Control pH During Method Development for Better Chromatography

Technical Overview

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Introduction

In reversed-phase liquid chromatography, pH and ionic strength of the aqueous portion of mobile phases are important in developing rugged methods not sensitive to small variations in conditions. With ionic compounds, retention of typical species shows significant changes with pH. It is very important to control pH in reversed-phase systems to stabilize retention and selectivity. A pH between 2 and 4 generally provides the most stable conditions for retention versus small changes in pH, and this pH range is recommended for starting method development with most samples, including basic compounds and typical weak acids. For reproducibility, the pH used should be ± one pH unit above or below the pKa or pKb of the solutes being separated.

The pKa of the analytes may not be known, therefore, testing more than one mobile phase pH may provide the best results. Most reversed-phase silica-based columns work well from pH 2 to pH 8 or more, providing a wide range when searching for the optimum mobile phase pH for a separation. Polymer-based HPLC columns have outstanding chemical stability and can be used from pH 1 to pH 14. For all separations, measure and adjust pH on the aqueous component, before mixing with organic modifiers, to give the most accurate and reproducible results.

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Managing pH with mobile phase modifiers

The pH of the mobile phase can affect chromatography in a number of ways. Depending on the compound being analyzed, pH can impact selectivity, peak shape, and retention. With a fairly nonpolar or neutral compound, the effect of pH will typically be insignificant for resolution and retention.

Figure 1 shows how pH can affect resolution. On the left hand side are examples of nonpolar samples run at pH values of 3 and 7. There is not much difference between the two chromatograms. The polar compounds, which can be seen in the middle panel, tend to be less retentive on C18 columns. Notice that pH has little or no effect on the retention time or peak shapes of the compounds.

Ionizable compounds, such as acids or bases, cause significant changes in retention factor and selectivity with changes in pH, as evident for benzoate and benzanilide in the right hand panel of Figure 1. Benzanilide, a neutral compound, shows almost no change in retention while benzoic acid has a very noticeable change in retention as the pH is shifted from 3 to 7. At pH 7, well above the pKa of benzoic acid, it exists as the ionized benzoate anion. This form is more ionic, prefers the aqueous mobile phase, and elutes from the column much faster than at pH 3, where it exists in the predominantly ion-suppressed form.

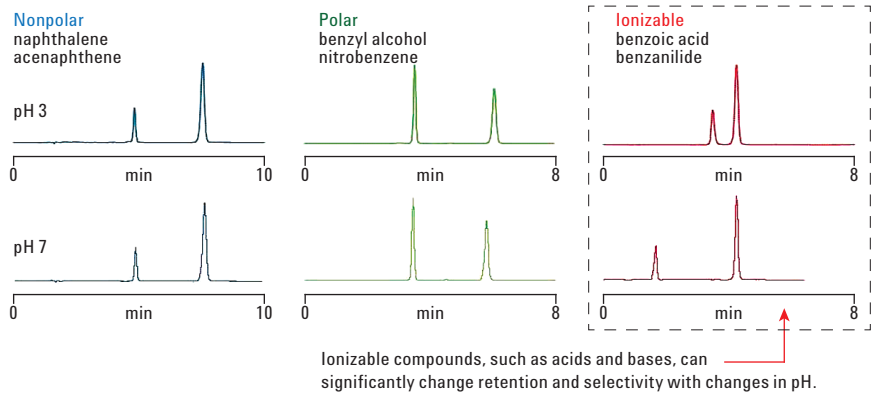

Figure 1. pH and resolution.

When considering method development with ionizable analytes, it is important to understand that ion-suppressed analytes have better retention than ionized analytes. For acidic analytes, choose a low-mobile-phase buffered pH to keep the analytes from being ionized. Knowing the pKa of the analytes permits an effective choice of mobile phase pH. A buffer is effective at ± one pH units from the pK of the buffering ion, giving some flexibility in optimizing the mobile phase. Acetate, for example, has a pKa of 4.8 and buffers from pH 3.8 to 5.8. Formate is more acidic and buffers from pH 2.8 to 4.8. There are additional buffer choices if acidic analytes would be not be ionizable at lower pH. For more details on buffers, refer to Table 1.

Table 1. UV cutoffs for common mobile phase modifiers.

