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Chromatography Solutions

A Guide to HPLC and LC-MS Buffer Selection

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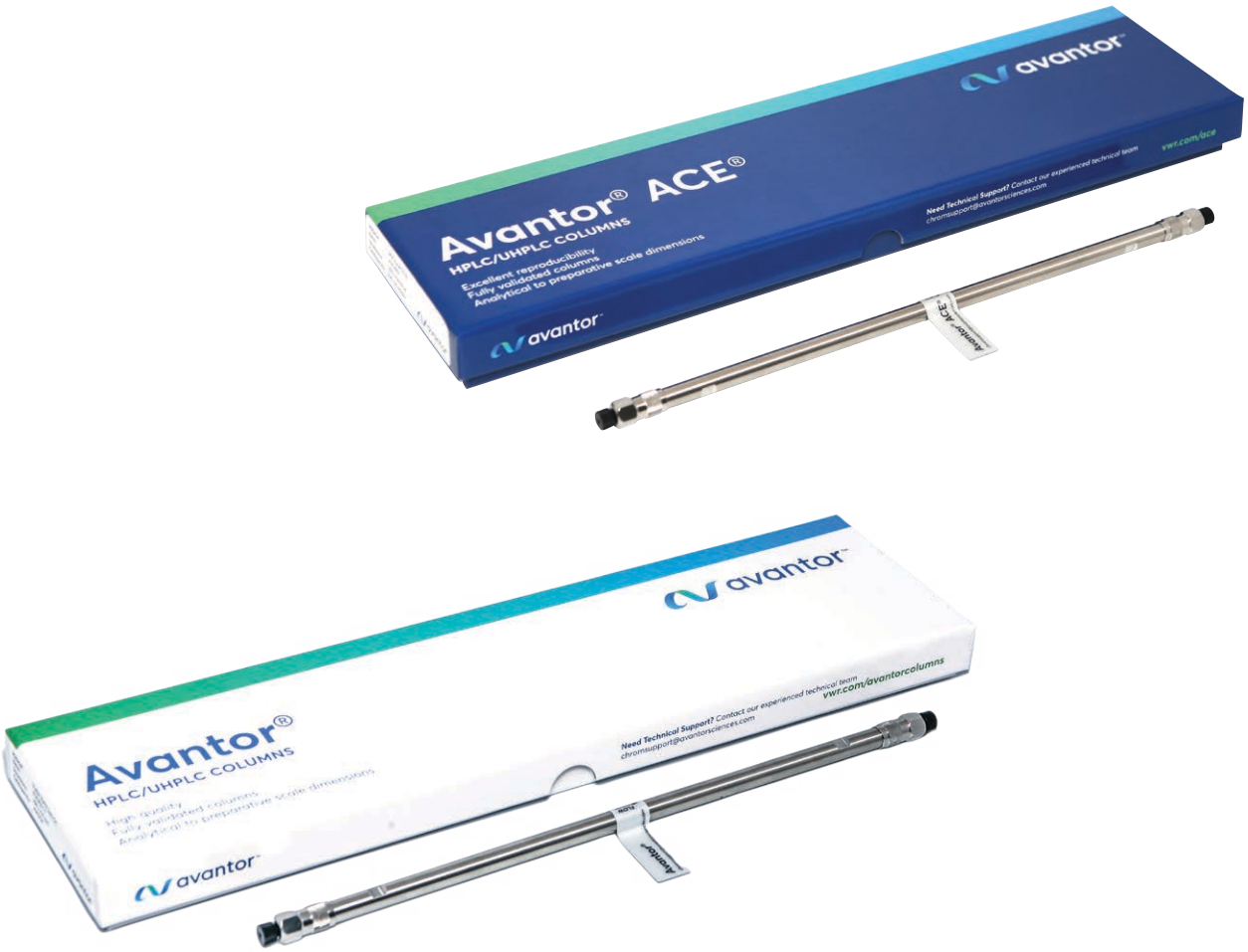
Introduction

When samples contain ionisable compounds, the mobile phase pH can be one of the most important variables in the control of retention in a reversed-phase HPLC (RP-HPLC) separation. However, if it is not controlled properly, pH can be a source of many problems. Since most compounds analysed by RP-HPLC contain one or more acidic or basic functional groups, most mobile phases require pH control. For this reason, buffers are widely used. This booklet highlights some of the important aspects of mobile phase pH for the practical chromatographer.




HiPerSolv CHROMANORM
HiPerSolv CHROMANORM
CM6430
CM6270
CM6310
3.0L

WHY CONTROL PH?

