ORIGINAL SCIENTIFIC PAPER

CROATICA CHEMICA ACTA

Croat. Chem. Acta **2026**, *99*(2)
Published online: May 21, 2026
DOI: 10.5562/cca4235

HKD 1926

Systematic Optimization of Micro-flow LC–QTOF Conditions for Data-Dependent Peptide Analysis

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RECEIVED: December 22, 2025 * REVISED: January 16, 2026 * ACCEPTED: January 17, 2026

Abstract: This study describes the systematic optimization of a micro-flow LC–MS / MS method on a QTOF platform for bottom-up protein analysis. Key chromatographic parameters were evaluated, and an injection mass of 2 µg combined with a shallow 3 –30 % B gradient provided the best separation efficiency and retention time reproducibility. Fragmentation performance was optimized by scaling the vendor-provided CE table to 80 %, which produced the most informative spectra under the argon collision gas conditions used in this study. Under the tested acquisition settings, dynamic MS / MS acquisition yielded a higher number of MS / MS spectra, peptide identifications, and sequence coverage than the fixed acquisition mode. The final method was validated using BSA, phosphorylase b, alcohol dehydrogenase, and their ternary mixture, demonstrating high sequence coverage and consistent chromatographic peak profiles across all samples. These results confirm that the optimized workflow is robust, reproducible, and suitable for routine proteomic applications, with future work aimed at evaluating its performance on complex proteomes such as yeast and HeLa.

Keywords: micro-flow LC-MS/MS, QTOF, bottom-up proteomics, data-dependent acquisition, collision energy optimization.

INTRODUCTION

PROTEOMICS aims to characterize the complete protein complement of a cell, tissue, or organism, including protein identities, abundances, modifications, and interactions. Modern proteomics relies predominantly on mass spectrometry–based workflows, which enable high-throughput, high-sensitivity analysis of complex biological samples and have become indispensable for large-scale discovery applications.[1,2] Among available approaches, bottom-up proteomics, in which proteins are enzymatically digested into peptides prior to LC–MS / MS analysis, remains the most widely adopted strategy due to the superior chromatographic behavior of peptides, their efficient ionization, and the rich structural information contained in their MS / MS spectra .[3] In recent years, data-independent acquisition (DIA) workflows have become the prevailing approach for large-scale quantitative proteomics, owing to their high reproducibility and depth of coverage. Nevertheless, data-dependent acquisition (DDA) continues to play a critical and complementary role in bottom-up proteomics, particularly in method development, optimization studies, spectral library generation, and targeted or mechanistic investigations, where precise control over precursor selection and fragmentation parameters is required. In such discovery-driven and methodological contexts, DDA remains an essential acquisition strategy in which precursor ions are selected in real time for MS / MS fragmentation and subsequent peptide identification.[1]

In bottom-up proteomics, liquid chromatography coupled with tandem mass spectrometry (LC–MS / MS) plays a central role by separating complex peptide mixtures, generating gas-phase ions, and acquiring their mass-to-charge and fragmentation information. Continuous advances in chromatographic performance, ion transmission,

scan speed, mass resolution, and fragmentation chemistry have dramatically increased proteome depth and quantitative accuracy over the past two decades.[4] Although nano-flow LC systems have historically dominated the field due to their excellent sensitivity, their widespread routine use is often limited by operational fragility, lower throughput, and demanding maintenance. In contrast, micro-flow LC–MS / MS has re-emerged as a robust and practical alternative, offering a favorable balance of sensitivity, reproducibility, and throughput. Micro-flow systems provide improved chromatographic stability, reduced susceptibility to clogging, and greater robustness, features that are highly attractive for routine proteomics and large-scale studies. Recent reports demonstrate that micro-flow LC–MS / MS can achieve substantial proteome depth while providing markedly enhanced robustness and quantitative precision compared with nano-flow configurations, making it particularly suitable for systematic method development and high-throughput applications.[5] Beyond nano- and micro-flow regimes, several studies have also demonstrated the feasibility of higher-flow and analytical-flow LC–MS / MS approaches for proteomics, emphasizing improved robustness, throughput, and long-term operational stability. In particular, work from Markus Ralser and colleagues has shown that analytical-flow LC–MS / MS, when combined with advanced acquisition strategies such as Scanning SWATH or Zeno SWATH, can deliver reproducible peptide and protein identifications while substantially reducing system complexity and maintenance demands.[6]

The performance of bottom-up proteomics workflows depends critically on the interplay between chromatographic separation and mass spectrometric acquisition parameters. Chromatographic peak shape and width fundamentally determine precursor ion statistics and thus influence MS1 detectability and MS / MS triggering efficiency. As shown in Lenco et al., sharper peaks generate higher apex intensities and improve MS / MS sampling at the chromatographic apex, directly enhancing identification rates and reducing co-isolation.[7] Conversely, broad or distorted peaks reduce precursor intensity, compromise MS / MS selection, and diminish overall proteome coverage.

Mass spectrometric acquisition parameters further influence identification performance. Efficient DDA requires synchronizing MS1 and MS / MS scan times with chromatographic peak widths to obtain enough data points across each peak. Defossez et al. highlight that at least 7–8 points per peak are required for robust quantification, and that insufficient MS1 sampling density or suboptimal cycle times can compromise MS / MS triggering efficiency in DDA experiments.[8] These findings underscore that optimal DDA performance requires careful balancing of scan duration, cycle time, and precursor-selection logic with chromatographic behavior.

