Supplemental Materials for

Improved discrimination of asymmetric and symmetric protein arginine dimethylation by optimizing the normalized collision energy in LC-MS proteomics

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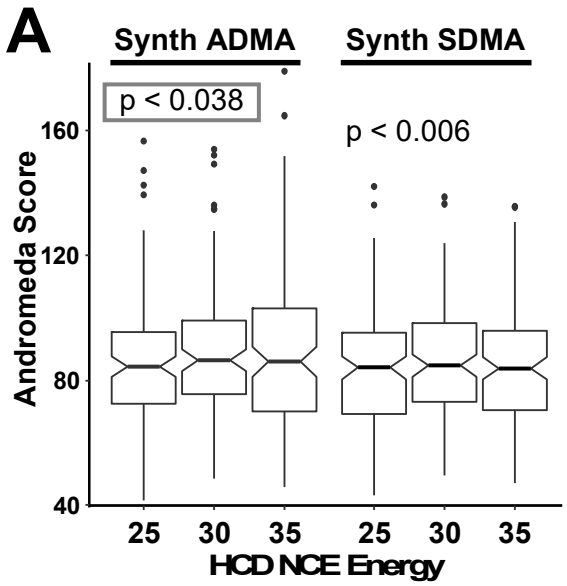

Figure S1. Higher NCE improves andromeda scores of synthetic ADMA and SDMA peptides

Boxplots of the andromeda scores of synthetic dimethyl peptides fragmented at each NCE energy. The difference in scores at 25 and 30 were significant by Student's paired t-test with $p < 0.038$ for ADMA and $p < 0.006$ for SDMA. MaxQuant was configured to consider neutral losses as part of its scoring algorithm, which occur more frequently at higher NCEs.

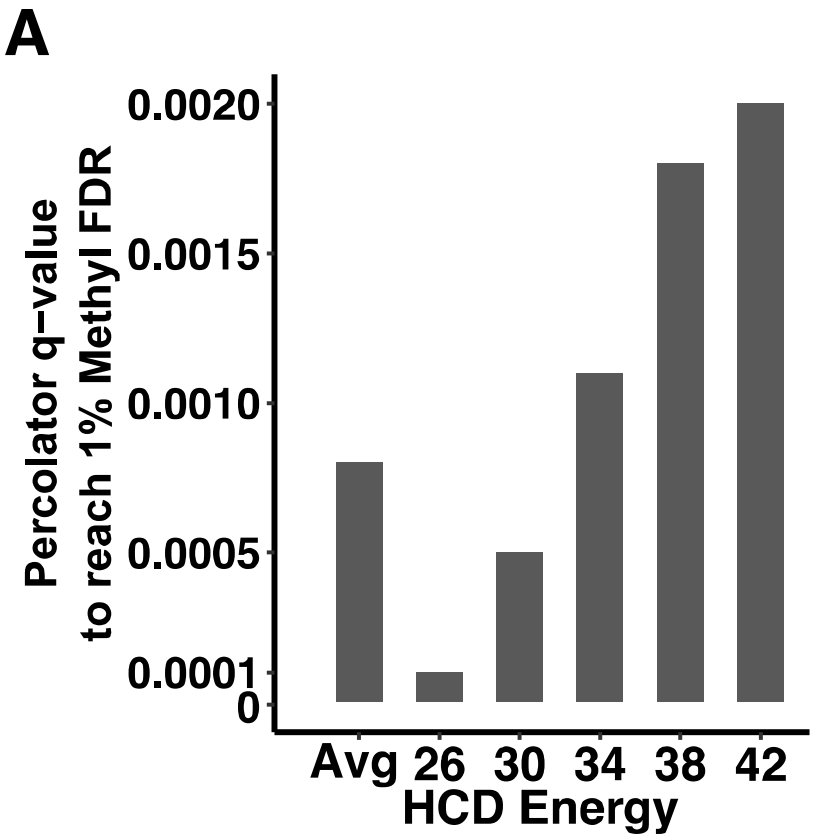

Figure S2. Higher NCE improves methyl PSM false discovery rate by increasing the percolator q-value needed to reach a 1% methyl FDR

Barchart of the q-value from the Percolator node in Proteome Discoverer 2.2 needed to attain a 1% methyl FDR for each energy. The q-values of the decoy PSMs from the "Decoy PSMs" tab of Proteome Discoverer were extracted and used to estimate the methyl FDR from the q-values in the target dataset. Higher NCEs reduced the number of methyl decoys present, leading to improved q-values for the 1% methyl FDR cutoff. For reference a percolator q-value of 0.01 represents a 1% peptide FDR which is not strict enough for methyl peptides which suffer from higher rates of FDR (1).

