Preparative Chromatography Focused Gradients, pH Control, and Ionizable Compounds

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Abstract

When applying a focused gradient after a scouting run, the desired compounds may not always elute at the expected time due to the effects of compound ionization and pH control. When using the PeakTrak® Focused Gradient Generator, the calculation includes a wide margin for variance, which allows most compounds to elute during the focused gradient portion of the run even if the retention shifts due to equilibration of ionized and non-ionized forms of the compound.

Other commonly used techniques, such as "compound-specific method optimization"[1] have the same deviation from the expected elution time which may prevent the compound from eluting within the focused gradient. Despite the name of the algorithm, the determined gradient isn't specific for any compound within a given gradient zone, so the compound of interest probably will not elute in the middle of the gradient, even if the compound remains in a single form. The PeakTrak Focused Gradient Generator calculation is designed to place a peak in the middle of a focused gradient to allow for some error due to compound interconversion.

Background

Ionizable compounds can convert between a polar form and a less polar form in solution (Figure 1). When running reverse phase columns, the ionized form elutes earlier, while the unionized moiety has later elution. A compound may elute as a narrow peak, a broad peak, or as multiple peaks depending on its ionization constant, and whether or not the ionized and unionized forms are readily interconvertible.

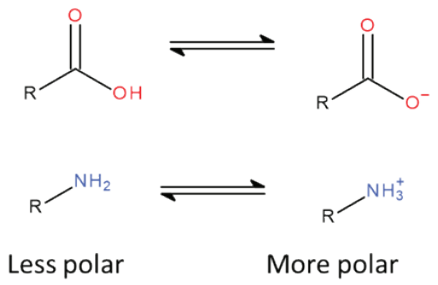

Figure 1 – examples of ionizable compound equilibrium between ionized and unionized forms.

Another effect of such interconversion is that when focused gradients are created from scouting runs, the compound retention time is significantly different than the expected retention time, as shown in Figure 2 for quinine.

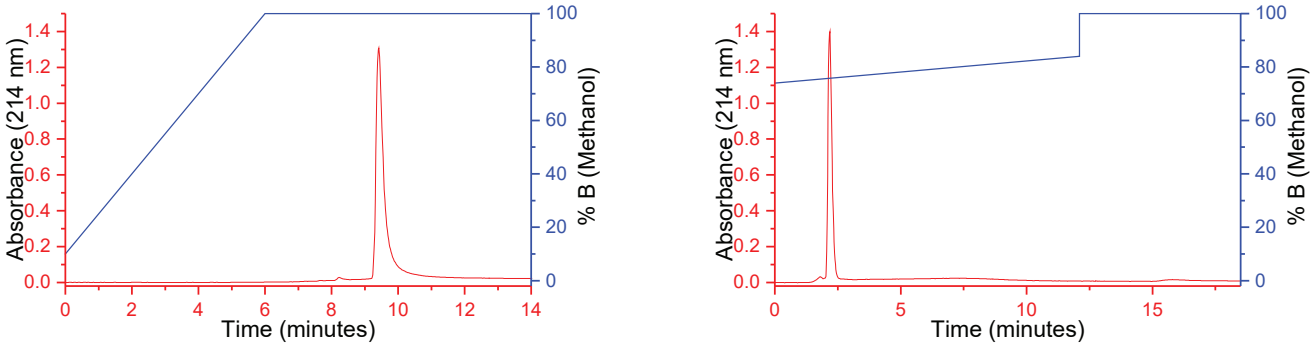

Figure 2 – Quinine run in water/methanol with no modifier on C18. The run on the left is a scouting run; that on the right is a calculated focused gradient.

In general, the use of a solvent modifier reduces such issues and produces a usable chromatogram; the retention time, however, may be different than expected.

Experimental Results

Basic Compounds

Adding 0.1% trifluoroacetic acid (TFA) causes the quinine to elute earlier in the scouting run, which translated to a more polar solvent composition for the focused gradient as compared to the run in Figure 2. Note that the peak shape in the scouting run in Figure 3 exhibits no tailing compared to the scouting run in Figure 2. This time, the compound did elute during the focused gradient, but at about 8 minutes, rather than the expected 6 minutes. However, the purification is usable without further changes.