Figure 1 illustrates the need for pH control when ionisable compounds are present. When an acid is more than 2 pH units above or below its pKa, it will be >99% ionised or non-ionised, respectively. Bases are ionised below their pKa and non-ionised above their pKa. The non-ionised form will be less polar (more hydrophobic), and thus more strongly retained in a reversed-phase system. Thus, at low pH, acids will be more retained (Fig. 1a) whereas bases will be more retained at high pH (Fig. 1b).

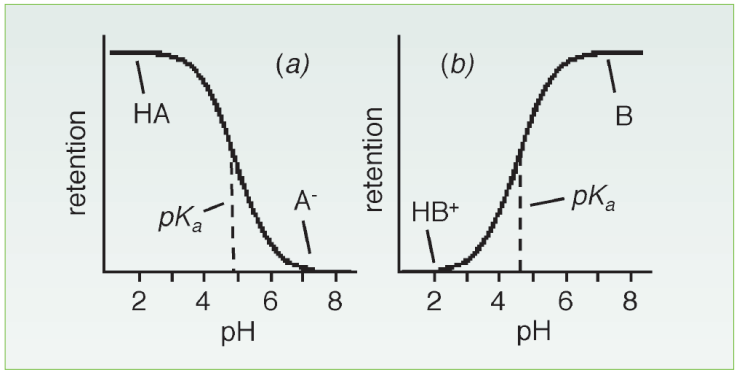


FIGURE 1. Retention vs. pH for a hypothetical acid (a) and base (b).

If the mobile phase pH is near the pK_a, you can see that small changes in pH can make large changes in retention – not what is desired for a robust separation. This is illustrated in Figure 2a, which shows the extreme sensitivity of some compounds to very small changes in pH. Here the resolution changes by a factor of two for a change of only 0.1 pH units – this is the amount of error in pH adjustment common to many laboratories. Figure 2b represents a plot of retention vs. pH for an acid, a base and a neutral compound. At pH 5, retention is less sensitive to pH than it is at pH 3 (for the acid) or pH≥6 for the base.

Besides the instability of retention times when the pH is near the pKa, relative peak spacing (selectivity) can change if compounds of similar structure are present.

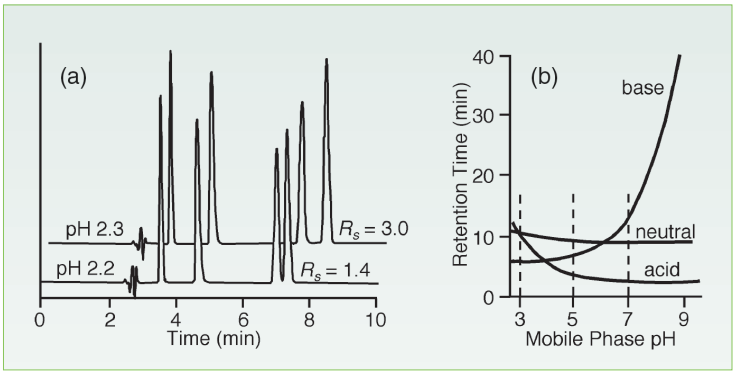


FIGURE 2. **The effect of small changes in mobile phase pH on the separation.** (a) Basic analytes: p-anisidine, m-toluidine, 4-chloroaniline, 3-aminobenzonitrile (in retention order); 27:73 methanol/phosphate buffer. (b) Acid: salicylic acid; base: methylamphetamine; neutral: phenacetin.

Another factor that should be considered when choosing the mobile phase pH is the stability of the column. As a general rule, silica-based columns should be operated at 2<pH<8. At pH<2, bonded phase loss due to hydrolysis can occur. Above pH 8, the silica backbone becomes increasingly soluble. Higher-purity silica tends to tolerate high pH better than lower-purity products. A further complicating matter is the potential for ionisation of unbonded silanol (-Si-OH) groups on the surface of the silica particle. For older, less-pure silica (often referred to as "Type-A" silica), the pK_a of these silanol groups is in the pH 4-5 region. This means that at pH>6, significant silanol ionisation can occur for these materials. Historically, this has been the major cause of peak tailing for basic compounds through cation exchange processes. The newer, high-purity ("Type-B") silica has a pK_a>7, so peak tailing due to cation exchange with ionised silanol sites is minimal. This is one reason why high-purity silicas give much better peak shape for bases than their older counterparts, as is illustrated dramatically for several basic components in the separations shown in Figure 3. In addition to peak shape improvement, the use of high purity silica leads to improved reproducibility compared to low purity silica due to the reduction of these unpredictable secondary silanol interactions.

