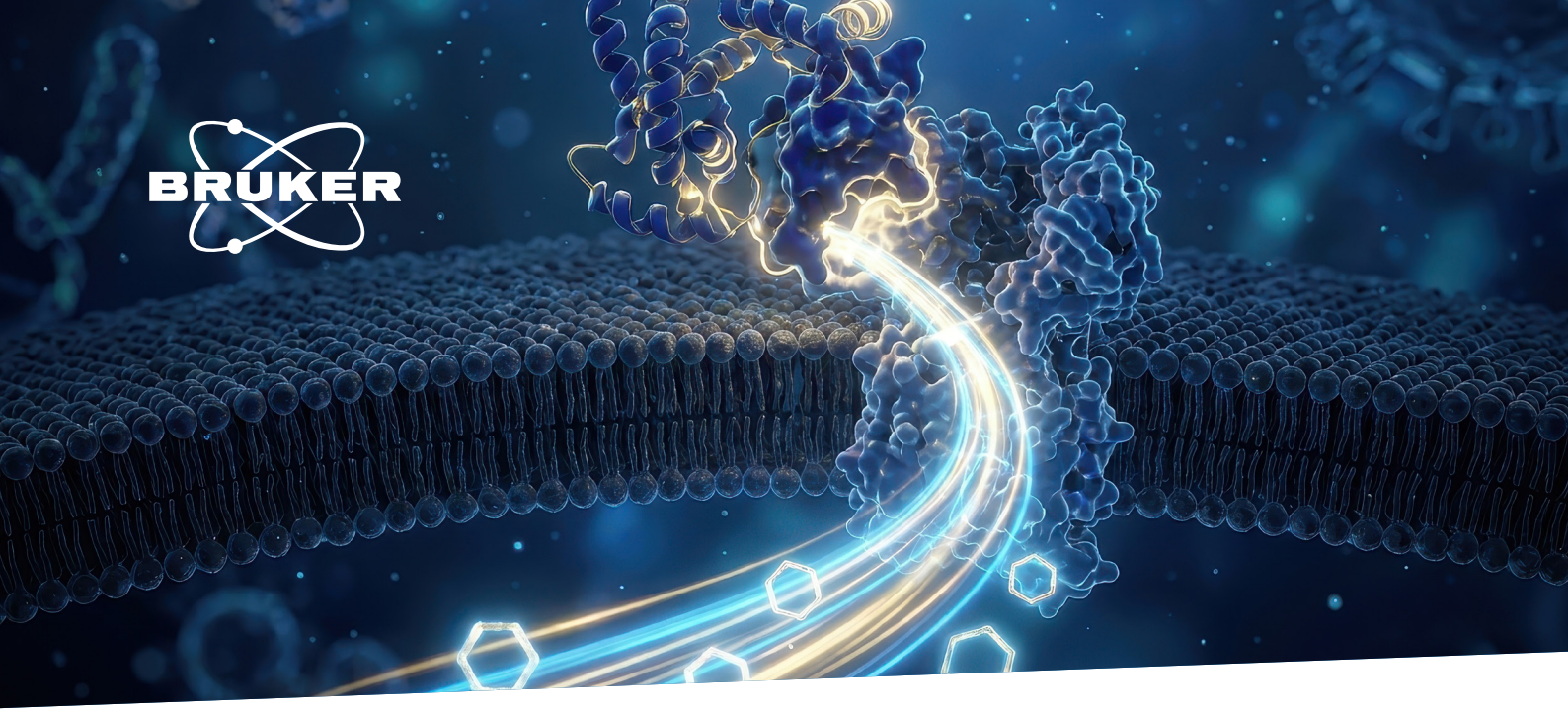

High Confidence Glycopeptide Structural Analysis Using timsOmni

Accurate glycan structure characterization is essential for understanding glycopeptide heterogeneity and site-specific modifications that influence biological function. The timsOmni™ platform combines ion mobility with complementary collision- and electron-based activation modes to resolve isomeric glycoforms and support confident structural interpretation.

