

Low or No TFA Reversed-Phase Polypeptide Separations on Discovery BIO Wide Pore Media

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 **SUPELCO**

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ETM

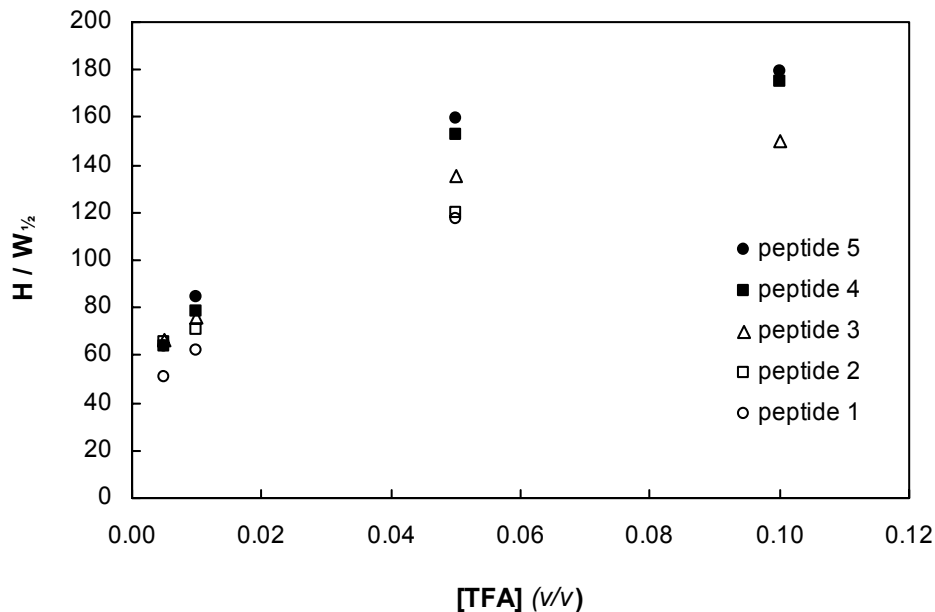


SIGMA-ALDRICH

Effect of TFA on Peak Sharpness

The interest in documenting the effect of TFA on column performance is prompted by the general knowledge that TFA has a detrimental effect on MS sensitivity by virtue of ionization suppression.

Peak Sharpness declines as TFA concentration declines.



Mobile Phase A: water / x %TFA

Mobile Phase B: 50:50, (water / x %TFA) :
(MeCN / x %TFA)

Column: Discovery BIO C18, 2.1 x 150mm, 3 μ m

Flow: 0.208mL/min

Temp: 35° C

Detection: 215nm

Injection: 0.2 μ L (~0.2 μ g ea peptide)

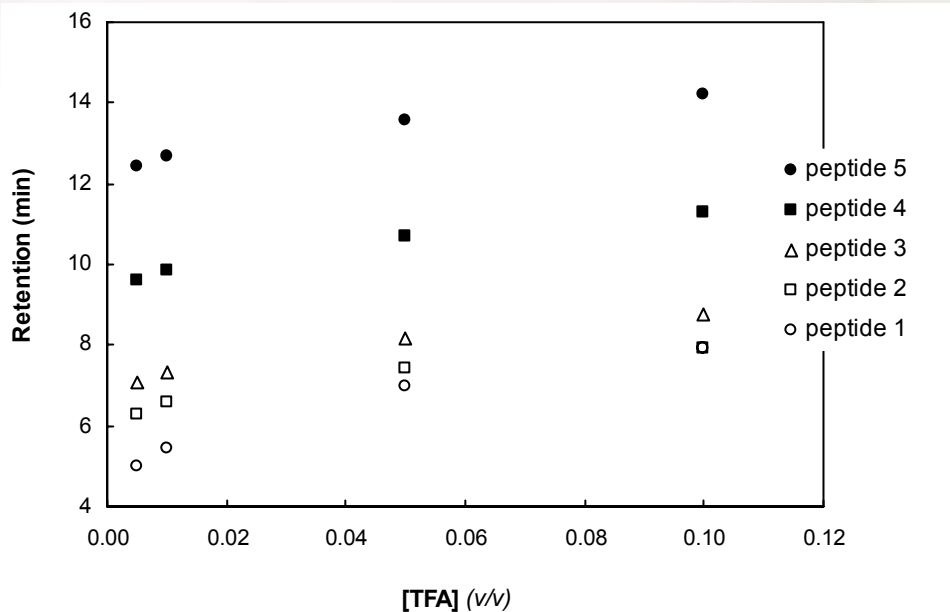
Gradient: 20 to 60%B in 20 min

Sample:

Peptide 1	RGAGGLGLGK-amide
Peptide 2	ac-RGGGGLGLGK-amide
Peptide 3	ac-RGAGGLGLGK-amide
Peptide 4	ac-RGVGGLGLGK-amide
Peptide 5	ac-RGVVGLGLGK-amide

Effect of TFA on Retention

As the concentration of TFA is increased (within the range investigated), peptide retention increases. This is purportedly because as the TFA concentration increases, ion pairing improves, and thus increased retention takes place by virtue of the additional hydrophobicity of the fluorine atoms.



