Improved Reversed-Phase Peptide Separations on High-Performance Silica Particles

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Introduction

Trifluoroacetic acid (TFA) is typically used for RPC of peptides (at 0.1% *v/v*) in conjunction with UV detection.

- TFA forms effective ion pair with basic moieties on the peptide.
- TFA keeps pH well below pK_a of side-chain carboxyls to maximize retention.

TFA alone, is not suitable for RPC of peptides when MS detection employed.

- Surface tension of 0.1% (*v/v*) solutions precludes efficient nebulization (spray formation) in ESI source [1].
- TFA ions in gas phase form strong ion-pair with basic moieties on peptide, masking charge [1].
- Need to use alternative ion-pairing agent, that is MS-compatible.
- Formic acid is the usual selection; acetic acid less common.

Problem: Generally much poorer peak shape and lower peak capacity with formic (or acetic) acid than when TFA is used.

Experimental Strategy

Compare and contrast column performance between various columns and as a function of specific mobile phase modifications.

Utilize peptide probes of varying basicity and hydrophobicity as probes:

1. ac-GGGLGGAGGLKG monoisotopic mass: 941.5
2. ac-KYGLGGAGGLKG monoisotopic mass: 1118.6
3. ac-GGAVKALKGLKG monoisotopic mass: 1139.7
4. ac-KYALKALKGLKG monoisotopic mass: 1330.8

Peptide probes shown in order of increasing basicity and hydrophobicity.

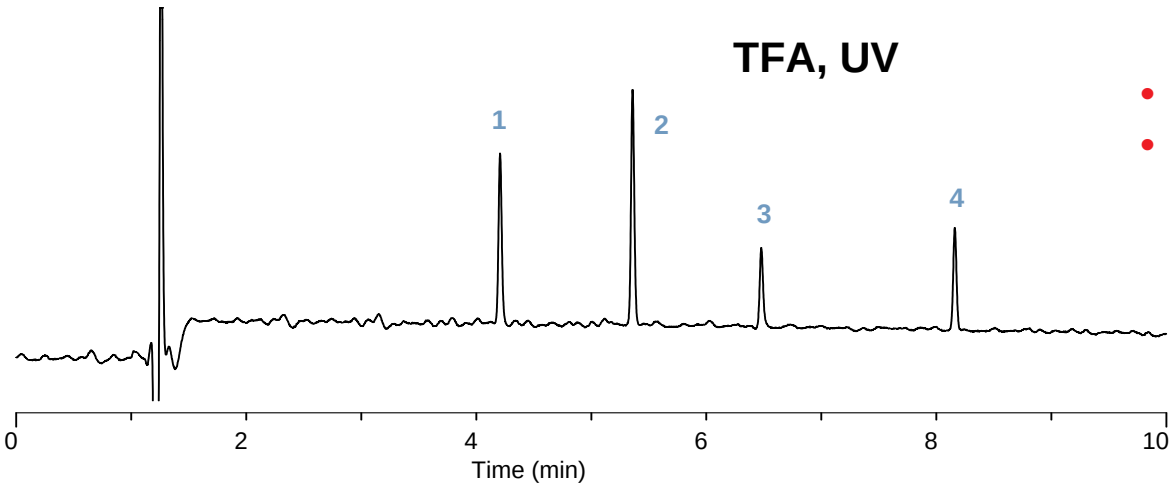
Utilize complex tryptic digests for further qualitative and quantitative comparisons.

Chromatographic Conditions for Basic Peptides

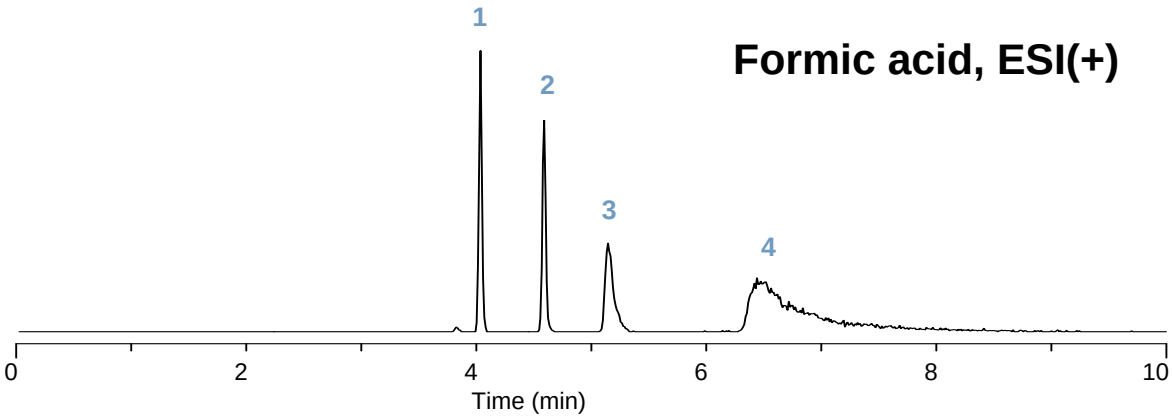
Columns are run under equivalent conditions of gradient slope (Δ %MeCN per column volume).