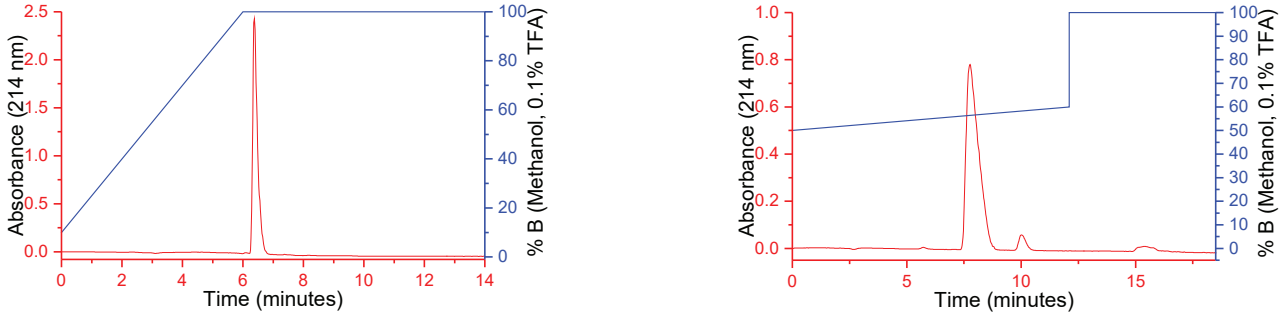

Figure 3 – Quinine run in water/methanol with 0.1% TFA with C18

Adding formic acid to a concentration of 0.1 % greatly improves the peak shape in both the scouting run and the preparative run.

Acidic Compounds

Acidic compounds also benefit from the use of solvent modifiers, as shown in Figure 4. In this case, the scouting run without a solvent modifier shows that the focused gradient will have poor resolution, since the peak shows considerable fronting, which is also observed in the focused gradient purification.

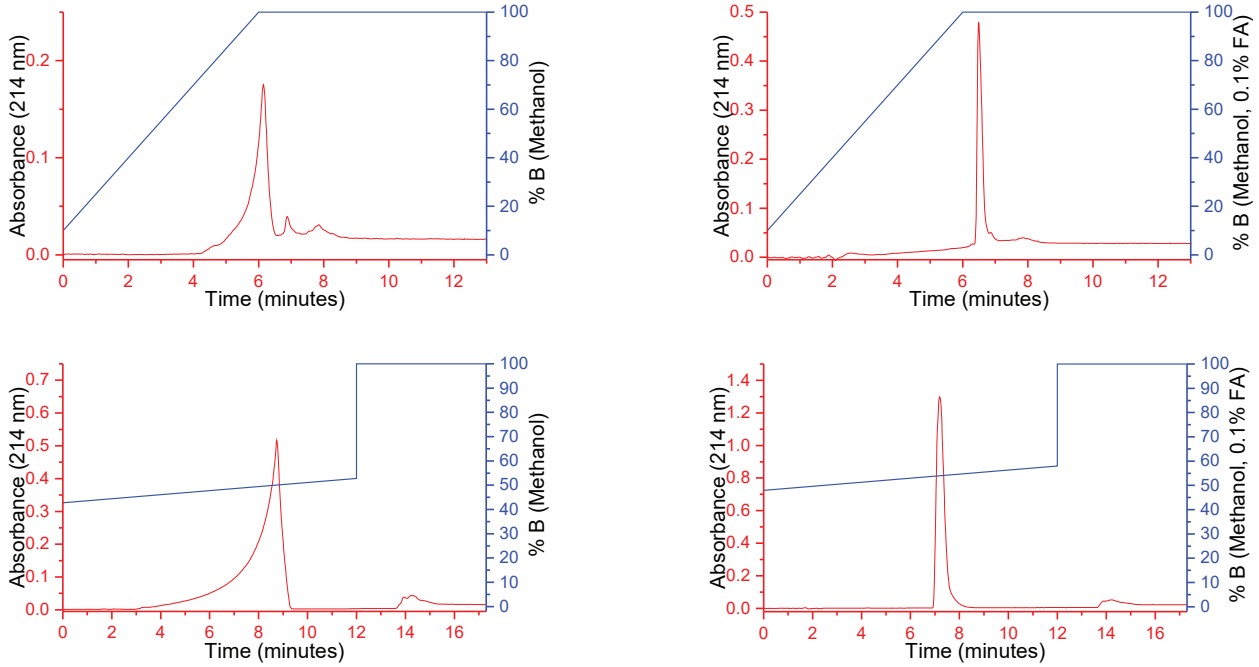

Figure 4 – Salicylic acid in water/methanol without modifiers (left) and in 0.1% FA (right)

Adding formic acid to a concentration of 0.1 % greatly improves the peak shape in both the scouting run and the preparative run. However, the salicylic acid and the quinine in the example above both elute slightly late.