Collision energy (CE) is an additional critical parameter that governs fragmentation quality. As demonstrated by Révész et al., optimal CE depends on precursor *m* / *z*, charge state, and structural features, and strongly influences the intensity and completeness of b / y ion series. Under-fragmentation results in sparse MS / MS spectra with low peptide-spectrum match scores, while over-fragmentation produces excessive low-mass ions and reduces sequence-informative content. The authors further emphasize that CE optimization is instrument-specific, as different collision-cell architectures and ion-acceleration profiles result in distinct fragmentation behavior across platforms.[9]

Despite extensive methodological development on Orbitrap-based systems, particularly in micro-flow LC–MS / MS workflows,[10–12] equivalent optimization studies are largely lacking for QTOF instruments. Existing QTOF literature focuses primarily on nano-flow LC and deep shotgun proteomics applications, such as high-resolution Impact II workflows,[13] or examines isolated aspects such as collision energy selection strategies[14] or peptide identification using UPLC–QTOF–MS / MS.[15] However, these studies typically use default instrument settings and do not address how QTOF-specific timing parameters – including transfer time, pre-pulse storage, or fixed versus dynamic MS / MS acquisition modes – affect precursor sampling efficiency or fragment ion quality, particularly under micro-flow chromatographic conditions. This represents a significant knowledge gap. Unlike Orbitrap instruments, which use automatic gain control (AGC) and ion accumulation strategies, QTOF platforms rely on real-time ion transmission and timing, making their performance highly sensitive to chromatographic peak widths, ion flux, and MS / MS duty cycle. Yet no published work systematically evaluates how chromatographic performance, DDA timing, and collision energy settings interact on QTOF instruments in the micro-flow LC regime.[16]

This study addresses this gap by systematically optimizing micro-flow LC–QTOF conditions for peptide analysis, focusing on chromatographic parameters, and QTOF-specific DDA parameters, including collision energies and fixed versus dynamic MS / MS acquisition. Performance was assessed using bovine serum albumin (BSA) digest and validated with a peptide standard mixture.

EXPERIMENTAL

Chemical and Reagents

MassPREP BSA Digestion Standard was purchased from Waters (Milford, MA, USA, Cat. No. 186002326). MassPREP phosphorylase b (from rabbit muscle) was obtained from Waters (Milford, MA, USA, Cat. No. 186002328), and

MassPREP yeast alcohol dehydrogenase was purchased from Waters (Milford, MA, USA, Cat. No. 186002329). Formic acid (98–100 %, LC–MS grade; LiChropur) was obtained from Merck (Darmstadt, Germany). Acetonitrile (LC / MS grade; BAKER ANALYZED ULTRA) was purchased from J.T. Baker (Phillipsburg, NJ, USA). Ultrapure water was produced using an ELGA purification system (ELGA LabWater, High Wycombe, UK).

Sample Preparations

MassPREP BSA Digestion Standard, MassPREP phosphorylase b, and MassPREP yeast alcohol dehydrogenase were each reconstituted according to the manufacturer's instructions. For individual analyses, each peptide standard (100 µg) was dissolved in 100 µL of 0.1 % (*v* / *v*) formic acid in water, vortexed, and briefly centrifuged before use. For preparation of the ternary peptide mixture, each standard (100 µg) was reconstituted in 33 µL of 0.1 % (*v* / *v*) formic acid, and all three solutions were then combined to obtain a total volume of 100 µL, ensuring equal volumetric contribution from each component. The final mixture was vortexed, centrifuged, and stored at –20 °C until analysis.

General LC–MS / MS Conditions

All experiments were performed using a Vanquish Flex UHPLC system (Thermo Fisher Scientific, Waltham, MA, USA) coupled to a Compact QTOF mass spectrometer (Bruker Daltonics, Bremen, Germany). Chromatographic separation was conducted on an ACQUITY CSH C18 column (1.7 µm, 1.0 × 150 mm; Waters, Milford, MA, USA) maintained at 55 °C, with the autosampler temperature set to 4 °C. The flow rate was 50 µL min^{-1} for all runs.

Mobile phase A consisted of 0.1 % (*v* / *v*) formic acid in water, and mobile phase B consisted of 0.1 % (*v* / *v*) formic acid in acetonitrile.

Electrospray ionization (ESI) in positive mode was used for all analyses with a mass range of 150 to 3500 *m* / *z*, the capillary voltage set to 4500 V, and the end-plate offset at 500 V. The nebulizer operated at 1.0 bar, while the drying gas was maintained at a flow rate of 5.0 L min^{-1} and a temperature of 200 °C. Ion optics were operated with Funnel 1 RF amplitude set to 400.0 Vpp and Funnel 2 RF amplitude at 600.0 Vpp. The ion source collision-induced dissociation (isCID) energy was set to 0.0 eV, and the ion energy prior to transfer into the collision cell was 5.0 eV. The collision cell operated with a fixed collision energy of 7.0 eV and a collision RF amplitude of 2000.0 Vpp. The hexapole RF amplitude was set to 400.0 Vpp, and the low-mass cutoff for MS / MS acquisition was defined at 150.0 *m* / *z*. A lower *m* / *z* cutoff of 150 was applied during MS / MS acquisition to reduce low-mass background and non-sequence-informative fragment ions. This empirical setting, although not inherent to QTOF operation, was kept constant across all experiments to ensure consistent spectral quality and comparability. Collision-induced dissociation was performed in a collision cell pressurized with argon, which served as the collision gas for all MS / MS experiments. Argon was used as the collision gas in all MS / MS experiments, consistent with the configuration of the Q-TOF instrument used in this study for low-energy CID.

All samples were analyzed under identical chromatographic and MS / MS conditions unless otherwise specified in the optimization sections. Each experimental condition was evaluated with three technical replicates, which were processed and analyzed independently.

Initial conditions

The initial LC–MS / MS conditions used as a reference throughout this study were selected based on a combination of instrument vendor recommendations and some preliminary experiments. The initial chromatographic conditions used as a reference throughout this study included the injection of 3.5 µg of peptides and a linear gradient from 3 % B at 0 min to 40 % B at 60 min, followed by re-equilibration at 3 % B from 63 to 73 min. Under these initial conditions, MS / MS acquisition parameters were set according to the values listed in Supplemental Table 1.

Optimization of LC Conditions

Optimization of the liquid chromatographic conditions was conducted in two sequential phases. In the first phase, the effect of injection volume on chromatographic performance and MS / MS identification efficiency was evaluated while in the second phase different gradient slopes were evaluated. The optimization strategy was designed to follow a logical progression from parameters that directly affect chromatographic load and separation space to those influencing peptide elution behavior and, subsequently, MS / MS acquisition efficiency. Injection volume was optimized first, as it directly determines column loading and can influence peak shape, retention time stability, and susceptibility to column overloading. Establishing an appropriate injection volume was therefore a prerequisite for meaningful evaluation of gradient conditions, which primarily affect peptide separation and co-elution patterns under a given load. MS / MS parameters were optimized only after chromatographic conditions were fixed, ensuring that observed effects on identification performance could be attributed to acquisition settings rather than chromatographic variability.