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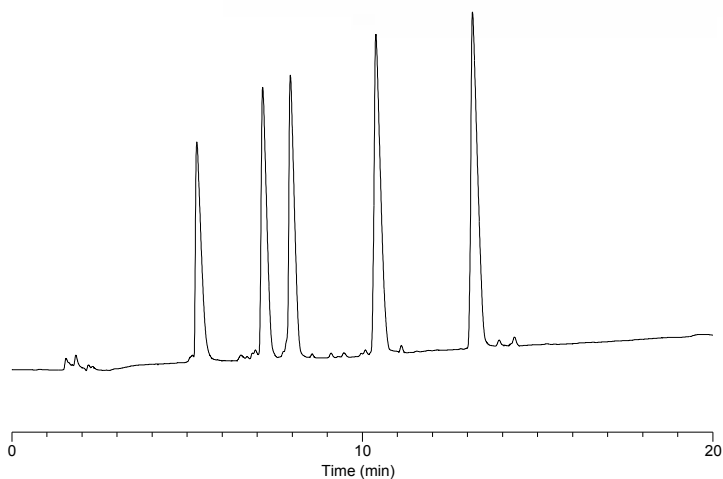
Peptide 1	RGAGGLGLGK-amide
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Peptide 5	ac-RGVVGLGLGK-amide

Formic Acid Can Be a Reasonable Substitute For TFA

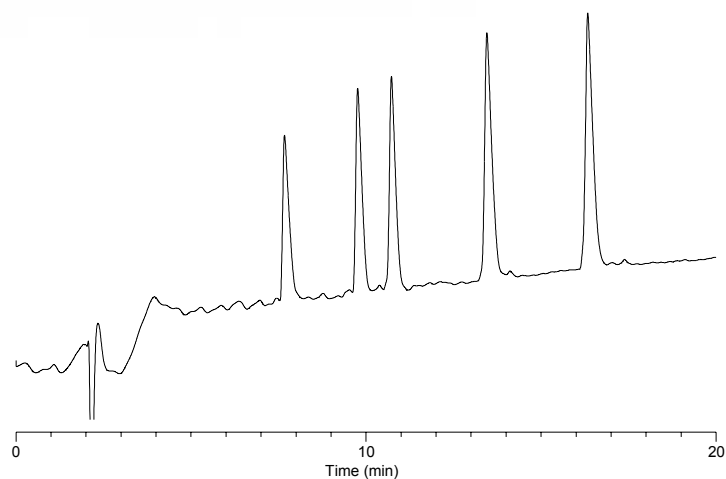
Since the presence of TFA may be detrimental to MS detection, an alternate volatile mobile phase additive is desired, which will minimally, control mobile phase pH. Formic acid fulfills this requirement: at an equivalent concentration, the mobile phase pH is not as low (2.6 vs 2.0), but still low enough to keep peptide side-chain carboxylates protonated. However, its ion-pairing properties are comparatively limited at this pH. Nevertheless, it does still permit useful chromatograms to be obtained.

Demonstrated below are similar elution profiles for a 5 peptide sample when chromatographed under a low TFA concentration vs formic acid.

0.005% TFA



25mM HCO₂H



Basic Peptide Probes

Worst-case scenarios of chromatographing peptides under low or no TFA conditions are realized with basic analytes. Basic peptides will interact strongly with residual silanols. Therefore a series of peptides (shown below) was selected as a sample to probe resolution and silanol activity. Lower silanol activity is expected to result in improved resolution and peak shape. Each peptide is successively more basic as well as more hydrophobic. All peptides are acetylated on the *N*-terminus and amidated on the *C*-terminus so as to derive all ionic interactions from lysine ϵ -amino groups.

