Comprehensive analysis of low abundant mannose glycopeptides in peptide mapping of adalimumab

Featuring CESI 8000 Plus and TripleTOF® 6600+ LC-MS/MS System

Zuzana Demianova[1], Miloslav Sanda[2], Zoe Zhang[3], Sean McCarthy[4], and Sahana Mollah[3]

1 SCIEX, Brea, CA; 2 Georgetown University, Washington, DC; 3 SCIEX, Redwood City, CA; 4 SCIEX, Framingham, MA

Glycosylation is a common modification that occurs during the production of antibodies or other protein therapeutics. The glycan structures attached to the proteins can directly affect protein stability, bioactivity, and immunogenicity. Therefore, glycan variants of a glycoprotein product must be adequately analyzed and controlled throughout development and production to ensure product quality. Reversed phase-liquid chromatography-mass spectrometry (RP-LC-MS) has become a primary technique for antibody and protein therapeutics characterization. However, it is challenging to chromatographically resolve some types of low abundant glycoforms, such as high mannose glycopeptides (Figure 1).

CESI-MS provides a separation mechanism orthogonal to LC-MS based approaches for separation, identification, and quantification. It provides the exceptionally high resolution power of capillary zone electrophoresis (CZE) in conjunction with the sample identification capability of mass spectrometry. CZE is uniquely able to separate peptides based on charge difference, unlike a reversed phase HPLC, and provides sensitive and reproducible results. The ultra-low flow rate of CESI (~20 nL/min) is highly beneficial in maximizing ionization efficiency and minimizing ion suppression. In addition, the open tubular format of CESI without any solid stationary phase, allows elution of all peptides from the capillary (both hydrophobic and hydrophilic), thus providing better sequence coverage of the antibody.

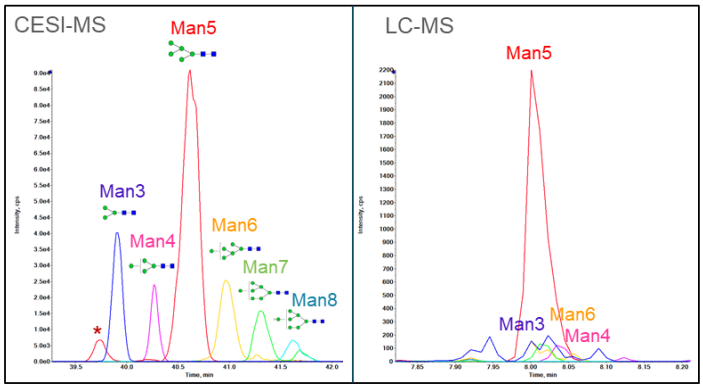

Figure 1. Separation of highly mannose glycopeptides by CESI-MS and LC-MS.

CESI 8000 Plus and TripleTOF 6600+ System with processing software SCIEX OS Software and BYOS

In this technical note, bottom-up peptide mapping of adalimumab (Humira) and its post-translational modifications (PTMs) were performed by CESI-MS analysis. We were able to electrophoretically separate various classes of glycopeptides and confirm using diagnostic ions in the MS/MS spectra.

Key features of CESI 8000 Plus coupled to the TripleTOF 6600+ System

- Capability of electrophoretically separate various glycopeptides including a high mannose species
- Minimum sample required (nanoliters) to obtain high sequence coverage
- High sensitivity of CESI allows for identification of low-level PTMs
- Data Processing using BYOS software (Protein metrics) for identification with MS/MS confirmation of short and hydrophilic peptides which are often missed in LC-MS

Methods

Sample preparation and mAb digestion: Adalimumab (1 mg/ml) was digested with trypsin (gold trypsin, Promega) in a 1:20 (v/v) enzyme-to-antibody sample ratio for 2h at 37°C. Prior to trypsin digestion, antibody was reduced with 20 mM dithiothreitol (DTT) at 56°C for 30 min and alkylated with 20 mM iodoacetamide (IAM) in the dark for 30 min. The alkylation reaction was quenched with 10 mM DTT. A detailed protocol is described in a CESI peptide digest preparation technical note.[1]

Capillary electrophoresis and mass spectrometry: *CESI method parameters:* Trypsin digested sample was diluted in a 1:2 (v:v) ratio with 100 mM ammonium acetate at pH 4. For the analysis, a Bare-Fused Silica Surface OptiMS Cartridge was used (P/N B07367). Sample was injected hydrodynamically at 5 psi for 60 s, resulting in approximately 44 nL injected volume, which is approximately 39 ng of adalimumab on the cartridge. Separation conditions are listed in Table 1.