Injection volumes of 1.0, 1.5, 2.0, and 3.5 µL were tested, corresponding to peptide mass of 1.0, 1.5, 2.0, and 3.5 µg, respectively. All analyses in this phase were performed using the same gradient conditions, with chromatographic and MS / MS settings identical to those described in the General LC–MS / MS Conditions section:

gradient 3 % B at 0 min → 40 % B at 60 min → 3 % B at 63–73 min. The MS / MS acquisition parameters are summarized in Supplemental Tables 1 and 2.

In the second phase, different gradient slopes were evaluated to determine their effects on peptide separation and identification efficiency. Three gradients were tested, each with an injection volume of 2 µL:

- Gradient 1: 3% B at 0 min → 40% B at 60 min → 3% B at 63–73 min
- Gradient 2: 3% B at 0 min → 35% B at 60 min → 3% B at 63–73 min
- Gradient 3: 3% B at 0 min → 30% B at 60 min → 3% B at 63–73 min

All LC experiments described in this section were conducted using the same instrumental settings and MS / MS acquisition parameters, as summarized in Tables 1 and 2. This ensured that any observed differences in analytical performance were attributable exclusively to the injection volume or gradient conditions being evaluated.

Table 1. Comparison of parameters used for fixed (Method 1) and dynamic (Method 2) MS/MS acquisition.

Category	Parameter	Value Method 1	Value Method 2
	Spectra rate (MS)	2.00 Hz	2.00 Hz
	Number of precursors per cycle	5	n/a
	Cycle time	n/a	2.5 s
MS1 Scan	Minimum intensity	70 counts	70 counts
	Preferred charge states	2–5	2–5
	Active exclusion	On	On
	Release after	0.50 min	0.50 min
Auto MS/MS Prefer-ences	Preferred charge range	2–5	2–5
	Exclude unknown charge	On	On
	Isolation width	2–5 *m/z* (*m/z*-dependent)	2–5 *m/z* (*m/z*-dependent)
	Collision energy	*m/z*-dependent list (300–1300 *m/z*)	*m/z*-dependent list (300–1300 *m/z*)
CID	Fixed MS/MS acquisition	On	Off
	Fixed MS/MS scan rate	1.00 Hz	n/a
	Dynamic MS/MS acquisition	Off	On
	Target Intensity (MS/MS TIC)	n/a	40.000 cts
	Min. MS/MS spectra acquisition	n/a	4.00 Hz
	Max. MS/MS spectra acquisition	n/a	16.00 Hz

Optimization of MS / MS Parameters

Optimization of MS / MS acquisition parameters was performed in two phases. All experiments in this section used identical chromatographic settings and the same instrument tune and source parameters described in the General LC–MS / MS Conditions, with a gradient of 3 % B at 0 min, 30 % B at 60 min, and 3 % B at 63–73 min, and an injection volume of 2 µL.

OPTIMIZATION OF COLLISION ENERGY (CE)

Collision energy was optimized to assess how varying fragmentation intensities influence peptide fragmentation quality and identification efficiency. The optimization used the BSA digest as the test sample. Four collision energy (CE) conditions were tested by adjusting the instrument's *m* / *z*-dependent CE table to 100 %, 90 %, 80 %, and 70 % of the default collision energy values listed in Supplemental Table 2. During CE optimization, all other MS / MS acquisition parameters remained the same as those in Table 1, including precursor selection rules, MS / MS scan rates, exclusion settings, and ion-optics tune parameters. This ensured that observed differences in identification performance were due solely to changes in collision energy.

FIXED VERSUS DYNAMIC MS / MS ACQUISITION

The effects of fixed and dynamic MS / MS acquisition strategies on precursor sampling and peptide identification efficiency were evaluated using collision energies set to 80 % of the values listed in Supplemental Table 2. The MS / MS acquisition parameters that differed between the two methods are summarized in Table 1.

Validation of Optimized LC–MS / MS Conditions

The optimized LC and MS / MS conditions were validated using two additional protein digests, Phosphorylase b and Yeast Alcohol Dehydrogenase, as well as their ternary mixture with BSA. Validation was performed using the conditions established during the optimization experiments, which included an injection volume of 2 µL and an LC gradient from 3 % B at the start of the run to 30 % B at 60 minutes, followed by a return to 3 % B between 63 and 73 minutes. Collision energies were set to 80% of the values listed in Table 2, and MS / MS acquisition was performed using the dynamic MS / MS acquisition mode described as Method 2 in Table 1. All other chromatographic and mass spectrometric parameters were as specified in the General LC–MS / MS Conditions section.

Data Analysis

Raw LC–MS / MS data were first processed using Bruker Compass DataAnalysis to inspect chromatographic quality,

Table 2. Final optimized LC conditions for micro-flow peptide analysis.

Category	Parameter	Optimized setting
Column	Stationary phase	ACQUITY CSH C18, 1.7 µm
	Dimensions	1.0 × 150 mm
	Column temperature	55 °C
Autosampler	Temperature	4 °C
	Injection volume	2.0 µL (optimized)
Flow properties	Flow rate	50 µL min^{-1} (micro-flow)
Mobile phase composition	Mobile phase A	0.1% formic acid in water
	Mobile phase B	0.1% formic acid in acetonitrile
Optimized gradient	Time (min)	%B
	0	3 %
	0–60	Linear to 30 % B
	60–63	Return to 3 % B
	63–73	Hold at 3 % B

peak shape, retention time stability, and fragmentation characteristics. Peptide and protein identification was performed using PEAKS Studio 11 (Bioinformatics Solutions Inc., Waterloo, Canada), with all datasets processed under identical search and filtering parameters to ensure full methodological comparability across optimization steps.

Database searches were performed against a focused protein sequence database containing BSA (SwissProt P02769), yeast alcohol dehydrogenase (SwissProt P00330), and phosphorylase b (SwissProt P00489), supplemented with a standard contaminant list. Trypsin was specified as the digestion enzyme, allowing up to two missed cleavages. Carbamidomethylation of cysteine was set as a fixed modification, while oxidation of methionine was included as a variable modification. Mass tolerances were set to 15 ppm for MS1 precursor ions and 0.05 Da for MS / MS fragment ions. Peptide-spectrum matches, peptide identifications, and protein assignments were filtered at a 1 % false discovery rate (FDR) at both the peptide and protein levels using a target–decoy strategy. For each experimental condition, the number of acquired MS spectra, MS / MS spectra, identified peptides, peptide-spectrum matches (PSMs), and sequence coverage were recorded. Chromatographic performance was evaluated based on retention time reproducibility, full width at half maximum (wh), and peak shape parameters extracted from extracted-ion chromatograms.

