크로마토그래피의 원리와 분석법

HPLC의 분석법-2

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Common HPLC buffers.

Buffer	pK_a	pH range	UV cut off (nm)
Phosphate	2.1 7.2 12.3	1.1-3.1 6.2-8.2 11.3-13.3	< 200
Acetate[#]	4.8	3.8-5.8	210 (10 mM)
Citrate	3.1 4.7 5.4	2.1-4.1 3.7-5.7 4.4-6.4	230
Carbonate	6.1 10.3	5.1-7.1 9.3-11.0	< 200
Formate[#]	3.8	2.8-4.8	210 (10 mM)
Ammonium bicarbonate	7.6	6.6-8.6	230
Borate	9.3	8.3-10.3	N/A

The definition of a buffer is a weak acid or base in co-solution with its salt – an example would be acetic acid (the weak acid) and sodium acetate (its salt). A known weight of the salt is usually added to the mobile phase to achieve a known concentration, the weak acid or base is then added to the mobile phase (with stirring), until the desired pH is achieved.

A particular buffer is only reliable in the pH ranges given – usually around 1 pH either side of the buffer pK_a value (note some buffers have more than one ionizable functional group and therefore more than one pK_a value).

pK_a values for common HPLC additives.

Compound	pK_a
Trifluoroacetic acid	0.3
Phosphoric acid	2.15 7.20 12.33
Citric acid	3.13 4.76 6.40
Formic acid	3.75
Acetic acid	4.76
Propionic acid	4.86
Carbonic acid	6.35 10.33
Tris	8.06
Boric acid	9.23
Ammonia	9.25
Glycine	9.78
Triethylamine	10.21[7]
Pyrrolidine	11.27
Methanesulfonic acid	-1.61[7]

pH value	Buffer	UV cut-off [nm]
2.0 – 3.0	Phosphate	210
3.5 – 5.5	Acetate	240
4.0 – 6.0	Citrate	250
6.0 – 8.5	Phosphate	210
7.0 – 9.0	TRIS	225
8.0 – 10.5	Borate	210

Phosphate has three pKa values that give it three buffering ranges: 1.1<pH<3.1, 6.2<pH<8.2, and 11.3<pH<13.3 (allowing for buffering of pKa ±1 pH units). Practical limits of column stability require that we truncate the lower range to 2.0<pH<3.1 and eliminate the highest range. Notice that there is a gap in buffering between pH 3.1 and pH 6.2 for phosphate. This means that, although it is possible to adjust the pH of phosphate to 5.0, there is negligible buffering capacity at this pH. To fill this buffering gap, another buffer is needed.

Fortuitously, acetate fills this need well, with a buffering range of 3.8<pH<5.8. With a slight extension of the buffering range from ±1 pH units from the pKa, phosphate and acetate can cover the entire pH range of 2<pH<8 normally used for silica-based columns.

Sometimes during method development, you may desire to have full control of the pH over the useful range of the column. In this case, a blend of phosphate and acetate buffer will allow continuous variation of the mobile phase from 2<pH<8. Once you find the desired pH, the buffer not needed can be eliminated. For example, if the final mobile phase pH is 4.3, acetate is all that is needed, so phosphate does not need to be used at all.

Additive	UV cut-off [nm]
Acetic acid	230
Diethylamine	210
Formic acid	210
Triethylamine	235
Trifluoroacetic acid	210

Some analysts like to use citrate for a buffer, because it has three overlapping pKa values that allow buffering over the 2.1<pH<6.4 range (Table 2). However, citrate does not have as low a UV-cutoff as acetate and phosphate, so work at wavelengths below 220 nm is not possible; in addition, some analysts find that they have more problems with check valves when citrate is used. So citrate buffer usually is a second choice to phosphate and acetate.

As most analysts know, acetonitrile (ACN) is a much poorer solvent for buffers and salts than methanol (MeOH), and tetrahydrofuran (THF) is even worse.

%B	*MeOH*	*ACN*	*THF*
50[2]	*>50 mM*	*>50 mM*	*25 mM*
60	*>50*	*45*	*15*
70	*35*	*20*	*10*
80	*15*	*5*	*<5*
90	*5*	*0*	*0*

Solubility of Potassium Phosphate, pH 7.0, in Common HPLC Solvents

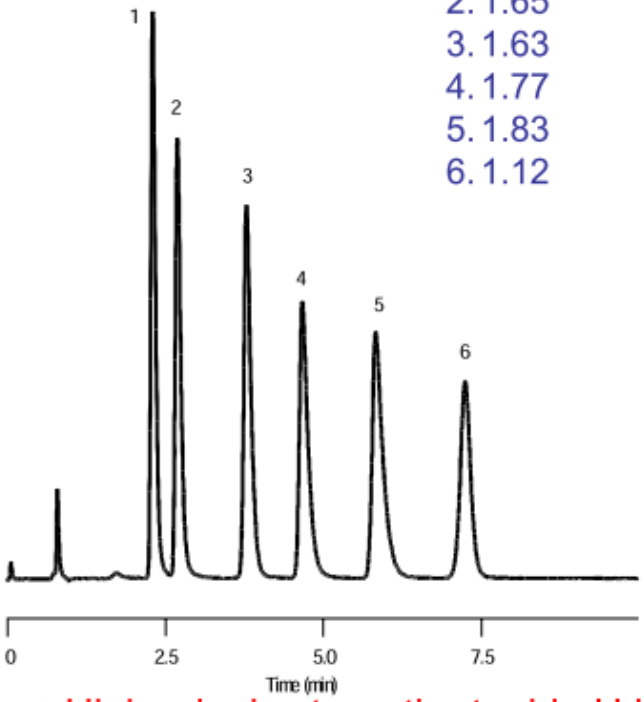
Solvent	UV cut-off [nm]
Acetone	330
Acetonitrile	190
Dichloromethane	233
Dimethoxy sulfoxide	268
Dioxan	215
Ethanol	210
Ethylacetate	256
Hexane	195
Heptane	200
Methanol	205
Methyl-ethyl ketone	329
Methyl t-butyl ether	210
N,N-Dimethylformamide	268
Propan-2-ol (IPA)	205
Tetrahydrofuran	210
Toluene	284

Buffer the sample as it is injected, so that it quickly attains the mobile phase pH.

