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Improving LC-MS Separations of Peptides with Difluoroacetic Acid Ion Pairing

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Introduction

Peptide mapping is an important and critical technique in the characterization of biopharmaceuticals. These separations are based upon reversed-phase liquid chromatography that can be coupled to mass spectrometry (RPLC-MS). Separations using spectroscopic detection often employ trifluoroacetic acid (TFA), a strong, hydrophobic acid and an effective ion pair for minimizing chromatographic secondary interactions.

Conversely, to avoid ion suppression, LC-MS analyses are often performed with a weaker ion pairing modifier, like formic acid (FA), but with a compromise to chromatographic resolution. Therefore, an alternative mobile phase modifier, difluoroacetic acid (DFA) may be used. DFA is less acidic and less hydrophobic than TFA and lessens the surface tension of droplets during electrospray. However, to give higher fidelity MS data, it was necessary to use a purified DFA for peptide separations.

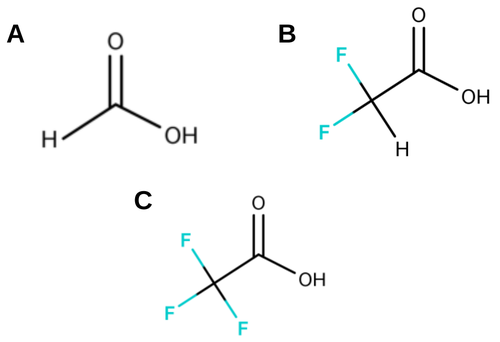

Figure 1. Structures of A) formic acid (FA), B) trifluoroacetic acid (TFA), and C) difluoroacetic acid (DFA).

Minimizing trace metal contamination in MS based workflows has become ever more critical in protein and peptide analyses as increasingly sensitive assays are being deployed throughout the development and manufacturing of biotherapeutics. Herein, using a purified DFA certified to low metals content grants unique selectivity and acted as a compromise between FA and TFA in terms of resolution and sensitivity while providing exceptionally high quality peptide mass spectra.

Methods

A reduced and alkylated tryptic digest of the NIST Reference Material 8671 (NIST mAb) was acquired in the form of the Waters mAb Tryptic Digestion Standard (p/n 186009126, Golden, CO). MS quality DFA was acquired in the form of Waters IonHance Difluoroacetic Acid (p/n 186009201, Golden, CO). LC-MS quality FA and TFA were purchased from Fisher Scientific.

Analyses were performed using an ACQUITY UPLC H-Class Bio, ACQUITY UPLC TUV detector, and a Xevo G2-XS QToF mass spectrometer. Separations were performed on a 1.7 µm, 130 Å, 2.1 x 150 mm ACQUITY UPLC Peptide BEH C18 or Peptide CSH C18 column at 80 ° C. Samples were run using 0.1% FA, DFA, or TFA in water (mobile phase A) and the same percent modifier in acetonitrile (mobile phase B). The gradient conditions are shown in **Table 1**. Analyses were performed with UV detection at 210, 214, or 219 nm using MassLynx 4.1 and UNIFI 1.8. LC-MS analyses were performed in full scan with fragmentation mode with a mass range from 100-2000 m/z, cone voltage of 50 V, capillary voltage of 3.5 KV, desolvation temperature of 500 ° C, and fragmentation from 20-40 V.

Gradient			
Time	%A	%B	Curve
Initial	99.0 or 99.5	0.5 or 1.0	Initial
10.00	99.0 or 99.5	0.5 or 1.0	6
75.00	60	40	6
76.00	20	80	6
80.00	20	80	6
81.00	99.0 or 99.5	0.5 or 1.0	6
100.00	99.0 or 99.5	0.5 or 1.0	6

Table 1. Gradient conditions for the separation of the mAb Tryptic Digestion Standard, where the lower starting percent of mobile phase B was used with the ACQUITY UPLC Peptide CSH C18 column and the higher starting percent was used with the ACQUITY UPLC Peptide BEH C18 column.

Results and Discussion

Recent work on the use of DFA for intact, subunit, and peptide levels of analysis has indicated that DFA is a promising alternative to FA or TFA.[1,2,3] To further expand upon its potential in peptide mapping, a reduced and alkylated tryptic digest of NIST mAb was analyzed using separations based on FA, DFA, and TFA modified mobile phases. Two hybrid silica columns, an ACQUITY UPLC Peptide BEH C18 column and an ACQUITY UPLC Peptide CSH C18 column, were chosen for their high performance in peptide mapping separations. The CSH particle technology differs from the BEH technology in that it was designed with a low level surface charge that improves sample loadability and peak asymmetry in low ionic-strength mobile phases.