All data analysis parameters were kept constant throughout the study to ensure that observed differences

Table 3. Final optimized MS and MS/MS parameters used for micro-flow LC–qTOF analysis.

Category	Parameter	Final setting
Ion source (ESI)	End plate offset	500 V
	Capillary voltage	4500 V
	Nebulizer pressure	1.0 bar
	Drying gas	5.0 L min^{-1}
	Drying temperature	200 °C
	Polarity	Positive
MS settings	Mass range	150–3500 m/z
	Spectra rate (MS)	2.0 Hz
	Abs. threshold	25 counts
Ion optics	Funnel 1 RF	400 Vpp
	Funnel 2 RF	600 Vpp
	Hexapole RF	600 Vpp
	Ion energy	5 eV
	isCID energy	0 eV
	Low mass	150 *m/z*
	Collision RF	2000 Vpp
	Transfer time	110 µs
	Pre-pulse storage	10 µs
Auto MS/MS settings	Acquisition mode	Dynamic MS/MS
	Precursor reconsideration	On
	MS repetitions	1×
	MS/MS repetitions	1×
	Active exclusion	On
	Release after	1.00 min
	Smart exclusion	5×
	Exclude ions below	70 counts
	Cycle time	2.5 s
	Precursor sorting	By intensity
	Preferred charge states	2–5
	Singly charged exclusion	On
Fragmentation (CID)	Collision gas	Argon
	CE mode	*m* / *z*-dependent
	Isolation widths	2–5 *m* / *z* (*m* / *z* dependent)
Acquisition control	Max MS/MS spectra	16 Hz
	Dynamic MS/MS	On
	Minimum MS/MS rate	4 Hz
	Target MS/MS TIC	40,000

in analytical performance reflected the impact of LC and MS / MS optimization rather than downstream computational variation.

Statistical comparisons were performed using a repeated-measures one-way ANOVA (randomized block design), with injection block as the repeated-measures factor. Post-hoc pairwise comparisons were conducted using Tukey's test, and statistical significance was defined as $p < 0.05$. Detailed ANOVA and post-hoc statistics (F-values, degrees of freedom and p-values) for all optimisation experiments are provided in Supplemental Table 4.

RESULTS AND DISCUSSION

The development of robust and reproducible LC–MS/MS methods is essential for high-quality peptide identification and consistent performance in bottom-up proteomics workflows. Although nano-flow LC is traditionally considered the most sensitive approach, recent studies have shown that micro-flow LC offers significant advantages in robustness, throughput, and long-term system stability, making it well suited for routine proteomic analyses.[5] Given these benefits, this work systematically evaluated and optimized micro-flow LC–MS / MS conditions on a QTOF platform to identify operational parameters that provide the best balance between chromatographic resolution, MS / MS sampling efficiency, and overall identification performance. A structured optimization strategy was used, in which LC parameters (injection volume and gradient), MS / MS parameters (collision energy and acquisition mode), and the performance of the optimized settings were evaluated using multiple protein digests. In all experiments, only one parameter was varied at a time, allowing clear attribution of the observed effects to the parameter under investigation.

All LC–MS / MS optimization experiments were conducted under constant chromatographic and mass-spectrometric parameters selected based on previously published evidence and instrument-specific recommendations. The choice of micro-flow LC operating conditions was guided by earlier systematic evaluations showing that micro-flow separation at 20–60 µL min^{-1} provides excellent robustness, retention time stability, and adequate sensitivity for proteomics applications, as reported in studies assessing the analytical potential of micro-flow LC–MS / MS in bottom-up workflows.[5] Similarly, the baseline MS / MS acquisition logic used throughout this work was derived from established optimization guidelines for Bruker QTOF instruments, particularly the Impact II platform, which provides documented evidence on precursor selection rules, fragmentation behavior, and cycle time optimization in DDA proteomics. These parameters served as the stable MS / MS framework against which all optimization experiments were compared.[12] Electrospray ionization (ESI) settings were kept constant and implemented according to the manufacturer's recommendations for operation at 50 µL min^{-1}, ensuring stable spray formation, efficient desolvation, and reproducible ion transmission. Together, these controlled LC, MS / MS, and ESI conditions ensured that each optimization step could be evaluated independently, providing a reliable foundation for interpreting the effects of the tested parameters on chromatographic and identification performance.

Optimization of LC Conditions

OPTIMIZATION OF INJECTION VOLUME

The influence of injection volume on chromatographic performance and peptide identification was evaluated using four tested volumes (1.0, 1.5, 2.0, and 3.5 µL), corresponding to peptide loads of 1.0, 1.5, 2.0, and 3.5 µg, respectively. As shown in Figure 1, the number of MS and MS / MS spectra remained constant across all conditions (844 MS and 4,215 MS / MS scans), indicating a stable LC–MS / MS duty cycle and allowing observed differences in identification performance to be attributed primarily to chromatographic effects rather than acquisition variability. A clear trend was observed in peptide identifications and sequence coverage (Figure 1). Increasing the injected peptide load from 1.0 to 2.0 µg resulted in progressively higher peptide identifications (47.00 ± 1.00, 51.53 ± 1.15, and 59.33±0.58 peptides, respectively) and increased sequence coverage from 69.33 ± 1.36 % to a maximum of 78.07 ± %. The highest identification performance was obtained at 2.0 µg, which yielded both the maximum peptide count and the highest number of PSMs (144 ± 2). At the highest injection volume (3.5 µg), identification performance declined, with reduced peptide identifications (52.67 ± 0.58), PSMs (135 ± 3), and sequence coverage (73.10 ± 1.03 %), indicating the onset of column overloading. This effect was further reflected in chromatographic reproducibility metrics, where injection volumes up to 2.0 µg showed excellent retention-time stability (RT RSD 0.3±0.05–0.4±0.05 %), while the 3.5 µg injection exhibited a markedly increased RT RSD of 2.0 ± 0.8 %. Chromatographic peak widths remained constant across all conditions (w_h 16.3 s; full width 0.28 min), suggesting that overall separation efficiency was maintained but became increasingly sensitive to overloading at higher sample loads.

