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Importance of Controlling Mobile Phase pH in Reversed Phase HPLC

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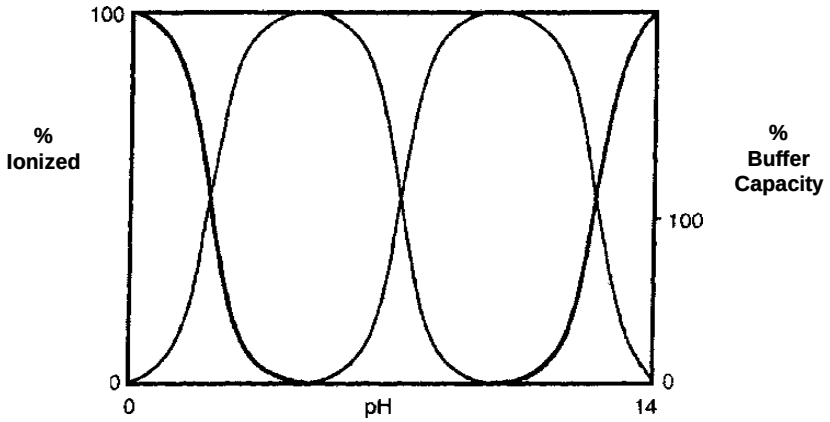
INTRODUCTION

The importance of controlling mobile phase pH when analyzing ionizable compounds by reversed phase (RP) HPLC is often recognized and easily understood, however it is often equally important to control pH when working with field samples of non-ionizable compounds due to the presence of ioniable impuritites.

Figure 1. Buffer Capacity



SELECTING THE RIGHT BUFFER

A partial list of common buffers and their corresponding pH values is shown in Table 1[1]. Perhaps the most common HPLC buffer is some form of phosphoric acid. A definition of buffer strength is given in Figure 1, where a plot of how conjugate forms of phosphoric acid change with pH. Note that buffer capacity (the ability to resist pH change when a sample is introduced at a different pH) is only 100% at the pK value of the acid or base. At pH 4, phosphate is a poor buffer and would change rapidly toward one of its pK_a values if a more acidic or basic sample were introduced. As a rule, one should work within ±1 pH unit of the buffer pK_a value for good pH control of the mobile phase. Adequate buffer concentrations for HPLC tend to be in the 10-100 millimolar level depending on the size and nature of the sample, as well as the column packing material. Phases that contain polar groups such as AQUASIL C18 and PRISM RP, are often more compatible with dilute buffers than traditional alkyl packings.

When control at a lower pH (2-3) is desired, phosphate, or stronger organic acids such as TFA or acetic acid when volatility is of concern, are commonly used. If control at pH 4-5 is desired, an organic acid buffer such as acetate or citrate should be considered in place of phosphate.

Table 1. Properties of Common Buffers

Common Buffers		pK_a	Useful pH Range
Phosphate	pK_1	2.1	1.1-3.1
	pK_2	7.2	6.2-8.2
	pK_3	12.3	11.3-13.3
Citrate	pK_1	3.1	2.1-4.1
	pK_2	4.7	3.7-5.7
	pK_3	5.4	4.4-6.4
Formate		3.8	2.8-4.8
Acetate		4.8	3.8-5.8
Tris		8.3	7.3-9.3
Ammonia		9.2	8.2-10.2
Borate		9.2	8.2-10.2
Diethylamine		10.5	9.5-11.5

WHEN IS pH CONTROL NECESSARY FOR STRONGLY IONIZABLE COMPOUNDS?