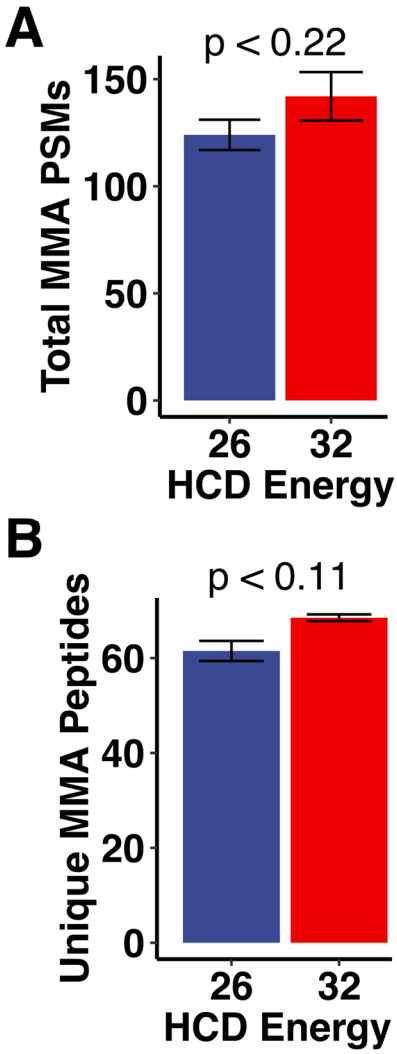

Figure S3: **Increased identification of MMA peptides at optimized NCE.**

A) Total MMA peptide spectrum matches (PSMs) from all SCX fractions fragmented at 26 and 32 NCE. Mean +/- standard deviation of two biological replicates is shown. Methyl peptides were removed until a 1% methyl-FDR was achieved for each fragmentation energy. **B)** Unique MMA peptides from all SCX fractions fragmented at 26 and 32 NCE. Only peptides with MMA sites and without DMA sites were counted in panels A & B.

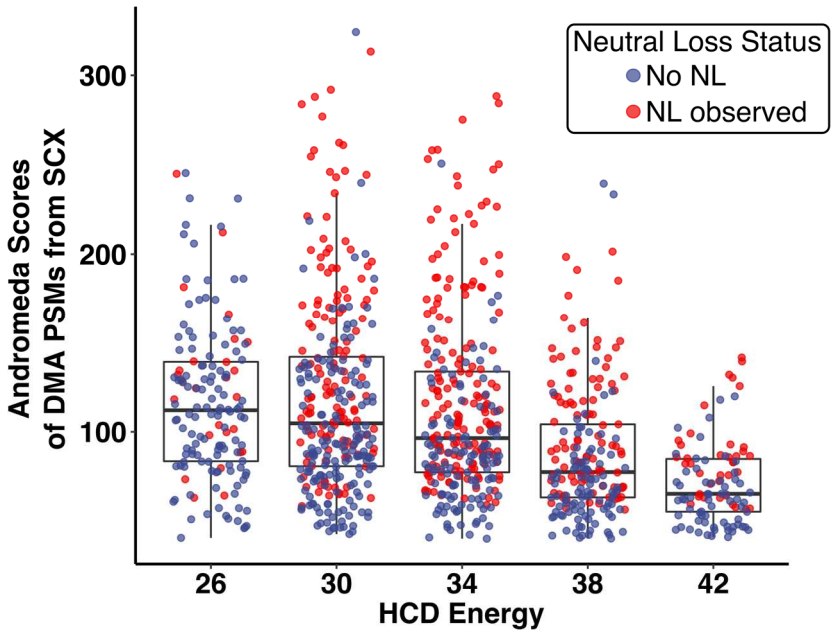

Figure S4: **Increased Andromeda scores at higher NCE for peptides with and without neutral loss.**

Boxplot of Andromeda scores of dimethyl arginine PSMs fragmented at the specified NCE. Points are colored by whether the spectrum contained neutral losses of ADMA/SDMA (red) or not (blue). Data from SCX fraction three with two biological replicates for each NCE.