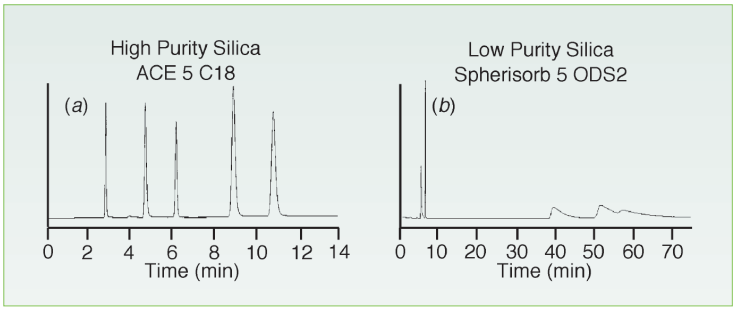


FIGURE 3. **The effectiveness of high purity silica in reduction of silanol tailing of bases.** Components: norephedrine, nortriptyline, toluene (neutral), imipramine, amitriptyline. Conditions: 250 x 4.6 mm, 5 µm columns; 80:20 methanol/25 mM phosphate (pH 6.0); 1.00 ml/min.

PRACTICAL IMPLICATIONS

The characteristics of the column and of samples in general, lead to a recommendation to start method development with a mobile phase in the pH 2-3 range. At this pH, the ionisation of most organic acids will be suppressed, as will the ionisation of any silanol groups on the column. Bases will be ionised under these conditions, but the pKa of most basic compounds is >7, so operating at a sufficiently high pH to suppress ionisation will be detrimental to most columns. So, all other things being equal, it is best to start out at a low pH. If you need to operate the column at a high pH, be sure to select a column known to be stable in the pH region you choose.

If low-pH ion suppression does not provide acceptable results, the mobile phase pH can be adjusted to help obtain the desirable separation. It usually is most fruitful to adjust the mobile phase

organic content (%B-solvent) to obtain acceptable retention for neutral and non-ionised compounds, then to adjust the pH to fine-tune retention of ionic analytes.

CONTROLLING PH

Since the retention of ionisable compounds is very sensitive to the mobile phase pH, it is necessary to control the pH of the mobile phase by the addition of a buffer. A buffer maintains the pH when a small amount of acid or base is added. Many different substances have been used for buffering in HPLC; some of these additives are listed in Table 1. A buffer is most effective when used within ±1 pH unit of its pK_a, but may provide adequate buffering ±2 pH units from the pK_a.

pK_a (25 °C)	Compound
0.3	trifluoroacetic acid[2]
2.15	phosphoric acid (pK_1)
3.13	citric acid (pK_1)
3.75	formic acid
4.76	acetic acid
4.76	citric acid (pK_2)
4.86	propionic acid
6.35	carbonic acid (pK_1)
6.40	citric acid (pK_3)
7.20	phosphoric acid (pK_2)

pK_a (25 °C)	Compound
8.06	tris
9.23	boric acid
9.25	ammonia
9.78	glycine (pK_2)
10.33	carbonic acid (pK_2)
10.72	triethylamine
11.27	pyrrolidine[3]
12.33	phosphoric acid (pK_3)

[1] data of [1]; [2] Merck Index; [3] CRC Handbook of Chemistry and Physics

TABLE 1. pK_a Values of Common Mobile Phase Additives[1].

The most popular buffers for HPLC with UV detection are phosphate and acetate. Phosphate and acetate are particularly useful buffers because they can be used at wavelengths below 220 nm. As can be seen from Table 2, phosphate has three pK_a values that give it three buffering ranges: 1.1<pH<3.1, 6.2<pH<8.2, and 11.3<pH<13.3 (allowing for buffering of pK_a ±1 pH units). Practical limits of column stability require that we truncate the lower range to 2.0<pH<3.1 and eliminate the highest range.

Notice that there is a gap in buffering between pH 3.1 and pH 6.2 for phosphate. This means that, although it is possible to adjust the pH of phosphate to 5.0, there is negligible buffering capacity at this pH. To fill this buffering gap, another buffer is needed. Fortuitously, acetate fills this need well, with a buffering range of 3.8<pH<5.8. With a slight extension of the buffering range from ±1 pH units from the pK_a, phosphate and acetate can cover the entire pH range of 2<pH<8 normally used for silica-based columns.

