Rapid Characterization of Biologics using a CESI 8000 – SCIEX TripleTOF® 5600+ System

Comprehensive Qualitative and Quantitative Analysis of Biopharmaceuticals Using CESI-MS Technology

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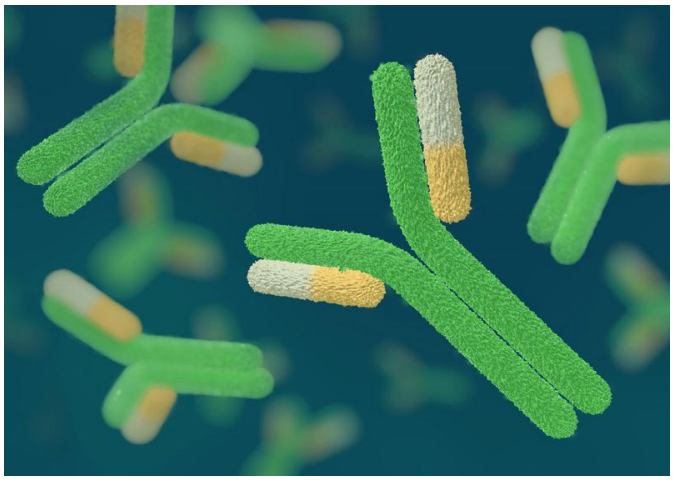
Monoclonal antibodies (mAbs) make up an important class of biotherapeutics undergoing significant growth in the pharmaceutical industry today. Currently, more than 30 mAbs have been approved for use in treatment of a number of indications ranging from various forms of cancer to autoimmune and infectious diseases. Therapeutic pipelines for mAbs and mAb-like molecules like bi-specific antibodies, single chain variable fragments (scFv), and antibody drug conjugates (ADC) are expanding. Due to approaching innovator patent expirations, a growing number of mAb biosimilar and biobetter products are also in development. Consequently, there has been a shift towards more comprehensive characterization of both innovator mAbs as well as the alternatives since changes in primary amino acid sequences, quality attribute modifications, and/or such post translational modifications as glycosylation may impact therapeutic efficacy, bioavailability, and biosafety.

Capillary electrophoresis (CE) technology using absorbance- or fluorescence-based detection methods has long been applied in the biopharmaceutical industry for the characterization of mAb purity and heterogeneity in the forms of capillary zone electrophoresis (CZE), capillary isoelectric focusing (cIEF) and capillary SDS gel electrophoresis (CE-SDS). Studies exploring the transferability of this technique illustrate excellent robustness when performed in different laboratories in various geographical locations by different instrument operators[1,2]. The exceptionally high separation power of capillary electrophoresis (CE) in conjunction with the sample identification capability of mass spectrometry fulfills Level-3 characterization requirements necessary to reveal mAB modifications and degradations. Some important attributes including primary sequence, presence of degradation hotspots like oxidation, deamidation, isomerization, cyclization, and post-translational modifications like glycosylation are only detected, localized, and quantified by peptide analysis.

To facilitate this work, SCIEX Separations has integrated capillary electrophoresis (CE) and electrospray ionization (ESI) into a single dynamic process, called CESI. This process was first described by Moini *et al.*[3], and further refined by Beckman Coulter to create the CESI 8000 High Performance Separation –



ESI module [4]. In this technical note we describe comprehensive characterization of a representative monoclonal antibody Trastuzumab (Herceptin), illustrating the benefits of CESI coupled with high resolution mass spectrometry. This is a continuation of the work previously published by Gahoul *et al.* in the journal mAbs[5]. Small and large peptides in the range of 3 – 65 amino acids have been separated, identified with 100% sequence coverage and quantified, including degradative hotspots such as asparagine-deamidation, methionine-oxidation, glutamic-acid-cyclization, and C-terminal lysine heterogeneity using a single separation of only 100 fmol of trypsin protease-digested sample. The low-flow rate of the system (~20 nL/min) maximized ionization efficiency and dramatically reduced ion suppression.

Benefits of CESI – MS technology

CESI provides a separation mechanism orthogonal to LC/MS-based approaches for separation, identification, quantification, and validation of sequence variants as well as post translational modifications (PTMs). The extremely low flow rate of CESI (~20 nL/min) is highly beneficial in maximizing ionization efficiency and minimizing ion suppression. An example of this is the strong ionization of such hydrophilic species as glycopeptides. The inherent separation efficiency of CE provides sharp peptide peaks for sensitive and reproducible identification, as well as relative quantitation. Using the open tubular format of CESI without any solid stationary phase, all peptides elute from the capillary (both hydrophobic and hydrophilic), supporting high mAb sequence coverage and essentially eliminating sample

losses and carry-over. Based on the 100% protein sequence coverage routinely obtained by this approach, qualitative and quantitative analysis of mAb purity, stability, and glycoform heterogeneity is possible from a single CESI-MS run with the use of a single protease in the digestion reaction (trypsin). The migration times of the separating peptide components vary less than 30 sec (≤ 0.83% RSD) over the 60 minute separation and the relative abundance measurements of modified and unmodified peptides vary less than 2%.

Experimental Design

Sample preparation: 100 µg of Trastuzumab was solubilized using Rapigest followed by reduction with dithiothreitol (DTT) and alkylation by iodoacetamide (IAM). The resulting sample was digested overnight with trypsin at 37ºC, dried, and resuspended in 300 µL of 133 mM ammonium acetate (pH 4), yielding a final concentration of 0.33 µg/µL of digested antibody. 50 µL was used for the sample injection.

CESI-MS and CES-MS/MS: Analysis was performed on the Beckman Coulter CESI 8000 High Performance Separation – ESI Module sold through SCIEX Separations, a part of SCIEX. Separations were performed in a 30 µm ID x 90 cm bare-fused-silica capillary housed in an OptiMS CESI cartridge, with recirculating liquid coolant set to 25°C, and coupled to the SCIEX TripleTOF® 5600+ mass spectrometer. 50 nL of sample (equivalent to 100 fmol of digested antibody) in 100 mM leading electrolyte was introduced into the bare-fused silica separation capillary and transient-isotachophoresis (t-ITP) was applied to focus the sample for the electrophoretic separation. The background electrolyte was 10% acetic acid and a voltage of 20 kV was applied for separation. Under the influence of the electric field, the analytes migrate within the separation capillary according to their charge to hydrodynamic volume ratio and upon reaching the porous sprayer tip of the capillary, are introduced into the mass spectrometer by ESI.