column:	2(2.1) x 100 mm; C18
mobile phase A:	0.1 % (*v/v*) additive
mobile phase B:	25:75, (0.4 % additive) : acetonitrile
gradient:	initial = 15% B, slope = 2% MeCN / column volume
flow rate:	0.3 mL/min
temp.:	35 °C
det.:	UV 210 nm OR ESI(+) TOF
injection:	1 µL
sample:	5 mg/L peptide 1 & 3, 1 mg/L peptide 2, 15 mg/L peptide 4
additive:	TFA, pH 2.0 @ 0.1% (pH unadjusted) *OR* formic acid, pH 2.6 @ 0.1% (pH unadjusted) *OR* formic acid, pH 3.5 (pH adjusted with ammonium hydroxide)

Basic Peptides: Typical Chromatograms



- greater retention
- better peak shape



- less retention
- poor peak shape for most basic peptides

Column Performance: Formic Acid vs TFA

David McCalley has published a series of peer-reviewed journal articles in which he details his line of investigation to uncover the mechanistic explanation as to why peak shape and peak capacity of peptides are so negatively impacted by the use of formic acid as the additive, compared to TFA [2 –5]. This is outlined as follows:

TFA 0.1% (v/v) aqueous solution (~ 13 mM)

- pH = 2.0
- 98% ionized (pKa = 0.3)
- TFA anion, ~ 12.7 mM

Formic acid, 0.1% (v/v) aqueous solution (~ 26 mM)

- pH = 2.6
- 7% ionized (pKa = 3.7)
- Formate anion, ~ 1.8 mM

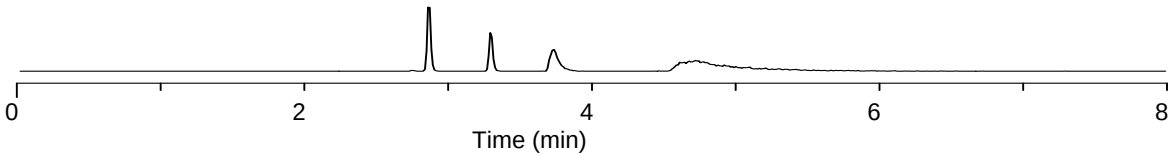
If, however, formic acid (0.1% v/v) is titrated to pH 3.5

- 36% ionized
- Formate anion, ~ 9.4 mM (>5x higher than at pH 2.6)
- Peak shape much improved

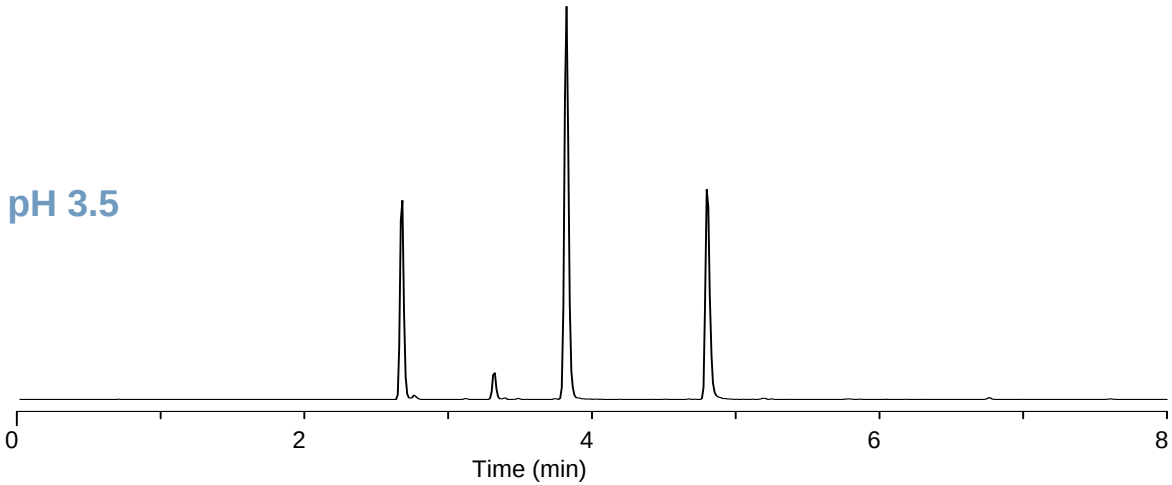
Ascentis® Express Peptide ES-C18, 160 Å, 2.7 µm