Table 1. CESI separation conditions used for the peptide mapping of adalimumab

	Event	Time (min)	Pressure (psi)	Voltage (kV)	Direction	Solution/Note
1	Rinse	2.5	100		Forward	0.1 M NaOH
2	Rinse	2.5	100		Forward	0.1 M HCl
3	Rinse	4	100		Forward	Water
4	Rinse	3	75		Reverse	10% Acetic acid
5	Rinse	4	100		Forward	10% Acetic acid
6	Injection	60 s	5		Forward	10% Acetic acid
7	Injection	25 s	0.5		Forward	10% Acetic acid
8	Separation	50	0	15	Forward	10% Acetic acid
9	Separation	5	0	1	Forward	5 min ramp down

MS method parameters: The TripleTOF 6600+ System was coupled with the NanoSpray® III Ion Source with CESI adapter (P/N B07366) and controlled by Analyst® Software 1.8.1. The peptide mapping of adalimumab was performed using data dependent acquisition (DDA) with 10 MS/MS cycles (150 and 50 ms accumulation times for MS and MS/MS, respectively). The acquisition mass range was from 100 to 2250 m/z with cycle time of 0.8 s. The precursors with charges from +1 to +5 and intensity above 100 cps were triggered for MS/MS fragmentation. Selected precursors were fragmented using a rolling collision energy (CE) with spread of ± 5 V. The CE curve slope (Table 2) was modified for better glycopeptide fragmentation.[2] Source parameters were set to ion spray voltage 1850 V, source temperature 30°C and curtain gas 5 psi.

Table 2. The CE curve slope used to calculate CE for rolling collision energy

Charge	Slope	Intercept
Unknown	*0.037*	*-1*
1	*0.038*	*5*
2	*0.037*	*-1*
3	*0.036*	*-2*
4	*0.038*	*-2*
5	*0.038*	*-2*

Liquid chromatography and mass spectrometry: The Waters CSH C18 column (2.1×150 mm, particles 1.7 µm) was used to separate 2 µg of trypsin digested adalimumab using ExionLC™ AD System coupled to a X500B QTOF system. The 0.1% formic acid in water (mobile phase A) and 0.1% formic acid in acetonitrile (mobile phase B) were used. The LC gradient was set accordingly, from 1 to 5 min, 1% of mobile phase B, from 5 to 6 min, 1 to 10% of mobile phase B, from 6 to 32 min, 10% to 25% of mobile phase B, 32 to 65 min, 25% to 40% of mobile

