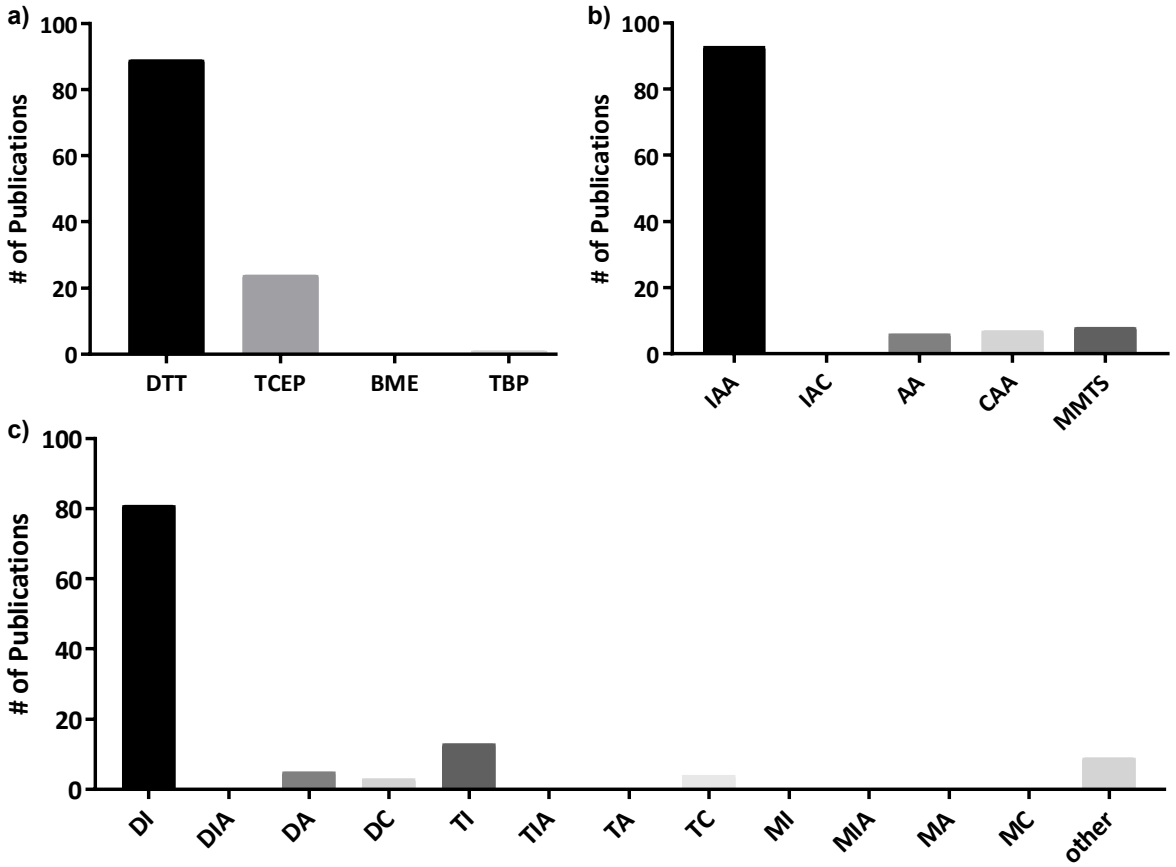
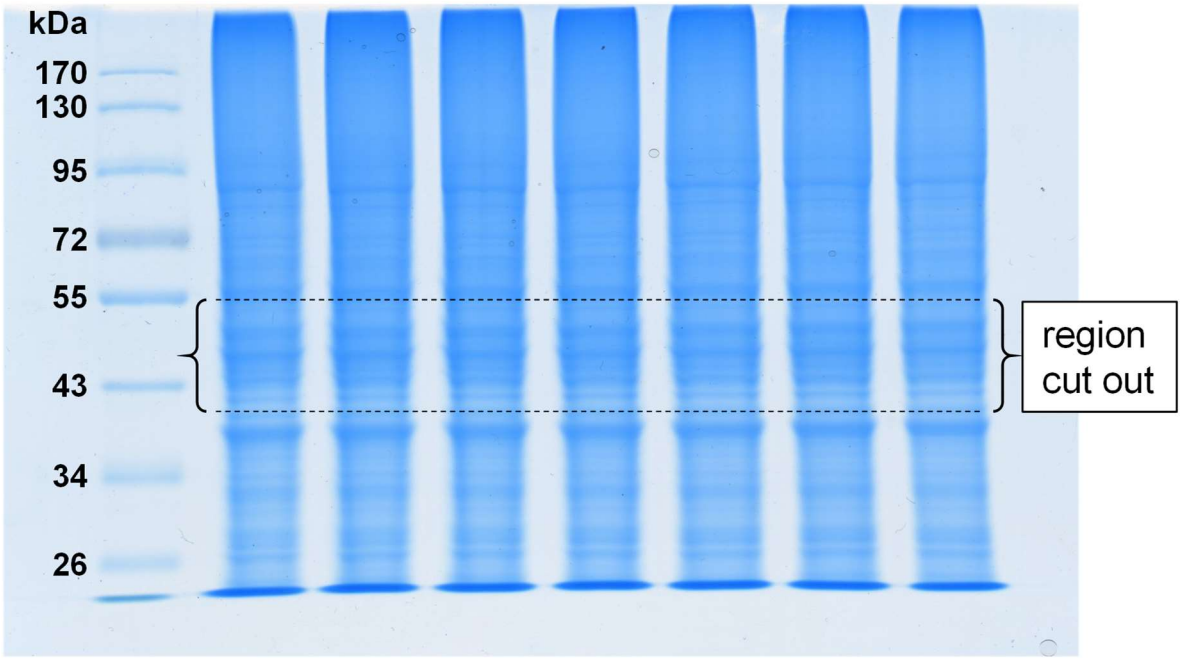


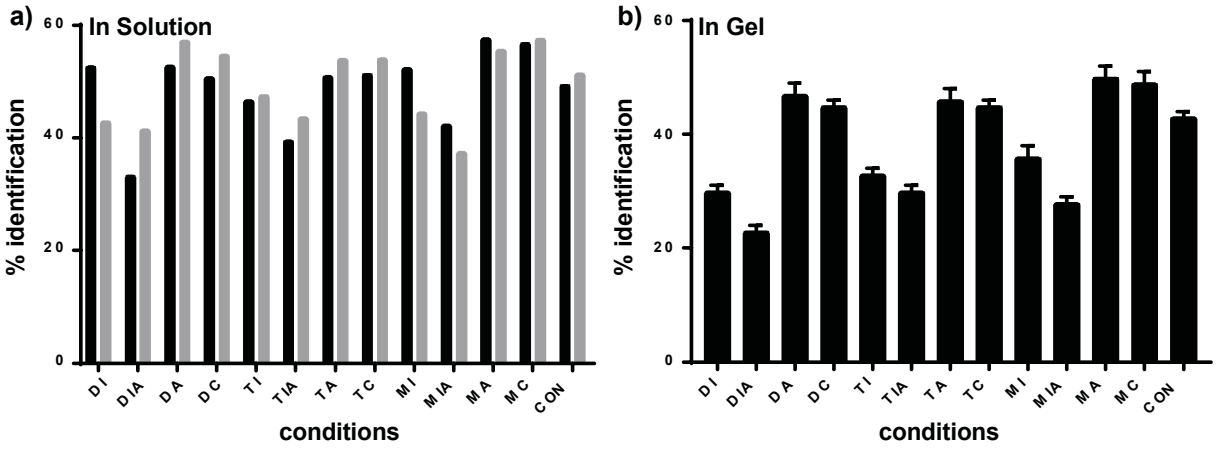
Supplementary Figure 1: Results from a literature survey investigating reduction and alkylation reagents used in proteomics studies: a) frequency of reduction reagents used; b) frequency of alkylation reagents used; c) frequency of combinations of reduction and alkylation reagents used ("other" refers to combinations not included in the indicated conditions, for details see Supplementary Table 1). Shown are the combined data extracted from 63 publications in Journal of Proteome Research and 51 publications from Molecular and Cellular Proteomics in 2016/2017 (for single references and numbers see Supplementary Table 1). DI: DTT+IAA, DIA: DTT+IAC, DA: DTT+AA, DC: DTT+CAA, TI: TCEP+IAA, TIA: TCEP+IAC, TA: TCEP+AA, TC: TCEP+CAA, BI: BME+IAA, BIA: BME+IAC, BA: BME+AA, BC: BME+CAA.

