

## Preparing Buffered Mobile Phases for Reversed Phase HPLC

### When Should a Mobile Phase be Buffered?

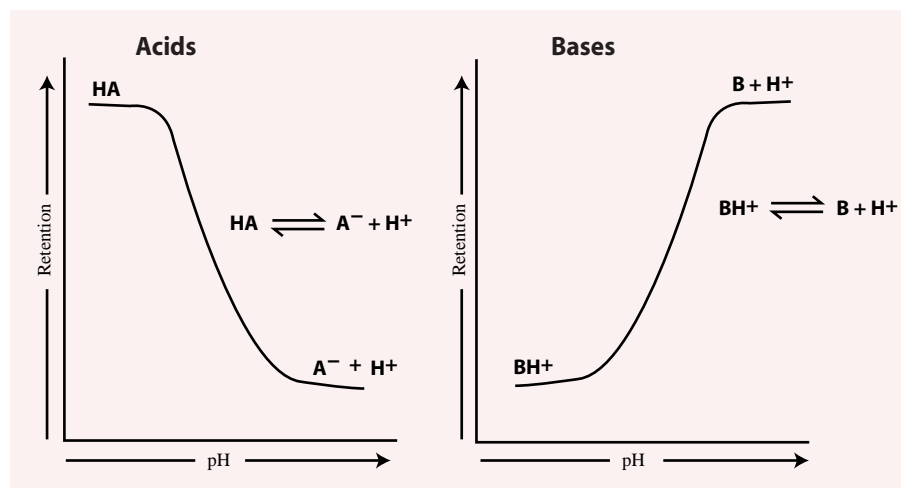
In reversed phase HPLC, the retention of analytes is related to their hydrophobicity. The more hydrophobic the analyte, the longer it is retained. When an analyte is ionized, it becomes less hydrophobic and, therefore, its retention decreases. Acids lose a proton and become ionized when pH increases and bases gain a proton and become ionized when pH decreases. (See Figure 1) Therefore, when separating mixtures containing acids and/or bases by reversed phase HPLC, it is necessary to control the pH of the mobile phase using an appropriate buffer in order to achieve reproducible results.

For the most robust methods, it is recommended that separations be developed at a mobile phase pH where the retention of analytes are little affected by changes in pH. When separating bases, for example, acidic mobile phases usually show better reproducibility than neutral mobile phases. As can be seen in Figure 2, methylamphetamine is fully protonated at a pH less than 3 and its retention is not affected by slight changes in mobile phase pH. However, at a pH of 7, closer to its pKa, a change in pH of only 0.2 units will shift retention by almost 9%.

It is often the case that an acceptable separation of ionic compounds can only be achieved at a mobile phase pH where retention and resolution are sensitive to pH. In fact, mobile phase pH can be a powerful variable to use to adjust retention and achieve separations of mixtures containing ionic compounds.

*A change in mobile phase pH from 7.0 to 7.2 will cause the retention of methylamphetamine to increase from 13.6 minutes to 14.8, an almost 9% increase. However, at a mobile phase pH of 2.8, there is a negligible change in retention with an increase of 0.2 pH units. When developing a reversed phase method for basic compounds, like methylamphetamine, you can expect a more robust method when using acidic mobile phases.*

FIGURE 1  
The Effect of pH on the Retention of Acids and Bases in Reversed Phase HPLC



As acids lose a proton and become ionized (with increasing pH), their retention decreases. As bases gain a proton and become ionized (with decreasing pH) their retention decreases.

Figure 2  
The Robustness of an HPLC Method Is Often Dependent on Mobile Phase pH