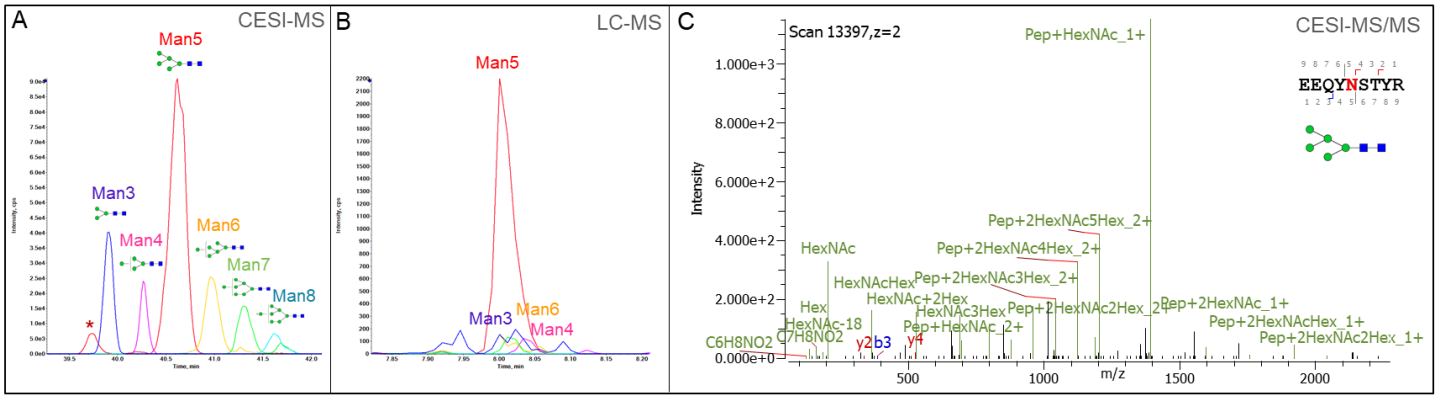

Figure 2. Separation of high mannose glycopeptides of adalimumab using CESI-MS and LC-MS techniques. A) CESI-MS separation, B) RP-LC-MS separation and C) fragmentation spectra of the Man5 glycoform with adjusted rolling CE curve (Table 2) for maximum recovery of the y-ions. * interfering ion

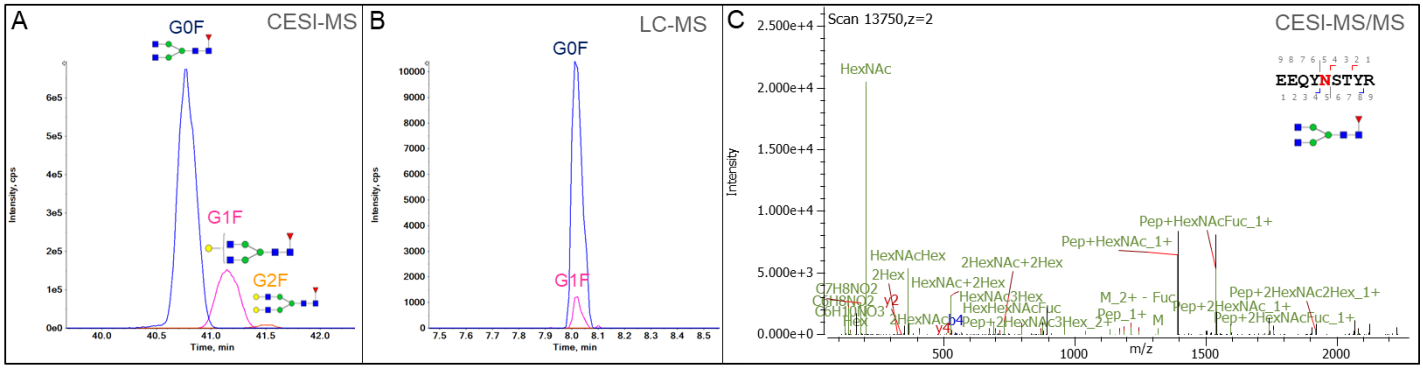

Figure 3. Separation of fucosylated glycopeptides of adalimumab using CESI-MS and LC-MS techniques. A) CESI-MS separation, B) RP-LC-MS separation and C) fragmentation spectra of the G0F glycoform using adjusted rolling CE (Table 2)

phase B, from 65 to 70 min, 40 to 60% of mobile phase B and from 70 to 70.5 min, 60 to 90% of mobile phase B. The analysis length was 75 min using DDA with 8 MS/MS cycles (250 and 40 ms accumulation times for MS and MS/MS, respectively). The MS parameters were ion spray voltage 5500 V, gas 1 60 psi, gas 2 60 psi, curtain gas 55 psi, source temperature 350°C, and rolling collision energy.

Data processing: Data were visualized using SCIEX OS Software and processed with a BYOS (Protein Metrics). Search parameters were set for two miss-cleavages, trypsin digestion and +/- 5 ppm mass error.

Results and discussion

The biologics segment is growing significantly within the therapeutic industry.[3] As biotherapeutic products are derived from recombinant DNA technology and produced in host organisms, monitoring of CQAs (Critical Quality Attributes) is crucial to ensure the consistency of product quality, safety, and efficacy. Glycosylation is considered as one of the crucial CQA of mAbs and biosimilars. A unique feature of CESI compare to LCMS analysis is the ability to separate various glycopeptides and characterize the mAb in a single run. In this study, we focus on full characterization of adalimumab.