Abstract

Glycopeptide characterization requires simultaneous peptide backbone cleavage and the production of structurally informative glycan fragments. The timsOmni platform combines TIMS with highly selective PASEF scan modes and the Omnitrap® architecture that supports tuneable ECD, hot ECD (hECD), ECID and EID. These electron based activation modes generate complementary peptide and glycan fragments for confident site localization and structural analysis. Here, we illustrate how electron-based fragmentation within the Omnitrap overcomes prior hurdles to comprehensive glycopeptide characterization, particularly for lower charge precursors, larger glycans and O-linked glycopeptides with several potential glycosylation sites.

Keywords: glycopeptide analysis, glycoproteomics, ECD, ECID, EID, electron-based dissociation, site-specific glycosylation, O-glycopeptide localization, glycan topology, timsOmni

Introduction

Glycopeptides exhibit extensive structural diversity arising from branching patterns, linkage variations, and positional isomers. The labile nature of glycosidic bonds complicates their measurement, as fragmentation methods must preserve key glycan information while also generating peptide backbone ions that enable site localization. Electron-based dissociation is well suited for this purpose, but until now, limitations were observed for lower charge states, glycopeptides carrying large or sialylated glycans and O-glycopeptides with closely spaced potential glycosylation sites.

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The timsOmni platform enables a wide range of energies and reaction times with a precisely controlled set of electron-driven fragmentation conditions. Within the Omnitrap region, electron kinetic energies can be tuned to produce electron capture dissociation (ECD), electron ionization dissociation (EID) or hybrid modes (hECD, ECID), each yielding distinct combinations of glycosidic, cross ring, and backbone fragments. In parallel, the dual TIMS system allows PASEF acquisition which concentrates ion flux to obtain high quality MS/MS spectra with mobility separated precursors at LC time scale. Finally, the AIP collision cell enables the detection of fragments across a broad *m/z* range, including high mass c/z ions that retain the intact glycan. Together, these capabilities make timsOmni the platform of choice to address both peptide centric and glycan centric questions.

Methods

This study evaluated a range of glycopeptide mixtures (>70% glycopeptide content), standard glycoprotein digests (AGP, transferrin, RNase B, and fetuin), and complex mixtures containing relatively low glycopeptide abundance. The samples were introduced with a nanoElute 2 and a CaptiveSpray Ultra 2, using an Aurora Ultimate C18 column (IonOpticks, 25cm x 75 µm, 1.7 µm particle size).

Acquisition strategies included targeted-PASEF EXD and dda-PASEF CID/EXD. Data were processed using MSFragger (PTM Shepherd and O-Pair), Byonic (Protein Metrics), and OmniScape where manual validation was required.

Electron based dissociation was evaluated using multiple energy regimes: ECD (~2 eV), hECD (~7 eV), ECID (~12–17 eV), and EID (~35 eV). The corresponding hybrid modes that apply CID activation after EXD are denoted by ECciD, hECciD, ECIciD and EIciD. Typical electron irradiation times of 15 to 30 ms were applied.