Overall, these results indicate that 2.0 µL is the optimal injection volume, providing the highest number of peptide identifications and PSMs, maximal sequence coverage, and excellent retention time reproducibility without causing column overloading. Although some optimization experiments yielded a relatively low number of MS / MS spectra, identification confidence was not compromised, as database searches were performed

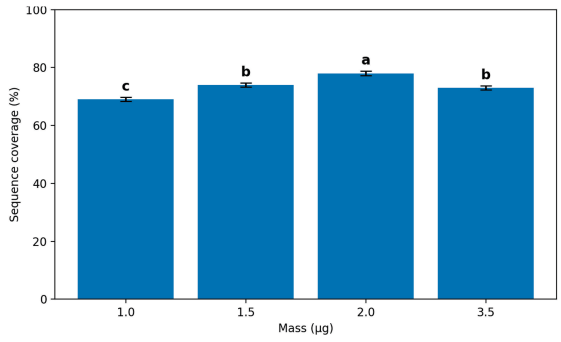

Figure 1. Sequence coverage (%) as a function of injection volume. Injection volumes of 1.0, 1.5, 2.0, and 3.5 µg. Mean values and measures of variability are shown as error bars while different letters indicate significant differences between conditions (Tukey's test, $p < 0.05$).

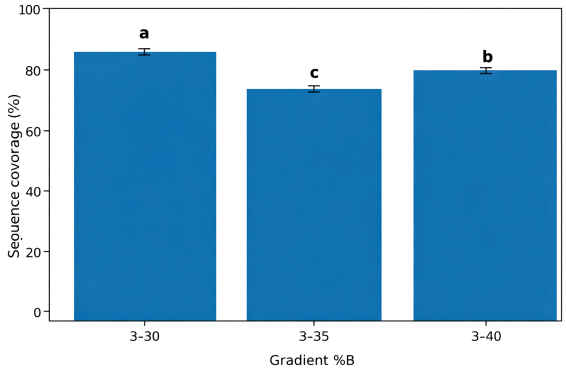

Figure 2 Influence of LC gradient slope on sequence coverage. Mean values and measures of variability are shown as error bars while different letters indicate significant differences between conditions (Tukey's test, $p < 0.05$).

against a highly constrained protein set and FDR control was supported by consistent target–decoy behavior and high reproducibility across technical replicates.

Optimization of LC Gradient

Three gradient slopes were evaluated to determine their impact on peptide identification efficiency and chromatographic performance. All gradients had identical starting and ending conditions, differing only in the final percentage of mobile phase B reached at 60 minutes (30 %, 35 %, or 40 % B). As in the injection volume optimization, the number of MS and MS / MS spectra remained constant across all conditions (844 and 4,215, respectively), confirming that the acquisition duty cycle did not influence the observed differences in identification outcomes.

The steepest gradient (3 → 40 % B) and the shallowest gradient (3 → 30 % B) yielded comparable but distinct performance characteristics. The 3 → 30 % B gradient produced the highest number of peptide identifications (62.00±1.00) and PSMs (158±3) and achieved the highest sequence coverage (83.33±0.77 %), indicating that the shallower slope improved chromatographic separation and enhanced sampling of lower-abundance peptides (Supplemental Figure 2). A more gradual gradient increases peptide residence time in the low-to-mid organic phase range, effectively enhancing separation space and reducing co-elution, which often results in higher MS / MS identification efficiency. The intermediate gradient (3 ⟶ 35 % B) showed the lowest performance among the three, yielding 56.67±0.58 peptide IDs, 146±2 PSMs, and 76.33±0.75 % sequence coverage (Figure 2). This suggests that at this slope, peptides eluting in the mid-retention range may experience more pronounced co-elution, slightly reducing fragmentation efficiency and MS/MS sampling depth. The steepest gradient (3 ⟶ 40 % B) produced 58.68±0.58 peptide IDs and 144 ± 2 PSMs with 78.34 ± 0.75 % sequence coverage, closely matching the performance observed with the 3 ⟶ 35 % gradient while remaining inferior to the shallow 3 ⟶ 30 % gradient. Although steeper gradients can shorten method development time and increase throughput, they compress the separation window and can reduce chromatographic resolution. Retention-time stability was excellent across all gradients (RT RSD = 0.4 %), and the w_h (16.3 s) and full peak width (0.28 min) remained unchanged, indicating that the chromatographic system was stable and that differences in performance stemmed primarily from separation efficiency rather than system variability.

Overall, these findings indicate that the 3 ⟶ 30 % B gradient provides the highest identification performance, offering superior peptide separation efficiency and the greatest sequence coverage while maintaining excellent chromatographic reproducibility.

Optimization of MS / MS Parameters

OPTIMIZATION OF COLLISION ENERGY

The reference collision energy (CE) values used in this study were taken from the vendor-provided m / z-dependent CE table recommended for Bruker QTOF instruments, which is calibrated using nitrogen as the collision gas.[17] Because the instrument used in this study operated with argon as the collision gas, these nitrogen-calibrated CE values were used only as a reference framework and were empirically scaled during optimization. Argon has a higher atomic mass (39.9 u) than nitrogen (28.0 u), which can lead to more efficient momentum transfer during ion–neutral collisions. Consequently, when applying a nitrogen-calibrated CE table under argon conditions, the effective collisional activation may be stronger for a given nominal CE value. Therefore, lowering the CE relative to the vendor-calibrated

values for N_2 was evaluated as a strategy to obtain more controlled and informative peptide fragmentation under the argon collision gas conditions used in this study.

To identify the optimal CE under these conditions, four energy levels corresponding to 70 %, 80 %, 90 %, and 100 % of the vendor-provided CE table were evaluated. The number of MS scans (844) and MS / MS scans (4,215) remained constant across all experiments, confirming that differences in identification performance resulted from fragmentation behavior rather than acquisition variability. A clear non-linear dependence between CE and identification efficiency was observed. The 80 % CE condition produced the best overall performance, yielding the highest number of peptide identifications (92.00 ± 1.00), the highest number of PSMs (265 ± 2), and the greatest sequence coverage (89.33 ± 0.78 %). This demonstrates that a moderate reduction in CE provides the most balanced fragmentation regime under argon, generating rich and interpretable b- and y-ion series without inducing excessive internal fragmentation. At 70 % CE, performance was slightly reduced (87.67 ± 0.58 peptides, 263 ± 4 PSMs, 85.00 ± 0.21 % coverage), indicating that fragmentation becomes suboptimal for some peptide populations at this lower activation energy (Supplemental Figure 3 and Figure 3). Increasing the CE to 90 % led to a noticeable decline in performance (85.00 ± 0.21 peptides, 225 ± 5 PSMs), consistent with the onset of over-fragmentation. The poorest performance was observed at 100 % CE (62.00 ± 1.00 peptides, 158 ± 2 PSMs), where extensive fragmentation likely yielded overly complex MS / MS spectra containing fewer sequence-informative ions.