Sometimes during method development, you may desire to have full control of the pH over the useful range of the column. In this case, a blend of phosphate and acetate buffer will allow continuous variation of the mobile phase from 2<pH<8. Once you find the desired pH, the buffer not needed can be eliminated. For example, if the final mobile phase pH is 4.3, acetate is all that is needed, so phosphate does not need to be used at all.

Some analysts like to use citrate for a buffer, because it has three overlapping pK_a values that allow buffering over the 2.1<pH<6.4 range (Table 2). However, citrate does not have as low a UV-cutoff as acetate and phosphate, so work at wavelengths below 220 nm is not possible; in addition, some analysts find that they have more problems with check valves when citrate is used. So citrate buffer usually is a second choice to phosphate and acetate.

Buffer	pH range	LC-MS compatible
phosphate (pK_1)	1.1 – 3.1	X
phosphate (pK_2)	6.2 – 8.2	X
phosphate (pK_3)	11.3 – 13.3	X
acetate[1]	3.8 – 5.8	YES
citrate (pK1)	2.1 – 4.1	X
citrate (pK_2)	3.7 – 5.7	X
citrate (pK_3)	4.4 – 6.4	X
trifluoroacetic acid (0.1%)	2.0	YES
phosphoric acid (0.1%)	2.0	X
formic acid (0.1%)	2.7	YES
ammonium formate	2.7 – 4.7	YES
ammonium bicarbonate	6.6 – 8.6	YES
borate	8.3 -10.3	X

[1] Suitable for LC-MS as ammonium acetate.

TABLE 2. Common HPLC Buffers.

WHAT ABOUT LC-MS?

When a mass spectrometer is used as the LC detector (LC-MS), the mobile phase must be volatile, because one of the functions of the LC-MS interface is to vaporize the mobile phase. This means that the most popular buffer for LC-UV work, phosphate, cannot be used. Ammonium acetate is sufficiently volatile for LC-MS use, but we are left with the 2.0<pH<3.8 and 5.8<pH<8.0 range to cover. Table 2 lists several additional buffers that are sufficiently volatile for LC-MS use. Ammonium formate (2.7<pH<3.7) does a fairly good job of filling the gap at the low-pH end. Ammonium bicarbonate (6.6<pH<8.6) works for higher pH values. These do not completely cover the desired buffering ranges, but for LC-MS applications they are what are available to work with.

JUST INTERESTED IN LOW PH?

If a low-pH mobile phase is all that is important, 0.1% v/v phosphoric acid (Table 2) provides reasonable buffering at pH 2 for LC-UV applications. Trifluoroacetic acid (TFA) also generates a mobile phase pH of ≈2 at 0.1% v/v (Table 2), and for many years was the additive of choice for LC-MS at low pH. TFA also acts as an ion-pairing reagent, and is widely used for protein and peptide separations. TFA, however, can suppress ionisation in the LC-MS interface, causing a drop in signal, so it has fallen out of favour in recent years. Instead, 0.1% formic acid (pH ≈2.7, Table 2) is the first choice for LC-MS at low pH. With the use of an acid for low-pH, it is assumed that the pH is more important than the buffering capability – if true buffering is needed, select a buffer from Table 2 that encompasses the desired pH.

OR HIGH PH?

As mentioned earlier, the solubility of silica increases as the mobile phase pH is increased above pH 8, so pH>8 generally is not recommended. However, it may be necessary to work with higher-pH mobile phases in order to obtain the desired separation. In such cases, you should select a column designed for work at high pH. As a general rule, high purity silica, high "carbon load" and endcapping reduce silica solubility. Phosphate buffer should be avoided, because it enhances silica dissolution at high pH. Instead use organic buffers, such as pyrrolidine, which can help extend column lifetime at high pH. Even with careful selection of conditions, column lifetimes at pH>8 are expected to be shortened. Of course columns which use polymeric, rather than silica, particles are not susceptible to silica dissolution, so may be used at high pH, but such columns are not available in as wide a range of stationary phases as silicabased columns.