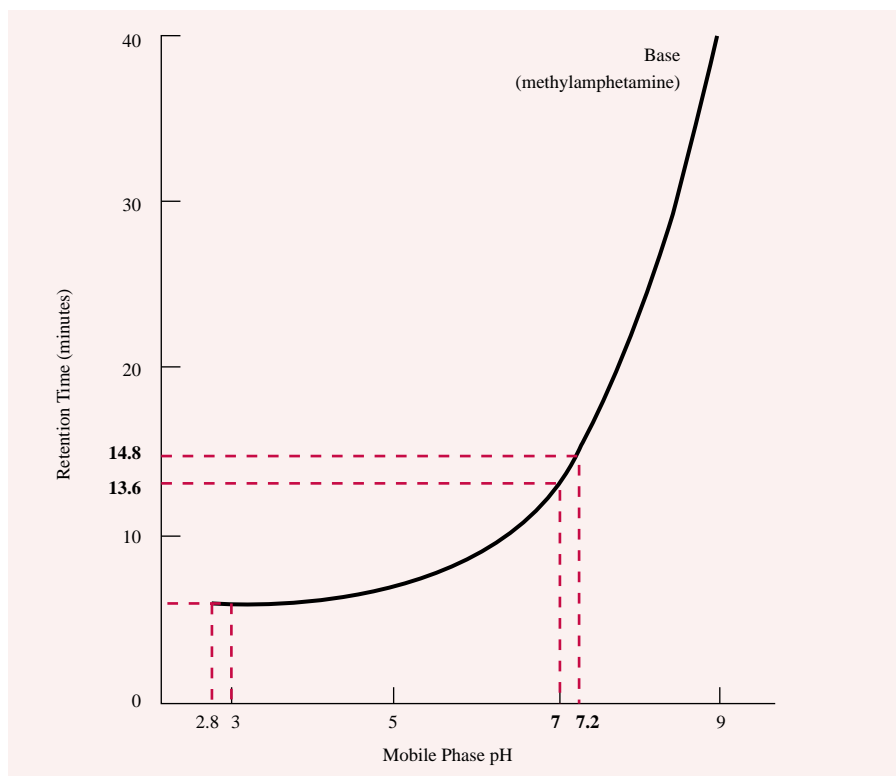
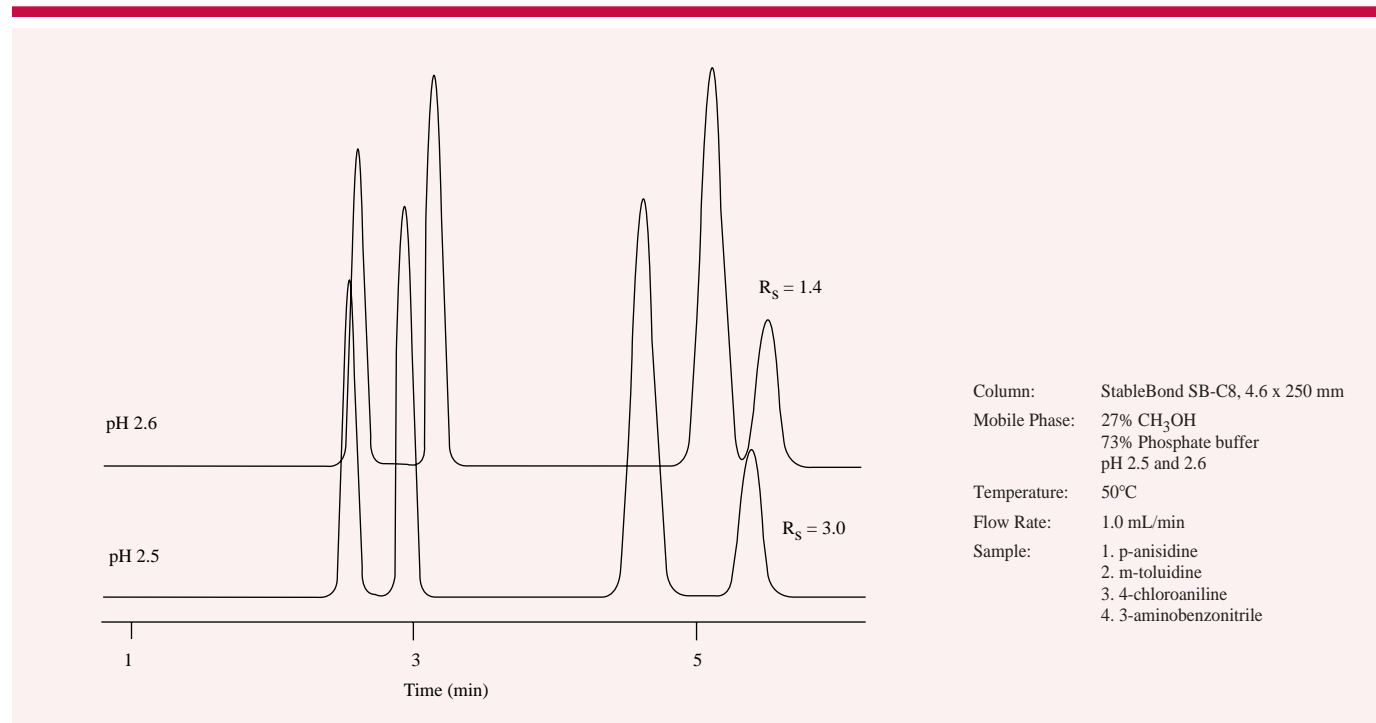