Increasing Buffer Concentration Decreases Tf

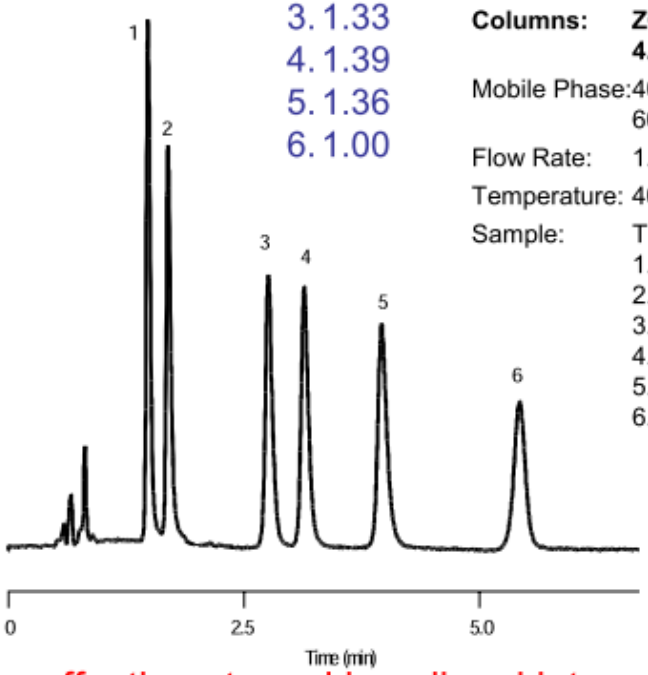
10 mM Phosphate
pH 7.0

	USP Tf
1.	1.62
2.	1.65
3.	1.63
4.	1.77
5.	1.83
6.	1.12



25 mM Phosphate
pH 7.0

	USP Tf
1.	1.41
2.	1.50
3.	1.33
4.	1.39
5.	1.36
6.	1.00



Columns: **ZORBAX Eclipse XDB-C8 4.6 x 150 mm, 5 µm**
Mobile Phase: 40% phoshpate buffer 60% ACN
Flow Rate: 1.5 mL/min.
Temperature: 40°C
Sample: Tricyclic Antidepressants
1. Desipramine
2. Nortriptyline
3. Doxepin
4. Imipramine
5. Amitriptyline
6. Trimipramine

- Higher ionic strength at mid pH is very effective at masking silanol interactions and reducing peak tailing.

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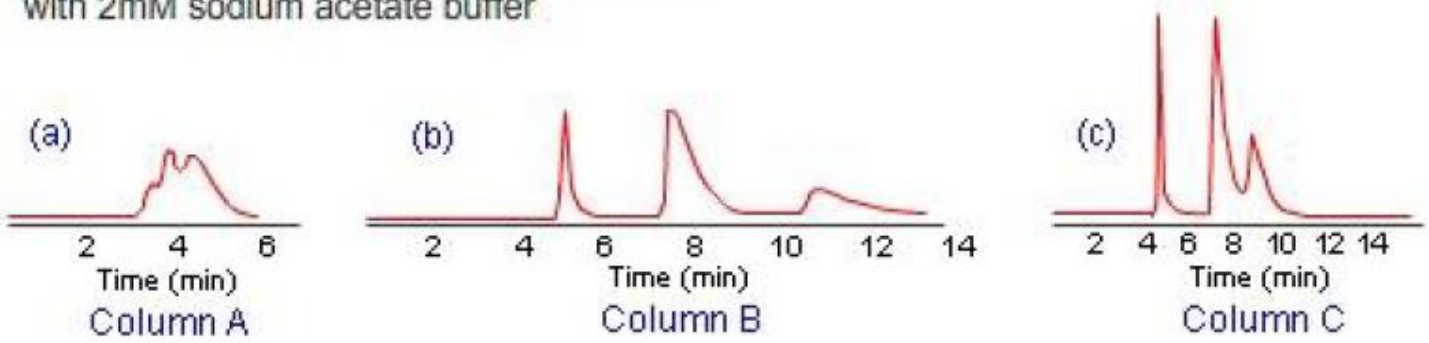
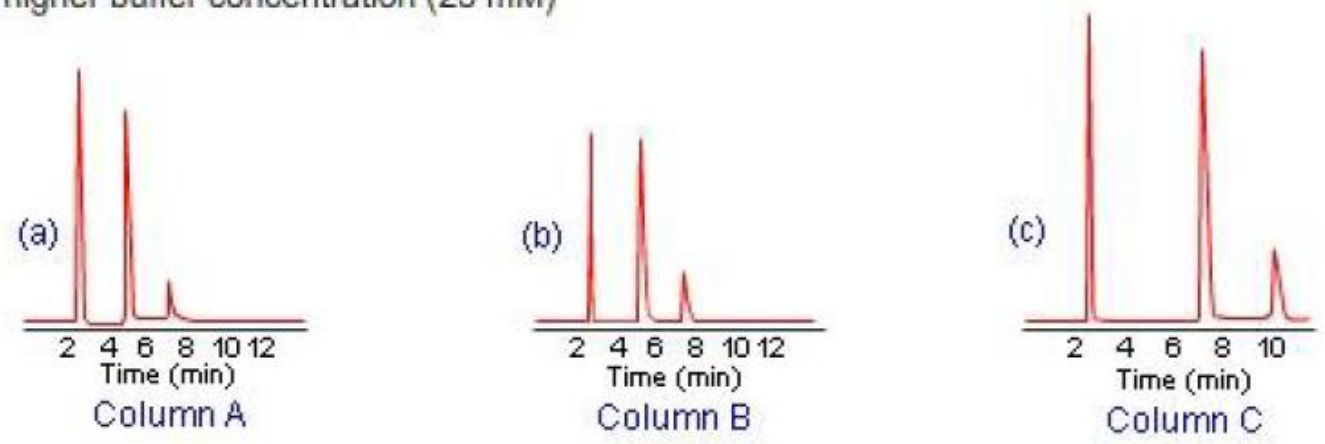
Buffers prepared below 10 mM can have very little buffering capacity and impact on chromatography, whilst those at high concentration (>50mM) risk precipitation of the salt in the presence of high organic concentration mobile phases (i.e. >60% MeCN), which may damage the internal components of the HPLC system.

Separation of Morphine, Codeine and Oxymorphone

Mobile phase : (a) 80 vol %, (b) 40 vol %, and (c) 40 vol % acetonitrile in water, all at pH 6 with 2mM sodium acetate buffer

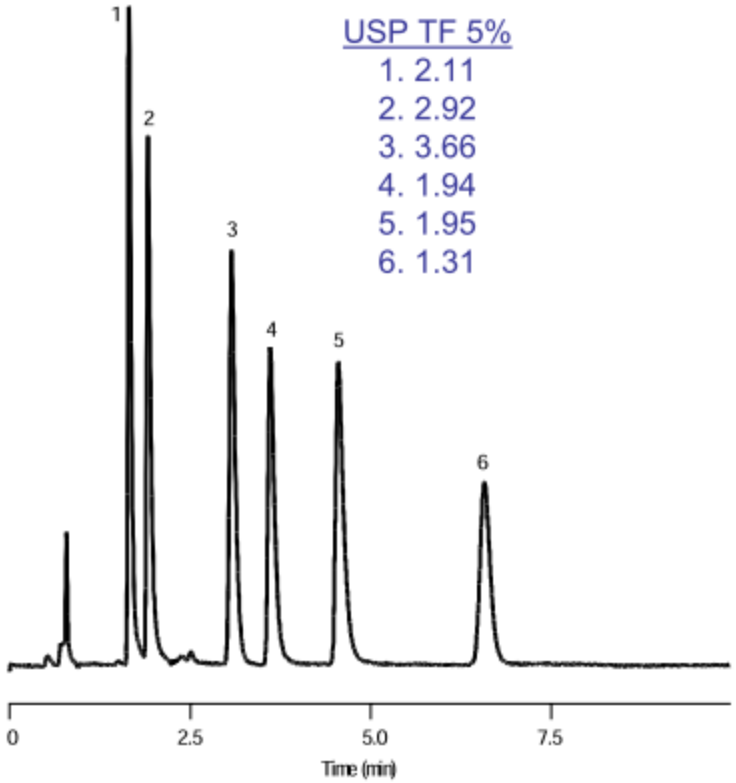


Mobile phase : (a) 16 vol %, (b) 10 vol %, and (c) 7 vol % acetonitrile in water, lower pH (3.5) and higher buffer concentration (25 mM)