Dataset	Modifications & Amino Acid Specificity	Suppl.Table
In-Solution / In-Gel	carbamidomethyl, carboxymethyl, and propionamide (C); Oxidation (M)	2 / 3
In-Gel	carbamidomethyl, carboxymethyl, and propionamide (all amino acids); Oxidation (M)	4
In-Solution / In-Gel	carbamidomethyl, carboxymethyl, and propionamide (C, Y, S, D, T, H, E, K, N-term); oxidation (M)	5 / 6
In-Solution / In-Gel	carbCarb, coxCox, and propProp (C, Y, S, D, T, H, E, K, N-term); oxidation (M)	7 / 8
In-Gel	dimethylation (K, N-term); carbamidomethyl, carboxymethyl, and propionamide (C); oxidation (M)	9
In-Gel	250 Da Mass Tolerance: carbamidomethyl, carboxymethyl, and propionamide (C); oxidation (M)	10
In-Solution / In-Gel	dethiomethyl (M); carbamidomethyl, carboxymethyl, and propionamide (C); oxidation (M)	11 / 12
In-Gel	dethiomethyl (M); carbamidomethyl, carboxymethyl, and propionamide (C, M); oxidation (M)	13

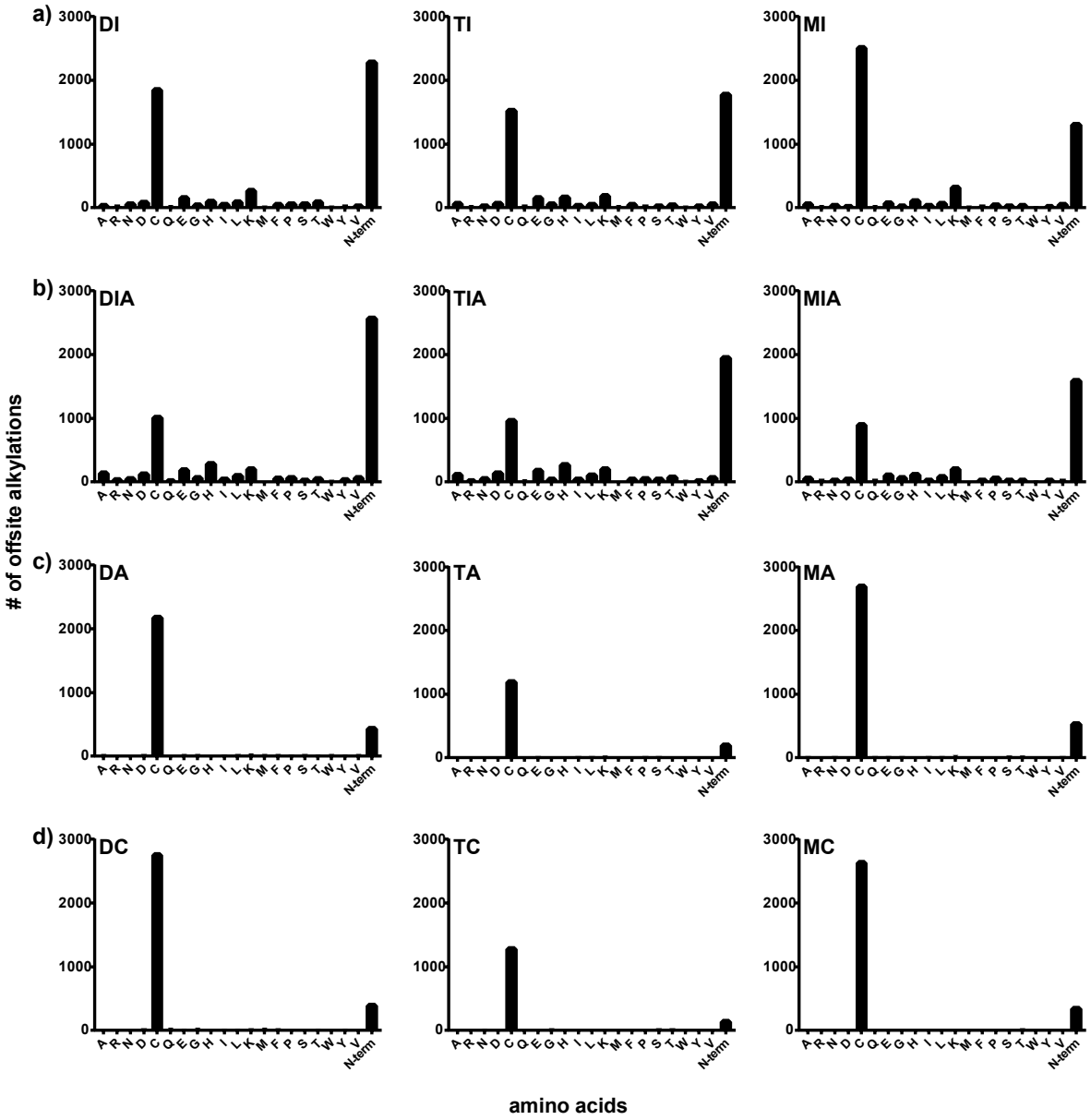
Supplementary Figure 2: Overview of the parameters used for Mascot database searching. Combinations of modifications as well as the defined amino acid specificity settings used for Mascot searches are shown, as well as the corresponding supplementary table. All modifications were searched as variable with the exception of alkylation of cysteine in supplementary tables 5, 6, and 10 in which it was set as fixed. The dimers of the respective alkylation-induced modifications were termed CarbCarb (dimer of 2 carbamidomethyl adducts), CoxCox (dimer of 2 carboxymethyl adducts), and PropProp (dimer of 2 propionamide adducts). For multi stage activation (MSA) scans, neutral losses of 105 (IAA and CAA), 106 (IAC), and 119 (AA) were defined.



