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MS^n Characterization of Chemical Modifications in Pharmaceutical Protein Byproducts Using the timsOmni Platform

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Introduction

Chemical modifications in pharmaceutical protein byproducts can significantly impact stability, efficacy, and safety. These modifications are often present at low abundance, occur on small or heterogeneous proteins, and include chemically similar mass shifts, making confident structural characterization challenging. Assignments based solely on intact-mass information (MS^1) can therefore be ambiguous or misleading. To reliably elucidate degradation pathways and support pharmaceutical quality control, structurally informative MS^n workflows are required that enable site-specific localization and unambiguous discrimination of closely related chemical modifications.

In this study, small synthetic pharmaceutical protein byproducts exhibiting multiple low-level chemical modifications were analyzed using targeted LC-MS^n experiments. A combination of collision-based and electron-based ion activation techniques was applied to systematically interrogate modified proteoforms and evaluate their impact on confident structural assignment.

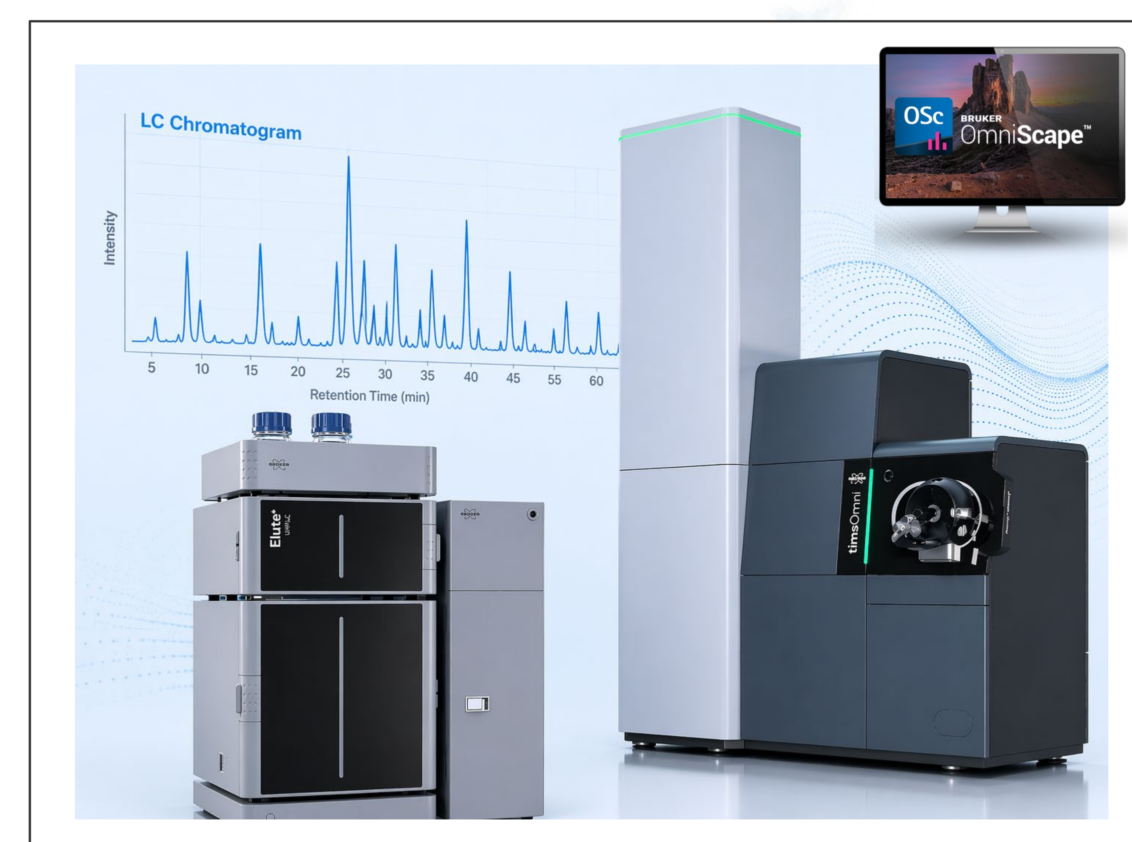

Fig. 1 Method overview: Instrumentation and data processing

Methods

Synthetic protein byproducts were analyzed by reversed-phase chromatography with an Elute+ LC (Bruker) coupled to a timsOmni™ MS system (Bruker). Targeted MS^2 experiments were performed using complementary ion activation techniques, including cCID, ECD, ECciD, and EID, with dynamic control of Omnitrap™ ion accumulation and defined electron irradiation times. MS^n workflows were applied to interrogate modified proteoforms. Data were processed and validated using OmniScape™ software, enabling systematic PTM screening and localization of modification sites. A method overview is shown in Fig.1.