chromatograms, equally scaled

pH 2.6

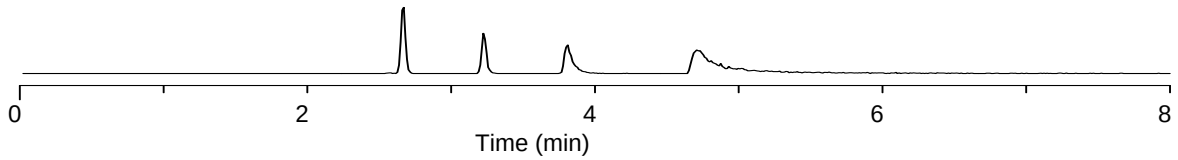


pH 3.5

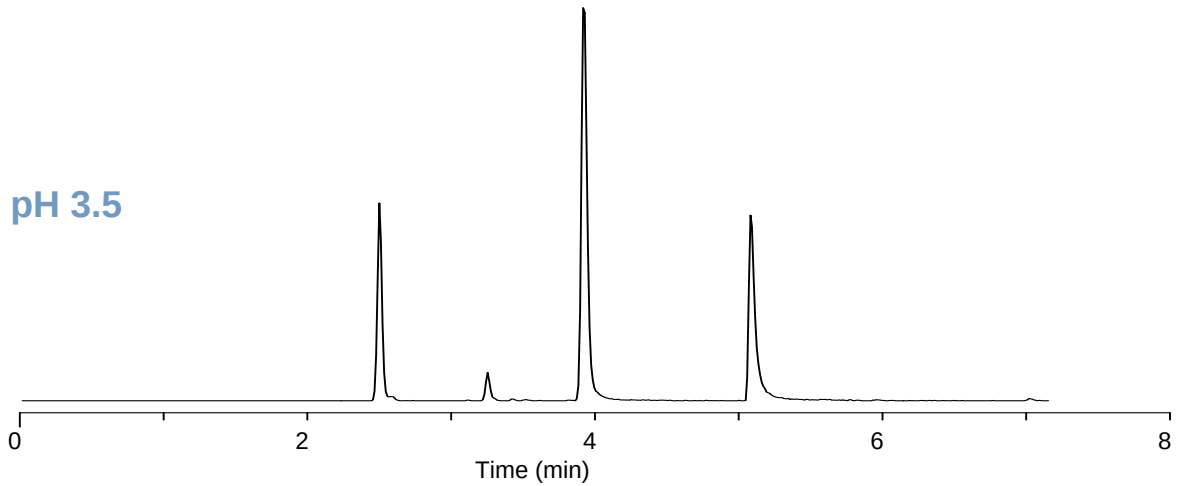


Competitor Z C18, 300 Å, 3.5 µm

pH 2.6

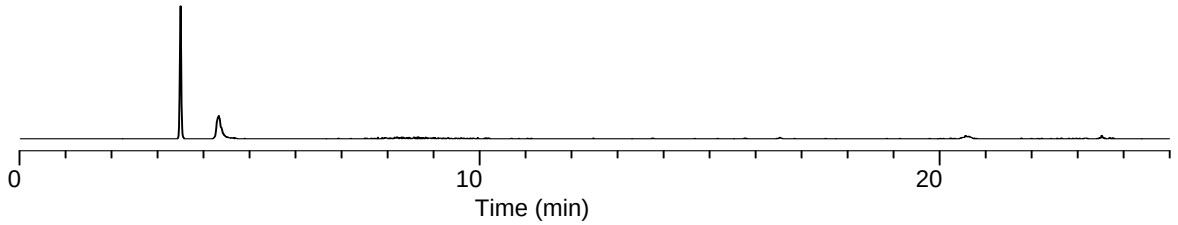


pH 3.5

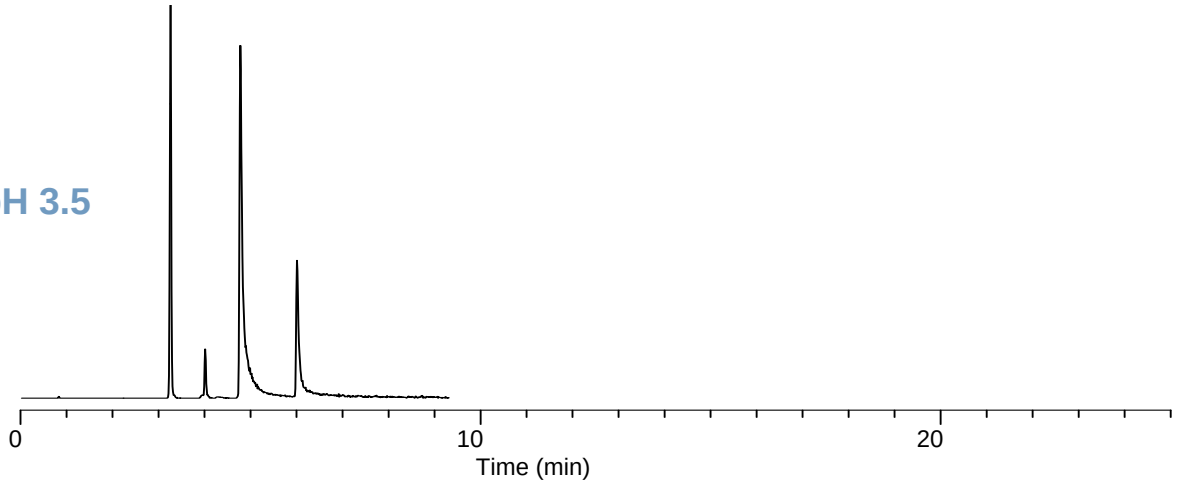


Competitor V C18, 120 Å, 1.5 µm

pH 2.6

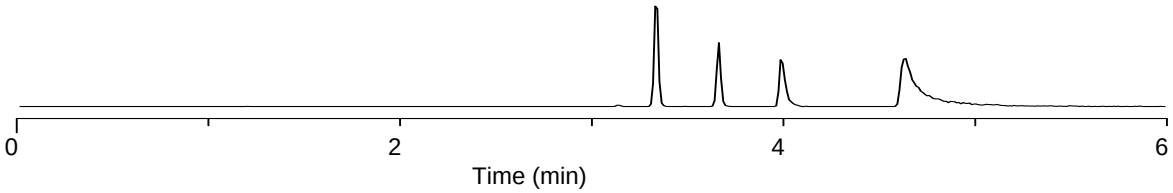


pH 3.5

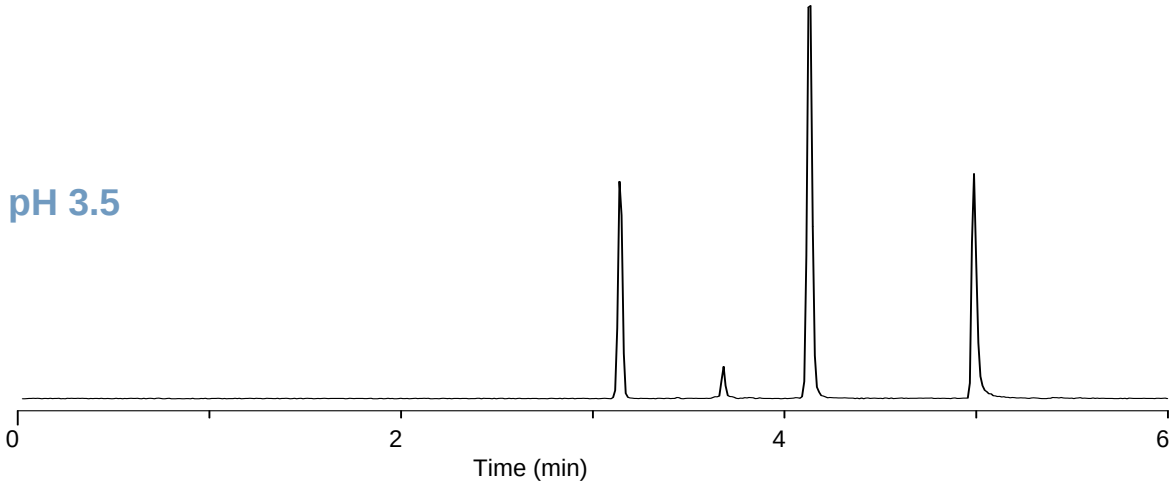


Competitor W C18, 130 Å, 1.7 µm

pH 2.6



pH 3.5



Some Quantitative Comparisons

Column		Peak 1 $w_{½}$*	Peak 1 $h/w_{½}$*	Peak 1 $TF_{0.1}$*	Peak 2 $w_{½}$	Peak 2 $h/w_{½}$	Peak 2 $TF_{0.1}$
Ascentis Express Peptide ES-C18							
	pH 2.6	0.0258	2981	1.00	0.0241	2005	1.24
	pH 3.5	0.0258	9725	1.20	0.0224	1520	1.00
Competitor Z C18, 3.5 µ							
	pH 2.6	0.0258	2267	1.00	0.0361	788	1.42
	pH 3.5	0.0344	4082	1.14	0.0344	572	1.25
Competitor V C18, 1.5 µ							
	pH 2.6	0.0344	1493	1.17	0.1118	80	1.50
	pH 3.5	0.0344	4418	1.03	0.0344	548	1.06
Competitor W C18, 1.7 µ							
	pH 2.6	0.0198	6065	1.00	0.0258	2216	1.00
	pH 3.5	0.0258	7568	1.10	0.0241	1203	1.20