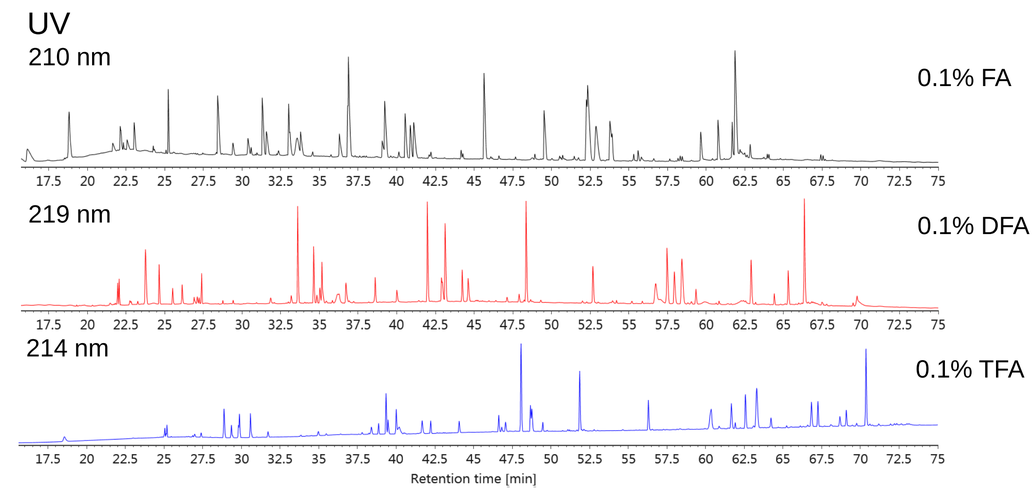

Figure 2. Representative UV chromatograms from a peptide mapping separation of the mAb Tryptic Digestion Standard using 0.1% FA, purified DFA, or TFA modified mobile phases and an ACQUITY UPLC Peptide BEH C18, 130 Å, 1.7 µm, 2.1 x 150 mm column.

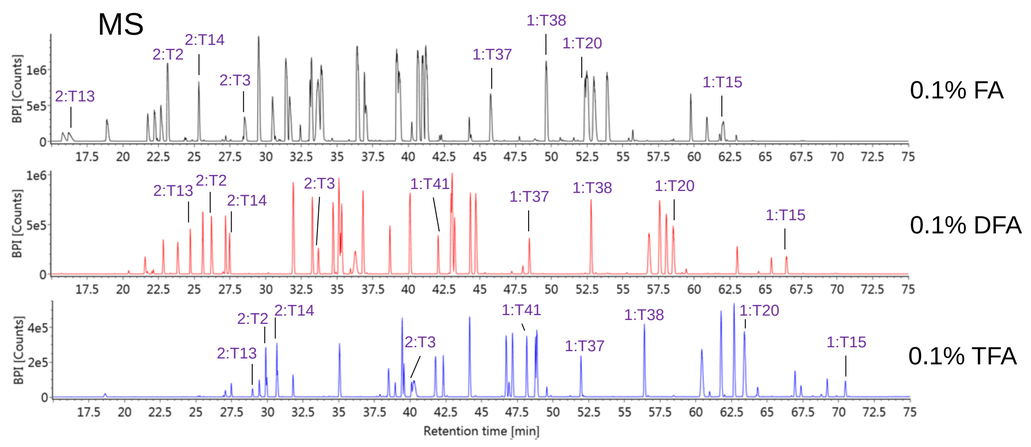

Figure 3. Representative BPI chromatograms from a peptide mapping separation of the mAb Tryptic Digestion Standard using 0.1% FA, purified DFA, or TFA modified mobile phases and an ACQUITY UPLC Peptide BEH C18, 130 Å, 1.7 µm, 2.1 x 150 mm column.

Figures 2 and **3** show the UV and BPI chromatograms of separations resulting from the BEH column. Due to the differences in UV absorptivity between the acids, different wavelengths were necessary to optimize the baseline properties of the separation, as seen in **Figure 2**. From these data, it is clear that each acidic modifier imparts a unique selectivity to the separation, which is a characteristic that could be beneficial when developing certain peptide maps.

The BPI chromatograms in **Figure 3** indicate that separations using DFA provide slightly lower MS sensitivity than separations using FA. In comparison, separations with TFA show a drastic decrease in sensitivity. This can be further confirmed when evaluating the peak areas of 8 peptides, as shown in **Figure 4** for both the BEH and the CSH columns. These peptides were chosen to offer a well distributed mix of sizes and hydrophobicities.

