# Improved separation and sensitivity of sialylated glycopeptide isomers on reversed-phase LC-MS/MS

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## ABSTRACT

Among HPLC methods used for protein/peptide separation, reversed-phase HPLC (RPLC) is most common because of its separation power for these modalities. However, the separation of glycosylated peptides, especially glycan isomers, by RPLC is in many cases compromised compared to HILIC and graphitized carbon LC. Here, a RPLC method with improved separation of sialylated glycopeptide isomers, by modifying additives is presented. It improves both the separation and sensitivity of sialylated glycopeptide isomers from a protein digest, while the good separation and high sensitivity of non-glycosylated peptides are still achieved. Combined with the novel fragmentation technique electron activated dissociation (EAD)[1] available on the ZenoTOF 7600 system, the established method allows the detection and characterization of more sialylated glycopeptide isomers.

## INTRODUCTION

Glycosylation is one of the most important and common post-translational modifications during protein synthesis. It has very diverse biological functions and involved in many pathology processes.[2] A comprehensive characterization of glycosylation in protein samples by mass spectrometry, both qualitatively and quantitatively, has gained great interest in an effort for better understanding the roles of glycosylation in biological processes.

Unlike other PTMs such as phosphorylation, glycosylation attaches to peptides with a huge structural diversity and consequently produces a large number of structural isomers. An analytical method which can resolve these structural isomers would be very helpful in characterization of protein glycosylation from different biological samples. While RPLC-MS/MS is the most popular method for peptide analysis, its ability to separate and detect glycopeptides is not as good as that of HILIC or graphitized carbon. This is especially true for analysis of sialylated glycopeptides.[3]

Here, a RPLC method with improved separation of sialylated glycopeptide isomers, by modifying additives, e.g. increasing the formic acid (FA) concentration from 0.1% to above 0.3% is shown. It does not only improve the separation, but also the sensitivity of sialylated glycopeptide isomers from fetuin digest. Good separation and high sensitivity of non-glycosylated peptides were still achieved. In combination with the novel fragmentation technique EAD[1], the detection and characterization of more sialylated glycopeptide isomers could be demonstrated.

## MATERIALS AND METHODS

**Preparation:**
Trypsin/Lys-C digested bovine fetuin was prepared in-house. α(2-3) sialidase was obtained from QA-BIO Canada.
**HPLC conditions:**
A Prominence LC system (Shimadzu) with an ACQUITY UPLC CSH C18 column (130Å, 1.7 µm, 2.1 mm × 100 mm, Waters) at 40ºC was used at a flow rate of 150 µL/min. Mobile phase were water (A) and acetonitrile (B) with different concentrations of FA (0.1, 0.3, 0.5, and 0.7%, respectively) added. A gradient from 2% to 40% B in 45 min with an increase to 80% in 1 min followed by a 4 min isocratic wash step, was used for fetuin digest analysis. A modified gradient from 2% to 10% B in 10 min and to 32% B at min 24 was used for sialidase-treated fetuin digest for improved separation of glycopeptides. Injection amount was 20 µL with 1 pmol/µL of fetuin digest.
**MS and MS/MS conditions:**
A research grade ZenoTOF 7600 system (SCIEX) with Turbo V ion source and electrospray ionization (ESI) probe was used. An MRM$^{HR}$ method with 67 transitions with 100 ms accumulation time per transition was used to acquire MS and MS/MS data of glycopeptides. Zeno EAD was used for all MS/MS experiments. Source and EAD MS/MS parameters are listed below:

| | | | | | |
|---|---|---|---|---|---|
| IS voltage | 5000 V | Curtain gas | 30 psi | Filament Voltage | 1.1 V |
| Gas 1 | 30 psi | CAD | 7 | Kinetic energy | 6.5 eV |
| Gas 2 | 60 psi | DP | 80 V | Electron transmission | 60% |
| Source temp | 200°C | CE | 10 V | Reaction time | 10 ms |

## RESULTS

Two peptides with different glycans were monitored to evaluate the separation and sensitivity. Their sequence, glycan structures and *m/z* are listed in Table 1.

**Table 1. Glycopeptide sequence, glycan structures and their m/z of selected peptides.**

| Glycopeptide sequence | Dominant *m/z* | *z* | A2G2S2 | A3G3S2 | A3G3S3 | A3G3S4 |
|---|---|---|---|---|---|---|
| LC[CAM]PDC[CAM]PLLAPLN[A2G2S2]DSR | 987.1587 | 4 | | | | |
| LC[CAM]PDC[CAM]PLLAPLN[A3G3S2]DSR | 1078.4418 | 4 | | | | |
| LC[CAM]PDC[CAM]PLLAPLN[A3G3S3]DSR | 1151.2156 | 4 | | | | |
| LC[CAM]PDC[CAM]PLLAPLN[A3G3S4]DSR | 1223.9895 | 4 | | | | |
| RPTGEVYDIEIDTLETTCHVLDPTPLAN[A2G2S2]CSVR | 1176.1139 | 5 | | | | |
| RPTGEVYDIEIDTLETTCHVLDPTPLAN[A3G3S2]CSVR | 1249.1403 | 5 | | | | |
| RPTGEVYDIEIDTLETTCHVLDPTPLAN[A3G3S3]CSVR | 1307.3594 | 5 | | | | |
| RPTGEVYDIEIDTLETTCHVLDPTPLAN[A3G3S4]CSVR | 1365.5785 | 5 | | | | |