Information dependent acquisition (IDA) mode consisting of a high resolution TOF MS survey scan followed by several MS/MS scans was utilized to acquire the data. The IDA parameters were as follows: 100 msec TOF MS survey scan, 50 msec IDA on the top 30 ions which exceed 200 cps, rolling collision energy to induce fragmentation. The dynamic exclusion time was set to 10 sec. The total cycle time was equal to 1.8 sec and the IDA parameters were optimized so that the duty cycle of the MS readily supported the high speed CE separation. By enabling auto calibration during CE-MS batches, the instrument was automatically calibrated once every 3 runs, limiting deviation of the mass measurement accuracy.

Data Analysis: Data analysis was performed using SCIEX BioPharmaView™, ProteinPilot™ and PeakView® software. Some confirmation was manually performed if necessary.

Results and Discussion

Although a number of monoclonal antibodies have been approved by regulators for commercial use, few of these have been as comprehensively characterized as Trastuzumab. This work utilized a bottom-up analysis approach in which Trastuzumab was digested using only a single enzyme, trypsin. Development of a 'universal' enzyme approach for all biologics would greatly simplify sample preparation and also help in overcoming matrix effects. From the viewpoint of workflow efficiency, the single enzyme approach can be beneficial under circumstances in which numerous molecules are analyzed in parallel and where it is unclear which choice of enzyme would provide the best results.

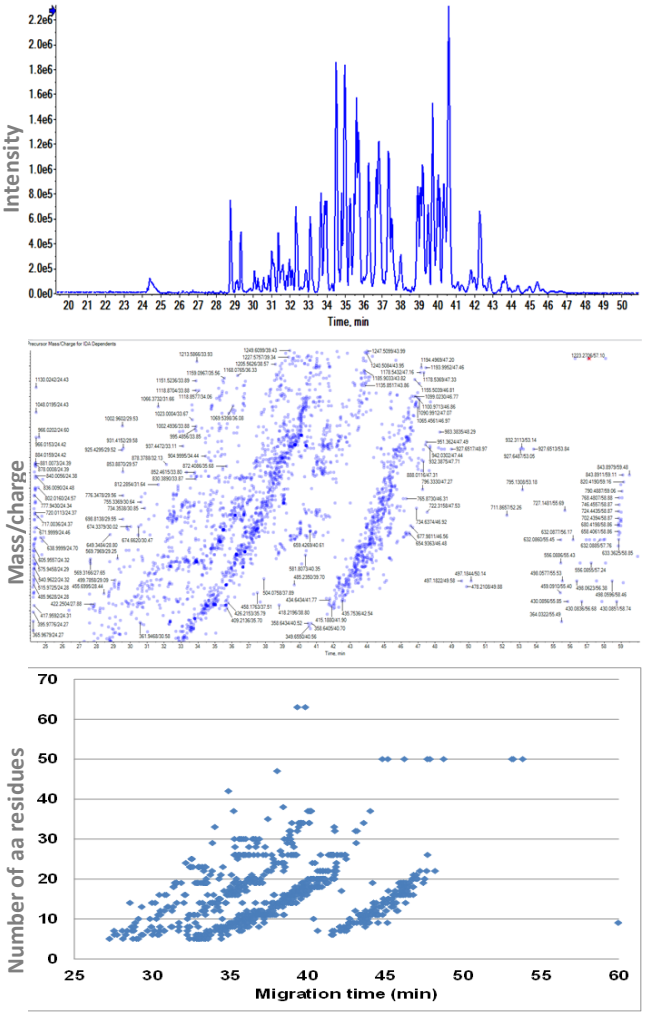


Figure 1: CESI-MS analysis of the tryptic digest of Trastuzumab a) Base peak electropherogram; b) Distribution of precursor m/z values over time; c) Peptide elution times according to their number of amino acid residues.

Figure 1 depicts the CESI-MS analysis of the tryptic digest of Trastuzumab. The upper panel shows the base peak electropherogram of the experiment. In the middle panel of Figure 1, the selected precursor ions from the information dependent acquisition (IDA) are observed. The abundance of these ions over all the m/z value range increases the probability of obtaining more peptide identifications. These ions are analyzed by MS/MS, making it possible to identify tryptic peptides with a high confidence, including very small and very large species (up to 63 amino acids) as shown in the bottom panel of Figure 1. The open tubular arrangement of the CE capillary allows the detection of hydrophobic and hydrophilic peptides avoiding discrimination due of lack of interactions with the capillary surface.

Achieving 100% sequence coverage

Figure 2 depicts the protein sequence coverage using the combined approach of ProteinPilot™ software. The upper and middle panels depict the sequence information of the heavy and light chains, respectively. Manual determination was used for short peptide sequences (lower panel) that are commonly excluded from protein database search parameters (grey sequence sections). Using this combined approach, 100% amino acid sequence coverage was obtained from this single set of data for both the heavy (HC) and light (LC) chains of Trastuzumab. The upper and middle panels show the two protein groups and representative peptide identifications that have been assigned to the heavy and light chains. Short peptide matches were further validated manually (lower panel) based on their MS/MS spectra. 100% sequence coverages were achieved in this way for each of the CESI-MS analyses performed, indicating the advantages of CE separations for comprehensive characterization of a mAb from a single digestion step using only trypsin and a single 60 minute separation. The lower panel of Figure 2 further highlights the excellent separation capability of the CESI-MS setup for small peptides.