Results

Protein A

For the minibinder synthetic protein A, the LC chromatogram revealed two distinct peaks corresponding to different deamidation products (Fig. 2). Each peak was subjected to targeted MS^2 analysis using ECD, EID, CID, and ECciD fragmentation. For the first LC peak, OmniScape PTM Screening strongly indicated deamidation in the C-terminal region, localizing the modification to either an asparagine or a proximal glutamine residue.

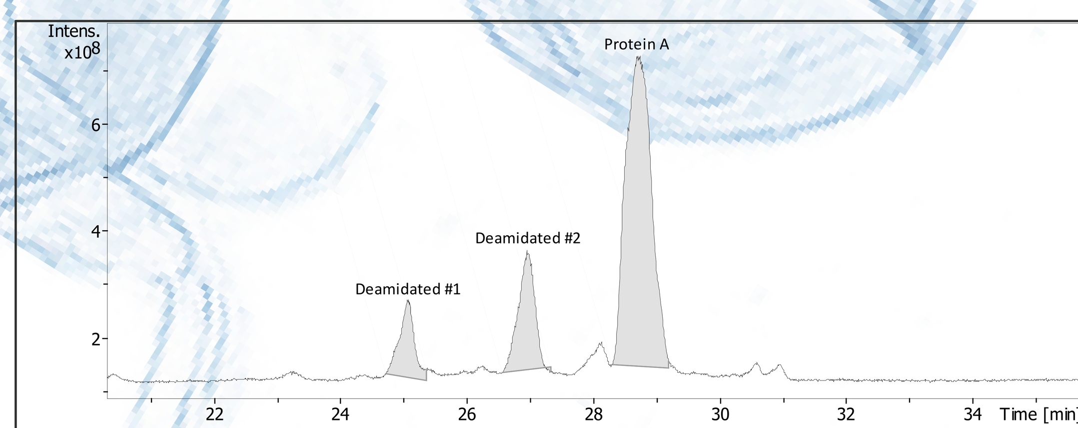

Fig. 2 Total Ion Chromatogram of minibinder protein A showing two deamidation products eluting prior to the main component.

Subsequent confirmation workflows focusing on these candidate sites demonstrated that deamidation at the asparagine residue consistently provided higher sequence coverage across all fragmentation methods. In addition, multiple confidently annotated fragment ions spanning the sequence region surrounding the modification supported localization to this asparagine residue, leading to its assignment as the primary site of modification (Fig. 3).

