	170	69-2203-385	170 gram Strong Anion Exchange RediSep Rf column, pkg. of 1.
≤344.3 mMol	313	69-2203-389	313 gram Strong Anion Exchange RediSep Rf column, pkg. of 1.
≤517 mMol	470	69-2203-386	470 gram Strong Anion Exchange RediSep Rf column, pkg. of 1.

a. Sample load = mMol × compound molecular weight/1000

Natural Products

Natural products chemistry is unique because the chemist generally doesn't know the structure of the compound until after the material is purified. Natural products must be removed from their matrix (plant, fermentation, or marine organism) and the desired compound isolated from a host of other materials without *a priori* knowledge of which compound is active.

Automated Flash chromatography helps the natural products chemist with well-packed columns that improve resolution, precise gradient control, large sample capacity, and systems such as the Combi*Flash* Rf that allow easy scale-up. UV detection also helps to find compounds easily, although this is of greater use later in the purification. Automated Flash chromatography systems can be used with a wide variety of pre-packed columns that work well on a wide range of compounds. Columns that are usually hand-packed due to variable swelling of the stationary phase in different solvents, such as LH-20 or CHP-20, are easily adapted to Combi*Flash* systems to provide the benefits of automation to these purifications as well. The parameters for a particular sized column can be entered manually into the system and saved for future use. Automated gradients avoid the need for pre-mixing solvents.

Purification of natural products starts with a chemical screen. Compounds that extract into ethyl acetate are good candidates for silica gel using hexane/ethyl acetate or hexane/acetone gradients. Compounds that adsorb onto XAD-16 can be purified with silica gel using more polar solvents systems including methylene chloride/methanol. The use of Redi*Sep* TLC plates also aids in determining which solvent system provides the best resolution of components in the mixture. Combi*Flash* Rf systems allow easy changing of solvent systems during the run with their four-solvent inlet lines which allows single-step purification of a wider range of compounds in a single run.

Plant alkaloids can be easily purified on Redi*Sep* C18 columns after extraction. The mixture is first dissolved in methylene chloride and extracted into acidic water. The water is adjusted to pH 10 and back-extracted into methylene chloride to yield only the free bases. The resulting mixture is often clean enough for C18 purification that yields clean compounds.

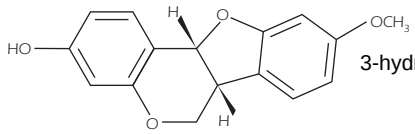
Figure 98: Photo of column mount part number 60-5394-432 (shown below valve) used to adapt a CHP-20 column to a CombiFlash Rf system.

Cytotoxic Constituents from Butea superba

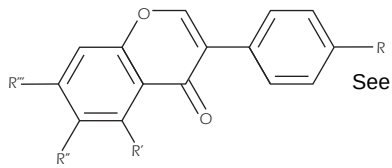
Butea superba[4] is a legume with the common name "red Kwao Krua" in Thailand. This species has shown anti-proliferation effects on the growth of MCF-7 and HeLa cells.

The dried tubers of *Butea superba* were extracted with methanol and the methanolic extract was re-extracted with hexane followed by extraction with CH_3Cl. The chloroform extract was separated by Flash chromatography on Redi*Sep* columns with 100% CH_2Cl_2 with 1% step gradients of methanol. Compounds A–E (Figure 99) were obtained from 1–2% MeOH in CH_2Cl_2. The compounds were identified by spectral comparison with reported values in the literature without further purification.

Cytotoxicity was determined by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay. Compounds B and D showed moderate cytotoxic activity on KB cell lines.



3-hydroxy-9-methoxypterocarpan (A)



See table below

R	R'	R''	R'''	Compound
OCH_3	H	H	OH	7-hydroxy-4'-methoxy-isoflavone (B)
OCH_3	H	H	OCH_3	7,4'-dimethoxyisoflavone (C)
OH	OH	H	OCH_3	5,4'-dihydroxy-7-methoxy-isoflavone (D)
OCH_3	H	OCH_3	OH	7-hydroxy-6,4'-dimethoxyisoflavone (E)

Figure 99: Diagram of compounds extracted from Butea superba

4. Ngamrojanavanich, N.; Loontaisong, A.; Pengpreecha, S.; Cherdshewasart, W.; Pornpakakul, S.; Pudhom, K.; Roengsumran, S.; Petsom, A. *J. of Ethnopharmacology* **2007**, 109, 354

Alkaloids of Banisteria caapi

The plant *Banisteria caapi*[5] is the source of the alkaloid harmine, known for its hallucinogenic properties, and the related harmaline. Harmaline was purified from Florisil using a chloroform-methanol gradient. The dried vine was refluxed in methanol. The extract was dried and extracted into chloroform with 5% ammonium hydroxide. In our labs, we found that harmine would elute readily from silica gel using methylene chloride, but harmaline would only elute slowly with a broad peak even with 100% methanol. We then used a Redi*Sep* Amine functionalized column using a gradient from 50 to 100% ethyl acetate in hexane. Although the harmaline still produces a broad peak, it is much more compact than the run from silica gel. Further optimization of the gradient could be employed to sharpen the harmaline peak.

The use of amine functionalized silica in this example illustrates the ability to purify even difficult samples.

Teledyne Isco produces a number of pre-packed columns containing other media such as acidic, basic, and neutral alumina, cyano, amine, and ion exchangers.

5. Hochstein, F. A. and Paradies, A. M. *J. Amer. Chem Soc.* **1957**, 79(21), 5735.

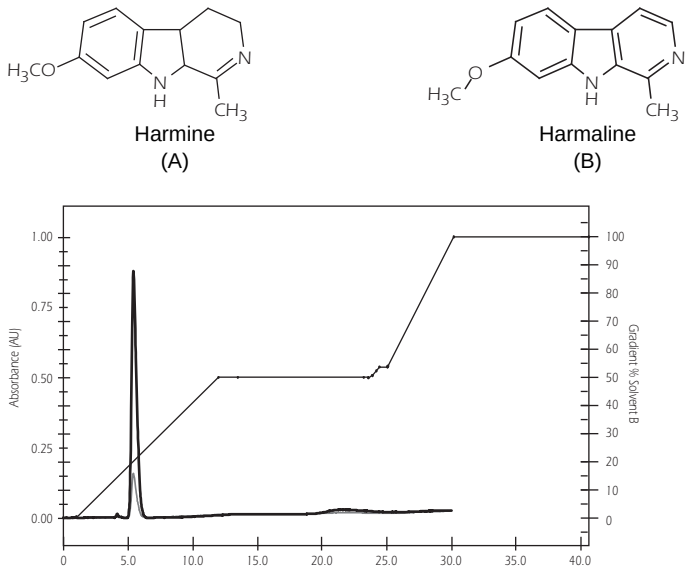


Figure 100: Illustration of harmine and harmaline separation on a silica gel column with a methylene chloride/methanol gradient solvent system

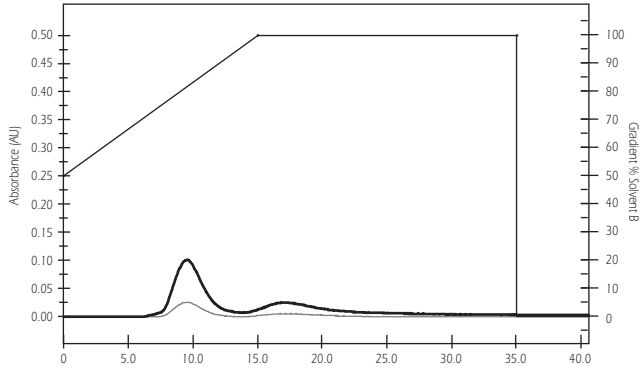
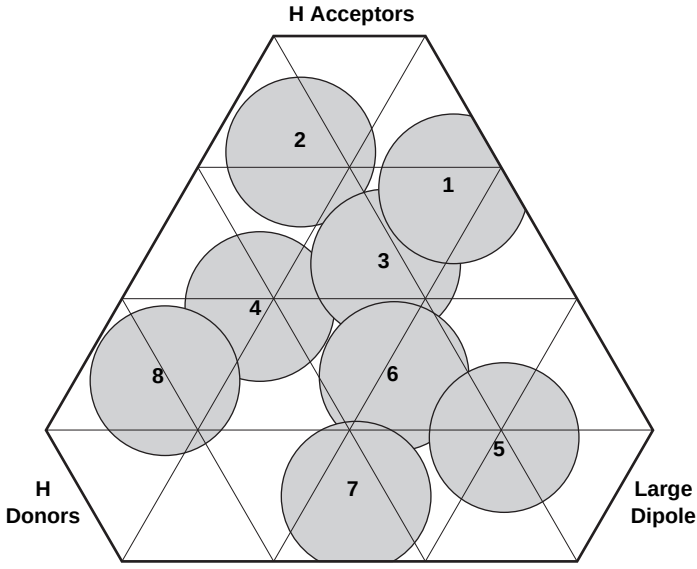


Figure 101: Illustration of harmine and harmaline separation on a RediSep amine column with hexane/ethyl acetate gradient solvent system

Advanced Solvent Strategies

Running various solvents on TLC plates allows a user to select a solvent system that would cause a good retention (measured by R_f), but poor resolution between compounds. Resolution can be improved by using solvents with different selectivity. Figure 102 shows a "selectivity triangle." This three-axis chart allows the scientist to achieve a change in solvent selectivity much more rapidly than testing various solvents randomly.