Detailed analysis of degradation hotspots

The characteristic highly resolved capillary electrophoresis peaks shown in Figure 1 were used for comparative peptide mass mapping, i.e., extracting peptide peak areas with the combined high separation power of CESI and high resolving power of MS in the mass dimension. To illustrate the capabilities of CESI-MS, identification and quantification of degradative hot spots on peptides within Trastuzumab are delineated in Table 1 as pyroglutamate formation (N-terminal glutamate cyclization), methionine oxidation, and C-terminal lysine loss. Additional less abundant sites of the same degradative PTMs, among a few others, such as lysine glycation and/or tryptophan dioxidation were also identified but not described here due to their very low level. Extracted ion electropherograms (EIEs) were analyzed to determine the presence of degradation hotspots, which can commonly occur during processing and storage of mAbs. N-terminal modification can result in cyclization of glutamine to form pyroglutamate. Although not deemed critical, pyroglutamate formation has been proposed to increase the *in vivo* half-life of antibodies and is an indicator of manufacturing process control[6]. MS/MS spectra revealed both cyclized and unmodified forms of the N-terminal peptide, which differ by 18 amu. Extracted ion data suggested only a partial modification (Figure 3) of 3.26% pyroglutamate. Pyroglutamate cyclization leads to loss of a positive charge resulting in lower electrophoretic mobility of the modified peptide relative to the unmodified one. This is advantageous since the modified and unmodified forms can be well separated by CESI as shown in Figure 3 in addition to structural confirmation using MS/MS.

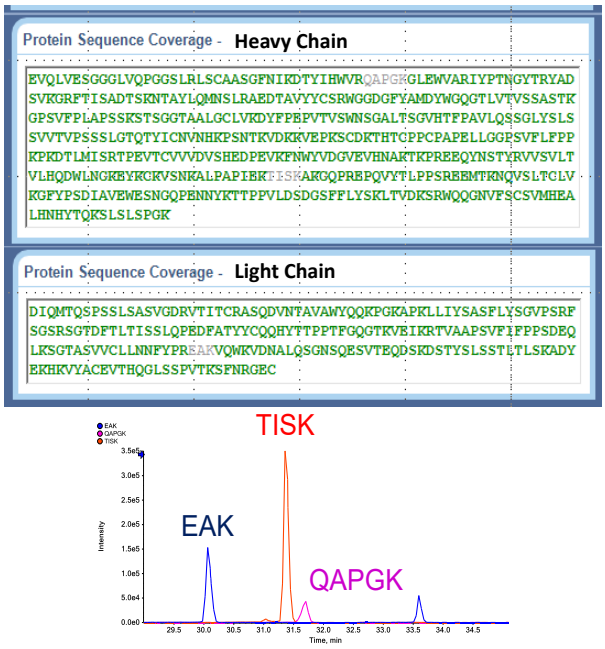


Figure 2. ProteinPilot™ Software Database Search Results of CESI 8000 – SCIEX TripleTOF 5600+ data and additional manual processing resulted in 100% sequence coverage of Trastazumab heavy and light chains.

Methionine (Met) oxidation is another common modification also associated with mAb manufacturing and storage but linked to a decrease in mAb stability and biological activity [7]. Analyzing extracted ion data revealed oxidation at Met255 and Met83 on the heavy chain (Table 1) and indicated the presence of both oxidized and unmodified methionine at both sites in the Trastuzumab sample. MS/MS diagnostic ions (Figure 4) confirmed the MetOx modification by a +16 amu molecular mass addition relative to unmodified Met in both sites. From EIEs, the relative abundances of MetOx255 and MetOx83 were determined to be 1.56% and 1.14%, respectively.

Table 1. Identification of degradation hotspots in Trastuzumab

Modification	Protein Localization	Sequence and associated mass shift	Monoisotopic mass $[M+H]^+$	Migration time (min)	Relative Abundance (%)
Pyroglutamate formation	*N-terminal*	*E(-18.010565)VQLVESGGGLVQPGGSLR*	*1863.9923*	*46.22*	*3.26*
Methionine Oxidation	*Met255*	DTLM(15.994915)ISR	*851.4291*	*35.79.*	*1.56*
Methionine Oxidation	*Met83*	*NTAYLQM(15.994915)N*	*970.4299*	*14.62*	*1.14*
Asparagine deamidation	*Asn55*	*R.IYPTN(0.984016)GYTR.Y*	*1085.5262*	*36.65*	*92.47*
	Asn387 *Asn387 & Asn392* *Asn387 & Asn393*	*K.GFYPSDIAVEWESN(0.984016)GQPENNY.K* *K.GFYPSDIAVEWESN(0.984016)GQPEN(0.984016)NY.K* *K.GFYPSDIAVEWESN(0.984016)GQPENN(0.984016)Y.K*	*2545.1154* *2546.0994* *2546.0994*	*40.82* *4169* *41.86*	*79.00**
	Asn30	*R.ASQDVN(0.984016)TAVAWYQQ.P*	*1708.8289*	*39.07*	*48.06*
	Asn152	*K.VDNALQSG N(0.984016)SQESVTEQDSK.D*	*2136.9527*	*41.14*	*13.00*
C-terminal lysine loss	*C-terminal*	*K.SLSLSPGK(128.094963)*	*660.3563*	*40.5*	*>99*