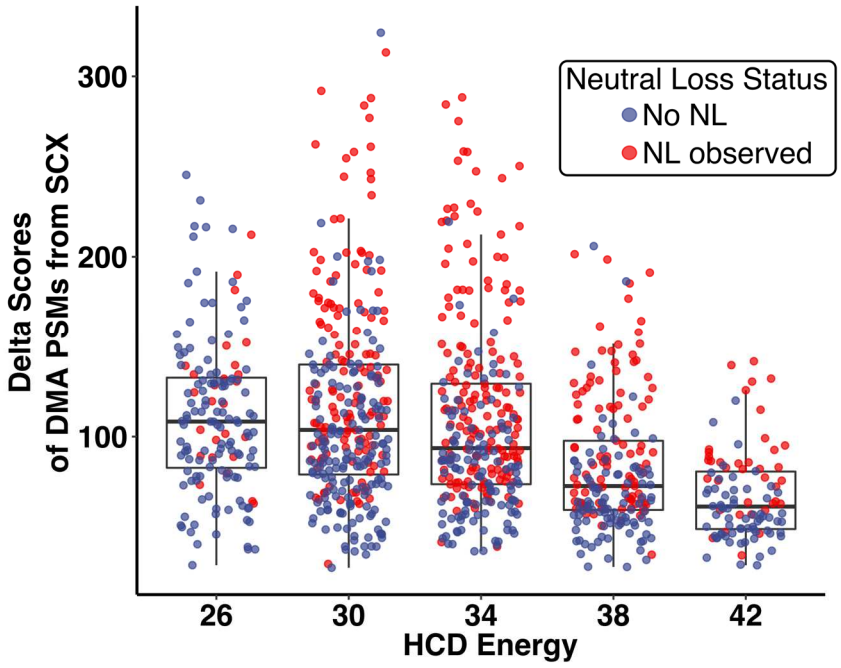

Figure S5: **Increased Andromeda delta scores at higher NCE for peptides with and without neutral loss.**

Boxplot of Andromeda delta scores of dimethyl arginine PSMs fragmented at the specified NCE. Points are colored by whether the spectrum contained neutral losses of ADMA/SDMA (red) or not (blue). Data from SCX fraction three with two biological replicates for each NCE.

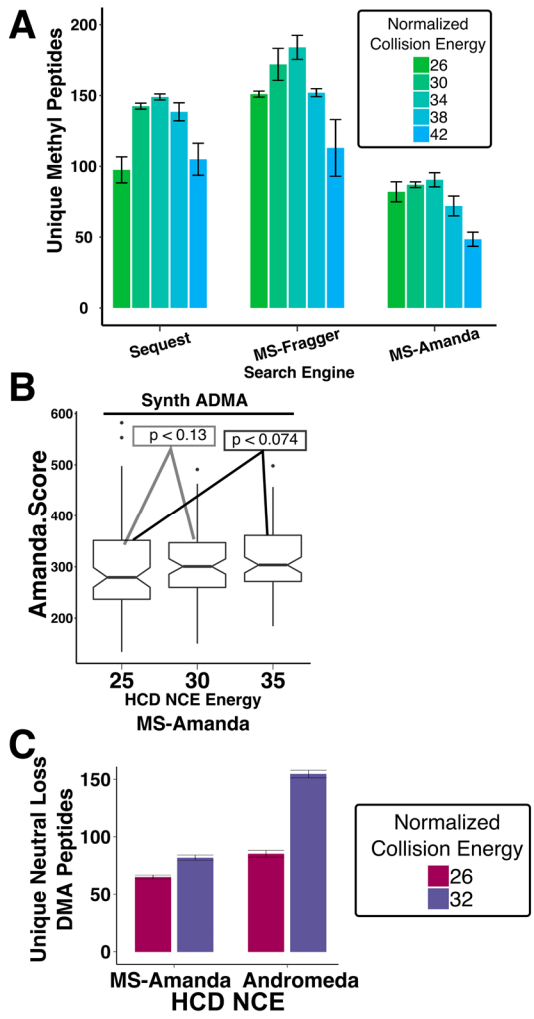

Figure S6: Search of data with alternative search engines.