Figure 3  
**An Example of the Effect of Changes in Mobile Phase pH on Resolution**



Some separations are extremely sensitive to changes in mobile phase pH. This example shows how resolution goes from an unacceptable 1.4 to an acceptable 3.0 when the mobile phase pH is decreased by only 0.1.

Figure 3 shows a chromatographic separation where resolution goes from an unacceptable 1.4 to an acceptable 3.0 when the mobile phase pH is decreased by only 0.1 units. When a separation is this sensitive to pH, extra care has to go into making sure that the mobile phase pH is what the method calls for. Depending on the separation, the mobile phase pH may have to be controlled to within  $\pm 0.02$  units in order to achieve acceptable reproducibility. To accomplish this, the proper selection and preparation of mobile phase buffer is required.

### Selecting the Right Buffer

It is surprising how often reversed phase methods are developed using buffers that have little or no buffering capacity at the specified mobile phase pH. Methods that specify a phosphate buffer in the pH range of 4 to 6, or an acetate buffer in the range of 6 to 7 are, unfortunately, not uncommon. These buffers are not just useless in these pH ranges, they complicate the preparation of mobile phase unnecessarily and give the analyst a false sense of controlling the reproducibility of the separation.

Optimum buffering capacity occurs at a pH equal to the pKa of the buffer. In general, you can expect most buffers to provide adequate buffering capacity for controlling mobile phase pH only within  $\pm 1$  unit of their pKa. Beyond that, buffering capacity will be inadequate. Table 1 lists some

commonly used buffers for reversed phase HPLC. Included in the Table are pKa values, optimal range for controlling pH, and uv cutoffs for the buffers listed. Since it is becoming more common to find HPLC interfaced to mass spectrometers, volatile buffers for LC/MS applications are indicated.

Table 1  
**Commonly Used Buffers for Reversed Phase HPLC**

Buffer	pKa	Buffer Range	UV Cutoff (nm)
Phosphate	2.1	1.1 – 3.1	200
	7.2	6.2 – 8.2	
	12.3	11.3 – 13.3	
Formic acid*	3.8	2.8 – 4.8	210
Acetic acid*	4.8	3.8 – 5.8	210
Citrate	3.1	2.1 – 4.1	230
	4.7	3.7 – 5.7	
	5.4	4.4 – 6.4	
Tris	8.3	7.3 – 9.3	205
Triethylamine*	11.0	10.0 – 12.0	200
Pyrrolidine	11.3	10.3 – 12.3	200

\* Volatile buffers

## Buffer Concentration

The concentration of the mobile phase buffer usually has little effect on retention in reversed phase HPLC, just as long as the buffer concentration is high enough to control pH. A buffer concentration in the range of 25 to 50 mM is adequate for most reversed phase applications.

This concentration is also low enough to avoid problems with precipitation when significant amounts of organic modifiers are used in the mobile phase and, in the

case of phosphate buffers, low enough to minimize the abrasive affect on pump seals. It is seldom advisable to use a buffer concentration less than 10 mM.