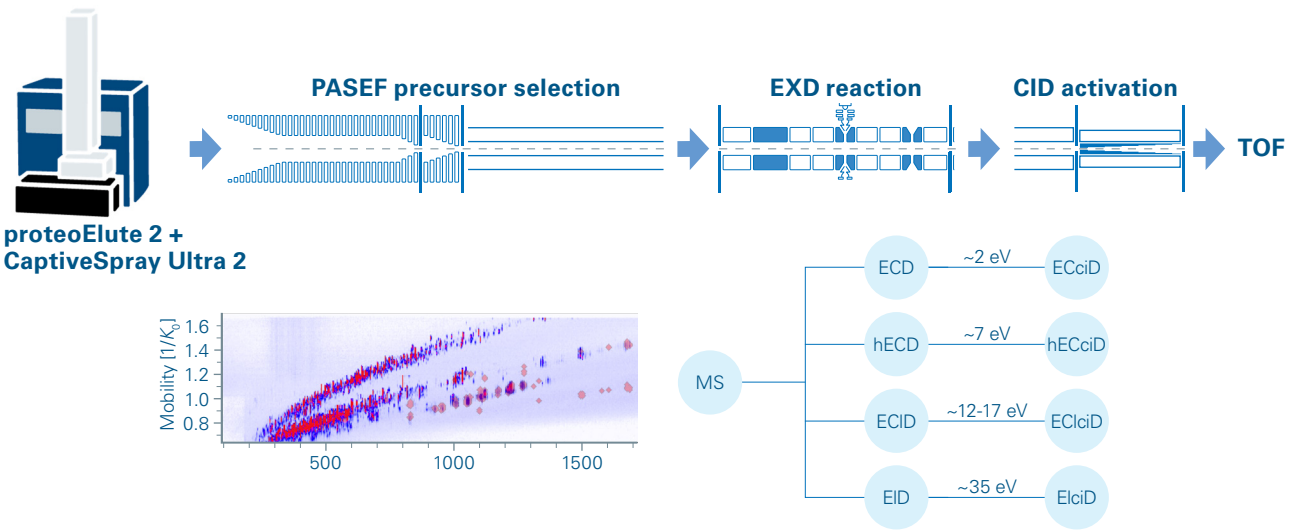


Figure 1. Integration of TIMS and the Omnitrap enables multiple electron-based dissociation strategies (ECD, hECD, ECID, EID and hybrid EXciD modes) that can be selected and tuned based on glycopeptide charge state, mass, and structural complexity.

Results and Discussion

Complementary modes for in-depth characterization

The tunable electron activation modes available on the timsOmni (Figure 1) provide complementary structural information for glycopeptide characterization. ECD generates extensive backbone c- and z-type ions while preserving labile glycan moieties intact, enabling confident localization of N- and O-linked glycosylation sites. The efficient implementation of trapped EXD (tEXD), in which ions confined within the Omnitrap are exposed to a high-flux electron beam, delivers high reaction yields within short reaction times of 10-30 ms.

Increasing the electron energy toward EID conditions enhances the formation of radical species in higher charge states, thereby enabling alternative dissociation pathways. In addition, CID activation following electron irradiation produces complementary information, including b- and y-type peptide backbone ions, as well as large glycan-derived fragments resulting from glycosidic bond cleavage (Y ions) and cross-ring cleavages (X ions). Figure 2 presents a fully annotated mass spectrum from a glycopeptide standard in which a series of X ions enables confirmation of linkage patterns within a complex glycan structure.

