Application Note

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Monoclonal Antibody Charge Variants Identification by Fully Automated Capillary Isoelectric Focusing – Mass Spectrometry

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Abstract

This Application Note describes a method for the direct mass spectrometric (MS) determination of monoclonal antibody (mAb) charge variants. The applied workflow featured an Agilent 7100A CE instrument equipped with a CMP Scientific EMASS-II capillary electrophoresis/mass spectrometry (CE/MS) ion source. MS was performed on either an Agilent 6230 accurate-mass TOF LC/MS or an Agilent 6545 Q-TOF LC/MS.

Introduction

mAb therapeutics exhibit charge heterogeneity as a result of post translational modifications such as deamidation, C-terminal lysine, glycosylation, lysine glycation, and N-terminal pyroglutamate. Isoelectric focusing gel electrophoresis, capillary isoelectric focusing (CIEF), and imaged capillary isoelectric focusing methods have routinely been used for the quantitative analysis of charge variants of biotherapeutics. However, MS identification of the underlying species of each charge variant has been challenging. We demonstrate the use of the CMP Scientific EMASS-II CE/MS ion source on an Agilent 7100A CE instrument and TOF/Q-TOF systems for the direct MS identification of therapeutic mAb charge variants.

Experimental

Materials

The carrier ampholytes, Pharmalyte with a pH range of 3.0 to 10.0, mAb standards, and SILu Lite SigmaMAb universal antibody standard human (catalog number MSQC4-1MG), were purchased from Sigma-Aldrich (St. Louis, MO, USA). A stock solution of the SigmaMAb was prepared at 5 mg/mL in 50 % glycerol, and stored at −20 °C. LC/MS grade reagents, including water, formic acid, ammonium hydroxide, acetonitrile, and methanol were also obtained from Millipore-Sigma (Burlington, MA).

Online CIEF-MS

Online CIEF-MS was performed on a 7100A CE instrument (Agilent Technologies, Santa Clara, CA, USA) coupled to a 6230 accurate-mass TOF LC/MS or a 6545 Q-TOF LC/MS using an EMASS-II CE/MS ion source (CMP Scientific Corp, Brooklyn, NY, USA). Electrospray emitters for online CIEF-MS analysis (1.0 mm od, 0.75 mm id, 30 µm tip size), and neutral coating PS1 capillaries (360 µm od and 50 µm id) for separation were from CMP Scientific. The catholyte was 0.2 N ammonium hydroxide in 15 % glycerol (v/v). The anolyte was 1 % formic acid in 15 % glycerol (v/v). A positive electrospray ionization voltage (between 2.2 and 2.4 kV) was supplied by an external benchtop high-voltage power supply that comes with the EMASS-II ion source. The sheath liquid was 20 % acetic acid, 25 % acetonitrile (v/v). The capillary voltage (Vcap) on the 6230 and 6545 was set at zero volts. A single bore in-line nanospray shield was used to divert the drying gas out of the MS to minimize interference with the nanospray out of the EMASS-II ion source. The distance from the emitter tip to the MS was adjusted to be between 3 to 4 mm, as measured by the microscope camera. Samples (0.1 to 0.5 mg/mL) were prepared in 5 mM ammonium acetate, 1 to 1.5 % ampholyte, and 15 % glycerol.

Sample injection and separation

Sample solution injection time was set as 75 seconds following 10 seconds of the catholyte solution injection under 950 mbar in a 75 cm long capillary (50 µm id). A positive CE separation voltage (18.8 kV) was then applied at the capillary inlet end to begin electric focusing. A constant pressure (5 to 10 mbar) was applied at the capillary inlet to generate hydrodynamic flow to shorten analysis time.

MS parameters

The Q-TOF capillary voltage was set at zero volts. The drying gas was 6 L/min at 365 °C. The fragmentor voltage was 380 V. The CE/MS method setup, data acquisition, and analysis were performed using Agilent MassHunter workstation software (v B.09). Table 1 summarizes this CIEF-MS method.

Table 1. CIEF-MS method parameters.