Group 1
i-Propyl ether (2.40)
Ethyl ether (2.90)

Group 2
Isobutyl alcohol (3.00)
n-Butanol (3.90)
n-Propanol (4.00)
i-Propanol (4.30)
Ethanol (4.30)
Methanol (6.60)

Group 3
Tetrahydrofuran (4.20)
Dimethyl formamide (6.40)

Group 4
Acetic acid (6.20)
Ethylene glycol (6.90)

Group 5
Methylene chloride (3.40)

Group 6
Ethyl acetate (4.30)
Dioxane (4.80)
Acetone (5.40)
Acetonitrile (6.20)

Group 7
Toluene (2.40)
Chlorobenzene (2.70)
Benzene (3.00)

Group 8
Chloroform (4.40)
Water (10.20)

Figure 102: Diagram of solvent selectivity *Solvent polarity is listed in parentheses. Refer to Figure 131 for additional solvent properties.*

As an example, assume separation trials on silica TLC plates suggest the correct solvent strength is obtained with a solvent system of 8:2 hexane:methylene chloride. Using the polarity values in Figure 116 on page 122, the total solvent system polarity can be determined using the following equation:

$$P_T = A \times P_A + B \times P_B$$

where A and B are the ratios of solvent A and B;
P_A and P_B are the polarities.

For our example, the polarity is $0.73 = 0.8 \times 0.06 + 0.2 \times 3.40$

To move the compound up the TLC plate in the same fashion, we need a solvent system with a polarity of 0.73.

Referring to Figure 102, methylene chloride is in solvent group 5. To significantly change the selectivity, the solvent group most distant from group 5 in should be chosen. Group 8 is distant from group 5; chloroform is a choice from this group. To determine the concentration of chloroform, use the equation:

$$B_{new} = B \times P_B / P_{new}$$

where B_{new} is the ratio of new solvent B;
P_{new} is the polarity of the new solvent B.

For our example: $0.16 = 0.2 \times 3.4 / 4.4$, so the new solvent system is 0.84 : 0.16 hexane : chloroform.

If this system failed to work well, a solvent from group 2 such as *n*-propanol could be used.

Choosing a solvent from the same group will make little difference to the selectivity. Changing from ethyl acetate to acetone, both in group 6, makes little difference in the selectivity. This knowledge can be used to choose a solvent in the same group that may have physical properties, such as absorbance, more appropriate for the purification.

For reverse phase, changing the less polar solvent (solvent B) is less effective in changing selectivity. Changing from methanol to acetonitrile generally results in little change to the chromatogram. A change to tetrahydrofuran sometimes produces good results.

In addition to determining the best solvent system for purifying compounds, the concept of solvent groups can also be used to find solvents that allow easier detection of compounds. When using

silica gel, hexane-ethyl acetate is the most common solvent pair used. These solvents purify a wide range of compounds and are commonly used for reactions and extractions, making them plentiful in labs. Many compounds show only end absorption of UV light, from 200–220 nm (the end of the detector range). Ethyl acetate adsorbs in this range causing sloping baselines which hide smaller peaks. Ethyl acetate is in solvent group 6 (Figure 102). Acetone is also in group 6 and has similar characteristics to ethyl acetate for chromatography—both solvents have similar polarity and solvating powers for most organic compounds.

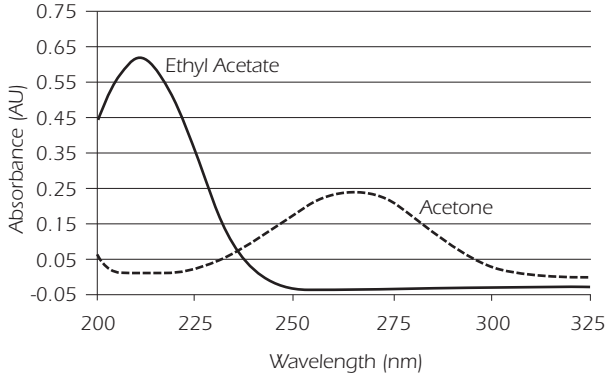
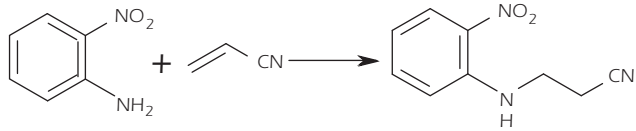


Figure 103: Chart of UV spectra of ethyl acetate and acetone.
Ethyl acetate absorbs UV light strongly at short wavelengths which masks compounds that show only end absorption.

Acetone is slightly more polar (see Figure 131 on page 135) so peaks may elute slightly earlier. Acetone is also readily available in most labs because it is commonly used as a solvent. Acetone absorbs UV light from 225 nm through 300 nm making this solvent an excellent choice for compounds that only absorb UV light at shorter wavelengths (Figure 103).

Acetone does have some drawbacks—it readily absorbs moisture from the air so containers should be capped when not in use. Acetone absorbs UV light with wavelengths longer than 220 nm. For this reason, it is not useful for detectors that operate only at 254 nm. Acetone also should not be used to purify compounds containing diols, or primary and secondary amines since there is a possibility of reactions with those compounds.

The synthesis and purification of 3-(2-nitrophenylamino) propionitrile provides an example of the utility of acetone as an alternative to ethyl acetate.



3-(2-nitrophenylamino) propionitrile absorbs most strongly at 230 nm, lower than the UV cutoff for ethyl acetate. Setting the detection wavelength to 230 nm will cause the detector to see the absorbance of both the ethyl acetate and the desired compound.

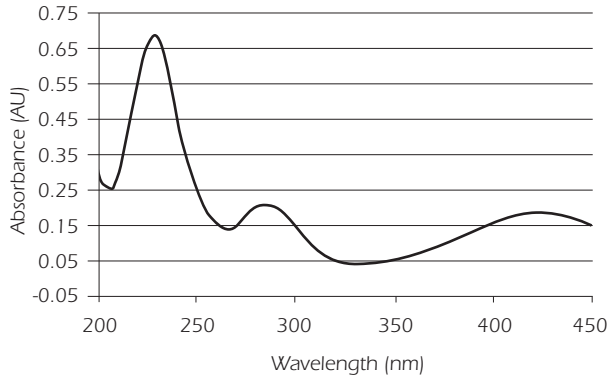


Figure 104: Chart of UV absorbance of 3-(2-nitrophenylamino) propionitrile

PeakTrak software's Gradient Optimizer on the Combi*Flash* Rf system was used to create a method using acetone and hexane as the elution solvents. The desired compound was purified from the starting compound and other impurities while maintaining the maximum sensitivity for detecting the compounds on the instrument.

Figure 105 shows the purification of 3-(2-nitrophenylamino) propionitrile at 229 nm (dashed absorbance trace) and at 285 nm (solid absorbance trace) The 285 nm trace is much smaller, reflecting the reduced absorbance at this wavelength. The stronger absorbing 229 nm trace is riding on the sloping baseline from the ethyl acetate.

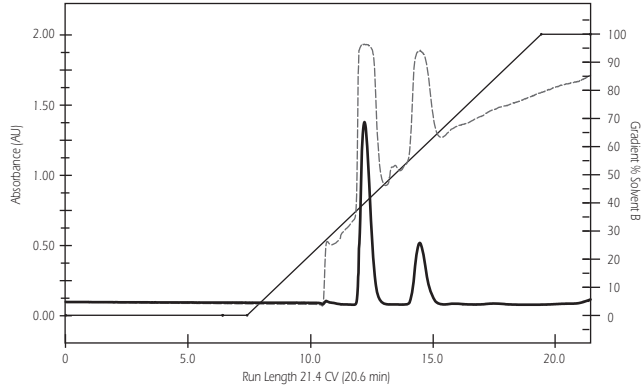


Figure 105: Chromatogram of 3-(2-nitrophenylamino) propionitrile purification in hexane/ethyl acetate *The dashed trace is absorbance at 229 nm; solid trace is absorbance at 285 nm.*

Running the same mixture in hexane/acetone provides the chromatogram in Figure 106. The baseline in the acetone run is flat allowing easy observation of minor impurities. The peaks are rounded at the top due to high loading on the column.

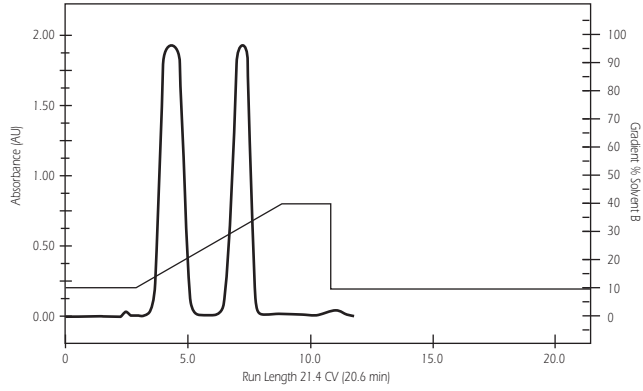


Figure 106: Chromatogram of 3-(2-nitrophenylamino) propionitrile purification in hexane/acetone *Absorbance measured at 229 nm.*

Another example is the purification of stigmasterol, a plant steroid. This compound, in common with many natural products, shows only weak end absorption (Figure 107).

The use of the traditional hexane/ethyl acetate solvent system would make this compound difficult to observe so that the fraction collector could collect the peak. Using acetone allows the compound to be observed. The alternative to the use of acetone in this situation would require the system to be set to "collect all" and manual TLC evaluation of each fraction by the chemist.

The baseline is still drifting slightly since acetone has a slight absorbance at 205 nm but the compound and impurities are still detected very well (Figure 108).