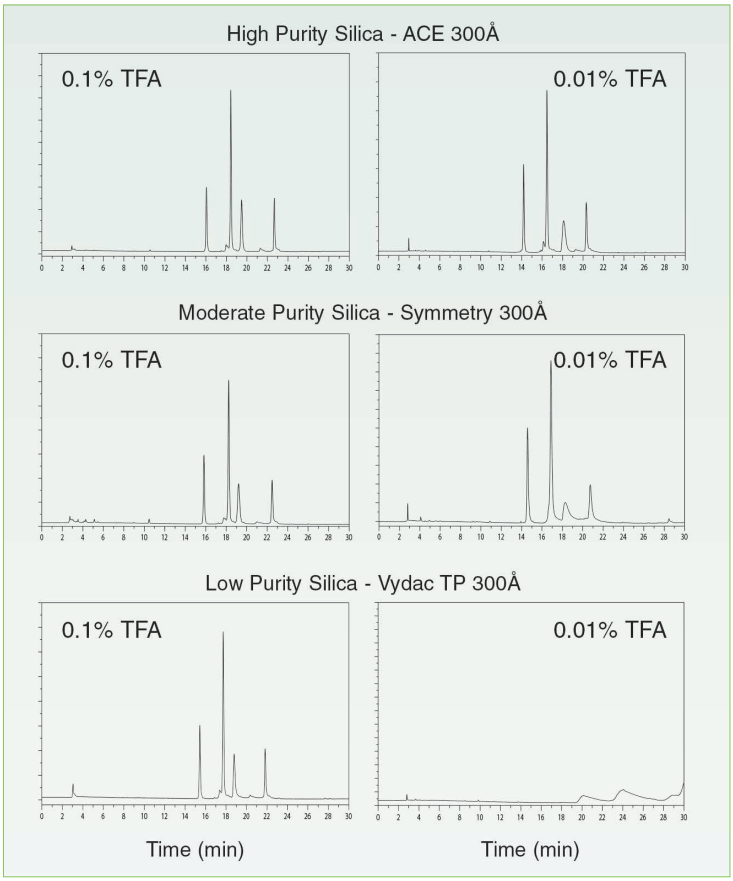
HOW MUCH?

One function of the buffer is to keep the analyte at the desired pH. If you consider just this aspect of buffering, very little buffer is needed. For analytical work, many samples are in the µg/ml to ng/ml range. So for injections of <100 µl, the mass on column will be no more than a few hundred nanograms. It takes very little buffer, even at 1 pH unit from the pKa of the buffer, to provide all the buffering that is needed for the sample.

The buffer also must buffer the column so that it stays at constant pH. A relatively large volume of buffered mobile phase passes through the column, so the stationary phase is constantly exposed to buffer. Newer, high-purity silica columns have fewer acidic silanol groups, so they need less buffer than the older, lower-purity silica columns. This property of the column is illustrated with the chromatograms of Figure 4. (In this application, trifluoroacetic acid serves to keep the pH low and also acts as an ion-pairing reagent, so the two effects are not isolated from each other.) Note in the left column of chromatograms that the peak shape is comparable for all three columns when 0.1% TFA is added to the mobile phase. However, when the concentration of TFA is dropped ten-fold to 0.01% as shown in the chromatograms on the right of Figure 4, the correlation of silica purity and peak shape is obvious.

The peak shape degrades slightly for the high-purity silica column when compared to that at 0.1% TFA, but there is a dramatic change in peak shape for the moderate- and low-purity silica columns. The only variable that has changed between the two sets of chromatograms is the concentration of TFA. It appears that the TFA acts, at least in part, to buffer the column so that the strong interactions responsible for peak tailing are minimized. When insufficient buffer is present, peak shape suffers on all columns. Although most new methods are developed on high-purity, Type-B silica, the level of silanol acidity may vary significantly from one manufacturer's product to another's. As a safety factor, it is a good idea to use more than the minimum amount of buffering required. This leads us to recommend acid additives in the range of about 0.1% v/v or buffers of at least 5-10 mM in the final solution.

A third, and perhaps most important, task of the buffer is to buffer the sample as it is injected, so that it quickly attains the mobile phase pH. It should be obvious that the job of the mobile phase buffer is much easier if a small-volume injection of sample is made in an injection solvent at the same pH as the mobile phase, as opposed to a large injection of sample in an injection solution with a pH that differs significantly from the mobile phase. One can overcome the need for a high concentration of buffer in the mobile phase by adjusting the sample pH prior to injection.



Column: 250 x 4.6 mm, 5 µm, C18 300Å. Conditions: A: 0.1% or 0.01% TFA in H_2O; B: 0.1% or 0.01% TFA in ACN; 5-70% B in 30 min; 1.00 ml/min, 280 nm. Components (in retention order): ribonuclease A, cytochrome C, holo-transferrin, apomyoglobin.

FIGURE 4. Effect of trifluoroacetic acid on peak shape.

SOLUBILITY ISSUES

We've just looked at the need to have enough buffer present to buffer the sample, both during injection and during the

separation, as well as buffering the column. On the other end of the scale, how much is too much? One critical problem to avoid is precipitation of the buffer in the system. If buffer precipitates in the HPLC pump, it can be a lot of work to clean out, and parts may have to be replaced. However, if the buffer precipitates inside the column, the column should be discarded, because it is nearly impossible to wash precipitated buffer from the pores in the stationary phase.

