In-Depth Characterization of Intact Protein Standards Using HRAM Top Down Mass Spectrometry with Multiple MSMS Strategies

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OVERVIEW -

Purpose: Demonstrate unique characteristics and effectiveness of various dissociation mechanisms for intact protein identification and characterization.

Methods: Collection and analysis of high resolution CID, HCD, ETD, and UVPD data on various proteins at various energies or reaction times.

Results: Each fragmentation mechanism generates unique data that, together, maximizes sequence coverage for improved protein identification and proteoform characterization. Considerations for optimizing each dissociation mechanism with respect to proteins representing a MW range from 9kDa to 50kDa are presented.

INTRODUCTION -

Complete and accurate characterization of intact proteins by mass spectrometry is both possible and increasingly popular today thanks to the latest technological developments made in LC and MS hardware, instrument control software, and data processing software. Here we demonstrate the dissociative behavior of four proteins from the recently released Pierce[™] Intact Protein Standard Mix representing a MW range of 9kDa to 50kDa, with four different modes of ion dissociation (CID, HCD, ETD, and UVPD) available on the Thermo Scientific[™] Orbitrap[™] Fusion Lumos[™] instrument. For each dissociation mode, we test three different normalized collision energies or reaction/ irradiation times. We aim to illustrate attributes of each of these modes on intact proteins, and ultimately inform method development for top down proteomics applications. While we focused here was on single mode techniques to highlight the specific uniqueness of each mode of dissociation, mixed mode dissociation techniques (e.g. EThcD) are also available and can be highly beneficial for both identification and structural characterization.

Ion trap CID employs m/z selective slow heating to produce b- and y- type product ions via many low energy-imparting collisions with He atoms, resulting in minimal secondary dissociation of product ions. This is advantageous, unless post translational modification (PTM) loss is the primary fragmentation pathway. HCD also produces b- and y- type ions through "fast heating" induced relatively fewer, but higher energy-imparting collisions with N₂ gas molecules in a non-m/z selective manner. This makes subsequent over-fragmentation of product ions a risk, but also overcomes the limitation presented by primary loss of labile PTMs. By contrast, ETD generates c- and z- type ions through the abstraction of electrons from a donor reagent anion. To accommodate the resulting radical site, the cation almost instantaneously undergoes rearrangement leading to bond cleavage without internal energy transfer. As such, PTMs are preserved by this mode of dissociation. Intact charge reduced dissociation products from lower charge state precursors can at times dominate spectra, however mild activation of these species through techniques such as EThcD can overcome this limitation. Finally, UVPD, the most recently introduced mode of dissociation on the Orbitrap Fusion Lumos MS is initiated by irradiation of the precursor ions with photons from a 213nm UV laser, proceeds though multiple dissociation pathways. This results in formation of a-, b-, c-, x-, y-, and z- type fragment ions, many only observed with this mode of dissociation.

MATERIALS AND METHODS -

Pierce intact protein standard mix (A33526) was purchased from Fisher Scientific and each vial was reconstituted in 100ul HPLC grade water prior to use. Proteins were separated over a 20minute gradient (Figure 1a) at 200ul/min using a Dionex Ultimate 3000 UHPLC system fitted with a 2.1 mm MabPac[™] RP LC column. Solvent A was 0.1% formic acid in LCMS grade water (Fisher Scientific LS118-1) and solvent B was 0.1% formic acid in LCMS grade acetonitrile (Fisher Scientific LS120-1). Full scan MS data was collected at 15k resolution in the Orbitrap analyzer, with alternating targeted MS2 scans at either 60k (CID, HCD) or 120k resolution (ETD, UVPD). A single charge state of each protein near the center of the charge envelope was selected at random for isolation and fragmentation. As such, precursor charge state selection within a protein is not considered here, though it can be a major variable affecting extent of dissociation. In all cases, precursors were isolated by the quadrupole using a 3Da window. For ETD, anion target value was reduced to 5e4 to reduce reaction kinetics in an attempt to avoid over-fragmentation of large highly charged precursors. Data was collected in a targeted fashion, and MS2 were manually averaged, then decovoluted using Xtract in QualBrowser. Xtracted raw files were submitted to ProSightPC 4.1 for fragment ion assignment. The Pierce Intact Protein Standard Mix database (.pscw) was downloaded directly from the Proteinacious database warehouse (http://proteinaceous.net/database-warehouse/).





