Mobile Phase Additive Selection for LC-MS

INTRODUCTION

Acids have always been preferred additives for use in HPLC methods because silica bonded phases typically exhibit highest performance under acidic conditions. Volatile acids and buffers are required for mass spectrometric detection. Selected properties of common acids and additives are shown in Table 1.

Table 1. Mobile Phase Additives for HPLC (UV and MS Detection)

Acidic Additive	pK_a	pH (aq. sol.)	BP (°C)	Ion-pairing
Phosphoric	2.1 (pK_1)	2	158	no
Formic	3.8	3	100	no
Acetic	4.8	4	118	no
3,3,3-Trifluoropropanoic	3.2	3	145	weak
Difluoroacetic	1.3	2	134	strong
Trifluoroacetic	0.3	<2	74	very strong
Formate/Formic acid	n. a.	3-5 (buffer)	volatile	no
Acetate/Acetic acid	n. a.	4-6 (buffer)	volatile	no

Table properties are approximate and were obtained from several sources. The pH values for acid solutions are estimates that will vary with concentration. Volatile organic reagents should always be used in a well-ventilated environment.

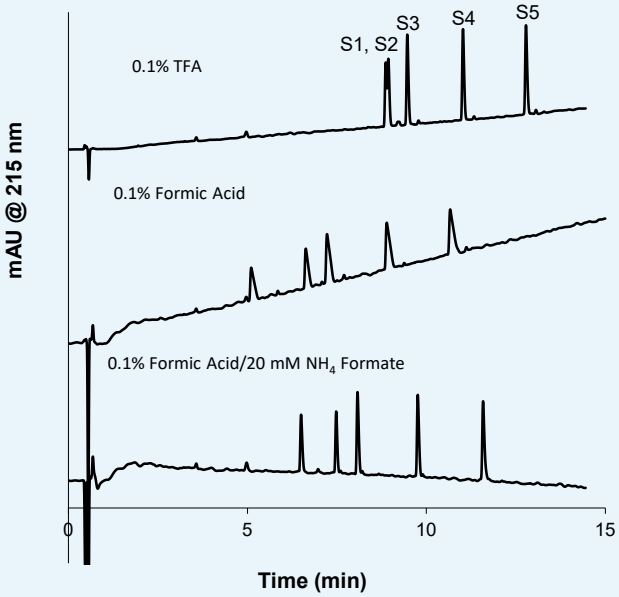
More information is readily available in modern texts (Practical HPLC Method Development by L.R. Snyder, J.J. Kirkland and J.L. Glajch, 2nd ed., John Wiley & Sons. Hoboken, NJ, 1997.) and also in A Guide to HPLC and LC-MS Buffer Selection (prepared by John Dolan) from http://mac-mod.com. Also refer to an oral presentation given by Barry Boyes at HPLC 2016 for more details.

DISCUSSION AND EXAMPLES

Figure 1 shows a comparison of low MW peptide standards separated on a HALO® 160 Å ES-C18, 2.7 µm column with three different mobile phase additives from Table 1 at similar concentration levels. HALO® ES-C18 is a sterically protected bonded phase that is stable at the low pH conditions that are typically used for peptide analysis. Note that the very low pH condition fully suppresses silanol interaction to create very sharp peaks; and causes maximum retention because TFA is a strong ion-pair reagent that interacts with peptides to make them seem more hydrophobic to the stationary phase. Since selectivity for peaks 1 and 2 is lost, more screening is needed. Changing to formic acid raises pH to 2.7 and brings possible silanol ionization into the picture. Peptides are well separated, but rather broad because of competing ionic and RP interactions at the higher pH, and the ionic strength is too low to suppress ionic effects. The ammonium formate/ formic acid gradient shows the most promise of the three conditions.

Although the threat of mixed ionic and RP retention is even greater at pH 3.3, the formate increases ionic strength and keeps peaks as sharp as TFA does at much lower pH. Although this study was done with low wavelength UV detection, note that all mobile phase additives are volatile. It has been reported by some analysts that combinations of TFA and FA are useful to offset weaknesses in both acids. Alternatively, in some cases, dilute TFA (0.03%) is as effective as 0.1% FA/0.05% TFA, yielding conditions that are compatible with MS detection.

Figure 1. Improving Retention and Peak Shape Using Ammonium Formate. Comparison of TFA, Formic Acid (FA), and FA/Ammonium Formate for Peptide Separations.