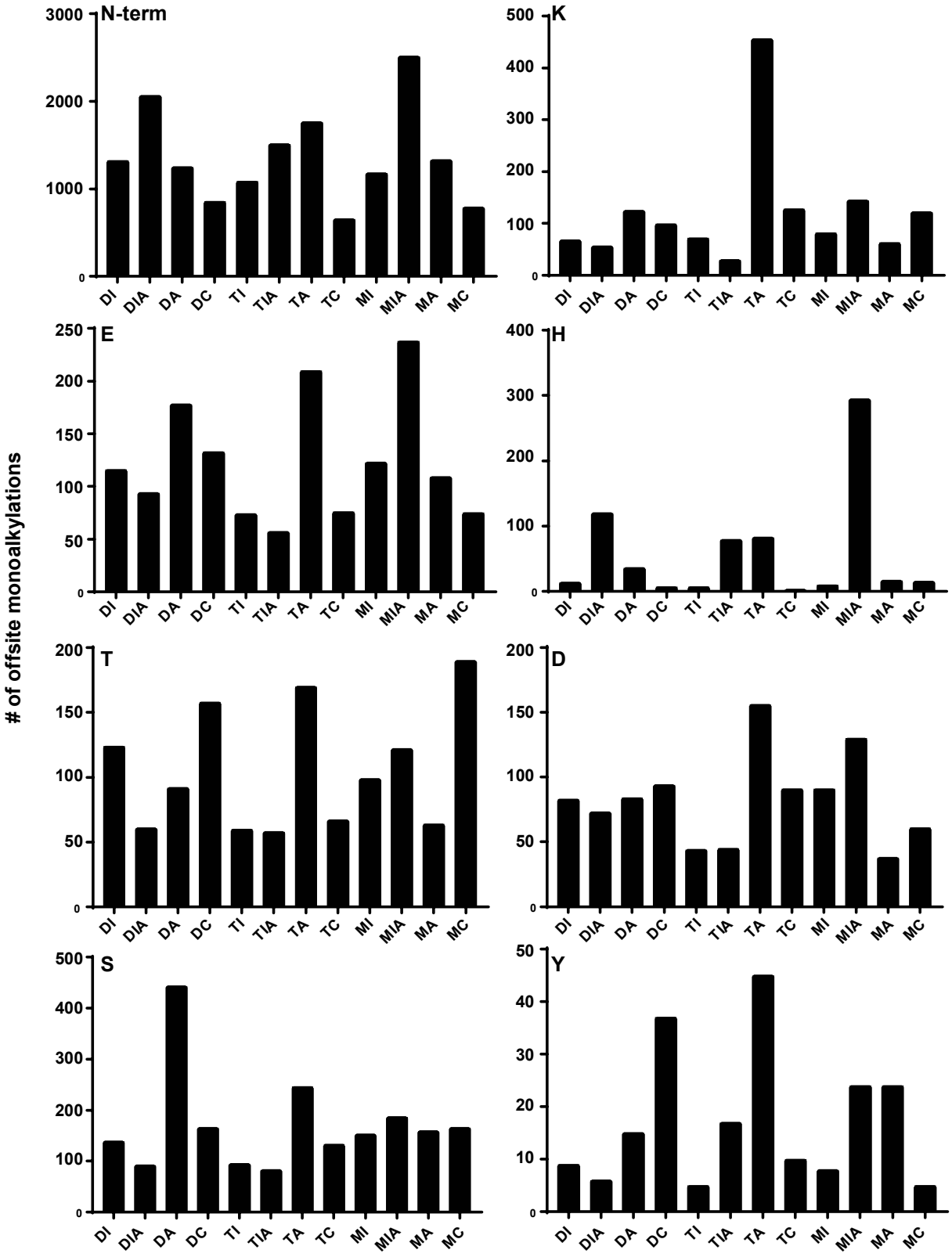
Supplementary Figure 3: Representative SDS-PAGE gel of 50 µg HeLa cytosolic fraction. The gel was stained with Coomassie brilliant blue. The area within the black bars was cut, reduced, and alkylated, and digested in-gel.



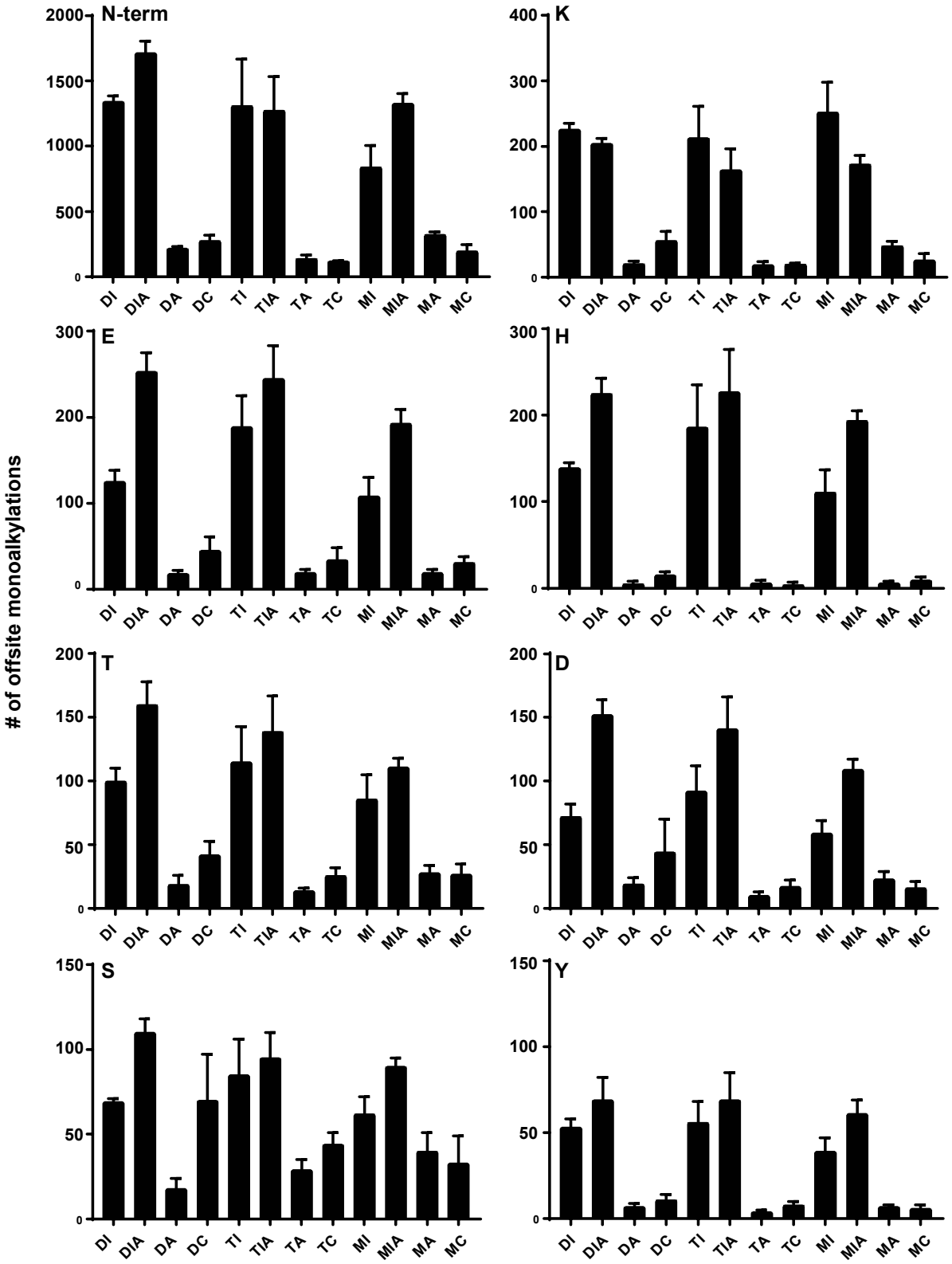
Supplementary Figure 4: Combined results of the analyses of 2 independent replicates of in-solution and 6 replicates of in-gel digested cytosolic fractions of HeLa cells using standard modifications for database searching. a) percentage of identified spectra for in-solution digests; b) percentage of identified spectra for in-gel digests (shown are average values + standard deviation). DI: DTT+IAA, DIA: DTT+IAC, DA: DTT+AA, DC: DTT+CAA, TI: TCEP+IAA, TIA: TCEP+IAC, TA: TCEP+AA, TC: TCEP+CAA, BI: BME+IAA, BIA: BME+IAC, BA: BME+AA, BC: BME+CAA.