Buffer	v/v (%)	pKa at 25 °C	Max. pH range	UV cutoff (nm)
Trifluoroacetic acid (TFA)	0.1	0.3		210
	0.05	0.3		210
	0.01	0.3		210
Phosphate, pK1		2.1	1.1.–3.1	<200
Phosphate, pK2		7.2	5.2–8.2	<200
Phosphate, pK3		12.3	11.3–13.3	<200
Citrate, pK1		3.1	2.1–4.1	230
Citrate, pK2		4.7	3.7–5.7	230
Citrate, pK3		6.4	5.4–7.4	230
Carbonate, pK1		6.1	5.1–7.1	<200
Carbonate, pK2		10.3	9.3–11.3	<200
Borate		9.2	8.2–10.2	n/a
Triethylamine (TEA)		10.8	9.8–11.8	<200
TRIS-HCl		8.3	7.3–9.3	205 (120 mM)

The selection of a mobile phase modifier is a balance between various requirements. Does it produce a stable pH solution within the safe range of the column that is appropriate to control the ionization (either by ion suppression, ion pairing, or a combination) of the analytes? Does it interfere significantly with the detector performance, including the disparate properties of volatility, MS ion suppression, and UV transparency? The challenge is to work with a smaller range of acceptable modifiers, often requiring the investigation of a more diverse range of stationary phases (embedded polar, alkyl phenyl, fluorinated RP, in addition to typical alkyl phases, such as C8 and C18) to enhance selectivity for specific analytes.

With basic compounds, the ion-suppressed form may be at a high pH that is not suitable for the column. However, many basic compounds are adequately retained at low pH. While greater retention can be achieved in an ion-suppressed form, this may not be practical or necessary for all basic compounds.

An additional issue impacting retention of acidic and basic compounds is the potential ionization of silanols on the silica surface at mid pH. Typically, these silanols become deprotonated and, hence, negatively charged. This may result in more retention for positively-charged, basic ions, which can result in ion-exchange interactions, a type of secondary interaction. The end result is often peak broadening or peak tailing due to an interaction other than the partitioning that is expected with a reversed-phase column. This does not happen at low pH and is another reason why acidic mobile phases are preferred for the separation of ionizable compounds by reversed-phase chromatography.

Table 2. Mobile phase modifiers for LC/MS.

Concentrated reagent	pKa at 25 °C	Max. pH range	Formula weight[1]	Density	Approx. strength (%)[2]	Molarity (M)	Normality (N)	Volume (mL) required to make 1,000 mL solution[3]	
								1 M	1N
Acetic acid	4.8	3.8–5.8	60.062	1.05	99.8	17.4	17.4	57.5	57.5
Formic acid	3.8	2.8–4.8	46.026	1.13	90.0	23.6	23.6	42.5	42.5
Ammonium hydroxide	9.2	8.2–10.2	35.046	0.90	56.5[4]	14.5	14.5	69.0	69.0

[1] Based on atomic weight table (32 °C = 12)

[2] Representative value, w/w %

[3] Rounded to nearest 0.5 mL

[4] Equivalent to 28.0% w/w NH_3

Choosing and using buffers

- Choose mobile phase pH to optimize retention and selectivity during method development.
- If using silica columns, avoid extreme pH values, very high or very low, to prevent shortening column life.
- Nonionized analytes have better retention (that is acids at low pH and bases at high pH). However, it is not necessary to fully suppress ionization for success with HPLC; 90% suppression is generally considered adequate when sufficient buffer capacity is employed in the mobile phase.
- Silanols on silica ionize at mid pH and higher, increasing retention of basic analytes (that is possible ion-exchange interactions).
- The buffering capacity of any mobile phase is related to the prepared molarity and how close the desired eluent pH is to the pK of the buffering ion.
- Buffering is typically effective at up to one pH unit above or below the pK of the buffering ion (Table 1).
- Choices are limited when using alkaline buffers. Triethylamine is not freely water soluble and has a high pK (11), and ammonia dissolves freely but also has a pK too high for most columns.

A word of caution regarding using buffers at high pH; depending on buffering capacity, these are very liable to absorb CO_2, and the pH will change as carbonate is added inadvertently to the buffer. The only protection against this is blanketing the mobile phase bottle to exclude air except through an Ascarite trap.

A nonbuffered mobile phase is also an option for pH modification. It is not unusual for acidic analytes to be chromatographed with simple acid solutions, where the concentration of acid is sufficient to create a much lower pH than needed.