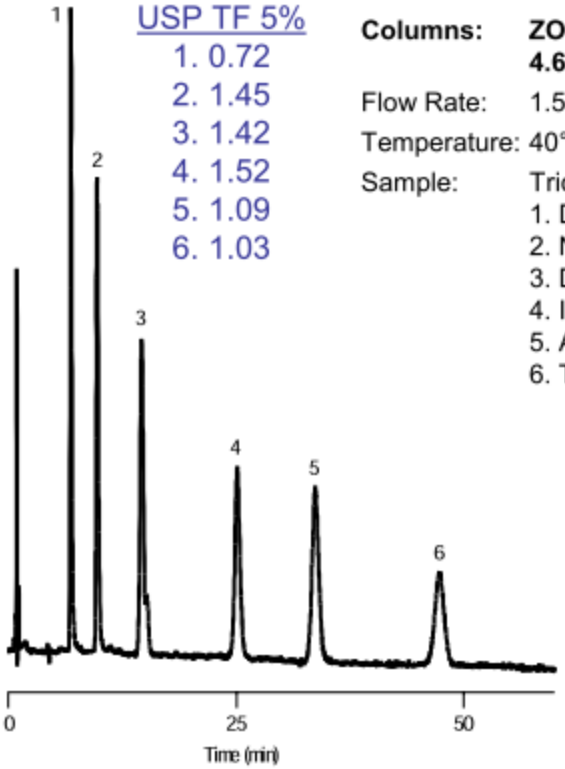
Mobile Phase Organic Modifier
Acetonitrile vs. Methanol

40% 25 mM Na_2HPO_4 pH 7.0
60% ACN



USP TF 5%
1. 2.11
2. 2.92
3. 3.66
4. 1.94
5. 1.95
6. 1.31

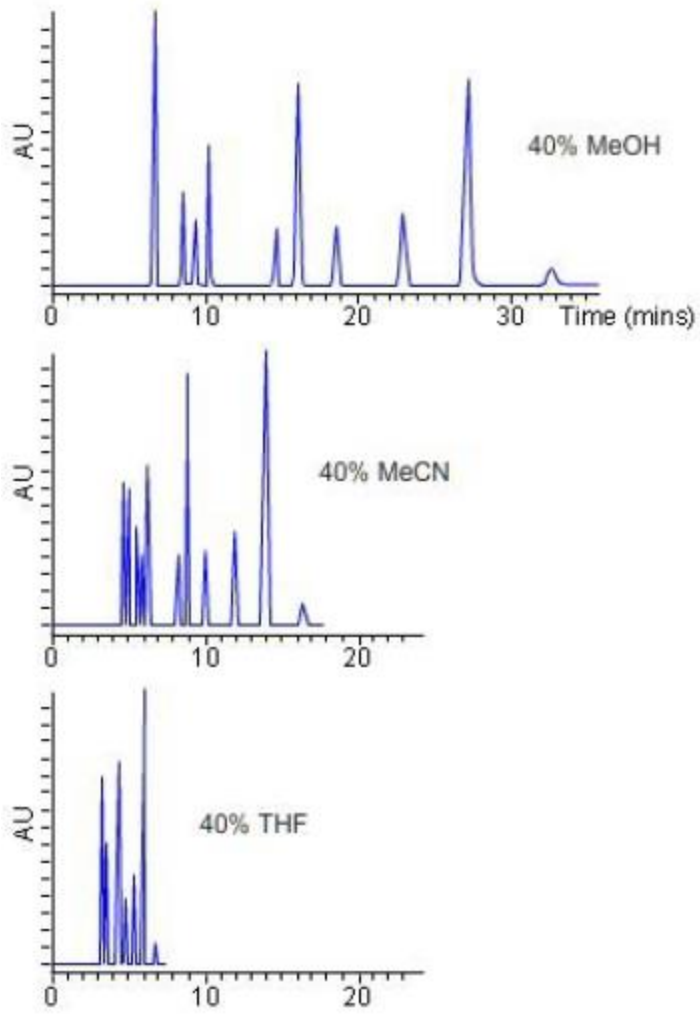
40% 25 mM Na_2HPO_4 pH 7.0
60% MeOH



USP TF 5%
1. 0.72
2. 1.45
3. 1.42
4. 1.52
5. 1.09
6. 1.03

Columns: **ZORBAX Eclipse XDB-C8 4.6 x 150 mm, 5 µm**
Flow Rate: 1.5 mL/min.
Temperature: 40°C
Sample: Tricyclic Antidepressants
1. Desipramine
2. Nortriptyline
3. Doxepin
4. Imipramine
5. Amitriptyline
6. Trimipramine

•Changing the organic modifier may improve peak shape due to secondary interactions.



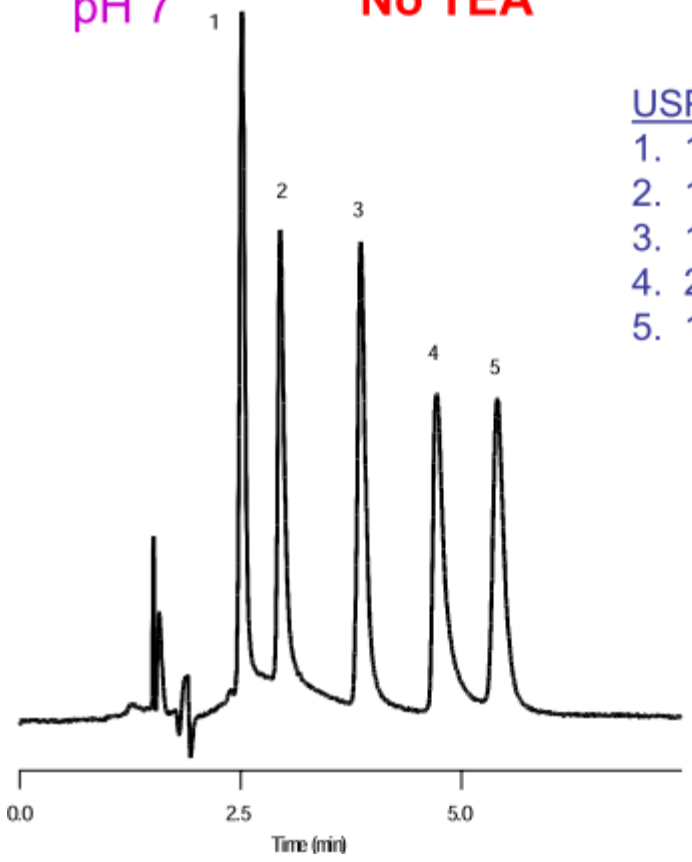
Relative solvent strength of some typical reversed phase HPLC solvents.

