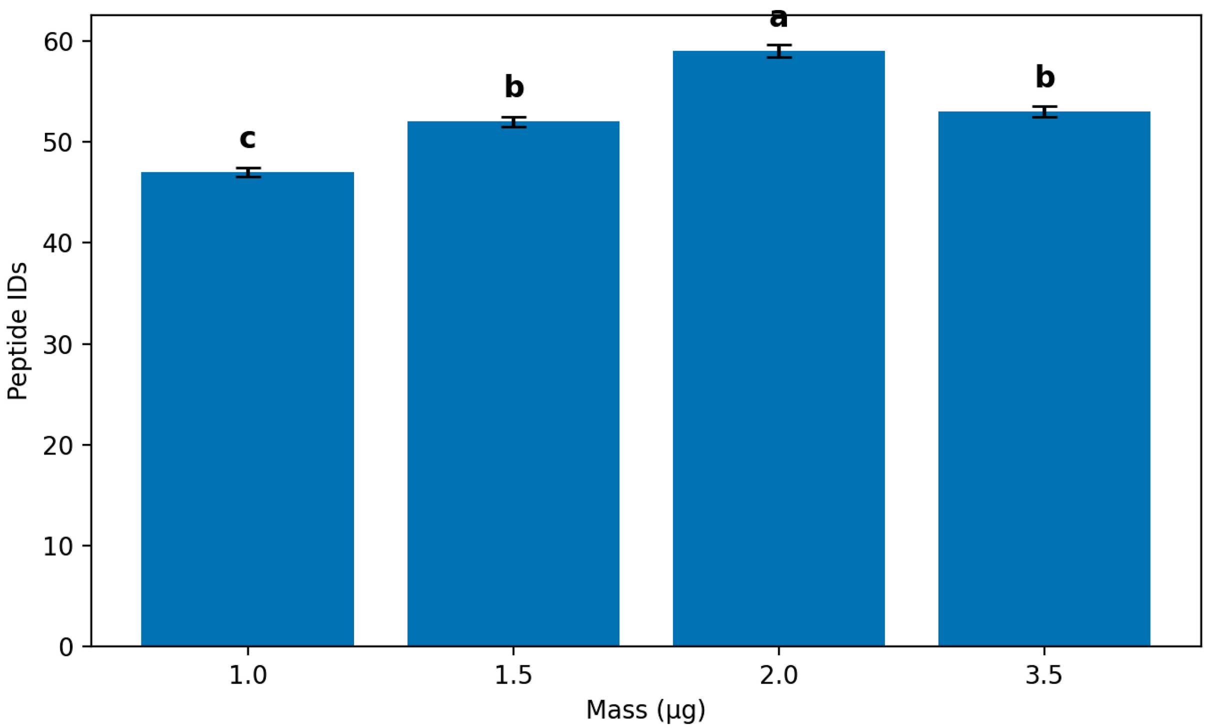
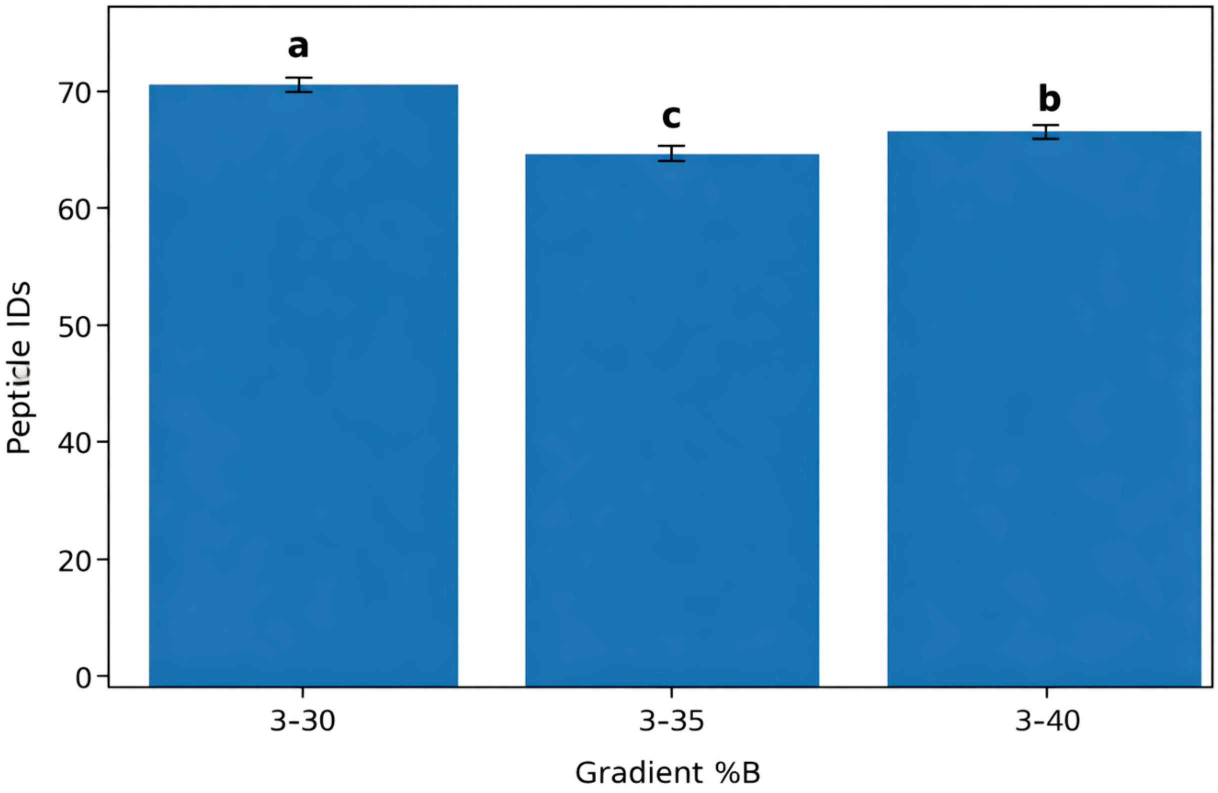
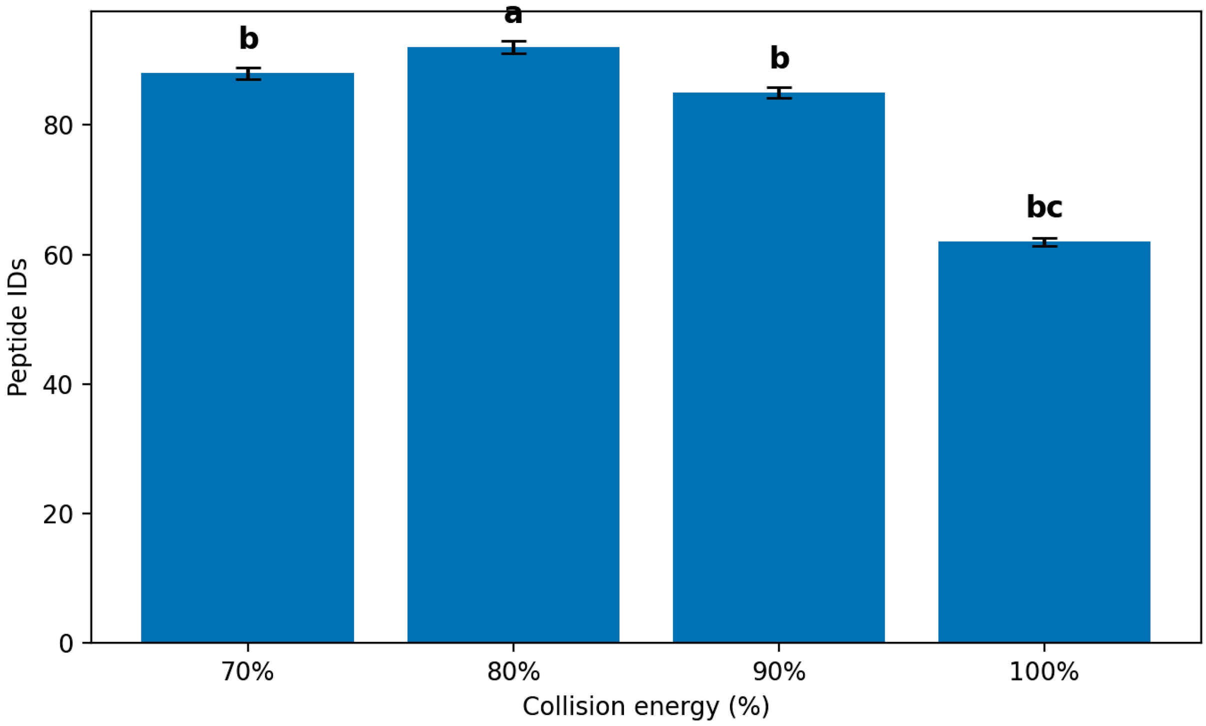