Peptide 1 ac-GGGLGGAGGLK-amide

Peptide 2 ac-KYGLGGAGGLK-amide

Peptide 3 ac-GGALKALKGLK-amide

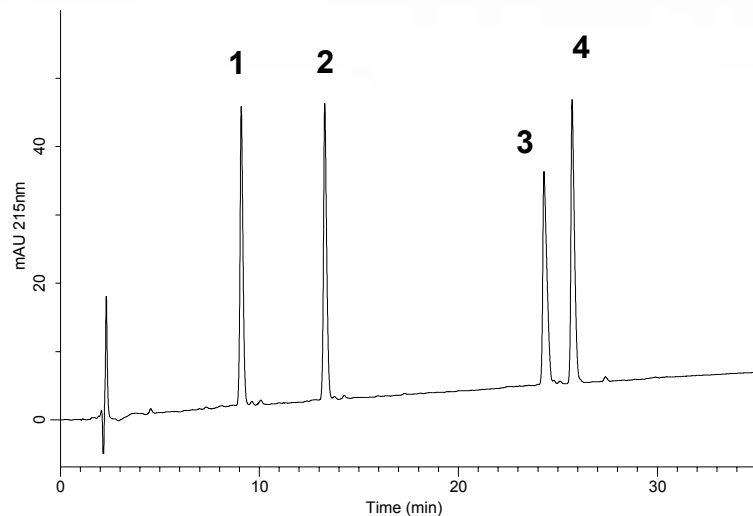
Peptide 4 ac-KYALKALKGLK-amide

Various competitor columns are subsequently compared to the new Discovery BIO Wide Pore media.

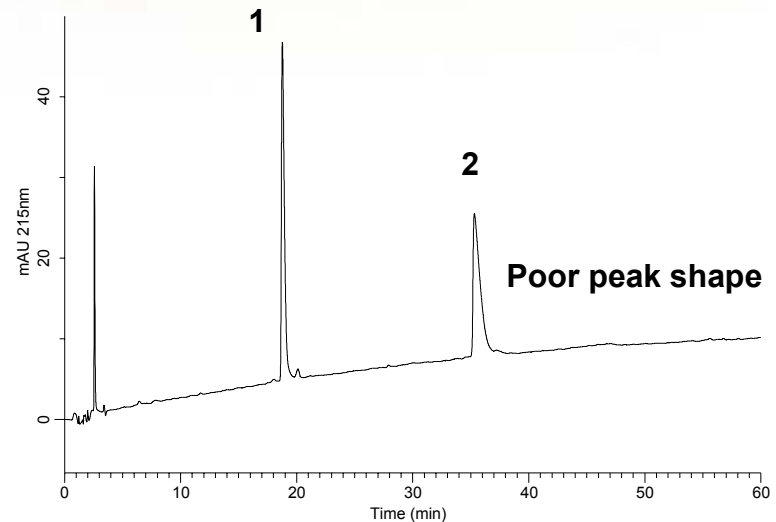
Basic Peptides: 0.05% TFA

Chromatographed under conditions of 0.05% (v/v) TFA, the previous basic peptide sample displays an elution pattern most similar for all competitors: all display baseline resolution and good peak shape. As a control, a column of known high silanol activity was compared. As shown below, under identical conditions, retention increased greatly in this case (due to silanol interactions) and the two most basic peptides didn't even elute.

Typical chromatogram of a modern, quality wide-pore column

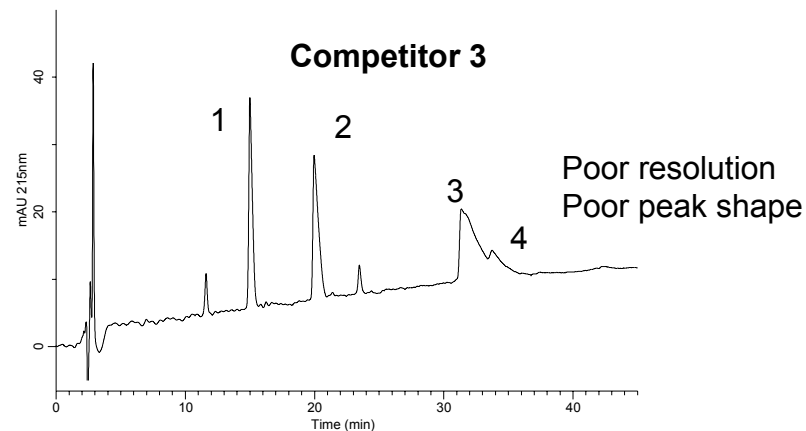
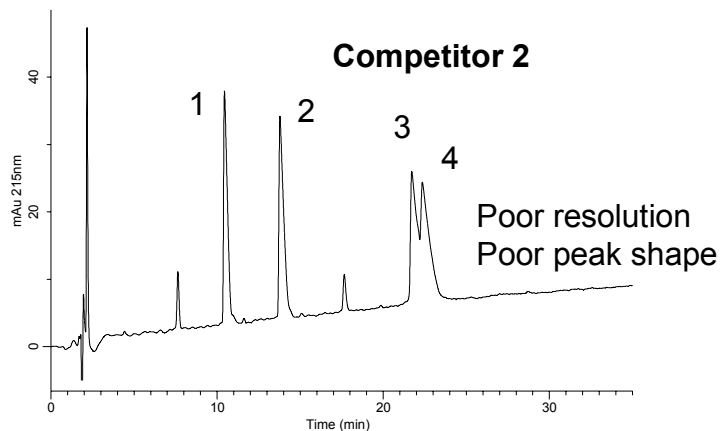
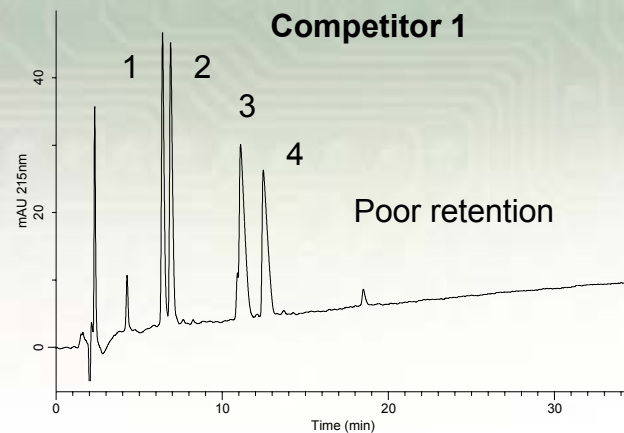
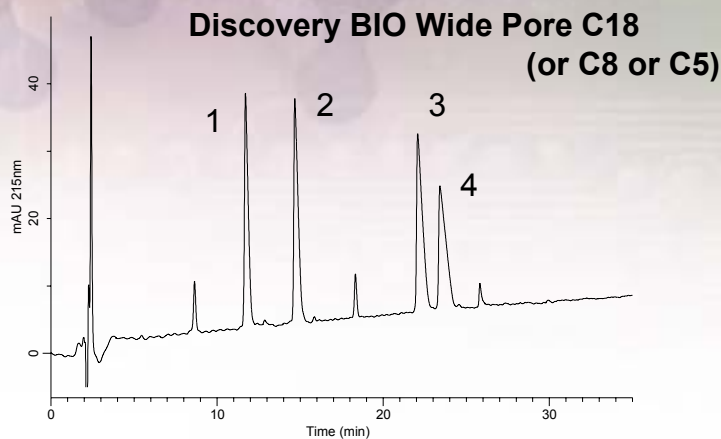