Overall, these findings indicate that scaling the vendor-provided CE table to approximately 80 % provides fragmentation conditions that yield the most informative spectra under the argon collision gas conditions used in this study.

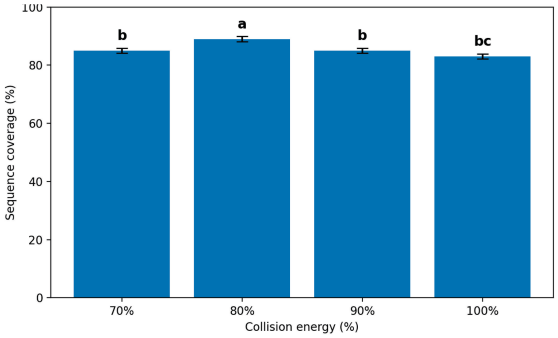

Figure 3 Collision energy–dependent changes in sequence coverage. Mean values and measures of variability are shown as error bars while different letters indicate significant differences between conditions (Tukey's test, $p < 0.05$).

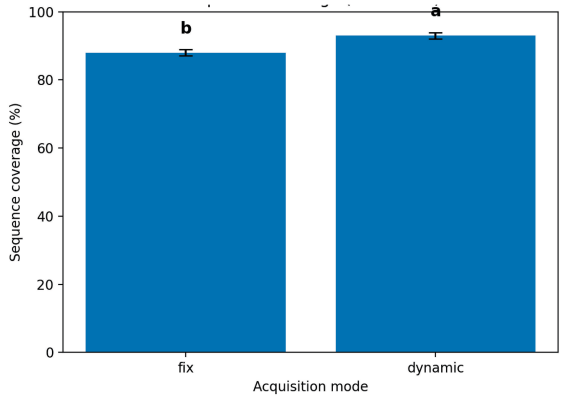

Figure 4 Effect of MS/MS acquisition strategy on sequence coverage. Mean values and measures of variability are shown as error bars while different letters indicate significant differences between conditions (one-way repeated measurements ANOVA $p < 0.05$).

Comparison of Fixed and Dynamic MS / MS Acquisition

Two MS / MS acquisition strategies, fixed and dynamic, were evaluated to assess their impact on MS / MS sampling behavior and peptide identification under the applied experimental conditions. On Bruker Q-TOF instruments, dynamic MS / MS acquisition represents an adaptive data-dependent approach in which the frequency and timing of MS / MS events are adjusted in real time in response to precursor ion availability. In contrast, fixed MS / MS acquisition operates at a constant MS / MS scan rate, independent of chromatographic precursor density. As a result, the two strategies differ primarily in how MS / MS scan capacity is distributed across the chromatographic time scale. Under fixed MS/MS acquisition, the number of MS scans (844) and MS / MS scans (4,215) remained constant and reflected the predefined cycle-time settings of the method. This acquisition mode yielded 92.00±1,00 peptide identifications, 265±2 PSMs, and 89.33±1.13 % sequence coverage (Supplemental Figure 4 and Figure 4), with excellent retention-time reproducibility (RT RSD = 0.4 %) and chromatographic peak widths consistent with previous optimization steps (w_h 16.3 s, full width 0.28 min). These results demonstrate that fixed-rate acquisition provides stable and predictable sampling but does not adjust MS / MS allocation in response to changes in precursor density. Dynamic MS / MS acquisition resulted in a substantially higher number of acquired spectra, with 1,868 MS scans and 15,227 MS / MS scans. This increase primarily reflects the higher effective MS / MS scan rate enabled by the dynamic mode under the applied conditions, rather than a direct algorithmic comparison at identical scan rates. Because the dynamic and fixed acquisition modes operated

with different effective MS / MS scan rates, the comparison presented here reflects practical performance under the tested configurations rather than a direct algorithmic comparison at identical scan-rate conditions. Correspondingly, dynamic acquisition yielded higher peptide identifications (116.00 ± 1.00), PSMs (521 ± 4.00), and sequence coverage (93.53 ± 1.13 %). The increased MS sampling frequency was also associated with narrower chromatographic features (w_h 7.8 s, full width 0.17 min), consistent with a higher density of data points across chromatographic peaks.

Taken together, these results show that, under the experimental configuration used in this study, dynamic MS / MS acquisition resulted in a higher number of MS / MS spectra and correspondingly higher peptide identification counts compared with the fixed acquisition mode. It should be noted that the BSA digest represents a low-complexity system, and the practical impact of this higher MS / MS sampling capacity may be particularly relevant in more complex proteomic samples, although this was not evaluated in the present study. Based on its favorable performance under realistic operating conditions, dynamic acquisition was selected for subsequent analyses in this study.

Optimized LC–MS / MS Conditions

Systematic optimization of chromatographic and MS / MS parameters resulted in a robust micro-flow LC–MS / MS workflow that consistently delivered the highest analytical performance on the QTOF platform. In the LC domain, reducing the injection volume from the initial 3.5 µL to 2.0 µL eliminated column overloading and produced significantly narrower peaks (w_h reduced from 16.3 s to 7.8 s; full width from 0.28 min to 0.17 min). These peak widths are fully consistent with published micro-flow LC performance benchmarks. Bian et al. demonstrated that 1 mm ID micro-flow columns operated at 20–60 µL min^{-1} typically yield peptide w_h values between 5 and 8 s, depending on gradient length.[11] The agreement between our w_h values and those reported for state-of-the-art micro-flow systems confirms that the optimized conditions (Table 2) effectively exploit the chromatographic advantages of this flow regime.