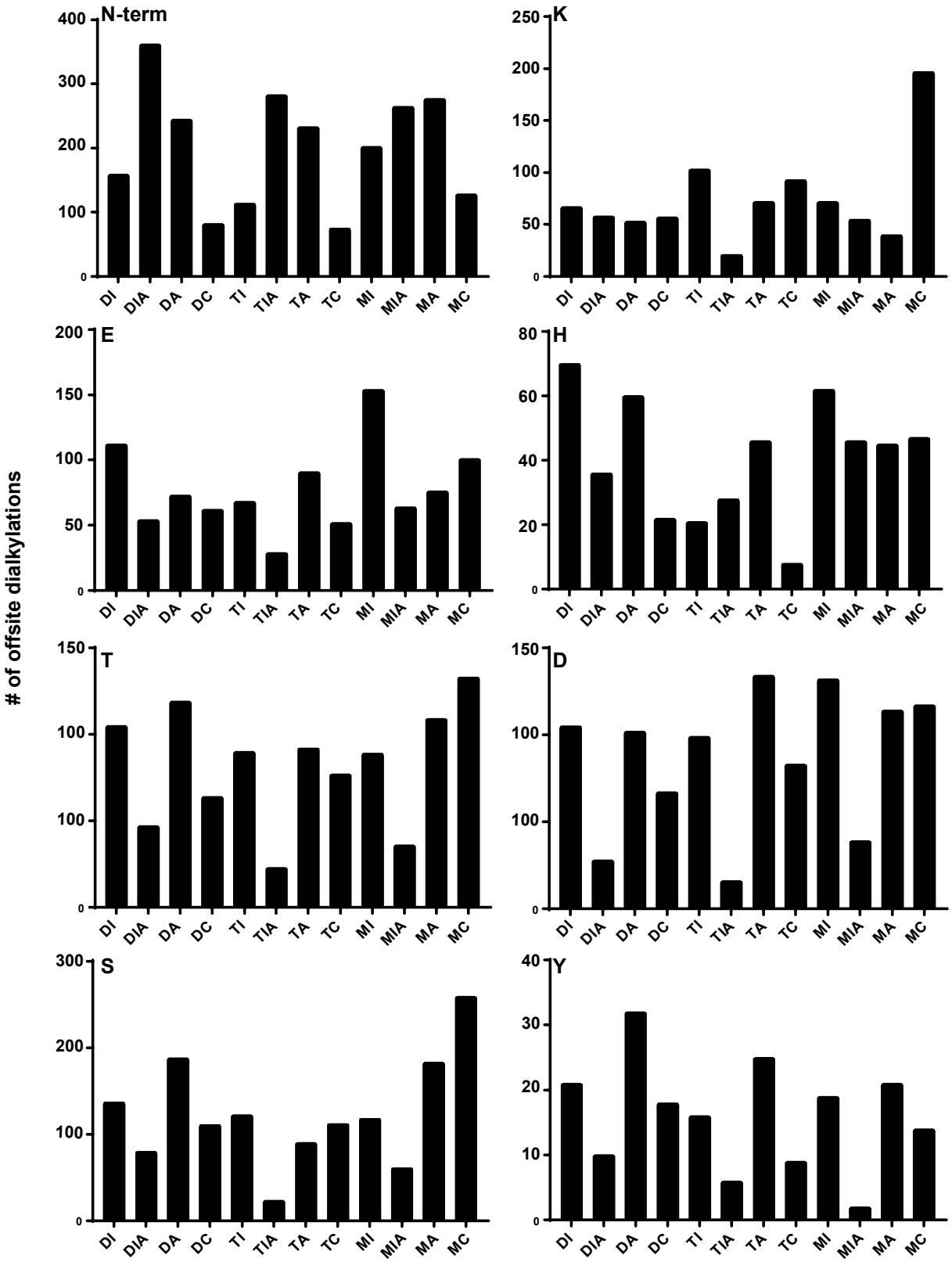
Supplementary Figure 5: Combined results of the analyses of 2 independent replicates of in-gel digested cytosolic fractions of HeLa cells allowing for alkylation at all amino acid residues. The number of offsite alkylation at the respective amino acids is shown for a) Iodoacetamide (DI: DTT+IAA, TI: TCEP+IAA, MI: BME+IAA); b) Iodoacetic acid (DIA: DTT+IAC, TIA: TCEP+IAC, MIA: BME+IAC); c) Acrylamide (DA: DTT+AA, TA: TCEP+AA, MA: BME+AA); and d) Chloroacetamide (DC: DTT+CAA, TC: TCEP+CAA, MC: BME+CAA).



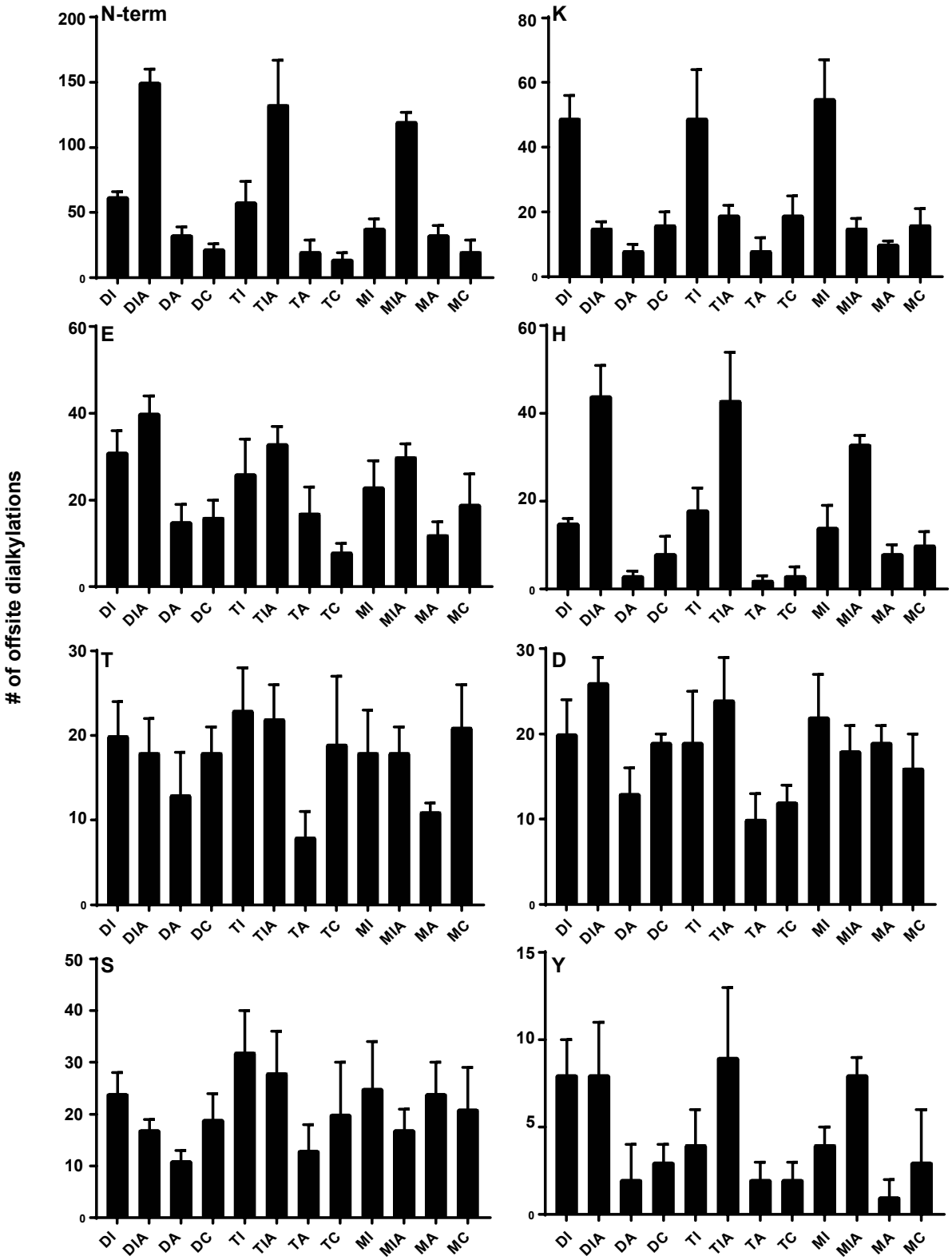
Supplementary Figure 6: Combined results of the analyses of 2 independent replicates of in-solution digested and SAX fractionated cytosolic fractions of HeLa cells using alkylation at the peptide N-terminus, K, E, H, T, D, S, and Y as variable modification as well as alkylation at C as fixed modification for database searching. DI: DTT+IAA, DIA: DTT+IAC, DA: DTT+AA, DC: DTT+CAA, TI: TCEP+IAA, TIA: TCEP+IAC, TA: TCEP+AA, TC: TCEP+CAA, BI: BME+IAA, BIA: BME+IAC, BA: BME+AA, BC: BME+CAA.