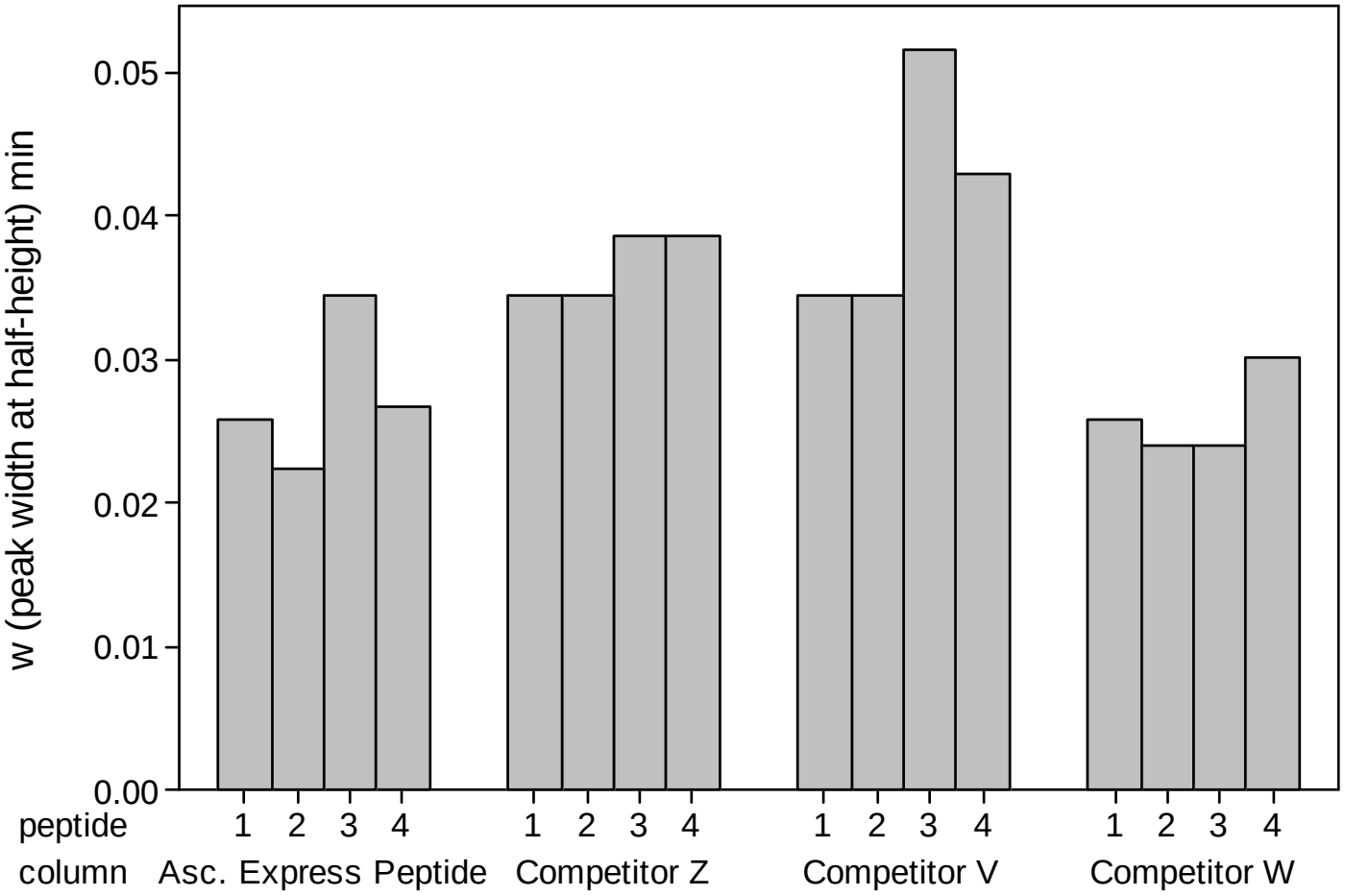
Column		Peak 3 $w_{½}$	Peak 3 $h/w_{½}$	Peak 3 $TF_{0.1}$	Peak 4 $w_{½}$	Peak 4 $h/w_{½}$	Peak 4 $TF_{0.1}$
Ascentis Express Peptide ES-C18							
	pH 2.6	0.0688	317	1.60	0.3183	35	2.92
	pH 3.5	0.0344	11321	1.00	0.0267	9706	1.30
Competitor Z C18, 3.5 µ							
	pH 2.6	0.0559	363	1.60	0.1419	118	4.64
	pH 3.5	0.0387	8267	1.08	0.0387	3871	1.74
Competitor V C18, 1.5 µ							
	pH 2.6	n/a	n/a	n/a	n/a	n/a	n/a
	pH 3.5	0.0516	3044	2.69	0.0430	1281	2.38
Competitor W C18, 1.7 µ							
	pH 2.6	0.0258	2006	1.37	0.0740	586	3.44
	pH 3.5	0.0241	1942	1.00	0.0301	6684	1.30

* $w_{½}$ peak width at half height (min)