As most analysts know, acetonitrile (ACN) is a much poorer solvent for buffers and salts than methanol (MeOH), and tetrahydrofuran (THF) is even worse. This is illustrated in the data of Table 3 for the least soluble common buffer, potassium phosphate, at pH 7.0 [2]. At 80% organic, phosphate is soluble at a 15 mM concentration in MeOH, but only at 5 mM in ACN; it is practically insoluble (<5 mM) in 80% THF. At mobile phases containing 50% organic, phosphate is soluble at >50 mM MeOH or ACN and 25 mM THF. This means that for isocratic separations that use high-organic (%B) mobile phases or gradient runs that end at high %B, care must be taken to avoid conditions in which buffer precipitation can occur.

%B	MeOH	ACN	THF
50[2]	>50 mM	>50 mM	25 mM
60	>50	45	15
70	35	20	10
80	15	5	<5
90	5	0	0

[1] data of [2]; [2] >50 mM in all solvents at lower %B.

TABLE 3. Solubility of Potassium Phosphate, pH 7.0, in Common HPLC Solvents[1].

The data shown in Table 3 are for bulk solutions; the situation can get even worse for on-line mixing. With either high-pressure-mixing or low-pressure-mixing HPLC systems, the A- and B-solvents come in direct contact with each other at full strength when they enter the mixer. This can create problems that you might not expect otherwise. Consider the case in which a mobile phase of 60% ACN containing 10 mM buffer is desired – this is much less than the 45 mM critical concentration for 60% ACN shown in Table 3. If the A-reservoir contained 25 mM buffer and the B-reservoir contained 100% ACN, a 40/60 blend of A/B should give the desired mobile phase. However, at the point the solvents are mixed, 100% ACN contacts 25 mM buffer and precipitation may occur. This may cause problems, or the precipitate may redissolve quickly, depending on specific equipment design characteristics.

The choice of salts also can make a significant difference in solubility. As is shown in Table 4, the ammonium salts of phosphate are much more soluble in ACN than are the potassium salts under comparable conditions. Acetate is much more soluble than phosphate. Generally, it is best to use potassium salts rather than sodium salts because of solubility differences.

%B	ammonium acetate pH 5.0	ammonium phosphate pH 3.0	potassium phosphate pH 3.0	ammonium phosphate pH 7.0	potassium phosphate pH 7.0
60[2]	>50 mM	>50 mM	>50 mM	50 mM	45 mM
70	>50	>50	>50	25	20
80	>50	35	20	5	0
90	25	5	0	0	0

[1] data of [2]; [2] >50 mM in all solvents at lower %B.

TABLE 4. Solubility of Various Buffers in Acetonitrile[1].

DILUTION EFFECTS

There are several possible ways to formulate a mobile phase comprising buffer and organic solvent. For isocratic work, the mobile phase can be mixed manually so that mobile phase is pumped from a single reservoir. Alternatively, the HPLC system can be used to blend the components. A concentrated buffer can be mixed with pure organic solvent or a diluted buffer can be blended with the organic solvent containing buffer at the same concentration. For gradient elution methods, only the last two techniques can be used (of course in either isocratic or gradient systems, the A- and/or B-solvents can be an aqueous/organic blend.) Each mixing technique has tradeoffs. Manual mixing is the least likely method to result in buffer precipitation in the HPLC system, because the mixture can be observed for clarity and filtered prior to use to remove any precipitate.

On-line mixing is more convenient for isocratic method development and is required for gradient elution. Blending aqueous buffer solution with pure organic solvent is simple, but has two potential problems. Firstly, because the buffer will be diluted by the organic solvent, the A-solvent must be at a higher concentration of buffer than that required in the mobile phase and secondly with gradients, a reverse-gradient in buffer concentration will exist. The higher the buffer concentration, the more likely it is that precipitation will occur when the buffer and organic solvent initially contact each other in the mixer. In the example cited earlier, a 10 mM potassium phosphate, pH 7.0 buffer in 60% ACN would be soluble in bulk solution, but would require 25 mM phosphate in the A-reservoir. When 25 mM phosphate contacts 100% ACN in the mixer, precipitation may occur.