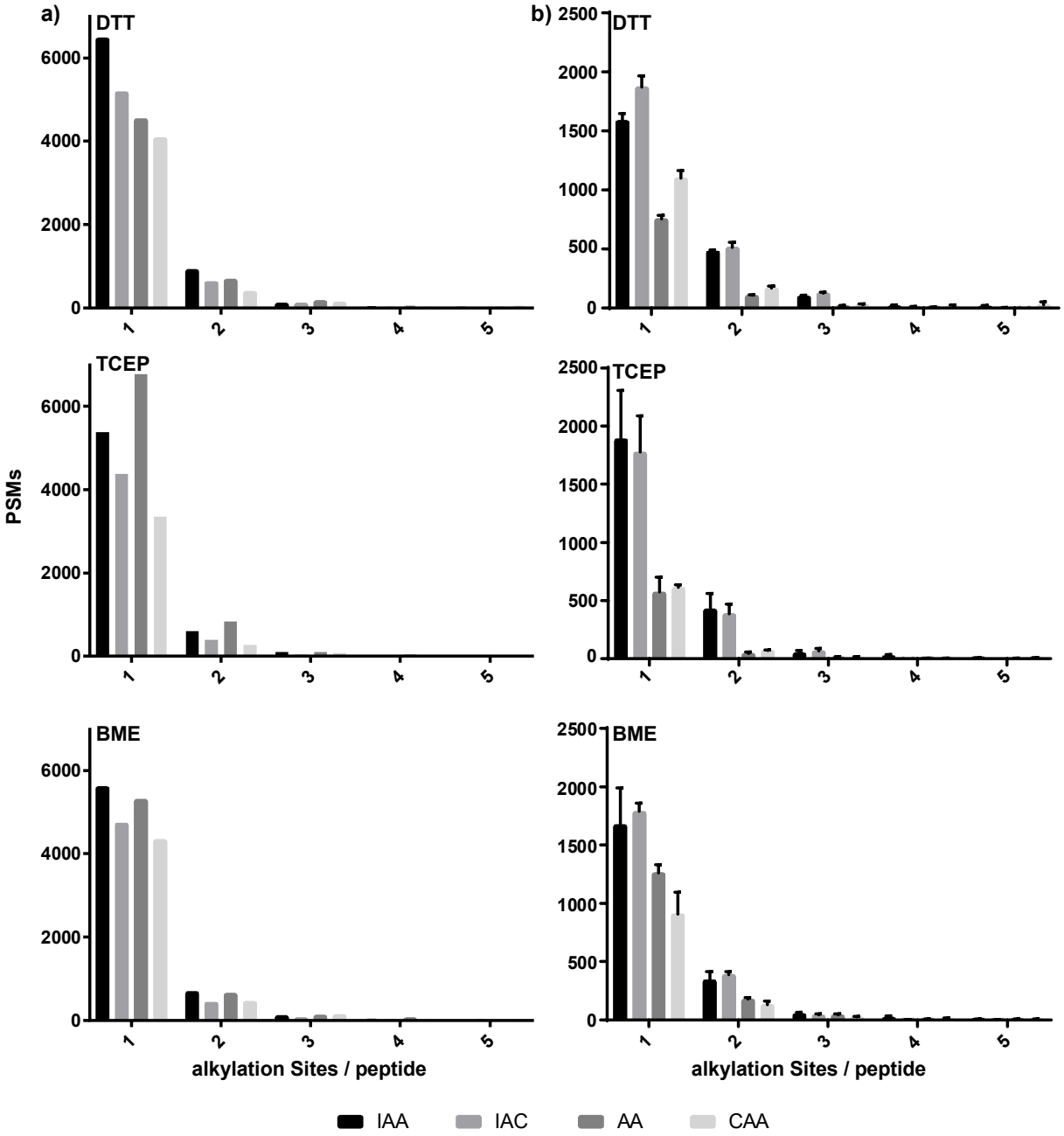
Supplementary Figure 7: Shown are average values + standard deviation of the analyses of 6 replicates of in-gel digested cytosolic fractions of HeLa cells using alkylation at the peptide N-terminus, K, E, H, T, D, S, and Y as variable modification as well as alkylation at C as fixed modification for database searching. DI: DTT+IAA, DIA: DTT+IAC, DA: DTT+AA, DC: DTT+CAA, TI: TCEP+IAA, TIA: TCEP+IAC, TA: TCEP+AA, TC: TCEP+CAA, BI: BME+IAA, BIA: BME+IAC, BA: BME+AA, BC: BME+CAA.



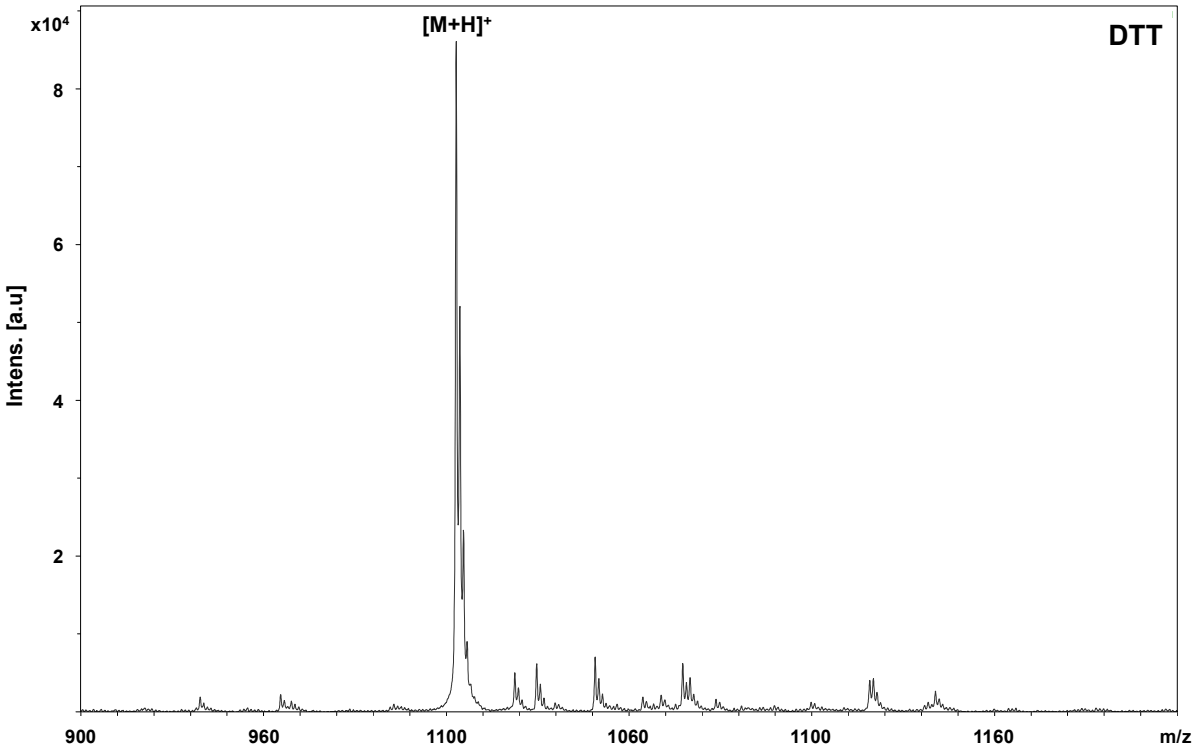
Supplementary Figure 8: Combined results of the analyses of 2 independent replicates of in-solution and SAX fractionated cytosolic fractions of HeLa cells using alkylation reagent dimers at the peptide N-terminus, K, E, H, T, D, S, and Y as variable modification for database searching. DI: DTT+IAA, DIA: DTT+IAC, DA: DTT+AA, DC: DTT+CAA, TI: TCEP+IAA, TIA: TCEP+IAC, TA: TCEP+AA, TC: TCEP+CAA, BI: BME+IAA, BIA: BME+IAC, BA: BME+AA, BC: BME+CAA.



