# Optimization of LC-MS peptide mapping

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## Introduction

Peptide mapping is an invaluable tool in analysis and quality control of biotherapeutic proteins. Peptide mapping has been widely used for purity testing, comparability testing and batch release of protein therapeutics. Mass spectrometry in recent years has revolutionized the field of protein chemistry. High resolution and accurate mass analysis provide an additional dimension to chromatographic separation. Tandem mass spectrometry yields important information about peptide sequence and can be used to detect and confirm post-translational modifications. Hence, mass spectrometry is now being routinely used in conjunction with conventional LC-UV peptide mapping.

Here we present a study for the optimization of LC-MS peptide mapping. Mobile phase considerations are of paramount importance during LC-MS method development. Mobile phase composition can affect ionization of analyses during MS analysis and at the same time also influence chromatographic separation. We studied the effect of different mobile phase modifiers such as trifluroacetic acid (TFA), formic acid, acetic acid, etc., on ion suppression during ESI-MS analysis. We found that formic acid showed the lowest levels of suppression. We also studied the effect of these modifiers on chromatographic separation of peptides on various stationary phases. Our study indicates that the difunctionally bonded silica based reversed-phase C18 material exhibits the best peak capacity when using formic acid as a modifier. We also optimized chromatographic variables such as flow rate, temperature and column length. We were able to obtain highly reproducible peptide maps for IgG, human serum albumin and yeast enolase using shallow 5 hour gradients on 2.1 mm X 25 cm columns packed with 3 µm particles at a flow rate of 0.2 ml/min.

## System Components

Waters® BioSuite™ Peptide Mapping MS System

- Waters® 2796 Separations module.
- Waters® 2487 Dual Wavelength Absorbance Detector
- Waters® ZQ™ Mass detector
- Waters® Q-Tof micro™

Columns:

- Waters® BioSuite™ $C_{18}$ PA-A columns
- Waters® BioSuite™ $C_{18}$ PA-B columns

## Experimental

MS Conditions:

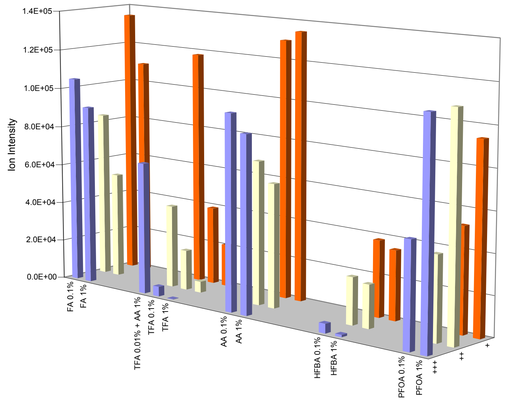
-Source = ESI(+)
-Capillary (kV) = 3.3
-Cone (V) = 30
-Temperature (°C)
  -Source = 150
  -Desolvation = 300
-Gas Flow (L/Hr)
  -Cone = 50
  -Desolvation = 500
-Scan Mode

HPLC Conditions:

Buffer A: 0.1% formic acid in water
Buffer B: 0.1% Formic acid in acetonitrile
Gradient 0-40% Buffer B in 60, 150 or 300 min.
Flow Rate: 0.2 ml/min
Column temperature: 50°C

### Figure 1: Suppression Effects of Mobile Phase Additives on Peptide Ionization.

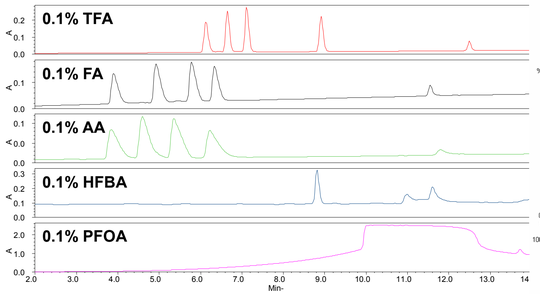
The suppression effects of formic acid (FA), trifluoroacetic acid (TFA), acetic acid (AA), pentadecafluorooctanic Acid (PFOA) and heptafluorobutyric acid (HFBA) on peptide analysis by ESI-MS was studied. Our results indicate that formic acid shows the least amount of ion suppression during ESI-MS analysis of peptides. TFA at the concentration of 0.1% shows the maximum suppression effects and should be avoided or used at lower concentration. These are in agreement with previously published results for the ESI-MS of small molecules (1).




### Figure 2: Chromatographic effects of mobile phase additives on peptide separation.

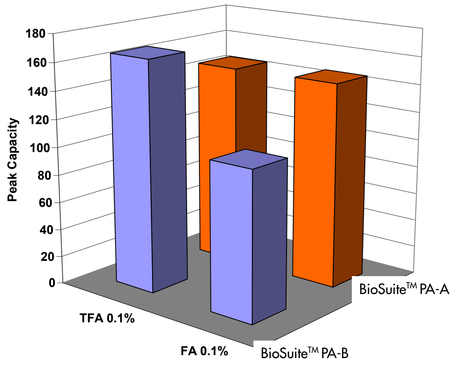
The effect of various mobile phase modifiers used in Figure 1 on the chromatographic resolution of peptides was analyzed. TFA with its superior ion-pairing ability showed better resolution and peak capacity than the other additives. Formic and acetic acids showed moderate separation and PFOA and HFBA were not able the resolve all the components.