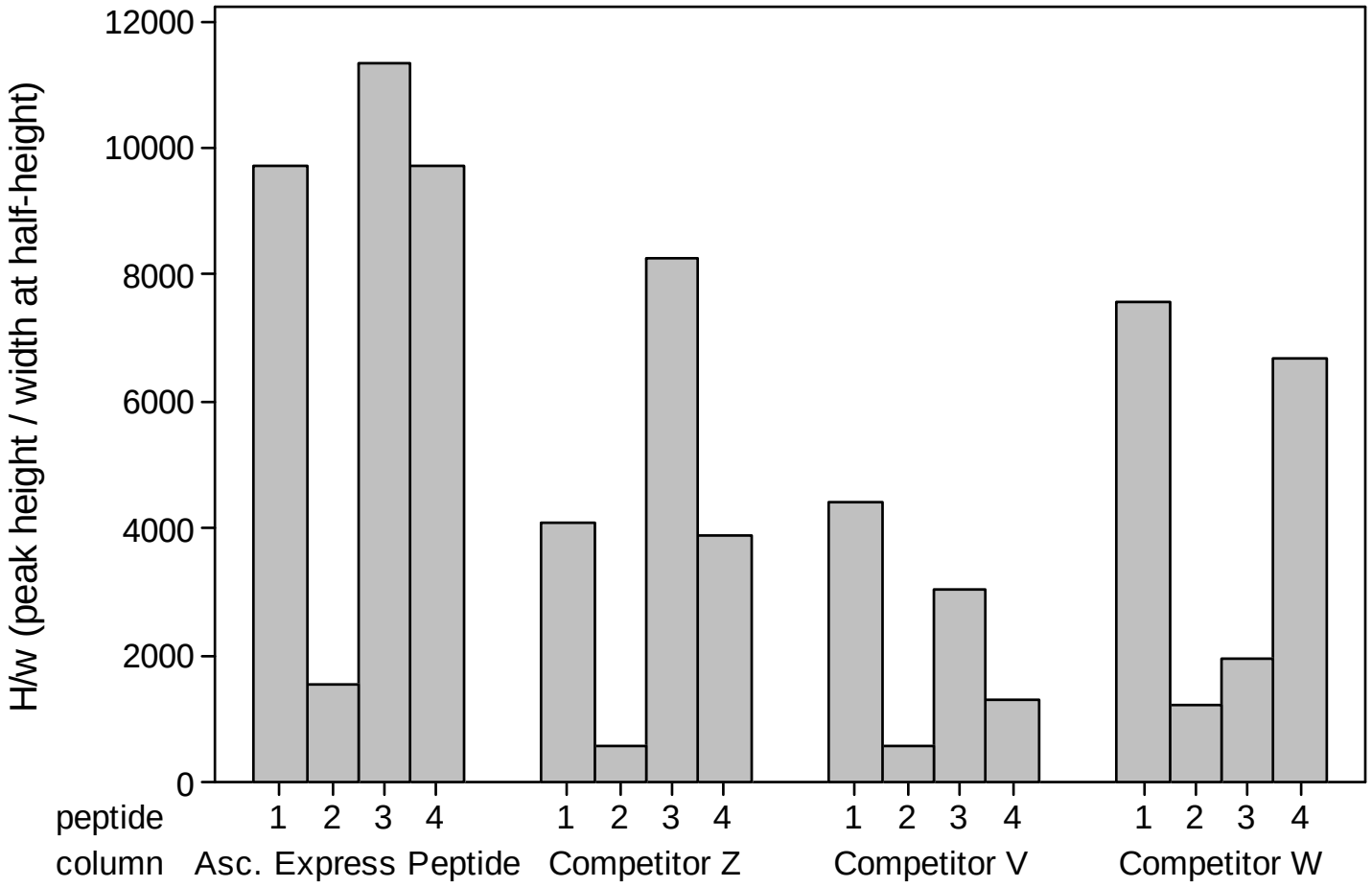
* $h/w_{½}$ peak height divided by width at half height; a measure of peak sharpness