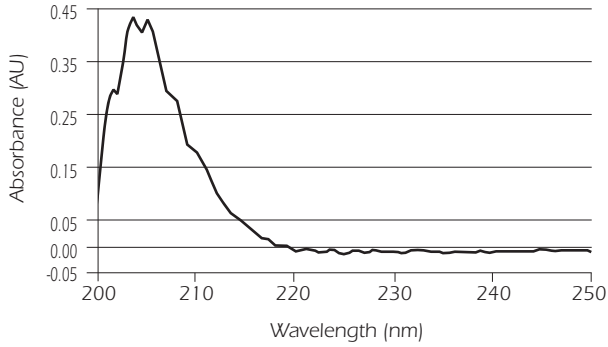


Figure 107: Chart of stigmasterol absorbance *shows absorbance only at shorter wavelengths*

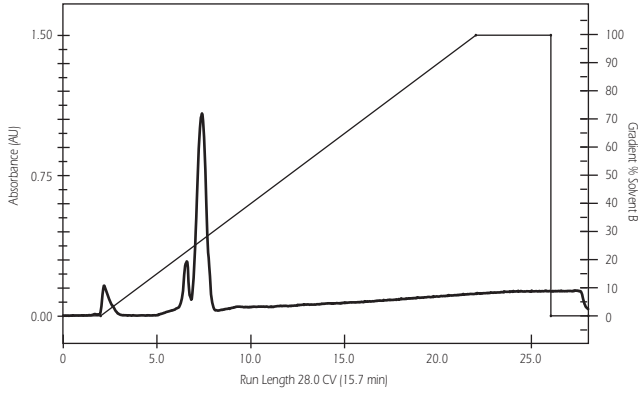


Figure 108: Chromatogram of stigmasterol purification *using hexane/acetone*

Chapter 6
Detection Techniques

Prior to the introduction of automated Flash chromatography systems, the user would collect all of the column eluent into fractions. The fractions would be spotted onto TLC plates and visualized to determine how to cut the fractions. Most compounds absorb ultraviolet (UV) light, so Flash systems use UV lamps and detectors. The detector generates a voltage proportional to the absorbance which is used by the fraction collector to cut fractions based on signal intensity and/or slope. Using the detector and fraction collector can eliminate the need to test the fraction with TLC to determine which fractions to combine and also saves time and test tubes because only eluent with an absorbing compound is collected.

UV Detection

UV detection is the most common detection technique for Flash chromatography. Most compounds can be detected within the range of 200–360 nm used by UV detectors.

The default wavelength commonly used is 254 nm because many compounds absorb at this wavelength. Since some compounds exhibit weak absorbance at this wavelength (Figure 109), they will only show a small peak on the detector.

For this reason, the absorbance spectrum of the compound should be known before starting the purification.

Redi*Sep* columns can be loaded with enough sample that the detector becomes saturated as the peak elutes. The detection wavelength can be moved to a different value during the elution on Combi*Flash* systems such as a minor absorbance (280 nm using the spectrum in Figure 109), or a shoulder of the major absorbance if saturation of the detector prevents fractionation of closely eluting peaks.

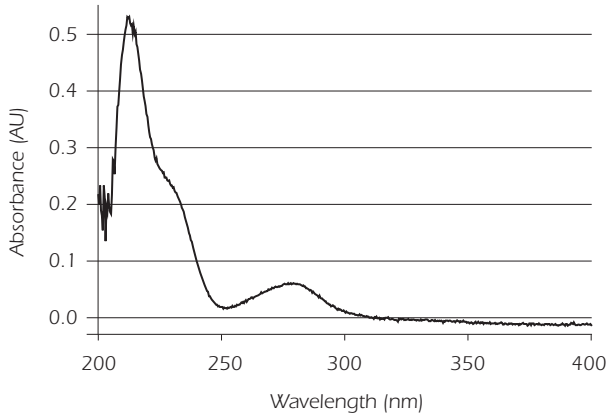


Figure 109: Chart of UV absorbance of catechin

Detection with UV-Vis

A detector sensitive to ultraviolet and visible (UV-vis) light is useful for fractionating compounds that absorb in the visible light range such as pigments and dyes. Since fewer compounds absorb in the visible range, the chromatogram is simpler allowing collection only of those compounds of interest. Some compounds may show their greatest absorbance in the UV range, but the absorbance of the purifying solvent may overlap the compound spectrum. An extended wavelength detector allows the detection of the compound peak at another wavelength. The Combi*Flash* Rf 200 (PN 68-5230-008) and the development-scale Combi*Flash* Torrent systems configured with UV-vis option (PN 68-5240-004) have a working range of 200–780 nm.

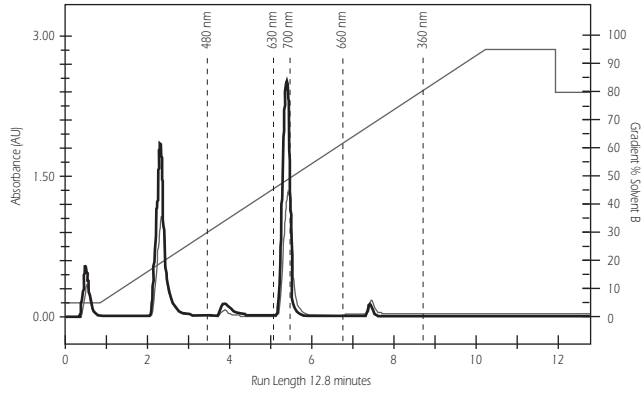


Figure 110: Chromatogram of hair dye compounds purification with UV-vis detection.

All-Wavelength Detection

All-Wavelength Collection in the Combi*Flash* Rf 200 and Torrent systems measures the average absorbance on all wavelengths detected on a photodiode array. The signal is processed to remove baseline drift caused by solvent absorbance. This creates a single voltage that allows the fraction collection program in the MPLC or Flash chromatography system to properly cut the peak. All-wavelength detection is useful when:

- The UV-vis spectrum is unknown, such as compounds purified from natural products.
- There is a mixture of compounds with various absorbances such that a single wavelength can't "see" all the compounds in the mixture.
- The elution solvent spectrum overlaps that of the desired compound.
- Compounds with similar spectra overload the detector, making it difficult to properly fractionate compounds.
- Only compounds within a specified range of absorbance are desired. This method would exclude some starting materials, of the products have a different absorbance spectrum.

All-wavelength detection enhances the ability for a Combi*Flash* system to purify compounds in an automated fashion.

Example with a compound mixture

Figure 111 shows the purification of chlorophyll, caffeine and catechins, and tannins using All-Wavelength collection with a diol column. All of these compounds have differing spectra, yet all were detected with the All Wavelength Collection.

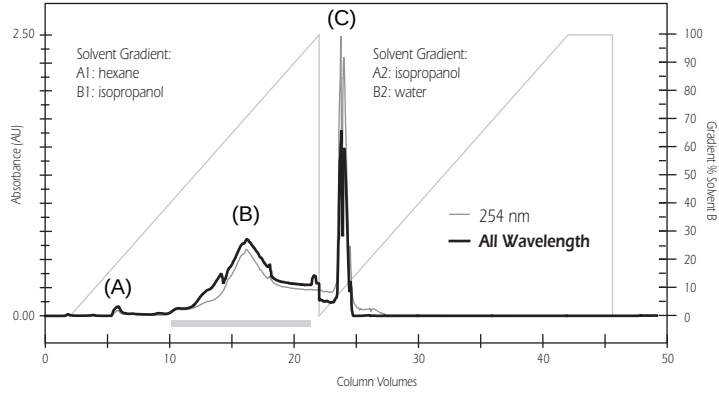


Figure 111: Chromatogram showing detection of chlorophyll (A), catechins and caffeine (B), and tannins (C) with an All-Wavelength Collection range of 200–360 nm.

Example of unknown spectrum

In Figure 112, catechin (compound A) is not detected with the commonly used 254 nm wavelength since this wavelength is at a minimum in its spectrum (Figure 113), but the All-Wavelength Collection was able to detect and fractionate the compound. This is an especially useful technique for natural products where the absorbance of the desired compound generally isn't known until after the final purification.

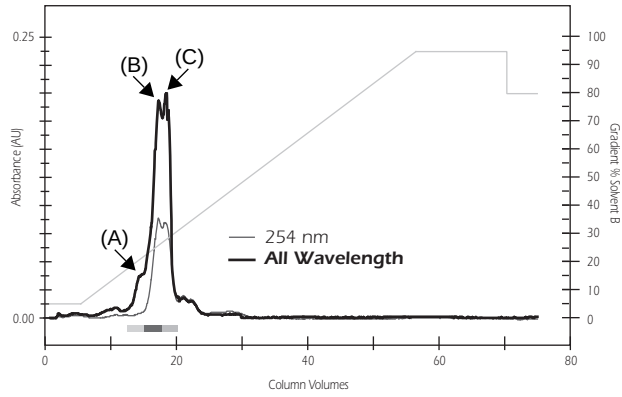


Figure 112: Chromatogram showing detection of catechin with an All-Wavelength Collection range of 200–360 nm.

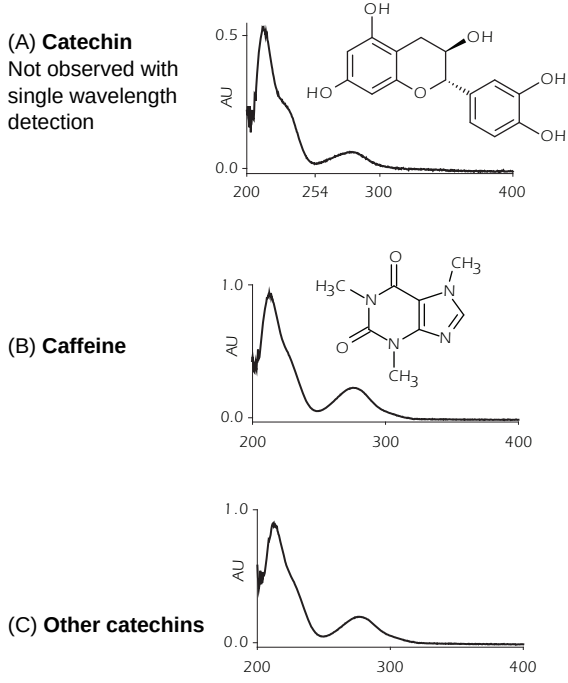


Figure 113: Diagram showing UV absorption of various compounds separated in Figure 112.

