# Liquid Chromatography Optimization Glycopeptides Analysis by Electron Transfer Dissociation

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## Overview

**Purpose:** Optimize the liquid chromatography for glycopeptides analysis by Electron Transfer Dissociation (ETD).

**Methods:** Glycoprotein digests were analyzed by nanoLC ESI using different types of HPLC columns and a Thermo Scientific LTQ XL™ mass spectrometer equipped with an ETD source option.

**Results:** Graphitic carbon column demonstrated excellent capabilities for glycopeptides analysis by mass spectrometry without any enrichment.

## Introduction

Glycosylation plays key roles in controlling various biological processes. It is one of the most widespread and complex post-translational modifications (PTMs) found on proteins and its characterization remains a great analytical challenge. LC-MS/MS is the most powerful and versatile technique for glycopeptide structure elucidation. However, commonly used collisional-induced dissociation (CID) has limitations on determining the modification site due to the labile nature of the glycan modification. Also sialic acid residue in glycans of glycoproteins add negative charges, and thus reduces the pI of the corresponding molecule. This additionally complicates the glycoprotein analysis by LC/MS. As a new dissociation technique, Electron Transfer Dissociation (ETD) preserves labile PTMs and provides a new and powerful tool that makes the identification of modification site possible. Since glycosylated proteins and the resulting peptides are often highly heterogeneous, the high quality liquid chromatography is critical for glycopeptides analysis.

In this study, two reasonably well characterized glycoproteins, bovine $\alpha_1$–acid glycoprotein and bovine fetuin [1,2] were analyzed using nano LC MS/MS with ETD. Liquid chromatography separation conditions were systematically optimized for glycopeptides analysis prior mass spectrometry using different stationary-phase columns. Two reversed phase columns, $C_8$ and $C_{18}$, and a graphitic carbon column were evaluated.

## Methods

**Samples:**

Reduced and alkylated enzymatic digests of bovine α1–acid glycoprotein, bovine fetuin. Glycoproteins were purchased from Sigma.

**LC/MS:**

| | |
|---|---|
| HPLC System: | Surveyor™ MS Pump with a flow splitter |
| Columns: | Agilent® ZORBAX 300SB C8 column ( 75 µm x 5 cm)<br>Microtech, C18 column (150 µm x 10cm)<br>Thermo Scientific, Hypercarb™ column (75 µm x 5cm) |
| Mobil Phase: | A: Water, 0.1% formic acid; B: Acetonitrile, 0.1% formic acid |
| Gradient: | 5-50% B in 30 minutes |
| Mass Spectrometer: | Thermo Scientific LTQ XL™ linear ion trap mass spectrometer with ETD and nano-ESI source |
| Spray Voltage: | 2 kV |
| Capillary Temp: | 160 °C |
| Capillary Voltage: | 35 V |
| Tube Lens: | 125 V |
| MSn Target: | 1e4 |
| Mass range : | 50-2000 *m/z* or 100-4000 *m/z* |
| Anion Reagent: | Fluoranthene |
| Anion Reagent Isolation: | On |
| Anion Target: | 2e5 |
| Max Anion Injection Time: | 50 ms |
| ETD Reaction Time: | 100 ms |
| Mass Spectrometer | Thermo Scientific LTQ Orbitrap XL™ |
| Mass range | 400-2000 m/z, resolution 100000 |

**Data Processing:**

Data were processed using BioWorks™ 3.3.1 with SEQUEST®. Xtract program (Thermo Scientific ) was used for deconvolution of multiply charged precursors.

## Results

Figure 1 shows LC/MS analysis of bovine α1–acid glycoprotein on $C_{18}$ and Hypercarb columns. The top panels are the base peak chromatograms and the bottom panels are the bi-antennary glycopeptide 91-99 MS profiles. One pmol of protein digest was injected into $C_{18}$ column versus 500 fmol on Hypercarb column resulting in a three times higher $C_{18}$ base peak intensity than the one from the Hypercarb column. However as shown in bottom panel of Figure 1, chromatography on Hypercarb column promotes additional higher-charge state precursor ions than $C_{18}$ which is instrumental in obtaining a high quality ETD MS/MS. Overall intensity of glycopeptide precursor ions with Hypercarb column are the same or higher than with $C_{18}$ column.

