# What is 0.1% TFA?

## Summary

- Changing the modifier concentration provides a way to vary peptide selectivity.
- Keeping it the same is important for reproducibility.
- Proper communication of concentrations is essential if experimental results are to be duplicated reliably.

## Introduction

For complex peptide separations, the key to success can be to vary selectivity. Peptides that coelute on one type of column may be resolved on another. Even on the same column, changing the mobile phase composition or temperature can change selectivity enough to resolve peptides that overlap.

Modifiers are substances added to the mobile phase, usually in relatively low concentration, that interact with both the stationary phase and sample constituents to alter retention. Interaction with sample components will vary, so selectivity can be adjusted by changing the modifier concentration.

## Experimental Results

Trifluoroacetic acid (TFA) is the modifier most frequently used for peptide separations in reversed-phase HPLC. The TFA concentration usually specified is 0.1%. Figure 1 shows the effects of changing TFA concentration from 0.1% to 0.3% on chromatography of tryptic peptides on Vydac's two different C18 TP columns. Peak displacement and changes in resolution are clearly seen in all regions. Coupled with two column types, two TFA concentrations produce four different selectivities for resolving peptides. (Note: The rising baseline absorbance is due to the extinction coefficient of TFA, which increases with increasing concentration of acetonitrile in ACN:water mixtures.)

A less complex separation shown in Figure 2 provides perhaps a clearer example of the effect of changing TFA concentration. In this example, a sample containing five standard peptides from Sigma was chromatographed on a 218TP54 column in the presence of 0.1% and 0.3% TFA.

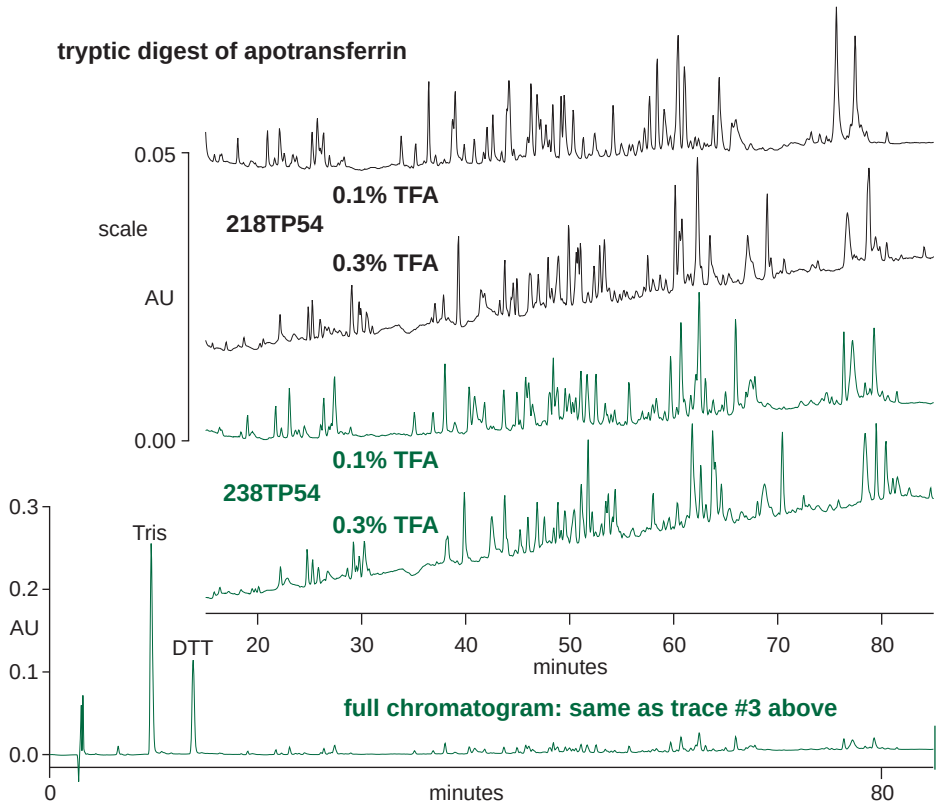


*Figure 1. Effects of reversed-phase column type and TFA concentration on complex peptide separations. The bottom trace is a complete chromatogram. The upper traces show the region from 15 to 85 minutes, in which virtually all tryptic peptides emerge, for four individual runs with traces offset from zero and the vertical scale expanded to facilitate comparison. Samples: Tryptic digests of apotransferrin. Conditions: 1.0 mL/min, absorbance at 215 nm, TFA concentration (w/v) as indicated, gradient from 0% to 50% ACN over 100 minutes. Both columns were 4.6mm ID x 250mm L.*

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At 0.1%, leu-enkephalin and angiotensin II are seen to coelute at about 24 minutes. In 0.3% TFA, angiotensin II is more strongly retained and completely resolved from leu-enkephalin due to TFA ion pairing to the arginine (R). Interestingly, this change in TFA concentration does not significantly alter retention of any of the other four peptides.