* Overall value

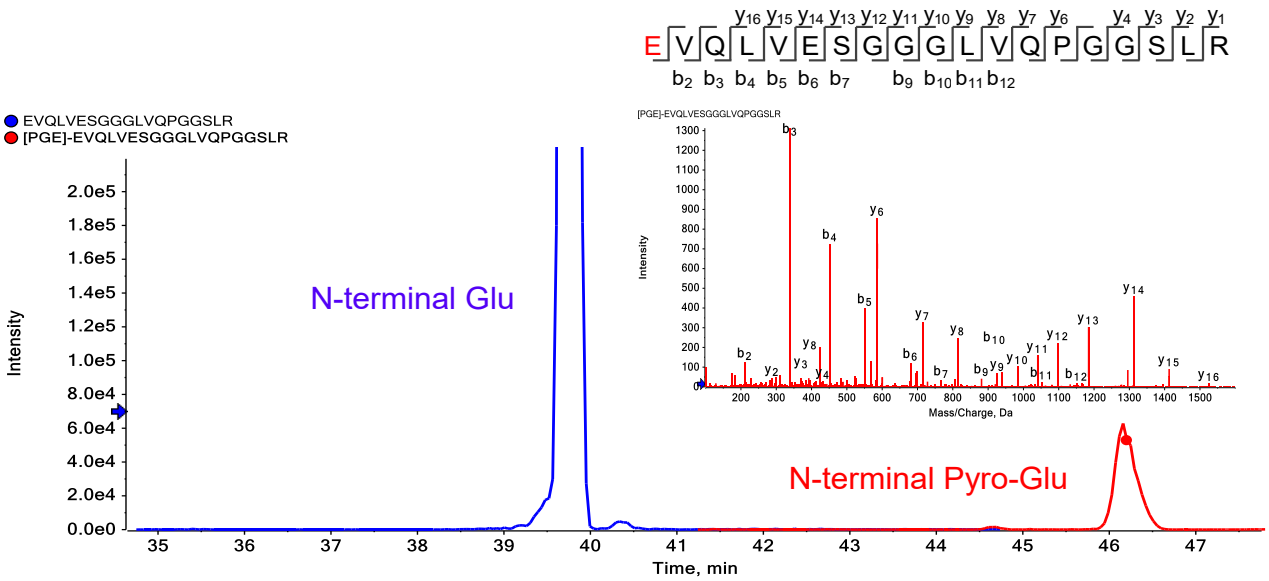


Figure 3. Extracted CESI-MS ion data revealed both cyclized (pyroGlu) and unmodified (Glu) forms of the N-terminal peptide.

'Extracted ion analysis of the data indicated quite a few incidences of Asp deamidation, specifically at Asn55, Asn387, Asn392, and Asn393 in the heavy chain and Asn30 and Asn152 in the light chain. MS/MS diagnostic ions for each of these identified the +1 amu difference confirming each instance of deamidation (Figure 5). With CESI, these two forms can be readily separated from each other, making their assignment and differentiation from ionization artifacts much easier. This is particularly important to emphasize since deamidated peptides are not always resolved by LC/MS analyses and may co-elute with unmodified peptides. Additionally, the isotopic envelopes of the peptides, only differing by 1 amu, can also overlap and be mis-assigned by automated identification softwares, even with high resolution MS and MS/MS data. Physical separation of these peptides by CESI-MS, can address both of these challenges with LC/MS.

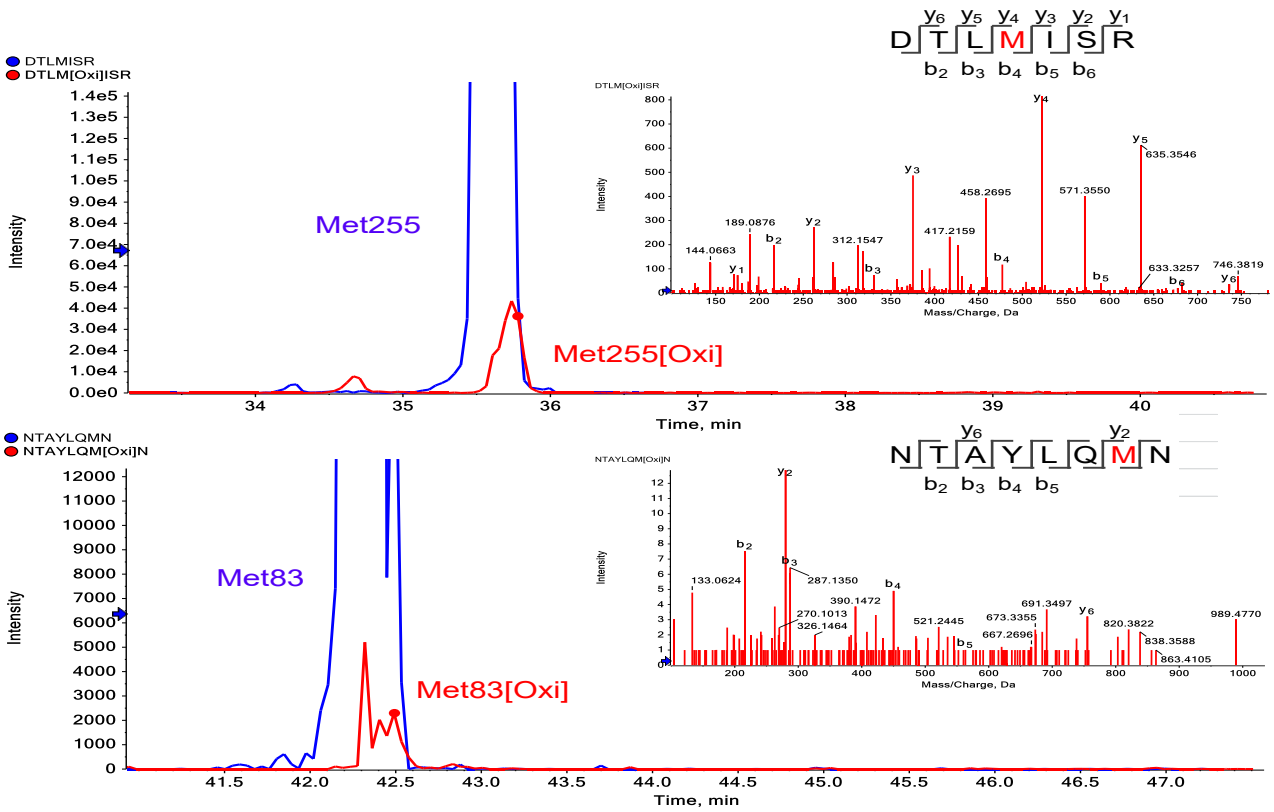


Figure 4. CESI-MS analysis of methionine oxidation (MetOx) at positions 255 and 83.