Separation and identification of glycopeptide of adalimumab

A high-resolution CESI-MS technique is well suited for the analysis of glycopeptides as it separates peptides based on their charge-to-mass ratio. Of these glycopeptides, high mannose glycopeptides are considered to be some of the most challenging to analyze due to their low abundance in the samples. Figure 1 and 2A show a comparison of CESI-MS and LC-MS separation of high mannose glycopeptides. Man3, Man4, Man5, Man6, Man7 and Man8 were electrophoretically separated from each other, which allowed for the identification of low abundant species compared to the LC analysis (Figure 2B). Furthermore, Figure 2C illustrates fragmentation spectra of Man5 glycopeptide. The y-ion 793 m/z represents the loss of five mannose residues, leaving the two core GlcNAc residues attached to the peptide backbone. The y-ions 1041 m/z and 879 m/z are losses of 2 an 4 mannose residues, respectively.

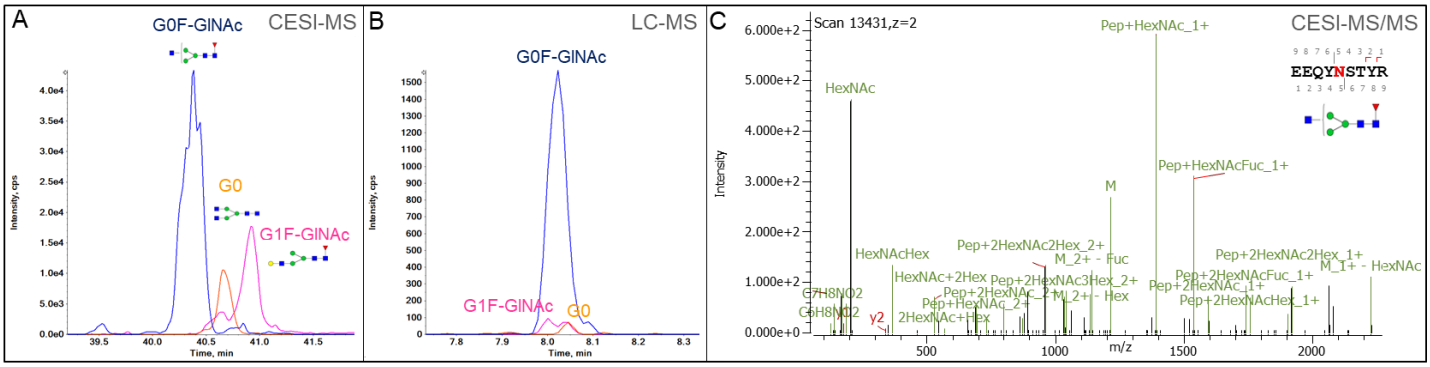

Figure 4. Minor glycopeptides of adalimumab separated using CESI-MS and LC-MS techniques. A) CESI-MS separation, B) RP-LC-MS separation and C) fragmentation spectra of the G0F-GlNAc

Table 3. Glycoforms observed with high confidence

	Mod. Names ↑	General name	Glycans ↑	Score ↑	%Mod
EEQYNSTYR	NGlycan/892.3172	M3	HexNAc(2)Hex(3)	*91.45 - 118.13*	1.43
	NGlycan/1054.3700	M4	HexNAc(2)Hex(4)	*30.00 - 185.00*	0.822
	NGlycan/1216.4229	M5	HexNAc(2)Hex(5)	*30.00 - 184.31*	7.68
	NGlycan/1241.4545	G0F-GlNAc	HexNAc(3)Hex(3)Fuc(1)	*96.01 - 200.92*	3.99
	NGlycan/1298.4760	G0	HexNAc(4)Hex(3)	*30.0018*	0.705
	NGlycan/1378.4757	M6	HexNAc(2)Hex(6)	*60.64 - 89.62*	2.12
	NGlycan/1403.5073	G1F-GlNAc	HexNAc(3)Hex(4)Fuc(1)	*30.00 - 88.31*	1.78
	NGlycan/1444.5339	G0F	HexNAc(4)Hex(3)Fuc(1)	*29.99 - 243.58*	60.4
	NGlycan/1540.5285	M7	HexNAc(2)Hex(7)	*29.99 - 46.53*	1.69
	NGlycan/1606.5867	G1F	HexNAc(4)Hex(4)Fuc(1)	*30.00 - 195.48*	17.8
	NGlycan/1702.5813	M8	HexNAc(2)Hex(8)	*29.99 - 30.00*	0.662
	NGlycan/1768.6395	G2F	HexNAc(4)Hex(5)Fuc(1)	*30.00 - 75.53*	0.914
TKPREEQYNSTYR	NGlycan/892.3172	M3	HexNAc(2)Hex(3)	*163.372*	0.244
	NGlycan/1216.4229	M5	HexNAc(2)Hex(5)	*56.81 - 153.52*	4.09
	NGlycan/1241.4545	G0F-GlNAc	HexNAc(3)Hex(3)Fuc(1)	*29.99 - 116.08*	2.23
	NGlycan/1298.4760	G0	HexNAc(4)Hex(3)	*29.99 - 30.00*	0.831
	NGlycan/1378.4757	M6	HexNAc(2)Hex(6)	*30.00 - 86.90*	1.7
	NGlycan/1403.5073	G1F-GlNAc	HexNAc(3)Hex(4)Fuc(1)	*30.00 - 30.00*	0.721
	NGlycan/1444.5339	G0F	HexNAc(4)Hex(3)Fuc(1)	*29.98 - 143.33*	39.5
		G0F	HexNAc(4)Hex(3)Fuc(1)	*142.943*	34.6
	NGlycan/1540.5285	M7	HexNAc(2)Hex(7)	*29.9984*	0.563
	NGlycan/1606.5867	G1F	HexNAc(4)Hex(4)Fuc(1)	*29.98 - 157.84*	14.8
	NGlycan/1768.6395	G2F	HexNAc(4)Hex(5)Fuc(1)	*30.00 - 30.00*	0.708