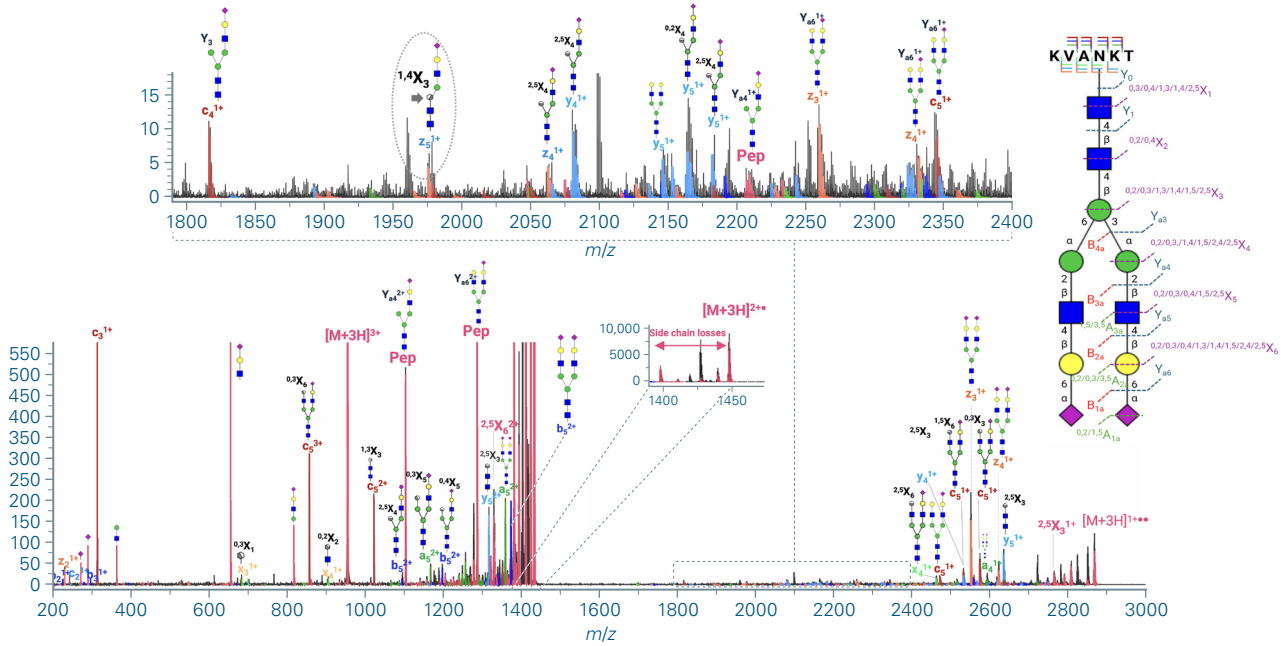


Figure 2. Comprehensive characterization of the SGP glycopeptide using an ECciD experiment (30 ms electron-irradiation time, 20 eV CID activation). Extensive backbone and glycan fragmentation is observed across the m/z range, including diagnostic glycan cross-ring cleavages.

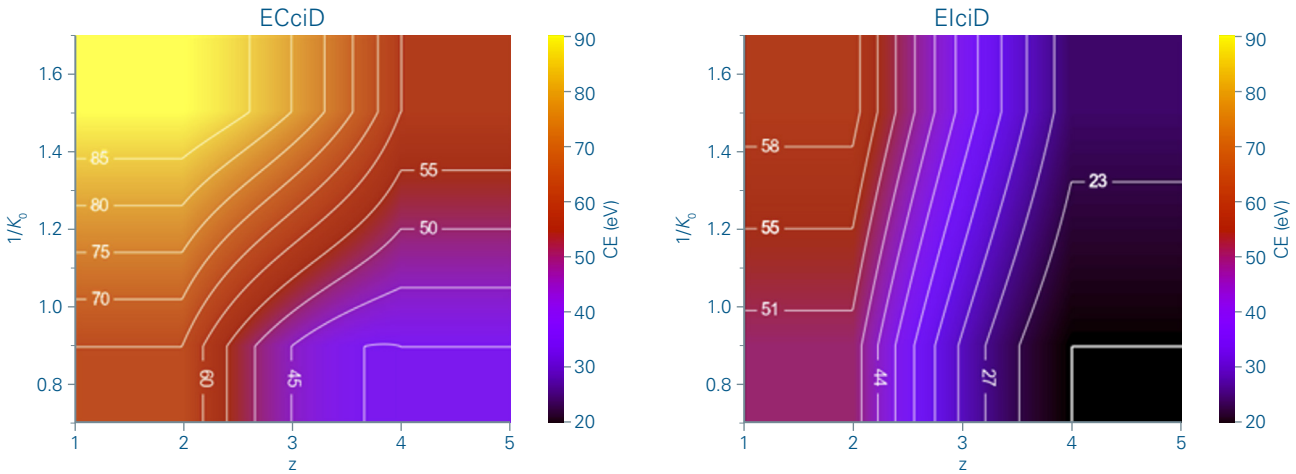


Figure 3. Post tEXD activation with CID can be normalized based on the precursor mobility and charge for optimal fragmentation

The precursor charge state and ion mobility also strongly influence the collision energy (CE) required to generate highly informative fragmentation spectra. PASEF-EXD methods enable dynamic CE calibration based on the precursor charge state and $1/K_0$ value determined during precursor selection. As shown in Figure 3, ECD generally requires higher collision energies than EID, reflecting the predominance of lower charge states formed upon electron capture compared with the higher charge states generated during electron ionization.

ECID introduces additional glycosidic and cross-ring cleavages, generating fragments that provide insights into glycan topology and linkage arrangements. EID further enhances the abundance of large Y ions and additional cross-ring fragments, facilitating differentiation of glycan isomers within chromatographic timescales. Detailed analysis of these fragmentation patterns revealed that ECciD is particularly well suited for the characterization of O-linked glycopeptides, whereas EIciD produces rich fragmentation signatures across multiple glycan classes, enabling structural characterization of complex and hybrid glycans (Figure 4). Access to a broad range of electron energies for glycopeptide analysis therefore enables structural characterization that previously required digestion with glycan-specific enzymes.