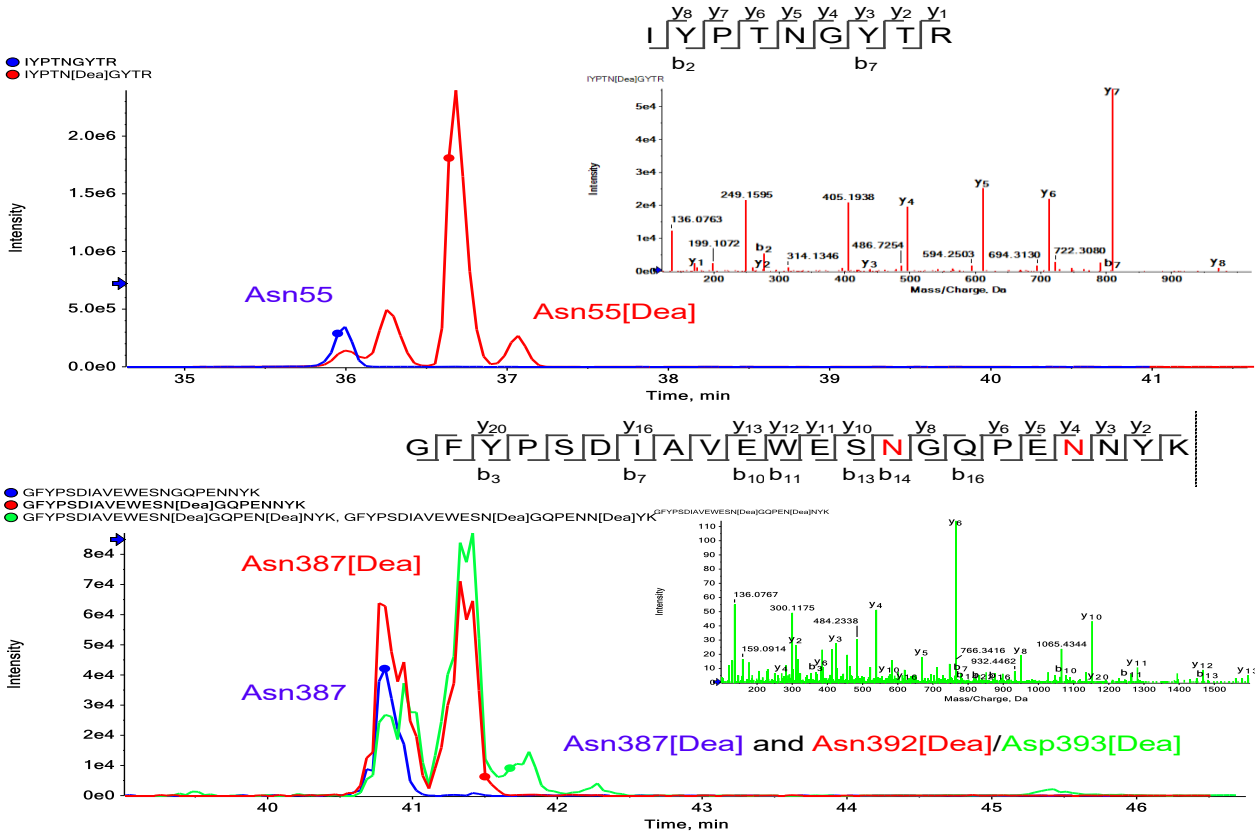


Figure 5. CESI-MS identification of asparagine deamidation sites.

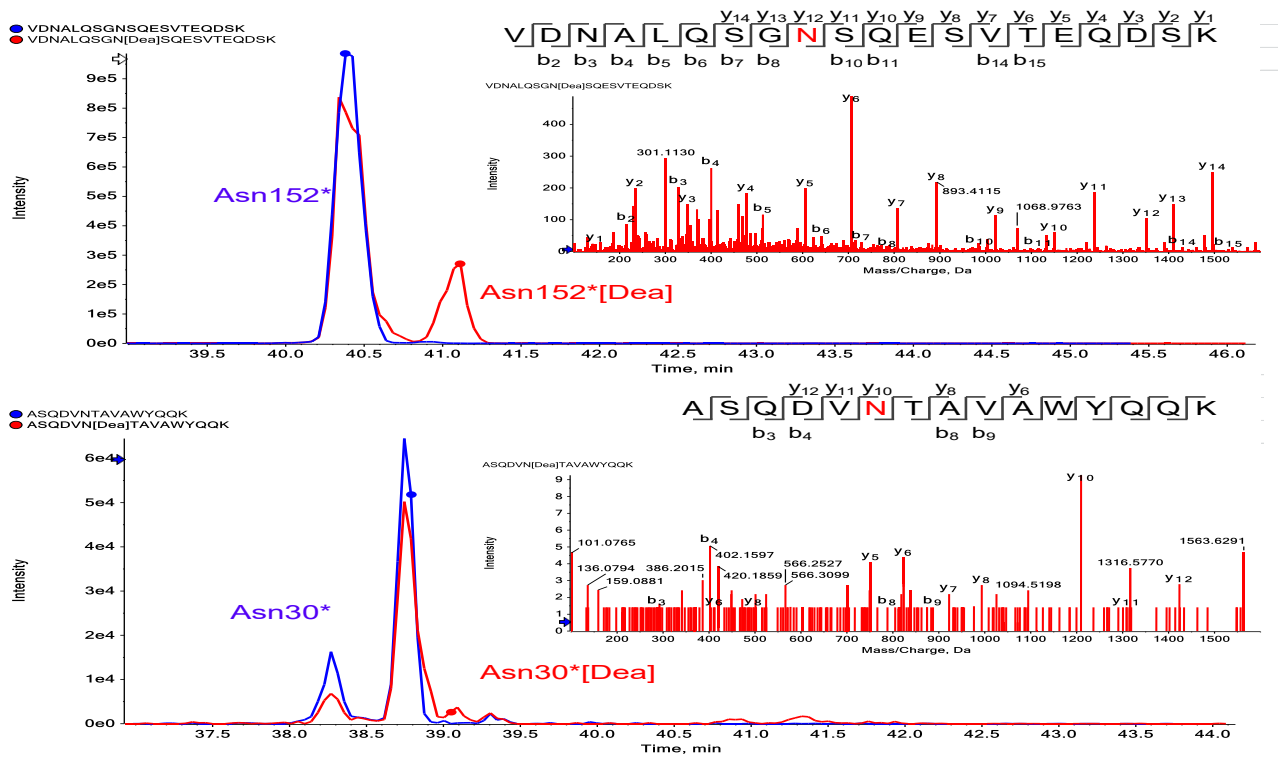


Figure 5 (Continued). CESI-MS identification of asparagine deamidation sites.