A similar trend was observed for peak variance and effective peak capacity. Compared with nano-flow LC, micro-flow LC is less sensitive to extra-column peak dispersion and allows more favorable matching between column dimensions and LC system volumes, thereby facilitating reproducible chromatographic performance under practical operating conditions, such.[10] The markedly narrower and more uniform peaks observed in our optimized micro-flow method therefore reflect both the advantages of reduced dispersion and the use of a shallow 3–30 % B gradient, which together contribute to increased resolving power. Since peak capacity in gradient LC scales inversely with peak width, the w_h values obtained in this study correspond to effective peak capacities comparable to those reported for optimized micro-flow proteomic workflows. It should also be noted that columns with a 1.0 mm inner diameter generally exhibit lower intrinsic chromatographic efficiency compared with larger-diameter micro-flow columns, which can partly be attributed to challenges in achieving optimal bed packing uniformity at smaller column diameters. While this may result in reduced theoretical plate numbers relative to wider formats, 1.0 mm i.d. columns are widely used in micro-flow proteomics due to their favorable balance between sensitivity, solvent consumption, and compatibility with standard UHPLC instrumentation. Recently, 1.5 mm i.d. columns have been reported as an alternative micro-flow format, offering improved packing efficiency and chromatographic performance at the expense of higher flow rates and sample consumption. The choice between these formats therefore represents a trade-off between chromatographic efficiency, robustness, and practical considerations, rather than a universally optimal solution[10,18] Supplemental figure 5 shows a representative BSA TIC chromatogram acquired under the final conditions.

In the MS / MS domain, replacing the initial nitrogen-calibrated collision energy settings (Supplemental Tables 1 and 2) with 80 % of the CE table, adjusted for argon's higher collisional efficiency, significantly improved fragmentation quality. The optimized *m* / *z*-dependent CE table is shown in Supplemental Table 3. Additionally, switching from fixed to dynamic MS/MS acquisition substantially increased the total number of acquired MS / MS spectra under the applied settings, which was associated with a higher number of peptide identifications. These improvements are demonstrated by the more than threefold increase in MS / MS spectra (Figure 5). Similar benefits of dynamic DDA acquisition in micro-flow LC–MS / MS workflows have been

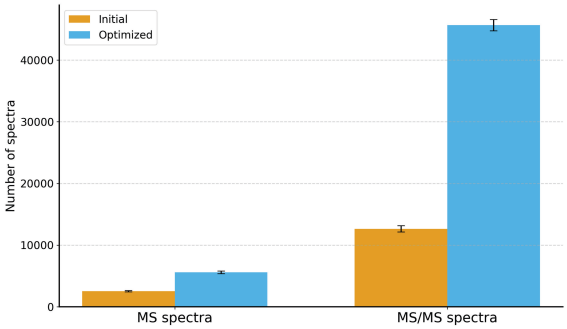

Figure 5 Comparison of the number of acquired MS and MS/MS scans under initial and optimized micro-flow LC–MS/MS conditions. Mean values and measures of variability are shown as error bars.

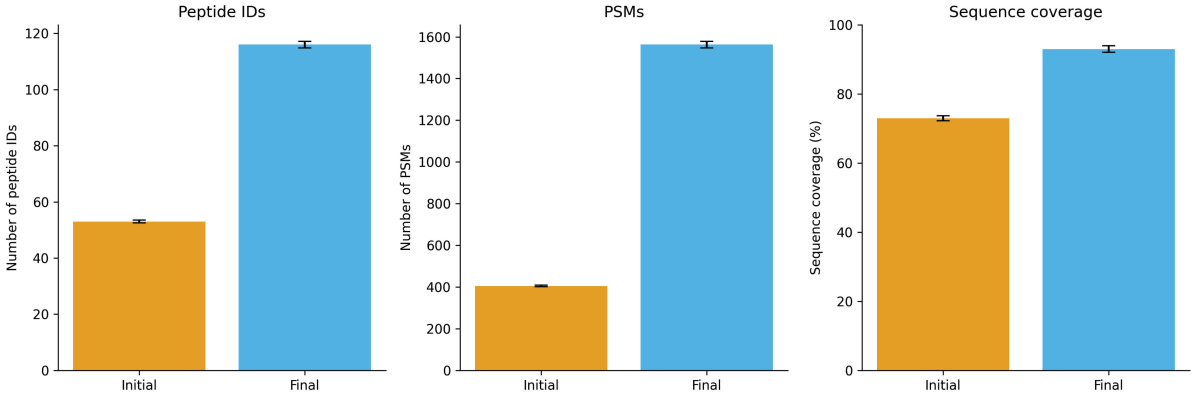

Figure 6. Comparison of peptide identifications (peptide IDs, PSMs and sequence coverage) obtained under initial and optimized micro-flow LC–MS/MS conditions. Mean values and measures of variability are shown as error bars.

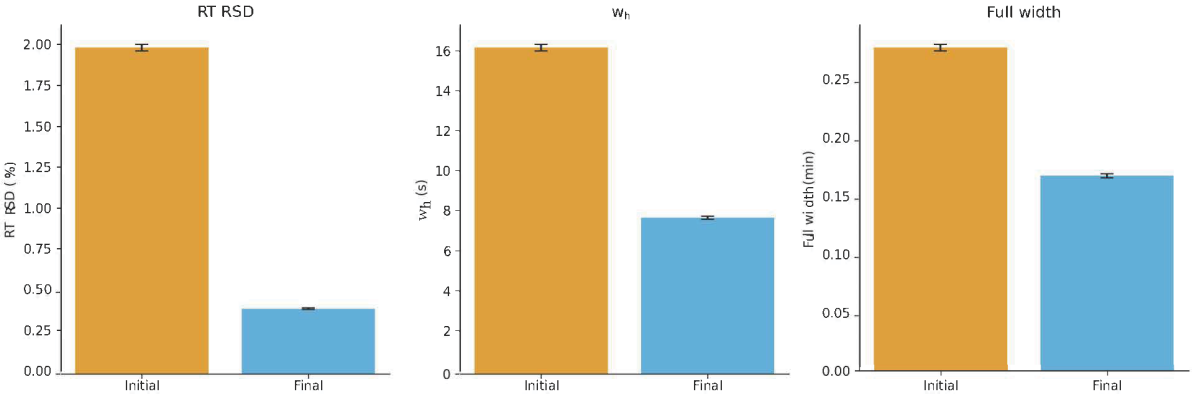

Figure 7. Comparison of chromatographic performance metrics (RT RSD, w_h and full peak width) under initial and optimized micro-flow LC–MS/MS conditions. Mean values and measures of variability are shown as error bars.

reported previously.[11] All optimized MS and MS / MS settings are summarized in Table 1.

