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Technical Report

Buffered Mobile Phases in Reversed-Phase Liquid Chromatography

Aqueous mobile phase preparation is perhaps the most critical factor in reversed-phase chromatography (RPC) method development of ionic analytes. This includes consideration of the effects of pH on analyte retention, what buffer to use, what concentration of buffer to use, the solubility of the buffer in the organic modifier, the effect of the buffer on detection, and probably other considerations as well.

Retention

Mobile phase pH fundamentally impacts retention of ionic analytes in RPC. In consideration of the ionic functional groups, the neutral (uncharged) form of the analyte is better retained. Thus, the dissociation properties of these ionic functional groups will affect the retention of the analyte. For acidic moieties (usu. carboxylates) a pH below the pK_a (within limits) enhances retention of the compound, while for basic moieties (usu. amines), a pH above the pK_a (within limits) enhances retention. Therefore retention of non-ionic analytes is effected minimally by mobile phase pH.

It is within a pH range near the pK_a of a given functional group that dramatic affects on retention is observed. This becomes apparent in consideration of a mathematical description of chemical dissociation.

For a weak acid: $HA \leftrightarrow H^+ + A^-$

and $$K_a = \frac{[H^+][A^-]}{HA}$$

Therefore, by algebraic rearrangements including definition of $pH = -log\,[H^+]$,

$$pH = pK_a + log\,\frac{[A^-]}{[HA]}$$

The corresponding equation for a weak base is

$$pH = pK_a + log\,\frac{[B]}{[BH^+]}$$

These last two equations are commonly known as the Henderson-Hasselbach equation. An important point to note is that in the last term, the unprotonated species is in the numerator and the protonated species is in the denominator, regardless of whether consideration is made of an acid or base.

From the Henderson-Hasselbach, it can be seen that at a pH far removed from the pK_a, the ratio of unprotonated-to-protonated forms is disparate, and a small change in the pH has minimal affect on this ratio. Thus, at pHs far removed from the pK_a of the analyte, moderate change in pH won't significantly affect retention. However, at pHs near the pK_a, small changes in pH will produce significant changes in the ratio of the two species. Recall that it is the uncharged form of the analyte that is better retained. Therefore, changing the pH within a range of values sufficiently close to the pK_a, will dramatically affect retention. Usually this range is pK_a±1.5 pH units.

Buffer Selection

Choice of buffer is typically governed by what pH is desired. With reference again, to the above Henderson-Hasselbach equation, when the pH is near the pK_a, significant changes in the ratio of unprotonated-to-protonated species can take place without major changes in the pH. It is when pH = pK_a, that the system is able to accommodate changes in proton (or hydroxide) concentration with minimal changes in pH. Thus, the term "buffer": the pH is buffered. We thus use the term "buffer" in common speech of aqueous solutions, to actually mean pH-buffer.

Therefore in order to provide a pH-buffer, it is necessary to select an ionizable chemical species whose pK_a is close to the desired pH. If the pK_a is too far removed from the desired pH, no significant pH-buffering will occur, and reproducibility of retention times may suffer. It is generally recommended to use a buffer within a pH range of its pK_a±0.5 pH units. However, the pK_a±1.0 pH units is acceptable. Table 1 lists some common buffers used for small molecule HPLC and their useful pH-buffer range.

Table 1. Common Buffers, their pH Ranges, and UV-Vis Range

Buffer	pK_a (25°C)	Maximum Buffer Range	UV Cutoff (nm)
TFA	0.3		210 (0.1%)
Phosphate, pK_1	2.1	1.1-3.1	< 200
Phosphate, pK_2	7.2	6.2-8.2	< 200
Phosphate, pK_3	12.3	11.3-13.3	< 200
Citrate, pK_1	3.1	2.1-4.1	230
Citrate, pK_2	4.7	3.7-5.7	230
Citrate, pK_3	6.4	5.4-7.4	230
Carbonate, pK_1	6.1	5.1-7.1	< 200
Carbonate, pK_2	10.3	9.3-11.3	< 200
Formate	3.8	2.8-4.8	210 (10mM)
Acetate	4.8	3.8-5.8	210 (10mM)
Ammonia	9.3	8.3-10.3	200 (10mM)
Borate	9.2	8.2-10.2	n/a
TEA	10.8	9.8-11.8	< 200

