

Why is pH so important in buffers and additives in reversed-phase HPLC or LC-MS?

When samples contain ionisable compounds, 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.

WHY CONTROL pH?

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. The non ionised form will be less polar (more hydrophobic), and so more strongly retained in a reversed-phase system. As a result, at low pH, acids will be more retained, whereas bases will be more retained at high pH.

If the mobile phase pH is near pKa, you can see that slight changes in pH can make substantial changes in retention – not what is desired for a robust separation. Some compounds are very sensitive 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. Besides the instability of retention times when the pH is near pKa, relative peak spacing (selectivity) can change if compounds of similar structure are present.

Another factor that should be considered when choosing the mobile phase pH is the stability of the column. As a rule, silica-based columns should be operated at $2 < \text{pH} < 8$. At $\text{pH} < 2$, bonded phase loss due to hydrolysis can occur. Above pH 8, the silica backbone becomes increasingly soluble.

CONTROLLING pH

Since the retention of ionisable compounds are 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 pKa, but may provide adequate buffering ± 2 pH units from pKa.

The most popular buffers for HPLC with UV detection are phosphates and acetates. Phosphates and acetates are particularly useful buffers because they can be used at wavelengths below 220 nm. As can be seen from Table 2, phosphate has three pKa values that give it three buffering ranges: $1,1 < \text{pH} < 3,1$; $6,2 < \text{pH} < 8,2$ and $11,3 < \text{pH} < 13,3$ (allowing for buffering of pKa ± 1 pH units). Practical limits of column stability require that we truncate the lower range to $2,0 < \text{pH} < 3,1$ and eliminate the highest

pKa (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)
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)

TABLE 1. pKa values of common mobile phase additives.

range. Notice that there is a gap in buffering between pH 3,1 and 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. Fortunately, acetate fills this need well, with a buffering range of $3,8 < \text{pH} < 5,8$. With a slight extension of the buffering range from ± 1 pH units from the pKa, phosphate and acetate can cover the entire pH range of $2 < \text{pH} < 8$ normally used for silica-based columns.

Sometimes during method development, you may need 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 $2 < \text{pH} < 8$. 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 pKa values that allow buffering over the $2,1 < \text{pH} < 6,4$ range (Table 2). However, citrate does not have as low a UV cut-off 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	No
Phosphate (pK 2)	6,2 – 8,2	No
Phosphate (pK 3)	11,3 – 13,3	No
Acetate ¹	3,8 – 5,8	Yes
Citrate (pK 1)	2,1 – 4,1	No
Citrate (pK 2)	3,7 – 5,7	No
Citrate (pK 3)	4,4 – 6,4	No
Trifluoroacetic acid (0,1%)	2,0	Yes
Phosphoric acid (0,1%)	2,0	No
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	Yes

¹ suitable for LC-MS as ammonium acetate.

TABLE 2. Common HPLC buffers.

KEY POINTS

- Buffers are used in RP-HPLC separations to control the retention of ionisable compounds. Usually we would like to suppress ionisation of analytes to maximise sample retention. This means that 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 to 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 online
- A general rule is no more than 50% organic should be used with a buffer. This will depend on the specific buffer as well as its concentration

Finally, take care to avoid contaminants, filter the buffers after preparation, and do not leave buffers in a system when not in use.

Description	100 ml/100 g	250 ml/250 g	500 ml/500 g
Acetic acid 99% LC-MS	84874.180		84874.260
Ammonia 32% HPLC	153312K		
Ammonium acetate 99% LC-MS	84885.180		84885.260
Ammonium acetate $\geq 98.0\%$ HPLC		153164R	
Ammonium formate $\geq 99\%$ LC-MS	84884.180		84884.260
Formic acid $\geq 99\%$ LC-MS	84865.180		84865.260
Potassium dihydrogen phosphate $\geq 99.5\%$ HPLC			153184U
Triethylamine $\geq 99.8\%$ LC-MS	84883.180		84883.260
Trifluoroacetic acid $\geq 99.9\%$ LC-MS	84868.180		84868.260

Formic acid and TFA LC-MS grade are also available in ampoules of 1 or 10 ml. See our website vwr.com