**FIGURE 1. Comparison of base peak chromatograms of bovine $\alpha_1$–acid glycoprotein using $C_{18}$ and Hypercarb columns.**

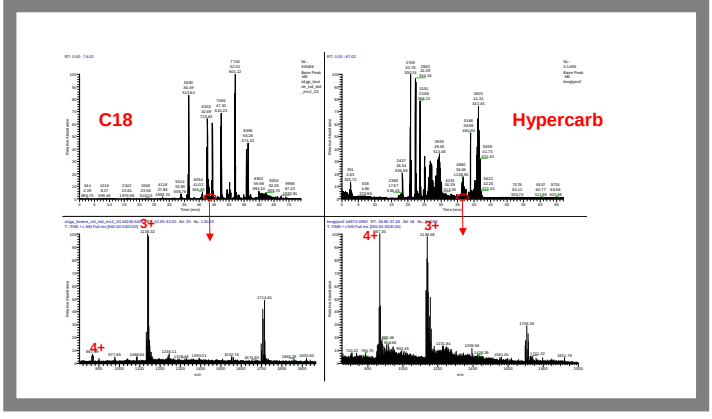
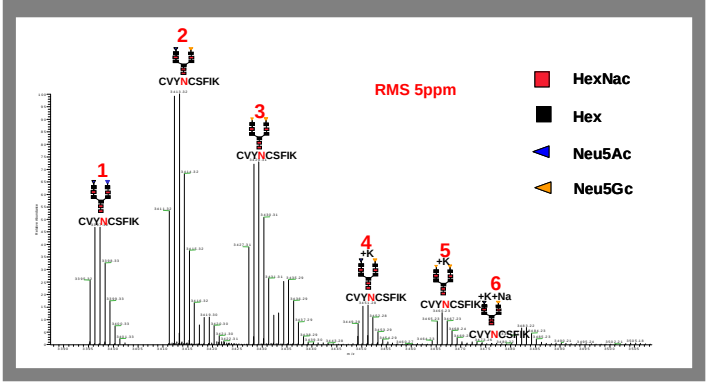


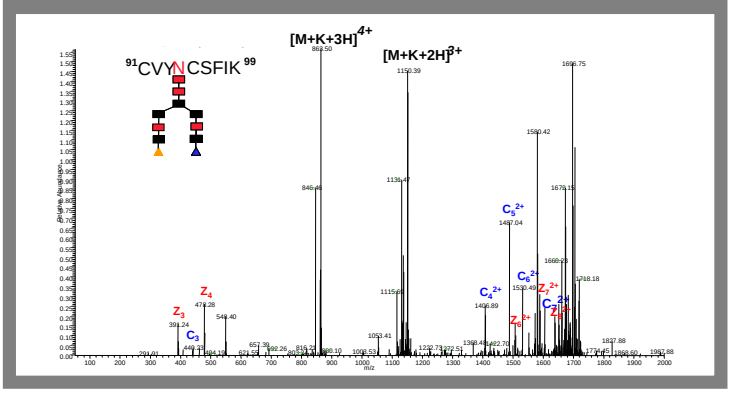

**FIGURE 2. Deconvoluted full MS spectrum of bovine α1–acid bi-antennary glycopeptide $^{91}$CVYNCSFIK$^{99}$**




In addition, as shown in Figure 2, chromatography on the Hypercarb column promotes metal adduct formation. Figure 2 shows a high resolution deconvoluted spectrum of 91-99 bovine glycopeptide obtained on an LTQ Orbitrap XL. At least one metal adduct was observed for each glycoform. As demonstrated in Figure 1, formation of metal adduct is likely responsible for producing high charge state ions: charge state 4+ (867.3) precursor was the dominant peak for glycopeptide 5 while charge state 3+ (1138.6) was the dominant peak for glycopeptide 2 . These results can be explained by partial neutralization of sialic acid negative charge by metal cations [3] and that the metal stabilizes the labile glycans and promotes formation of a higher charge state precursor [4] .