Sometimes a buffer species is used in a mobile phase, but the pH is well outside of its ability to buffer it. An example is ammonium acetate, pH ~7. In this case, solid ammonium acetate is dissolved in water with no further pH adjustment. Ammonium acetate does not buffer pH between pH 6-8. It is usually in such cases that the use of the ammonium acetate is to provide some pH control (but not buffer) while taking advantage of its volatility downstream. It is also not unusual to see TFA or formic acid used alone at pHs outside of their buffering ranges: TFA as an ion-pairing reagent, formate for reasons of its volatility. Again, there are other reasons for their selection including that they do provide for pH control given the concentration of the analyte as compared to the acid (TFA, formate) in the bulk mobile phase.

Buffer Concentration

As indicated above, better buffering occurs when the pH is closer to the pK_a. Additionally, this buffer capacity is enhanced by a higher concentration of the buffer itself. Higher buffer concentrations will give more reproducible separations of compounds partially ionized at the pH of the mobile phase, by reducing local perturbations of the pH of the migrating analyte peak. Generally, a buffer concentration of 10-50mM is adequate for small molecules. Note that the buffer concentration will decline upon mixing with the organic modifier.

Buffer Solubility

An immediate concern regarding buffer concentration is consideration of its solubility upon addition of the organic modifier. This is especially important when performing gradient separations. Solubilities in mixtures of aqueous solution and a given organic solvent are not readily predictable. A suggested approach is to determine it empirically: try a given volume fraction of the buffer of interest (at a given concentration) with a given volume fraction of the organic solvent. Thoroughly mix the two phases and observe whether the solution remains transparent. If there is a solubility problem, a precipitate will form, or the solution may turn opaque. This sort of test must be done for the final conditions of a gradient run, to avoid consequential instrument and column problems related to buffer precipitation. While this is not foolproof, a general rule is no more than 50% organic should be used with a buffer. However, keep in mind, this will depend on the specific buffer as well as its concentration.

Buffer Effects on Detection

Often the choice of buffer is determined by the means of detection. For traditional UV detection, the buffer needs to be effectively transparent in this region. This too becomes more critical for gradient separations. All the buffers listed in Table 1 have low enough absorption below 220nm, with the exception of citrate, which generally must be used above 230nm. Several of them can be used considerably lower than 220nm.

More common today, are issues related to compatibility with mass spectral (MS) detection. Though some common bench-top MS detectors have considerable rugged inlet systems, buffer volatility permits less intensive maintenance and thus less downtime of the MS detector. Preferred buffers addressing this issue of volatility are formate, acetate, carbonate, and ammonia. Furthermore, the issue of suppression of ionization is the more fundamental concern as regards to buffer selection in LC-MS method development. In this regard, formate and acetate are ideal choices for positive-ion mode detection. TFA, however, can negatively impact detector response even in positive-ion mode (see references 3 & 4), while with negative ion mode, strongly suppresses ionization. Acetic acid is good for negative-ion mode. Also for issues of mass detection, high concentrations of buffer can simply swamp-out any signal one might have wished to detect. In general, the less buffer needed the better, and 5-10mM is a suggested starting point. Thus, application of LC-MS does further limit buffer selection and buffer concentration. This often compromises the available options to develop an optimal separation, but is usually offset by the power of the MS.

Practical Notes

1) In preparing the buffer, dissolve the solid in ~95% of the final volume desired. After the solution is adjusted to the pH desired, bring the solution to volume.
2) Always calibrate the pH meter either at the final adjusted pH (the final adjusted pH must match the pH of a standard) or calibrate the pH meter at values above and below the final adjusted pH. This is the only way to reliably measure pH with a pH-meter. You cannot reliably measure a value outside the range for which you have calibrated an instrument.
3) For consistent results, pH adjustments to the aqueous solution should be made before addition of organic. True, the actual pH will change after addition of the organic, but there is no good method for reliable measurement of pH after such addition. This way, at least the laboratory practice is consistent.
4) All buffer solutions should be filtered before use as mobile phase in HPLC. This is to remove any particulates that may already be in the water or introduced by the solid buffer when dissolved. A hydrophilic 0.45µ filter is recommended. Filtration should be a last step before use as is or before mixing with organic.

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