In-depth characterization of glycopeptides by combination of CID and ETD fragmentation after charge state enhancement

**Introduction**

The analysis of protein glycosylation is an important major topic in recent research. Changes in glycosylation patterns can be affected by factors such as disease and therefore variously influencing biological processes. Beside the overall glycosylation pattern the detection of the glycosylation sites and their specific glycosylation patterns is the focus of research. Here, we present a workflow for the analysis of N- and O-glycopeptides combining enhanced glycopeptide intensities with the CaptiveSpray nanoBooster ionization source, the analysis of glycan patterns with CID and GlycoQuest and the identification of glycopeptide sequences and glycosylation sites by ETD.

**Methods**

Several standards e.g. MOPC-21, hCG and EPO were reduced, carbamidomethylated and digested with different proteases. The generated (glyco)peptides (200 fmol-1pmol) were separated on an Ultimate 3000 nanoRSLC system (column: Acclaim® PepMap C18). The amaToN speed ETD ion trap system, equipped with a CaptiveSpray nanoBooster ionization source (Bruker) was used for MS experiments. Acetonitrile-enriched nitrogen was used as sheath gas to enhance glycopeptide intensities and charge states.

Spectra were acquired in AutoMS mode (CID & ETD) using Enhanced resolution for MS and MS/MS acquisition. MS data were processed with DataAnalysis 4.1. Glycopeptide spectra were classified in ProteinScape 3.1 and finally searched against GlycomeDB using the integrated search engine GlycoQuest. In-depth interpretation of ETD spectra was carried out in BioTools 3.2.

**Results**

Even nowadays the analysis of glycopeptides is still a challenge. The generally lower ionization efficiency in comparison to non-glycosylated peptides and a high micro-heterogeneity (different glycan structures attached to a single glycosylation site) makes the analysis of N- and O-glycopeptides difficult. In particular ETD fragmentation yield can suffer from low charge states in combination with high m/z of glycopeptides. In order to further improve precursor intensities and also ETD efficiency the new CaptiveSpray nanoBooster with sheath gas enrichment has been used to enhance glycopeptide charge states on the one hand and increase their overall signal intensities on the other hand. We compared intensities and charge states of glycopeptides measured with CaptiveSpray nanoBooster with and without acetonitrile enriched sheath gas.

Figure 1 illustrates extracted ion chromatograms (EICs) of N-glycopeptides analyzed with CaptiveSpray nanoBooster. This figure gives an impression on signal enhancement when using CaptiveSpray nanoBooster as ionization source. Figure 2 gives a detailed overview on the intensity gain of different glycopeptides. Beside overall signal intensity enhancement, additional higher charge states could be observed.

Charge distribution and also signal enhancement are dependent on glycan structure, peptide sequence, glycosylation site and finally LC-solvent composition at individual retention times. Due to the clearly improved signal intensities when using the nanoBooster option more glycopeptides with better quality fragmentation spectra could be detected and identified. The improvement after charge state enhancement is in particular striking when regarding low abundant glycopeptides. The additional higher charge states led to even higher qualities of ETD spectra since ETD fragmentation works best on precursor with lower m/z-values and higher charge states.

Figures 3a and 3b show the results of the combined CID-ETD workflow. The CID spectra (top) are annotated with glycan fragments derived from the identified glycan structures with GlycoQuest. The ETD spectra (bottom) show the annotated glycopeptide sequence including the modified glycosylation sites.

**Summary**

We have demonstrated that the nanoBooster has a crucial influence on improvement of glycopeptide signal intensities. This simplifies the detection of low abundant glycopeptides and also identification of glycan structures due to higher quality MS/MS spectra.

The enhanced charge states even improve ETD spectra quality. Based on this methodology this study shows the successful application of the different fragmentation techniques – CID and ETD – in combination with improved ionization for glycopeptide sequencing and glycosylation site.

**Conclusions**

- nanoBooster with acetonitrile enriched sheath gas improves glycopeptide intensities significantly.
- Charge enrichment provides higher quality ETD spectra and enables glycopeptide sequencing and identification of glycosylation sites.
- The combination of CID and ETD delivers the full glycopeptide picture: glycan structure, sequence and glycosylation site.

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**Fig. 1:** EICs of hCG N-glycopeptides shown in Fig 2.

**Fig. 2:** Charge distribution and intensities of N-glycopeptides with different peptide sequences (A: CRPINATLAVEK and B: NVTSESTCCVAK) measured with CaptiveSpray and nanoBooster.

**Fig. 3a:** hCGa [M+4H]+ = 757.47 m/z Top: (CID) Identification of N-glycan structure with GlycoQuest. Bottom: (ETD) Elucidation of N-glycopeptide sequence and glycosylation site (N2).

**Fig. 3b:** hCGb [M+3H]+ = 728.05 m/z Top: (CID) Identification of O-glycan structure with GlycoQuest. Bottom: (ETD) Elucidation of O-glycopeptide sequence and glycosylation site (S1,2,3).