

# Use of Ammonium Formate to Modify Reversed-Phase LC-MS Analyses of Peptides and Tryptic Digests

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## Objectives

- Compare acid modifiers of mobile phases for high resolution reversed-phase gradient separations of peptides.
- Evaluate the separation benefits of modifying typical formic acid mobile phases by addition of ammonium formate.
- Examine the compatibility of ammonium formate as an additive to formic acid mobile phase for LC-MS analysis of tryptic digests.
- Define the practical value of the use of ammonium formate modified mobile phase in a proteomic workflow.

## Introduction

Recent developments in HPLC instruments and column packing materials are permitting faster separations, particularly for reversed-phase analyses of peptides and protein fragments. The recent popularity of sub-2  $\mu\text{m}$  diameter particles and the new development of small diameter superficially porous particles designed for biomolecules is permitting faster separations of peptides and protein fragments.

Better materials and instruments for conducting high efficiency separations need to be combined with appropriate methods of use. It has long been appreciated that peptide separations using typical "MS friendly" formic acid acidified mobile phases yield separations that are inferior to "MS unfriendly" trifluoroacetic acid (TFA) modified mobile phases. The Halo Peptide ES-C18 column is prepared using fused-core particles that are surface modified with an extremely stable steric-protected C18 bonded phase, appropriate for the low pH conditions preferred for peptide separations. We show, consistent with many previous observations, that TFA is a most effective acid modifier for separations, and that formic acid exhibits significant band broadening when used with the Halo Peptide ES-C18 columns; this is universally observed for high performance column packing materials of use for peptide separations, and is thought to result from the poor dissociation of this weak acid, particularly in acetonitrile/water mixtures. Ammonium formate is an effective modifier, previously shown to reduce band broadening of bases, including peptides. The compatibility of this additive with LC-MS has not been fully explored for proteomic applications, and there has been no systematic analysis of the potential benefit to protein IDs for complex proteomic samples.