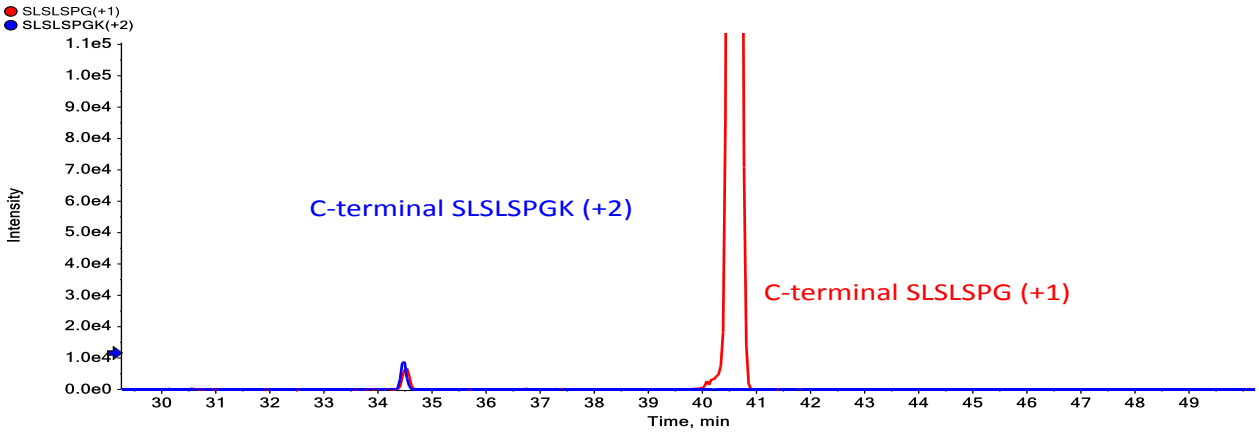


Figure 6. C-terminal lysine heterogeneity analysis by CESI-MS.

The analysis of C-terminal lysine heterogeneity is depicted in Figure 6. Due to the loss of a positively charged lysine on the heavy chain, it can be confirmed by a large migration shift. From the extracted ion electropherograms (XIEs), greater than 99% of the heavy chain had C-terminal lysine loss.

Glycopeptide mass mapping

One of the most important effector function-related post translational modifications common to mAbs is glycosylation. The presence or absence of specific glycan residues can significantly alter the efficacy, stability, and immunogenicity of mAbs. Trastuzumab contains a consensus glycosylation site at Asn300 of the heavy chain on which various glycoforms can associate. Because mAbs are made up of two heavy chains, various combinations of glycoforms may be present, resulting in significant complexity. With CESI, very high ionization efficiency of the glycopeptides was achieved, generating strong signals and allowing for MS/MS identification of low abundance species. As the migration time of these glycans differs depending upon structure, the mobility of the peptide becomes a valuable aid in structural assignment. Since tryptic peptides separated by CESI-MS retain all associated glycosylation, specific amino acid linkage can be identified allowing confirmation that linkage occurs at Asn300. As Tables 2A and 2B depict, in this Trastuzumab sample 14 different glycoforms were identified on the EEQYNSTYR peptide (Figure 7A) and 7 on the TKPREEQYNSTYR peptide (Figure 7B). The relative abundances of the glycans ranged from 47% to as low as 0.29%. Approximately half of these glycan species were present in low relative abundance but were still ionized and identified by CESI-

MS. Since capillary electrophoresis has long been a robust technique for oligosaccharide analysis, application of this fundamental capability of CE to separate glycosylated species based on charge and hydrodynamic radius (i.e., differential electromigration) provides another dimension of analysis by which glycoform identity can be readily determined (Figure 7). Similarly to degradative PTM analysis, there are notable benefits from CESI-MS analysis of glycopeptides. First, with the low nL/min flow rates, glycopeptides are readily ionized among other peptides with better ionization efficiencies, illustrated by the comprehensiveness and dynamic range of glycopeptides identified. Additionally, since glycopeptides regularly co-elute with LC/MS analysis, CESI-MS separation of glycopeptide is advantageous. The migration-based separation of glycopeptides helps to confirm glycan structures. As expected, the identified glycans on peptides increase in size and negative charge as migration time increases. Any unwanted fragmentation of glycans prior to MS and MS/MS (i.e. in-source fragmentation) can be identified as electrophoretic peak shoulders and non-gaussian peak shapes. Thus the separation of glycopeptides by CESI-MS adds both sensitivity and confidence to glycan characterization