RESULTS





Figure 6: UVPD analysis of 4 different proteins ranging in MW from 9kDa to 50kDa, at 3 different Figure 3: HCD analysis of 4 different proteins ranging in MW from 9kDa to 50kDa, at 3 different rradiation times. Inset numbers in red are ProSightPC P-scores. Dissociation here happens at a collision energies. Inset numbers in red are ProSightPC P-scores. Provided that energies are speed proportional to the MW of the precursor, and as such we see rich spectra produced for the carefully chosen to avoid over-fragmentation, this mode of fragmentation is efficient across the smaller proteins, but a high unresolved baseline for ProteinAG, indicating over fragmentation. mass range, and provides well resolved fragments regardless of presence of PTMs.



Figure 5: ETD analysis of 4 different proteins ranging in MW from 9kDa to 50kDa, at 3 different reaction times. Inset numbers in red are ProSightPC P-scores. ETD spectra of the smaller proteins are extremely rich, however because reactions proceed at rates proportional to the square of the precursor charge state, overfragmentation of larger proteins is common and evidenced by the high, unresolvable baseline seen in all ProtAG spectra.



	EID
26 NGFIQSLKDDPSQSANVLGEAQKLN 50	²⁶ NGFIQSLKDDPSQSANVLGEAQKLN ⁵⁰
⁵¹ D S Q A P K A D A Q Q N N F N K D Q Q S A F Y E I ⁷⁵	51 D S Q A P K A D A Q Q N N F N K D Q Q S A F Y E I 75
76 L N M P N L N E A Q R N G F I Q S L K D D P S Q S 100	76 LNMPNLNEAQRNGFIQSLKDDPSQS 100
101 TNVLGEAKKLNESQAPKADNNFNKE 125	101 TNVLGEAKKLNESQAPKADNNFNKE 125
126 OONAFYEILNMPNLNEEORNGFIOS 150	126 OONAFYEILNMPNLNEEORNGFIOS 150
ADN KFN KEQQNAFYEILHLPNLNEE 200	ADNEFNEQQNAFYEILHLPNLNEE 200
201 Q R N G F I Q S L K D D P S Q S A N L L A E A K K 225	201 Q R N G F I Q S L K D D P S Q S A N L L A E A K K 225
226 L N D A Q A P K A D N K F N K E Q Q N A F Y E I L 250	226 L N D A Q A P K A D N K F N K E Q Q N A F Y E I L 250
251 H L P N L T E E Q R N G F I Q S L K D D P S V S K 275	251 H L P N L T E E Q R N G F I Q S L K D D P S V S K 275
276 E I L A E A K K L N D A Q A P K E E D N N K P I E 300	276 E I L A E A K K L N D A Q A P K E E D N N K P I E 300
301 G R N S R G S V D A S E L T P A V T T Y K L V I N 325	301 G R N S R G S V D A S E L T P A V T T Y K L V I N 325