High-silanol control column



Basic Peptides: 25mM (~0.1%) Formic Acid

When TFA is substituted with formic acid, under these conditions of very limited ion pairing (to mask silanol interactions), differences between competitors (all their best wide-pore C18) are revealed:



Comparative Runs with Proteolytic Digest

The next evaluation consisted of taking a real sample and doing comparative runs under varying conditions of ion-pair reagents and with different competitor columns. All competitor columns are 300Å, 5µm, C18.

For this evaluation, the sample was a carboxymethylated hemoglobin tryptic digest.

Run conditions were constant except for the additive that was included in the mobile phases and at what concentration.

Method:

Mobile Phase A: water / TFA or HCO₂H

Mobile Phase B: 50:50, (water / TFA or HCO₂H) : (MeCN / TFA or HCO₂H)

Column: 2.1 (or 2.0) x 150mm, 5µm

Flow: 0.208 (or 0.189) mL/min

Temp: 35° C

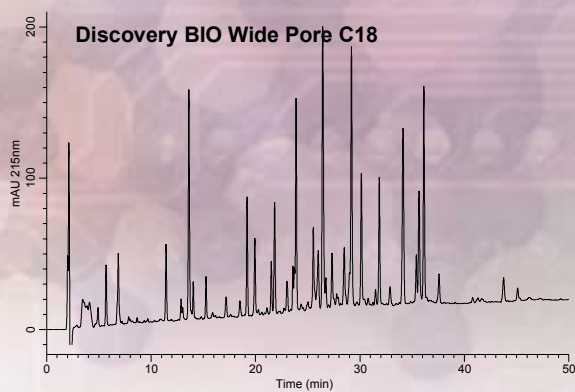
Detection: 215nm

Injection: 10µL (<50µg)

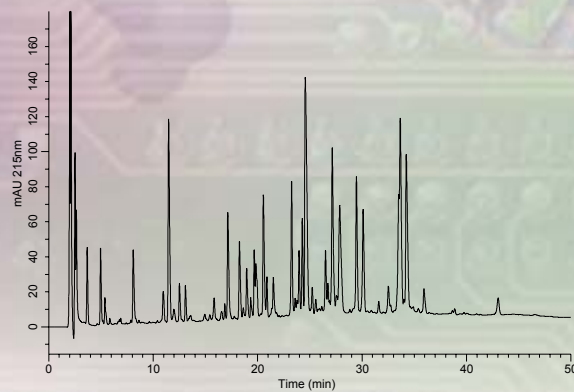
Gradient: 0 to 100%B in 50 min

Comparative Runs Cont.