Solvent spectrum overlaps compound

Ethyl acetate and dichloromethane are two of the most commonly used solvents for Flash chromatography. Both of these solvents absorb UV light below 250 nm which interferes with detection of compounds that also absorb in this wavelength range when gradients are used. The constantly changing baseline interferes with the ability of the fraction collector to properly cut fractions.

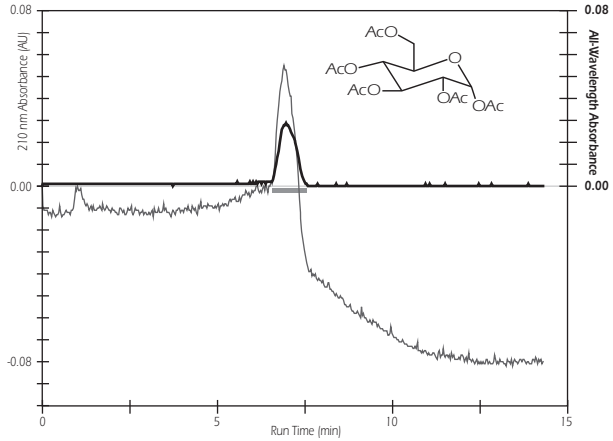


Figure 114: Chromatogram of glucose pentaacetate purification *using a dichloromethane/methanol gradient and an All-Wavelength Collection range of 200–250 nm.*

Glucose pentaacetate shows only weak end adsorption which is further suppressed by the absorption of the dichloromethane (Figure 114). As the dichloromethane concentration is decreased, the baseline drifts downwards. This drift tends to confuse common fraction collection programs but is not an issue with All-Wavelength Collection. All-wavelength collection filters out the baseline drift to create a baseline usable by the fraction collector in the Combi*Flash* system.

Sample overloads detector

High sample loads that cause the absorbance to saturate the detector are common in Flash chromatography. If compounds are closely eluting, the saturation prevents the fraction collector from separating the compounds properly since the saturated peak is seen as a single large peak.

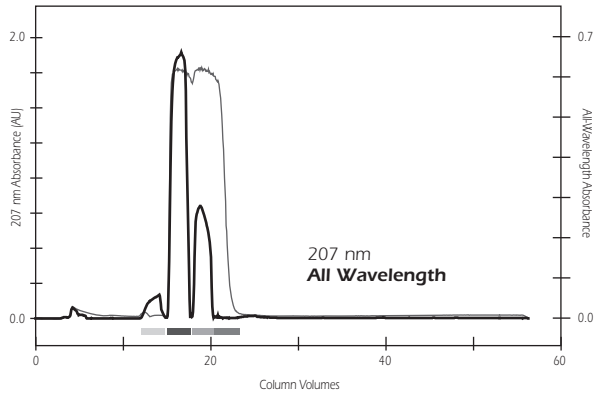


Figure 115: Chromatogram showing purification of closely-eluting, saturated peaks *using All-Wavelength Collection with a range of 200–360 nm.*

All-Wavelength Collection measures all absorbance within the range selected by the user and can cut the peaks since the program detects an absorbance change at non-saturating wavelengths. In Figure 115 catechol and resorcinol are purified from an overloaded, overlapping peak with All-Wavelength Collection.

Other Detectors

Occasionally, other detectors are used to purify compounds such as refractive index (RI), fluorescence, or evaporative light scattering (ELSD) detectors. External detectors may be connected to Combi*Flash* Rf and Torrent systems. These systems will then cut peaks using an input from the external detector.

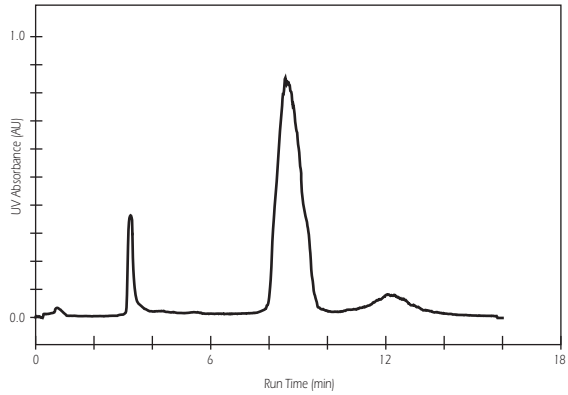


Figure 116: Chromatogram showing ELSD detection of 2,3-O-isopropylidene-D-ribofuranose
using a CombiFlash Rf system

Appendix A
Column Media Selection

Media Selection

The following charts and figures can assist with the selection of stationary phase media based on sample properties and size.

- Figure 117: *Chart for column media selection*
- Figure 118: *Table of RediSep Rf Gold Silica Gel Disposable Flash Columns, 20–40 microns*
- Figure 119: *Table of RediSep Rf Silica Gel Disposable Flash Columns, 40–60 microns*
- Figure 120: *Table of Reusable RediSep Rf Gold C18 Reversed Phase columns, 20–40 microns*
- Figure 121: *Table of Reusable RediSep Rf C18 Reversed Phase columns, 40–60 microns*
- Figure 122: *Table of Reusable RediSep Rf Gold Amine Columns, 20–40 microns*
- Figure 123: *Table of Reusable RediSep Rf Amine Columns, 40–60 microns*
- Figure 124: *Table of Reusable RediSep Rf Gold Cyano Columns, 20–40 microns*
- Figure 125: *Table of Reusable RediSep Rf SAX Columns*
- Figure 126: *Table of Reusable RediSep Rf SCX Columns*
- Figure 127: *Table of Reusable RediSep Rf Gold Diol Columns, 20–40 microns*
- Figure 128: *Table of RediSep Rf Alumina Acidic Columns*
- Figure 129: *Table of RediSep Rf Alumina Neutral Columns*
- Figure 130: *Table of RediSep Rf Alumina Basic Columns*

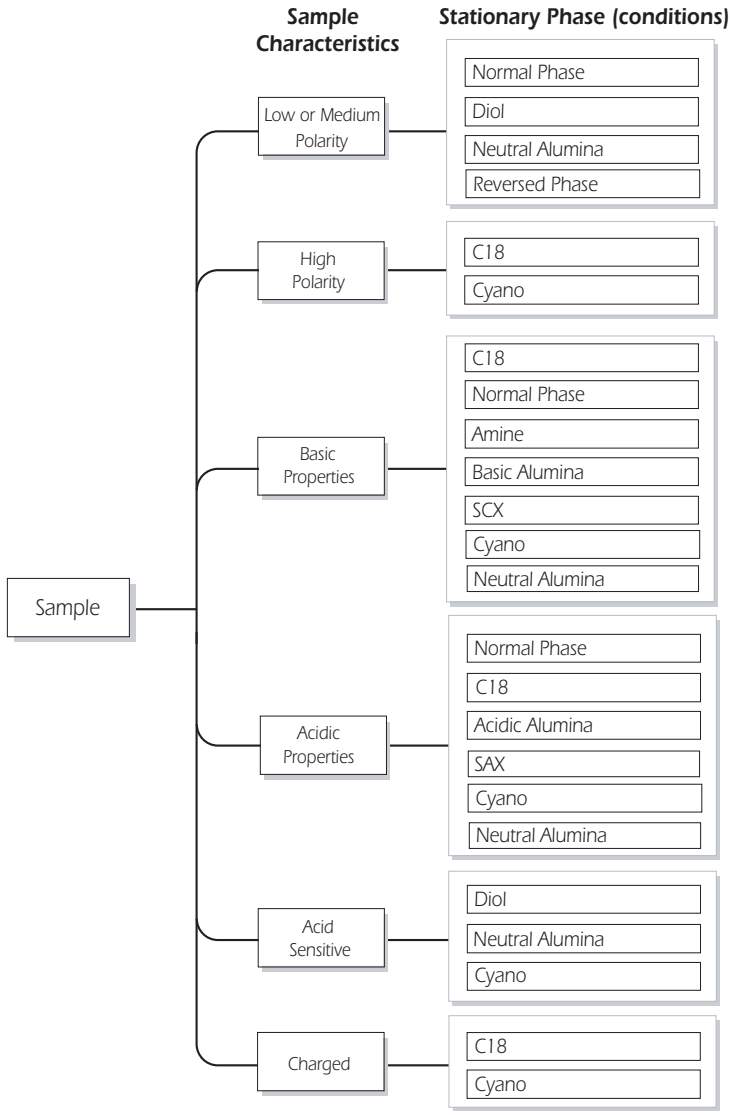


Figure 117: Chart for column media selection
For purification of small molecules (MW<2000)

Figure 118: Table of RediSep Rf Gold Silica Gel Disposable Flash Columns, 20–40 microns Sample loading capacities and ordering information

Sample Size	Column Size (g)	Teledyne Isco Part Number	Description
20 mg - 0.4 g	4	69-2203-344	4 gram RediSep Rf Gold Silica Gel Disposable columns, pkg. of 14.
60 mg - 1.2 g	12	69-2203-345	12 gram RediSep Rf Gold Silica Gel Disposable columns, pkg. of 14.
120 mg - 2.4 g	24	69-2203-346	24 gram RediSep Rf Gold Silica Gel Disposable columns, pkg. of 10.
200 mg - 4 g	40	69-2203-347	40 gram RediSep Rf Gold Silica Gel Disposable columns, pkg. of 10.
400 mg - 8 g	80	69-2203-348	80 gram RediSep Rf Gold Silica Gel Disposable columns, pkg. of 6.
600 mg - 12 g	120	69-2203-349	120 gram RediSep Rf Gold Silica Gel Disposable columns, pkg. of 6.
1.1 - 22 g	220	69-2203-359	220 gram RediSep Rf Gold Silica Gel Disposable columns, pkg. of 4.
1.65 - 33 g	330	69-2203-369	330 gram RediSep Rf Gold Silica Gel Disposable columns, pkg. of 3.
3.8 - 75 g	750	69-2203-427	750 gram RediSep Rf Gold Silica Gel Disposable columns, pkg. of 3.
7.5 - 150 g	1500	69-2203-428	1.5 kg RediSep Rf Gold Silica Gel Disposable columns, pkg. of 2.
15 - 300 g	3000	69-2203-529	3 kg RediSep Rf Gold Silica Gel Disposable columns, pkg. of 1.