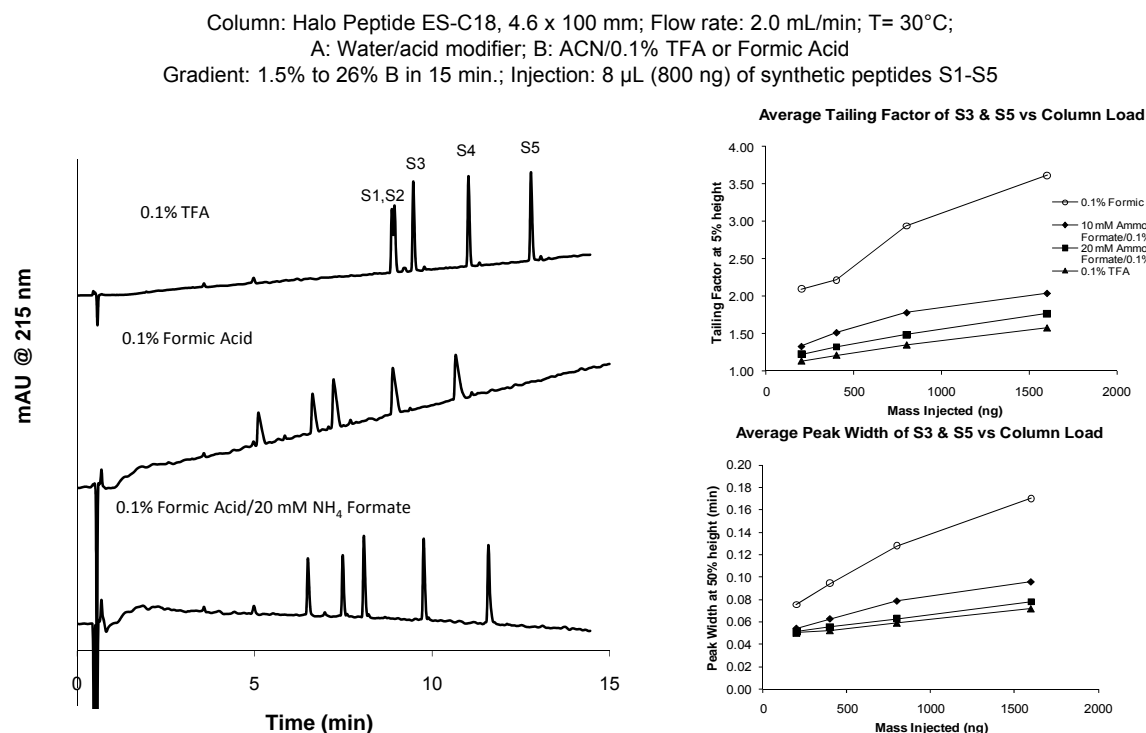
## Materials and Methods

Columns of HALO Peptide ES-C18 were produced at Advanced Materials Technology Inc. (Wilmington, DE). HPLC separations used the quaternary Agilent 1100, binary 1200 SL or capillary 1100 LC systems controlled with ChemStation software. The capillary LC was connected to the ThermoFisher LTQ ion-trap mass spectrometer via the Michrom Bioresource Advance spray source. Samples from the autoinjector were captured on the EXP Stem Trap (2.6  $\mu\text{L}$ ) cartridge packed with Halo Peptide ES-C18 (Optimize Technologies), using the LTQ automated valve. Data collection for the narrow peaks obtained in this and an associated study (See Poster MP 226.) required optimization of the DDA and Minimum Signal setting for the LTQ. Data was searched with Mascot, and ProteoIQ (Nusep, Bogart, GA) was used for data comparisons. Synthetic peptides were obtained from AnaSpec (Freemont, CA) or from ThermoFisher, in the case of the Retention Standard Mix (Mant and Hodges), the S1-S5 sequences are:

S1 RGAGGLGLGK-Am  
S2 Ac-RGGGLGLGK-Am  
S3 Ac-RGAGGLGLGK-Am  
S4 Ac-RGVGLGLGK-Am  
S5 Ac-RGVVGLGLGK-Am

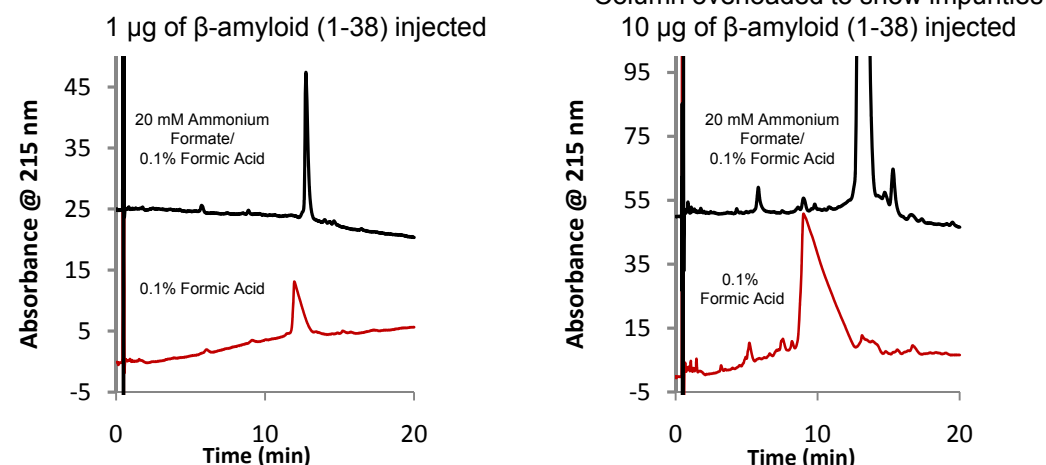
## Mobile Phase Modifiers for LC Separations

- TFA and Formic acid have disadvantages for LC/MS. A mix of Ammonium Formate/Formic Acid is an attractive mobile phase, showing narrow and symmetrical peaks, compared to Formic Acid alone.