Parameter	Value
Capillary Electrophoresis	7100A CE
Mass Spectrometers	6230 accurate-mass TOF LC/MS; 6545 Q-TOF LC/MS
CE/MS Coupling	CMP Scientific EMASS-II CE/MS ion source
Separation Capillary	75 cm PS1 capillary (CMP Scientific Corp)
Anolyte	1 % formic acid, 15 % glycerol
Catholyte	0.2 N ammonium hydroxide, 15 % glycerol
Sheath Liquid	20 % acetic acid, 25 % acetonitrile
Sample Buffer	5 mM ammonium acetate, 1 to 1.5 % Pharmalyte 3-10, 15 % glycerol
Injection	Flush, 10 seconds (catholyte) Flush, 75 seconds (sample solution)
Separation	15.0 to 18.8 kV, 5 to 10 mbar
Electrospray Emitter	30 µm tip size (CMP Scientific Corp)
ESI Voltage	2.4 kV
Distance from Emitter to Mass Spectrometer	4 mm
Distance from Capillary End to Emitter Tip	0.8 mm
Fragmentor Voltage	380 V
Vcap	0 V
Drying Gas Flow Rate	6 L/min
Drying Gas Temperature	365 °C

Results and Discussion

Principle of a fully automated CIEF-MS method

The details of this online CIEF-MS method can be found in the papers listed in the References section[1–4]. Briefly, to establish a pH gradient inside the separation capillary, a small plug of ammonium hydroxide is first injected through the capillary inlet (Figure 1, Step 1). A long plug of sample mixed with ampholyte is then injected from the capillary inlet. The sample solution pushes the ammonium hydroxide plug towards the capillary outlet, forming the catholyte end for subsequent isoelectric focusing (Figure 1, Step 2). While the pH gradient is formed inside the capillary, the acidic sheath liquid gradually titrates the ammonium hydroxide plug. For monoclonal antibodies, the optimized condition is 20 % acetic acid, 25 % acetonitrile. Once the catholyte solution is fully consumed, the focused protein zones start to migrate towards the capillary outlet. This migration follows the same principle of chemical mobilization as in traditional two-step CIEF-MS methods (Figure 1, Step 3). Therefore, the focusing (with ammonium hydroxide inside the capillary) and mobilization (ammonium hydroxide completely titrated by acidic sheath liquid) happen in a consecutive manner. This greatly simplifies the operation and maximizes the success rate of charge variants analysis.

Agilent-CMP CE/MS system performance check by basic protein mixture

We recommend evaluating the Agilent-CMP CE/MS system performance by injecting a small amount of lysozyme and ribonuclease A mixture. The sample is prepared as 0.1 mg/mL of lysozyme and 0.3 mg/mL of ribonuclease A in 100 mM ammonium acetate, 4 % acetic acid (v/v). The background electrolyte is 10 % acetic acid (v/v). The sheath liquid is 5 % acetic acid, 10 % methanol (v/v). Sample injection is 100 mbar, seven seconds. The separation voltage is 30 kV. Figure 2 shows the capillary zone electrophoresis (CZE)-MS result of this basic protein mixture on a good performing system.

The following criteria should be examined: no obvious peak tailing observed on both protein peaks; the separation window between lysozyme and ribonuclease A is over one minute; peak heights are similar; peak width is less than 30 seconds.