* $TF_{0.1}$ tailing factor at 10% peak height

pH 3.5 Peak Widths



pH 3.5 Peak Sharpness



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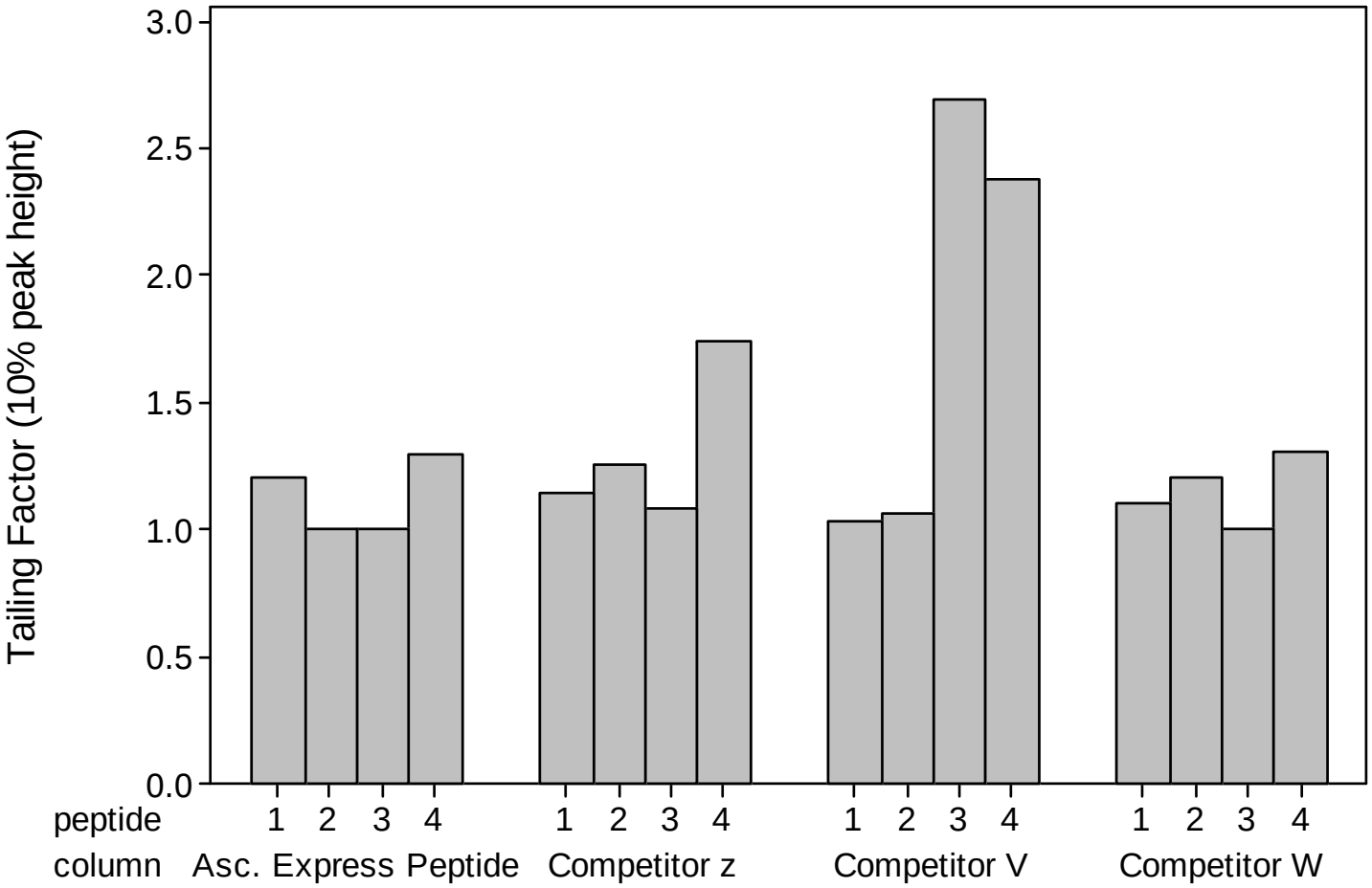
pH 3.5 Peak Symmetry



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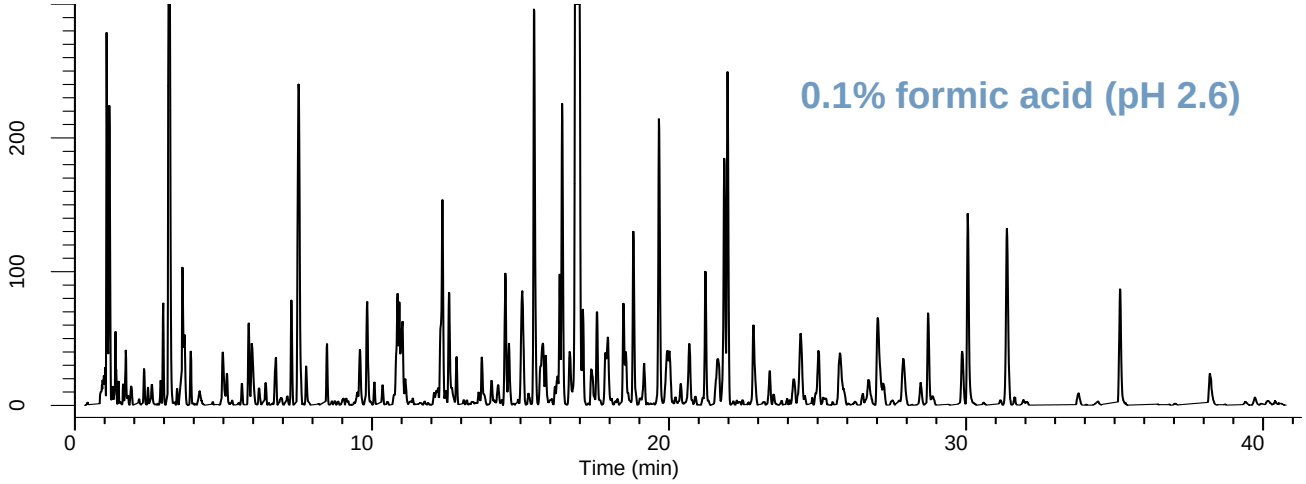
Results from Formic Acid pH Adjustment with Basic Peptides