Mobile Phase Modifiers – Effect of TEA on Peak Shape of Basic Compounds

Column: ZORBAX Eclipse XDB-C8, 4.6 x 150 mm, 5 µm Mobile Phase: 85% 25 mM Na_2HPO_4 : 15% ACN
Flow Rate: 1.0 mL/min. Temperature:35°C Sample: Amphetamines 1. Phenylpropanolamine 2. Ephedrine 3. Amphetamine 4. Methamphetamine 5. Phenteramine

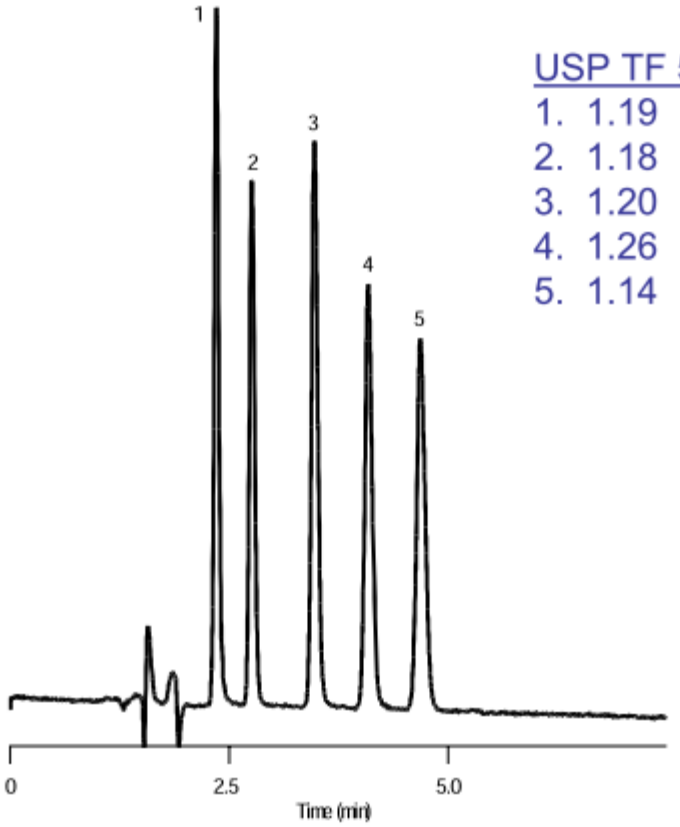
pH 7

No TEA



USP TF 5%
1. 1.29
2. 1.33
3. 1.63
4. 2.35
5. 1.57

10 mM TEA

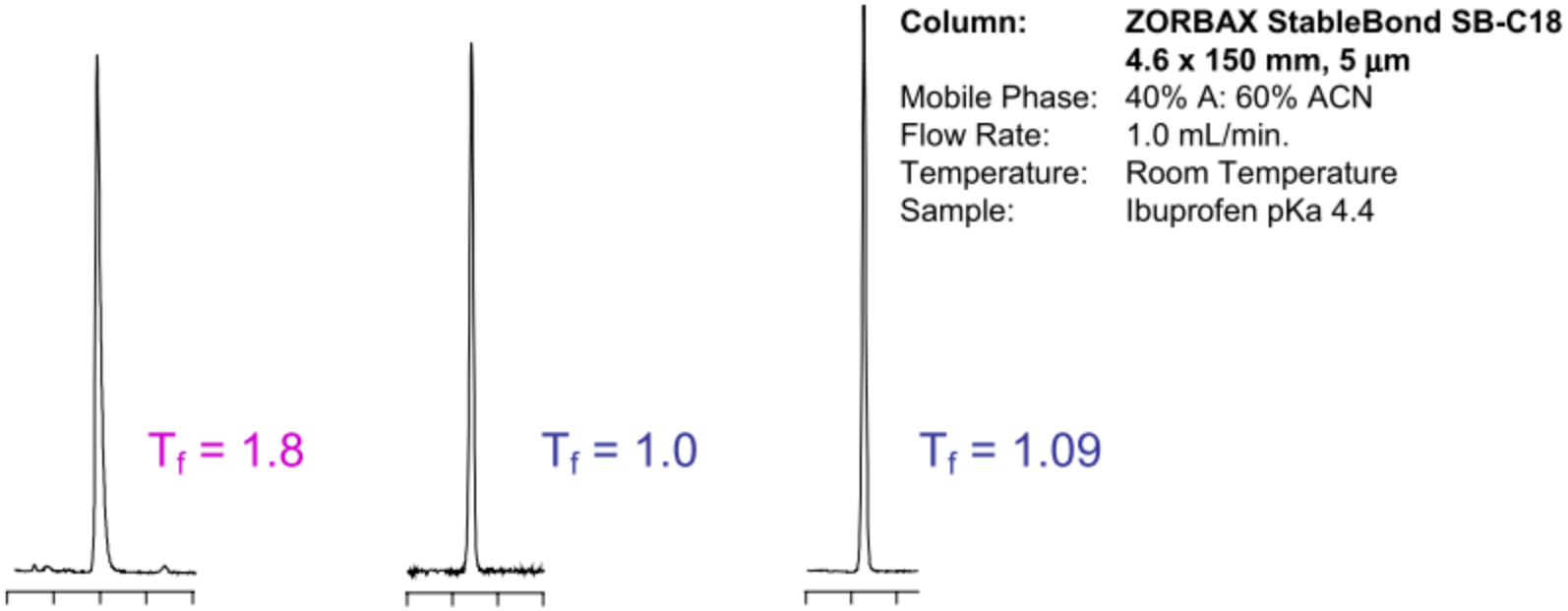


USP TF 5%
1. 1.19
2. 1.18
3. 1.20
4. 1.26
5. 1.14

Mobile Phase Modifiers – Effects of Competing Acids on the Peak Shape of Acidic Compounds

A:

pH 3 5 mM NaH_2PO_4	pH 3 5 mM NaH_2PO_4 1% acetic acid	pH 2.5 0.1% TFA



Column:	**ZORBAX StableBond SB-C18 4.6 x 150 mm, 5 µm**
Mobile Phase:	40% A: 60% ACN
Flow Rate:	1.0 mL/min.
Temperature:	Room Temperature
Sample:	Ibuprofen pKa 4.4

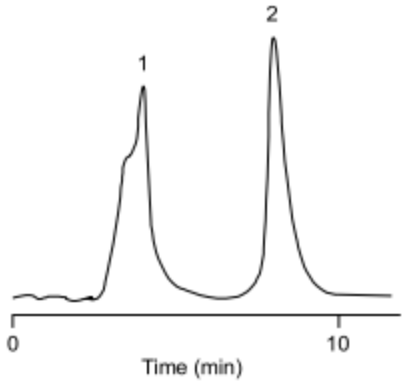
- Both acetic acid and TFA (trifluoroacetic acid) act as competing acids.
- TFA can be used at a lower concentration and is the preferred choice.