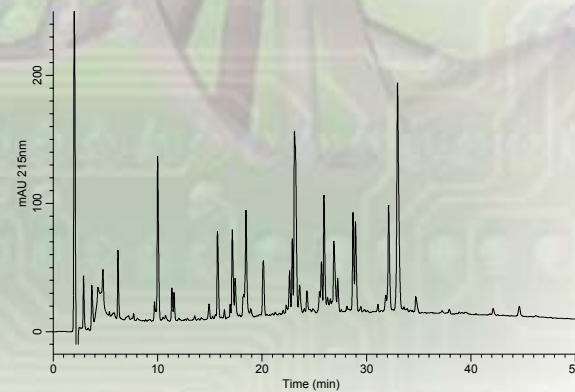
0.1% TFA



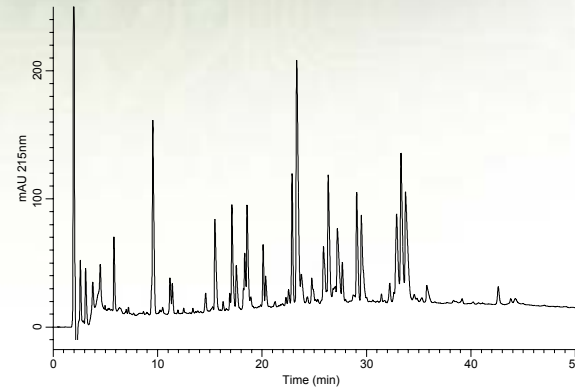
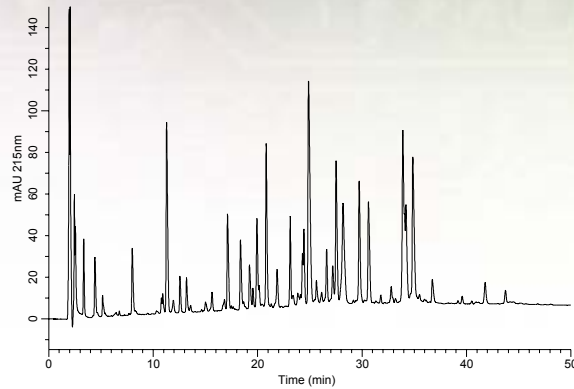
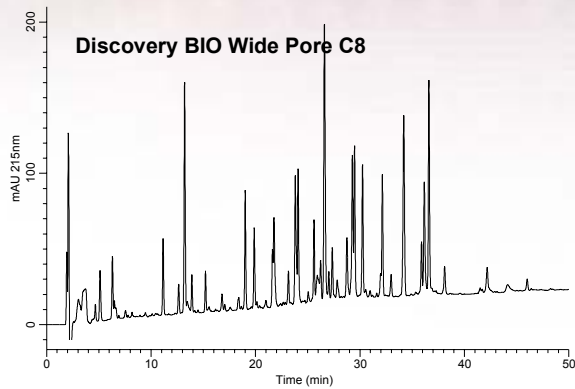
0.01% TFA



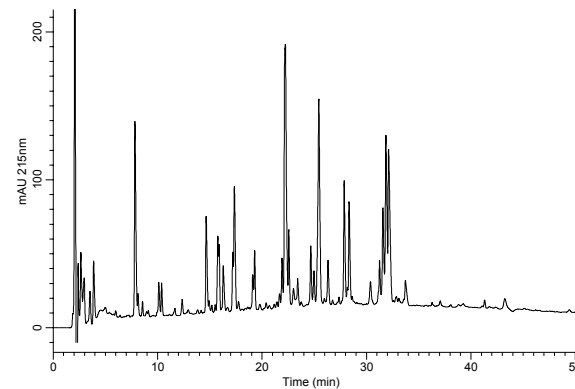
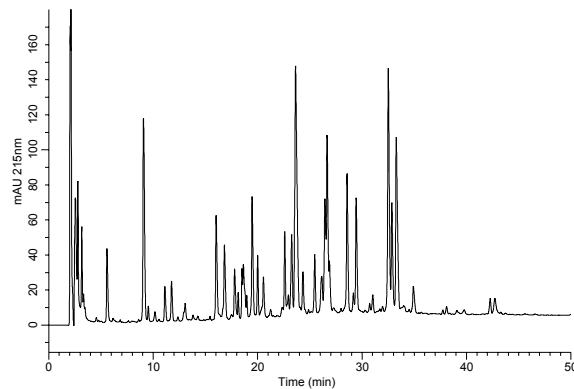
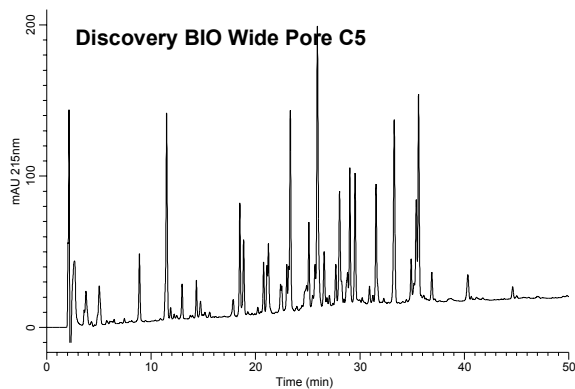
25mM HCO₂H