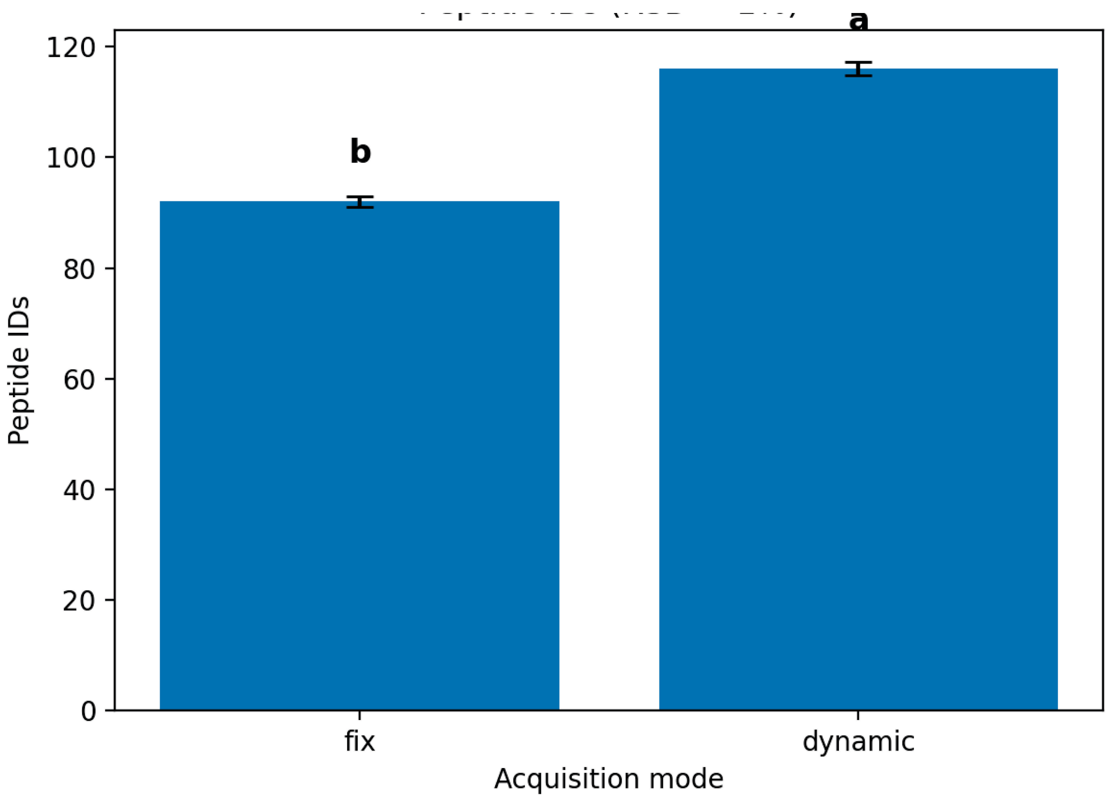
Supplemental Figure 1. Effect of injection volume on the number of peptide identifications. Mean values and measures of variability are shown as error bars while different letters indicate significant differences between conditions (Tukey's test, $p < 0.05$).



