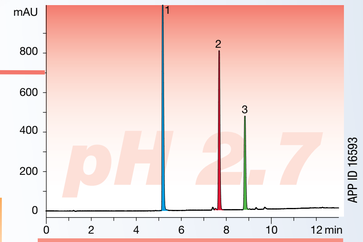
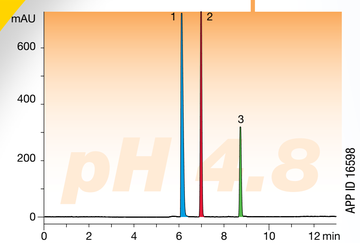
pH HPLC METHOD DEVELOPMENT

Adjust pH to Alter Selectivity

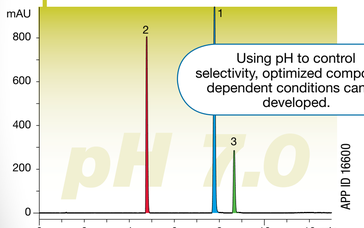
1 2 3 4 5 6 7 8 9 10 11 12



Mobile Phase
A: 0.1 % Formic Acid in Water pH 2.7
B: 0.1 % Formic Acid in Acetonitrile

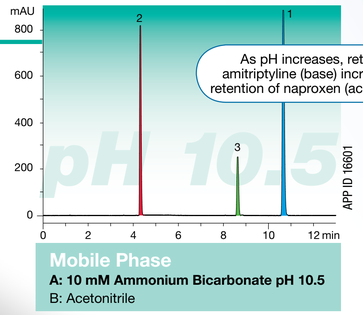


Mobile Phase
A: 10 mM Ammonium Acetate pH 4.8
B: Acetonitrile



Using pH to control selectivity, optimized compound-dependent conditions can be developed.

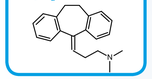
Mobile Phase
A: 20 mM Potassium Phosphate pH 7.0
B: Acetonitrile



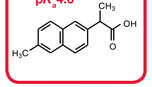
As pH increases, retention of amitriptyline (base) increases while retention of naproxen (acid) decreases.

Mobile Phase
A: 10 mM Ammonium Bicarbonate pH 10.5
B: Acetonitrile

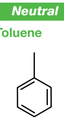
Basic
1. Amitriptyline
pK_a 9.4



Acidic
2. Naproxen
pK_a 4.0



Neutral
3. Toluene

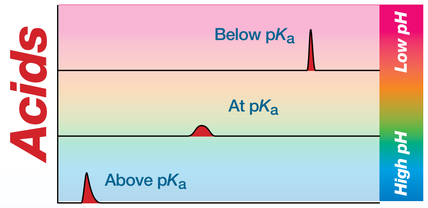


Column: Gemini-NX 5 µm C18
Dimensions: 150 x 4.6 mm
Part No.: 00F-4454-E0
Mobile Phase: *(see chromatograms)*
Gradient: A/B (95:5) to (5:95) for 10 min, hold for 2 min
Flow Rate: 1.5 mL/min
Temperature: Ambient
Detection: UV @ 254 nm
Sample Analytes:
1. Amitriptyline
2. Naproxen
3. Toluene

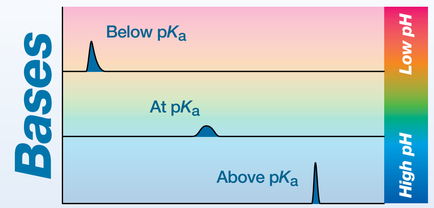
Method Development Tips

- Compounds being analyzed should be neutral for reversed phase chromatography
- For acidic and basic compounds, keep the mobile phase 2 pH units above or below the pK_a value
- For increased column lifetime, use a < 20 mM buffer concentration
- Be sure to stay within the buffering range of the buffer being used