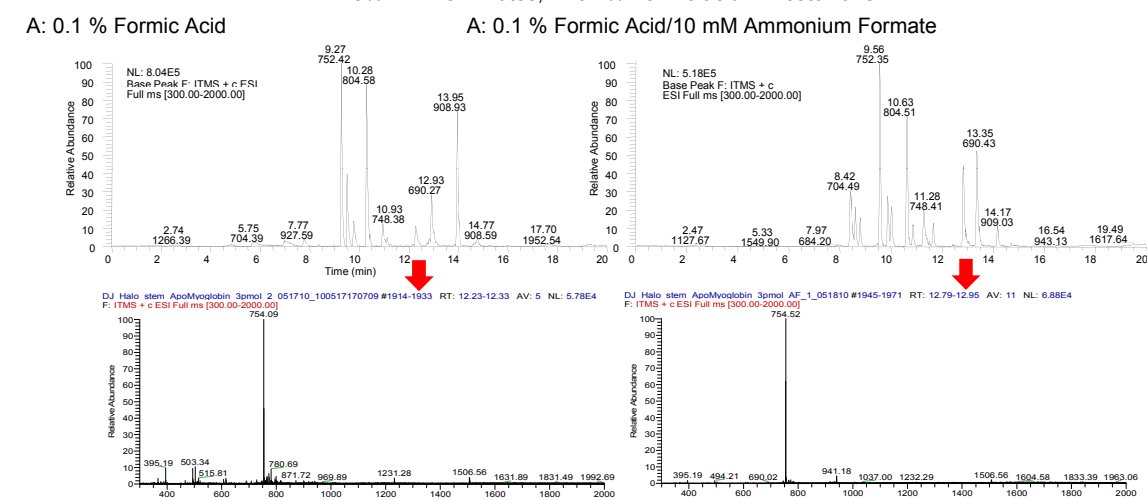
- Band broadening and tailing using Formic Acid is driven by surface overload for basic peptides, even at low quantities. Peptides with a blocked N-terminus, and without basic residues, show very high load tolerance, narrow width and good symmetry, compared to the example of S1-S5 peptides or  $\beta$ -amyloid (1-38).
- Detection of trace impurities is enhanced with Ammonium Formate/Formic Acid mobile phase.

Column: Halo Peptide ES-C18, 2.1 x 100 mm; Flow rate: 0.5 mL/min; T= 60°C;  
A: Water/acid modifier; B: ACN/0.1% Formic Acid  
Gradient: 20 mM Ammonium Formate/0.1% Formic Acid : 24% to 27% B in 20 min.;  
0.1% Formic Acid : 20% to 24% B in 20 min.



## Ammonium Formate as an Additive for LC/MS Analyses

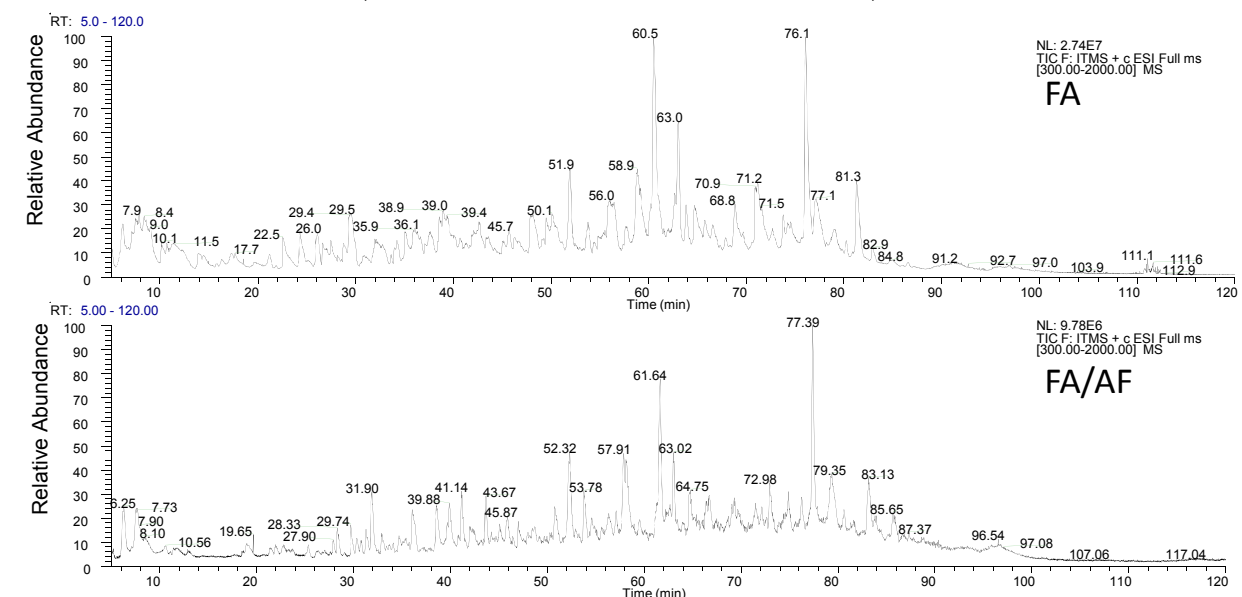
Halo Peptide ES-C18, 0.2 mm ID x 50 mm, Flow Rate 9  $\mu\text{L}/\text{min}$ ., T=25C, 3 pmol apoMyoglobin digest in 2  $\mu\text{L}$ ;  
2-45% B in 15 minutes, B: 0.1% Formic acid in Acetonitrile