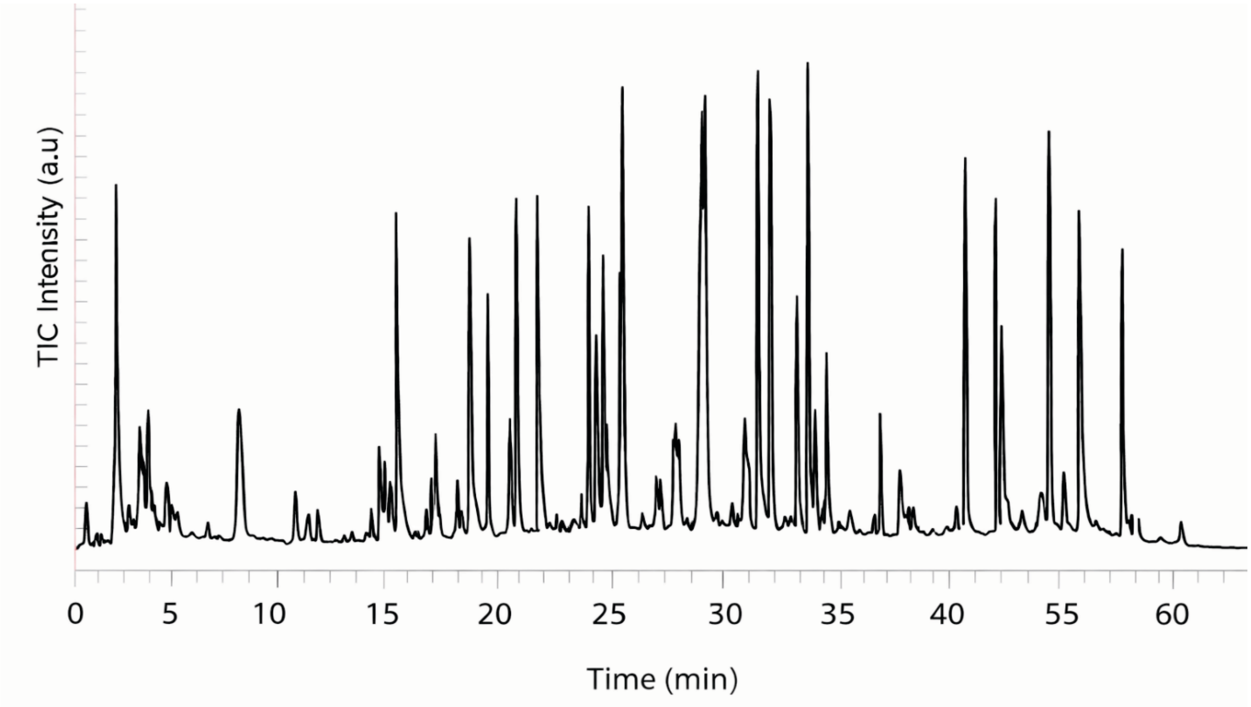
Supplemental Figure 2. Comparison of peptide identification performance across three LC gradients. Mean values and measures of variability are shown as error bars while different letters indicate significant differences between conditions (Tukey's test, $p < 0.05$).