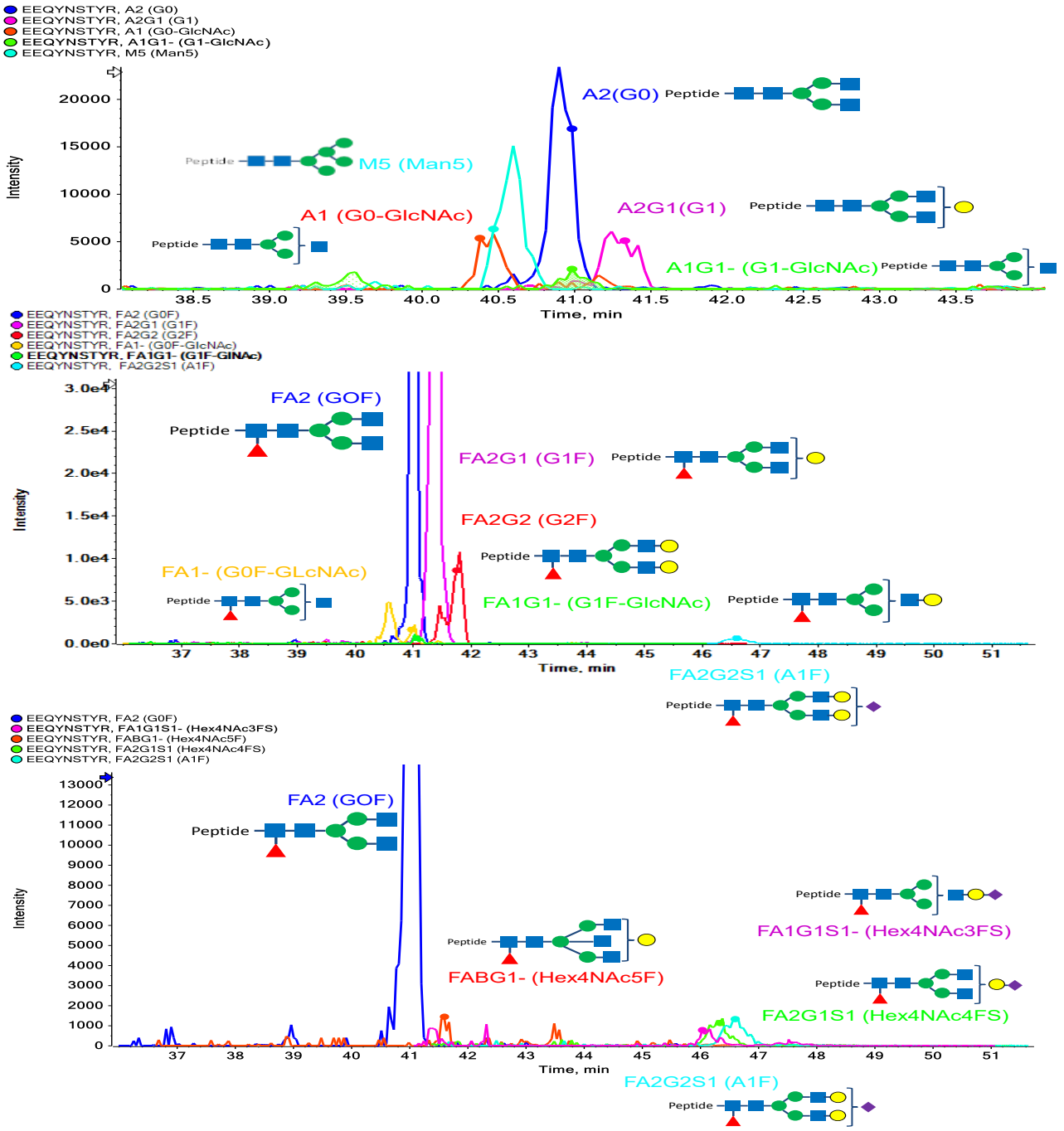


Figure 7A. Glycosylation structures on the EEQYNSTYR peptide identified by CESI-MS. Further glycopeptide characterization is shown in Table 2B. Glycan structure interpretation followed the CFG protocol.

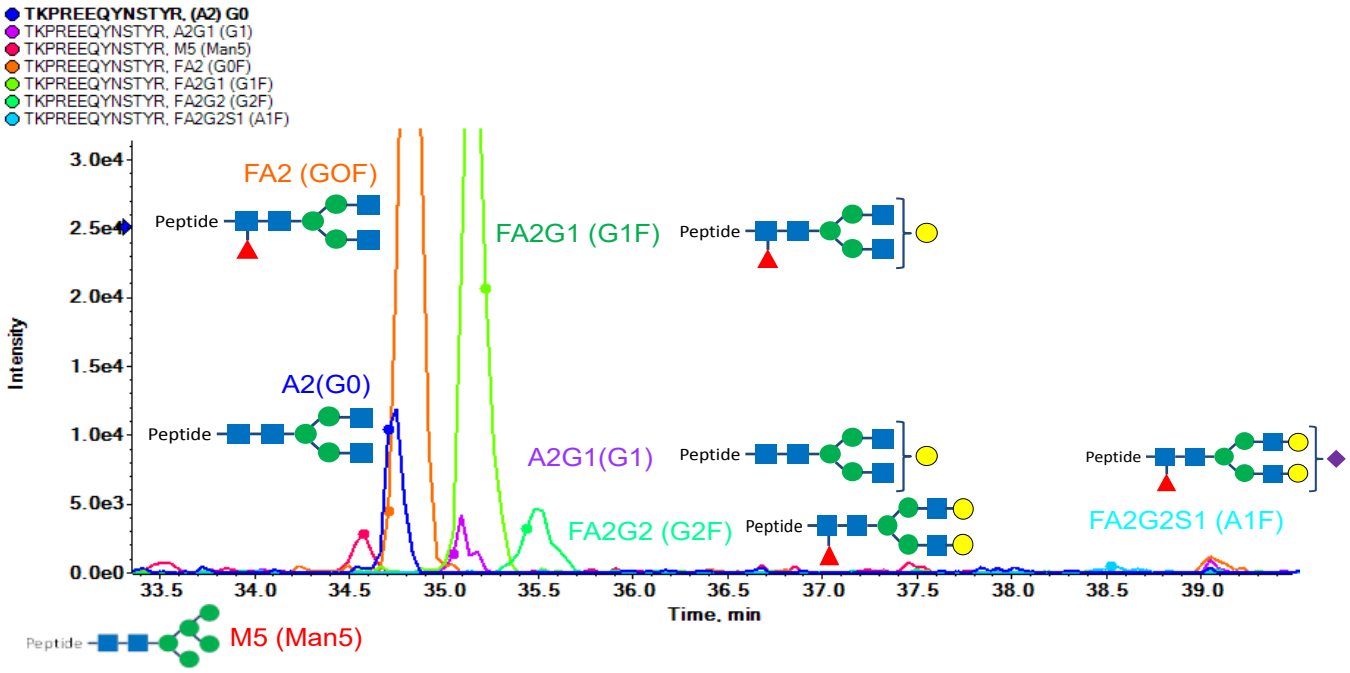
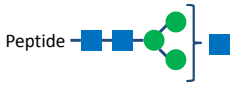
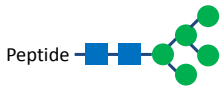
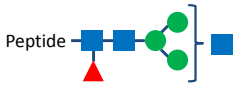
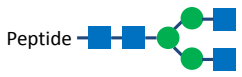
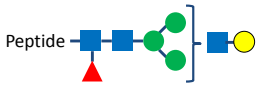