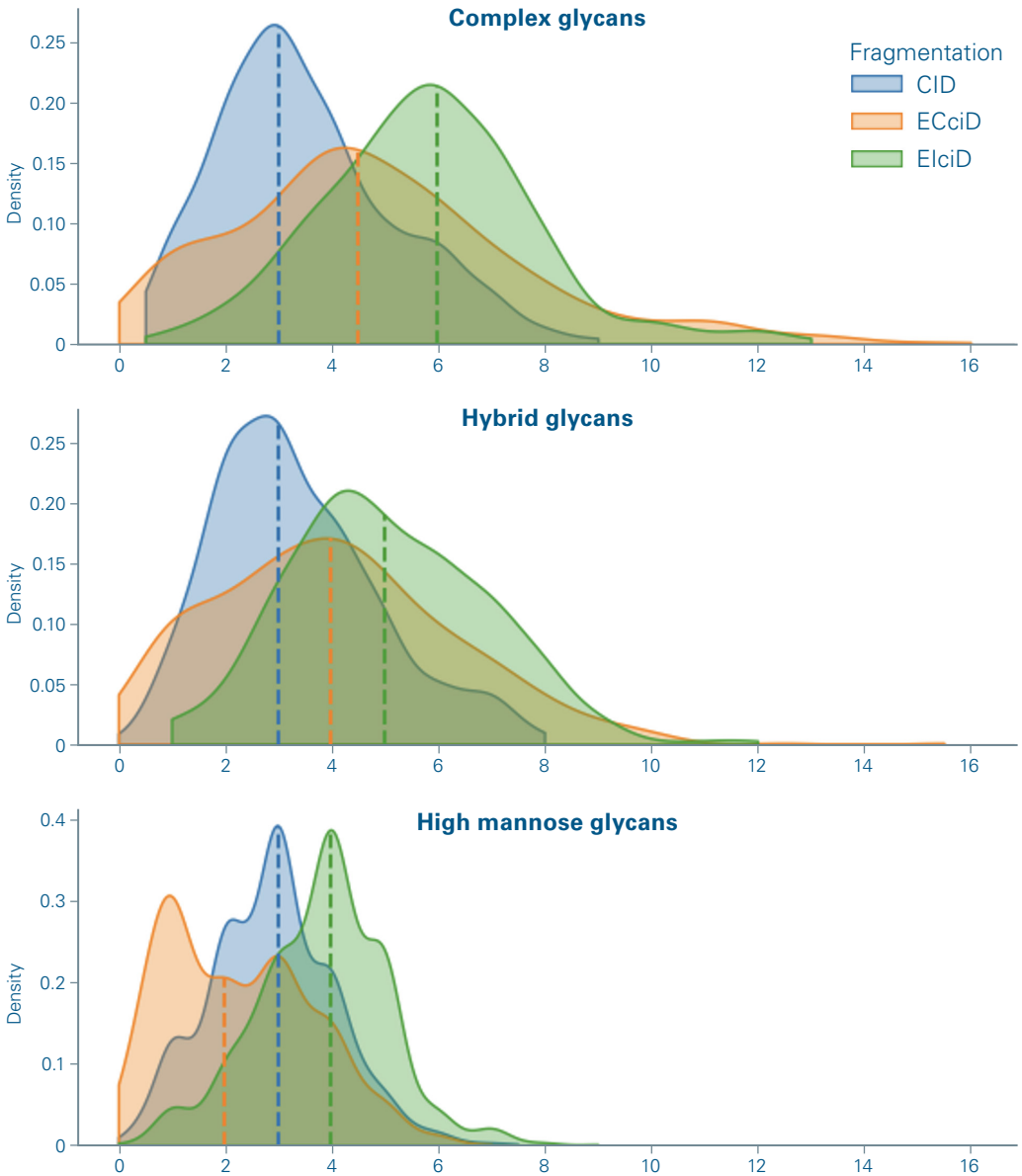


Figure 4. Median length of Y ions observed in MS2 data for glycopeptides from a milk proteins digest

O-Glycopeptide Characterization and Site Localization

The application of ECciD fragmentation provides complementary structural information for O-linked glycopeptide analysis, particularly in peptides with multiple adjacent serine and threonine residues that complicate site localization. ECciD generates a robust combination of a/z and c/z backbone fragments with intact glycan moieties, effectively resolving ambiguity in glycan positioning. Additionally, the method produces b/y fragments that confirm both peptide sequence and glycan composition at the peptide level, enabling comprehensive characterization of O-glycopeptides.

