# The Effect of Mobile Phase Additives on the Analysis of Peptides and Proteins

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## Abstract

New polymeric RP-HPLC columns, Amberchrom Profile, were used for the separation of peptide and protein mixtures. These columns were packed with a new 10µm, monosized, styrene/divinylbenzene polymer. The separations were conducted under a variety of mobile phase conditions where the type and concentration of mobile phase additives were varied. The results were compared to those obtained on conventional, pre-packed, silica RP-HPLC columns. Amberchrom Profile columns demonstrated superior separations over a wider range of mobile phase conditions. This allowed simplified mobile phases and more flexibility in separation conditions.

## Introduction - Effect of TFA Concentration

Analysis of peptides by reversed phase chromatography has historically been done using various mobile phase additives to improve the separations. This is due to the ionizable nature of silica packing materials which can cause tailing of basic compounds such as certain peptides. Trifluoroacetic acid (TFA) is one of the most common ion pairing reagents for reversed phase chromatography because of UV transparency, volatility, ability to improve peptide solubility, ability to suppress residual silanol ionic interaction, and ability to interact with basic amino acids.[1-4] We investigated the effect of TFA concentration on the separation of four basic, bioactive peptides on either polymeric or silica reversed phase packings. A new polymeric RP-HPLC column, Amberchrom Profile, was compared to two conventional silica RP-HPLC columns, Competitor K C4 and Competitor B C5.

## Experimental Methods – Effect of TFA Concentration

The effect of TFA concentration was studied using a mixture of Angiotensin peptides, Angiotensin I, Angiotensin III, Val4-Angiotensin III, and Ile7-Angiotensin III (Sigma, St. Louis, MO). The peptides were separated using either 0.1%, 0.5%, or 0.01% TFA in a Milli-Q® water/acetonitrile gradient on three different 10 micron reversed phase HPLC packing materials Amberchrom™ Profile™ (PN HP1010250), Competitor K C4, and Competitor B C5. All separations were done using an Agilent 1100 HPLC system, and the conditions are listed below.

**Conditions**

| | |
|---|---|
| Column | 1 cm ID x 25 cmL |
| Flow rate | 2 mL/minute |
| Sample | 100 µl of 1 mg/mL each<br>Angiotensin I (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu)<br>Angiotensin III (Arg-Val-Tyr-Ile-His-Pro-Phe)<br>Val[4]-Angiotensin III (Arg-Val-Tyr-Val-His-Pro-Phe)<br>Ile[7]-Angiotensin III (Arg-Val-tyr-Ile-His-Pro-Ile) |
| Mobile Phase | A: 0.1%, 0.05% or 0.01% TFA in Milli-Q® Water<br>B: 0.1%, 0.05% or 0.01% TFA in acetonitrile |
| Gradient | Hold for 10 minutes at 10% B<br>10% to 70% B in 60 minutes |
| Detection: | UV @ 214 nm |