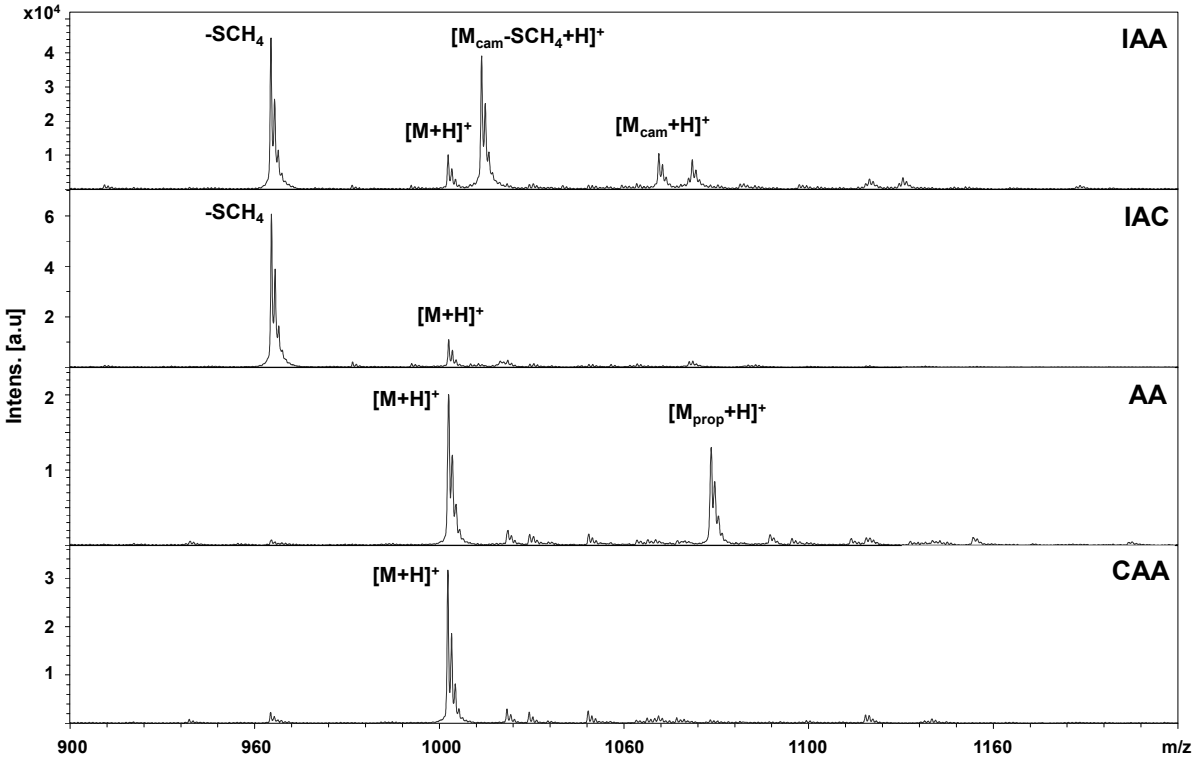
Supplementary Figure 9: Shown are average values + standard deviation of the analyses of 6 replicates of in-gel digested cytosolic fractions of HeLa cells using alkylation reagent dimers at the peptide N-terminus, K, E, H, T, D, S, and Y as variable modification for database searching. DI: DTT+IAA, DIA: DTT+IAC, DA: DTT+AA, DC: DTT+CAA, TI: TCEP+IAA, TIA: TCEP+IAC, TA: TCEP+AA, TC: TCEP+CAA, BI: BME+IAA, BIA: BME+IAC, BA: BME+AA, BC: BME+CAA.



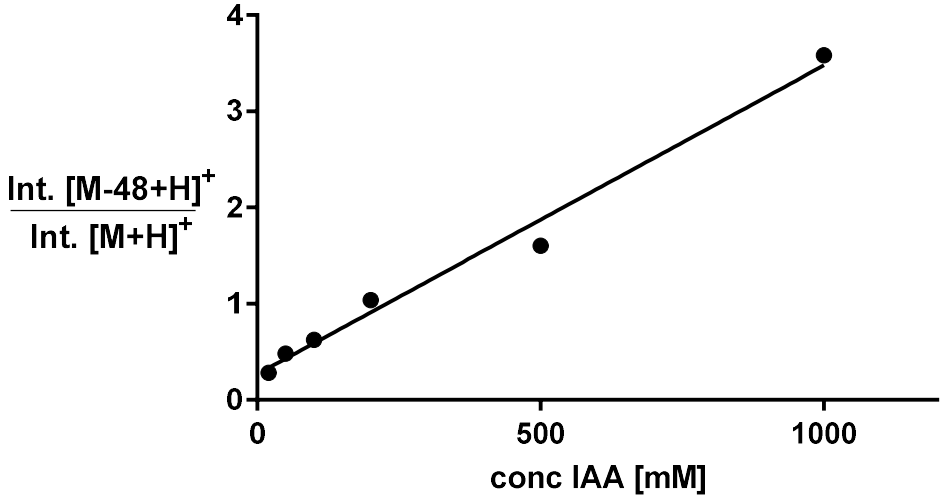
Supplementary Figure 10: Combined results for the analyses of 2 independent replicates of in-solution and 6 replicates + standard deviation of in-gel digested cytosolic fractions of HeLa cells allowing for alkylation at Y, S, D, T, H, E, K, and the peptide N-terminus for database searching. The number of alkylation sites per peptide is shown for a) in-solution digests and b) in-gel digests reduced with DTT, TCEP, and BME, and alkylated with IAA, IAC, AA, and CAA respectively.



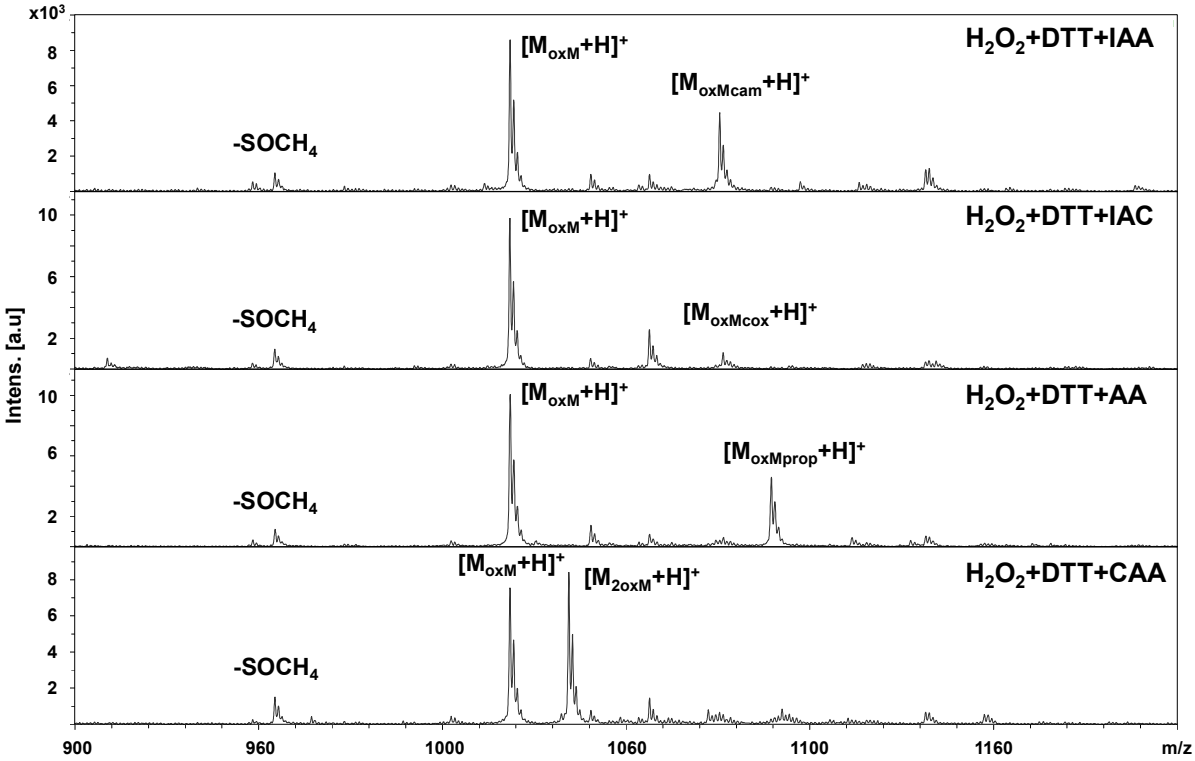
Supplementary Figure 11: MALDI MS measurement of the synthetic peptide APEIMLNSK incubated with DTT. No modification of the peptide due to DTT treatment was observed.