Figure 119: Table of RediSep Rf Silica Gel Disposable Flash Columns, 40–60 microns *Sample loading capacities and ordering information*

Sample Size	Column Size (g)	Teledyne Isco Part Number	Description
20 mg - 0.4 g	4	69-2203-304	4 gram RediSep Rf Disposable Flash columns, pkg. of 20.
60 mg - 1.2 g	12	69-2203-312	12 gram RediSep Rf Disposable Flash columns, pkg. of 20.
120 mg - 2.4 g	24	69-2203-324	24 gram RediSep Rf Disposable Flash columns, pkg. of 15.
200 mg - 4 g	40	69-2203-340	40 gram RediSep Rf Disposable Flash columns, pkg of 15.
400 mg - 8 g	80	69-2203-380	80 gram RediSep Rf Disposable Flash columns, pkg. of 12.
600 mg - 1 2g	120	69-2203-320	120 gram RediSep Rf Disposable Flash columns, pkg. of 10.
N/A	125	69-2203-314	125 gram RediSep Rf Disposable Filter column, pkg. of 6.
1.1 - 22 g	220	69-2203-422	220 gram RediSep Rf Disposable Flash column, pkg. of 6.
1.65 - 33 g	330	69-2203-330	330 gram RediSep Rf Disposable Flash columns, pkg. of 4.
3.8 - 75 g	750	69-2203-275	750 gram RediSep Disposable Flash columns, pkg. of 4.
7.5 - 150 g	1500	69-2203-277	1.5 kg RediSep Disposable Flash columns, pkg. of 3.
15 - 300 g	3000	69-2203-527	3 kg RediSep Silica Gel Disposable columns, pkg. of 1.

Figure 120: Table of Reusable RediSep Rf Gold C18 Reversed Phase columns, 20–40 microns *Sample loading capacities and ordering information*

Sample Size	Column Size (g)	Teledyne Isco Part Number	Description
5.5 - 110 mg	5.5	69-2203-328	5.5 gram RediSep Rf Gold C18 columns, pkg. of 2.
15.5 - 310 mg	15.5	69-2203-334	15.5 gram RediSep Rf Gold C18 column, pkg. of 1.
30 - 600 mg	30	69-2203-335	30 gram RediSep Rf Gold C18 column, pkg. of 1.
50 mg - 1.0 g	50	69-2203-336	50 gram RediSep Rf Gold C18 column, pkg. of 1.
100 mg - 2 g	100	69-2203-337	100 gram RediSep Rf Gold C18 column, pkg. of 1.
150 mg - 3 g	150	69-2203-338	150 gram RediSep Rf Gold C18 column, pkg. of 1.
275 mg - 5.5 g	275	69-2203-339	275 gram RediSep Rf Gold C18 column, pkg. of 1.
415 mg - 8.3 g	415	69-2203-341	415 gram RediSep Rf Gold C18 column, pkg. of 1.
0.95 - 19 g	950	69-2203-492	950 gram RediSep Rf Gold C18 column, pkg. of 1.
1.9 - 38 g	1900	69-2203-493	1.9 kg RediSep Rf Gold C18 column, pkg. of 1.
3.8 - 76 g	3800	69-2203-528	3.8 kg RediSep Rf Gold C18 column, pkg. of 1.

Figure 121: Table of Reusable RediSep Rf C18 Reversed Phase columns, 40–60 microns *Sample loading capacities and ordering information*

Sample Size	Column Size (g)	Teledyne Isco Part Number	Description
4.3 - 86 mg	4.3	69-2203-410	4.3 gram Reverse Phase (C18) RediSep Rf columns, pkg. of 2.
13 - 260 mg	13	69-2203-411	13 gram Reverse Phase (C18) RediSep Rf column, pkg. of 1.
26 - 520 mg	26	69-2203-412	26 gram Reverse Phase (C18) RediSep Rf column, pkg. of 1.
43 - 860 mg	43	69-2203-413	43 gram Reverse Phase (C18) RediSep Rf column, pkg. of 1.
86 mg - 1.72 g	86	69-2203-416	86 gram Reverse Phase (C18) RediSep Rf column, pkg. of 1.
130 mg - 2.6 g	130	69-2203-414	130 gram Reverse Phase (C18) RediSep Rf column, pkg. of 1.
240 mg - 4.8 g	240	69-2203-418	240 gram Reverse Phase (C18) RediSep Rf column, pkg. of 1.
360 mg - 7.2 g	360	69-2203-415	360 gram Reverse Phase (C18) RediSep Rf column, pkg. of 1.

Figure 122: Table of Reusable RediSep Rf Gold Amine Columns, 20–40 microns Sample loading capacities and ordering information

Sample Size	Column Size (g)	Teledyne Isco Part Number	Description
5.5 - 110 mg	5.5	69-2203-504	5.5 gram Amine RediSep Rf Gold columns, pkg. of 2.
15.5 - 310 mg	15.5	69-2203-505	15.5 gram Amine RediSep Rf Gold column, pkg. of 1.
30 - 600 mg	30	69-2203-506	30 gram Amine RediSep Rf Gold column, pkg. of 1.
50 mg - 1.0 g	50	69-2203-507	50 gram Amine RediSep Rf Gold column, pkg. of 1.
100 mg - 2 g	100	69-2203-508	100 gram Amine RediSep Rf Gold column, pkg. of 1.
150 mg - 3 g	150	69-2203-509	150 gram Amine RediSep Rf Gold column, pkg. of 1.
275 mg - 5.5 g	275	69-2203-510	275 gram Amine RediSep Rf Gold column, pkg. of 1.
415 mg - 8.3 g	415	69-2203-511	415 gram Amine RediSep Rf Gold column, pkg. of 1.
0.9 - 19 g	950	69-2203-512	950 gram Amine RediSep Rf Gold column, pkg. of 1.
1.9 - 38 g	1900	69-2203-513	1.9 kg Amine RediSep Rf Gold column, pkg. of 1.
3.8 - 76 g	3800	69-2203-534	3.8 kg Amine RediSep Rf Gold column, pkg. of 1.

Figure 123: Table of Reusable RediSep Rf Amine Columns, 40–60 microns *Sample loading capacities and ordering information*

Sample Size	Column Size (g)	Teledyne Isco Part Number	Description
23 - 235 mg	4.7	69-2203-350	4.7 gram Amine RediSep Rf columns, pkg. of 2.
70 mg - 0.7 g	14	69-2203-351	14 gram Amine RediSep Rf column, pkg. of 1.
140 mg - 1.4 g	28	69-2203-352	28 gram Amine RediSep Rf column, pkg. of 1.
235 mg - 2.35 g	47	69-2203-353	47 gram Amine RediSep Rf column, pkg. of 1.
470 mg - 4.7 g	94	69-2203-356	94 gram Amine RediSep Rf column, pkg. of 1.
0.7 - 7 g	140	69-2203-354	140 gram Amine RediSep Rf column, pkg of 1.
1.3 - 12.8 g	260	69-2203-358	260 gram Amine RediSep Rf column, pkg. of 1.
1.92 - 19.25 g	385	69-2203-355	385 gram Amine RediSep Rf column, pkg. of 1.

Figure 124: Table of Reusable RediSep Rf Gold Cyano Columns, 20–40 microns *Sample loading capacities and ordering information*

Sample Size	Column Size (g)	Teledyne Isco Part Number	Description
5.5 - 110 mg	5.5	69-2203-494	5.5 gram Cyano RediSep Rf Gold columns, pkg. of 2.
15.5 - 310 mg	15.5	69-2203-495	15.5 gram Cyano RediSep Rf Gold column, pkg. of 1.
30 - 600 mg	30	69-2203-496	30 gram Cyano RediSep Rf Gold column, pkg. of 1.
50 mg - 1.0 g	50	69-2203-497	50 gram Cyano RediSep Rf Gold column, pkg. of 1.
100 mg - 2 g	100	69-2203-498	100 gram Cyano RediSep Rf Gold column, pkg. of 1.
150 mg - 3 g	150	69-2203-499	150 gram Cyano RediSep Rf Gold column, pkg. of 1.
275 mg - 5.5 g	275	69-2203-500	275 gram Cyano RediSep Rf Gold column, pkg. of 1.
415 mg - 8.3 g	415	69-2203-501	415 gram Cyano RediSep Rf Gold column, pkg. of 1.
0.9 - 19 g	950	69-2203-502	950 gram Cyano RediSep Rf Gold column, pkg. of 1.
1.9 - 38 g	1900	69-2203-503	1.9 kg Cyano RediSep Rf Gold column, pkg. of 1.
3.8 - 76 g	3800	69-2203-533	3.8 kg Cyano RediSep Rf Gold column, pkg. of 1.