Samples containing ionizable compounds are strongly influenced by pH of the mobile phase as illustrated by the separations of sorbic and benzoic acids shown in Figure 2. While organic acids are typically separated under ion suppression conditions where the pH is adjusted to 2 or 3 (Figure 2A), this sample required a pH of 7 (Figure 2B) due to the tendency of the sample matrix to precipitate at lower pH and higher organic concentrations. Low pH decreases the solubility of organic acids in water and requires the use of a higher organic percentage in the mobile phase for practical elution under RP conditions. For acids, the retention time decreases as the pH of the mobile phase is increased . Greater charge can be thought of as an extreme case of polarity. At pH's above the analyte's pK_a, the acidic analyte carries a negative charge and behaves as an extremely polar molecule. In order to achieve adequate retention, the mobile phase should be highly aqueous. Below its pK_a, the acidic analyte is neutral and much more hydrophobic. Under ion suppresion RP retention can be more easily achieved and analysis times can be longer.

When the acid standards were introduced to unbuffered, neutral mobile phase of 10% methanol (Figure 2C), poor peak shape resulted. This result can be traced to a mismatch caused by the acidic nature of the sample and zero buffer strength in the neutral mobile phase. The sample therefore experiences a pH gradient during the first part of the separation, which usually causes ionizable compounds to exhibit broad peak shape and poor retention reproducibility. As shown in Figure 2B, addition of a phosphate buffer at pH 7 eliminated the broad tailing peaks and created rugged conditions suitable for successful assay. Because the assay of sorbic and benzoic acids was related to a food product containing protein, the wide pores and neutral pH prevented precipitation and prolonged column life. Essentially, the solutes are instantly ionized and the weak acids are separated in their anionic forms. This pH gradient effect is difficult to control and can almost guarantee problems with reproducibility.

CONCLUSION: While it is not always strictly necessary to operate under buffered conditions, one should recognize that poor peak shape and variable retention can result when the sample pH differs significantly from the pH of the non-buffered mobile phase and when ionizable compounds are present in the sample.

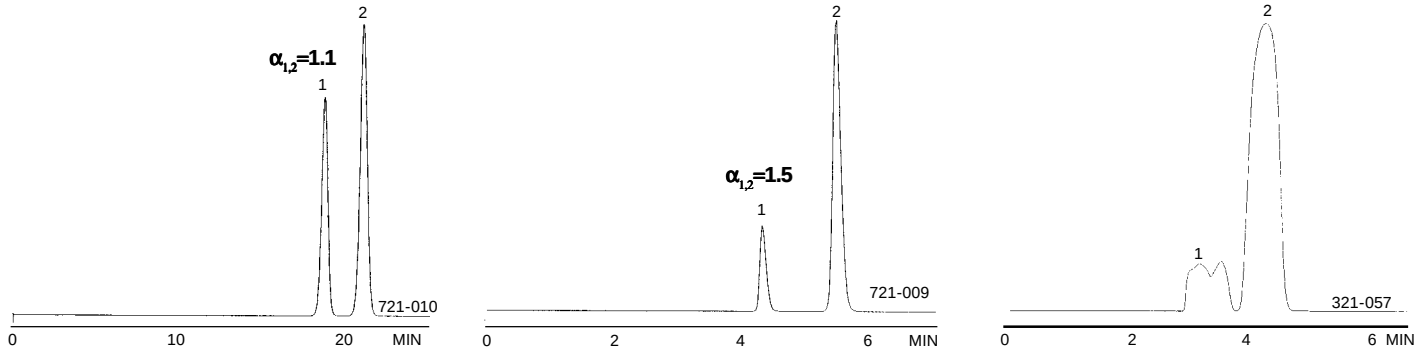
Figure 2. Effect of pH Control on Separation of Ionizable Compounds

A: pH 3.5, buffered

B: pH 7.0, buffered

C: pH 7.0, not buffered

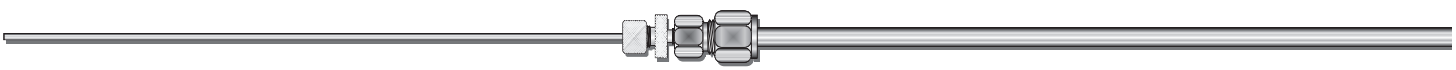
Sample:
1. Benzoic Acid
2. Sorbic Acid



BioBasic 18, 5µm, 150x4.6mm
Part No: 155-721
Eluent: 20% MeOH/80% 0.05M KH_2PO_4, pH 3.5
Flow: 1.0 mL/min
Detector: UV @ 235