With 0.1% FA in the mobile phases, the fetuin peptides were separated well and detected by LC-MS/MS. However, some sialylated glycopeptide isomers were partially separated as wide and tailing peaks. For example, a peptide with a glycan containing 3 sialic acids, LCPDCPLLAPLN[A3G3S3]DSR was detected as two partially separated peaks with tailing (Figure 1 top left). When 0.3% FA was used, the major isomers were as detected baseline separated peaks, as well as a few minor peaks. In addition, the sensitivity increased significantly (e.g. 10x for 1534.618 *m/z*, see Figure 1, right).
Zeno EAD MS/MS spectra confirmed glycopeptides isomers for all chromatographic peaks. One example for precursor ion at *m/z* 1151.21 [M+4H]$^{4+}$ with Rt = 13.84 min is shown in Figure 2. It confirms the amino acid sequence and the glycosylation site as evidenced by the observation of C and Z series ions with intact glycan attached.
The glycopeptide containing 4 sialic acids (LCPDCPLLAPLN[A3G3S4]DSR) was not detected with 0.1% FA in the mobile phases, while it could be detected as two major peaks, when 0.3% FA was used (Figure 3).

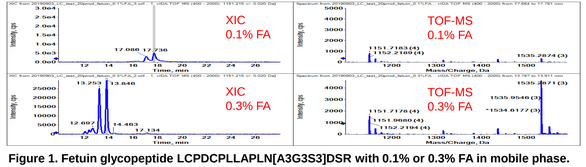


**Figure 1. Fetuin glycopeptide LCPDCPLLAPLN[A3G3S3]DSR with 0.1% or 0.3% FA in mobile phase.** Left: XIC of *m/z* 1151.215. Sharper peaks with higher intensity were observed for 0.3% FA. Right: Averaged spectra for indicated retention time.

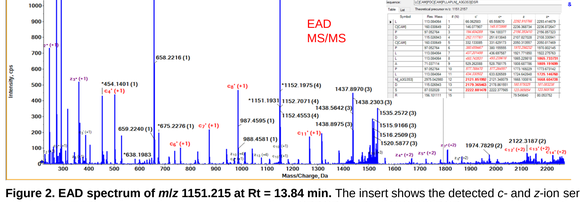


**Figure 2. EAD spectrum of *m/z* 1151.215 at Rt = 13.84 min.** The insert shows the detected *c*- and *z*-ion series obtained by EAD confirming the sequence for peptide LCPDCPLLAPLN[A3G3S3]DSR and providing position information for the glycosylation.

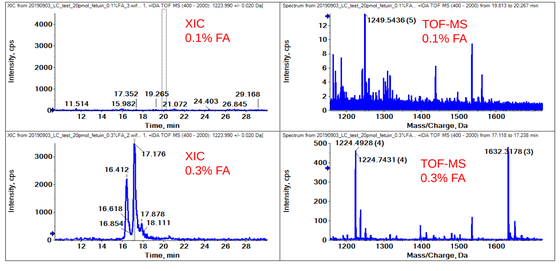


**Figure 3. Fetuin glycopeptide LCPDCPLLAPLN[A3G3S4]DSR with 0.1% or 0.3 % FA in mobile phase.** Left: XIC of *m/z* 1223.990. No peak was observed for 0.1% FA. Right: Averaged spectra for indicated retention time.

It has been reported that fetuin contains many sialic acid linkage isomers, i.e., α(2,3) or α(2,6) linkages.[3] To verify the separated peaks are linkage isomers, the fetuin digest was treated by site specific α2-3 sialidase (Figure 4). In the untreated control sample, two major peaks and one minor peak were detected for the doubly sialylated peptide at m/z 987.16 (Figure 4A), corresponding to α(2-6)/α(2-6), α(2-3)/α(2-6) and α(2-3)/α(2-3) linkage isomers at 15.42, 15.73 and 16.1 min, respectively. After treatment with α(2-3) sialidase, the peaks corresponding to α(2-3)/α(2-6) and α(2-3)/α(2-3) were not observed and only the peak corresponding to α(2-6)/α(2-6) was observed at 15.45 minutes (Figure 4C). Meanwhile, the single sialylated peptide at 914.38 *m/z*, which was only detected as a minor peak at 13.5 min in control sample (Figure 4 B), was detected at 13.48 min after treatment (Figure 4D) with peak height similar to doubly sialyated species at 15.7 min (Figure 4A). The enzyme treatment converted the double sialylated glycopeptide (peak at 15.7 min in Figure 4A) to single sialylated glycopeptide (peak at 13.48 min in Figure 4D). The EAD spectra for both *m/z* 914. 38 and 987.16 are shown in Figure 5 confirming the glycosylation site and glycan structures.