Effects of Mobile Phase pH on Acids

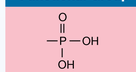
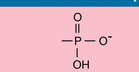
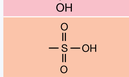
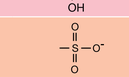
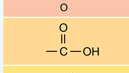
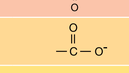
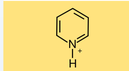
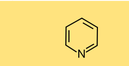
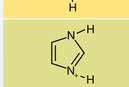
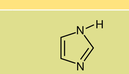
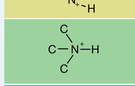
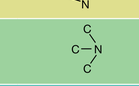
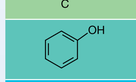
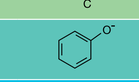
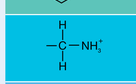
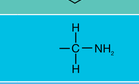
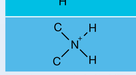
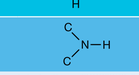


Effects of Mobile Phase pH on Bases



Retention of Different Functional Groups

Analytes contain functionalities which will affect retention on a reversed phase column. Whether a functionality is protonated or deprotonated will usually affect the chromatographic behavior of a compound; altering the pH of a separation will often change an elution profile. A general observation is that an acidic functionality will tend to have greater retention and efficiency at a pH below its pK_a value and less retention at a pH above its pK_a value. Basic compounds follow the opposite trend: basic functionalities are often less retained at pH's below their pK_a values and demonstrate greater retention and better peak shape at pH's above their pK_a values.

Protonated Functional Group	Deprotonated Functional Group	Approx. pK_a
		1.5
		2.0
		4.7
		5.3
		7.0
		9.8
		10.0
		10.6
		10.7

Acids

Bases

These values are approximate at best. Other nearby functionalities can DRAMATICALLY affect pK_a of functional groups.
Source: *Introduction to Organic Chemistry* - Streitwieser and Heathcock, MacMillan Publishing, 1981.

Buffer Selection For Method Development

Next to organic solvent, buffer selection is the most important variable in HPLC method development. There are several criteria in selecting the appropriate buffer. The first is choosing a buffer that has a pK_a near the desired working pH. Other criteria like ionic strength, ion-pairing properties, as well as mass spectrometer compatibility should be considered before selecting any mobile phase modifier.

Buffer†	pK_a	Buffer Range (pH)	MS Compatible
Trifluoroacetic Acid	**< 2**	**< 2.5**	•‡
Phosphoric Acid (pK_1)	2.1	1.1 - 3.1	
Citric Acid (pK_1)	3.1	2.1 - 4.1	
Formic Acid	**3.8**	**2.8 - 4.8**	•
Citrate (pK_2)	4.7	3.7 - 5.7	
Acetic Acid	**4.8**	**3.8 - 5.8**	•
Citrate (pK_3)	5.4	4.4 - 6.4	
Carbonate (pK_1)	**6.4**	**5.4 - 7.4**	•
Phosphate (pK_2)	**7.2**	**6.2 - 8.2**	
Triethanolamine	7.8	6.8 - 8.8	•
TRIS	8.3	7.3 - 9.3	
Diethanolamine	8.9	7.9 - 9.9	•
Ammonia	**9.2**	**8.2 - 10.2**	•
Ethanolamine	9.5	8.5 - 10.5	•
Carbonate (pK_2)	**10.3**	**9.3 - 11.3**	•
Diethylamine	10.5	9.5 - 11.5	•
Triethylamine	**11.0**	**10.0 - 12.0**	•
Piperidine	11.1	10.1 - 12.1	
Phosphate (pK_3)	12.3	11.3 - 13.3	

Sources: Practical HPLC Method Development; L.R. Snyder, JJ Kirkland, and JL Glajch. Wiley Interscience, 1997, and *Introduction to Protein and Peptide HPLC*; TP Bradshaw, Phenomenex, 1998.

† Common buffers are in bold.

‡ TFA can be used at low concentrations for LC/MS applications but can affect MS sensitivity.

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