Best practice for mixing buffers

- Use a good pH meter, such as the Agilent G4383A. Calibrate the pH meter, bracketing the target pH. This is key for reliably measuring pH with a pH meter.
- Ensure your buffers are as fresh as possible.
- Start by dissolving the solid in the liquid, very close to the final volume. After adjusting the mixture to the required pH, add additional liquid to bring the solution to the appropriate volume.
- pH adjustments should be made to the aqueous solution before addition of the organic. There is no reliable way to measure pH after adding the organic.
- When the buffer solution is complete, filter it before use to remove particulates. Use a 0.45 µm filter for most HPLC applications; use a 0.22 µm filter for UHPLC applications.

pH and column selection

Chromatographic resolution between two or more peaks depends upon three factors; column efficiency, selectivity, and retention. With ionizable analytes, all of these factors change dramatically with pH. Low, mid, and high pH are the three general regions for chromatographic separations, as summarized in Table 3. Method development proceeds by investigating chromatographic separations at low pH, and then higher pH, until optimum results are achieved. Finding the optimum pH for a separation is a key part of method development. Most separations will take place between pH 2 and 8. Columns are available for each pH region, as shown in Table 4.

Table 3. Method development at different pH for silica columns.

Low pH < 3	**Mid pH 7**	**High pH > 9**
Start method development at low pH, where silanols on a reversed-phase HPLC column are protonated, to minimize peak tailing by eliminating silanol/base interactions.	Develop methods at least one pH unit above or below the pKa to minimize changes in retention with small changes in pH.	Basic compounds may be in their free-base form.
At low pH, basic compounds are positively charged and their retention may be reduced.	Some silica surface SiOH groups become SiO^- above pH 4 to 5; tailing interactions may occur.	Increased retention and resolution of basic compounds is likely.
Acidic compounds may be protonated and have increased retention.	Minimize interactions by selecting a well-designed and endcapped column, using additives such as TEA (triethylamine) or using polar-linked bonded phases.	Retention changes little in this region, thus robust methods can be developed.
Retention times are usually stable with small changes in pH, producing a robust method.	Silica breakdown is prevented by innovative bonding chemistry, heavy endcapping, and use of very high purity silica with lower silanol activity due to low metal content (Rx-SIL).	Silica breakdown is prevented by innovative bidentate column chemistry, heavy endcapping, use of Rx-SIL, and optimum mobile phase.
Volatile mobile phase additives, such as formic acid or TFA, are often used at low pH with LC/MS.		Ammonium hydroxide is an excellent volatile mobile phase modifier at high pH.

Table 4. pH ranges of Agilent LC reversed-phase silica columns. (The pH range of individual phases within a column family may differ slightly.)

Column	pH range
Agilent ZORBAX 80Å StableBond	0.8–8
Agilent Poroshell 120	1–8
Agilent ZORBAX Rx	1–8
Pursuit	1.5–10
Polaris	1.5–10
Original Agilent ZORBAX columns	< 3
Original Agilent ZORBAX ODS Classic	2–7
Bonus RP	2–7
Agilent ZORBAX Eclipse Plus	2–9
Agilent ZORBAX Eclipse XDB	2–9
Agilent ZORBAX Bonus-RP	2–9
Agilent ZORBAX 80Å Extend-C18	2–11.5

Agilent PLRP-S polymer-based columns for pH extremes

When a column is needed for use at very low or very high pH, polymeric packings provide an alternative to silica-based materials. Agilent polymeric particles are ideal for small-scale chromatography, particularly LC/MS, as they are chemically stable and do not leach soluble or particulate species.

Polymeric columns offer significant advantages for analyses of difficult samples. They provide chemical and extreme pH stability, from pH 1 to 14. If high pH must be used, select a stationary phase designed for high pH work, such as PLRP-S. These columns have no reactive sites and, due to their polymeric nature, the stationary phase will not dissolve in extreme pH environments. A good starting point for reversed-phase gradient separations with polymeric columns is to use ACN/water + 0.1% TFA mobile phase screening gradient that is 5% to 95% ACN. The gradient can be modified to improve resolution of all components depending on where the analytes elute.

Polymeric media can be used with acidic, neutral, and basic eluents. For example, a synthetic peptide can be screened using ACN eluents at four different pH levels: 0.1% TFA, 20 mM ammonium acetate at pH 5.5, 20 mM ammonium carbonate at pH 9.5, and 20 mM ammonium hydroxide at pH 10.5. For more complex samples, it may be difficult to obtain the desired purity or recovery, or both, due to limited solubility or co-eluting species. The net charge on the peptide depends on the pH of the buffer, and it will have zero net charge at its isoelectric point (pI). Therefore, in reversed-phase HPLC, changing the pH alters the net charge of the sample and any closely related components, and hence changes the retention and selectivity of the separation.

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