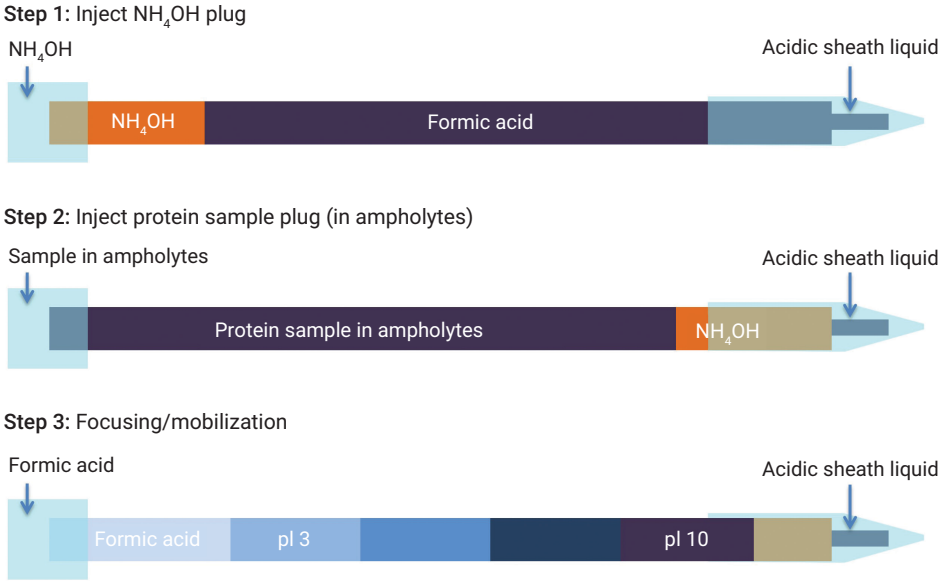

Figure 1. CIEF-MS method principle.

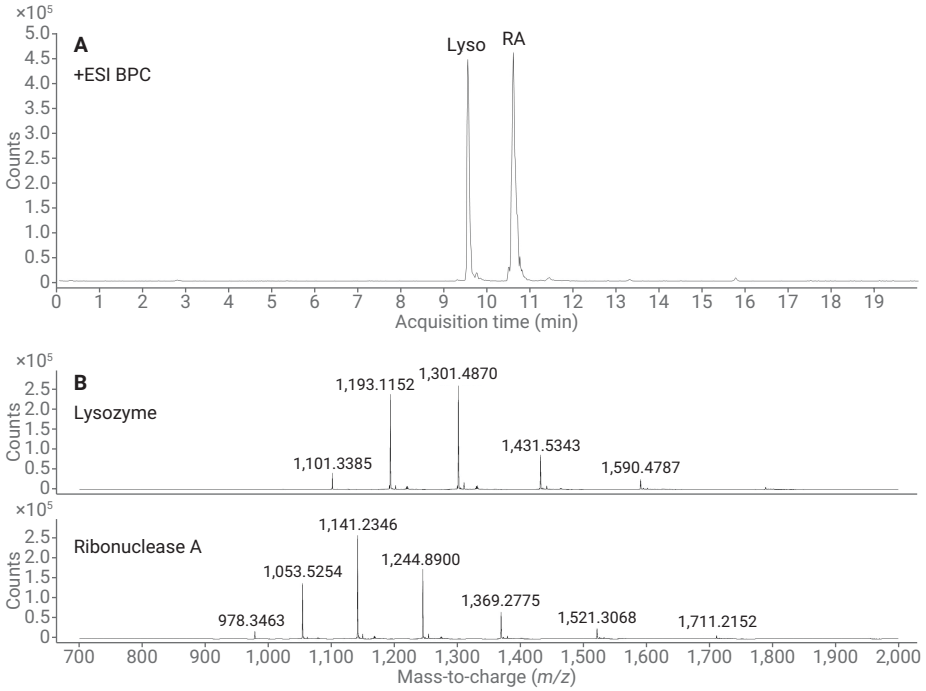

Figure 2. Agilent-CMP CE/MS system performance check by lysozyme and ribonuclease A protein mixture.

Agilent-CMP CE/MS system performance verification by monoclonal antibody standards

Before starting the CIEF-MS experiments for mAb charge variants analysis, it is good practice to further test the system performance by injection of an mAb sample. Taking SigmaMAb as an example, the IgG molecule is expected to migrate after 20 minutes (Figure 3). This long migration time indicates that minimal electro-osmotic flow exists on the PS1 capillary. No presence of peak tailing suggests minimal protein absorption on the capillary wall. The experimental conditions are:

- **Sample:** 0.2 mg/mL of SigmaMAb in 100 mM ammonium acetate, 4 % acetic acid (v/v)
- **Background electrolyte:** 30 % acetic acid (v/v)
- **Sheath liquid:** 20 % acetic acid, 25 % acetonitrile
- **Injection:** sample injection at 950 mbar for three seconds, then background electrolyte injection at 950 mbar for three seconds
- **Separation:** 30 kV

Agilent-CMP CIEF-MS system performance verification by protein standards

After the CZE-MS evaluation, the CIEF-MS method can be tested using a mixture of cytochrome C (pI value 10.3) and myoglobin (pI values 7.2 and 6.8). Figure 4 shows the CIEF-MS result of such a mixture on a 100 cm PS1 capillary. The experimental conditions are:

- **Sample:** 0.1 mg/mL cytochrome C, 0.08 mg/mL myoglobin in 1.2 % Pharmalyte 3-10, 4 mM ammonium acetate, 12 % glycerol
- **Anolyte:** 1 % formic acid, 15 % glycerol
- **Catholyte:** 0.2 N ammonium hydroxide, 15 % glycerol
- **Sheath liquid:** 30 % acetic acid
- **Injection:** catholyte at 950 mbar for 25 seconds, then sample at 950 mbar for 110 seconds
- **Focusing and mobilization:** 15 kV, 10 mbar

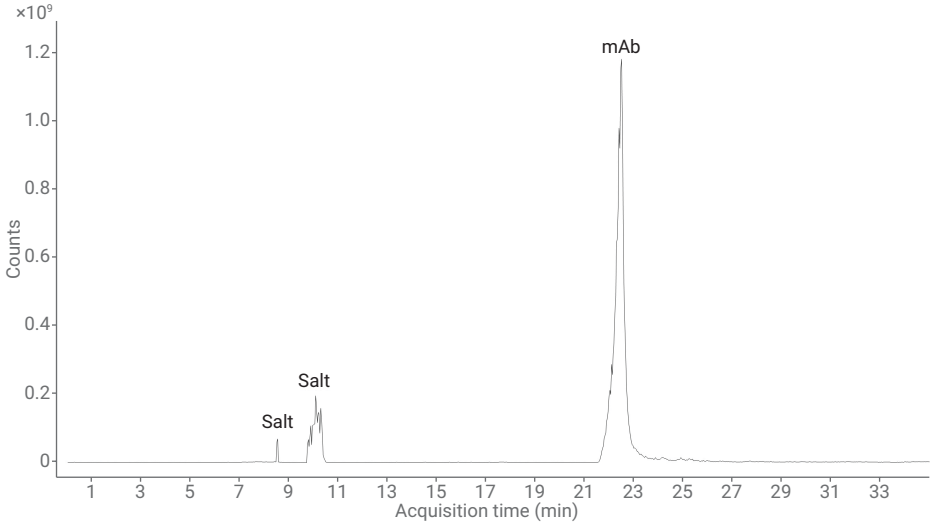

Figure 3. Separation capillary performance check by monoclonal antibody standards.

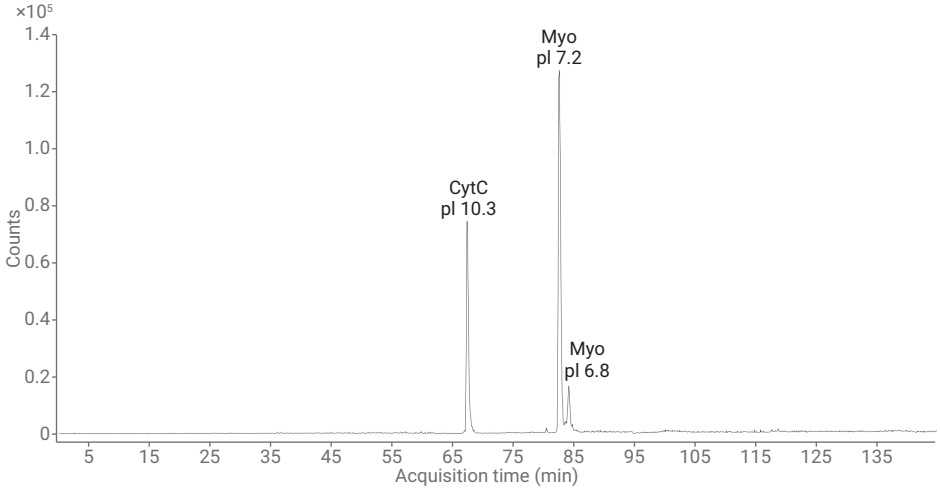

Figure 4. CIEF-MS system performance check by cytochrome C and myoglobin mixture.

