

# In-depth Characterization of Neutral and Acidic Glycopeptides by ZIC-HILIC Enrichment and Mass Spectrometry

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## Introduction

- Analysis of **glycopeptides** is challenging due to their high heterogeneity and low abundance in tryptic digests.
- Ion suppression effects require selective methods for **glycopeptide enrichment**.
- In this study, we used **ZIC-glyco-capture resins** with a dedicated buffer system.
- We analyzed a tryptic digest of bovine  **$\alpha$ -1-Acid-protein (AGP)**, a protein with complex di-, tri- and tetra-antennary, highly sialylated glycans, N-linked to 5 sites containing both N-acetylneuraminic and N-glycolylneuraminic acid.
- For fast QC of the glycopeptide pool **ESI-ION-Trap** was used.
- LC-MALDI-TOF/TOF-MS** was used for deep analysis for the peptide and the glycan parts of each glycosylation site.

## Methods

- Sample:** tryptic digest of bovine AGP ( $\alpha$ -1-Acid-protein)
- ESI Infusion :** nanomate (Advion)
- ESI-Ion-Trap:** amaZon ETD (Bruker)
- Glycoenrichment:** ProteoExtract® Glycopeptide Enrichment Kit (Art. No. 72103, EMD Chemicals Inc.).
- LC-MALDI Analysis:** Easy-nLC for LC separation, FC II for fraction collection, ultrafleXtreme in linear ion mode for detection of acidic glycopeptides and in LIFT mode for MS/MS analysis (all Bruker).
- Software:** Mascot 2.2 for peptide identification. Glycans were identified using the freeware GlycoWorkbench (<http://www.ebi.ac.uk/eurocarb/gwb/home.action>).

## Results

### ESI-ION-TRAP Infusion-MS:

- Enrichment performance: non-glycosylated peptides largely removed, glycosylated peptides clearly increased (Fig. 1)
- Follow-up experiments show high reproducibility of the method (Fig. 2-3)

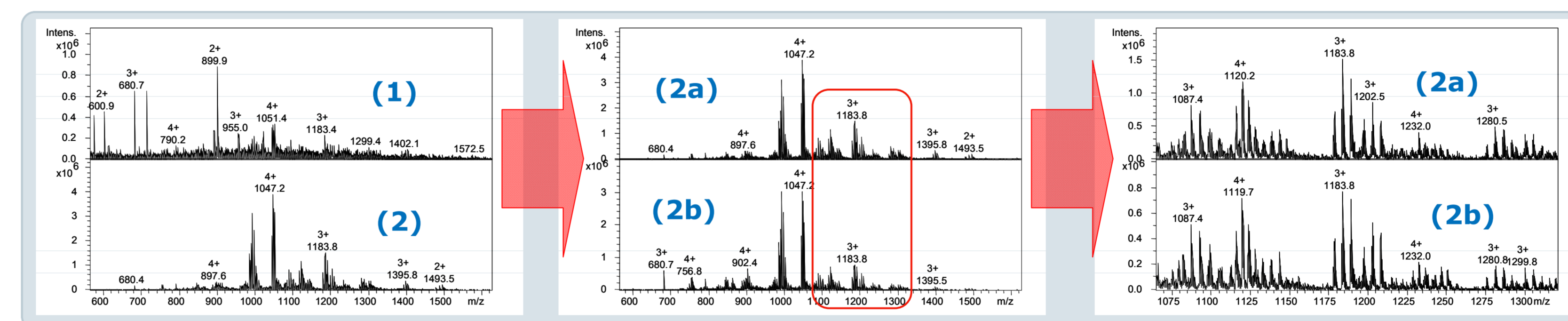


Fig.1 ESI-Ion-Trap-MS comparison bovine AGP digest prior (1) and after (2) enrichment of glycopeptides.

Fig.2 Reproducibility of enrichment procedure: the AGP digest were enriched with different lots (2a, 2b) of the enrichment kit.

Fig.3 Zoom into a narrow m/z range of Fig.2. High reproducibility of the enrichment experiment shown with almost identical MS pattern.