Strong Injection Solvent May Cause Poor Peak Shape

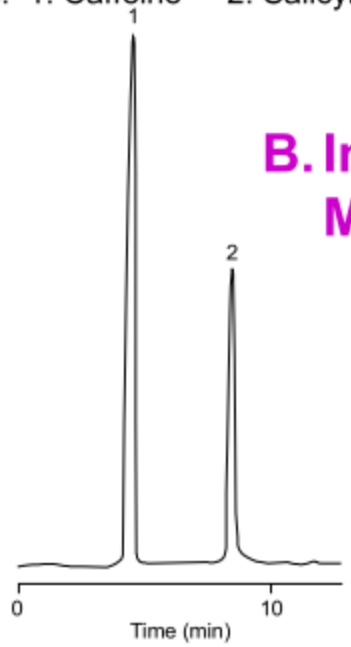
Column: ZORBAX StableBond SB-C8, 4.6 x 150 mm, 5 µm Mobile Phase: 82% H_2O : 18% ACN

Injection Volume: 30 µL Sample: 1. Caffeine 2. Salicylamide

A. Injection Solvent 100% Acetonitrile



B. Injection Solvent Mobile Phase



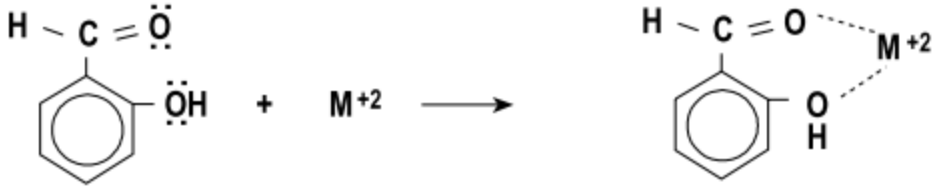
- Injecting in a solvent stronger than the mobile phase can cause peak shape problems, such as peak splitting or broadening.

Metal Complexation May Cause Poor Peak Shape

- Analytes that may complex with metals may show poor peak shape
- Both tailing and fronting may result from metal complexation
- Metals are present in LC systems from solvents, tubing, and stainless steel frits
- High purity silica eliminates silica as a source of metals

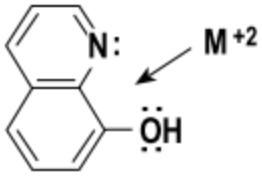
Metal Sensitive Compounds Can Chelate

Hint: Look for Lone Pair of Electrons on :O: or Ṅ Which Can Form 5 or 6 Membered Ring with Metal

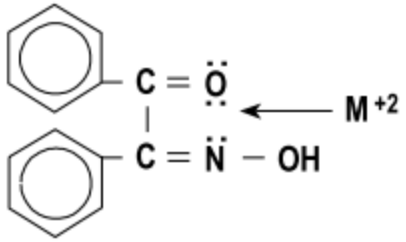


Salicylaldehyde

6-membered ring complex



8-hydroxyquinoline 5-membered ring complex

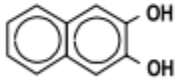


α-benzoinoxomine 5-membered ring complex

Acid Wash Can Improve Peak Shape

Before Acid Wash

1. 

2. 

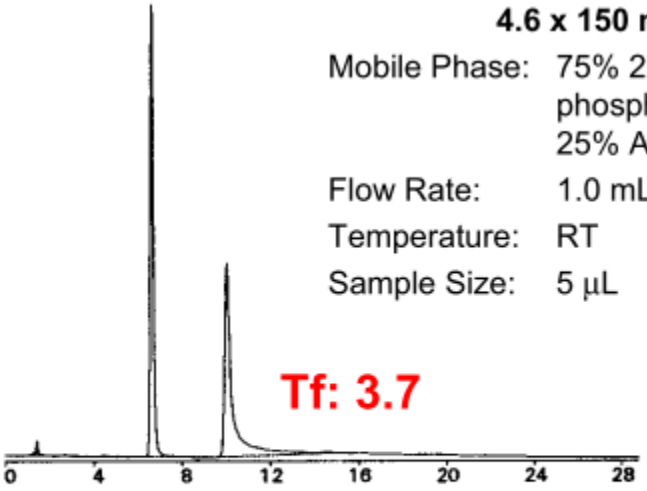
Columns: ZORBAX SB-Phenyl
4.6 x 150 mm

Mobile Phase: 75% 25 mM ammonium phosphate buffer
25% ACN

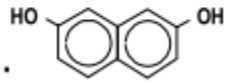
Flow Rate: 1.0 mL/min.

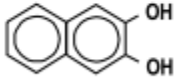
Temperature: RT

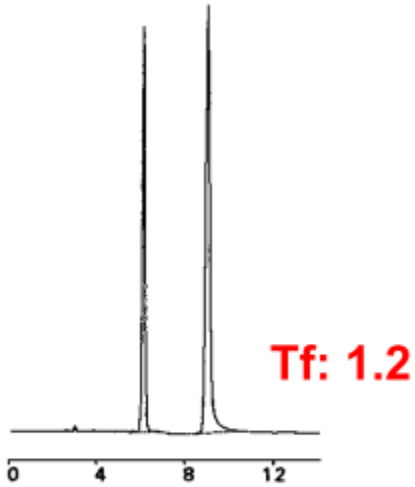
Sample Size: 5 µL



After Acid Wash 50 – 100 mLs 1% H_3PO_4

1. 

2. 



- A 1% H_3PO_4 solution is used on SB columns, 0.5 % can be used on endcapped columns.

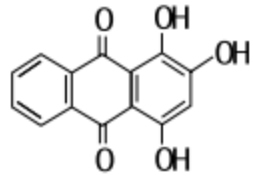
Purpurin

Column: Zorbax SB-C8, 4.6 x 250 mm, 5 µm Mobile Phase: 20% 0.02% TFA in water: 80% MeOH
Flow Rate: 1 mL/min Detection: UV 254 nm Temperature: 24°C