Comparing Formic Acid PLUS Ammonium Formate versus Formic Acid for LC/MS analyses of peptides and tryptic digests of proteins reveals:

- Retention increases with ammonium concentration, accompanied by selectivity shifts, and improved peak shape.
- Improved sample mass load tolerance at 10 or 20 mM ammonium formate. Even at the minute quantities detected by online MS, peak shapes are superior with the added salt.
- Global MS signal is lower, but S/N is very similar. For a small percentage of peptides (c. 15%) up to 10-fold differences in either direction of relative signal strength are observed.
- Ionization charge state differences are dependent on flow rate (column ID) and are highly sequence dependent. Determination of a systematic trend to higher or lower ionization state will require analysis of a larger dataset.

Halo Peptide ES-C18, 0.2 mm ID x 150 mm, Flow Rate 4  $\mu\text{L}/\text{min}$ ., T=25C, 3  $\mu\text{g}$  synthetic protein standard digest in 5  $\mu\text{L}$ ;  
2-45% B in 120 minutes, A: 0.1 % Formic Acid +/- 10 mM Ammonium formate, B: 0.1% Formic acid in Acetonitrile



## Effect of Ammonium Formate on Proteomic Analysis by LC/MS

Proteins from procyclic *T. brucei* were analyzed via LC-MS/MS using the DDA optimized experimental conditions using Halo Peptide ES-C18 0.2 x 150 mm columns (see also Poster Number MP 226). Gradient elution with mobile phase combinations with and without ammonium formate (in Eluent A and B) were examined for peptide identifications. The number of unique peptide identifications for each experimental condition reflects a total collected from duplicate runs. The combination of the Halo Peptide column with ammonium formate in both eluents yielded the most peptide identifications using a 5% peptide false discovery rate.

- Experiments were performed in duplicate using a 90 minute gradient
- Protein IDs are number of protein groups using a 5% protein FDR
- Total protein groups across all experiments: 131 (87 groups commonly identified)
- Peptide IDs are number of unique peptides using a 5% peptide FDR
- Total unique peptides across all experiments: 1061 (287 peptides commonly identified)

Eluent A	Eluent B	Proteins IDs	Peptide IDs
99.9% H <sub>2</sub> O, 0.1% Formic Acid, 10 mM Ammonium Formate	80% ACN, 0.1% Formic Acid, 10 mM Ammonium Formate	118	825
99.9% H <sub>2</sub> O, 0.1% Formic Acid	99.9% ACN, 0.1% Formic Acid	118	639
99.9% H <sub>2</sub> O, 0.1% Formic Acid, 10 mM Ammonium Formate	99.9% ACN, 0.1% Formic Acid	92	457

## Conclusions and Future Directions

- High throughput RP HPLC separations were conducted to examine the utility of adding ammonium formate salt to standard formic acid eluents.
- Ammonium formate addition to formic acid eluent was beneficial for reversed phase separations, improving peak shape, band width and column load tolerance for peptides and mixtures of protein digests.
- Trace impurity detection was improved with the use of ammonium formate modified formic acid mobile phase. We are coupling this with online MS as a standard approach with synthetic peptides used in our labs.
- Capillary columns packed with these Fused-core particles can be operated at very high linear velocities to permit rapid and efficient proteomic analyses using standard LC/MS instruments.
- The ammonium formate additive is fully compatible with ion trap LC-MS operation.
- In a proteomic workflow higher Peptide IDs are obtained with the ammonium formate additive for a complex digest sample (22.5% increase). Ongoing work will reveal whether potential problems could arise with identifying post translational modifications.

## Acknowledgements

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