2. Optimizing selectivity

The first step in successful FLASH purification is maximizing ΔCV. Accomplish this by evaluating various solvent mixtures by TLC. Look for a binary mixture that provides the largest ΔCV between the compound of interest and all the impurities.

All solvents fall into a selectivity group. Each group has a different impact on a sample component's relative retention to another compound (selectivity). In Table 2, the most frequently used flash solvents and their selectivity groups are shown.

When possible, selectivity optimization should include mixtures of hexane with ethyl acetate (VIa), methylene chloride (V), toluene (VII), tetrahydrofuran (III), and ether (I). For more polar compounds and amines, mixtures of methylene chloride (V) with methanol (II) or acetonitrile (VIb) should be evaluated. These solvent combinations provide a broad range of separation selectivity and will help define the correct solvents for a sample's purification (Figure 3). For more discussion regarding solvent selectivity in chromatography (see Introduction to Modern Chromatography by L.R. Snyder and J.J. Kirkland[1]).

Solvent	Selectivity Group
Ether	I
Methanol	II
Ethanol	II
Isopropanol	II
Tetrahydrofuran	III
Dichloromethane	V
Acetone	VIa
Ethyl acetate	VIa
Acetonitrile	VIb
Toluene	VII
Chloroform	VIII
Hexane	-----
Heptane	-----
Isooctane	-----

Table 2. Solvent selectivity chart[1]

Hexane/EtOAc (VIa) (2:1)   Dichloromethane (V)

Figure 3. Impact of solvent selectivity on a chromatographic separation. In hexane/ethyl acetate the compound of interest (B) is poorly resolved from its major impurities (A and C). In dichloromethane, the retention of impurities A and C has been dramatically altered, providing a better purification of B.

3. Solvent strength optimization

When the correct solvents have been determined, the next step is to adjust the solvent composition (solvent strength) so the compound of interest elutes within the Rf range 0.15 – 0.35 (6.7 – 2.8 CV). By adjusting solvent strength to provide elution within this window, the chances for optimal purification are greatly enhanced.

As with selectivity, each solvent has its own polarity (Table 3). Each solvent mixture or mobile phase then has its own unique solvent strength. Calculation of a solvent mixture's strength is useful for comparison to other solvent mixtures. Solvent mixtures with the same strength but different selectivity can then be evaluated.
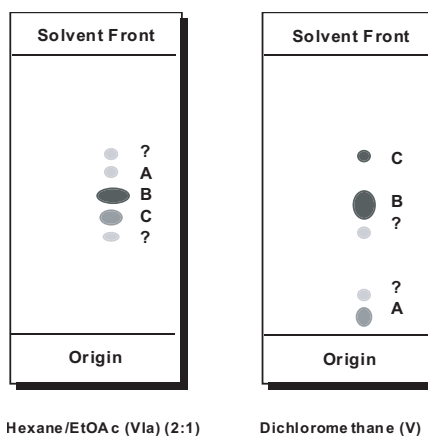
[1] L. R. Snyder and J. J. Kirkland, *Introduction to Modern Liquid Chromatography*, Wiley, 1979.

To bring the Rf of the compound of interest into the optimal range, reduce the amount of polar solvent in the mobile phase. As an example, in Figure 4, the results of a solvent selectivity study show a mobile phase of 50% hexane and 50% ethyl acetate (solvent strength = 0.30), providing adequate selectivity for a crude sample (Figure 4, top). The Rf for the compound of interest (B) is 0.4 (2.5 CV) and the Rf of the impurity (A) is 0.55 (1.8 CV), providing a ΔCV of 0.7. With a ΔCV this low, only a small sample amount can be FLASH purified before overload (resolution loss, low purity fractions) occurs. By weakening the solvent strength to 60% hexane and 40% ethyl acetate (solvent strength 0.24) (Figure 4, middle); the Rf of compound B falls to 0.2 (5 CV) and impurity A's Rf is lowered to 0.3 (3.3 CV) with a resulting ΔCV of 1.7, enabling a potential fivefold increase in sample load on a FLASH cartridge (Table 1).

Solvent	Strength
Methanol	0.95
Ethanol	0.88
Isopropanol	0.82
Acetonitrile	0.65
Ethyl acetate	0.58
Tetrahydrofuran	0.57
Acetone	0.56
Dichloromethane	0.42
Chloroform	0.40
Ether	0.38
Toluene	0.29
Hexane	0.01
Heptane	0.01
Isooctane	0.01

Table 3. A solvent mixture's strength is calculated using volume proportions and the individual solvent's strength.