PEAK IDENTITIES

1. S1 RGAGGLGLGK-Am
2. S2 Ac-RGGGGLGLGK-Am
3. S3 Ac-RGAGGLGLGK-Am
4. S4 Ac-RGVGGLGLGK-Am
5. S5 Ac-RGVVGLGLGK-Am

	TFA 0.1%	Formic Acid 0.1%	Ammonium Formate	AF/FA
Concentration	0.013 M	0.026 M	0.020 M	20 mM AF 26 mM FA
pH	1.8	2.7	c. 7	3.3
Ionic strength	26 mM	4.4 mM	40 mM	c. 44 mM

TEST CONDITIONS

Column: HALO 160 Å ES-C18, 2.7 µm, 4.6 x 100 mm
Mobile Phase A: Water/acid modifier
Mobile Phase B: ACN/0.1% TFA or Formic Acid
Flow rate: 2.0 mL/min
Gradient: 1.5% to 26% B in 15 min
Temperature: 30 °C
Detection: UV @ 215 nm
Injection: 8 µL (800 ng) of synthetic peptides S1-S5

McCalley, D. V., Effect of buffer on peak shape of peptides in reversed-phase high performance liquid chromatography. J Chromatogr A 2004, 1038 (1-2), 77-84. Schuster, S. A.; Boyes, B. E.; Wagner, B. M.; Kirkland, J. J., Fast high performance liquid chromatography separations for proteomic applications using Fused-Core® silica particles. J Chromatogr A 2012, 1228, 232-241.

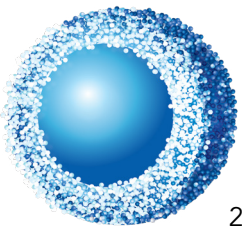
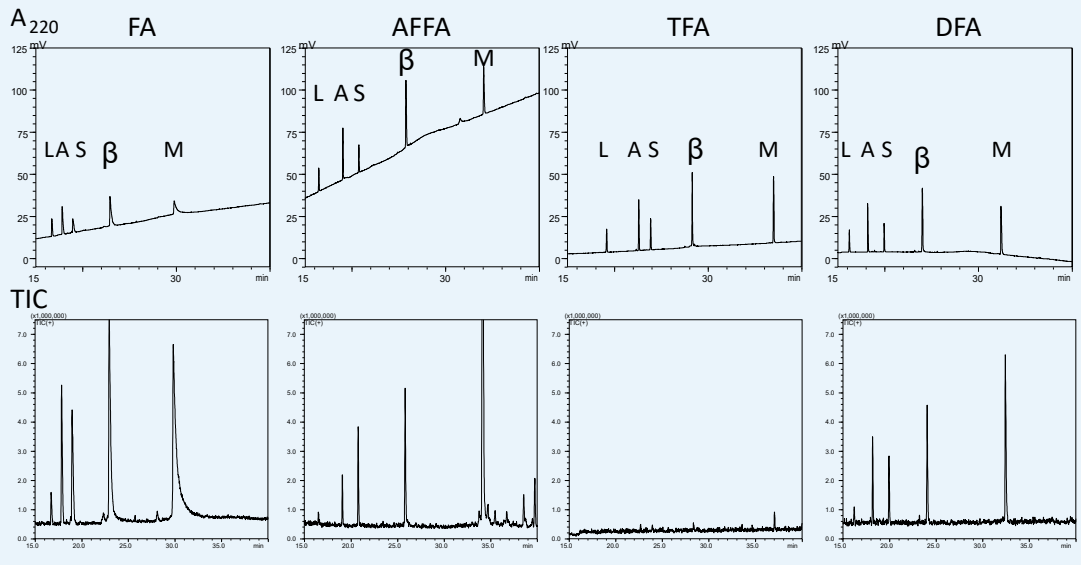


Figure 2 shows five natural peptides screened using the same HALO® Peptide ES-C18 column and the same three mobile phase additives with a stronger gradient and higher temperature using both UV (220 nm) and MS (ESI) detection. TFA, formic acid (FA) and ammonium formate/formic acid (AFFA) buffer were compared for retention, resolution, sensitivity and baseline stability to a fourth additive from Table 1, Difluoroacetic acid (DFA), that is growing in popularity for MS detection. Tailing is excessive with FA because ionic interactions are not adequately suppressed by lower pH and higher ionic strength, but it has highest MS sensitivity; AFFA yields excellent results and next highest MS sensitivity, but some baseline drift with UV is seen at 220 nm; TFA shows high performance and excellent baseline stability with UV, but the MS signal is completely suppressed due presumably to the formation of strong ion-pairs between peptides and TFA anion [1]; DFA offers an excellent combination of performance and baseline stability with both UV and MS. With DFA, pH is very low like TFA to suppress all silanol ionization and improve peak shape, while the ion-pairing is strong enough to maximize retention and resolution, but not so strong as to completely suppress the MS-ESI signal. This latter MS advantage may be related to a much higher boiling point (lower volatility) for DFA. With lower volatility, DFA also should not evaporate as quickly as TFA and change chromatographic conditions during use.