Figure 3 shows ETD spectrum of a Lysine adduct of bovine α1–acid glycoprotein peptide 91-99 charge 4+ (marked as 4 on Figure 2). Glycosylation site and peptide sequence were identified based on almost complete series of c/*z* ions. No significant loss of carbohydrate was detected.

**FIGURE 3 . ETD spectrum of CVYNCSFIK (charge 4+, *m/z* 863)**



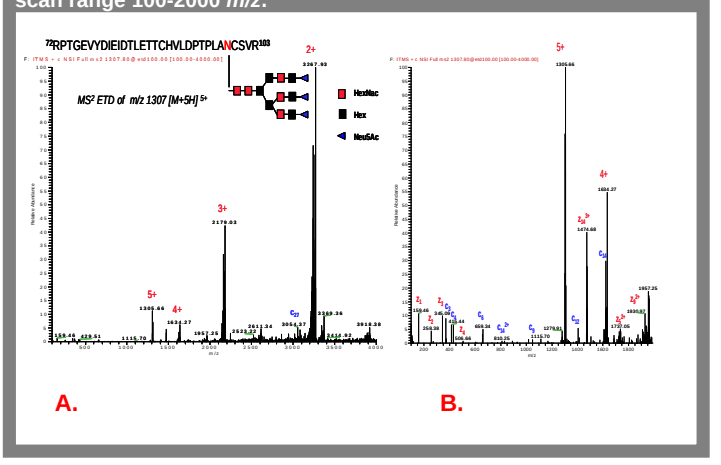

Overall difference in performance of reversed phase chromatography and porous graphitic carbon column can be explained by higher trapping efficiency of graphite column for hydrophilic peptides. Bovine α1–acid glycoprotein contains five N-glycosylation sites with complex-type glycan structures [1] . Four out of five glycopeptides were detected and identified using a Hypercarb column compared to two on $C_{18}$ and three on $C_8$ columns (Table 1) without any enrichment. Only the largest and most hydrophobic peptide was not detected.

**TABLE 1. Bovine α1–acid glycoprotein glycopeptides detected by nanoLC/MS/MS**

| Peptides/ Type of LC column | Graphite | $C_{18}$ | $C_8$ |
|---|---|---|---|
| $^{103}$QNGTLSK$^{109}$ | + | | |
| $^{53}$NPEYNK$^{58}$ | + | | |
| $^{91}$CVYNCSFIK$^{99}$ | + | + | + |
| $^{128}$TFMLAASWNGTK$^{139}$ | + | + | + |
| $^{19}$ QSPECANLMTVAPITNATMD LLSGK$^{43}$ | + | | + |

Similar results were obtained for bovine fetuin digest. All bovine fetuin glycopeptides can be detected after $C_{18}$ [2] or $C_8$ chromatography. Previously Alley et al [5] reported that fetuin glycopeptides could not be observed after separation on graphite HPLC ChipCube. We indeed found that larger hydrophobic peptides required higher organic gradient . Nevertheless three out of four fetuin peptides were identified after separation on the Hypercarb column. Only the largest O- glycosylated peptide 246-306 was not observed. Figure 4 shows an example of an ETD spectrum of charge 5+ of tri-antennary peptide 72-103, which was successfully identified by BioWorks 3.3.1.

**FIGURE 4. ETD spectrum of bovine fetuin peptide 72-103 (charge 5+) Panel A; Full MS/MS ETD spectrum. Panel B; Magnified region for scan range 100-2000 *m/z*.**




## Conclusions

- Graphitic carbon column (Hypercarb, Thermo Scientific) demonstrated excellent capabilities for glycopeptides analysis especially for short hydrophilic peptides containing bi- or tri-antennary glycan chains without any enrichment.
- Formation of metal adducts on Hypercarb column promote higher charge species and as a result promotes ETD fragmentation of glycopeptides.
- Optimization is required for successful glycoprotein nano LC-ETD MS analysis .
- As a gentle fragmentation technique, ETD preserves labile glycans, facilitating the identification of both the peptide of interest and its site of modification.

## References

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