Figure 125: Table of Reusable RediSep Rf SAX Columns Sample loading capacities and ordering information

Sample Size[a]	Column Size (g)	Teledyne Isco Part Number	Description
≤6.27 mMol	5.7	69-2203-381	5.7 gram Strong Anion Exchange RediSep Rf columns, pkg. of 2.
≤18.7 mMol	17	69-2203-382	17 gram Strong Anion Exchange RediSep Rf column, pkg. of 1.
≤37.4 mMol	34	69-2203-383	34 gram Strong Anion Exchange RediSep Rf column, pkg. of 1.
≤62.7 mMol	57	69-2203-384	57 gram Strong Anion Exchange RediSep Rf column, pkg. of 1.
≤125.4 mMol	114	69-2203-387	114 gram Strong Anion Exchange RediSep Rf column, pkg. of 1.
≤187 mMol	170	69-2203-385	170 gram Strong Anion Exchange RediSep Rf column, pkg. of 1.
≤344.3 mMol	313	69-2203-389	313 gram Strong Anion Exchange RediSep Rf column, pkg. of 1.
≤517 mMol	470	69-2203-386	470 gram Strong Anion Exchange RediSep Rf column, pkg. of 1.

a. Sample load = mMol × compound molecular weight/1000

Figure 126: Table of Reusable RediSep Rf SCX Columns *Sample loading capacities and ordering information*

Sample Size[a]	Column Size (g)	Teledyne Isco Part Number	Description
≤3.5 mMol	5	69-2203-390	5 gram Strong Cation Exchange RediSep Rf columns, pkg. of 2.
≤10.5 mMol	15	69-2203-391	15 gram Strong Cation Exchange RediSep Rf column, pkg. of 1.
≤21 mMol	30	69-2203-392	30 gram Strong Cation Exchange RediSep Rf column, pkg. of 1.
≤35 mMol	50	69-2203-393	50 gram Strong Cation Exchange RediSep Rf column, pkg. of 1.
≤70 mMol	100	69-2203-396	100 gram Strong Cation Exchange RediSep Rf column, pkg. of 1.
≤105 mMol	150	69-2203-394	150 gram Strong Cation Exchange RediSep Rf column, pkg. of 1.
≤192 mMol	275	69-2203-398	275 gram Strong Cation Exchange RediSep Rf column, pkg. of 1.
≤287 mMol	410	69-2203-395	410 gram Strong Cation Exchange RediSep Rf column, pkg. of 1.

a. Sample load = mMol × compound molecular weight/1000

Figure 127: Table of Reusable RediSep Rf Gold Diol Columns, 20–40 microns *Sample loading capacities and ordering information*

Sample Size	Column Size (g)	Teledyne Isco Part Number	Description
5.5 - 110 mg	5.5	69-2203-514	5.5 gram Diol RediSep Rf Gold columns, pkg. of 2.
15.5 - 310 mg	15.5	69-2203-515	15.5 gram Diol RediSep Rf Gold column, pkg. of 1.
30 - 600 mg	30	69-2203-516	30 gram Diol RediSep Rf Gold column, pkg. of 1.
50 mg - 1.0 g	50	69-2203-517	50 gram Diol RediSep Rf Gold column, pkg. of 1.
100 mg - 2 g	100	69-2203-518	100 gram Diol RediSep Rf Gold column, pkg. of 1.
150 mg - 3 g	150	69-2203-519	150 gram Diol RediSep Rf Gold column, pkg. of 1.
275 mg - 5.5 g	275	69-2203-520	275 gram Diol RediSep Rf Gold column, pkg. of 1.
415 mg - 8.3 g	415	69-2203-521	415 gram Diol RediSep Rf Gold column, pkg. of 1.
0.9 - 19 g	950	69-2203-522	950 gram Diol RediSep Rf Gold column, pkg. of 1.
1.9 - 38 g	1900	69-2203-523	1.9 kg Diol RediSep Rf Gold column, pkg. of 1.
3.8 - 76 g	3800	69-2203-535	3.8 kg Diol RediSep Rf Gold column, pkg. of 1.

Figure 128: Table of RediSep Rf Alumina Acidic Columns *Sample loading capacities and ordering information*

Sample Size	Column Size (g)	Teledyne Isco Part Number	Description
5.5 - 110 mg	8	69-2203-430	8 gram Alumina Acidic RediSep Rf columns, pkg of 20.
15.5 - 310 mg	24	69-2203-431	24 gram Alumina Acidic RediSep Rf columns, pkg. of 20.
30 - 600 mg	48	69-2203-432	48 gram Alumina Acidic RediSep Rf columns, pkg. of 15.
50 mg - 1.0g	80	69-2203-433	80 gram Alumina Acidic RediSep Rf columns, pkg. of 15.
100 mg - 2 g	160	69-2203-436	160 gram Alumina Acidic RediSep Rf columns, pkg. of 12.
150 mg - 3 g	240	69-2203-434	240 gram Alumina Acidic RediSep Rf columns, pkg. of 10.
415 mg - 8.3 g	440	69-2203-438	440 gram Alumina Acidic RediSep Rf columns, pkg. of 6.
0.9 - 19 g	660	69-2203-435	660 gram Alumina Acidic RediSep Rf columns, pkg. of 4.

Figure 129: Table of RediSep Rf Alumina Neutral Columns *Sample loading capacities and ordering information*

Sample Size	Column Size (g)	Teledyne Isco Part Number	Description
40 - 320 mg	8	69-2203-440	8 gram Alumina Neutral RediSep Rf columns, pkg. of 20.
120 - 960 mg	24	69-2203-441	24 gram Alumina Neutral RediSep Rf columns, pkg. of 20.
240 mg - 1.92 g	48	69-2203-442	48 gram Alumina Neutral RediSep Rf columns, pkg. of 15.
400 mg - 3.2 g	80	69-2203-443	80 gram Alumina Neutral RediSep Rf columns, pkg. of 15.
800 mg - 6.4 g	160	69-2203-446	160 gram Alumina Neutral RediSep Rf columns, pkg. of 12.
1.2 - 9.6 g	240	69-2203-444	240 gram Alumina Neutral RediSep Rf columns, pkg. of 10.
2.2 - 17.6 g	440	69-2203-448	440 gram Alumina Neutral RediSep Rf columns, pkg. of 6.
3.3 - 26.4 g	660	69-2203-445	660 gram Alumina Neutral RediSep Rf columns, pkg. of 4.

Figure 130: Table of RediSep Rf Alumina Basic Columns Sample loading capacities and ordering information

Sample Size	Column Size (g)	Teledyne Isco Part Number	Description
40 - 320 mg	8	69-2203-450	8 gram Alumina Basic RediSep Rf columns, pkg. of 20.
120 - 960 mg	24	69-2203-451	24 gram Alumina Basic RediSep Rf columns, pkg. of 20.
240 mg - 1.92 g	48	69-2203-452	48 gram Alumina Basic RediSep Rf columns, pkg. of 15.
400 mg - 3.2 g	80	69-2203-453	80 gram Alumina Basic RediSep Rf columns, pkg. of 15.
0.8 - 6.4 g	160	69-2203-456	160 gram Alumina Basic RediSep Rf columns, pkg. of 12.
1.2 - 9.6 g	240	69-2203-454	240 gram Alumina Basic RediSep Rf columns, pkg. of 10.
2.2 - 17.6 g	440	69-2203-458	440 gram Alumina Basic RediSep Rf columns, pkg. of 6.
3.3 - 26.4 g	660	69-2203-455	660 gram Alumina Basic RediSep Rf columns, pkg. of 4.

Appendix B
Solvent and UV-Vis Wavelength Selection Guide

Solvent Selection

Figure 131 lists typical chromatography solvents and their properties by increasing polarity. Figure 132 may be used to select miscible solvents.

Wavelength Selection

Figure 133 lists substances and the wavelength at which they typically may be detected. Figure 134 lists wavelengths that are available with Teledyne Isco optical detection units and substances that have good absorbance at these wavelengths.

Figure 131: Table of liquid chromatography solvents and their characteristics (by increasing polarity)

SOLVENT	Polarity	Viscosity (cp 20°)	Boiling Point (°C)	UV Cutoff (nm)	Selectivity Group (Fig. 102)
Pentane	0.00	0.23	36	210	—
Petroleum ether	0.01	0.30	30—60	210	—
Hexane	0.06	0.33	69	210	—
Cyclohexane	0.10	1.00	81	210	—
Isooctane	0.10	0.53	99	210	—
Trifluoroacetic acid	0.10	—	72	—	—
Trimethylpentane	0.10	0.47	99	215	—
Cyclopentane	0.20	0.47	49	210	—
n-Heptane	0.20	0.41	98	200	—
Trichloroethylene	1.00	0.57	87	273	—
Carbon tetrachloride	1.60	0.97	77	265	—
i-Propyl ether	2.40	0.37	68	220	I
Toluene	2.40	0.59	111	285	7
Chlorobenzene	2.70	0.80	132	—	7
o-Dichlorobenzene	2.70	1.33	180	295	—
Ethyl ether	2.90	0.23	35	220	I
Benzene	3.00	0.65	80	280	7
Isobutyl alcohol	3.00	4.70	108	220	2