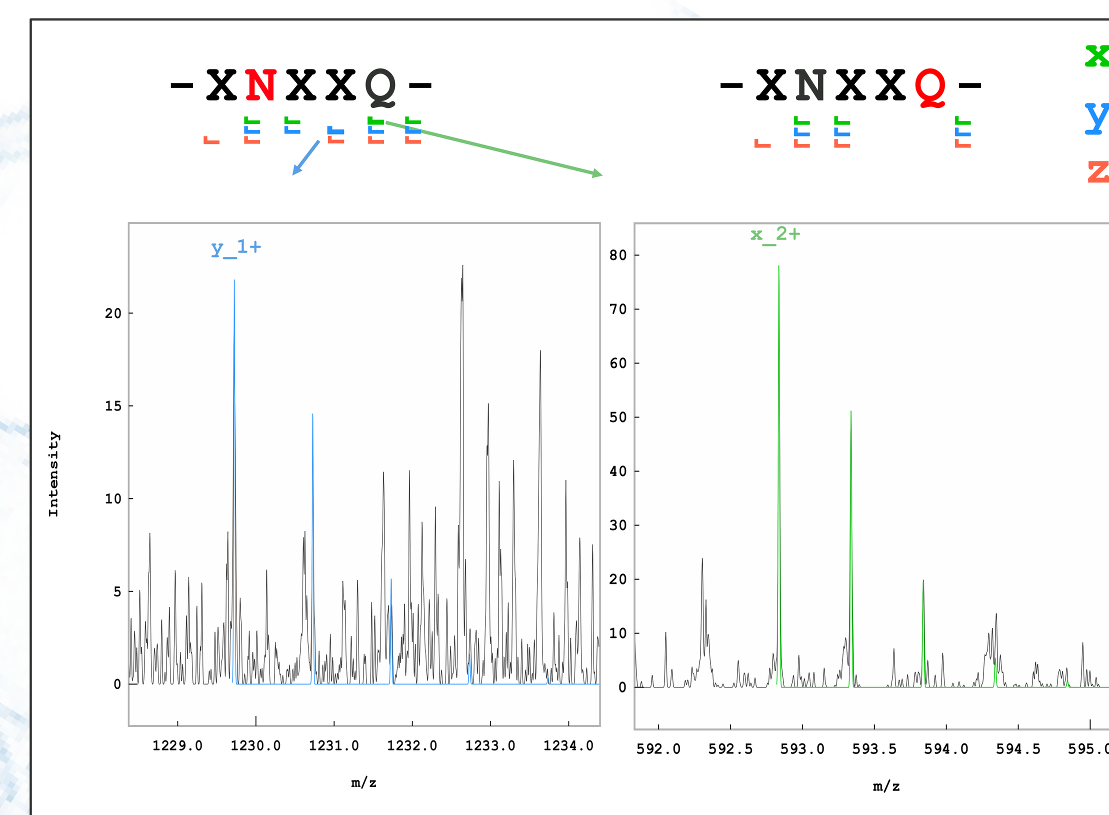

Fig. 3 Sequence map cutouts of the two top-scoring proteoforms based on EID fragmentation (top, deamidation site in red). Inspection of unique fragment ions confirms site-specific deamidation at the asparagine residue (bottom).

Analysis of the second LC peak, including detailed examination of fragment ion patterns, revealed highly similar scores for deamidation in the N-terminal region at two neighboring asparagine residues across all applied MS^2 techniques. The comparable fragmentation behaviour and overlapping evidence indicate the presence of a mixture of two proteoforms (Fig. 4).

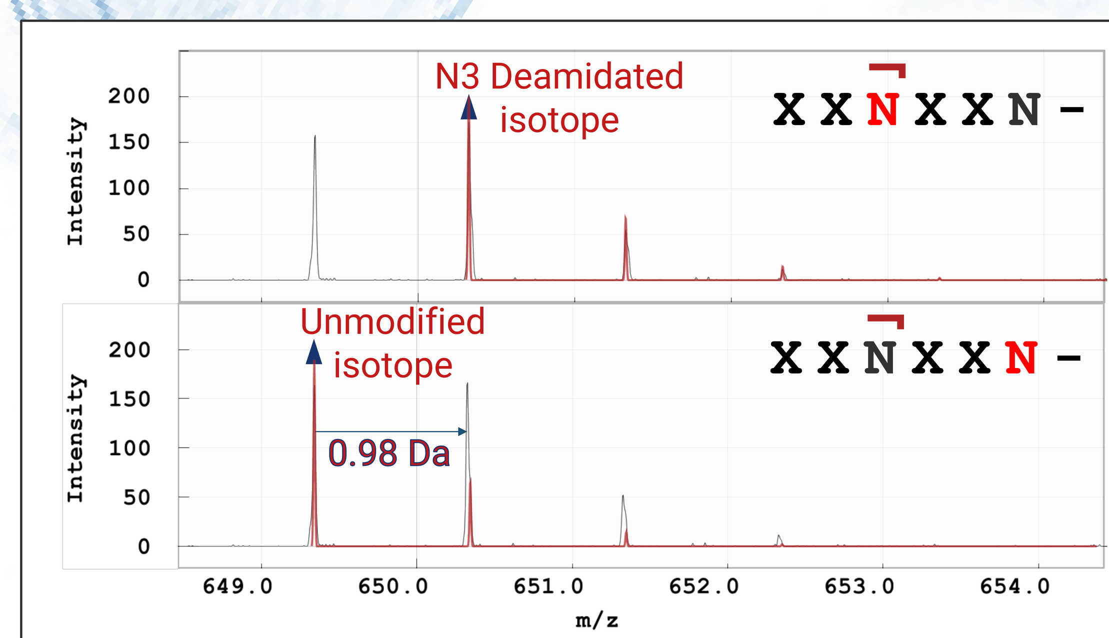

Fig. 4 c-ion fragment from ECD experiment evidences the presence of two distinct deamidation events close to the N-terminus (deamidation site in red).