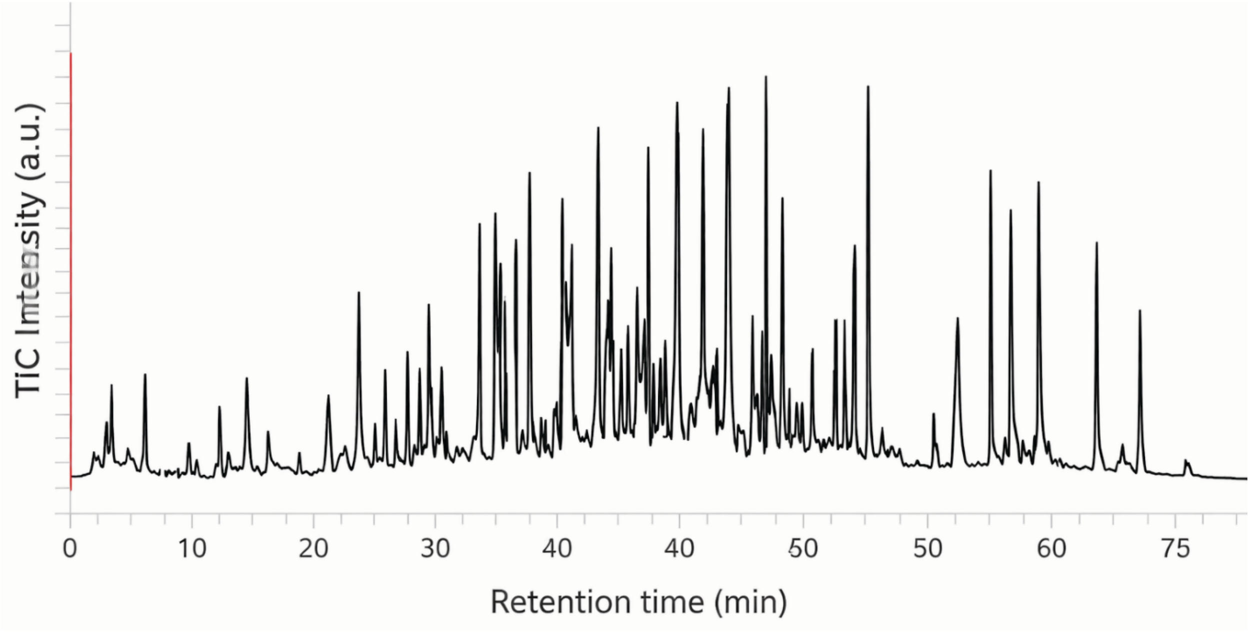
Supplemental Figure 3. Effect of collision energy reduction on peptide identification. Mean values and measures of variability are shown as error bars while different letters indicate significant differences between conditions (Tukey's test, $p < 0.05$).



