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We covered why pH is critical in mobile phase development, along with its effects on chromatography and analyte ionization in the first chapter of our blog series on mobile phase. Now I am going to discuss the significance and role of choosing correct buffers in mobile phase.

Buffer Definition

Simplest definition of buffer is – a solution which can resist the change in pH upon addition of small amounts of acid or a base in to it. By definition buffers can be of two types: acidic or basic buffer.

Acidic Buffer:

An acidic buffer is a mixture of a weak acid and it's salt with a strong base, for example.

Sodium Acetate Buffer = CH_3COOH + CH_3COONa, buffer range between 3.76 ~ 5.76

Basic Buffer:

Similarly, a basic buffer is a mixture of a weak base and it's salt with a strong acid, for example.

Ammonium Chloride Buffer = NH_4OH + NH_4Cl, buffer range between 8.24 ~ 10.24

Buffering Capacity

Buffering Capacity is the ability of the buffer to resist the change in pH and it is directly proportional to the molar concentration of the buffer salt an it's corresponding acid or base. Closer the pKa of the buffer to the desired mobile phase pH, greater will be its buffering capacity. For e.g., if any acidic analyte has a pKa of 4.5, the pH of the mobile phase need to keep it in deprotonated state will be approx. 2.0 and in this pH range the suitable buffer will be a phosphate buffer with pKa of 2.1 and with effective pH range of 1.1-3.1.

Need for buffers

We need buffers to control and maintain the pH. pH is a highly variable factor, it can change very easily, even when the solution was let to stand overnight, with the help of dissolved carbon di oxide, or volatile acids such as Trifluoroacetic acid (TFA) can evaporate over time.

One important point to consider is, during a chromatographic run if the sample diluent's pH is not controlled, the final pH of the sample injected on to the column inlet head, can be very

different from the original pH of the mobile phase solution. This will result in issues like peak distortion, retention shifts and even resolution issues in some critical separations. This will be especially true when either a large sample injection volume is being used or when very large or very less concentration of buffer is used for adjusting the pH of the mobile phase. Thus, in order to keep the pH as stable as possible an addition of buffer not just in to the aqueous part of the mobile phase but ideally in to the sample diluent as well is markedly recommended.

Buffer Functioning

In a buffer solution there always exists an equilibrium between the weak solvent and it's corresponding conjugate acid or a base. For e.g. here we have taken an equilibrium between weak acid and it's conjugate base.

$HA + H_2O \leftrightharpoons [H_3O^+] + [A^-]$

When an acid or base is added to this solution, the equilibrium either shift to right or to left.

And in order to regain that equilibrium state, the added acid or base will be utilized to maintain the concentration of H^+ and OH^- ions. Thus, the equilibrium remains stable in the solution and added acid or base will be inefficient in changing the pH of such a solution. In other words, added H^+ or OH^- ions sort of get neutralized when comes in contact with a buffered solution. Therefore, if the solution is not buffered there will be no equilibrium and added H^+ or OH^- ions can easily be able to drop or increase the pH of the solution.

