

# Strategies for acquisition and processing of N-term acetylated peptides on the timsTOF Ultra 2

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## Introduction

N-terminal acetylation is a prevalent post-translational modification that significantly influences protein stability, localization, and function. Despite its ubiquity, the dynamic regulation and functional implications of N-terminal acetylation remain underexplored. Investigating this modification in plant leaves is particularly complex due to the abundance of plastid-localized proteins that frequently undergo partial N-terminal acetylation and proteolytic processing, including N-terminal trimming. Additionally, the proteome exhibits a broad dynamic range, dominated by highly abundant proteins such as Rubisco and various components of the photosynthetic apparatus. Herein, enrichment via SCX fractionation, high sensitivity mass spectrometry and a dedicated processing pipeline were applied to overcome these challenges.

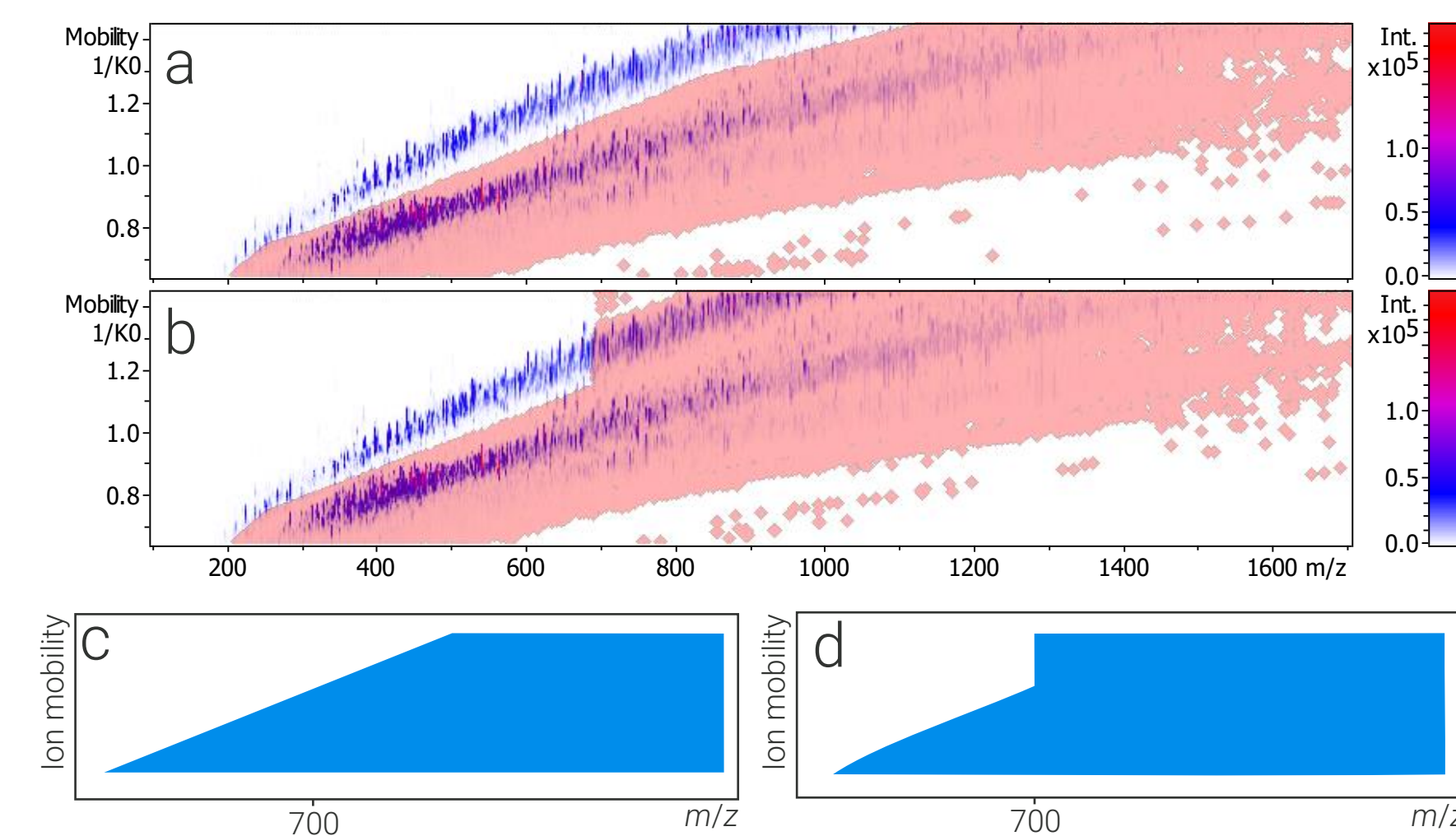


Fig. 1a: Selected precursor using the default selection polygon for tryptic digests. b: Selected precursor using an adapted selection polygon including singly-charged peptides > 700 Da. c: Scheme of default selection polygon. d: Scheme of adapted selection polygon.

## Methods

A protein extract from *Arabidopsis thaliana* young shoots was split into two samples: one aliquot was tryptically digested and cleaned-up (Crude); the second (SCX Pool) was chemically labeled with acetic anhydride-d<sub>6</sub>.

This induces a +3 Da mass shift of non-naturally labelled acetylation sites of lysines and protein N-termini. After trypsin cleavage, the sample was fractionated via SCX and the 10 first fractions were subsequently pooled [1].

All samples were analyzed using a nanoElute® 2 (Bruker Daltonics) equipped with an Aurora Ultimate CSI 25 cm column (IonOpticks) coupled to a timsTOF Ultra 2 (Bruker Daltonics). Chromatographic separation was done at 50°C on a 45 min active gradient and a flow rate of 250 nL/min. The mass spectrometer was operated in PASEF® using two different precursor selection schemes in DDA. Raw data processing was performed using MSFragger and IonQuant via FragPipe 22 [2,3] and the results were further processed in a dedicated Python-based pipeline, namely Nta-Quant.