Discovery BIO Wide Pore C8

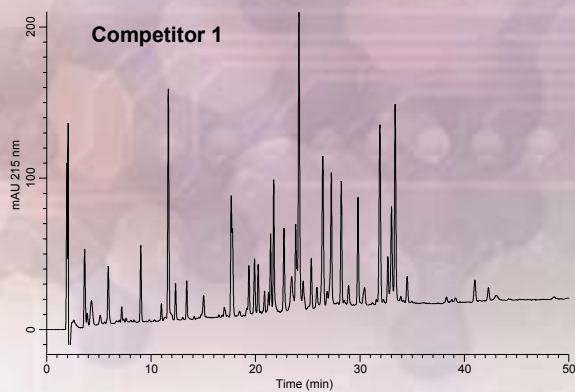


Discovery BIO Wide Pore C5

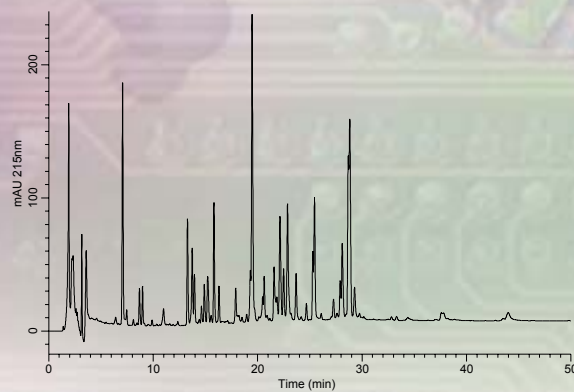


Comparative Runs Cont.

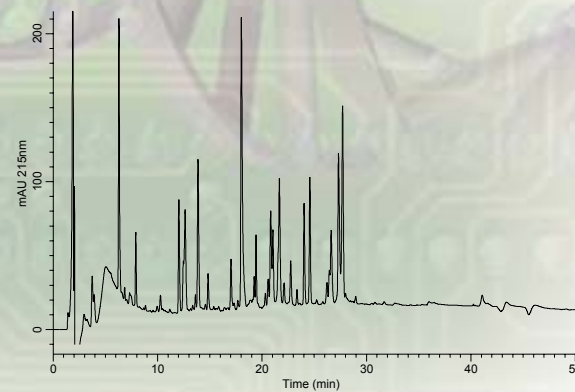
0.1% TFA



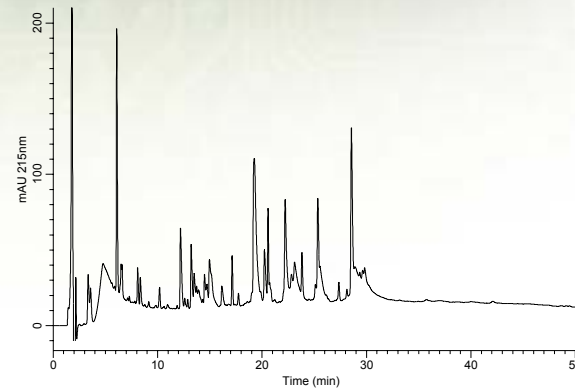
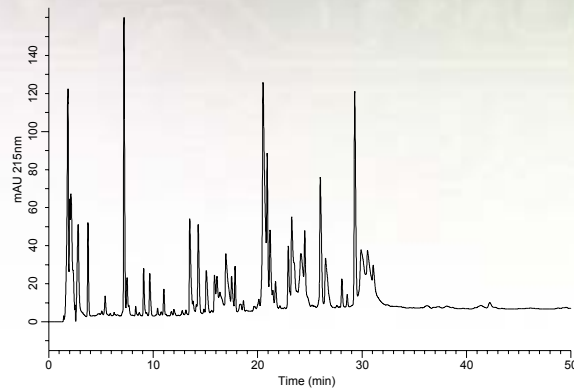
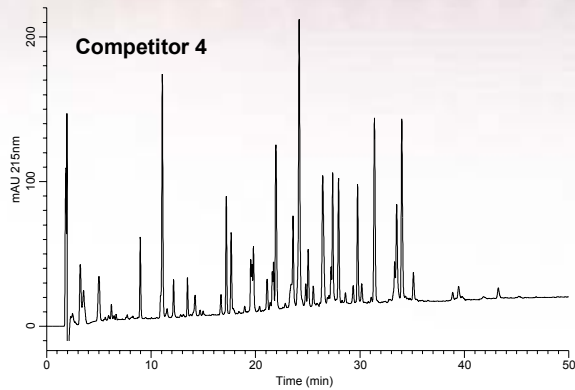
0.01% TFA