Protein B

For the second protein, targeted MS^2 experiments focused on a pyroglutamylated species observed with and without an additional 32 Da adduct. Based on MS^1 data alone, this mass shift was initially assigned as dioxidation.

However, data analysis in OmniScape of the corresponding MS^2 spectra revealed systematic ppm mass error shifts exclusively for fragment annotations containing two oxygen atoms. Reassignment of the 32 Da modification to a sulfur addition, consistent with trisulfide bridge formation, resulted in uniform mass accuracy across the entire fragment ion series (Fig. 5). This correction confirmed the trisulfide bridge as the true modification and highlighted the limitations of MS^1-based assignments.

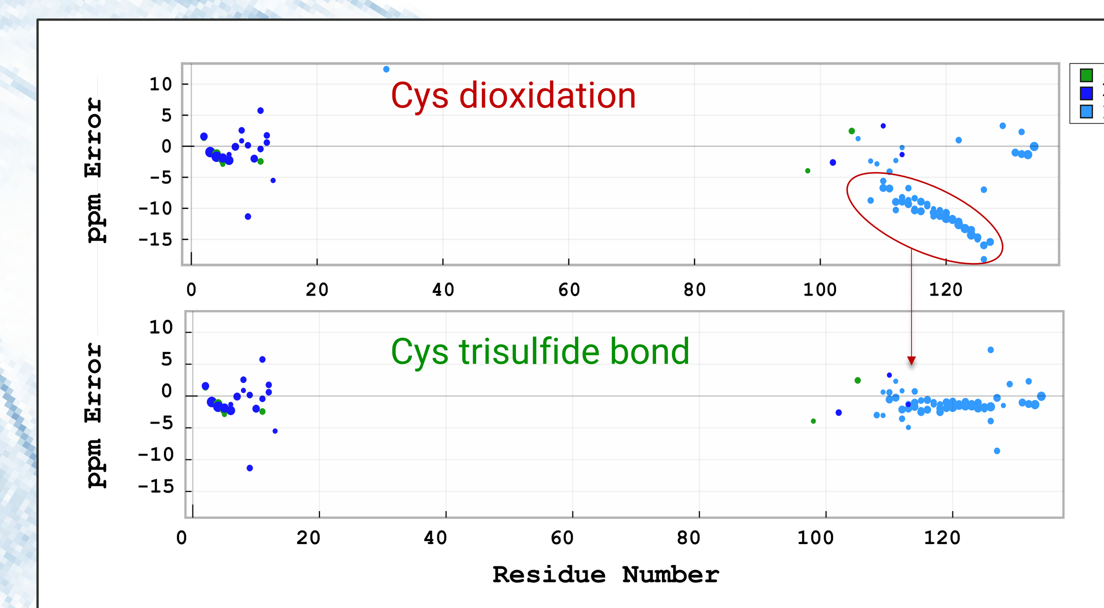

Fig. 5 Trisulfide bond identification using MS^2 y-fragments' ppm trend.

Conclusions

- Targeted MS^n workflows on LC timescale enable confident structural characterization of pharmaceutical protein byproducts.
- Electron-based dissociation reveals diagnostic fragments beyond MS^1 and CID capabilities.
- Complementary CID + ExD strategies distinguish chemically similar modifications with high confidence.
- Trisulfide formation was correctly identified by MS^2 after misassignment as dioxidation in MS^1.

Structural Characterization

Authors from Novo Nordisk have chosen the compounds and questions, evaluated the raw data and have no conflict of interest. Authors from Bruker have built the instrumentation and analysed the samples and may have a conflict of interest as they commercialize the timsOmni.

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