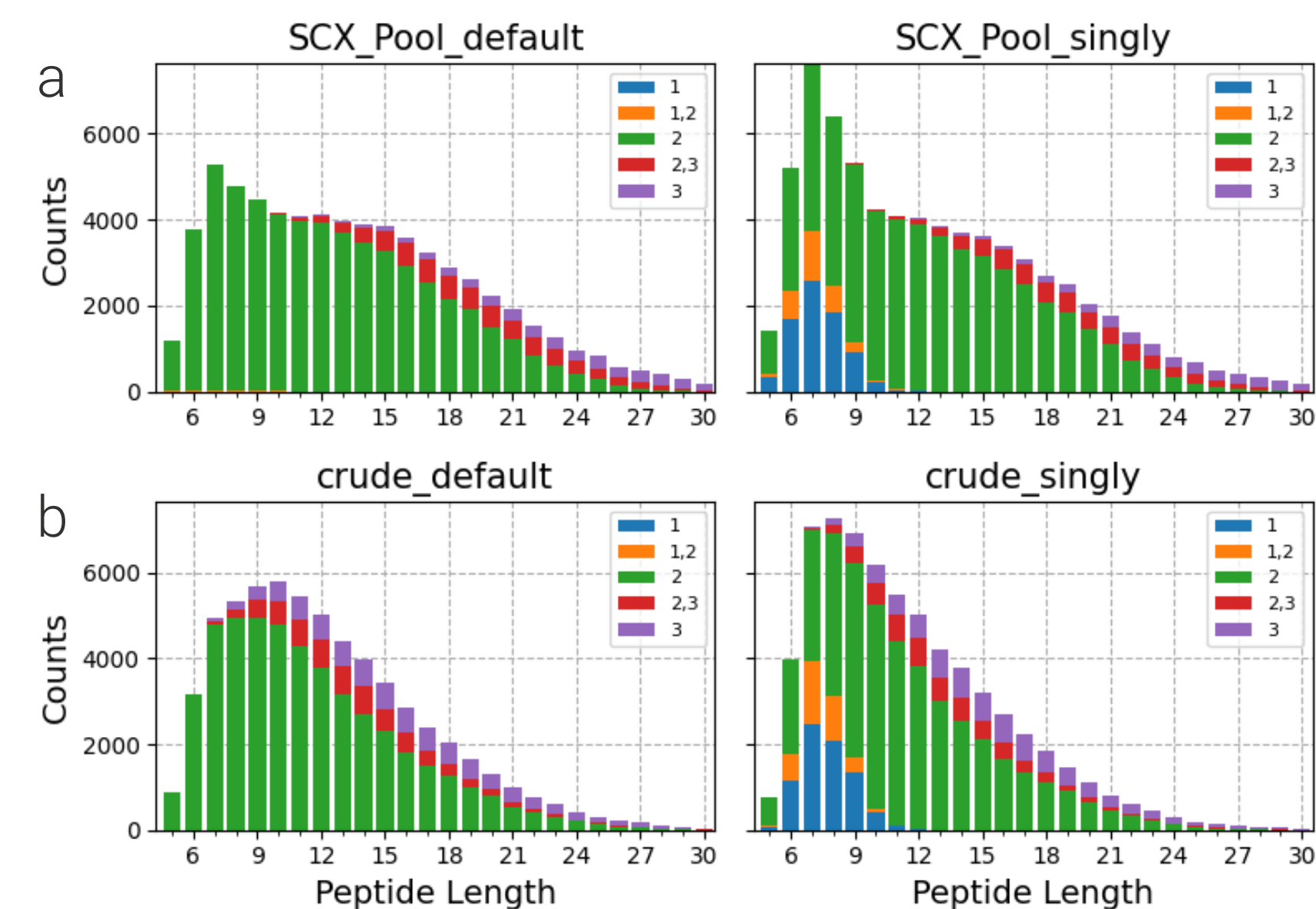


Fig. 2 Plots of charge states per peptide length. a: Showing data for the SCX Pool sample and both precursor selection schemes, default and modified (singly). b: Showing data for the Crude Extract sample and both precursor selection schemes, default and modified (singly).

## Results

The distribution of peptides in the heatmap of ion mobility dependent on the mass-to-charge ratio shows a grouping into charge states, in which the singly-charged peptides are clearly separated from multiply-charged peptides. Both the SCX\_Pool sample and the Crude extract were analyzed using the default polygon for precursor scheduling, which includes only multiply charged peptides, and a modified one, which includes singly charged peptides larger than 700 Da, additionally (Fig. 1).

Figure 2 shows the peptide length distribution and the corresponding charges states per sample and acquisition method.

For both samples, the adapted polygon allows to identify singly-charged peptides with lengths between 5 and 11 amino acids. In comparison, the ratio of multiply-charged peptides is higher in the Crude extract sample.