BioBasic 18, 5µm, 150x4.6mm
Part No: 155-721
Eluent: 10% MeOH/90% KH_2PO_4, pH 7
Flow: 1.0 mL/min
Detector: UV @ 235

BioBasic 18, 5µm, 150x4.6mm
Part No: 155-721
Eluent: 10% MeOH/90% H_2O
Flow: 1.0 mL/min
Detector: UV @ 235



WHEN IS pH CONTROL NECESSARY FOR NON-IONIZABLE SAMPLES?

DELTABOND AK was engineered for the assay of aldehydes and ketones present in auto emissions. Since then, other groups have employed DELTABOND AK for the assay of aldehydes and ketones in ambient air samples and samples from other sites where aldehyde and ketone pollution is present. Traditionally, the samples are first derivatized with DNPH for easy detection by UV-Vis, then assayed with mobile phase consisting of H_2O/ACN. The DNPH-derivatized aldehydes and ketones are not ionizable and therefore do not require a buffered mobile phase.

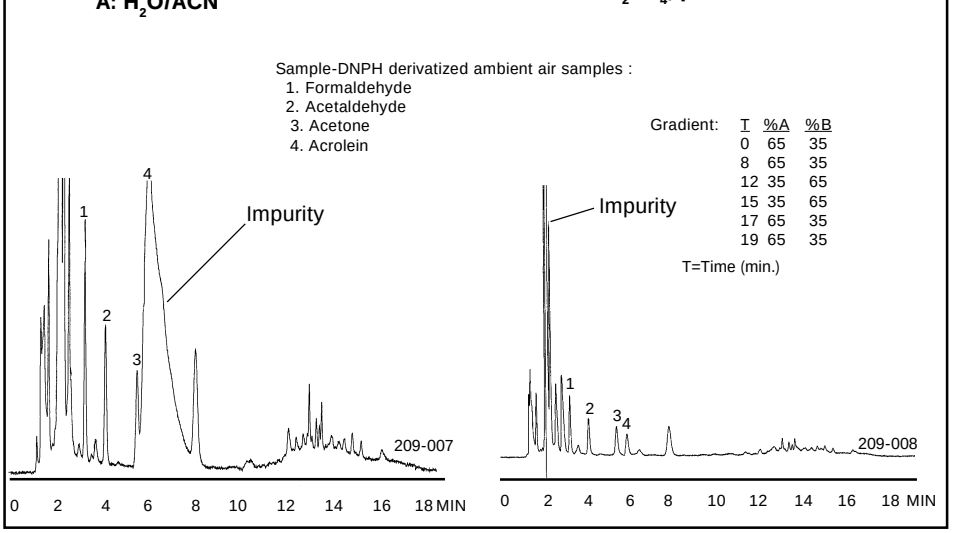
Auto emissions generally are free of ionizable compounds; however, recently the usual method was used with DELTABOND AK for the analysis of aldehydes and ketones in ambient air samples surrounding a facility which manufactures nylon (Figure 3A). Although the same method had been used successfully for this analysis at other manufacturing facilities, suddenly a large ghost peak was observed that interfered with the quantitation of formaldehyde. The ghost peak had poor peak shape and was not present in blank runs or standards. Although the peak shape was always broad, the retention time varied column-to- column and lot-to-lot, indicating that the ghost peak was interacting strongly with the residual silanol groups on the surface of the silica.

The H_2O component of the mobile phase was replaced with a buffer (KH_2PO_4 pH 2.5 with H_3PO_4) (Figure 3B). The large spurious peak shifted to the solvent front, making accurate quantitation of the formaldehyde peak possible. The retention times of the aldehydes and ketones were unaffected since they are not ionizable and therefore are unaffected by changes in pH.