A) Unique methyl peptides identified across a range of NCEs from SCX fraction 3, searched using multiple search engines. A 1% methyl FDR was achieved for each collision energy individually. **B)** Amanda Scores of Synthetic ADMA peptides at 25, 30, and 35 NCE searched using MS-Amanda. A student's paired t-test was used to assess statistical significance. **C)** Endogenous methyl peptides showing neutral loss from SCX fraction 3, fragmented at 26 and 32 NCE, searched using MS-Amanda and Andromeda.

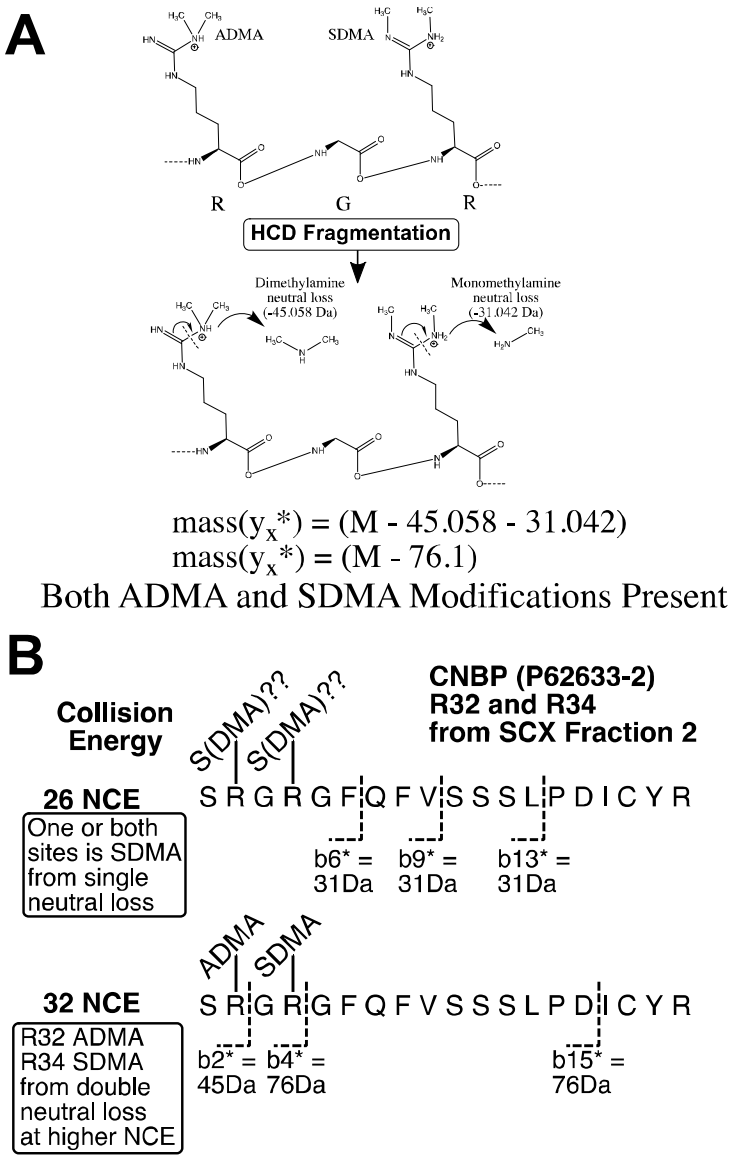

Figure S7: Optimized NCE enables ADMA/SDMA assignment of a doubly dimethylated peptide.

A) Mechanism of double neutral loss where a peptide harboring both ADMA and SDMA can simultaneously exhibit neutral loss of the dimethylamine from ADMA and monomethylamine from SDMA. The resulting deficit mass of the ion is 76.1 Da which, if observed indicates a “mixed” peptide that must include both modifications. **B)** A real peptide with two dimethyl sites was fragmented at 26 and 32 NCE. At 26 NCE, only

single neutral loss ions were observed (b6, b9, and b13), and we were unable to assign either dimethyl site since both sites could generate a loss of 31 Da. At NCE 32, the same peptide showed a double neutral loss occurring on b4 and b15 with a loss of 76 Da. This information combined with the neutral loss of b2 (45 Da) allowed assignment of R32 as ADMA and R34 as SDMA.

Supplemental References

1. Hart-Smith, G., Yagoub, D., Tay, A. P., Pickford, R., and Wilkins, M. R. (2016) Large Scale Mass Spectrometry-based Identifications of Enzyme-mediated Protein Methylation Are Subject to High False Discovery Rates. *Mol. Cell. Proteomics*. **15**, 989–1006