### LC-MALDI analysis

- RP-HPLC separation according to peptide moieties
- Specific fragmentation pattern of MALDI-MS/MS spectra allows automatic MW determination of peptide
- Spectra can be used for protein and for glycan identification via database searches
- Complete workflow is demonstrated for Asn-136 in Fig. 4-6

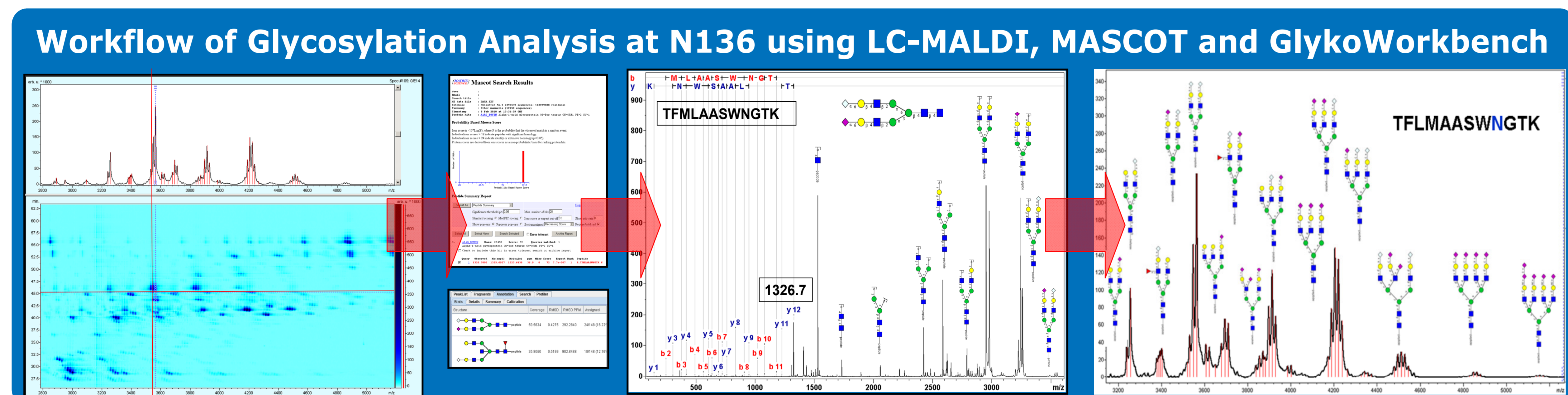


Fig.4 LC-MALDI analysis of the enriched glycopeptide fraction of AGP (Rt vs. m/z). Horizontal line: MS fraction at 45 min showing the heterogeneity at one glycosylation site. Vertical line: Precursor (3547 Da) was selected automatically for MS/MS analysis and mass of the pure peptide was provided: 1326.7 Da!

Fig.5 A Mascot MS/MS ions search of the MS/MS-spectrum of m/z 3547 (Fig.4, vertical cursor), using the aglycone as parent ion m/z 1326.65 Da, provided the peptide sequence. A subsequent glycan search via GlycoWorkbench used the peptide as modification and identified the glycan. The peptide m/z was well assigned in the MS/MS-spectrum just like the peptide sequence below the peptide mass and the glycan fragmentation above it. All this information was derived from this single MALDI-TOF/TOF spectrum.

Fig.6 MS Survey of the main glycoforms of the peptide TFMLAASWN<sup>136</sup>GTK (MALDI-TOF, linear mode). MS/MS spectra of all glycopeptides were analyzed and glycans identified using GlycoWorkbench. Several numbers of antennae and different neuraminic acid types (N-acetyl- and N-glycolyl) give rise to high heterogeneity and require further work to resolve remaining structural uncertainties in the spectrum.

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## Summary

- Glycopeptide enrichment increased the ability to analyze glycosylation sites
- Glycopeptide profiles can be analyzed fast and reproducibly by direct infusion ESI-Ion-Trap-MS
- LC-MALDI of glycopeptides allowed to identify peptide sequences and glycan structures from a single MS/MS spectrum
- LC-MALDI allowed the in depth analysis of all glycosylation sites of a protein in one experiment.

## Conclusions

- A reproducible glycopeptide enrichment method is described
- ESI-Ion-Trap-MS profiles allowed to monitor the reproducibility of the enrichment
- MALDI-MS/MS spectra of glycopeptides allowed to
  - ID the aglycone
  - Localize the N-glycosylation site
  - ID the glycan