The ghost peak is most likely a amine compound that has been partially derivatized with DNPH. A diamine is used in the manufacturing process of nylon, which could be the source of the spurious peak. The DNPH derivatization of one of the amino groups renders the other amino group inactive, resulting in a partially derivatized compound that is still ionizable and consequently does not behave well under the non-buffered chromatographic conditions. Although unbuffered, the current mobile phase is approximately pH 7, resulting in partial ionization. Mobile phase pH will change slightly as the percent organic in the mobile phase changes, which in turn affects the percent ionization of the ghost peak. The retention mechanism of this peak is probably due in part to ion exchange effects with the residual silanol groups, which can vary from lot-to-lot and even column-to-column. This effect can be eliminated by buffering the mobile phase at about pH 2.5. Under these conditions the amino compound will be extremely ionized, move to the solvent front, and behave consistently and the silanols will not dissociate and impart negative charge to the packing.

CONCLUSION: Mobile phase pH should be controlled when assaying non-ionizable or neutral analytes in the presence of ionizable contaminants or impurities. Ionizable compounds are easily recognized by their inconsistent run-run and sample-sample behavior under non buffer conditions.

FIGURE 3. Effect of pH Control on Separation of Non-Ionizable Compounds



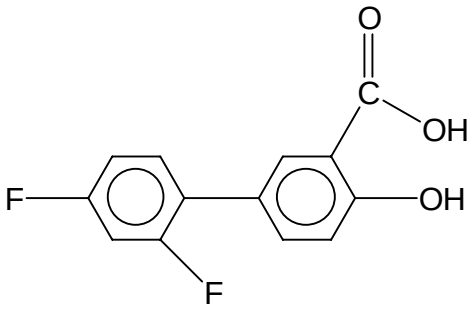
DELTABOND AK, 5µm, 150x4.6mm
Part No: 155-209
Eluent: A H_2O
B: ACN
Flow: 1.5 mL/min
Detector: UV @ 365

DELTABOND AK, 5µm, 150x4.6mm
Part No: 155-209
Eluent: A: 0.05M KH2PO4 in H2O, pH 2.5
B: ACN
Flow: 1.5 mL/min
Detector: UV @ 365

IMPORTANCE OF CAREFUL pH CONTROL?

Small changes in the mobile phase pH can also have a dramatic effect on the selectivity of weakly ionizable compounds. A sample of 7 common antiinflammatory drugs was separated at pH 2.1 (Figure 4A) and pH 2.5 (Figure 4B). Although 6 of the 7 analytes behaved very similarly under both conditions, Diflunisal eluted approximately 1 minute earlier at pH 2.5 than at pH 2.1, indicating that it is more ionized at the higher pH. This behavior indicates the presence of a carboxylic acid group in the molecule that was sensitivie to pH in this range

CONCLUSION: Adequate pH control should always be employed when working with mildly ionizable compounds to ensure maximum run-run reproducibility.

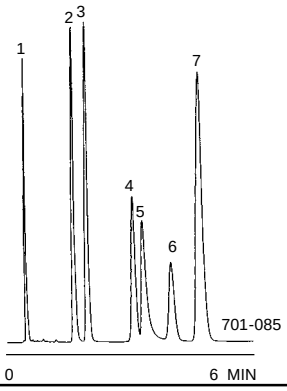


Diflunisal

FIGURE 4. Effect of Small Changes in pH on the Separation of Mildly Ionizable Compounds

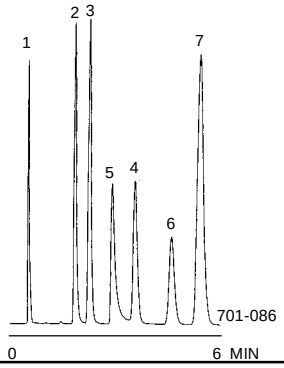
Sample:
1. Uracil
2. Tolmetin
3. Naproxin
4. Fenoprofen
5. Diflunisal
6. Indometacin
7. Ibuprofen