326 G K T L K G E T T T E A V D A A T A E K V F K Q Y 350	326 G K T L K G E T T T E A V D A A T A E K V F K Q Y 350
351 ANDNGVDGEWTYDDATKTFTVTEKP 375	351 ANDNGVDGEWTYDDATKTFTVTEKP 375
376 E V I D A S E L T P A V T T Y K L V I N G K T L K 400	376 E V I D A S E L T P A V T T Y K L V I N G K T L K 400
401 G E T T T K A V D A E T A E K A F K Q Y A N D N G 425	401 G E T T T K A V D A E T A E K A F K Q Y A N D N G 425
426 V D G V W T Y D D A T K T F T V T E M V T E V P L 450	426 V D G V W T Y D D A T K T F T V T E M V T E V P L 450
451 E S T A C	451 E S T A C
UCD ^Ν Α Q H D E A Q Q N A FÌΥÌQÌVÌLÌN MÌP N L N A D Q R ²⁵	N A Q H D E A Q Q N A F]Y]Q V L N M]P N L N A D Q R ²⁵
HCD NAQHDEAQQNAFYQVLNMPNLNADQR 25 26 NGFIQSLKDDPSQSANVLGEAQKLN 50	N AQHDEAQQNAFÌYÌQVLNMÌPNLNADQR ²⁵ 26 NGFIQSLKDDÌPSQSANVLGEAQKLN 30 UVPD
HCD N AQHDEAQQNAFYQVLNMPNLNADQR 25 26 NGFIQSLKDDPSQSANVLGEAQKLN 50 51 DSQAPKADAQQNNFNKDQQSAFYE] 75	N AQHDEAQQNAFÌYÌQVLNMÌPNLNADQR ²⁵ 26 NGFIQSLKDDÌPSQSANVLGEAQKLN ³⁰ UVPD 51 DSQAPKADAQQNNFNKDQQSAFYEI ⁷⁵
HCD N AQHDEAQQNAF]Y]Q]V]L]NM]PNLNADQR 25 26 NGFIQSLKDDPSQSANVLGEAQKLN 50 51 DSQAPKADAQQNNFNKDQQSA]FYE]I 75 76]L]N]MPNLNEAQRNGFIQSLKDDPSQS 200	N AQHDEAQQNAFÌYÌQVLNMÌPNLNADQR ²⁵ 26 NGFIQSLKDDÌPSQSANVLGEAQKLN ³⁰ UVPD 51 DSQAPKADAQQNNFNKDQQSAFYEI ⁷⁵ 76 LNMÌPNLNEAQRNGFIQSLKDDÌPSQS ¹⁰⁰
HCD N AQHDEAQQNAFYQVLNMPNLNADQR 25 26 NGFIQSLKDDPSQSANVLGEAQKLN 50 51 DSQAPKADAQQNNFNKDQQSAFYE] 75 76 LNMPNLNEAQRNGFIQSLKDDPSQS 100 101 TNVLGEAKKLNESQAPKADNNFNKE 125	N AQHDEAQQNAFÌYÌQVLNMÌPNLNADQR ²⁵ 26 NGFIQSLKDDÌPSQSANVLGEAQKLN ³⁰ UVPD 51 DSQAPKADAQQNNFNKDQQSAFYEI ⁷⁵ 76 LNMÌPNLNEAQRNGFIQSLKDDÌPSQS ¹⁰⁰ 101 TNVLGEAKKLNESQAÌPKADNNFNKE ¹²⁵
HCD N AQHDEAQQNAF]Y]Q]V]L]NM]PNLNADQR N GFIQSLKDDPSQSANVLGEAQKLN D SQAPKADAQQNNFNKDQQSA]FYE]I NMPNLNEAQRNGFIQSLKDDPSQS INVLGEAKKLNESQAPKADNNFNKE 2000NAFYE]]]LNM]PNLNEEQRNGFIOS 2000NAFYE]]]LNM]PNLNEEQRNGFIOS	N AQHDEAQQNAFÌYÌQVLNMÌPNLNADQR ²³ 26 NGFIQSLKDDÌPSQSANVLGEAQKLN ³⁰ UVPD 51 DSQAPKADAQQNNFNKDQQSAFYEI ⁷⁵ 76 LNMÌPNLNEAQRNGFIQSLKDDÌPSQS ²⁰⁰ 201 TNVLGEAKKLNESQAÌPKADNNFNKE ²²⁵ 2020 ONAFYEILNMPNLNEEORNGFIOS ²⁵⁰