SOLVENT	Polarity	Viscosity (cp 20°)	Boiling Point (°C)	UV Cutoff (nm)	Selectivity Group (Fig. 102)
Methylene chloride	3.40	0.44	40	245	5
Ethylene dichloride	3.50	0.79	84	228	—
n-Butanol	3.90	2.95	117	210	2
n-Butyl acetate	4.00	—	126	254	—
n-Propanol	4.00	2.27	98	210	2
Tetrahydrofuran	4.20	0.55	66	220	3
Ethanol	4.30	1.20	79	210	2
Ethyl acetate	4.30	0.45	77	260	6
i-Propanol	4.30	2.37	82	210	2
Chloroform	4.40	0.57	61	245	8
Dioxane	4.80	1.54	102	220	6
Acetone	5.40	0.32	57	205, 225–300	6
Acetic acid	6.20	1.28	118	230	4
Acetonitrile	6.20	0.37	82	210	6
Dimethyl formamide	6.40	0.92	153	270	3
Methanol	6.60	0.60	65	210	2
Ethylene glycol	6.90	19.90	197	210	4
Dimethyl sulfoxide	7.20	2.24	189	268	—
Water	10.20	1.00	100	—	8

Figure 132: Chart of Solvent Miscibility

Values provided are solubility in water, %w/w

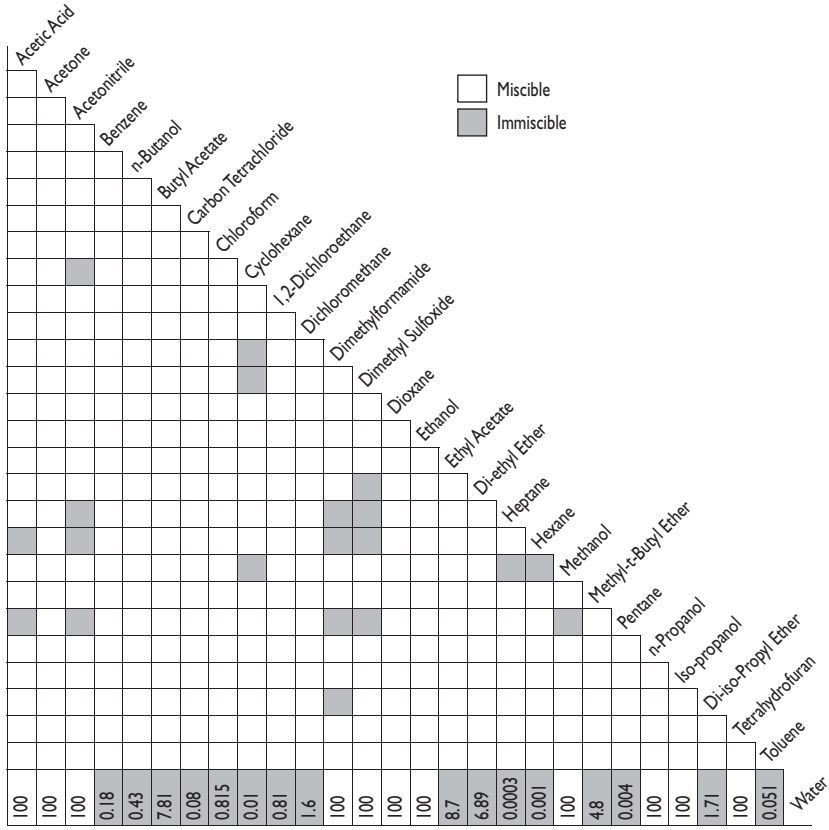


Figure 133: Table of compound absorbance wavelengths (by compound)

Compound	Wavelength (nm)	Compound	Wavelength (nm)
acridine	365	napthalenes	365
amino acids	214, 280[a]	ninhydrin	435
aromatic amino acids	254	ninhydrin-amino acid reaction product (DYDA)	580
amino acids, ninhydrin-primary and secondary	365	non-aromatic peptides	214
antibiotics	310	nucleic acids	254
aromatics	254	nucleoproteins	254
benzophenones, substituted	310	nucleotides	214
carotenoids	435, 546	o-napthaquinone	365
chlorophylls and derivatives	546, 580	porphyrin derivatives	365
enzymes; NADH, NADPH	340	porphyrins	435, 546, 580
ferretin	310	proline	435
ferricyanide	365	proteins	254, 280
ferroproteins	365	pterins	254
flavoproteins	470	pyr-heme a_2 hemachrome	620
hemachrome	620	rubredoxin, oxidized	326
heme proteins, oxidized	636	steroids	214, 365
hydroxyproline	435	tropolene derivatives	365
lactase	620	tropolene, natural	310
lipids	214	vitamins	254, 310

a. Amino acids which have greater absorbance at 280 nm are too dependent on pH for adequate accuracy at 254 nm.

Figure 134: Table of compound absorbance wavelengths (by wavelength)

Wavelength (nm)	Compound
214	non-aromatic peptides, amino acids, nucleotides, and lipids, steroids.
254	aromatics, proteins, nucleic acids, nucleoproteins, aromatic amino acids, pterins, and vitamins.
280	proteins and amino acids[a].
310	natural tropolene, ferretin, vitamins, antibiotics, and substituted benzophenones.
326	oxidized rubredoxin.
340	enzymes; NADH, NADPH.
365	acridine, tropolene derivatives, steroids, porphyrin derivatives, o-napthaquinone, naphthalenes, ferricyanide, ferroproteins, ninhydrin-primary and secondary amino acids.
435	porphyrins, chlorophyll a, carotenoids, ninhydrin, proline, and hydroxyproline.
470	flavoproteins.
546	porphyrins, chlorophylls and derivatives, and carotenoids.
580	porphyrins, chlorophylls, and ninhydrin-amino acid reaction product (DYDA).
620	lactase and pyr-heme a_2 hemachrome.
636	heme proteins, oxidized.

a. Amino acids which have greater absorbance at 280 nm are too dependent on pH for adequate accuracy at 254 nm.

Appendix C

Theory & Application of Flash Chromatography

Elementary theory

Given a compound X in a Flash column:

$(X)_s$ = concentration of X in stationary phase (g/mL or g/g)

$(X)_m$ = concentration of X in mobile phase (g/mL)

$K = (X)_s/(X)_m$ where K is the distribution coefficient between the stationary and mobile phases

The larger value of K, the longer the retention time. Differences in K values for different compounds lead to differential migration through the column.

Compound X is characterized by two parameters:

t_r = retention time (in units of time)

t_w = baseline bandwidth (same units as t_r)

$t_r = V_r/F, \quad t_w = V_w/F$

where: V_r = retention volume of band

V_w = baseline bandwidth (in mL)

F = solvent flow rate in mL/sec

The relationship between t_r (or V_r) and K can be obtained as follows.

$$k' = \frac{V_s(X)_s}{V_m(X)_m} = \left[\frac{V_s}{V_m}\right]K$$

where: k' = capacity factor

V_s = volume of stationary phase in column

V_m = volume of mobile phase in column

The velocity of compound X in the column is given as v_x

$$v_x = v_s/(1 + k')$$

where: v_s = velocity of solvent in column (cm/sec)

Since the distance traveled by X through the column is constant and since the velocity of a non-retained band is equal to that of the mobile phase,

$$L = t_r v_x = t_o v_s$$

where:

L = column length

t_o = retention time of non-retained band

Therefore:

$$t_r = t_o(1 + k') \text{ and } k' = \frac{(t_r - t_o)}{t_o}$$

$$\text{and } V_r = V_m(1 + k') = V_m + V_s K$$

The extent of broadening (t_w or V_w) of the band of compound X is defined in terms of the number of theoretical plate (N) of the column, which is a measure of column performance.

$$N = 16(t_r/t_w)^2 = 16(V_r/V_w)^2 = 5.54(t_r/t_{0.5w})^2$$

where:

$t_{0.5w}$ = bandwidth at one-half the peak height.

Application

The basic function of LC is to separate a mixture of two or more substances. Given two compounds, X and Y, in a column, their relative separation or resolution is defined as:

$$R_s = \frac{t_{r,y} - t_{r,x}}{1/2(t_{w,y} + t_{w,x})}$$

The resolution can also be related to the conditions of separation:

$$R_s = 1/4[k'/(1+k')]\sqrt{N}(\alpha - 1)$$

where:

k′ is the capacity factor of either compound

N is the number of theoretical plates

α is the separation factor, and is equal to $\frac{k'_y}{k'_x}$

The efficiency with which a column resolves or separates compounds X and Y is a function of N. It is useful to determine this efficiency as a proportionality constant (HETP) which is the height equivalent to a theoretical plate.

$$HETP = L/N$$

The first attempt to separate a mixture by liquid chromatography rarely gives the desired results. However, these results can give some clues as to what changes must be made to achieve an adequate separation. The Standard Resolution Curves (Figure 135) give the R_s values for band ratios of 1:1, 4:1, and 16:1. These charts give some idea how much R_s has been achieved and how much it must be increased to give the desired resolution.

Generally a large increase in R_s or decrease in t_r is most readily accomplished by varying k′. Small values of k′ give low R_s values and t_r values near t_o. Large k′ values give long t_r and large t_w (or V_w) values. If exclusion chromatography is being employed, k′ values are altered by changing the column packing. For other methods, the solvent strength is varied. Stronger solvents give smaller k′ values and weaker solvents give larger k′ values. Following this discussion is a list of various solvents arranged in their order of eluting strength. For separations involving several compo-

nents, k′ must be varied during separation. In this case gradient elution techniques are employed.

Where a change in k′ does not result in the desired change in R_s, resolution can be improved by increasing N. this involves changing the column length, solvent velocity, and/or temperature of the column.

The easiest method of increasing N is to reduce the mobile phase flow rate. This however, increases t_r.

Once it is understood how various parameters affect each other, experimental can be predicted for a desired change in R_s or t_r. Following this discussion is a table showing how the resolution can be changed by varying time, pressure, and/or column length. This table was derived from:

$$H = D\mu^n$$

where D is a column efficiency parameter, and n=0.4. Lower and higher values are sometimes encountered and tables for n=0.3 and n=0.5 are available.