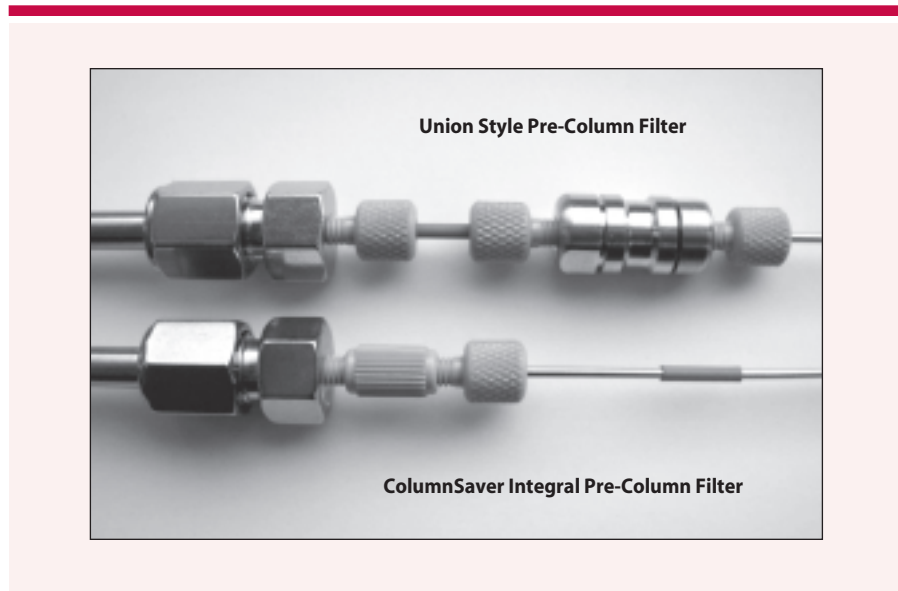
There are cases where solute retention in reversed phase HPLC is affected by buffer concentration. These cases are usually confined to situations where there are ion exchange interactions taking place between basic solutes and acidic silanols on the surface of silica stationary phase supports. Generally this will occur when

separating basic compounds using reversed phase columns packed with stationary phases that have significant silanol activity and is most often observed when using mobile phases with pH values greater than 3. Increasing the concentration of the mobile phase buffer, and thereby increasing the ionic strength of the mobile phase, will sometimes suppress this ion exchange interaction and reduce this “secondary retention” affect.

Table 2  
**Some Considerations in the Selection of Mobile Phase Buffers for Reversed Phase HPLC**

- Phosphate is more soluble in CH<sub>3</sub>OH/water than in CH<sub>3</sub>CN/water or THF/water.
- NH<sub>4</sub> salts are more soluble in organic/water mobile phases than potassium salts, and potassium salts are more soluble than sodium salts.
- TFA and TEA degrade with time and increase their uv absorbance. Mobile phases containing these buffers should be made fresh often.
- Citrate buffers attack stainless steel. When using these buffers, be sure to flush them out of the system as soon as you complete your assay.
- Microbial growth can quickly occur in buffered mobile phases that contain little or no organic modifier. This growth will accumulate on column inlets and damage chromatographic performance. These mobile phases should be made fresh daily and pre-column filters should be used to protect columns (See Figure 4).
- Using boiled water to prepare buffered mobile phase and storing it under refrigeration will help reduce the problem of microbial growth.
- At pH greater than 7, phosphate buffers accelerate the dissolution of silica and severely shorten the lifetime of silica-based HPLC columns. Organic buffers should be used at pH higher than 8 (See Figure 5).

Figure 4  
**Use Pre-column Filters to Protect Columns from Microbial Growth in the Mobile Phase**



Place a pre-column filter just before the column to prevent microbial growth, or any other particulate matter in the mobile phase, from reaching the column and damaging its performance. Integral pre-column filters add the least amount of dead volume to an HPLC system and are preferred over union-style filters for high performance applications. **The part number for a 10-pack of ColumnSaver pre-column filters is MMCS210.**

## Preparing a Buffered Mobile Phase

The following steps are suggested for preparing a buffered mobile phase:

1. Select an appropriate buffer.

Refer to Tables 1 and 2 to determine the right buffer for your application.

2. Prepare an aqueous buffer solution of the desired concentration and pH.
3. Measure the pH of the solution and adjust, if necessary, to the desired pH.

When adjusting the pH of a buffer solution, make sure to wait until the solution reaches equilibrium after adding acid or base before measuring the pH.