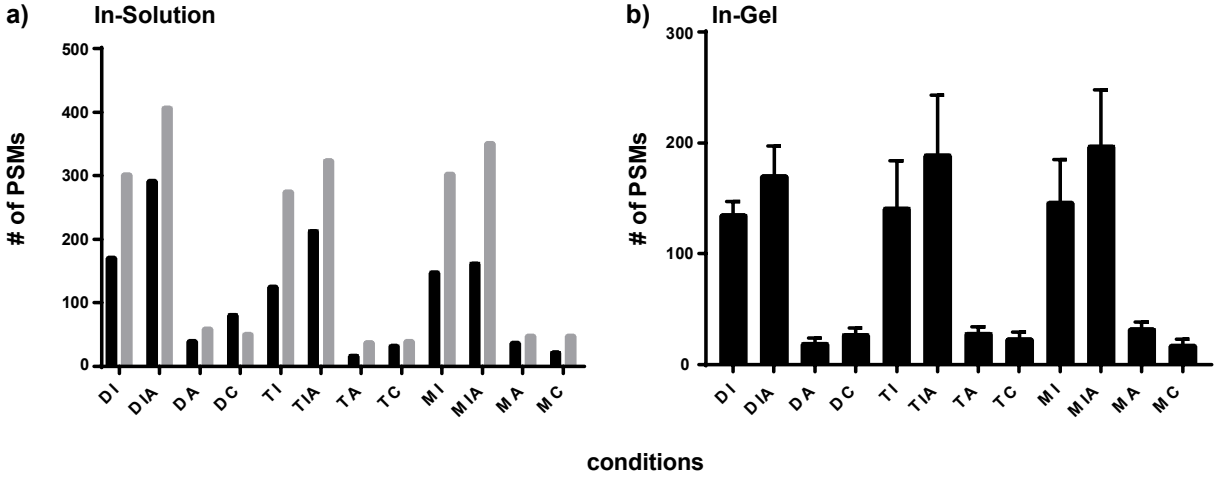
Supplementary Figure 12: MALDI MS measurements of the synthetic peptide APEIMLNSK with IAA, IAC, AA, and CAA without prior incubation by DTT. In combination to treatment after reduction by DTT higher levels of off-target alkylation were observed. "-SCH4": loss of the methionine side chain; cam: carbamidomethylation; prop: propionamide modification



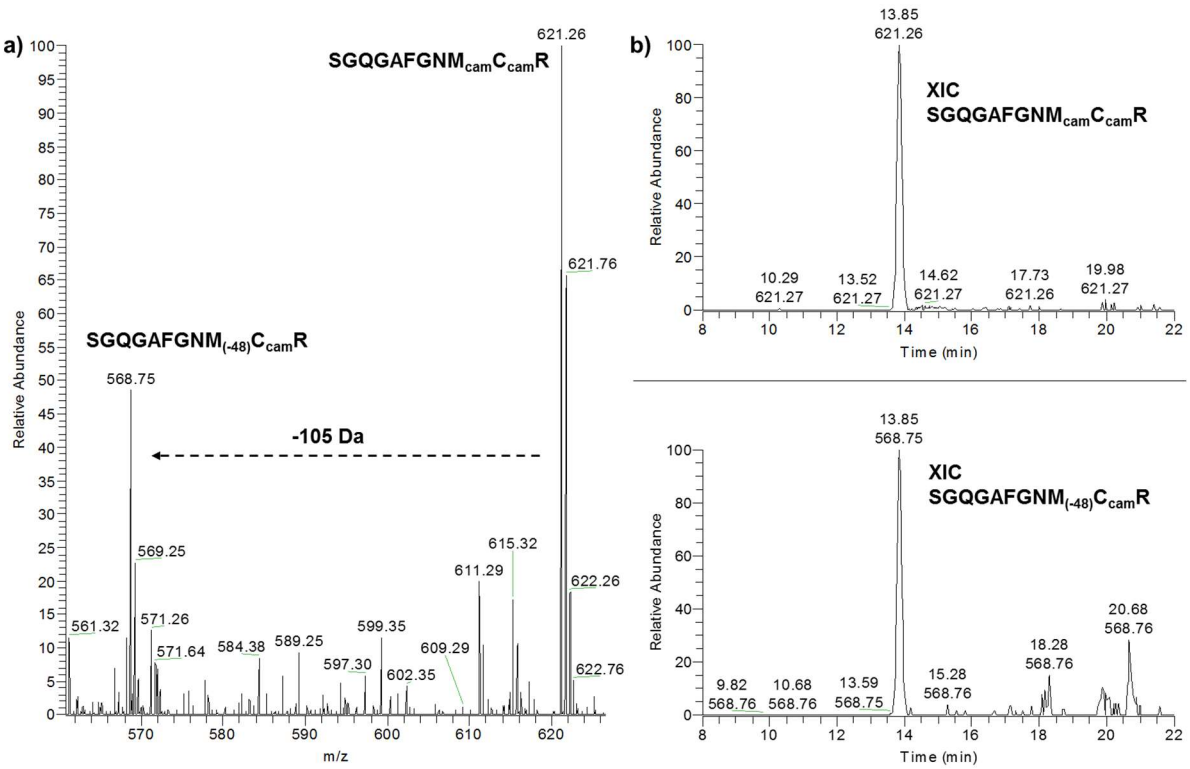
Supplementary Figure 13: Determination of the influence of IAA concentration on the degree of methionine carbamidomethylation of the synthetic peptide APEIMLNSK. Shown are the data from MALDI-MS measurements of the synthetic peptide APEIMLNSK alkylated with IAA at different concentrations ranging from 20 to 1000 mM. Comparison of the signal intensities of the $[M-48+H]^+$ relative to the signal of the $[M+H]^+$ show a linear relationship for the loss of the methionine side chain to the concentration of IAA.