In the example above, diluting a solvent mixture with a less polar solvent (hexane) from 50% to 60% reduces solvent strength, increasing compound retention and resolution (ΔCV). Also, solvent combinations of similar strength but different selectivity can also be compared. Both hexane/ethyl acetate (50:50) and hexane/dichloromethane (30:70) have solvent strength of 0.3, but ethyl acetate and dichloromethane provide different selectivity.

Formula:

$$\frac{(\text{Solvent A\% x solvent A strength})}{100} + \frac{(\text{Solvent B\% x solvent B strength})}{100}$$

Examples:

Hexane/ethyl acetate (50:50)
Solvent strength = (0.5 x 0.01) + (0.5 x 0.58) = 0.30

Hexane/ethyl acetate (60:40)
Solvent strength = (0.6 x 0.01) + (0.4 x 0.58) = 0.24

Hexane/dichloromethane (30:70)
Solvent strength = (0.3 x 0.01) + (0.7 x 0.42) = 0.30

If you find adequate component retention with a particular solvent mixture, you can prepare other solvent mixtures of similar strength but different selectivity for comparison (Figure 4, bottom).
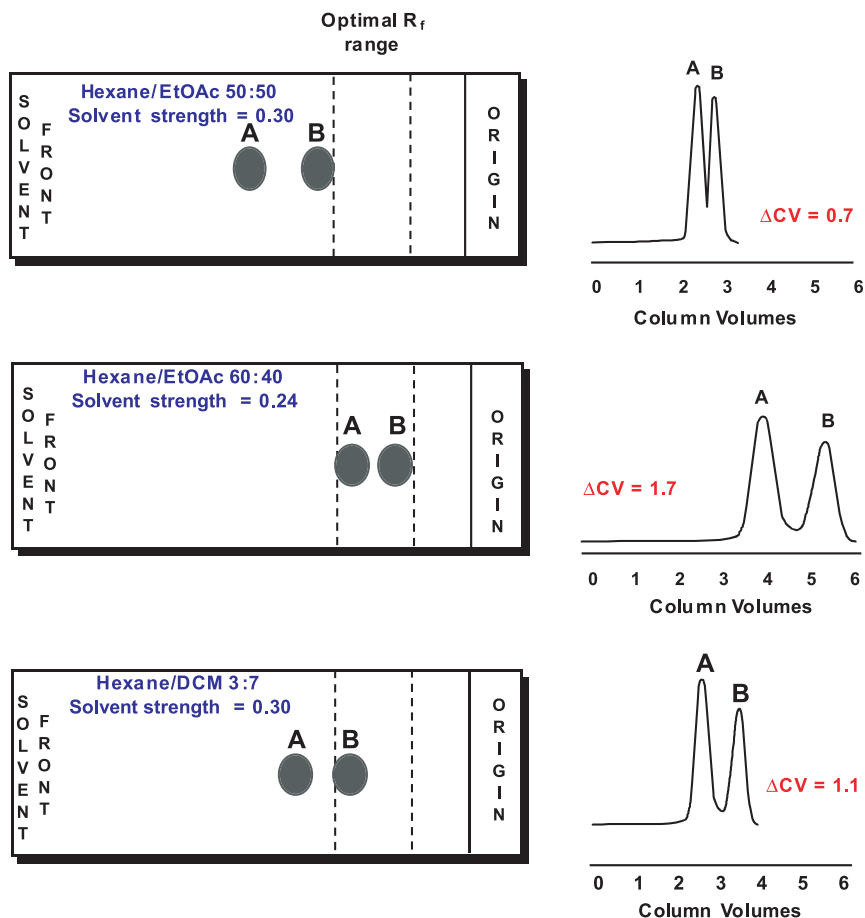

Figure 4. Examples of solvent strength on compound retention and resolution. The top TLC shows two sample components resolved with a 50:50 hexane/ethyl acetate solvent system (ΔCV = 0.7). Neither the component of interest (B) nor the impurity (A) has an Rf value within the optimal 0.15–0.35 range. This leads to poor flash purification (top chromatogram). After adjusting the solvent to 60% hexane/40% ethyl acetate, the Rf values for both A and B fall into the optimal zone (middle TLC). FLASH chromatography with these conditions (middle chromatogram) shows increased compound retention and greatly improved resolution (ΔCV = 1.7). Replacing 50:50 hexane/ethyl acetate with 30:70 hexane/dichloromethane (both 0.30 solvent strength) alters both selectivity and resolution (ΔCV = 1.1).

Once a solvent system has been selected, Rf values measured, and ΔCV values calculated, use Table 1 on page 230 to select the correct cartridge for your sample size and ΔCV. The data generated from your TLC method development efforts are applicable to any sized Biotage cartridge.