In separations where R_s for bands of components X and Y is <0.5 and k′ >2, an increase in N may not be practical. Resolution in this case can be improved by using gradient elution.

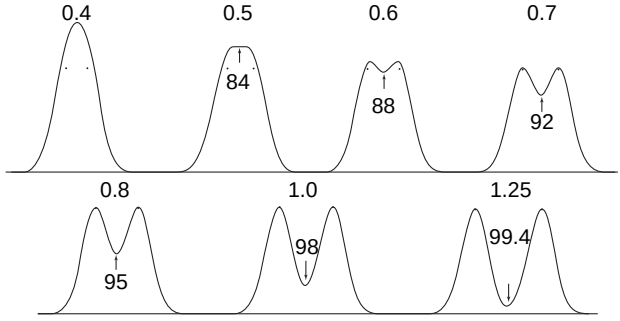
The foregoing discussion has been oversimplified as it is only our purpose to provide a quick reference to the basic theory of liquid chromatography and some data for its application. More detailed information may be obtained from the following references:

Snyder, L.R.; Kirkland, J.J.; *Introduction to Modern Liquid Chromatography*, Wiley-Interscience, N.Y. (1979)

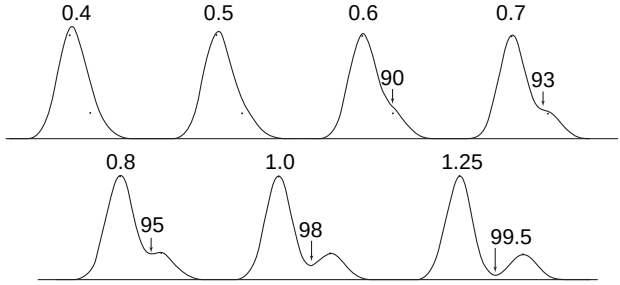
Runser, D.J., *Maintaining and Troubleshooting HPLC Systems*, Wiley-Interscience, N.Y. (1981)

Krstulovic, A.M.; Brown, P.R.; *Reversed-phase High Performance Liquid Chromatography*, Wiley-Interscience, N.Y. (1982)

1:1 ratio of two bands



4:1 ratio of two bands



16:1 ratio of two bands

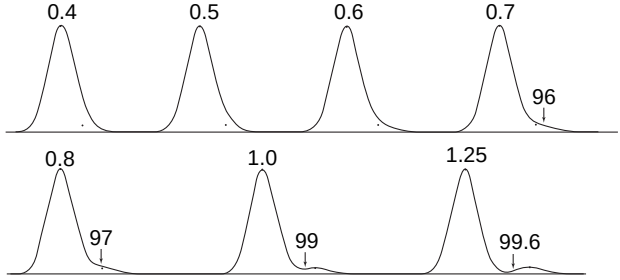


Figure 135: Standard resolution charts

Numbers above the bands refer to R_s. The arrows indicate the cut point which will yield bands of equal purity while the number above or below refers to the purity of each of the two resulting bands.

Charts adapted by permission of L.R. Snyder and Preston Technical Abstracts Co., from *Journal of Chromatographic Science* **10**, 364 (1972)

Appendix D
Troubleshooting LC Systems

Basic checklist

The list below summarizes common problems that can be quickly checked and remedied.

- Instrument(s) not plugged in
- Instrument(s) not turned on
- Fuse(s) blown
- No mobile phase
- Air lock in pump lines
- Leaks
- No sample being introduced
- Temperature gradients across system
- Contaminated or plugged column
- Wrong column type or size
- Flow through column reversed
- Wrong detector setting
- Dirty detector cell
- Indicator light or gauge malfunction
- Sample chemistry misinterpreted

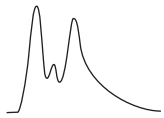
Troubleshooting

The following figures list common LC problems and solutions.

- Figure 136: *Table for troubleshooting peak problems*
- Figure 137: *Table for troubleshooting baseline problems*

- Figure 138: *Table for troubleshooting recovery and retention problems*
- Figure 139: *Table for troubleshooting pressure problems*
- Figure 140: *Table for troubleshooting leaks*

Figure 136: Table for troubleshooting peak problems

Symptom	Cause	Remedy
Broad peaks 	Retention time too long	Use stronger mobile phase, increase flow rate, select different column type.
	Too much sample injected	Decrease injection volume.
	Compounds eluted too early due to sample overloading.	Use larger-volume column, smaller injection volumes, or dilute the sample.
	Late elution from previous injection	Extend run times, finish runs with stronger percentage of mobile phase B, use longer column equilibration times before run.
Peak tailing 	High pH	Change to reversed-phase column, change to column with a better pH stability.
	High temperature	Maintain temperatures below 40° C.
	Interactions with silica stationary phase	Use reversed-phase column, decrease mobile phase pH, strengthen mobile phase, add triethylamine to mobile phase, or buffer sample.
	Interfering peak	Improve sample purification before injection, alter mobile phase, select an affinity column.
Peak fronting 	Poor column packing	Replace column.
	Column overloading	Use larger-capacity column or smaller sample volumes.

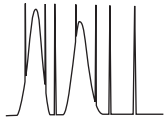
Symptom	Cause	Remedy
Peak doubling 	Interfering peak	Improve sample purification before injection, alter mobile phase, select an affinity column.
	interfering compound from previous run	Extend run times, finish runs with stronger percentage of modifier solvent, use longer column equilibration times before run.
	Column overloading	Use larger-capacity column or smaller sample volumes.
	Poor column packing	Replace column and decrease mobile phase solvent strength.
	Injection solvent too strong	Prepare sample with weaker solvent, then adjust mobile phase strength.
	Sample and mobile phase incompatible	Use miscible solvents.
Inverted peaks 	Mobile phase absorbance greater than that of elute.	Select mobile phase with lower absorbance, verify purity of mobile phase.
	Air injected with sample	Ensure solid sample loop or liquid injection is free of air.
Ghost peaks 	Impure mobile phase	Verify purity of mobile phase or use HPLC grade solvents.
	interfering compound from previous run	Extend run times, finish runs with stronger percentage of modifier solvent, use longer column equilibration times before run.
	Sample preparation	Improve sample purification before injection.
Spikes 	Bubbles in mobile phase	Degas solvents, check for bad fluid line fittings.
	Faulty UV detector	Test detector operation, replace lamp.

Figure 137: Table for troubleshooting baseline problems

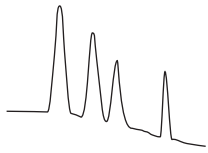
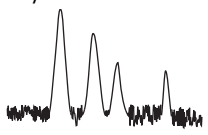
Symptom	Cause	Remedy
Drifting baseline 	Column contaminated	Purge column with strong solvent, replace column.
	Temperature effects	Control environment, allow system to warm up and stabilize.
	Leak in system	Locate and repair.
Rising baseline 	Absorbance of mobile phase B	Change to low- or non-absorbing mobile phase, add absorbing compound to mobile phase A, select a different wavelength.
	Sample preparation	Improve sample purification before injection.
Falling baseline 	Absorbance of mobile phase A	Change to low- or non-absorbing mobile phase, add absorbing compound to mobile phase B, select a different wavelength.
	interfering compound from previous run	Extend run times, finish runs with stronger percentage of modifier solvent, use longer column equilibration times before run.
Noisy baseline 	Residual compounds in system	Purge system with strong solvent, replace column.
	Mobile phase contamination	Use HPLC grade solvents.

Figure 138: Table for troubleshooting recovery and retention problems

Symptom	Cause	Remedy
Poor recovery of sample	Low yield (less than 90%)	Acidify the mobile phase for acidic compounds, add competing base for basic compounds, change to ion-exchange column.
	Interaction with stationary phase	Change to ion exchange or affinity column, eliminate possible reactive groups.
	Adsorption on column media	Increase mobile solvent strength.
Varying retention times	Equilibration volume too small	Increase column equilibration before run.
	Column contaminated	Purge column with strong solvent, replace column.
	Interaction with active sites in stationary phase	Manage interaction by modifying the mobile phase, inject large volume of sample onto column to condition it before use.

Figure 139: Table for troubleshooting pressure problems

Symptom	Cause	Remedy
Increasing or high pressure	Column blocked, sample	Improve sample purification before injection, purge column with strong solvent, replace column.
	Column blocked, microbial growth	Add organic solvent or growth inhibitor to mobile phase. If column is installed but not in use, purge regularly. Store column filled with sufficient organic solvent and sealed end-caps.
	Tubing blocked	Refer to flow diagram and disconnect components to locate obstruction.
	Precipitation	Use miscible solvents and buffers, use less-ionic mobile phase, avoid rapid changes to characteristics of mobile phase.
Low pressure	Leaks	Inspect and correct system tubing and fittings.
	Low flow rate	Check pump and tubing from pump.
	Pump inlet	Check solvent containers and pump inlet tubing, degas solvent.

Figure 140: Table for troubleshooting leaks

Leak Location	Cause	Remedy
Injection	Loose fitting	Re-seat sample cartridge or injection needle in fitting, inspect fitting for damage or wear, replace fitting.
	Injection valve damaged	Replace injection valve, prepare sample and solvents to avoid precipitation or formation of salts.
Column	Loose fitting	Re-seat column in fitting, inspect fitting for damage or wear, replace fitting.
System tubing and components	Loose fitting or seal	Tighten or replace.
	Damaged tubing	Replace tubing
	Leak from system component such as damper, detector, or pump.	Service or replace the component.

Teledyne Isco • 4700 Superior Street • Lincoln, NE 68504 • USA
Toll free: (800) 228-4373 (USA & Canada)
Telephone: (402) 464-0231 • Fax: (402) 465-3022
E-mail: iscoinfo@teledyne.com • Web: www.isco.com

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