CIEF-MS analysis of daratumumab

Daratumumab is a commercial therapeutic IgG1 monoclonal antibody. Figure 5 shows the screenshot of Agilent MassHunter data acquisition software during a CIEF-MS analysis of a daratumumab sample. In the left middle panel, the blue trace is the total ion electropherogram (TIE), the red is the CE current plot. Cytochrome C was spiked into the sample, showing up as the first peak in the TIE. Cytochrome C has a pI value of 10.3; therefore, it works well as the front marker for ampholytes within pH range 3 to 10. As shown in Figure 5, where daratumumab appeared, there was minimal ampholyte background, suggesting electrospray ionization conditions favoring the antibody molecule. It has been found that 20 % acetic acid and 25 % acetonitrile as a sheath liquid works well for many mAb therapeutics[2]. The experimental conditions are:

- **Sample:** 0.33 mg/mL daratumumab, 0.11 mg/mL cytochrome C in 5 mM AA, 1 % Pharmalyte 3-10, 15 % glycerol
- **Catholyte:** 0.2 N ammonium hydroxide, 15 % glycerol
- **Anolyte:** 1 % formic acid, 15 % glycerol
- **Sheath liquid:** 20 % acetic acid, 25 % acetonitrile
- **Injection:** anolyte injection at 950 mbar for 10 seconds, then sample injection at 950 mbar for 75 seconds
- **Focusing and mobilization:** 18.8 kV, 5 mbar

On a 75 cm PS1 capillary, under these conditions, the cytochrome C peak usually occurs after 40 minutes. The daratumumab peak then appears within five minutes.

