New Classification Workflow for $N$-Glycopeptides using Bruker oTOF Instruments and the Glycopeptide Instant Expertise™ Acquisition Method

Mass spectrometry has become the method of choice for glycopeptide characterization. Beside the instrumental setup an appropriate software solution is necessary to support glycopeptide identification and reduce time-consuming processing and de novo analysis time. GlycoQuest (search algorithm in ProteinScape) is Bruker’s well-established solution and provides reliable results in terms of glycopeptide identification.

This technical note describes a new optimized classification workflow for glycopeptides analysed with the Glycopeptide Instant Expertise™ acquisition method (QToF series). Glycopeptide fragmentation can be achieved under standard collision induced dissociation (CID) conditions, where the glycosidic bonds between carbohydrate bonds are preferentially cleaved. However, this rarely produces peptide b- and y-type ions (cleavage of peptide bond), which makes peptide identification in many cases impossible. Higher-energy CID (HCD), in contrast, mainly

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results in b/y-type peptide ions as well as glycan oxonium ions and fewer Y-type ions from fragmentation of the glycosidic linkage. Hinneburg et al.\textsuperscript{1,2} described a novel glycopeptide instant expertise method which uses multiple collision energies to generate glycan fragments and peptide fragments within the same MS/MS spectrum. With this fragmentation method and the new classification workflow in ProteinScape we are able to identify glycan composition and peptide sequence at the same time and reduce the number false or wrong classified spectra.

![Graph showing collision energy stepping](image)

\textbf{Figure 1}: Collision energy stepping implemented in the Glycopeptide Instant Expertise\textsuperscript{™} method.

### Experimental

**Sample**
All types of glycopeptide sample can be used in this workflow – low complex and high complex, with and without glycopeptide enrichment.

**Instrumentation**
The measurement can be performed using a direct infusion setup or LC separation.

**Mass Spectrometry**

\textbf{Instrument:} Compact, Impact II, MaXis II

\textbf{Method:} Glycopeptide Instant Expertise\textsuperscript{™}

**Data Processing**
Compound classification and subsequent glycopeptide identification is performed using ProteinScape 4.0 and the embedded search algorithm GlycoQuest.
Results and Discussion

Until now, classification of N-glycopeptide spectra in ProteinScape (detection of glycopeptide spectra and calculation of glycan and peptide moiety mass) has been based on glycan fragment distances and oxonium ion detection when using standard CID conditions on ion trap or qToF instruments (see application note LCMS-66). Whereas ion trap spectra (resonant fragmentation) always have [peptide + GlcNAc + H]^+ as the last fragment in the glycan cleavage chain, qToF fragmentation tend to deliver spectra with either [peptide + GlcNAc + H]^+ or [peptide + H]^+ as the last fragment in the chain, depending on size of peptide and glycan moiety and charge distribution over the molecule. Although the classification workflow in ProteinScape has a specific parameter to overcome this problem, the peptide mass of low abundant N-glycopeptides can sometimes be miscalculated and lead finally to no or wrong identifications. To overcome this, we further investigated the fragmentation of N-glycopeptides using Bruker oTOF instruments. Hinneburg et al. used synthetic and thus well-defined N-glycopeptides for the optimization of CID energy parameters to obtain maximum information on both the glycan and peptide moiety within a single tandem MS experiment. The optimum collision energies are shown in Figure 1C. The implementation of these results into a standardized acquisition method was established by using a timed stepping of collision energies and transfer settings (Figure 1A and B). The higher collision energies, which are mandatory for peptide moiety fragmentation, additionally induce a characteristic fragmentation pattern in the case of N-glycopeptide fragmentation, which has previously been described for MALDI fragmentation. Under these conditions, (0,2)X-ring fragmentation of the innermost N-acetylgalactosamine of the chitobiose core (+83 Da), and a loss of the complete N-glycan moiety by cleavage of both the N-glycosidic bond and the side-chain amide group of the N-glycosylated asparagine, yields a characteristic peak doublet with a mass difference of 17 Da. A typical CID N-glycopeptide CID spectrum measured with the Glycopeptide Instant Expertise™ acquisition method is shown in Figure 2. The zoom highlights the fragmentation pattern described above.

![Figure 2](image-url)
Since ProteinScape also supports the classification of MALDI glycopeptide fragment spectra, the software already includes a classification workflow based on the typical fragments shown in Figure 2 and described above (-17 Da, [Peptide + H]^+; +83 Da, +120 Da). This workflow can also be selected for qToF fragmentation, and adds another characteristic trait of N-Glycopeptide spectra that increases the reliability of automated glycopeptide spectra classification (see Figure 3). An example for a classified (new workflow) and identified (by GlycoQuest and Mascot) N-glycopeptide spectrum is shown in Figure 4.
Conclusions

N-glycopeptide analysis can be improved by adjusting CID fragmentation parameters as described by Hinneburg et al.\textsuperscript{1,2} in combination with a targeted classification workflow in ProteinScape. Due to the inclusion of a specific diagnostic fragmentation pattern, the number of wrong calculated peptide masses is dramatically reduced, which leads to highly confident N-glycopeptide identification results.

Figure 4: ProteinScape: Annotated glycopeptide CID spectrum.
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References


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