Buffer Selection

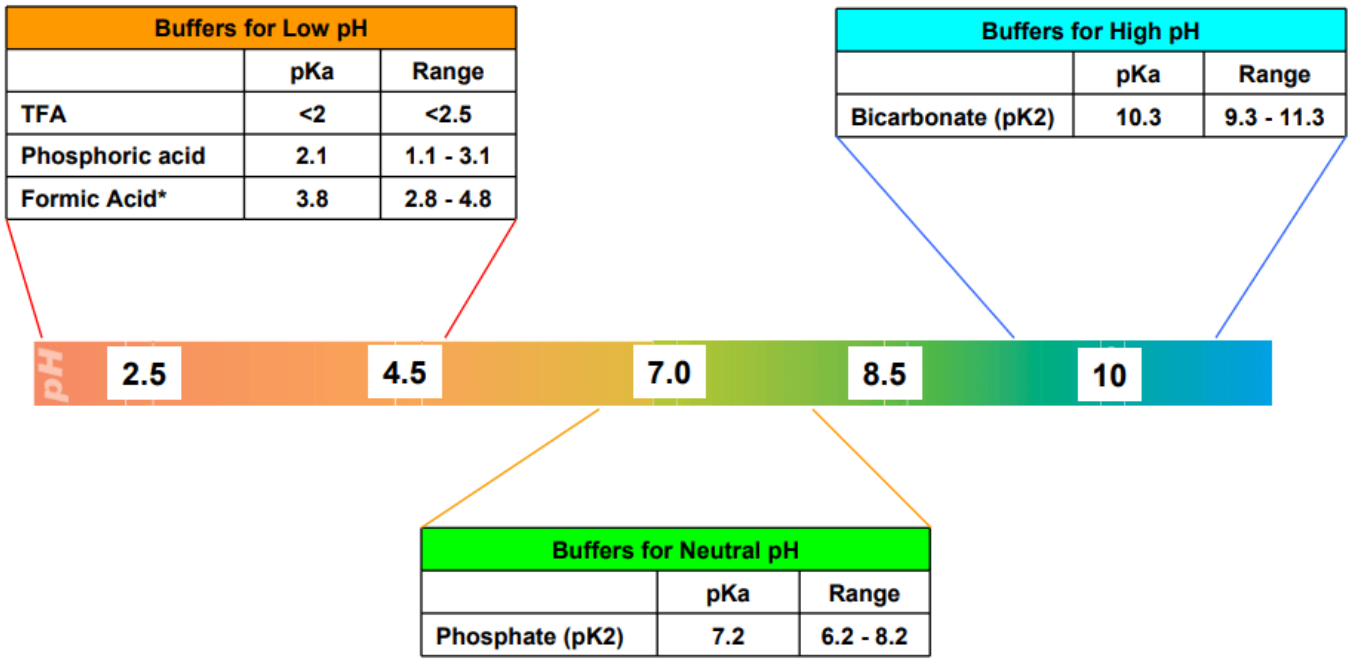
- Buffer selection mainly depends on the desired pH of the mobile phase and which in turn depends upon the analyte's pKa value. Thus, ideally you should choose a buffer which has it's pKa value as close as possible to the required mobile phase pH value.
- Compatibility with the detector – particularly when working with Mass as a detector. The pre-condition to work with Mass is that the elutes coming out of the column should be volatile to be able to generate enough number of ions in Ion-source in order to get detected by Mass detector. For this reason, the inorganic buffers like phosphate salts does not work with Mass spectrometer. It is a common practice to add modifiers in LC-MS, aim is to improve analyte signal or suppress unwanted signal or selectively enhance the signal of a particular compound in a mixture. Therefore, the buffers or additives used with Mass Spectrometer should always be volatile for e.g. Ammonium salts, Formic Acid and Di or Tri ethyl amine are the most common additives when working with Mass detector.
- Another important point to keep in mind when selecting the right buffer is their UV cut-off limit especially when working at lower wavelengths. Some buffers might have an absorbance at lower wavelengths which interferes with the analytes' absorbance range and they can also reduce the detector sensitivity which will then be visible in the form of baseline noise.
- Solubility of buffers is an important factor when choosing the right buffer, solubility of the buffer salts decreases as the counter ion is changed in the above order.

$NH_4 < K < Na$

- Sometimes when working with acidic or basic analytes buffers are not always used, a small percentage of acids or bases like TFA, Formic Acid and Ammonium Hydroxide are sufficient to neutralize the ionic species in the samples. These mobile phase additives

are called pH modifiers and they can only effectively work at their specific pKa/pKb values.

Below fig. categorize the most common additives or buffers used to maintain/control the pH at different values.



Below is the list of commonly used buffers in liquid chromatography:

Table of Common HPLC/UHPLC Buffers and Additives

Name	Chemical Formula	Molecular Weight	pKa	UV (nm) Cutoff @ 1 AU	Buffer Range
Hydrochloric Acid	HCl	36.46	-7.00		
Sodium Hydroxide	NaOH	40.00	13.80		
Potassium Hydroxide	KOH	56.11	15.70		
Trifluoracetic Acid**	$C_2HF_3O_2$	114.03	0.23	210 nm (0.1 %)	< 1.5
Acetic Acid*	$C_2H_4O_2$	60.05	4.76	210 nm (10 mM)	3.76 ~ 5.76
Sodium Acetate	$C_2H_3NaO_2$	82.03	4.54		3.76 ~ 5.76
Potassium Acetate	$C_2H_3KO_2$	98.13	4.54	210 nm (10 mM)	3.76 ~ 5.76
Phosphoric Acid	H_3O_4P	98.00	2.15 7.20 12.32	< 200 nm (0.1 %)	1.15 ~ 3.15 6.20 ~ 8.20 11.15 ~ 13.15
Monosodium Dihydrogen Phosphate	H_2NaO_4P	119.98	2.15 7.20 12.32		1.15 ~ 3.15 6.20 ~ 8.20 11.15 ~ 13.15
Monosodium Dihydrogen Phosphate Monohydrate	H_4NaO_5P	137.99	2.15 7.20 12.32		1.15 ~ 3.15 6.20 ~ 8.20 11.15 ~ 13.15
Monosodium Dihydrogen Phosphate Dehydrate	H_6NaO_6P	156.01	2.15 7.20 12.32		1.15 ~ 3.15 6.20 ~ 8.20 11.15 ~ 13.15
Disodium Monohydrogen Phosphate	HNa_2O_4P	141.96	2.15 7.20 12.32		1.15 ~ 3.15 6.20 ~ 8.20 11.15 ~ 13.15
Monopotassium Dihydrogen Phosphate	H_2KO_4P	136.08	2.15 7.20 12.32		1.15 ~ 3.15 6.20 ~ 8.20 11.15 ~ 13.15
Dipotassium Monohydrogen Phosphate	HK_2O_4P	174.18	2.15 7.20 12.32		1.15 ~ 3.15 6.20 ~ 8.20 11.15 ~ 13.15
Citric Acid	$C_6H_8O_7$	192.12	3.13 4.76 6.40	230 nm (10 mM)	2.13 ~ 4.13 3.76 ~ 5.76 5.40 ~ 7.40
Formic Acid*	CH_2O_2	46.02	3.74	210 nm (10 mM)	2.74 ~ 4.74
Sodium Formate	$CHNaO_2$	68.01	3.74		2.74 ~ 4.74
Potassium Formate	$CHKO_2$	84.11	3.74	210 nm (10 mM)	2.74 ~ 4.74
Ammonium Hydroxide	H_5NO	35.05	9.24	200 nm (10 mM)	8.24 ~ 10.24
Ammonium Chloride	ClH_4N	53.49	9.24		8.24 ~ 10.24
Ammonium Acetate*	$C_2H_7NO_2$	77.08	4.76 9.24	205 nm (10 mM)	3.76 ~ 5.76 8.24 ~ 10.24
Ammonium Formate*	CH_5NO_2	63.06	3.74 9.24		2.74 ~ 4.74 8.24 ~ 10.24
Tris	$C_4H_{11}NO_3$	121.14	8.08		7.08 ~ 9.08
Tris Hydrochloride	$C_4H_{12}ClNO_3$	157.60	8.08		7.08 ~ 9.08
Triethylamine*	$C_6H_{15}N$	101.19	10.72	< 200 nm (10 mM)	9.72 ~ 11.72
Triethylamine Hydrochloride	$C_6H_{16}ClN$	137.65	10.72		9.72 ~ 11.72
Pyrrolidine	C_4H_9N	71.12	11.30		10.30 ~ 12.30

* Volatile buffer, compatible with Mass Spectroscopy analysis.

** Compatible with Mass Spectroscopy but at low concentrations due to ion suppression effects.

General Instructions when using buffers

- Always use high purity chromatographic grade buffers for preparing the mobile phase solutions.
- It is recommended to filter the buffer solutions/mobile phase with a 0.45µ filter prior to using it in LC systems.
- Temperature directly affects the ionization and in turn pH of a solution, thus temperature should be maintained even while preparing a buffer because it will ultimately can change the pH.
- Concentration of buffer is directly linked to the polarity and ionization in mobile phase, therefore choose the buffer concentration wisely. 25 50mM is a good starting point for buffer concentration when developing a new method.
- Always measure the buffers by weight (gravimetrically) rather than volumetrically, start with the lowest concentration required, because high buffer amounts can deposit inside the HPLC pumps, and column and can result in higher back pressures.
- Increased buffer concentration improves peak shape and efficiency but may also result in change in selectivity and again higher amounts of buffer salts will get deposited inside the pumps and columns.
- Always adjust pH with only the aqueous part of the mobile phase to be consistent and accurate with pH measurement.
- Always try to prepare fresh buffer solutions, buffer salts can lead to microbial growth in the aqueous solutions, which will result in false chromatographic results also Triethylamine and Tri-fluoro acetic acid degrade over time and their UV cut-off increases.
- Citrate buffers can corrode stainless steel tubing and HPLC system parts over prolonged periods of contact, ensure you flush your HPLC system and column thoroughly to free it from citrate buffers prior to storage.
- Generally, buffers have no significant direct effect on analyte retention times, except when secondary interactions take place with ionizable analytes (e.g. secondary silanol interactions).
- Tri-fluoro acetic acid when used as a buffer behaves as an excellent ion-pairing agent as well and can drastically alters the analyte retention when used at wrong

concentrations.

- Buffers and additives are certainly important to achieve the desired selectivity and efficiency in reverse phase chromatography, but they are also harmful for columns if they are allowed to remain inside them. Thus it is recommended to remove these additives from the column especially before storing the column for mid or long term duration. High back pressure, irreproducible results, bad asymmetry and loss of retention are some of the signs of column contamination and degradation through these additives.
- Since buffers are salts and salts can be best removed by water, thus a thorough wash with water can easily remove the buffers from the column. Applying a bit of temperature while column cleaning will also help.
- When washing hydrophobic phases make sure you keep at least 10 15% organic in wash solvent to ensure there will be no hydrophobic collapse inside the column, however, you can run 100% water in phases that are aqueous stable.

Buffer is substantiated as one of the critical components of mobile phase, therefore choose it appropriately.

If you have any questions regarding this article or are looking for technical assistance of any kind, reach out to our technical specialist nearly 24/7 through Chat Now. You might even get to chat with Namrata herself!

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