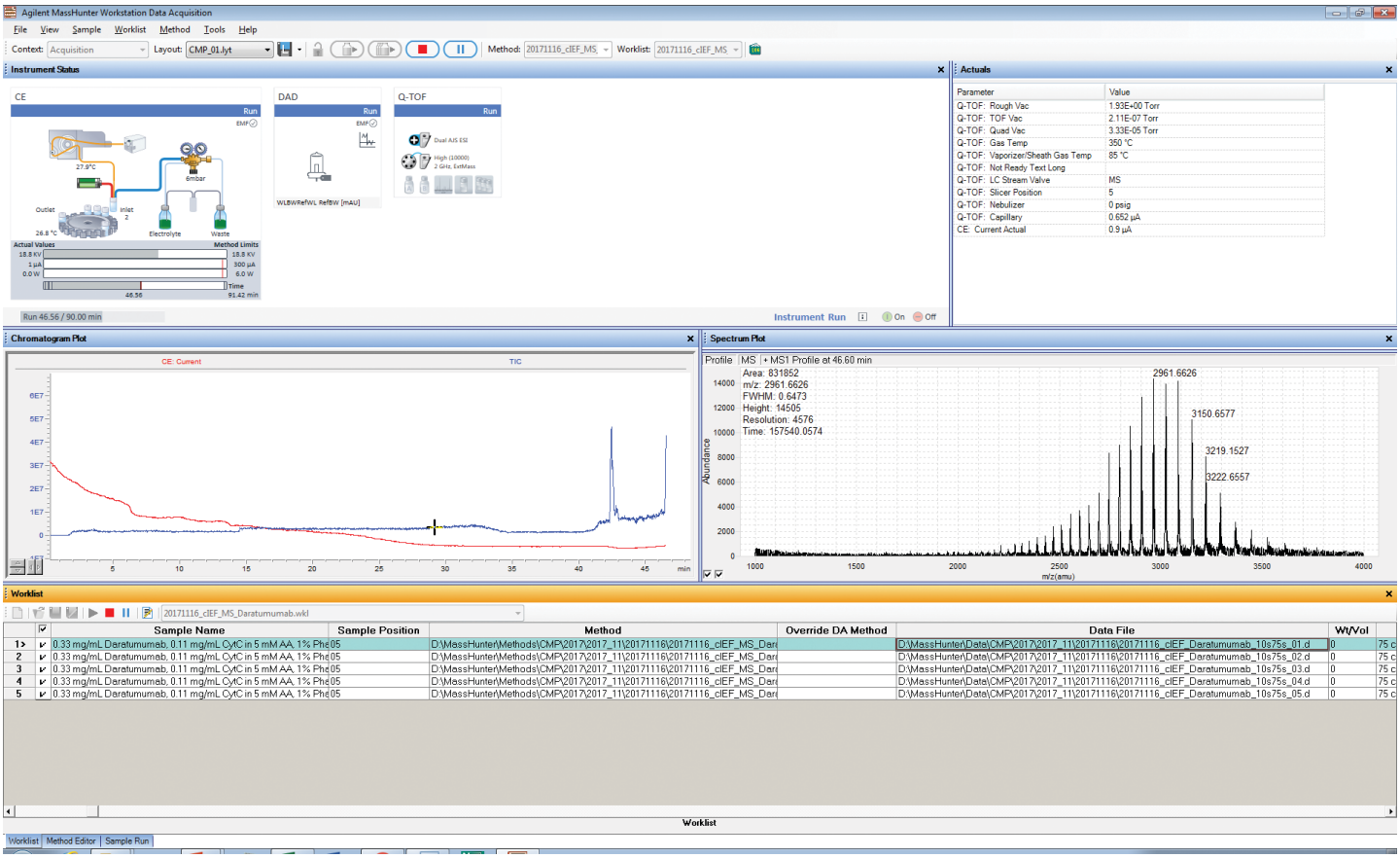

Figure 5. Screenshot of MassHunter data acquisition software during a CIEF-MS analysis of daratumumab.

Charge variants of daratumumab identified by CIEF-MS analysis

Figure 6 shows the identified charge variants of daratumumab. There is one basic variant, and two acidic variants. After deconvolution by MassHunter BioConfirm software, based on the differences of the intact masses of the charge variant peaks and the main peak, the charge variants were identified as N-terminal pyroglutamate (basic variant, peak B1), deamidation (acidic variant, peak A1), and lysine glycation (acidic variant, peak A2).