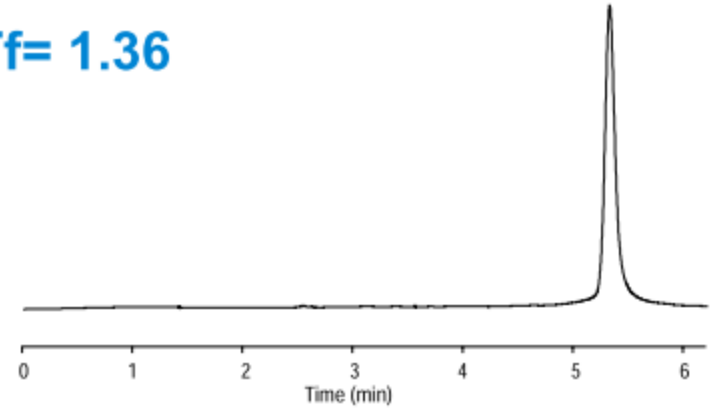
A. Before Acid Wash

B. After Acid Wash
1% phosphoric acid, 30 min.

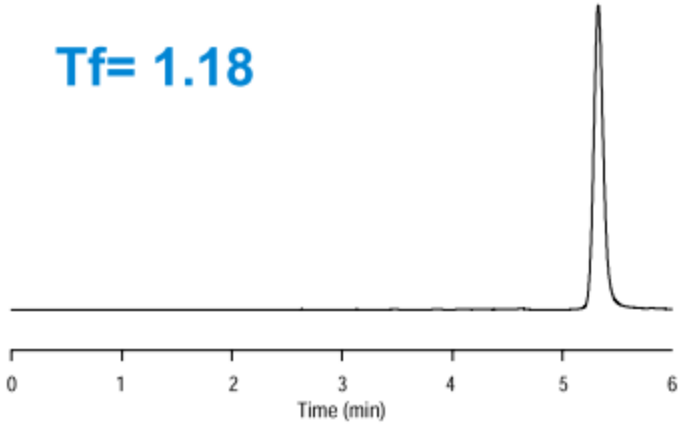
Purpurin



Tf= 1.36



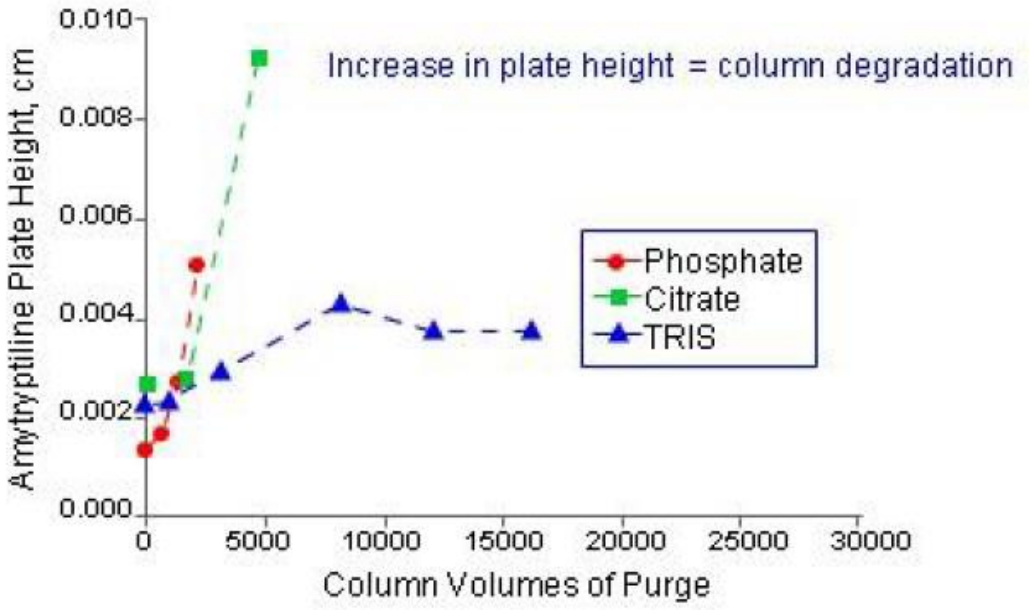
Tf= 1.18



- Both fronting and tailing are evident on purpurin before the acid wash

Buffers and Column Degradation

Buffer type, concentration, and temperature can all affect the column lifetime in HPLC. Ensure all buffers are flushed from the column after use. Citrate may seem to be a more attractive buffer, however, it will attack and cause corrosion of the stainless steel components of the HPLC system.



Why Control pH?

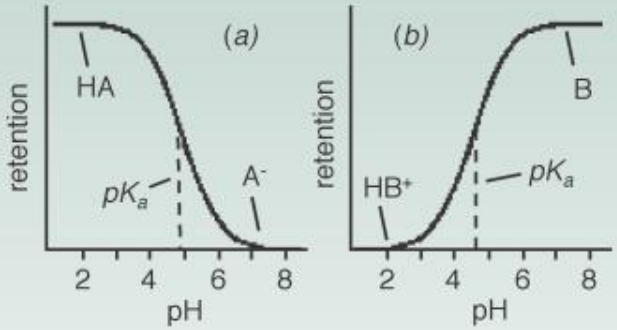


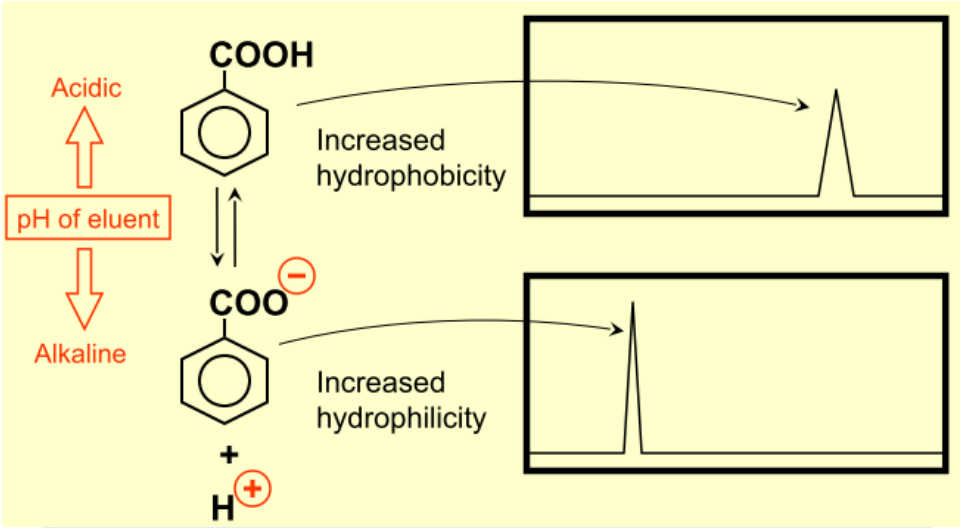
Figure 1. Retention vs. pH for a hypothetical acid (a) and base (b).

The non-ionized form will be less polar (more hydrophobic), and thus more strongly retained in a reversed-phase system. Thus, at low pH, acids will be more retained (Fig. 1a), whereas bases will be more retained at high pH (Fig. 1b).

If the mobile phase pH is near the pKa, you can see that small changes in pH can make large changes in retention – not what is desired for a robust separation.

As a general rule, silica-based columns should be operated at 2<pH<8. At pH<2, bonded phase loss due to hydrolysis can occur.

The potential for ionization of unbonded silanol (-Si-OH) groups on the surface of the silica particle: the pKa of these silanol groups is in the pH 4-5 region. This means that at pH>6, significant silanol ionization can occur for these materials. Historically, this has been the major cause of peak tailing for basic compounds through cation exchange processes.