Buffering

Compounds elute slightly differently from the expected elution time in preparative chromatography, probably due to concentration effects. The concentration of 0.1% modifier such as TFA is about 13 mMol. A peak containing 100 mg of a desired compound with a molecular weight of 300 g/mol will elute in approximately 20 mL of mobile phase, making a local concentration of 17 mMol, greater than the local concentration of solvent modifier. Running the quinine sample with 100 mMol ammonium acetate at pH 3 with water and methanol caused elution at the expected time.

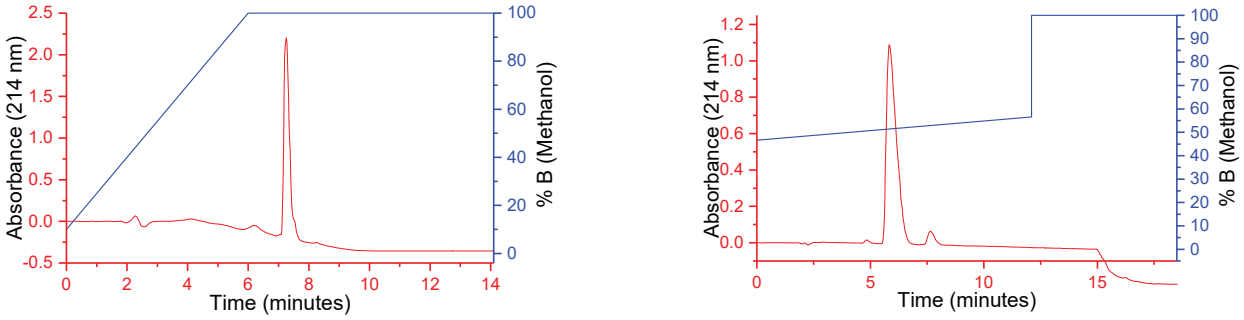

Figure 5 – Quinine, water (100 mMol ammonium acetate, pH 3)/ methanol scouting gradient and focused gradient with elution time targeted for 6 minutes. Buffered solvent shows expected elution at 6 minutes.

Modifiers such as TFA, acetic acid, or formic acid are soluble in the organic B solvent used for reverse phase chromatography and are added to both the A and B solvent. Buffers are usually only added to the aqueous solvent. Methanol is also better able to dissolve buffers and is used in favor of acetonitrile to avoid precipitation.

Strongly ionized Compounds

Bromocresol purple presented an interesting challenge. The compound converts between an ionized sulfonate and a sultone bearing no charge, creating very different retention for the two species.

The scouting run for bromocresol purple shows poor peak shape, giving a hint that the preparative run will also show poor peak shape.

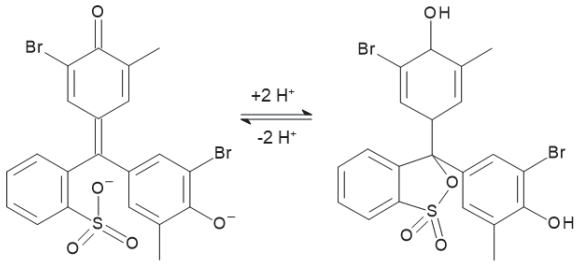

Figure 6 – Bromocresol purple converts from a polar sulfonate to a relatively non-polar sultone at low pH

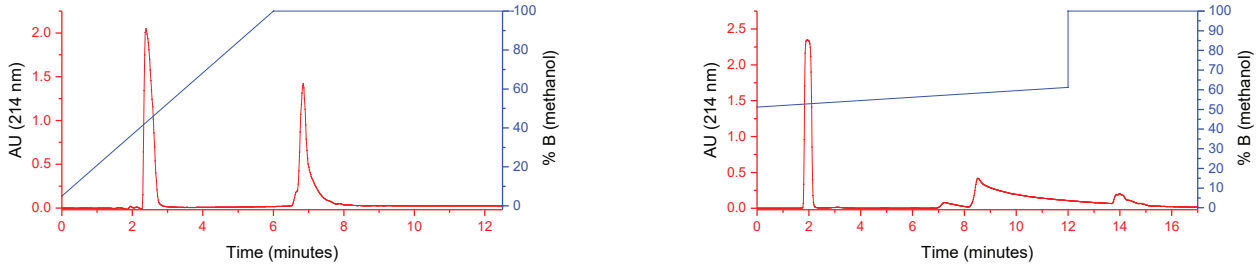

Figure 7 – Bromocresol purple run in water/methanol containing 0.1% formic acid; scouting gradient (left) and focused gradient (right).The DMSO used to dissolve the sample appears as the first eluting peak in both runs.