A: pH 2.1



BETASIL C18, 5µm, 50x4.6mm
Part No.: 155-701
Eluent: 50% ACN/ 50% 25mM H_3PO_4, pH=2.1
Flow: 0.8 mL/min
Detector: UV @ 220 nm

B: pH 2.5



BETASIL C18, 5µm, 50x4.6mm
Part No.: 155-701
Eluent: 50% ACN/ 50% 25mM KH_2PO_4, pH=2.5
Flow: 0.8 mL/min
Detector: UV @ 220 nm

USE OF ACID MODIFIERS TO ADJUST pH

It is also common to employ strong or weak acids alone to control pH at low values (Figure 6), as shown in Table 2, for commonly used trifluoroacetic (TFA) and acetic (HAC) acids. For a more thorough treatment of this topic please see reference 2. Equations used to calculate approximate pH values are shown in Figure 5 for strong (nearly dissociated) and weak acids, where C_a is the concentration of the acid in mol/L and K_a is the acid-dissociation constant.

As shown in the case of TFA, calculated values can differ significantly from measured values when the acid has properties between that of a weak and strong acid. Equation 2 is a more rigorous estimation of pH than equation 4 and offers a better approximation of pH for moderate acids such as TFA.

When TFA and HAC are used, this method of pH control does not provide a buffered mobile phase and may not be as effective for all types of samples, especially basic ones. However, it has become popular for adjusting the pH of mildly ionizable compounds such as peptides and proteins. As Figure 6 illustrates, TFA can be used to not only control mobile phase pH but also the selectivity as well. An order of magnitude change in concentration of TFA results in a significant change in pH and a dramatically different chromatogram. At 0.1% TFA (pH 2.0) (Figure 6A), Angiotensins II and III coelute and at 0.01% TFA (pH 2.4) (Figure 6B), they are baseline resolved. While some of the change can be attributed to ionization differences at the two pH values, TFA also has unique properties which may result from its reported ability to form strong ion-pairs with positively charged species.

Table 2. Properties of Acid Modifiers

Modifier (v/v)	Concentration (Moles/L)	Measured pH	Calculated pH [1]	Calculated pH [2]	Constant K_a	Buffer pK_a
0.1% TFA	1.35×10^{-2}	2.04	1.09	1.89	0.50	0.30
0.05% TFA	6.75×10^{-3}	2.20	1.23	2.18	0.50	0.30
0.01% TFA	1.35×10^{-3}	2.44	1.58	2.87	0.50	0.30
1.0% HAC	1.75×10^{-1}	3.01	2.75	2.74	1.85×10^{-5}	4.74