HCD N AQHDEAQQNAFYQQVLNMPNLNADQR 25 N GFIQSLKDDPSQSANVLGEAQKLN 50 S1 DSQAPKADAQQNNFNKDQQSAFYE]I 76]LNMPNLNEAQRNGFIQSLKDDPSQS 101 TNVLGEAKKLNESQAPKADNNFNKE 125 126 QQNAFYE]I]LNMPNLNEEQRNGFIQS 120 151 LKDPPSSANLSSAKKLNESQAPK 155 LKDPPSSANLSSAKKLNESQAPK 175	N AQH DE AQQNA FÌYÌQ V L NMÌPN L NADQR ²⁵ 26 NG F I Q S L K D DÌP S Q S AN V L G E AQ K L N ³⁰ UVPD 51 D S Q A P K A D AQQN N F N K D QQ S A F Y E I ⁷⁵ 76 L NMÌPN L N E AQ R N G F I Q S L K D DÌP S Q S ¹⁰⁰ 101 T N V L G E A K K L N E S Q AÌP K A D N N F N K E ¹²⁵ 126 QQN A F Y E I L NMPN L N E E Q R N G F I Q S ¹³⁰ 131 L K D D P S Q S A N L I S F A K K I N F S Q A P K ¹⁷⁵
HCD N AQHDEAQQNAFYQQVLNMPNLNADQR 25 N GFIQSLKDDPSQSANVLGEAQKLN 50 S1 DSQAPKADAQQNNFNKDQQSAFYEJI 75 76]LNMPNLNEAQRNGFIQSLKDDPSQS 100 101 TNVLGEAKKLNESQAPKADNNFNKE 125 126 QQNAFYEJILNMPNLNEEQRNGFIQSS 100 151 LKDDPSQSANLLSEAKKLNESQAPK 150 152 LKDDPSQSANLLSEAKKLNESQAPK 150 154 LKDDPSQSANLLSEAKKLNESQAPK 150	N AQHDEAQQNAFÌYÌQVLNMÌPNLNADQR ²⁵ 26 NGFIQSLKDDÌPSQSANVLGEAQKLN ⁵⁰ UVPD 51 DSQAPKADAQQNNFNKDQQSAFYEI ⁷⁵ 76 LNMÌPNLNEAQRNGFIQSLKDDÌPSQS ¹⁰⁰ 101 TNVLGEAKKLNESQAÌPKADNNFNKE ¹²⁵ 126 QQNAFYEILNMPNLNEEQRNGFIQS ¹⁵⁰ 151 LKDDPSQSANLLSEAKKLNESQAPK ¹⁷⁵ 126 ADNKENKENQNAEYEILHLPNNENE ²⁰⁰
HCD N AQHDEAQQNAFJYQVLJNMJPNLNADQR 25 N GFIQSLKDDPSQSANVLGEAQKLN 50 S1 DSQAPKADAQQNNFNKDQQSAJFYEJI 75 T6]LJNJMPNLNEAQRNGFIQSLKDDPSQS 100 101 TNVLGEAKKLNESQAPKADNNFNKE 125 126 QQNAFYEJJLNMJPNLNEEQRNGFIQS 150 151 LKDDPJSQSANLLSEAKKLNESQAPK 175 176 ADNKFNKEQQNAFYEILHLPNLNEEX	N AQHDEAQQNAFÌYÌQVLNMÌPNLNADQR ²⁵ 26 NGFIQSLKDDÌPSQSANVLGEAQKLN ⁵⁰ UVPD 51 DSQAPKADAQQNNFNKDQQSAFYEI ⁷⁵ 76 LNMÌPNLNEAQRNGFIQSLKDDÌPSQS ¹⁰⁰ 101 TNVLGEAKKLNESQAÌPKADNNFNKE ¹²⁵ 126 QQNAFYEILNMPNLNEEQRNGFIQS ¹⁵⁰ 151 LKDDPSQSANLLSEAKKLNESQAPK ¹⁷⁵ 176 ADNKFNKEQQNAFYEILHLPNLNEE ²⁰⁰
HCD N AQHDEAQQNAFJYQVLJNMJPNLNADQR 25 26 NGFIQSLKDDPSQSANVLGEAQKLN 50 25 DSQAPKADAQQNNFNKDQQSAJFYEJI 75 76 LJNJMPNLNEAQRNGFIQSLKDDPSQS 100 101 TNVLGEAKKLNESQAPKADNNFNKE 125 126 QQNAFYEJJLNMJPNLNEEQRNGFIQS 150 151 LKDDPJSQSANLLSEAKKLNESQAPK 175 176 ADNKFNKEQQNAFYEILHLPNLNEE 200 201 QRNGFIQSLKDDPSQSANLLAEAKK 255	N AQHDEAQQNAFÌYÌQVLNMÌPNLNADQR ²⁵ 26 NGFIQSLKDDÌPSQSANVLGEAQKLN ⁵⁰ UVPD 51 DSQAPKADAQQNNFNKDQQSAFYEI ⁷⁵ 76 LNMÌPNLNEAQRNGFIQSLKDDÌPSQS ¹⁰⁰ 101 TNVLGEAKKLNESQAÌPKADNNFNKE ¹²⁵ 126 QQNAFYEILNMPNLNEEQRNGFIQS ¹⁵⁰ 151 LKDDPSQSANLLSEAKKLNESQAPK ¹⁷⁵ 176 ADNKFNKEQQNAFYEILHLPNLNEE ²⁰⁰ 201 QRNGFIQSLKDDPSQSANLLAEAKK ²⁵⁵