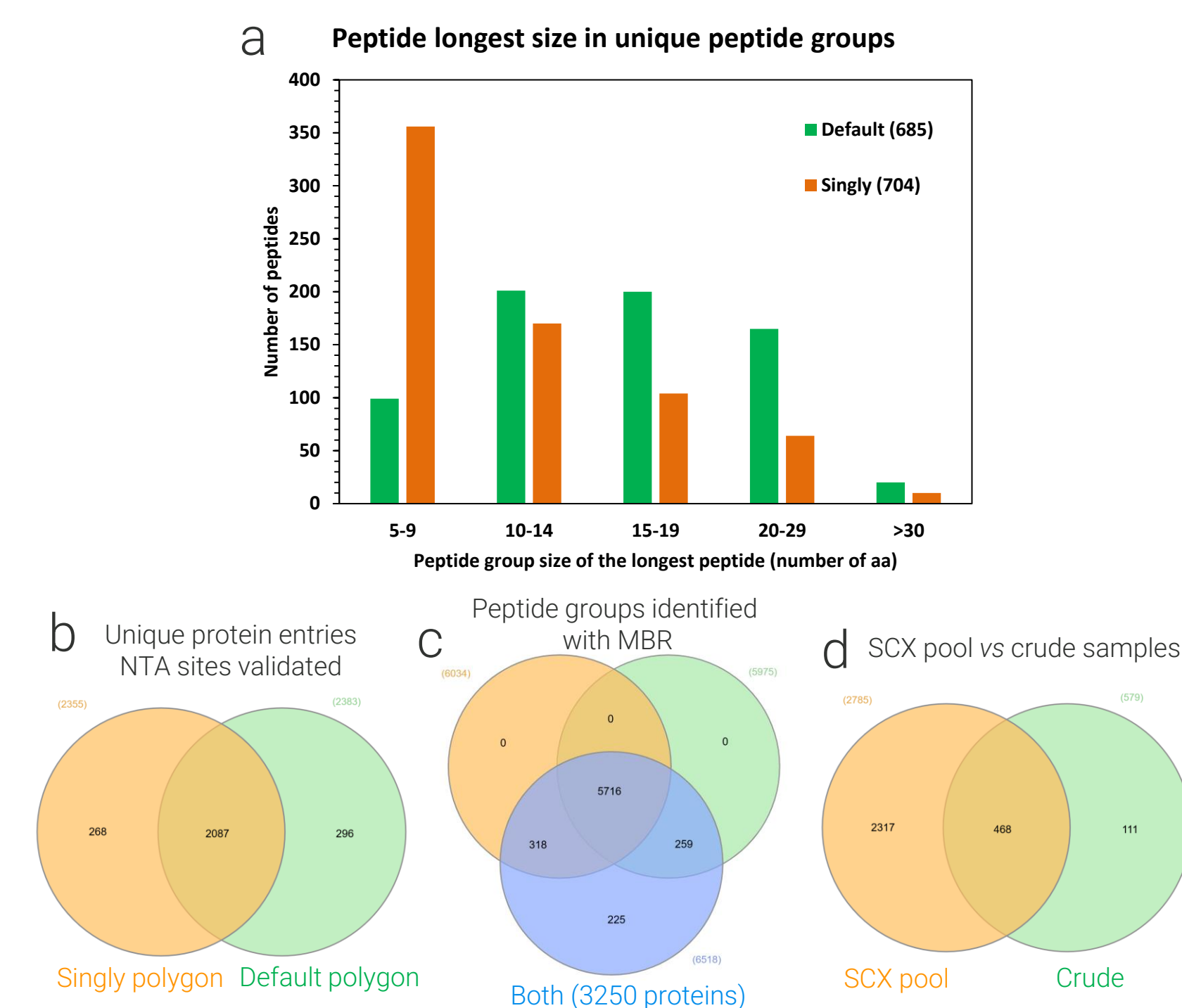


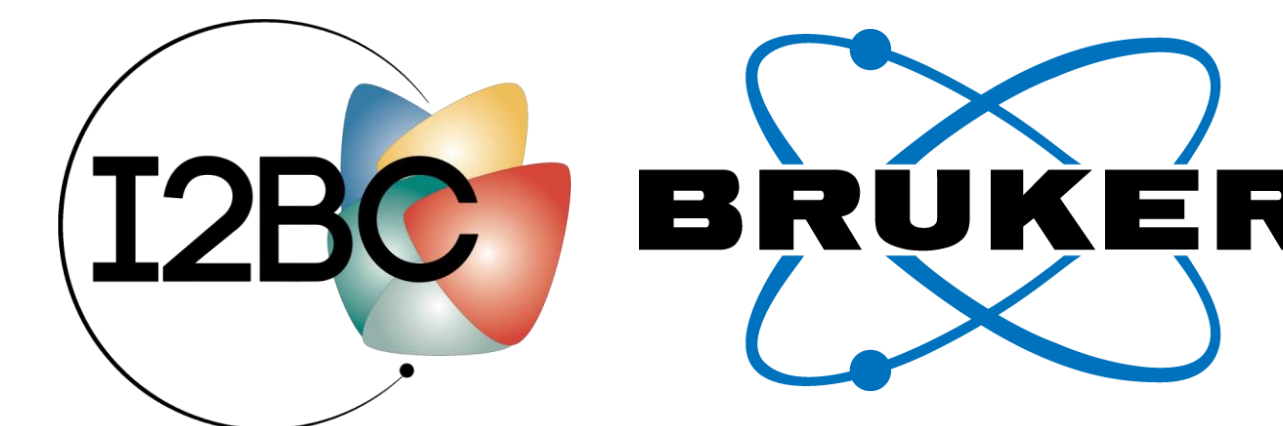
Fig. 3a: Distribution of the longest peptides per Peptide group. Venn diagrams: b: Identified N-terminal acetylation per acquisition method (SCX\_Pool). c: Peptide groups quantified per acquisition method and including MBR (SCX\_Pool). d: N-terminal acetylation sites per sample.

Figure 3a shows the distribution of the longest peptide of the validated peptide groups, which were uniquely identified by the respective method (in total: 685 with default polygon and 704 with adapted one). Nearly half the peptide groups uniquely identified with the singly method were characterized by peptides shorter than 10 amino acids, strongly underlining the benefit of including larger, singly charged precursors.

While both acquisition modes share the majority of identified N-terminal acetylation (Nta) sites, approximately 13% of the sites were uniquely identified using the adapted polygon, particularly due to short peptides (Fig 3b). This underlines that both methods should be applied per sample and merged to achieve deeper insight into the N-terminome.

When using the IonQuant module in FragPipe, match-between-runs (MBR) can be employed to increase the number of quantified peptides. Subsequently, the Nta-Quant script quantifies peptide groups, which consist of peptides belonging to the same entry and sharing the same N-terminus, but vary in

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peptide length (here: 5-44 amino acids) and modification profile. Figure 3c shows that the total number of peptide groups quantified increases by approximately 8% compared to processing without MBR.