## Peptide Separation with 0.1% TFA Background

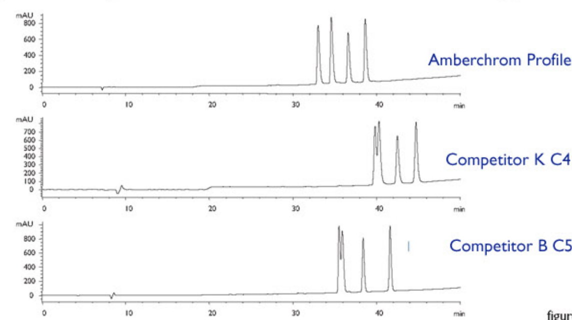


figure 1

## Peptide Separation with 0.05% TFA Background

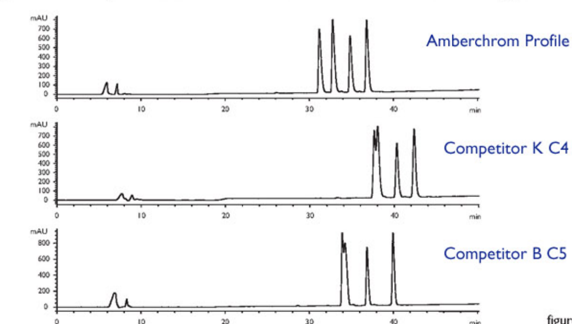


figure 2

## Peptide Separation with 0.01% TFA Background

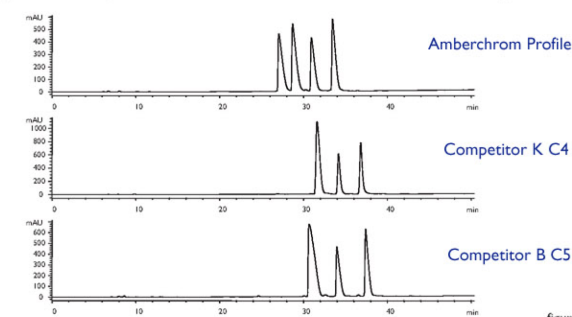


figure 3

## Results and Discussion – Effect of TFA Concentration

In general, the selectivity of the polymeric column provides a better separation of the two Angiotensin III variants, Ile$^{7}$-Angiotensin III and Val$^{4}$-Angiotensin III. As can be seen in Figures 1 and 2, the retention of the four Angiotensin peptides decreases on all three columns as the TFA concentration decreases from 0.1% to 0.05%. In addition, the Ile$^{7}$-Angiotensin III and Val$^{4}$-Angiotensin III variants begin to co-elute on the C4 and C5 silica columns as the TFA concentration decreases. As shown in Figure 3, when the TFA concentration is further decreased to 0.01%, Ile$^{7}$-Angiotensin III and Val$^{4}$-Angiotensin III totally co-elute on the two silica columns. Although there is a significant shift in retention time and increase in peak asymmetry, all four angiotensins are fully resolved on the polymeric column using the 0.01% TFA concentration. The decreased TFA concentration led to an overall decrease in peptide retention on all three columns. This is most likely due to an increase in the overall polarity of the peptides as the TFA concentration decreased. This effect on peptide retention is fairly well known.[1,3] However, this does not explain the loss of resolution of the two Angiotensin III variants on the C4 and C5 silica columns. The two Angiotensin III variants differ by two amino acids, a valine versus an isoleucine at position four and a phenylalanine versus an isoleucine at position seven. All of these are nonpolar amino acids, and therefore should not be affected by changes in TFA concentration. Because this effect is observed on the silica columns and not on the polymeric column, one possible explanation for the loss in resolution could be the loss of silanol ionization suppression. Therefore, polymeric reversed phase packings may provide a wider operating range for mobile phase conditions.

## Experimental Methods – Effect of Acid Type

The effect of acid type and concentration was studied using a mixture of proteins, cytochrome c, ribonuclease a, lysozyme, and bovine serum albumin (Sigma, St. Louis, MO). The proteins were separated using either 0.1% TFA, 0.5% TFA, 0.01% TFA, 0.1% acetic acid, or 0.1% sulfuric acid in a Milli-Q® water/acetonitrile gradient on two different 10 micron reversed phase HPLC packing materials, Competitor KC4 and Amberchrom Profile (PN HP1010250). All separations were done using an Agilent 1100 HPLC system, and the conditions are listed below.

**Conditions:**

| | |
|---|---|
| Column: | 1cm ID x 25cm L |
| Flow Rate: | 1 mL/minute |
| Sample: | 100µl of 1mg/mL each<br>Cytochrome c<br>Ribonuclease a<br>Lysozyme<br>Bovine serum albumin |
| Mobile Phase: | A: 0.1% TFA, 0.05% TFA, or 0.1% sulfuric acid in Milli-Q Water<br>B: 0.1% TFA, 0.05% TFA, or 0.1% sulfuric acid in acetonitrile |
| Gradient: | Hold for 10 minutes at 20% B<br>20% to 60% B in 40 minutes |
| Detection: | UV @ 280nm |