HCD N AQHDEAQQNAF]Y]QÌVÌLÌN MÌPNLNADQR 25 26 NGFIQSLKDDPSQSANVLGEAQKLN 50 51 DSQAPKADAQQNNFNKDQQSAÌFYEÌI 75 76 LÌNÌM PNLNEAQRNGFIQSLKDDPSQS 100 101 TNVLGEAKKLNESQAPKADNNFNKE 125 126 QQNAFYEÌIÌLNÌPNLNEEQRNGFIQS 150 151 LKDDP]SQSANLLSEAKKLNESQAPK 175 176 ADNKFNKEQQNAFYEILHLPNLNEE 200 201 QRNGFIQSLKDDPSQSANLLAEAKK 225 226 LNDAQAPKADNKFNKEQQNAFYEIL 250	N AQHDEAQQNAFÌYÌQVLNMÌPNLNADQR ²⁵ 26 NGFIQSLKDDÌPSQSANVLGEAQKLN ⁵⁰ UVPD 51 DSQAPKADAQQNNFNKDQQSAFYEI ⁷⁵ 76 LNMÌPNLNEAQRNGFIQSLKDDÌPSQS ¹⁰⁰ 101 TNVLGEAKKLNESQAÌPKADNNFNKE ¹²⁵ 126 QQNAFYEILNMPNLNEEQRNGFIQS ¹⁵⁰ 151 LKDDPSQSANLLSEAKKLNESQAPK ¹⁷⁵ 176 ADNKFNKEQQNAFYEILHLPNLNEE ²⁰⁰ 201 QRNGFIQSLKDDPSQSANLLAEAKK ²²⁵ 226 LNDAQAPKADNKFNKEQQNAFYEIL ²⁵⁰
HCD N AQHDEAQQNAF]Y]QÌVÌLÌN MÌPNLNADQR 25 26 NGFIQSLKDDPSQSANVLGEAQKLN 50 51 DSQAPKADAQQNNFNKDQQSAÌFYEÌI 75 76 LÌNÌM PNLNEAQRNGFIQSLKDDPSQS 100 101 TNVLGEAKKLNESQAPKADNNFNKE 125 126 QQNAFYEÌILNMÌPNLNEEQRNGFIQS 150 151 LKDDP]SQSANLLSEAKKLNESQAPKA 152 LKDDP]SQSANLLSEAKKLNESQAPK 175 176 ADNKFNKEQQNAFYEILHLPNLNEE 200 201 QRNGFIQSLKDDPSQSANLLAEAKK 225 226 LNDAQAPKADNKFNKEQQNAFYEIL 250 251 HLPNLTEEQRNGFIQSLKDDPSVSK 275	N AQHDEAQQNAFÌYÌQVLNMÌPNLNADQR 25 26 NGFIQSLKDDÌPSQSANVLGEAQKLN 50 UVPD 51 DSQAPKADAQQNNFNKDQQSAFYEI 75 76 LNMÌPNLNEAQRNGFIQSLKDDÌPSQS 100 101 TNVLGEAKKLNESQAÌPKADNNFNKE 125 126 QQNAFYEILNMPNLNEEQRNGFIQS 180 151 LKDDPSQSANLLSEAKKLNESQAPK 175 176 ADNKFNKEQQNAFYEILHLPNLNEE 200 201 QRNGFIQSLKDDPSQSANLLAEAKK 225 226 LNDAQAPKADNKFNKEQQNAFYEIL 250 251 HLPNLTEEQRNGFIQSLKDDPSVSK 275
HCD N AQHDEAQQNAF]Y]QÌVÌLÌN MÌPNLNADQR 25 26 NGFIQSLKDDPSQSANVLGEAQKLN 50 51 DSQAPKADAQQNNFNKDQQSAÌFYEÌI 75 76 LÌNÌM PNLNEAQRNGFIQSLKDDPSQS 100 101 TNVLGEAKKLNESQAPKADNNFNKE 125 126 QQNAFYEÌILNÌPNLNEEQRNGFIQS 150 151 LKDDP]SQSANLLSEAKKLNESQAPK 175 176 ADNKFNKEQQNAFYEILHLPNLNEE 200 201 QRNGFIQSLKDDPSQSANLLAEAKK 225 226 LNDAQAPKADNKFNKEQQNAFYEI 250 251 HLPNLTEEQRNGFIQSLKDDPSVSK 275 276 EILAEAKKLNDAQAPKEEDNNKPI 200	N A Q H D E A Q Q N A F Y Q V L N M P N L N A D Q R 25 26 N G F I Q S L K D D P S Q S A N V L G E A Q K L N 50 UVPD 51 D S Q A P K A D A Q Q N N F N K D Q Q S A F Y E I 75 76 L N M P N L N E A Q R N G F I Q S L K D D P S Q S 100 101 T N V L G E A K K L N E S Q A P K A D N N F N K E 125 126 Q Q N A F Y E I L