The experiment was carried out using Waters® BioSuite™ $C_{18}$ 5µm PA-B 5µ (4.6 X 50). Elution was achieved by acetonitrile gradient from 5 to 70 % in 15 min. The flow rate was maintained at 1 ml/min.



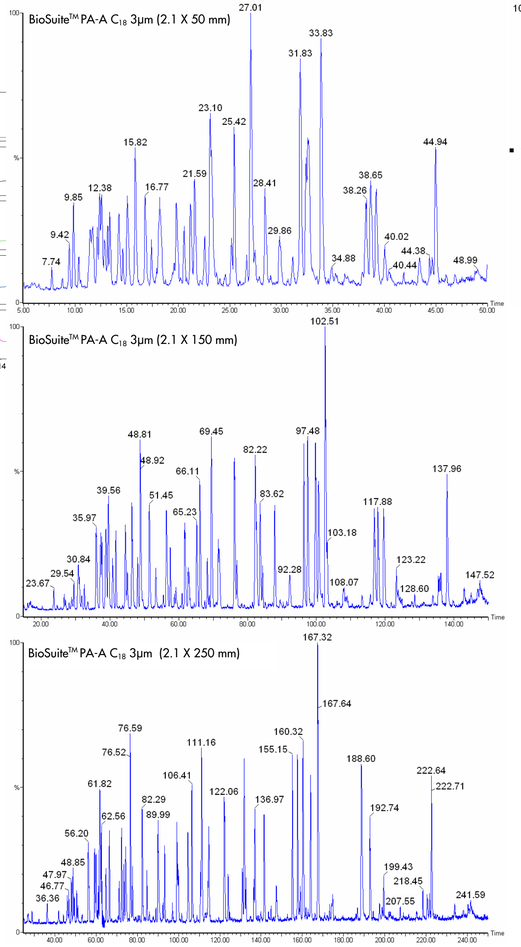

### Figure 3: Effect of formic Acid Vs TFA on peak capacity obtained with different column chemistries.

The effect of TFA and formic acid on the peak capacity of peptides on Waters® BioSuite™ PA-B and Waters® BioSuite™ PA-A is shown. It can be seen that Waters® BioSuite™ $C_{18}$ 5µm PA-B 5µ (4.6 X 50) shows higher peak capacity under TFA conditions while Waters® BioSuite™ $C_{18}$ 5µm PA-A 5µ (4.6 X 50), which is a difunctionally bonded silica based reversed-phase C18 material, exhibits god peak capacity under TFA as well as formic acid conditions



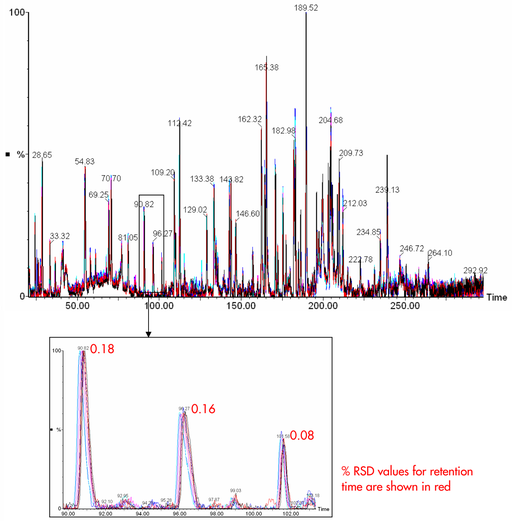

### Figure 4: Effect of column on peak capacity.

Waters® BioSuite™ PA-A columns with varying lengths were studied for optimal peak capacity. Increase in column length leads to increased theoretical plate number and hence improved peak capacity. It can be seen that 2.1 X 250 Waters® BioSuite™ PA-A column shows higher peak capacity and better separation than the 2.1 X 50 and 2.1 X 150 columns. Waters® BioSuite™ PA-A 2.1 X 250 shows moderate back pressure at flow rates of 0.2 ml/min and hence is very efficient in peptide mapping.




### Figure 5: Retention time reproducibility on BioSuiteTM PA-A C18 3 µm (2.1 X 250 mm)

8 chromatogram overlay of peptide map of monoclonal IgG2 is shown. It can be seen that highly reproducible peptide maps were obtained despite long 5 hr chromatographic runs. The inlay shows the 8 chromatogram overlay of a smaller section of the peptide map.




% RSD values for retention time are shown in red

## Conclusions

- Formic acid when used as mobile phase modifier shows the least levels of ion suppression.
- TFA cause upwards of 80% suppression and should be avoided or used in low concentration during LC-MS peptide mapping.
- BioSuiteTM PA-A column exhibits good peak capacity under TFA and formic acid conditions and hence is the ideal column for LC-MS peptide mapping .

## References

Claude R. Mallet , Ziling Lu. and Jeff Mazzeo. *Rapid Commun.Mass Spectrom.* 2003, **18**, 49-58