The comparison between the SCX\_Pool sample and the crude extract indicates that enrichment via fractionation remains indispensable for deep N-terminomics (Fig. 3d). However, including the crude extract in the analysis contributes approximately 4% additional quantified Nta sites.

## Summary

Our analysis demonstrates that combining acquisition strategies enhances N-terminome coverage. The adapted polygon enables identification of short, singly-charged peptides (5–11 amino acids), contributing uniquely to N-terminal acetylation (Nta) site detection. Approximately 13% of Nta sites associated to unique protein entries were exclusively identified using this approach. Match-between-runs (MBR) further improves quantification, increasing peptide group counts by 8%. While SCX-based fractionation remains essential for deep profiling, including crude extracts adds ~4% more quantified Nta sites. Together, these strategies provide a more comprehensive view of the N-terminome.

## References

1. Bienvenut et al. (2017) Methods Mol Biol 1574, 17-34.
2. Kong et al. (2017) Nat Methods 14, 513-20.
3. Yu et al. (2020) Mol Cell Proteomics 19, 1575-85.

## Conflict of Interest

P. S., B. F., L. S. and P.-O. S. are currently employed by Bruker.

- N-terminal acetylation in *A. thaliana* extract was analyzed using two different precursor selection schemes
- Samples were either enriched via SCX fractionation (first 10 fractions pooled) or analyzed as crude extracts
- Data processing using a dedicated pipeline demonstrates the value of including larger, singly charged peptides to gain deeper insights into short N-terminal peptides
- Though limited in depth as compared to SCX enrichment, crude extract analysis yields significant complementary results.

Technology