The preparative elution shows three peaks which are very broad.

Changing to a phosphate buffer allows the pH to be controlled at pH 2, and the ability to maintain the pH as the compound elutes.

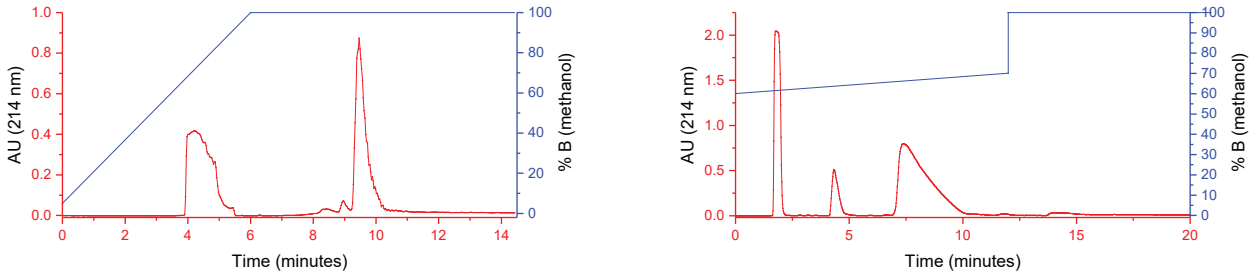

Figure 8 – Bromocresol purple purified in 50 mMol sodium phosphate at pH 2 and methanol.

Although the peaks are still fairly broad, the peak shape is greatly improved compared to the use of formic acid alone. Methanol needs to be used because acetonitrile won't dissolve the buffer. Using acetonitrile may cause clogs as the buffer precipitates. Also, phosphate is non-volatile. A desalting step is required to remove the salt prior to drying the compound[2]. The pump seals will also show reduced life span because the phosphate salt causes increased wear; the seals will need more frequent replacement.

Conclusion

Good pH control is needed to purify ionizable compounds to improve peak shape, resolution, and sample loading. For most compounds, a simple modifier such as TFA, formic acid, or acetic acid will suffice. A clue that a modifier is required is the peak shape observed from a scouting run. Any fronting or tailing during a scouting run will become worse during a preparative run which has a smaller change in solvent composition during the run.

Column care

When using silica-based columns, do not exceed pH 7.5 to 8.0 unless the column manufacturer instructions explicitly state the column may be run under such conditions. Aside from columns made specifically for high pH, the silica used in most columns will dissolves when exposed to polar, basic solvent systems.

A recommended lower pH limit for most bonded-phase columns, unless the manufacturer's instructions say otherwise, is pH 2.0. Lower pH tends to accelerate hydrolysis of the silyl ethers that hold the bonded phase in place.

If the column will not be used for some time, wash the column with mobile phase without modifiers. This reduces hydrolysis of the linkages holding the bonded phase in place and eliminates the chance for any precipitation and subsequent column or pump damage that may occur when a column containing buffer is washed with an organic solvent.

Modifier considerations

One of the most important considerations in preparative chromatography is how to remove the solvent modifier from the purified compound. Volatile modifiers such as TFA, formic acid, acetic acid, and ammonia are easily evaporated or lyophilized. Likewise, ammonium acetate and ammonium formate are volatile, especially when used at modest concentrations. Salts of mineral acids, such as phosphates, are removed in a desalting procedure.

Detectors may be sensitive to solvent modifiers. Some modifiers absorb UV light at some wavelengths, causing baseline drift. Mass spectrometer and evaporative light scattering detectors must only use volatile modifiers.

As mentioned earlier, the pH should be kept within the operating limits of the column. Non-volatile buffers will shorten the life span for pump seals because the crystals will act like sandpaper grit to abrade the surface of the softer seal material.

Some modifiers, such as TFA, acetic acid, formic acid, and ammonia are miscible in organic solvents and miscible in water. Others, notably salts and buffers, are not soluble in acetonitrile, and require the use of methanol as the strong solvent.

1. Blom, K. F.; Sparks, R.; Doughty, J.; Everlof, J. G.; Haque, T.; Combs, A. P. Optimizing Preparative LC/MS Configurations and Methods for Parallel Synthesis Purification. *J. Comb. Chem.* **2003**, 5, 670–683, DOI: 10.1021/cc020086n

2. Teledyne ISCO Chromatography Application Note AN95, August 2020, "Desalting Samples with RediSep Rf Gold® C18Aq Columns."

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