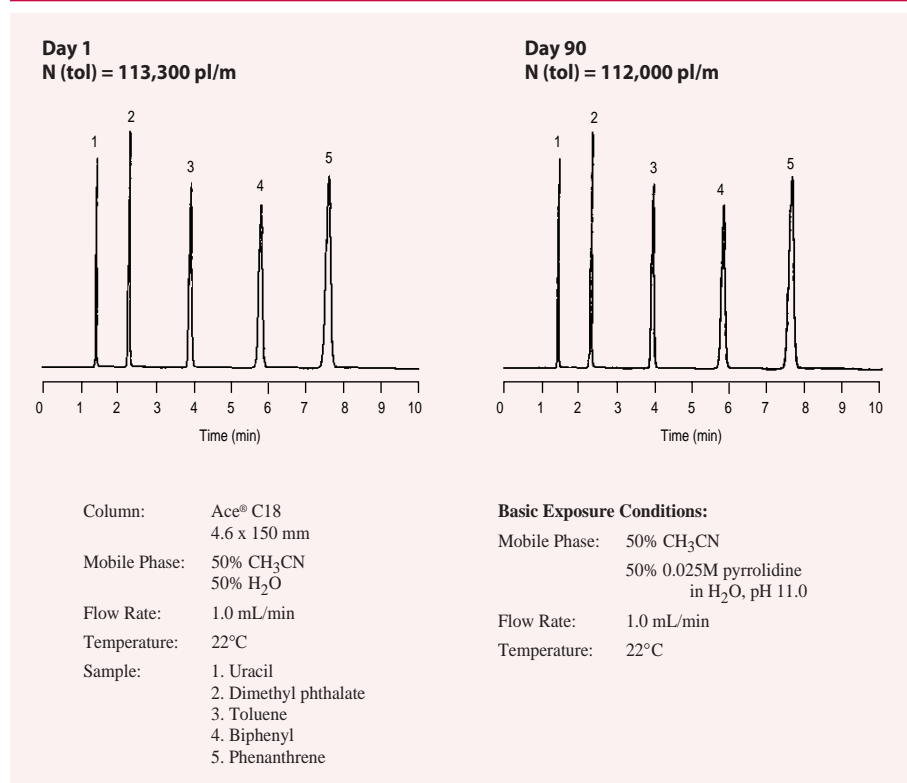
4. Combine the aqueous buffer solution with the appropriate organic modifier, e.g., methanol or acetonitrile, to produce the desired mobile phase.

*Continued on page 4*

You may choose to measure and adjust the pH of the final mobile phase rather than the aqueous buffer solution. However, measuring the pH of an aqueous/organic solution is not as accurate as measuring the pH of a purely aqueous solution, so it is highly recommended that you measure and adjust pH before adding the organic modifier. But if you do decide to measure and adjust the pH of the mobile phase, you should always do it the same way each time you prepare mobile phase including using the same glass and reference electrodes.

Zirchrom Separations provides a tool on their web site that is very helpful in preparing buffer solutions. It is called the "Buffer Wizard." The user inputs the desired pH and concentration of a buffered solution and then selects from a list of 19 acids and 19 bases to use for the buffer. The "Buffer Wizard" then automatically makes the required calculations and gives you a prescription for preparing the buffer. Zirchrom Separations provides the use of the "Buffer Wizard" online at no charge. Go to [www.zirchrom.com](http://www.zirchrom.com) and click on the "Buffer Wizard" calculator.

Figure 5  
**Some Silica-based HPLC Columns Can Be Used at High pH if Organic Buffers Are Used**



*At a mobile phase pH greater than 7, dissolution of silica can severely shorten the lifetime of columns packed with silica-based stationary phases. Phosphate or carbonate buffer and elevated temperature accelerate this dissolution. However, by using organic buffers and densely bonded stationary phases, surprisingly good column lifetime can be achieved with high pH mobile phases. In this example, the combination of a densely bonded C18 stationary phase and a pyrrolidine buffer permitted this separation to be run at pH 11 for over 90 days without any deterioration in performance.*



MAC-MOD Analytical, Inc.  
 P.O. Box 2600  
 127 Commons Court  
 Chadds Ford, PA 19317  
**Phone: 1-800-441-7508**  
**FAX: 1-610-358-5993**  
[www.mac-mod.com](http://www.mac-mod.com)  
[info@mac-mod.com](mailto:info@mac-mod.com)