For example, the podocalyxin peptide (**SS**H**S**V**TT**DL**T**S**T**KAEHL) presents eight potential glycosylation sites due to numerous adjacent serine and threonine residues. Characterization of this peptide requires a comprehensive ladder of fragments with intact glycans to accurately determine post-translational modification (PTM) distribution at the residue level. As illustrated in Figure 5, the PASEF-EXD spectrum enables localization of the modifications through glycan-retaining and complementary fragments. Specifically, b5 and c7 ions with intact glycan restrict the position of H1N1 to the –TT– region, while the b6 fragment, observed only in glycan-loss spectra, indirectly pinpoints the glycosylation site at T6. For the second glycan, c10 and x5 ions retaining the H2N2 constrain its localization to the –ST– motif. Applying this analytical approach to other precursors, five unique glycan distributions were identified on the peptide backbone under LC-MS conditions (Figure 6), demonstrating the effectiveness of ECciD in resolving complex glycosylation patterns.

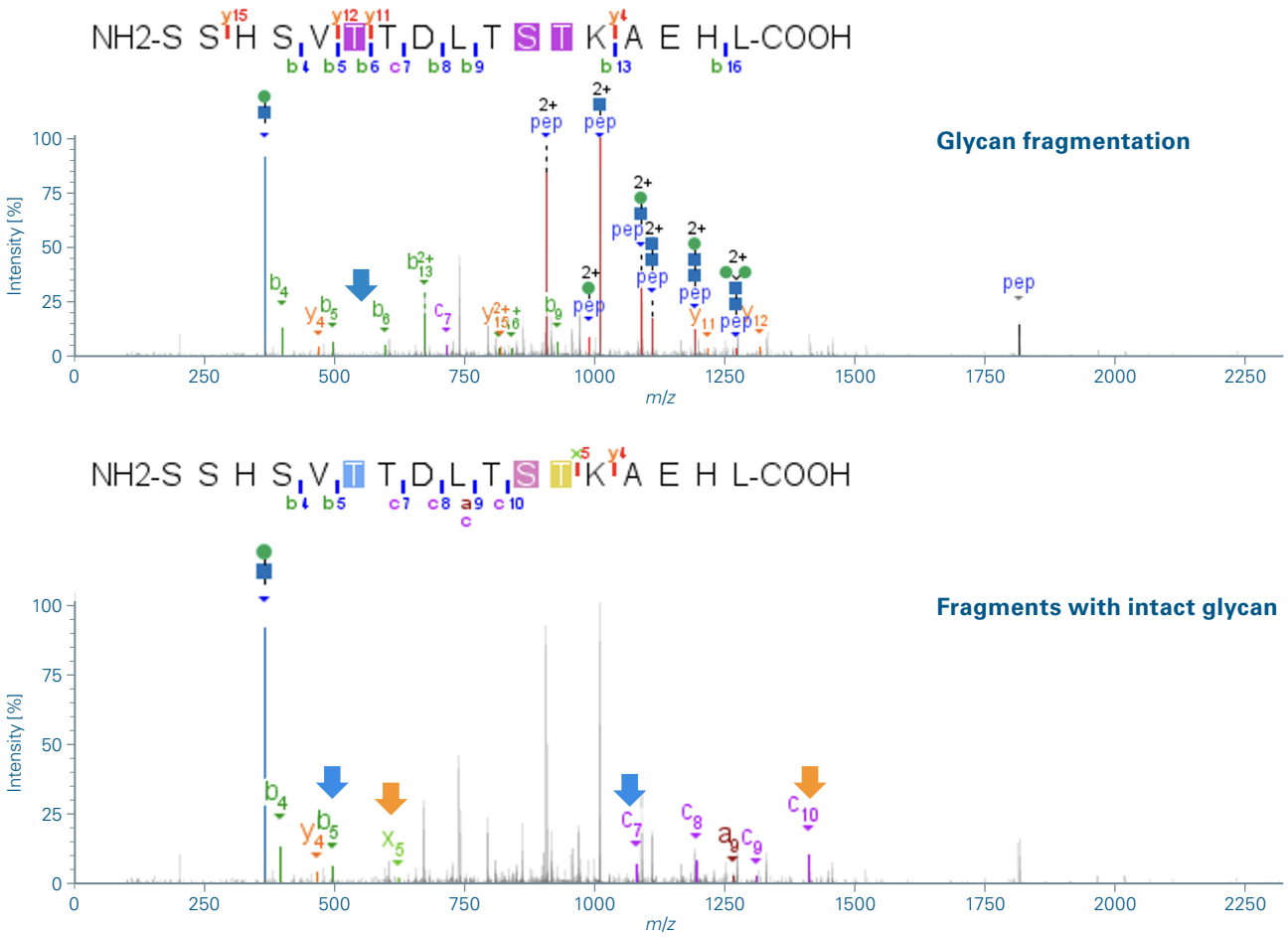
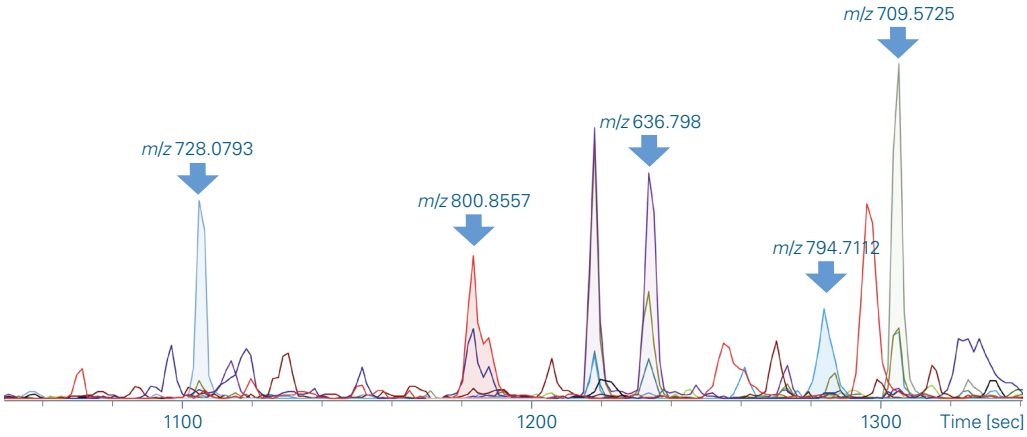


Figure 5. Localization of O-linked glycosylation sites in a podocalyxin-derived peptide using glycan-retaining c and z ions generated by an ECciD experiment.



728.0793	4+	SSHSVT[H1N1]TDLTST[H2N2]KAEHL
800.8557	4+	S[N1]SHSVT[H1N1S1]TDLTST[H2N1]KAEHL
636.798	4+	SSHSVTTDLTS[N1H1]T[N1H1]KAEHL
794.7112	3+	SSHSVTTDLTS[N2H1]TKAEHL
709.5725	4+	SSHSVTTDLTS[N2H1S1]TKAEHL

Figure 6. Identified podocalyxin O-linked glycopeptides with ECciD.

Conclusion

The timsOmni platform brings together mobility resolved precursor selection, PASEF acquisition, and tunable electron based fragmentation to enable comprehensive structural analysis of glycopeptides. By supporting multiple electron energies, the system provides access to peptide backbone ions, glycosidic cleavages, and cross ring fragments within a unified workflow. These capabilities allow confident localization of both N- and O-glycosylation sites, facilitate analysis of precursors across a wide range of charge states and provide structural information necessary to distinguish glycan isomers and to localize N- and O-linkded glycans even in densely modified peptides. The combination of TIMS, PASEF, and the Omnitrap technlogy establishes a unique approach for detailed glycoproteomic characterization in both enriched and complex biological samples.

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