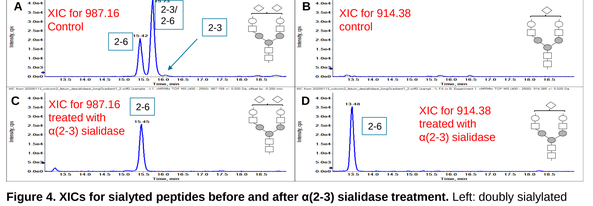


**Figure 4. XICs for sialyted peptides before and after α(2-3) sialidase treatment.** Left: doubly sialylated peptide LCPDCPLLAPLN[A2G2S2]DSR at *m/z* 987.16, [M+4H]$^{4+}$. Right: singly sialylated peptide LCPDCPLLAPLN[A2G2S1]DSR at *m/z* 914.38 [M+4H]$^{4+}$.

It was found that FA addition above 0.3%, did not result in any significant increase of separation nor sensitivity. Use of other additives such as TFA, ammonium formate and acetic acid also did not show any improvement despite similar pH ranges. It is possible that the sialic acids bind to specific sites on the stationary phase favoring an elution with > 0.3 % FA in the mobile phases. Table 2 summarizes the sensitivity gains for the above mentioned 8 glycopeptides (Table 1).

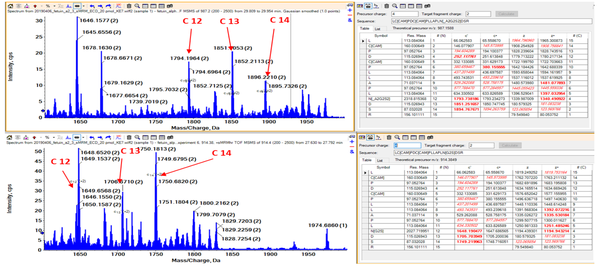


**Figure 5. EAD MS/MS spectra of selected glycopeptides.** Top: LCPDCPLLAPLN[A2G2S2]DSR at *m/z* 987.16. Bottom: LCPDCPLLAPLN[A2G2S1]DSR at *m/z* 914.38. Fragments with intact glycosylation, proving the position, are annotated.

**Table 2. Sialylated glycopeptide isomers and sensitivity gain for 0.3%FA vs. 0.1%FA.**

| Glycopeptide sequence | Dominant *m/z* | *z* | # of isomeric peaks | sensitivity gain |
|---|---|---|---|---|
| LC[CAM]PDC[CAM]PLLAPLN[A2G2S2]DSR | 987.1587 | 4 | 2 | 1 x |
| LC[CAM]PDC[CAM]PLLAPLN[A3G3S2]DSR | 1078.442 | 4 | 5 | 1 x |
| LC[CAM]PDC[CAM]PLLAPLN[A3G3S3]DSR | 1151.216 | 4 | 6 | 3 x |
| LC[CAM]PDC[CAM]PLLAPLN[A3G3S4]DSR | 1223.99 | 4 | 3 | > 10x |
| RPTGEVYDIEIDTLETTCHVLDPTPLAN[A2G2S2]CSVR | 1176.114 | 5 | 4 | 1 x |
| RPTGEVYDIEIDTLETTCHVLDPTPLAN[A3G3S2]CSVR | 1249.14 | 5 | 4 | 1 x |
| RPTGEVYDIEIDTLETTCHVLDPTPLAN[A3G3S3]CSVR | 1307.359 | 5 | 5 | 1 x |
| RPTGEVYDIEIDTLETTCHVLDPTPLAN[A3G3S4]CSVR | 1365.579 | 5 | 4 | >10x |

## CONCLUSIONS

- EAD revealed the glycosylation site information by generation of *c* and *z* fragment ions with intact glycan attached
- The simple RPLC method improved separation and increased sensitivity
- Detection and in-depth characterization of more sialylated glycopeptide isomers, especially for multiply sialylated glycopeptides was achieved

## REFERENCES

1 Takashi Baba et al. *J. Am. Soc. Mass Spectrom.* 2021, 32, 8, 1964–1975.
2 Anna Illiano, et al., *Cells.* 2020 Aug 28;9(9):1986. doi: 10.3390/cells9091986.
3 Yining Huang, et al. *Biomol Tech.* 2016, Sept; 27 (3) 98-104. doi: 10.7171/jbt.16-2703-003

## TRADEMARKS/LICENSING