1. pH calculated using Equation 4
2. pH calculated using Equation 2

Figure 5

Weak Acids

(1) $$[H^+] = \frac{-K_a \pm \sqrt{K_a^2 + 4K_aC_a}}{2}$$

(2) $$pH \cong -\log\left[\frac{-K_a \pm \sqrt{K_a^2 + 4K_aC_a}}{2}\right]$$

(3) $$[H^+] = \sqrt{K_aC_a}$$

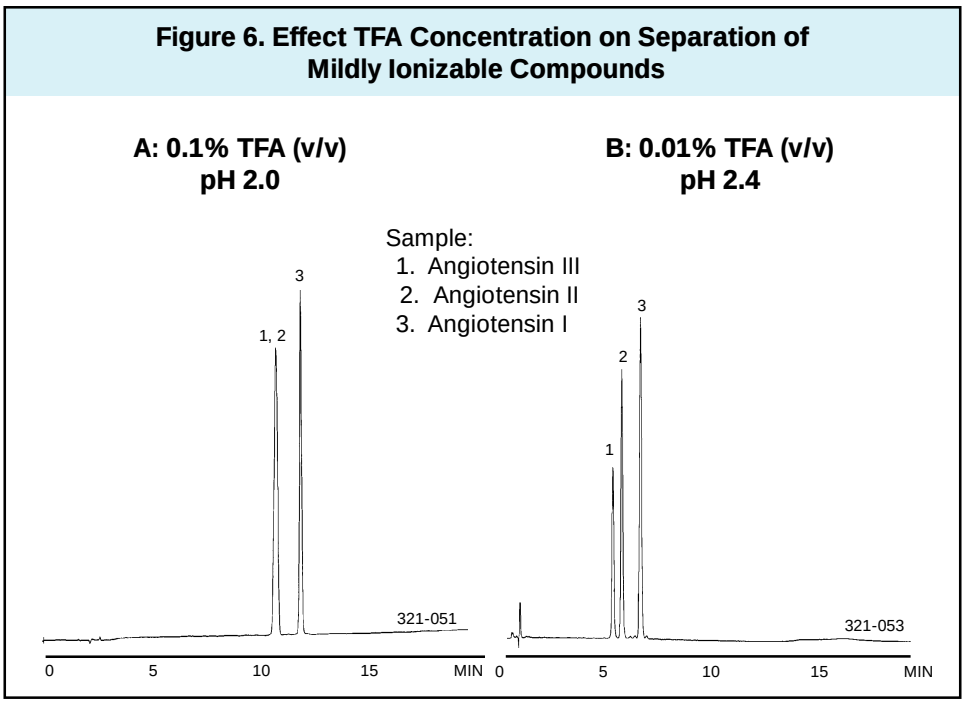
(4) $$pH \cong \left[\frac{-\log(K_aC_a)}{2}\right]$$

Strong Acids

(5) $$[H^+] = C_A$$

(6) $$pH = -\log C_a$$

Figure 6. Effect TFA Concentration on Separation of Mildly Ionizable Compounds

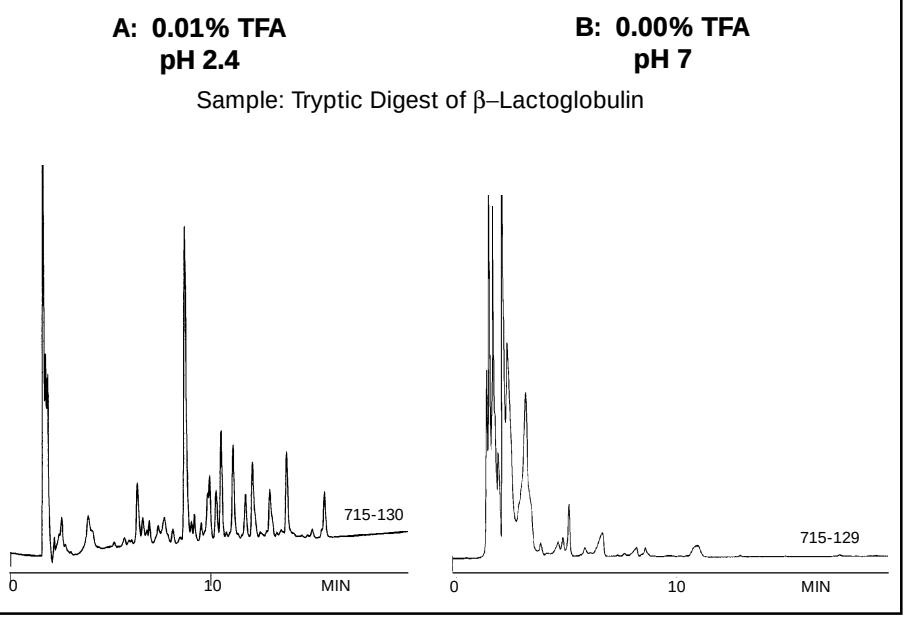


PRISM RP, 5µm, 150x4.6mm
Part No.: 155-321
Eluent: A: 0.01% TFA in H_2O
B: 0.01% TFA in ACN
10%→50% B in 20 min.
Flow: 1.0 mL/min
Detector: UV @220

PRISM RP, 5µm, 150x4.6mm
Part No.: 155-321
Eluent: A: 0.01% TFA in H_2O
B: 0.01% TFA in ACN
10%→50% B in 20 min.
Flow: 1.0 mL/min
Detector: UV @220