N M P N L N E E Q R N G F I Q S 150 151 L K D D P S Q S A N L L S E A K K L N E S Q A P K 175 176 A D N K F N K E Q Q N A F Y E I L H L P N L N E E 200 201 Q R N G F I Q S L K D D P S Q S A N L L A E A K K 225 226 L N D A Q A P K A D N K F N K E Q Q N A F Y E I L 250 221 H L P N L T E E Q R N G F I Q S L K D D P S V S K 275 276 E I L A E A K K L N D A Q A P K E E D N N K P I E 300
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HCD N A Q H D E A Q Q N A F Y Q V L N M P N L N A D Q R 25 N G F I Q S L K D D P S Q S A N V L G E A Q K L N 50 S D S Q A P K A D A Q Q N N F N K D Q Q S A F Y E I I 75 T L N M P N L N E A Q R N G F I Q S L K D D P S Q S A S D S Q A P K A D A Q Q N N F N K D Q Q S A F Y E I I T N V L G E A K K L N E S Q A P K A D N N F N K E 125 Q Q N A F Y E I I L N M P N L N E E Q R N G F I Q S 1 S L K D D P S Q S A N L L S E A K K L N E S Q A P K A D N N F N K E 125 Q R N G F I Q S L K D D P S Q S A N L L A E A K K 225 24 Q N A F Y E I C L H L P N L N E E 200 25 Q R N G F I Q S L K D D P S Q S A N L L A E A K K 225 26 L N D A Q A P K A D N K F N K E Q N A F Y E I L H L P N L N E E 200 25 H L P N L T E E Q R N G F I Q S L K D D P S V S K 275 27 E E I L A E A K K L N D A Q A P K E E D N N K P I E 300 30 G R N S R G S V D A S E L T P A V T T Y K L V I N 325 32 G G K T L K G E T T T E A V D A A T A E K V F K Q 350	N A Q H D E A Q Q N A FÌYÌQ V L N MÌP N L N A D Q R 25 N G F I Q S L K D DÌP S Q S A N V L G E A Q K L N 30 UVPD L D S Q A P K A D A Q Q N N F N K D Q Q S A F Y E I 75 L N MÌP N L N E A Q R N G F I Q S L K D DÌP S Q S 200 L N N V L G E A K K L N E S Q AÌP K A D N N F N K E 25 Q N A F Y E I L N M P N L N E E Q R N G F I Q S 20 L K D D P S Q S A N L S E A K K L N E S Q A P K 175 L K D D P S Q S A N L S E A K K L N E S Q A P K 175 L G R N G F I Q S L K D D P S Q S A N L L A E A K K 225 L N D A Q A P K A D N K F N K E Q N A F Y E I L 230 L N D A Q A P K A D N K F N K E Q N A F Y E I L 250 L N D A Y Y Y Y Y Y Y Y Y Y Y Y Y Y Y Y Y Y
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Figure 7: Sequence coverage maps for each of 4 proteins analyzed by each of the 4 modes of dissociation. Each map represents the results from the spectra to the left with the best P-score.