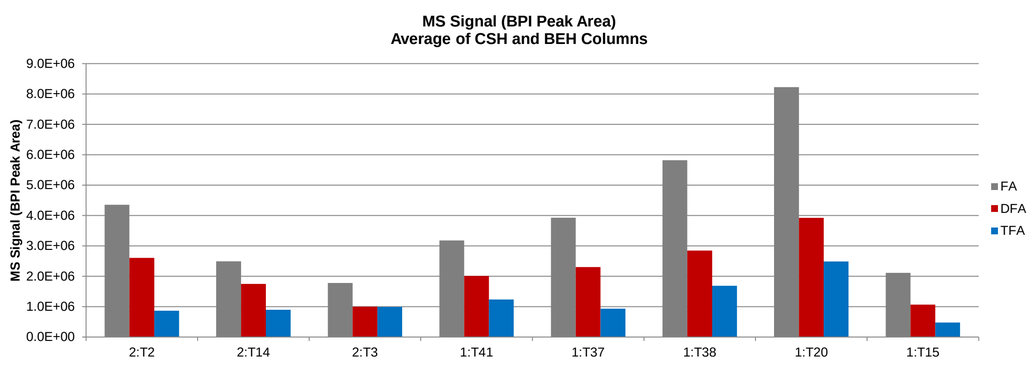

Figure 4. Average peak areas taken from the TIC chromatograms of 8 chosen peptides from the separation of the mAb Tryptic Digestion Standard using 0.1% FA, purified DFA, or TFA modified mobile phases and an ACQUITY UPLC Peptide BEH C18, 130 Å, 1.7 µm, 2.1 x 150 mm column and ACQUITY UPLC Peptide CSH C18, 130 Å, 1.7 µm, 2.1 x 150 mm column.

Peptide	m/z	Charge (FA)	Charge (DFA)	Charge (TFA)	Sequence	Modifications
2:T2	541.269	1.8	1.5	1.8	VTIT**C**SASSR	Carbamidomethyl C
2:T14	712.7153 1068.488 (TFA)	2.6	2.5	2.2	VDNALQSGNSQESVTEQDSK	
2:T3	541.3097 811.3977 (TFA)	3.2	2.4	2.3	VGYMHWYQQKPGK	
1:T41	561.1008 (FA) 701.12171 (DFA) 934.422 (TFA)	4.1	3.4	3.2	WQQGNVFS**C**SVMHEALHNHYTQK	Carbamidomethyl C
1:T37	848.7787 1272.5622 (TFA)	2.6	2.5	2.3	GFYPSDIAVEWESNGQPENNYK	
1:T38	937.4609	2.1	1.9	2.0	TTPPVLDSDGSFFLYSK	
1:T20	711.9565 948.8188 (TFA)	3.6	3.2	2.9	THT**C**PP**C**PAPELLGGPSVFLFPPKPK	Carbamidomethyl C (2)
1:T15	1343.5933 1679.0841 (TFA)	5.4	5.5	5.3	DYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYI**C**NVNHKPSNTK	Carbamidomethyl C

Table 2. List of the 8 tryptic digestion peptides from NIST mAb analyzed for peak area and peak capacity, where modification sites are labeled in red.

Table 2 lists the peptides analyzed and their weighted average charge states. The charge states for DFA are typically between those seen for FA and TFA. With subunit separations, similar charge states have been seen between FA and DFA, suggesting that their ionization efficiencies may be more similar than DFA and TFA, at least for subunit separations.[1] With peptides, the most abundant charge state is generally similar between FA and DFA. An example of this can be seen in the mass spectra of the 2:T14 peptide that are shown in **Figure 5**, where the relative abundances differ significantly with separations using TFA versus FA and DFA. Conversely, when assessing LC resolution between the three acidic modifiers, DFA offers high peak capacities nearer to separations using TFA rather than separations with FA (**Figure 6**).