A direct comparison with the initial method highlights the magnitude of improvement achieved. Under the initial conditions, only 52.67 ± 0.58 peptides, 135 ± 3 PSMs, and 73.10 ± 1.03 % sequence coverage were obtained, whereas the optimized workflow yielded 116±1 peptides, 521 ± 4 PSMs, and 93.53 ± 1.13 % sequence coverage, as shown in Figure 6. Retention time precision improved fivefold (RT RSD reduced from 2 ± 0.8 % to 0.4 ± 0.05 %), and chromatographic peak width narrowed substantially (Figure 7). These gains result from synergistic improvements in separation efficiency, precursor sampling, and fragmentation performance. The increased numbers of MS and MS / MS scans (Figure 5) further support the enhanced sampling efficiency achieved under optimized conditions. Although complete sequence coverage was not attained, this could be due peptide physicochemical constraints rather than limitations of the LC–MS / MS method. The coverage obtained under optimized conditions therefore represents the maximal performance achievable for the given digestion.

Collectively, the optimized LC and MS / MS parameters establish a high-performance workflow that matches the upper range of published micro-flow LC–MS / MS capabilities and offers a reliable platform for sensitive, reproducible peptide analysis.

Validation of the Optimized LC–MS / MS Method

The optimized LC–MS / MS conditions were validated by analyzing three individual protein digests – BSA, phosphorylase b, and yeast alcohol dehydrogenase (ADH)—as well as their ternary mixture. The primary evaluation metrics were sequence coverage and chromatographic peak profile (w_h and full width), enabling assessment of method robustness, reproducibility, and applicability beyond the model protein used during optimization.

All three proteins analyzed individually showed consistently high sequence coverage, with values of 93 % for BSA, 90 % for Phosphorylase b, and 91 % for ADH. These results confirm that the optimized method enables efficient detection and fragmentation across proteins with different molecular masses and peptide compositions. The minor differences in coverage reflect intrinsic digestibility variations rather than instrumental limitations, as previously discussed during the optimization steps. Chromatographic behavior was highly stable for all proteins. For each digest, the w_h was 7.8 s and the full peak width was 0.17 min, demonstrating uniform peak shape and retention-time behavior regardless of protein type. This uniformity indicates that, under optimized micro-flow LC conditions, peak width is primarily determined by column efficiency, gradient slope, and flow-dependent mass-transfer properties – not by the identity of the protein being digested. Because all three digests were prepared at comparable peptide loads, using factory-prepared peptide standards with certified concentrations, analyzed under identical LC–MS / MS conditions, and eluted across a similarly distributed hydrophobicity range, their chromatographic peaks experienced equivalent dispersion and exhibited nearly identical peak kinetics. Consequently, protein-specific peptide composition has little influence on w_h, which explains the highly consistent values observed for BSA, ADH, and phosphorylase b.

In the ternary mixture, sequence coverage for all three proteins was identical to the values obtained for the individual digests (BSA 93 %, phosphorylase b 90 %, ADH 91 %). Chromatographic peak metrics also remained unchanged (w_h 7.8 s; full width 0.17 min), and the overall chromatographic profile is shown in Supplemental figure 6, illustrating that peak capacity and separation quality were preserved despite increased sample complexity. As expected for a more complex sample, the mixture generated a proportional increase in analytical load: the numbers of acquired MS1 scans, MS / MS spectra, identified peptides, and PSMs all increased relative to the individual digests, reflecting the higher total number of detectable precursor ions and the corresponding increase in data-dependent sampling. Importantly, this scaling occurred without deterioration in chromatographic or fragmentation quality, demonstrating that the method accommodates increased sample complexity without compromising performance.

Together, these findings confirm that the optimized micro-flow LC–MS / MS method is robust, reproducible, and transferable across protein systems with different biochemical characteristics, performing equally well in both single-protein and multi-protein contexts. This establishes the method as suitable for broader proteomic applications beyond the initial model system.

CONCLUSION

This study developed a robust and reproducible micro-flow LC–MS / MS method for peptide analysis on a QTOF platform by systematically optimizing chromatographic and fragmentation parameters. Key LC variables, such as injection volume and gradient slope, significantly affected chromatographic performance and peptide identification, with an injection volume of 2 µL and a shallow 3–30 % B gradient providing the best overall results. MS / MS optimization showed that reducing the collision energy to approximately 80 % of the vendor-provided CE table produced the most informative fragmentation spectra under the argon collision gas conditions used in this study. Under the tested acquisition settings, dynamic MS / MS acquisition produced higher numbers of MS / MS spectra and peptide identifications than the fixed-rate acquisition mode.

The optimized method achieved high sequence coverage for three test proteins – BSA, phosphorylase b, and yeast alcohol dehydrogenase – and maintained excellent chromatographic reproducibility, as indicated by consistent w_h and peak-width metrics. Performance remained strong in a ternary protein mixture, with increases in MS1 and MS / MS scan counts, peptide identifications, and PSMs scaling proportionally with sample complexity, without compromising chromatographic or spectral quality. These results confirm that the method is robust, transferable, and suitable for analyzing both simple and moderately complex peptide samples under micro-flow conditions. In the next phase of method development, the optimized workflow should be further evaluated with more complex proteomes, such as yeast and HeLa cell lysates, to assess its scalability, depth of coverage, and performance in high-complexity biological matrices.

The optimized LC–MS / MS workflow provides a stable, high-performing platform for routine bottom-up proteomic analyses on QTOF instruments and serves as a valuable alternative to nano-flow approaches, especially in applications that require greater robustness and long-term operational stability. While the optimized LC–MS / MS settings reported here provided robust and reproducible performance for the model protein digests used in this study, further adjustment of selected parameters may be required when applying the method to more complex proteomic samples.

Acknowledgment. This research was funded by source 581 – recovery and resilience mechanism within the framework of the Program Financing of Public Higher Education Institutions and Public Scientific Institutes in the Republic of Croatia project „Proteomics of PIWI cultivars: an innovative

approach to understanding the molecular basis of their resistance and biochemical profile".

Supplementary Information. Supporting information to the paper is attached to the electronic version of the article at: https://doi.org/10.5562/cca4235.

PDF files with attached documents are best viewed with Adobe Acrobat Reader which is free and can be downloaded from Adobe's web site.

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