## Protein Separations

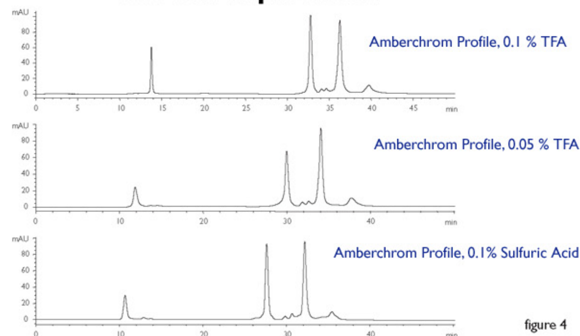


figure 4

## Protein Separations

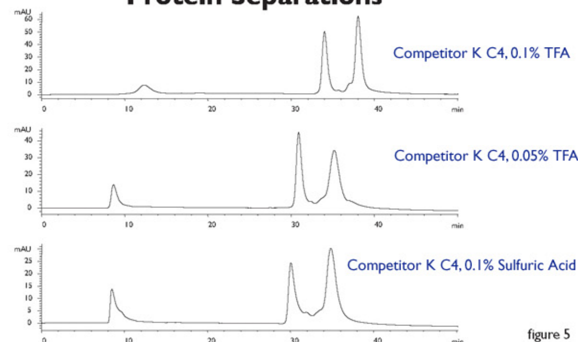


figure 5

## Results and Discussion – Effect of Acid Type

As can be seen when comparing Figures 4 and 5, the overall separation of the four proteins is better using the polymeric column under all of the conditions shown. This is probably due to the basic nature of the proteins used in the separation. Cytochrome c (10.7), Ribonuclease a (9.5), and lysozyme (11.0) all have pIs greater than 7, and therefore their peak shapes can be affected by residual silanols. BSA has a pI of 4.8 but is a much larger protein (67 kDa), and it's retention and separation may be more affected by the pore size and distribution of the chromatographic packings.

The retention of the four proteins decreases on both columns as the TFA concentration decreases from 0.1% to 0.05%. There is a further decrease in retention of the four proteins on the polymeric column when 0.1% sulfuric acid is used as the additive. In addition, the peaks broaden on the silica column as the TFA concentration is decreased or the acid type is changed to sulfuric acid. Little or no decrease in retention of the four proteins is observed on the silica column when comparing 0.05% TFA and 0.1% sulfuric acid chromatograms.

Neither column was able to separate the protein mixture using either 0.01% TFA nor 0.1% acetic acid as the mobile phase modifier (that data is not shown). Because acetic acid is a weak acid, and the concentration of TFA was so low, the pH of these two mobile phases was insufficiently low enough to effect a reasonable separation.

## Conclusions

Amberchrom Profile polymeric RP-HPLC columns were able to better separate peptides and proteins under a wider range of conditions than silica-based RP-HPLC columns. Additionally, these polymeric packings are more robust and are able to work under a full pH range of 1 to 14. Therefore, use of polymeric reversed phase packings may allow simplified mobile phases and more flexibility in separation conditions.

## References

Chen, Y., Mehok, A.R., Mant, C.T., Hodges, R.S., 2004. Optimum concentration of trifluoroacetic acid for reversed phase liquid chromatography of peptides revisited. J. Chromatogr., 1043, 9-18.

Chen, Y., Mant, C.T., Hodges, R.S., 2004. Selectivity differences in the separation of amphipathic a-helical peptides during reversed phase liquid chromatography at pHs 2.0 and 7.0 – Effects of different packings, mobile phase conditions and temperature. J. Chromatogr., 1043, 99-111.

Corradini, D., Kalghatgi, K., Horvath, C., 1996. Effect of mobile phase additives on peptide retention in reversed phase chromatography with pellicular and totally porous sorbents. J. Chromatogr., 728, 225-233.

Gritti, F., Guiochon, G., 2004. Role of buffer in retention and adsorption mechanism of ionic species in reversed-phase liquid chromatography. J. Chromatogr., 1038, 53-66.

Gritti, F., Guichon, G., 2004. Effect of the ionic strength of salts on retention and overloading behavior of ionizable compounds in reversed-phase liquid chromatography. J. Chromatogr. 1033, 43-55.

McCalley, D.V., 2004. Effect of buffer on peak shape of peptides in reversed phase high performance liquid chromatography. J. Chromatogr., 1038, 77-84.

McCrossen, S.D., Simpson, C.F., 1995. Effect of some mobile phase additives on the retention characteristics of different solute types on reversed phase media II. J. Chromatogr., 697, 53-58.