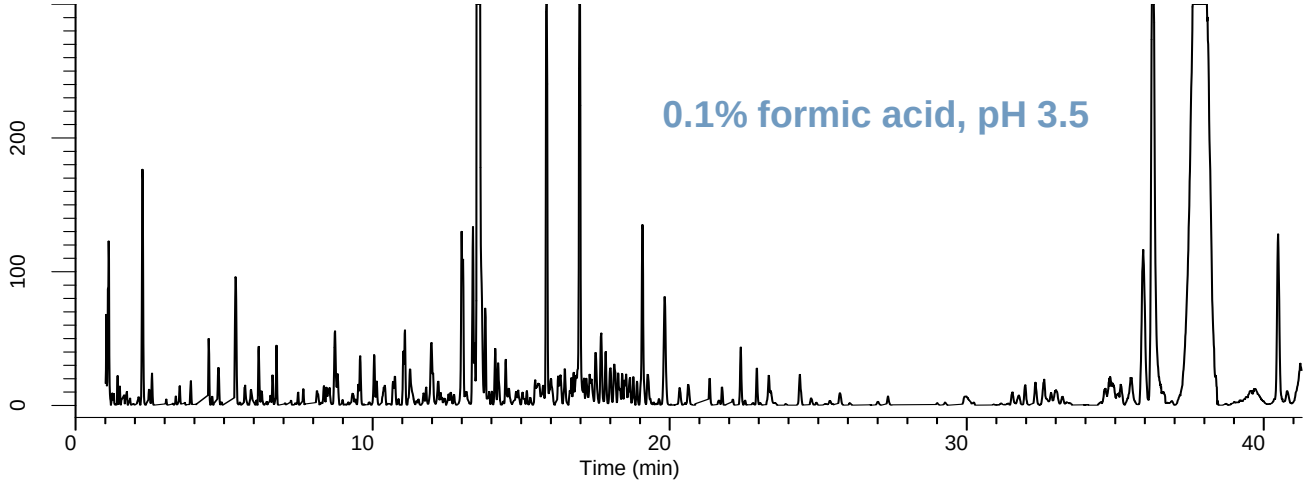
The raised pH (3.5 vs 2.6) yields improvement in peak shape of basic peptides; the greater the basicity (high pI), the more dramatic the improvement.

Compared to other high performance silica columns, Ascentis Express Peptide generally displays narrower, sharper peaks, without suffering from adverse peak tailing.

Increasing the pH further to 4.0, doesn't result in improvement in peak shape (though the formic acid is 64% ionized), but can significantly improve selectivity (data not shown).

Tryptic Digests: Formic acid pH 2.6 vs 3.5





Gradient

CV	%B
0	6
2	6
46	65

Chromatographic Conditions for Tryptic Digests

column:	Ascentis Express Peptide ES-C18, 2.1 x 150 mm
mobile phase A:	0.1 % (*v/v*) additive
mobile phase B:	25:75, (0.4 % additive) : acetonitrile
gradient:	initial = 6% B, hold @ 6%B 2 CV, slope = 1% MeCN / CV
flow rate:	0.3 mL/min
temp.:	35 °C
det.:	ESI(+) TOF
injection:	2 µL
sample:	2 µmol/L
additive:	formic acid, pH 2.6 @ 0.1% (pH unadjusted) *OR*
	formic acid, pH 3.5 (pH adjusted with ammonium hydroxide)

Results from Formic Acid pH Adjustment with Tryptic Digests

The raised pH (3.5 vs 2.6) does not yield dramatic improvements in peak shape as observed with particularly basic peptides.

The higher pH does affect selectivity, sensitivity, and likely ionization efficiency.

Conclusions

Improved peak shape of basic peptides with formic acid at pH 3.5 (vs formic acid with pH unadjusted – pH 2.6) fits with McCalley's hypothesis of formate anion functioning to mitigate ionic repulsion via ion pairing with basic moieties of the peptides.

Ion-pairing of formate anion with peptide basic moieties may also mitigate ion-exchange interaction with silanols.

The pH 3.5 mobile phase is suggested for pharmaceutical peptides.

While dramatic improvements with pH 3.5 mobile phase may not be apparent with tryptic digests, it will nevertheless have application with other digests (for example, Glu-C or cyanogen bromide) that potentially generate more basic peptides.

- *pH 3.5 mobile phase with tryptic digests may still prove useful when dealing with incomplete digestions*

Further studies will attempt to differentiate the impact of higher pH on the peptide chromatography vs MS source effects.

Ascentis Express Peptide ES-C18 is a high-performance platform for peptide separations.

References

1. Apffel, A., et. al. 1995. *J Chrom A* 712:177
2. Buckenmaier, S.M.C., D.V. McCalley, M.R. Euerby. 2002. *Anal Chem* 74:4672
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