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Application Note 168

Eliminate TFA and Improve Sensitivity of Peptide Analyses by LC/MS

TFA (trifluoroacetic acid) is a commonly used mobile phase additive for reversed-phase HPLC (RP-HPLC) separations of proteins and peptides. However, TFA interferes with and significantly reduces the LC/MS signal, lowering sensitivity. The ideal column for modern RP-LC/MS analysis should provide symmetrical peak shape without TFA in the mobile phase. The highly inert surface of Discovery BIO silica results in columns that give symmetrical and efficient peaks for peptides without TFA for maximum LC/MS sensitivity.

Key Words:
● TFA ● peptides ● RP-HPLC ● LC/MS

A major challenge facing biotechnology and proteomics researchers and others working with peptides or peptide maps is the need to detect and identify single peptides often at very low concentrations in extremely complex samples. Liquid chromatography coupled with mass spectroscopy (LC/MS) has become an invaluable tool to meet this challenge. Because of its high resolving power, RP-HPLC is the preferred separation mode for peptides. Traditionally, TFA is used in the mobile phases for RP-HPLC peptide separations. Ionic mobile phase additives like TFA serve one or more of the following functions: pH control (buffering), complexation with oppositely charged ionic groups to enhance RP retention (ion pairing), or suppression of adverse ionic interactions between peptides and silanol groups on the silica. The latter function is necessary when using RP-HPLC phases with high silanol activity.

While TFA has little effect on UV detection, it has serious disadvantages for LC/MS detection. First, typical concentrations of TFA (0.1% *v/v*) have high surface tension and prevent efficient spray formation (nebulization). Second, TFA ions in the gas phase ion-pair with the peptide's basic groups suppressing their ionization and reducing sensitivity. A demonstration of TFA's adverse effect on LC/MS sensitivity is shown in Figure A. Without TFA, the MS is able to detect much lower concentrations of these peptides. An added benefit is that at low TFA concentrations, resolution is improved because small differences in peptide retention are not masked. This is shown in the increased separation of peptides 1 and 2 in Figure A as the TFA concentration is decreased. At 0.1% TFA, they co-elute. Therefore, from the mobile phase standpoint, the best LC/MS method employs ionic additives other than TFA that are still volatile, can provide pH control, and do not strongly ion-pair with the peptides.

However, as previously mentioned, it is necessary to add TFA to the mobile phase when using RP-HPLC columns that exhibit

Figure A. Effect of Chromatographic Conditions on MS Signals of Peptides

Column: **Discovery BIO Wide Pore C18, 15cm x 2.1mm, 3µm**
Cat. No.: **567202-U**
Mobile Phase: (**A1**) A: 25mM formic acid in H_2O, B:50:50 (25mM formic acid in H_2O):(20mM formic acid in CH_3CN)[a];
(**A2**) A:0.01% TFA, B:0.01% TFA in 50:50 (CH_3CN:H_2O);
(**A3**) A:0.1% TFA, B:0.1% TFA in 50:50 (CH_3CN:H_2O)
Flow Rate: 0.208mL/min[b]
Det.: +ES
Temp.: ambient
Inj.: 1µL or 3µL
Sample: RP Peptide Performance Standard, p/n RPS-P0010 (Alberta Peptide Institute)

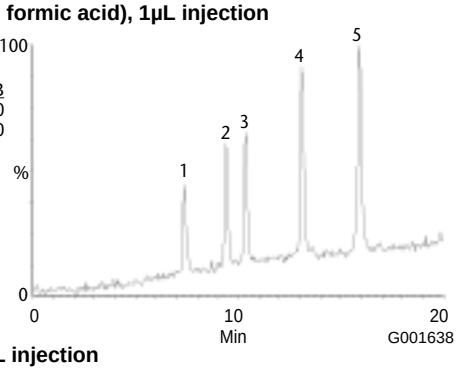
a) molarity of formic acid adjusted to provide minimum baseline drift
b) linear velocity equal to 1mL/min on 4.6mm ID columns

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

A1: 0% TFA (25mM formic acid), 1µL injection

Gradient:

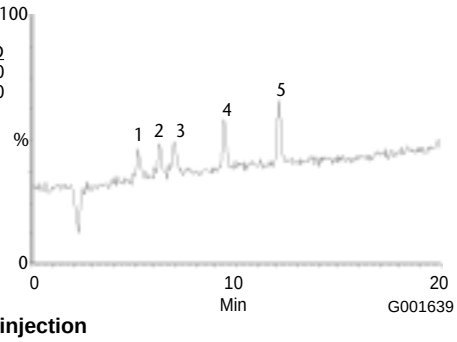
Min	%A	%B
0	90	10
20	50	50



A2: 0.01% TFA, 3µL injection

Gradient:

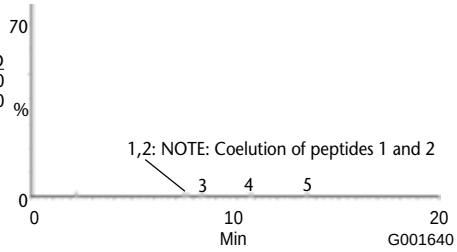
Min	%C	%D
0	80	20
20	40	60



A3: 0.1% TFA, 3µL injection

Gradient:

Min	%C	%D
0	80	20
20	40	60



high silanol activity. Without TFA, peptides, especially basic peptides, elute with low efficiency and tailing peaks and concurrently decreased sensitivity. Figure B shows a mixture of basic peptides on two columns that exhibit differing degrees of silanol activity. Without TFA, peptides on the popular brand column in Figure B2 show very asymmetrical, low efficiency peaks. However, because of its high degree of silanol deactivation and silica purity, the Discovery BIO Wide Pore C18 column shown in Figure B1 provides efficient and symmetrical peaks. Therefore, from the column standpoint, for the best LC/MS method choose a column that has low surface silanol activity.

There are several TFA-alternatives for LC/MS of proteins and peptides. For low pH operation, the most common are formic acid (HCOOH) and acetic acid (CH_3COOH). Ammonium acetate (CH_3COONH_4 or NH_4OAc) is commonly used for neutral pH operation, and ammonium bicarbonate (NH_4HCO_3) for basic pH. However, ionic additives not only control the pH, they can also influence the selectivity (peak order or spacing between peak apices). When choosing an ionic additive for LC/MS, it is important to consider its volatility, purity, pK_a, and degree to which it suppresses or expresses ionization of the analyte.

Of the myriad considerations for optimizing the LC/MS separation of proteins and peptides, two of the most important are to avoid TFA, and to use an RP-HPLC column that provides high efficiency and peak symmetry under those difficult conditions. Discovery BIO Wide Pore columns and capillaries provide sensitive, efficient, stable and reproducible LC/MS analyses of proteins and peptides without TFA in the mobile phase.

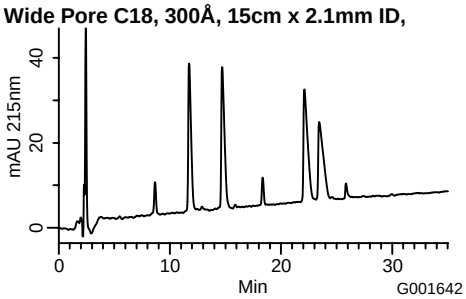
Figure B. Column Performance Differences Toward Basic Peptides without TFA

Columns:	**C18, 300Å, 15cm x 2.1mm or 2.0mm ID, 5µm**
Mobile Phase:	**A:** 25mM formic acid in water **B:** 50:50 (25mM formic acid in water):(20mM formic acid in CH_3CN)
Flow Rate:	0.208 (or 0.189) mL/min
Det.:	215nm
Temp.:	35°C
Inj.:	0.5µL (~0.25µg ea peptide)
Sample:	RP Peptide Ionic Interactions Standard, p/n RPS-I0020 (Alberta Peptide Institute)
Peptide 1:	ac-GGGLGGAGGLK-amide
Peptide 2:	ac-KYGLGGAGGLK-amide
Peptide 3:	ac-GGALKALKGLK-amide
Peptide 4:	ac-KYALKALKGLK-amide

B1: Discovery BIO Wide Pore C18, 300Å, 15cm x 2.1mm ID, 5µm, 0.208mL/min

Gradient:

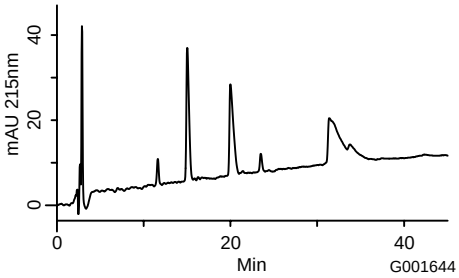
Min	%C	%D
0	80	20
20	40	60



B2: Competitor C18, 300Å, 15cm x 2.0mm ID, 5µm, 0.189mL/min

Gradient:

Min	%C	%D
0	80	20
20	40	60



Ordering Information

(Other dimensions available. Please call or visit our web site.)

Description	Cat. No.
Discovery BIO Wide Pore C18 HPLC Columns	
15cm x 2.1mm, 3µm	567202-U
15cm x 2.1mm, 5µm	568202-U
15cm x 4.6mm, 5µm	568222-U
Discovery BIO Wide Pore C18 Guard Columns	
2cm x 2.1mm, 3µm, Kit	567271-U
2cm x 2.1mm, 3µm, 2 cartridges	567270-U
2cm x 2.1mm, 5µm, Kit	568271-U
2cm x 2.1mm, 5µm, 2 cartridges	568270-U
2cm x 4mm, 5µm, Kit	568273-U
2cm x 4mm, 5µm, 2 cartridges	568272-U

The following literature is available upon request, or by downloading from our website sigma-aldrich.com/supelco

Discovery BIO Wide Pore Brochure	T402038
Discovery BIO Wide Pore C18	T401097
Discovery BIO Wide Pore C8	T401098
Discovery BIO Wide Pore C5	T401099
Discovery BIO Wide Pore Capillary and Microbore Dimensions	T402051

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