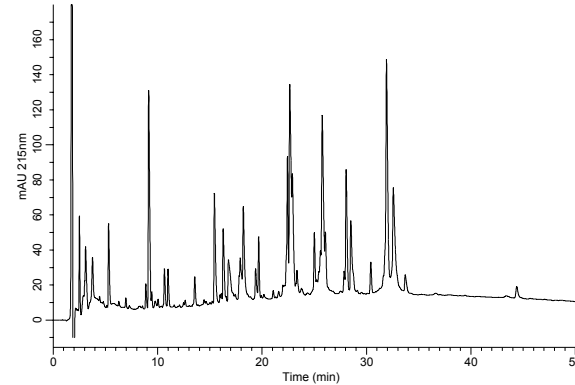
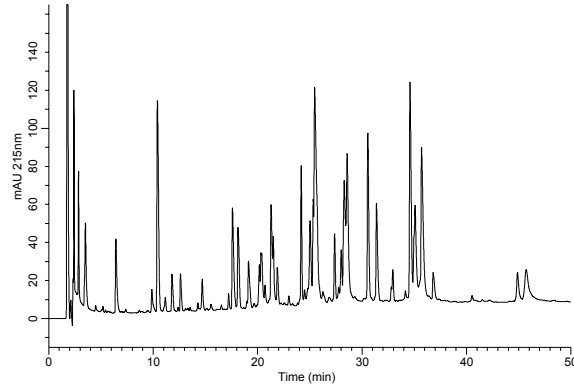
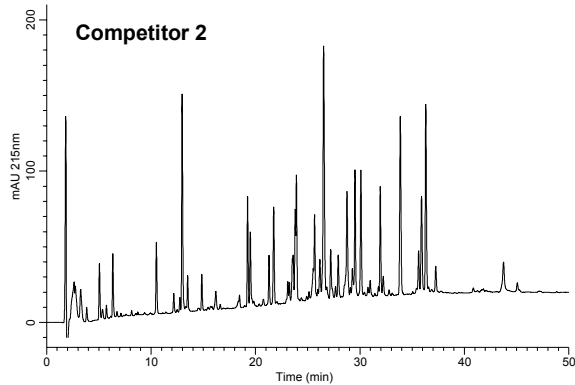
25mM HCO2H



Competitor 4

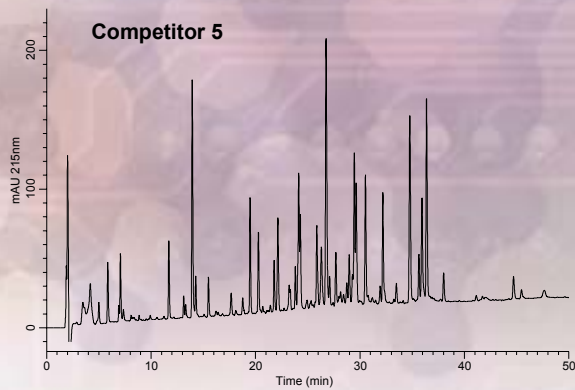


Competitor 2

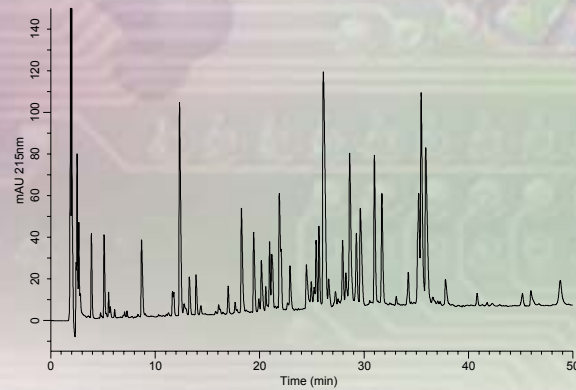


Comparative Runs Cont.