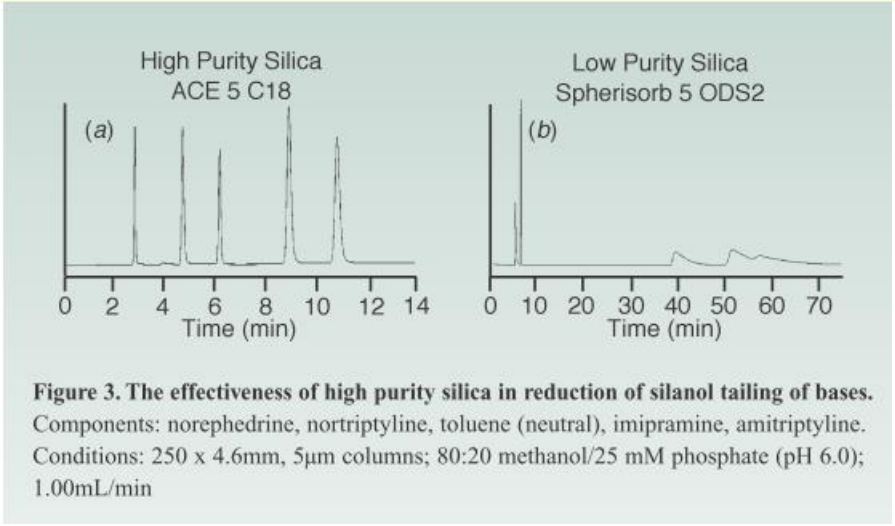


Figure 3. The effectiveness of high purity silica in reduction of silanol tailing of bases. Components: norephedrine, nortriptyline, toluene (neutral), imipramine, amitriptyline. Conditions: 250 x 4.6mm, 5µm columns; 80:20 methanol/25 mM phosphate (pH 6.0); 1.00mL/min

Recommendation to start method development with a mobile phase in the pH 2-3 range. At this pH, the ionization of most organic acids will be suppressed, as will the ionization of any silanol groups on the column.

It usually is most fruitful to adjust the mobile phase organic content (%B-solvent) to obtain acceptable retention for neutral and non-ionized compounds, then to adjust the pH to fine-tune retention of ionic analytes.

Table 1. pK_a Values of Common Mobile Phase Additives[1]

pK_a (25°C)	compound
0.3	trifluoroacetic acid[2]
2.15	phosphoric acid (pK_1)
3.13	citric acid (pK_1)
3.75	formic acid
4.76	acetic acid
4.76	citric acid (pK_2)
4.86	propionic acid
6.35	carbonic acid (pK_1)
6.40	citric acid (pK_3)
7.20	phosphoric acid (pK_2)
8.06	tris
9.23	boric acid
9.25	ammonia
9.78	glycine (pK_2)
10.33	carbonic acid (pK_2)
10.72	triethylamine
11.27	pyrrolidine[3]
12.33	phosphoric acid (pK_3)

[1] data of [1]; [2] Merck Index; [3] CRC Handbook of Chemistry and Physics

The most popular buffers for HPLC with UV detection are phosphate and acetate. Phosphate and acetate are particularly useful buffers because they can be used at wavelengths below 220 nm.

0.1% v/v phosphoric acid (Table 2) provides reasonable buffering at pH 2 for LC-UV applications. Trifluoroacetic acid (TFA) also generates a mobile phase pH of ≈2 at 0.1% v/v.

TFA, however, can suppress ionization in the LC-MS interface, causing a drop in signal, so it has fallen out of favour in recent years. Instead, 0.1% formic acid (pH ≈2.7, Table 2) is the first choice for LC-MS at low pH.

Very little buffer is needed (0.001~0.1 M). For analytical work, many samples are in the µg/mL to ng/mL range.

A buffer is most effective when used within ±1 pH unit of its pKa, but may provide adequate buffering ±2 pH units from the pKa.

즉, pH 2.1정도에서 분석을 하고 싶으면 phosphoric aicd를 이용한 buffer를 mobile phase로 사용하는 것이 좋다.

Table 2. Common HPLC Buffers

buffer	pH range	LC-MS compatible
phosphate (pK_1)	1.1 – 3.1	X
phosphate (pK_2)	6.2 – 8.2	X
phosphate (pK_3)	11.3 – 13.3	X
acetate[1]	3.8 – 5.8	YES
citrate (pK_1)	2.1 – 4.1	X
citrate (pK_2)	3.7 – 5.7	X
citrate (pK_3)	4.4 – 6.4	X
trifluoroacetic acid (0.1%)	2.0	YES
phosphoric acid (0.1%)	2.0	X
formic acid (0.1%)	2.7	YES
ammonium formate	2.7 – 4.7	YES
ammonium bicarbonate	6.6 – 8.6	YES
borate	8.3 -10.3	YES

[1] suitable for LC-MS as ammonium acetate

It should be obvious that the job of the mobile phase buffer is much easier if a small-volume injection of sample is made in an injection solvent at the same pH as the mobile phase.

As most analysts know, acetonitrile (ACN) is a much poorer solvent for buffers and salts than methanol (MeOH).

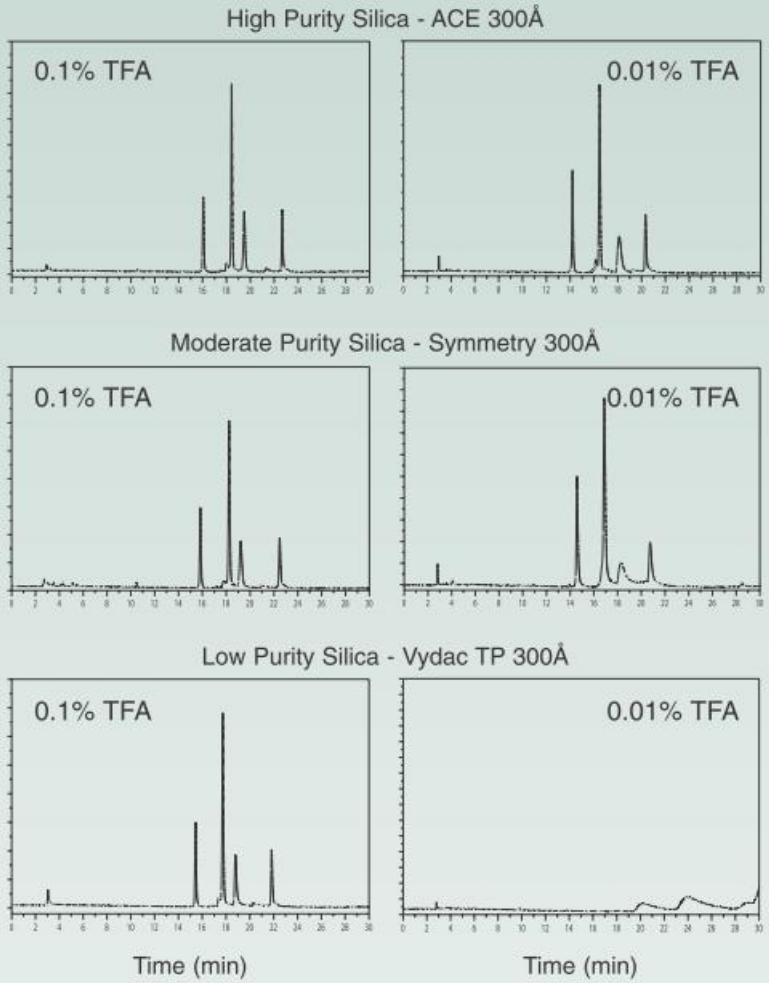
The improper technique of buffer preparation should be cited. This is to adjust the pH after organic solvent is added. Although it is possible to obtain a pH reading with organic solvent present, it cannot be compared directly to aqueous pH measurements, so the numeric value of the pH is not very meaningful.