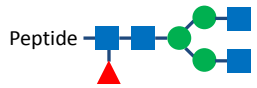
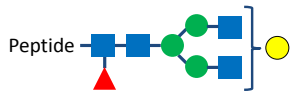
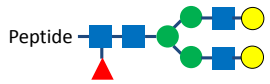
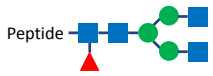
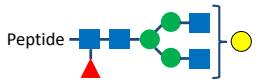
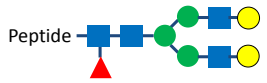
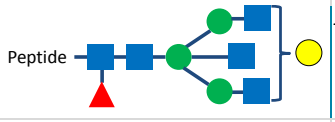
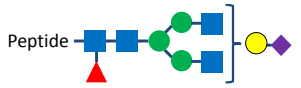
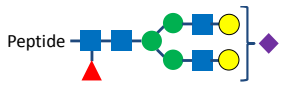
Figure 7B. Glycosylation structures on the TKPREEQYNSTYR peptide identified by CESI-MS. Further glycopeptide characterization is shown in Table 2A. Glycan structure interpretation followed the CFG protocol.

Table 2A. Characterization of Trastuzumab heavy chain glycosylation hot spot N300 on the TKPREEQYNSTYR peptide with structural identification and relative quantification. Glycan structure interpretation followed the CFG protocol.

Glyan abbreviation (Structural name)	Glycopeptide identified as K. TKPREEQYN(Glycan)STYR.V Asn300	Glycan mass (Da)	Monoisotopic mass $[M+H]^+$	Migration time (min)	Relative Abundance (%)
A1 (G0-GlcNAc)		*1095.3966*	*2284.9086*	*40.42*	*1.39*
M5 (Man5)		*1216.4228*	*2405.9349*	*40.5*	*3.52*
FA1 (G0F-GlcNAc)		*1241.4545*	*2430.9665*	*41.02*	*2.41*
A1G1 (G1-GlcNAc)		*1257.4494*	*2446.9614*	*41.02*	*0.48*
A2 (G0)		*1298.4760*	*2487.9880*	*41.02*	*5.50*
FA1G1 (G1F-GlcNAc)		*1403.5073*	*2593.01931*	*41.08*	*0.33*

* As a quantitative approximation, relative abundances were calculated using peak areas of glycosylated peptides of the same sequence and charge state detected (+4).

Table 2B. Characterization of Trastuzumab heavy chain glycosylation hot spot Asn300 on the EEQYNSTYR glycopeptide with structural identification. Glycan structure interpretation followed the Consortium of Functional Glycomics (CFG) protocol.

Glycan Abbreviation (Structural name)	Glycopeptide identified as R.EEQYN(Glycan)STYR.V Asn300	Glycan mass (Da)	Monoisotopic mass $[M+H]^+$	Migration time (min)	Relative Abundance (%)*
M5 (Man5)		*1216.4228*	*2888.2313*	*34.61*	*2.03*
(A2) G0		*1298.4760*	*2970.28452*	*34.73*	*7.49*
FA2 (G0F)		*1444.5339*	*3116.34242*	*34.74*	*47.37*
A2G1 (G1)		*1460.5288*	*3132.33732*	*35.07*	*2.25*
FA2G1 (G1F)		*1606.5867*	*3278.39522*	*35.24*	*35.38*
FA2G2 (G2F)		*1768.6395*	*3440.44802*	*35.47*	*4.94*
FA2G2S1 (A1F)		*2059.7349*	*3731.54342*	*38.56*	*0.54*
FA2 (G0F)		*1444.5339*	*2634.0459*	*41.00*	*39.06*
A2G1 (G1)		*1460.5288*	*2650.0408*	*41.35*	*1.86*
FA2G1 (G1F)		*1606.5867*	*2796.0987*	*41.36*	*36.68*
FA1G1S1 (Hex4NAc3FS)		*1694.6027*	*2884.1147*	*46.06*	*0.30*
FA2G2 (G2F)		*1768.6395*	*2958.1515*	*41.79*	*6.80*
FA2BG1 (Hex4NAc5F)		*1809.6661*	*2999.1781*	*41.62*	*0.29*
FA2G1S1 (Hex4NAc4FS)		*1897.6821*	*3087.1785*	*46.37*	*0.63*
FA2G2S1 (A1F)		*2059.7349*	*3249.2469*	*46.63*	*0.74*

* As a quantitative approximation, relative abundances were calculated using peak areas of glycosylated peptides of the same sequence at the charge state detected (+2 or +3).

Conclusions

In this application note, we illustrated the use of CESI-MS for rapid characterization of the therapeutic antibody Trastuzumab using the CESI 8000 - SCIEX TripleTOF 5600+ Platform. Starting with efficient sample preparation using a single enzyme, single digestion protocol followed by a single separation step, we were able to rapidly attain 100% primary sequence coverage for both the heavy and light chains of this important mAb therapeutic. In addition, identification of key amino acid modifications was accomplished, resulting in the elucidation of glutamine cyclization, methionine oxidation, asparagine deamidation, and C-terminal lysine heterogeneity. From the same CESI-MS separation, glycopeptide analysis was performed, resulting in the identification of glycan-amino acid linkage position for as many as 14 glycoforms, many in low abundance. Strong ionization signals for accurate mass measurement and subsequent MS/MS coupled with structure-based mobility separations greatly simplified the determination and assignment of the bound glycan structures. Because of its ultra-low-flow capability, CESI-MS provides remarkable sensitivity enhancements for poorly ionizing molecular species like glycopeptides, and when coupled to a fast accurate MS platform such as the TripleTOF 5600+, efficiently delivers broad mAb characterization from a single protease digestion and single CESI-MS run.

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