An alternative way to blend the solvents in the example above would be to put 10 mM phosphate in the A-reservoir. For the B-solvent, hand-mix 70% ACN with 30% of a 33 mM phosphate buffer solution to achieve 70% ACN containing 10 mM phosphate (half the critical concentration of Table 3). Now the A- and B-solvents can be blended to achieve 60% ACN with a final concentration of 10 mM phosphate. This technique avoids the potential of precipitation of buffers within the HPLC and allows the flexibility to mix online any mobile phase containing 70% ACN or less. It has the added advantage for gradient work that the concentration of buffer will be constant throughout the gradient, so dilution of the buffer during the gradient should not be a concern.

BUFFER PREPARATION

There are four common techniques to prepare buffers that are reviewed briefly here. Buffer recipes are plentiful, so they have not been reproduced. Many biochemistry and chemistry texts contain tables of buffers; one handy compilation of buffer recipes for HPLC is contained in the appendix of [3]. An excellent on line tool [4] prompts for the target buffer pH, concentration and volume and then generates a recipe.

The best way to prepare buffers is to weigh the buffer components according to the recipe and dilute them with water to the desired volume. At first this may seem like a tedious procedure, but it is more accurate, usually is faster than pH adjustment techniques and is easy to reproduce over time and from lab to lab. This technique delivers a buffer of the desired concentration and pH. It is a good idea to check the pH after formulation to ensure no mistakes were made. An alternative buffer preparation technique is to make separate solutions of the acidic and basic buffer components (e.g. phosphoric acid and the phosphate salt at 10 mM each). The two solutions are blended and the pH is monitored with a pH meter until the desired pH is reached. This gives a buffer of the desired concentration and with a pH as close as the pH meter can read (usually ±0.1 pH units in general use).

A variation on blending equimolar solutions of acid and base is to make up one component at the desired concentration (e.g. the phosphate salt at 10 mM) and titrate with concentrated acid (e.g. phosphoric acid) until the desired pH is reached. This buffer will be more concentrated than the equimolar blend, but the target pH should be correct (within the pH meter limits).

Finally, the improper technique of buffer preparation should be cited. This is to adjust the pH after organic solvent is added. Although it is possible to obtain a pH reading with organic solvent present [5-7], it cannot be compared directly to aqueous pH measurements, so the numeric value of the pH is not very meaningful. This technique also is more susceptible to other factors (e.g. %-organic, temperature) than the other methods of buffer preparation and therefore should be avoided.

Although, for most methods, it will not matter if you prepare buffer by weight, equimolar blending, or titration with concentrated acid, some methods will be sensitive to the subtle differences. For this reason, it is important to include a statement of the buffer preparation technique in the documentation for a method.

PRECAUTIONS

To consistently obtain high-quality results, a few techniques should be practiced routinely. First, use the best reagents available – most buffer components are available in HPLC grade. Buffer purity is more important for gradient elution separations than isocratic ones. Some reagent-related problems become more of an issue as the detection limits are lowered. This is illustrated in Figure 5 [8]. One way of checking for impurities is to run a non-injection, blank gradient. In the case of Figure 5a, the baseline showed several peaks larger than 10 mAU. For an assay with peaks of 0.8 to 1 AU in size range, such noise would be of little concern, but for this application, the sample contained peaks of 5-10 mAU that required quantification. Dirty blank gradients, as in Figure 5a, typically point to problems due to dirty water or reagents. However, in this case, the problem was isolated to the method of buffer preparation. If the pH was checked by dipping the pH meter probe into the bulk mobile phase, the results of Figure 5a were typical. An alternative procedure – removing an aliquot of mobile phase, checking its pH, and then discarding the aliquot – yielded the blank gradient shown in Figure 5b. You can see that the pH probe was the primary source of the extra peaks in the run of Figure 5a. For maximum sensitivity runs, particularly at UV wavelengths of <220 nm, it is a good idea to avoid contacting the bulk mobile phase with the pH probe.

Another good practice when using buffers is to always filter the buffer after it has been prepared. Although the buffer may be chemically pure, dust, particulate matter from an abraded buffer bottle-cap liner, or precipitation of salts may be present by the time the buffer is formulated. It is a good idea to filter the buffer through a 0.5 µm porosity filter prior to use.