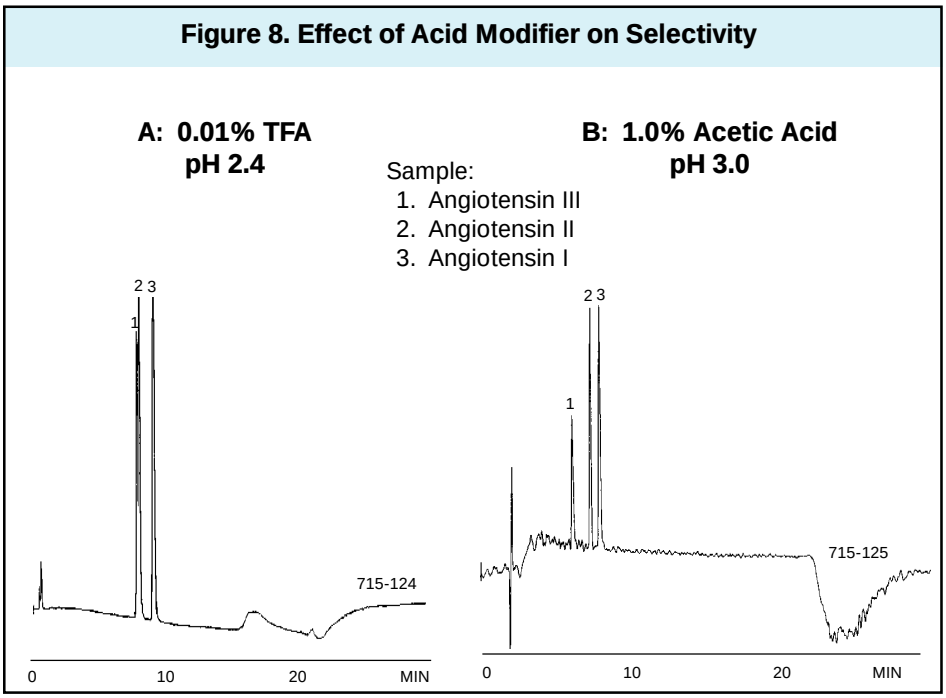
Figure 7 is another example of the importance of the acid concentration. Figure 7A shows a separation of a tryptic digest of β-Lactoglobulin with 0.01% TFA while Figure 7B shows the same separation without TFA present in the mobile phase. In this case there was dramatic loss of retention and selectivity for all of the peptide fragments. A trace amount of acid is usually required to maintain adequate pH control and improve the separation. Generally the lowest concentration possible should be employed as long as results show ruggedness and reproducibility. Lower concentrations of buffers and additives can reduce maintenance requirements, be more compatible with detectors, and improve the lifetime of columns and other system components.

Figure 7. Tryptic Digest of β–Lactoglobulin on BetaBasic 18



BetaBasic 18, 5µm, 150x4.6mm
Part No.: 155-715
Eluent: A: TFA in H_2O
B: TFA in ACN
10%→50% B in 20 min.
Flow: 1.0 mL/min
Detector: UV @ 220

Figure 8. Effect of Acid Modifier on Selectivity



BetaBasic 18, 5µm, 150x4.6mm
Part No.: 155-715
Eluent: A: 0.01% TFA in H_2O
B: 0.01% TFA in ACN
10%→50% B in 20 min.
Flow: 1.0 mL/min
Detector: UV @ 220 nm

BetaBasic 18, 5µm, 150x4.6mm
Part No.: 155-715
Eluent: A: 1.0% HAC in H_2O
B: 1.0% HAC in ACN
10%→50% B in 20 min.
Flow: 1.0 mL/min
Detector: UV @ 220 nm

Figure 8 illustrates the effect of different acid modifiers on selectivity. Although the pH of the two mobile phases varies by less than 1 pH unit, the acetic acid mobile phase offers much greater selectivity for the same pair of components that are only partially resolved with TFA. This is another indication that the mechanism of separation, especially with organic acids, can involve specific interactions between the solute and acid, such as ion-pairing mentioned above or simply pH effects.