Figure 2. Comparison of UV and MS Intensities Using Peptide Synthetic Mixture (10 mM Acid; 50 pmol of a 5 peptide mix)



PEAK IDENTITIES

Peptide	Abbrev.	MW (g/mol)
[Leu5]-enkephalin	L	555.6
angiotensin I, human acetate hydrate	A	1297
substance P acetate salt hydrate	S	1348
Melittin, honey bee venom	M	2847
beta-endorphin, human	β	3465

TEST CONDITIONS

10 mM Acid; 50 pmol of a 5 peptide mix
Column: HALO 160 Å ES-C18, 2.7 µm, 2.1 x 150 mm
Flow Rate: 0.3 mL/min
Mobile Phase Gradient: 2-47% ACN in 40 min
Temperature: 60 °C
Detection: UV @ 220 nm 300-1800 m/z, 4kV, 0.33s

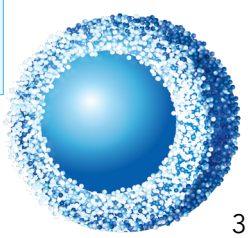
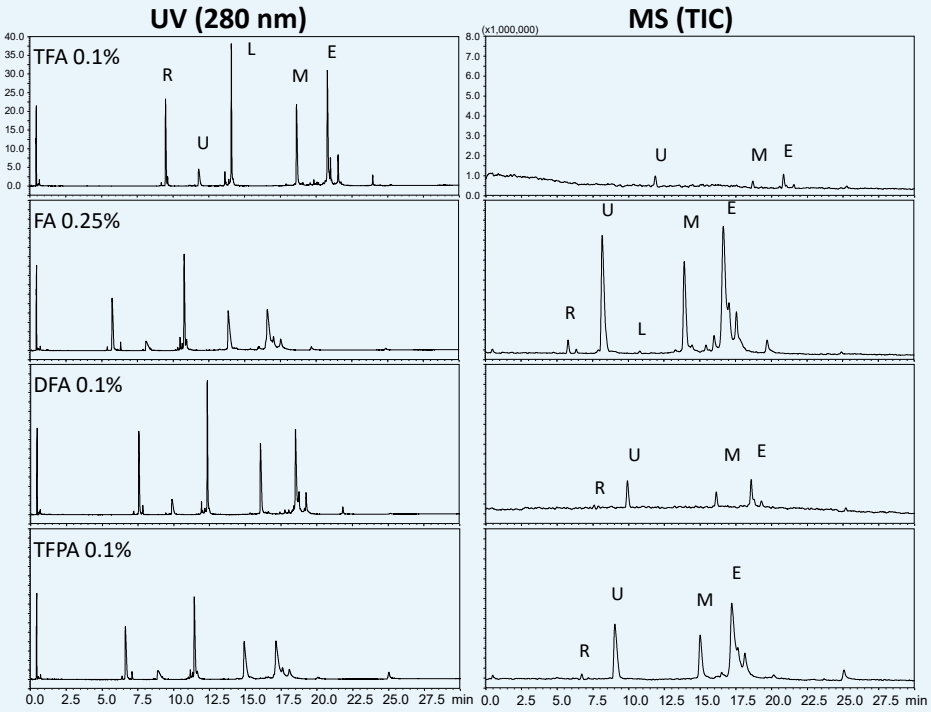


Figure 3 compares mobile phase additives from Table 1 for separating small proteins (under 50 kDa) using UV (this time at 280 nm) and MS (ESI) detection. In this case, pore-size has been eliminated as a performance factor by choosing the HALO® 400 Å C4 Protein column where the average pores are more than 10 times as large as the largest sample molecule to allow free diffusion within the particle. Similar to peptides, TFA shows highest retention and performance, but suffers from signal suppression in MS-ESI; higher boiling fluorinated acids DFA and 3,3,3-trifluoropropanoic acid (TFPA) can often improve MS sensitivity over TFA. The highest overall performance for this sample is shown with FA and TFPA.