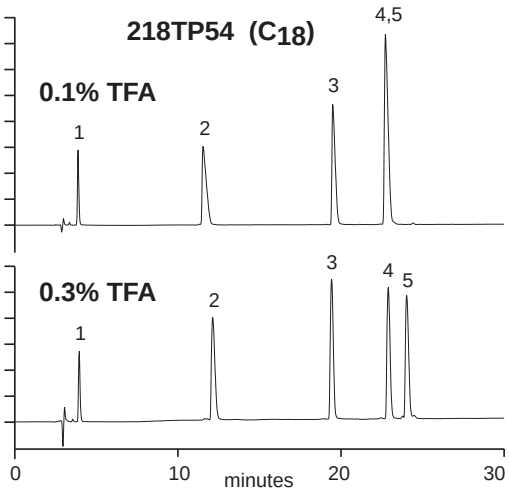


*Figure 2. Effect of TFA concentration on separation of five standard peptides. Ticks on vertical axis mark 0.1 AU intervals. Conditions: 1.0 mL/min, absorbance at 220 nm, gradient 10% to 50% ACN with indicated TFA concentration (w/v) over 40 minutes. Column: C18, 4.6mm ID x 250mm L. Peaks: 1>GY, 2>VYV, 3>met-enkephalin (YGGFM), 4>leu-enkephalin (YGGFL), and 5>angiotensin II (DRVYIHPF)*

## TFA Retention on Reversed Phase

The baselines in Figure 3 demonstrate that the TFA modifier is actually retained by the stationary phase. The bottom trace shows a baseline run with no column in place. A smooth increase in the absorbance of TFA corresponding to the gradient of acetonitrile concentration is seen. Returning the acetonitrile concentration to 0% at the end of the gradient returns the absorbance of TFA smoothly to its initial value.

Performing the same baseline analysis with a reversed-phase column, either C4 or C18, reveals a non-linear increase in the absorbance at about six minutes due to release of TFA from the stationary phase as the equilibrium of its interaction with the alkane chains changes between pure water and 3-5% ACN. (Timing of the absorbance increase is delayed somewhat due to the volume in the column and tubing.) Note that the increase is larger for C18 than for C4, indicating that more TFA is associating with the longer alkane chains.

At the end of the run when acetonitrile is returned to zero, the baseline dips below its initial value, recording a deficit in the column effluent as TFA from the mobile phase loads back onto the column. Significantly, neither the absorbance increase at six minutes nor the negative dip are seen in tubing-only runs.



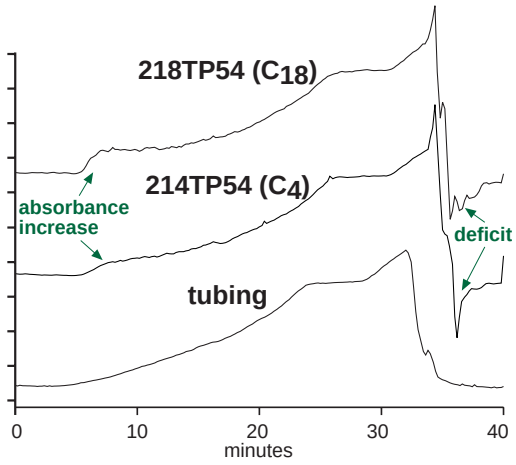


*Figure 3. Effect of reversed-phase columns on baseline absorbance with 0.1% (v/v) TFA. Ticks on vertical axis are 0.01 AU intervals. Conditions: 1 mL/min, absorbance at 220 nm, All buffers 0.1% TFA (v/v). Gradient from 0% to 80% ACN in 20 minutes, hold 80% five minutes, 80% to 100% in 5 minutes, return to 0% ACN in 0.1 minutes and hold 10 minutes to re-equilibrate.*

## The Importance of Communication

There is a corollary to understanding that TFA concentration can be changed to generate differences in selectivity: For highly reproducible separations from run to run, fingerprinting for example, or from lab to lab, it is essential to make TFA concentrations the same.

So 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. A positive-displacement pipette, graduated cylinder, or burette should produce an accurate measurement. But be sure to correct for density if TFA concentration was specified "w/v".

Authors who do not specify either "w/v" or "v/v" are leaving out important information.

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