Buffer purity is more important for gradient elution separations than isocratic ones. It is a good idea to avoid contacting the bulk mobile phase with the pH probe.

Always filter the buffer after it has been prepared. Although the buffer may be chemically pure, dust, particulate matter, filter the buffer through a 0.5 µm porosity filter prior to use.

Buffers are a good source of nutrition for micro-organisms; it is important to avoid conditions that support microbial growth. Wash the reservoir rather than refilling it.

Figure 4. Effect of trifluoroacetic acid on peak shape.



Column: 250x4.6mm, 5µm, C18 300Å. Conditions: A: 0.1% or 0.01% TFA in H_2O; B: 0.1% or 0.01% TFA in ACN; 5-70% B in 30 min; 1.00mL/min, 280nm. Components (in retention order): ribonuclease A, cytochrome C, holo-transferrin, apomyoglobin.

What is 0.1% TFA, anyway?

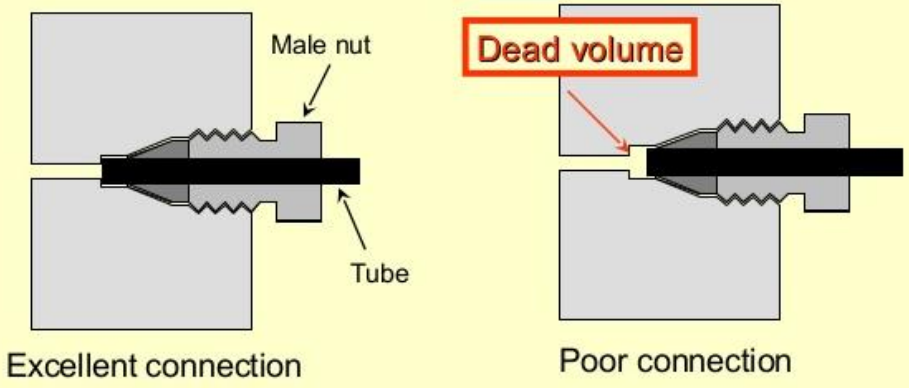
TFA concentration is usually specified with the notation “w/v”, for “weight/volume.” This indicates that TFA is to be weighed and added to a desired volume of mobile phase, for example, one gram per liter.

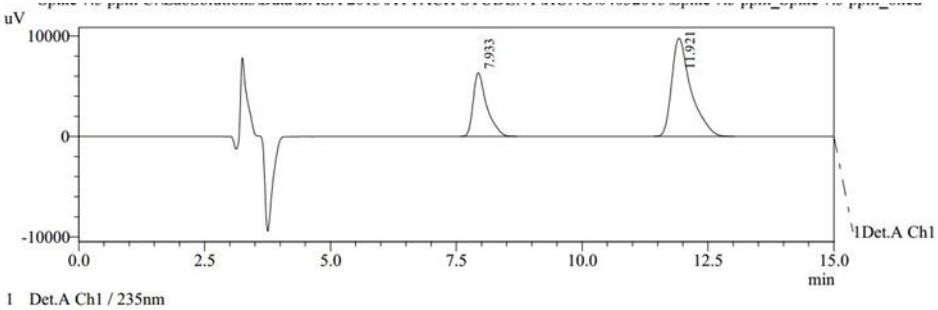
Some authors, however, may specify “v/v”, indicating that TFA, a liquid, is to be measured by volume. Caution applies here: the difference between 0.1% TFA (w/v) and 0.1% TFA (v/v) is more than 50%. The density of TFA is 1.53 grams/mL at 0°C.

It is important for the person writing the method and the one using it speak the same concentration language. A 50% difference in TFA can change peptide retention patterns significantly. Measuring TFA by volume can be subject to other errors. For example, using air-displacement pipettes is not recommended because the density, viscosity, and vapor pressure of TFA are significantly different from water, for which such pipettes are calibrated.

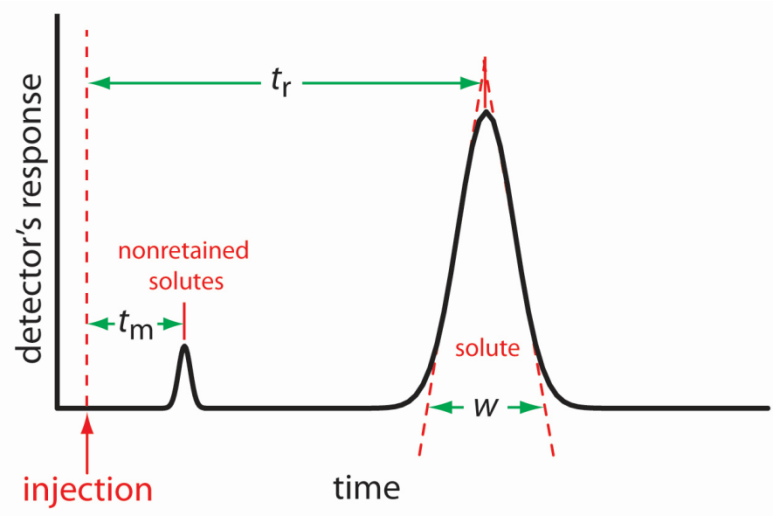
Dead Volume (Extra-column volume)

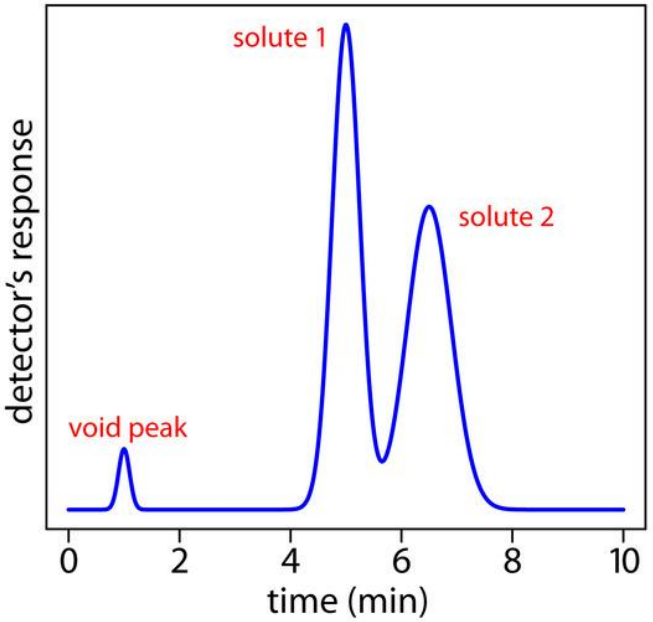
- Dead volume can cause peaks broadening.





Giant air bubble accompanying the unretained solvent.





This peak is for **nonretained solutes**. Because these solutes do not interact with the stationary phase, they move through the column at the same rate as the mobile phase. The time required to elute nonretained solutes is called the column's **void time**, t_m.

The End.