The increased noise observed in Figure 8B is caused by higher background UV absorbance of 1.0% acetic acid compared to 0.01% TFA.

GUIDELINES FOR PREPARING MOBILE PHASES

Because slight variations in pH and acid concentration can have a dramatic impact on separation, consistent certain techniques should be employed when preparing mobile phases to ensure good reproducibility. As described in the literature[3], it is generally a good idea to measure an appropriate amount of pure water into a volumetric flask with an accurate amount of salt or acid. The pH of the mobile phase should be adjusted, if required, by adding reagent before diluting to final volume and prior to blending of any organic solvents. For example, blending 25% methanol will raise the apparent, measure pH of the combined mobile phase by about 0.5 pH units. Alternatively, equimolar solutions of different ionic forms of the same buffer (i.e. mono and dibasic phosphate) can be blended to reach the desired pH.

When developing a rugged method, it is desirable to select a mobile phase with a final pH at least one pH unit away from any analyte's pK value to cause ionization or suppression of the analytes. There is often some guesswork in this because the effect of type and concentration of organic solvent on either mobile phase pH of solute pK values is not accurately known. During method development, it is important to monitor chromatographic (k', α) reproducibility with several batches of mobile phase, as it can be difficult to consistently reproduce pH precisely.

The equations in Figure 5 are a good starting point for calculating the concentration of acid required to achieve a desired pH for separation. However, as Table 2 shows, pH can vary significantly from those calculations. It is therefore very important to experimentally determine and report the value of the mobile phase pH with a calibrated pH meter to ensure reproducible results.

The use of pre-mixed mobile phase (pumping from a single reservoir) is essential to ensure accurate and reproducible mobile phase composition. However, it has become popular to prepare an aqueous buffer and program the instrument to blend organic solvent with aqueous buffer for gradient elution or fast isocratic method development. This practice can result in poor accuracy and incomplete mixing, depending on system maintenance and calibration, magnitude of dwell volume, flow rate and other factors. Isocratic methods that have been developed using instrument blending should be confirmed by pre-mixed mobile phases, and gradient methods should be compared between more than one instrument when possible.

SUMMARY AND RECOMENDATIONS

Controlling the separation of ionizable compounds can be difficult, and careful attention must be paid to all experimental details in order to accomplish a rugged method. Slight variations in mobile phase preparation can result in pH changes that can have dramatic effects on selectivity, capacity factor (retention factor), peak shape, resolution, and reproducibility. Optimum pH control will usually result in mobile phase containing buffer and acid compositions that will resist change when the sample is introduced and force ionizable analytes into predominantly one form (ionized or neutral) as they enter the column. Good laboratory practice in preparing mobile phases should be followed to ensure that results can be reproduced within and between laboratories. While instrument solvent blending has become very convenient for fast method development, it is best to evaluate pre-mixed solvent whenever possible to ensure accuracy and equilibration before completing and publishing an HPLC method. This extra step can eliminate the possibility that instrument factors could make separation results difficult for others to reproduce.

Mobile phase pH should be selected so that it is at least $\pm$ 1.5 pH units from the analyte's pK_a. This assures that the analytes are either 100% ionized or 100% non-ionized and should help control run-run reproducibility. At high pH, acidic compounds are ionized and are much more hydrophilic than under ion suppression conditions. These conditions should be selected when fast analysis and low retention are desired. BioBasic 18 is a good choice under high pH conditions.

REFERENCES:

1) R.C. Weast, ed., Handbook of Chemistry and Physics, (CRC Press: Cleveland, OH), 1974. D112-D114.

2) R.A. Henry, D Gahagan, Design of Volatile Buffer Systems for LC Applications, (Keystone Scientific, Inc. Bellefonte, PA)

3) "Separations Solutions: Mobile Phase pH," U.D. Neue, American Laboratory, March 1999, p. 60.