Sequential loss of oligosaccharides together with precursor provided confirmatory evidence for the presence of the Man5 glycoform. To obtain a good fragmentation pattern for glycopeptides as well as peptides with the DDA, the rolling collision energy curve slope was lowered compared to instrument pre-set values (Table 2).[2] In addition to y-ions, common glycan fragments (m/z 204, 366 and 528) are found in the spectra (Figure 2C, 3C and 4C). These fragments have a high sensitivity, but low specificity as they are common for all N-glycopeptides.[4]

Fucosylate glycopeptides is another class that is important to monitor as a CQA. This group is normally identified by LC-MS, but are challenging to separate using a RP column, which is commonly used for peptide mapping. In Figure 3A, CESI-MS shows three fucosylate glycopeptides (G0F, G1F and G2F) well separated in comparison to the LC-MS analysis (Figure 3B), which missed the low abundant G2F glycopeptide. Figure 3C represents the MS/MS spectrum of G0F glycopeptide.

In addition to higher abundant glycopeptides, we were able to confirm a few minor species, such as G0, G0F-GlNAc and G1F-GlNAc (Figure 4). Altogether, we have confidently identified 12 different glycopeptides of adalimumab with their relative abundances (Table 3).

Our findings are in good alignment with results from release N-glycan analysis using the SCIEX C100HT Biologics Analyzer.[5]

CESI-MS peptide mapping and post-translational modifications

CESI-MS is an effective and complementary technology to characterize mAbs on the peptide level with a high sequence coverage; in the case of adalimumab, we confidently map 100% of its sequence and have confirmed 12 various glycopeptides.

For the next step, we focused other PTMs, such as deamidation and spontaneous cyclization of N-terminal glutamic acid. Figure 5 illustrates examples of the separation between the unmodified and deamidated form of VGNALQSGNSQESVTEQDSK peptide. The deamidated peptide was determined to be 10.8% of the total amount of peptide in the sample. Deamidation is one of the most common PTMs occurring in therapeutic proteins produced using recombinant DNA technology.

Another example of common PTM is pyro-glutamate. Its presences is frequently detected and mainly attributed to spontaneous cyclization during fermentation, purification and storage. An example is shown in Figure 6, the unmodified