DISCUSSION

The versatility afforded by the Orbitrap Fusion Lumos instrument with respect to the multiple types of available dissociation modes is a clear advantage for top-down analyses. Additionally, the Pierce Intact Protein Standard Mix provides an ideal sample for method development (both data acquisition and data analysis) and quality control. In using this sample for method optimization we have highlighted both strengths and weaknesses of our current technology. We are able to obtain extensive sequence coverage for the proteins in the sample up to 30kDa on a chromatographic time scale. We do, however, still struggle with MS2 analysis of larger proteins. Multiple challenges contribute to this problem. First, by both ETD and UVPD, larger proteins dissociate much faster than smaller proteins, whether due to their higher charge state, or higher cross section, respectively. In this work, we decreased the anion target value in an attempt to reduce ETD reaction rate (the kinetics of the ETD reaction as we perform it here are first order with respect to anion concentration) and minimize over fragmentation of Protein AG (50kDa), though this helped only marginally. Other ion manipulation techniques such as ion parking have been shown to address this problem. Second, larger proteins can of course fragment at more positions, thereby diluting signal among more potential product ions. CID and HCD benefit here from preferential fragmentation at weaker bonds, concentrating signal to fewer possible product ions. Because ETD and UVPD are democratic in their bond cleavage, this is a significant challenge that currently can only be overcome with significant



Figure 8: ETD and UVPD of enolase (~46kDa); ~500 averaged transients.

CONCLUSIONS

- The multiple modes of dissociation available on the Orbitrap Fusion Lumos instrument present a clear advantage for intact protein identification and characterization, enabling extensive sequence coverage and PTM mapping capabilities.
- The Pierce Intact Protein Standard Mix is an ideal sample for top-down method development, optimization, and quality control.
- Many challenges remain in top-down analysis, particularly with respect to large proteins. We are actively working to address these.

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Figure 8 demonstrates the efficiency of ETD and UVPD on enolase, a 46kDa protein, when ~500 transients are averaged. An added challenge presented by over fragmentation is the production of internal ions. These low abundance, unresolved overlapping product ions create a high baseline that varies across the m/z range. Deconvolution algorithms generally use the averagine model to assign monoisotopic mass, but the large number of overlapping peaks confound such algorithms due to experimental isotopic distributions that deviate too far from theoretical. We continue to work toward addressing these challenges.

signal averaging.