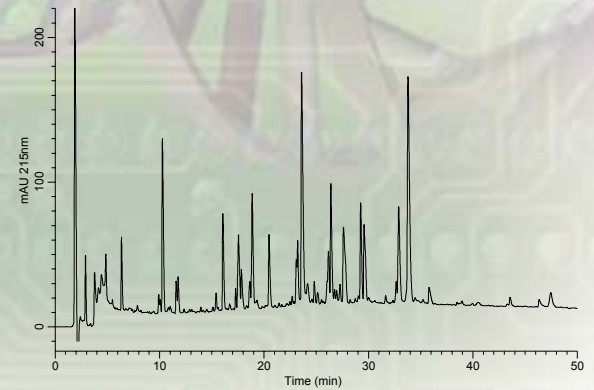
0.1% TFA



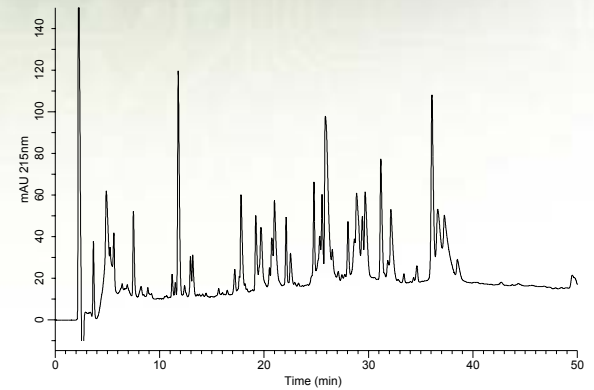
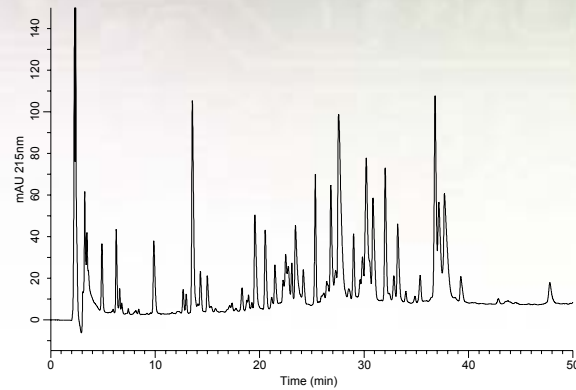
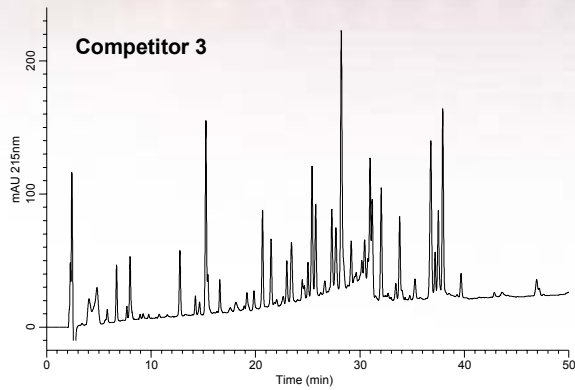
0.01% TFA



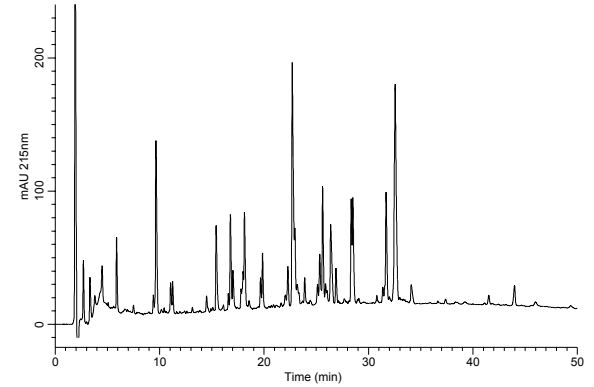
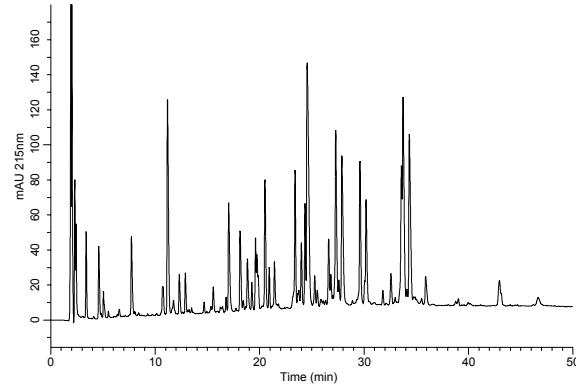
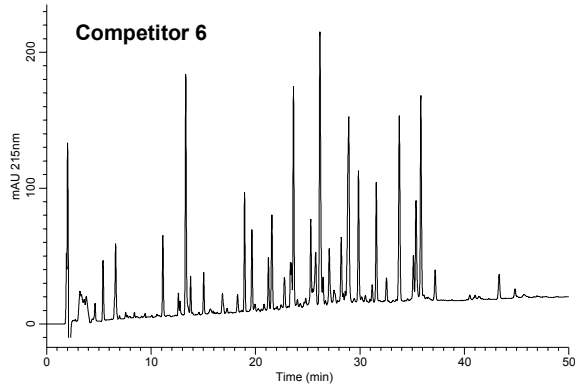
25mM HCO₂H



Competitor 3



Competitor 6



Conclusions

- Within the concentration range investigated, TFA enhances peak shape, resolution, and retention of peptides when chromatographed by reversed-phase.
- Formic acid can provide a reasonable substitute for TFA, yet permit sensitive MS detection.
- Column inertness (lack of silanol activity) is a key criteria for optimal chromatographic performance under low or no TFA conditions.
- Basic peptide probes reveal Discovery BIO Wide Pore to be a superior silica-based chromatographic matrix for operation under preferred conditions for MS-detection.
- Evaluation with a typical complex peptide sample reveals Discovery BIO Wide Pore columns to compare most favorably with the competition.
- The Discovery BIO Wide Pore reversed-phase family provides an ideal product for polypeptide chromatography under low or no TFA conditions.