Figure 3. Comparison of the Effect of TFA, FA, DFA, and TFPA on UV and MS Signals



PEAK IDENTITIES

R – Ribonuclease A:	13.7 kDa
U – Ubiquitin:	10.7 kDa
L – Lysozyme:	14.4 kDa
M – apo-Myoglobin:	17 kDa
E – Enolase:	46 kDa

25 pmol each protein

TEST CONDITIONS

Column: HALO 400 Å C4, 3.4 µm, 2.1 x 100 mm
Gradient: 15-55% AcN in 30 min
Flow Rate: 0.35 mL/min
Temperature: 50 °C
Detection: Nexera LC system
UV @ 280 nm
MS-2020 Single Quad
400 – 2000 m/z 3 pps, 3.8 kV ESI

Figure 4 compares three popular acid additives for a monoclonal antibody protein separation with the HALO® 1000 Å C4 Protein column using UV (280 nm) detection. The ability for this very large (150 kDa) protein to enter 1000 Å particle pores should be relatively unhindered. As seen for many proteins and peptides, FA does not completely suppress interaction with acidic silanols and shows peak broadening; however, both DFA and TFA operate at much lower pH and introduce extra retention from ion-pairing. If MS detection were needed, DFA would be preferred for higher MS sensitivity due to lower volatility and weaker ion-pair formation.

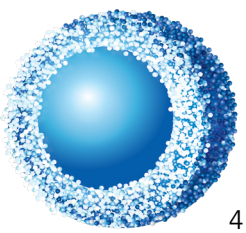
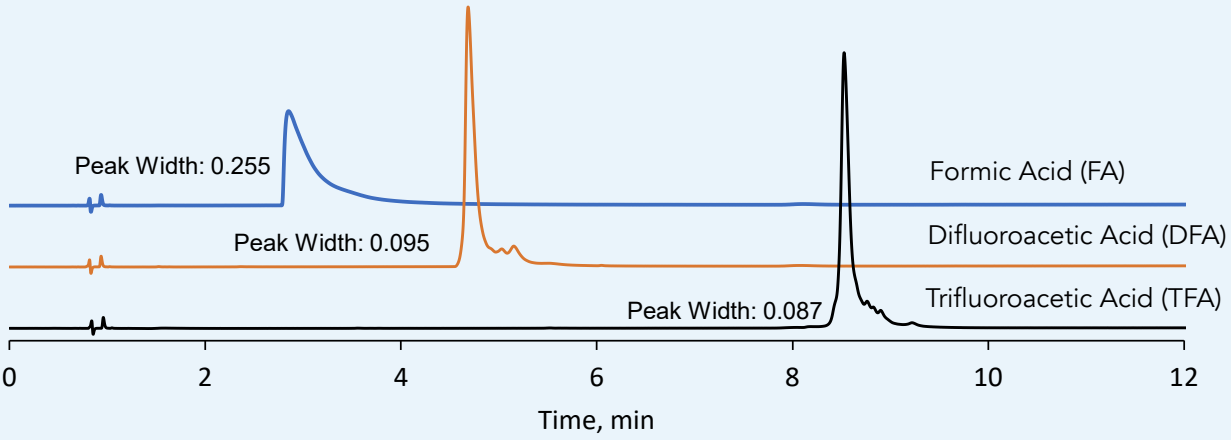


Figure 4. Mobile Phases for Improved mAb LC
Effect of FA, DFA, and TFA on Retention and Peak Shape



TEST CONDITIONS
Column: HALO 1000 Å C4, 2.7 µm, 2.1 x 150 mm
Gradient: 35-47.5% AcN/0.1% acid as indicated in 12 min
Flow Rate: 0.4 mL/min
Temperature: 80 °C
Sample: 2 µL of trastuzumab - 2 µg/µL (30/70 ACN/H2O)

PEAK IDENTITY
trastuzumab

CONCLUSIONS

For LC-MS of peptides and proteins, a compromise must be made between sharp peak shape and adequate ionization efficiency. While TFA gives sharp peaks and good retention, the ionization efficiency is poor. With FA, the ionization efficiency is high, but the peak shapes are poor. The use of either FA/AF or DFA are good options for a balanced result between good peak shape and good ionization efficiency.

REFERENCE

[1] U.A. Mirza, B.T. Chait, Effects of anions on the positive ion electrospray ionization mass spectra of peptides and proteins, Anal. Chem., 66 (1994) 2898-2904.