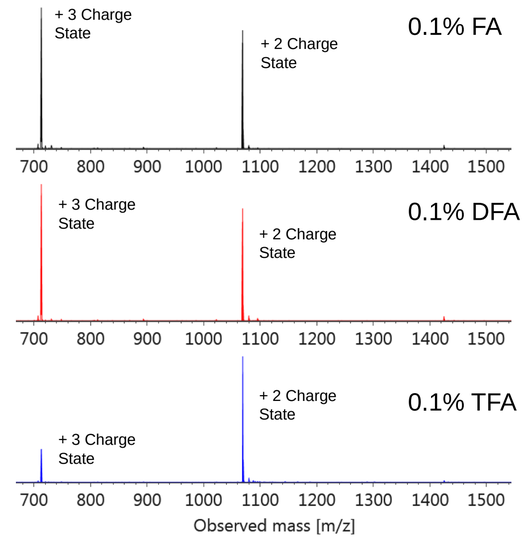

Figure 5. Mass spectra of the 2:T14 peptide from a peptide mapping separation of the mAb Tryptic Digestion Standard using 0.1% FA, purified DFA, or TFA modified mobile phases and an ACQUITY UPLC Peptide BEH C18, 130 Å, 1.7 µm, 2.1 x 150 mm column.

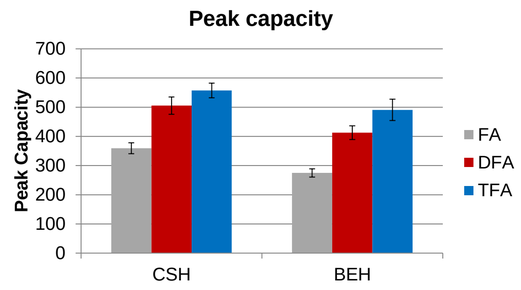

Figure 6. Effective peak capacities based on 8 tryptic digestion peptides.

Differences in peptide ion relative abundances also occur when using commercial, reagent quality DFA versus purified DFA and FA. **Figure 7** indicates that commercial DFA contains a high concentration of sodium. While trace level metal contaminants generally do not affect protein or peptide separations, they can disrupt the interpretability of the mass spectra by distorting relative abundances of protonated species and causing spectral crowding. Purifying DFA reduces the metal content to allow for the acquisition of high quality MS data.

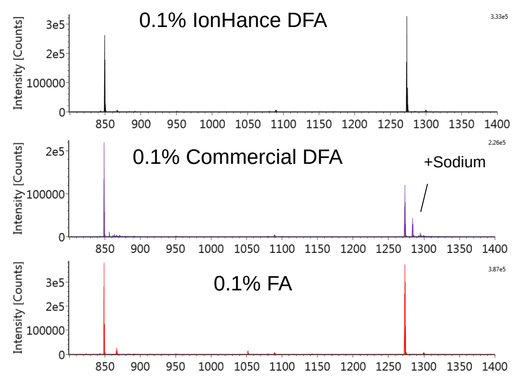

Figure 7. Mass spectra of the 1:T37 peptide from a peptide mapping sepration of the mAb Tryptic Digestion Standard using 0.1% purified DFA, commercial DFA, or FA modified mobile phases.

Sodium and potassium adducts can be particularly difficult to minimize as they can originate from multiple sources of contamination. **Figure 8** shows the importance of mobile phase container selection for low adduct MS. These data suggest that sodium can potentially leach from certain types of glass into the mobile phase, and that preference should be given to the use of certified polymer containers.

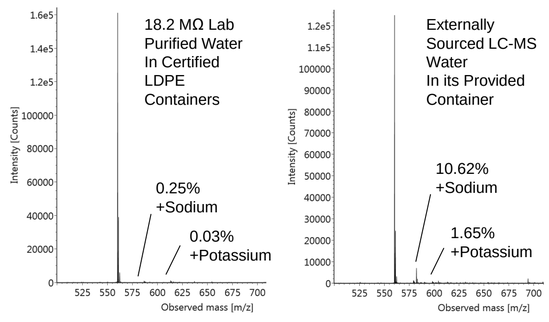

Figure 8. Mass spectra of the 2:T13 peptide as acquired with 0.1% purified DFA modified mobile phases.

CONCLUSIONS

- **DFA offers unique selectivity for peptide RPLC separations while also giving strong MS sensitivity gains over TFA and improved peak capacities over FA.**
- **Purified DFA in the form of IonHance DFA is certified to contain less than 100 ppb sodium and potassium, which is essential to minimizing adduct formation in peptide mass spectra. It is advised to pair this trace metal additive with trace metal certified, polymer mobile phase containers to optimize MS quality.**

References
1. *Manuscript in review.*
2. *Kellett, J., Birdsall, R., and Yu, Y. Application of Difluoroacetic Acid to Improve Optical and MS Performance in Peptide LC-UV/MS. (2018). Waters Technical Brief (PN: 720006482EN).*
3. *Nguyen, J. M., Liu, X., and Lauber, M. A. Low Adduct Peptide LC-MS Obtained with IonHance DFA and Certified LDPE Containers. (2019). Waters Application Note (PN: 720006596EN).*