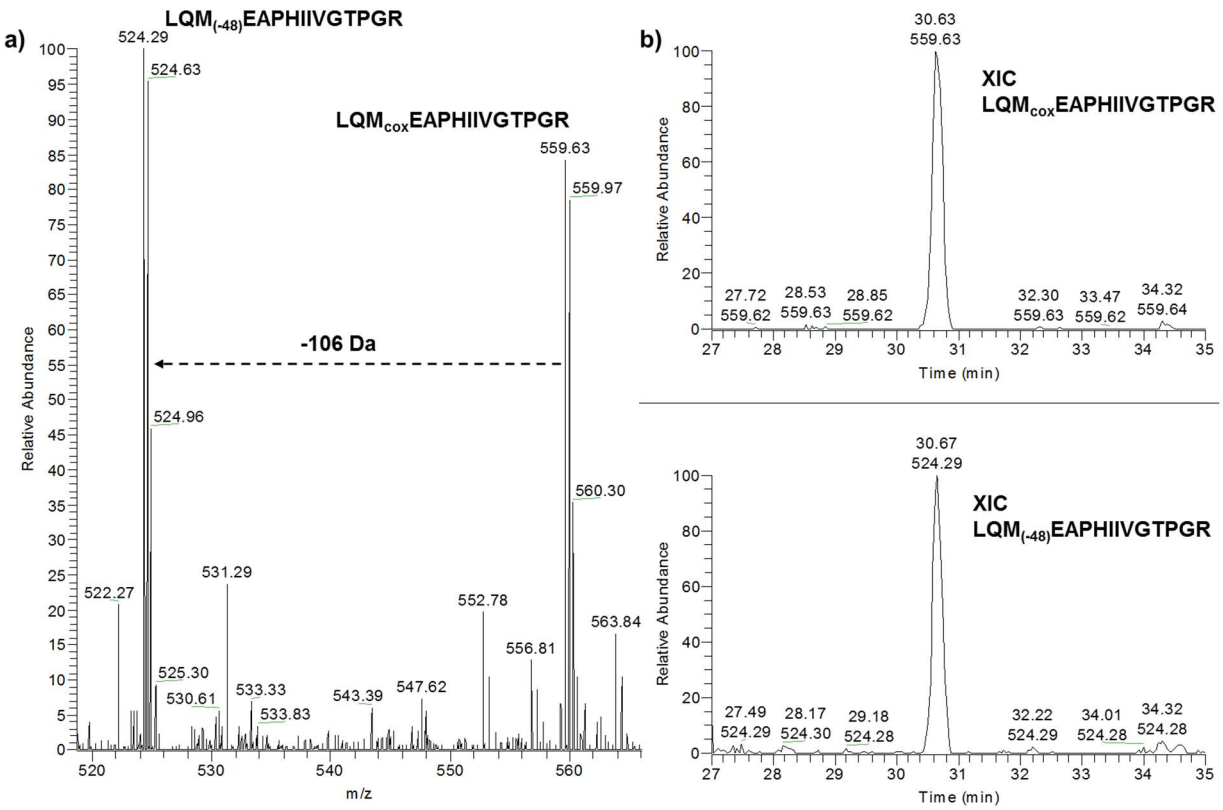
Supplementary Figure 14: MALDI-MS measurements of the synthetic peptide APEIMLNSK oxidized by H_2O_2 followed by reduction with DTT and alkylation by IAA, IAC, AA, and CAA. Oxidation of methionine residues prevented alkylation and resulted in a minor loss of the oxidized side chain of the amino acid. Additional offsite alkylation was observed, but to a lower degree than the alkylated unmodified peptide (compare to Supplementary Figure 12), and a higher degree than the reduced and alkylated unmodified peptide. cox: carboxymethylated; cam: carbamidomethylated; prop: propionamide modified; oxM: oxidized methionine; 2oxM: double oxidized methionine; "-$SOCH_4$": loss of the oxidized side chain of methionine.



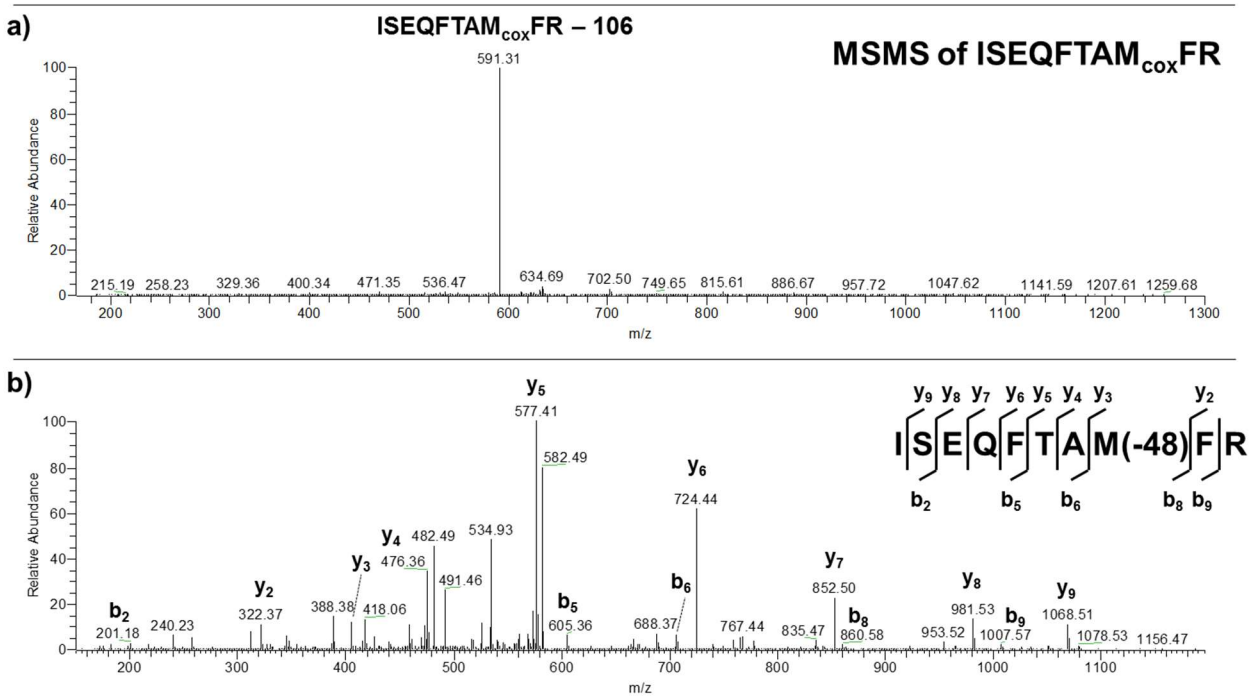
Supplementary Figure 15: Combined results for the analyses of 2 independent replicates of in-solution and 6 replicates + standard deviation of in-gel digested cytosolic fractions of HeLa cells using standard modifications for database searching. The number of peptide spectrum matches (PSMs) annotated with a loss of –SHCH3 (-48 Da) from methionine are shown for a) in-solution and b) in-gel digests. DI: DTT+IAA, DIA: DTT+IAC, DA: DTT+AA, DC: DTT+CAA, TI: TCEP+IAA, TIA: TCEP+IAC, TA: TCEP+AA, TC: TCEP+CAA, BI: BME+IAA, BIA: BME+IAC, BA: BME+AA, BC: BME+CAA.



Supplementary Figure 16: a) Survey scan showing the neutral loss of the carbamidomethylated side chain of methionine from the in-source fragmented peptide SGQGAFGNM(cam)C(cam)R identified in the in-gel digested samples reduced with DTT and alkylated with IAA. b) Extracted ion chromatograms (XICs) showing perfect co-elution of the monoisotopic peaks of SGQGAFGNM(cam)C(cam)R (upper panel) and SGQGAFGNM(cam)C(cam)R exhibiting a neutral loss of 105 (lower panel) which represents the loss of the side chain of methionine (48 Da relative to the unmodified peptide). M(cam): carbamidomethylated methionine; C(cam): carbamidomethylated cysteine.



Supplementary Figure 17: a) Survey scan showing the neutral loss of the carboxymethylated side chain of methionine from the in-source fragmented peptide LQM(cox)EAPHIIVGTPGR identified in the In-gel digested samples reduced with DTT and alkylated with IAA. b) Extracted ion chromatograms (XICs) showing perfect co-elution of the monoisotopic peaks of LQM(cox)EAPHIIVGTPGR (upper panel) and LQM(cox)EAPHIIVGTPGR exhibiting a neutral loss of 105 (lower panel) which represents the loss of the side chain of methionine (48 Da relative to the unmodified peptide). M(cox): carboxymethylated methionine.



Supplementary Figure 18: a) MS/MS fragment ion spectrum of the carboxymethylated peptide ISEQFTAM(cox)FR and b) MS/MS of the neutral loss peak of in-source fragmented ISEQFTAM(cox)FR. Fragmentation of the carboxymethylated peptide results in an abundant neutral loss of 106 Da relative to the precursor ion but no further fragment ions from which the peptide sequence can be determined. Fragmentation of the neutral loss, however, results in an information-rich fragment ion spectrum allowing annotation of the full sequence.