Supplemental Figure 4. Comparison of peptide identification depth between fixed and dynamic MS/MS modes. Mean values and measures of variability are shown as error bars while different letters indicate significant differences between conditions (one-way repeated measurements ANOVA $p < 0.05$).



Supplemental Figure 5. Total ion chromatogram of BSA obtained using the optimized micro-flow LC–QTOF method.



Supplemental Figure 6. TIC chromatogram of the BSA/phosphorylase b/ADH mixture obtained with the optimized LC–MS/MS workflow.

Supplemental Table 1. Consolidated MS1, MS/MS and CID Acquisition Parameters

Category	**Parameter**	**Value**
MS1 Scan	Spectra rate (MS)	2.00 Hz
	Number of precursors per cycle	5
	Minimum intensity	70 counts
	Preferred charge states	2–5
	Active exclusion	On
	Release after	0.50 min
Auto MS/MS Preferences	Preferred charge range	2–5
	Exclude unknown charge	On
CID	Isolation width	2–5 *m/z*
	Collision energy	*m/z*-dependent list (300–1300 *m/z*)
	Fixed MS/MS acquisition	On
	Fixed MS/MS scan rate	1.00 Hz

Supplemental Table 2 Collision Energy Matrix for CID Fragmentation

m/z	**CE for 1+ (eV)**	**CE for 2+ (eV)**	**CE for 3+ (eV)**
300	23	23	23
400	23	23	23
500	27	27	25
600	33	33	27
700	33	33	27
800	45	45	40
900	50	50	45
1000	55	55	50
1100	65	65	55
1300	65	65	55

Supplemental Table 3. *m/z*-dependent collision energy table used for optimized MS/MS fragmentation

m/z	**CE for 1+ (eV)**	**CE for 2+ (eV)**	**CE for 3+ (eV)**
300	20.0	20.0	20.0
400	23.0	23.0	23.0
500	27.0	27.0	25.0
600	26.4	26.4	21.6
700	26.4	26.4	21.6
800	36.0	36.0	32.0
900	40.0	40.0	36.0
1000	44.0	44.0	44.0
1100	52.0	52.0	44.0
1300	52.0	52.0	44.0

Supplemental Table 4. Summary of repeated-measures ANOVA and post-hoc statistics for method optimization experiments.

Effect tested	**Outcome**	**F**	**df1**	**df2**	**p-value**
Injection volume	Sequence Coverage	23.75	3	6	0.0010
	Peptide ID	88.08	3	6	<0.0001
	PSM	94.64	3	6	<0.0001
	RT RSD	16.39	3	6	0.0027
Gradient	Sequence Coverage	350910.10	2	4	<0.0001
	Peptide ID	35.64	2	4	0.0028
	PSM	516.00	2	4	<0.0001
Collision energy	Sequence Coverage	230.62	3	6	<0.0001
	Peptide ID	3863.20	3	6	<0.0001
	PSM	481.46	3	6	<0.0001
Acquisition mode	Sequence Coverage	20.49	2	4	0.0024
	Peptide ID	9.95×10^5	2	4	<0.0001
	PSM	221.70	2	4	<0.0001