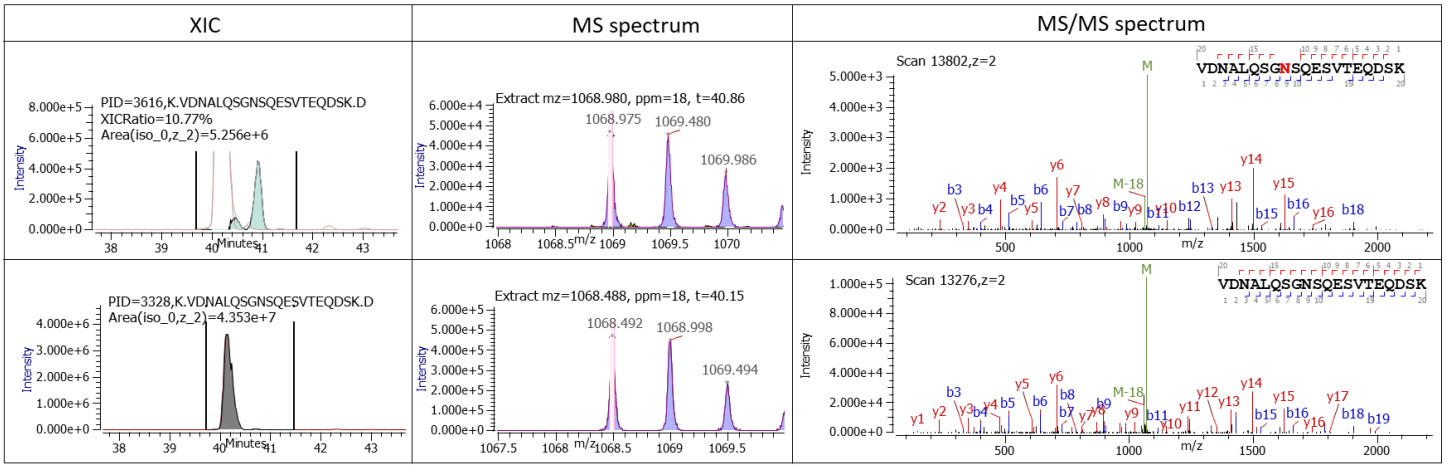

Figure 5. Unmodified vs. peptide with deamidated asparagine are separated by CESI-MS.

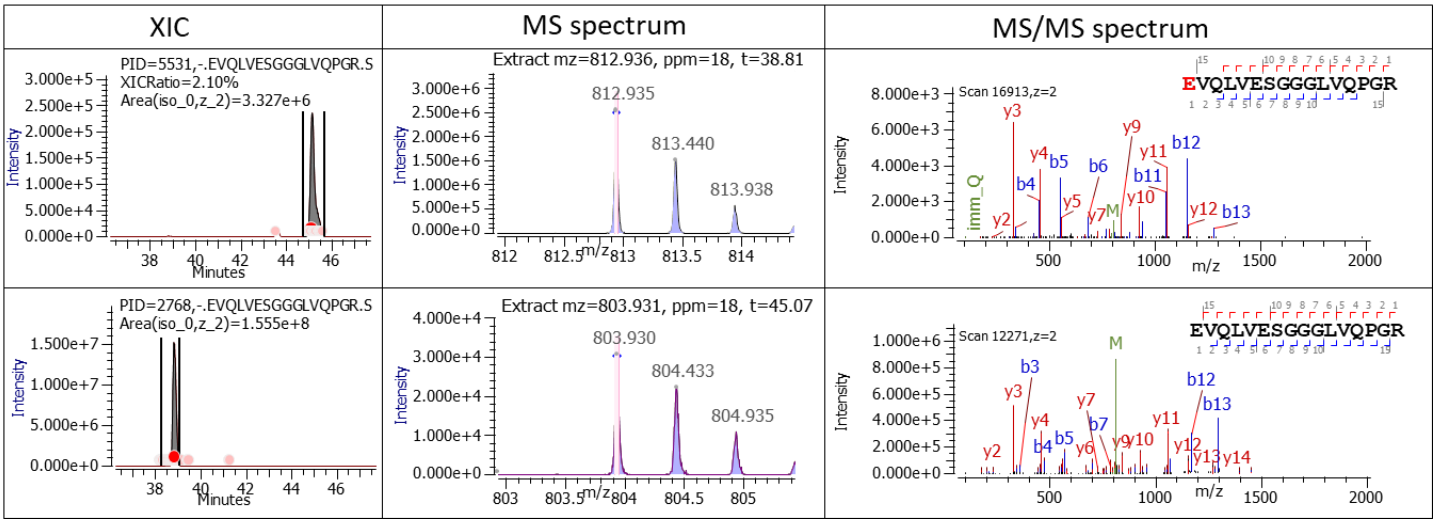

Figure 5. Unmodified N-terminal glutamic acid vs. peptide with N-terminal pyroglutamate are separated by CESI-MS.

EVQLVEGGGLVQPGR peptide contains 2.1% of the N-terminal pyro-glutamate.

In addition, CESI-MS is capable of analyzing challenging short peptides or hydrophilic peptides, that could be easily missed by LC-MS. [6] Figure 7 shows identification with MS/MS confirmation of short and hydrophilic peptide (VSNK $[M+H]^{+1}$).