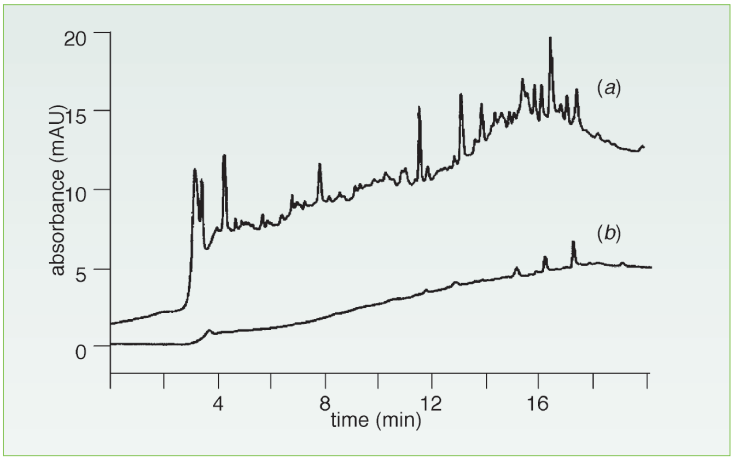


FIGURE 5. **Effect of pH-probe contamination of buffer.** Blank gradients with (a) buffer prepared in contact with pH probe; (b) no contact with probe. Adapted from [8].

Buffers are a good source of nutrition for micro-organisms; it is important to avoid conditions that support microbial growth. Wash the reservoir rather than refilling it. Set reasonable expiration dates for discarding unused buffer. Although you may be able to get longer stability, many laboratories specify one-week expiration dating on dilute (e.g. <50 mM) buffer so as to avoid problems with microbial growth.

Volatile buffers used for LC-MS (e.g. ammonium bicarbonate) are more likely to change pH over time, because of evaporative loss. It is a good idea to make up such buffers on a daily basis.

It is best to never shut off an HPLC system for an extended time (e.g. more than a few hours) without washing out the buffer. Leaving buffer in an unused HPLC system can result in buffer precipitation or microbial growth within the pump or other components. Switch to water/organic mobile phase (e.g. replace 50/50 buffer/MeOH with 50/50 water/MeOH) and flush 10-20 ml through the system to remove buffer prior to a strong-solvent flush to remove strongly retained material from the column. With reversed-phase columns, flushing with 100% water is not an effective way to remove buffer – many stationary phases will undergo dewetting (sometimes called phase collapse) with 100% water, defeating the washing process. However "high aqueous" or "AQ" type phases can be flushed with 100% water without dewetting concerns.

SUMMARY

- Buffers are used in RP-HPLC separations to control the retention of ionisable compounds. Usually we would like to suppress ionisation of analytes in order to maximize sample retention. This means the mobile phase pH should (ideally) be at least 2 pH units below or above the sample pKa, for acids or bases, respectively.
- For the most effective buffering, a buffer should be used within ±1 pH unit of the buffer's pKa. These characteristics of the sample and buffer mean that the choice of buffer should be made carefully, using Tables 1 and 2 for guidance.
- For LC-UV assays, phosphate and acetate buffers are most popular; the acetate-phosphate combination can cover the 2<pH<8 range, which spans the stability of most RP-HPLC columns.
- For LC-MS applications, the buffer must be volatile, so the choice of buffers is more limited. Various combinations of formate, acetate, ammonia, and bicarbonate (Tables 1 and 2) are most popular for LC-MS work.
- For many LC-UV and LC-MS methods, a low pH is more important than the presence of a true buffer, so 0.1% phosphoric (UV) or formic (MS) acid can be used to satisfy this requirement. The low silanol activity of the current high-purity columns, plus low concentrations of analyte molecules mean that buffers of 5-10 mM in the final mobile phase are satisfactory for most applications.
- If you blend mobile phase by placing buffer in the A-reservoir and organic solvent in the B-reservoir, it is a good idea to keep the buffer concentration less than ≈25 mM to avoid precipitation when the buffer and organic solvent are blended. Some HPLC systems are more effective than others at avoiding precipitation during mixing. A good test of potential for precipitation is to add buffer drop-wise to a test tube of organic solvent (and vice versa) – if precipitation is observed, dilute the buffer before trying to mix buffer and organic solvent on-line.
- Finally, take care to avoid contaminants, filter the buffer after preparation, and do not leave buffers in a system when not in use.

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Dr. John Dolan is best known for his monthly LC Troubleshooting column in LCGC and LCGC Europe. In addition to over 250 installments of his column, Dr. Dolan has published more than 100 papers related to HPLC. His research interests are method development, column characterization and gradient elution. He has worked in all aspects of HPLC from instrument design, to writing software, to managing a contract laboratory and to teaching HPLC techniques. Currently John is a principal in LC Resources, a company dedicated to training chromatographers and providing consultation for chromatographic problems. He shares some of his expertise with us in this guide for buffer usage. Further information on courses taught by John may be found on the LC Resources web site: **www.LCResources.com**

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