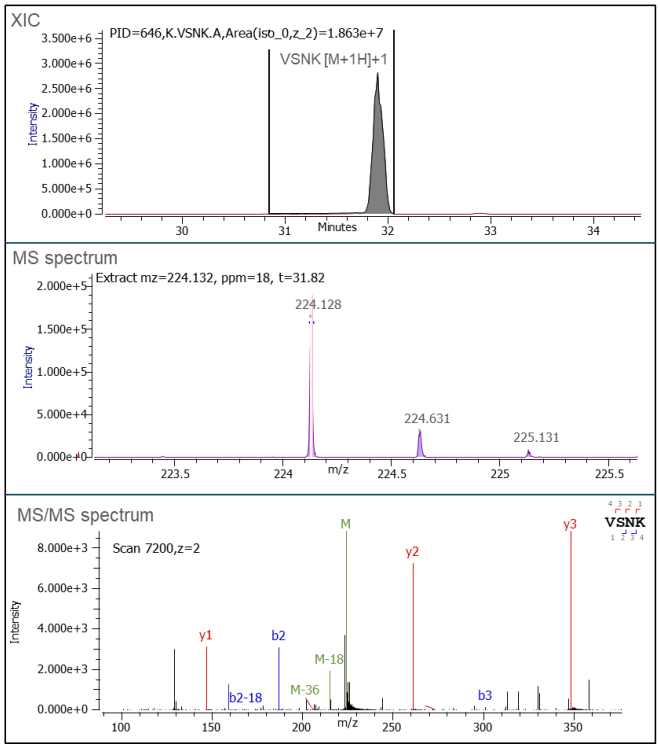

Figure 7. Identification of hydrophilic peptide VSNK $[M+H]^{+1}$.

Conclusions

Peptide mapping remains a key analytical methodology to characterize biotherapeutics from drug discovery through production and it provides the ability to assign specific locations to product attributes. It allows monitoring of amino acid substitution as well as post-translation modifications that are critical for a biotherapeutic's safety and efficacy.

CESI-MS peptide mapping is valuable orthogonal technology that provides additional sequence coverage compared to LC-MS. In this study, we characterized several CQAs, such as glycosylation, deamidation and oxidation of adalimumab peptides, within the same run. A high sequence coverage (>95%) was obtained from less than 40 ng of adalimumab loaded into the CESI cartridge which seamless data processing using BYOS software (Protein Metrics)

CESI-MS additionally separated 12 diverse glycopeptides including 6 low abundant high mannose glycopeptides of adalimumab. These are in good agreement with literature examples.[5] Adjusted CE slope allowed MS/MS confirmation of glycopeptides based on their specific y-ions as well as N-glycan diagnostic fragments without sacrificing results of adalimumab peptide mapping.

References

1. RUO-MKT-02-11835-A, Optimized digestion procedure and characterization for monoclonal antibodies and proteins by CESI-MS

2. https://sciex.com/community/application-discussions/proteomics/swath/data-

acquisition/crowdsourcing-optimized-rolling-collision-energy-curves-for-id-and-swath-acquisition

3. Jognson D. E., Biotherapeutics: Challenges and Opportunities for Predictive Toxicology of Monoclonal Antibodies, Int J Mol Sci., 2018, 19 (11), pp. 3685
4. Sanda M., Goldman R., Data Independent Analysis of IgG Glycoforms in Samples of Unfractionated Human Plasma, Anal Chem, 2016, 88, pp.1018
5. RUO-MKT-02-9755-A, Analysis of Fluorophore Labeled N-glycans by the Multicapillary C100HT Biologics Analyzer and HILIC-UPLC: Emphasis on Neutral Structure, https://sciex.com/Documents/tech%20notes/applications/pharma/c100ht-vs-uplc-neutralpdf.pdf
6. Lew C., Gallegos-Perez JL., et al., Rapid Level-3 Characterization of Therapeutic Antibodies by Capillary Electrophoresis Electrospray Ionization Mass Spectrometry, J Chrom Science, 2015, 53(3), pp.443

SCIEX

Headquarters
500 Old Connecticut Path | Framingham, MA 01701 USA
Phone 508-383-7700
sciex.com

International Sales
For our office locations please call the division headquarters or refer to our website at
sciex.com/offices