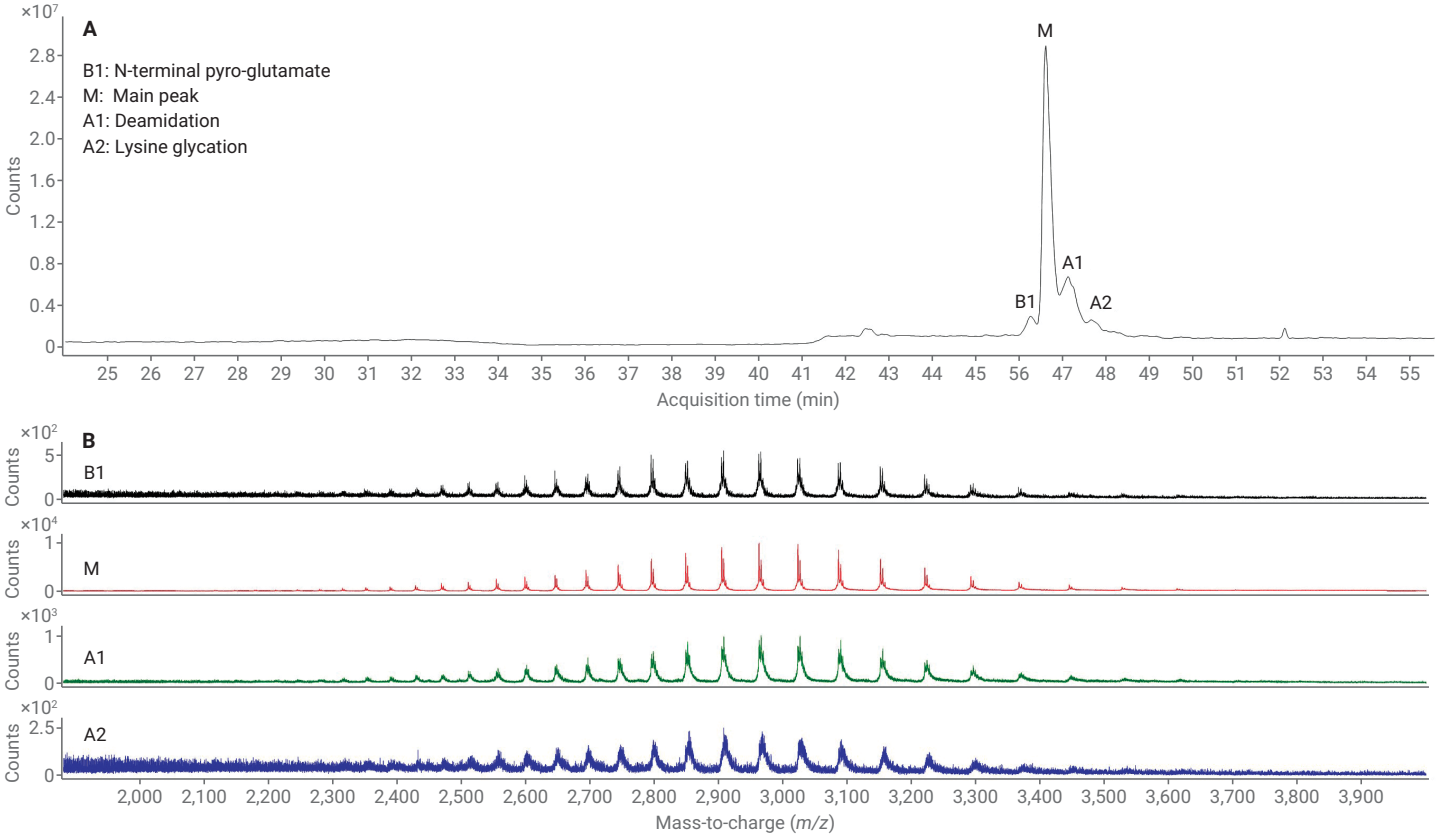

Figure 6. Charge variants analysis of daratumumab with the fully automated CIEF-MS method.

Repeat CIEF-MS injections of daratumumab

Figure 7 shows the results of five overnight CIEF-MS injections of daratumumab sample. Extended retention of ammonium hydroxide inside the capillary would compromise the capillary neutral coating. Therefore, we used high pressure (950 mbar) for catholyte and sample injection to quickly move the ammonium hydroxide plug from the capillary inlet to the outlet. This high velocity catholyte and sample injection may generate laminar flow, resulting in diffusion of the ammonium hydroxide front end, and the mixing of sample plug with the ammonium hydroxide fluid. As a result, the length of effective ammonium hydroxide and sample zones may vary slightly between injections. This may lead to the variation of peak migration time. Using the cytochrome C peak as a front marker, the relative migration time of the daratumumab main peak is rather stable. The RSD of the relative migration time is calculated to be 5.8 %.

Conclusions

This Application Note shows a fully automated online CIEF-MS method implemented on a 7100 CE instrument and TOF/Q-TOF mass spectrometers. This CIEF-MS workflow is enabled using electrokinetically pumped sheath liquid nanospray CE/MS coupling technology. The CZE-MS of basic proteins, monoclonal antibody, and the CIEF-MS of daratumumab for charge variants analysis demonstrates the excellent performance of the EMASS-II CE/MS ion source on Agilent systems. Details of this CEIF-MS method are provided, including capillary performance validation and system performance checkup. This Agilent-CMP CIEF-MS workflow enables direct mass spectrometry identification of intact mAb charge variants separated by capillary isoelectric focusing.

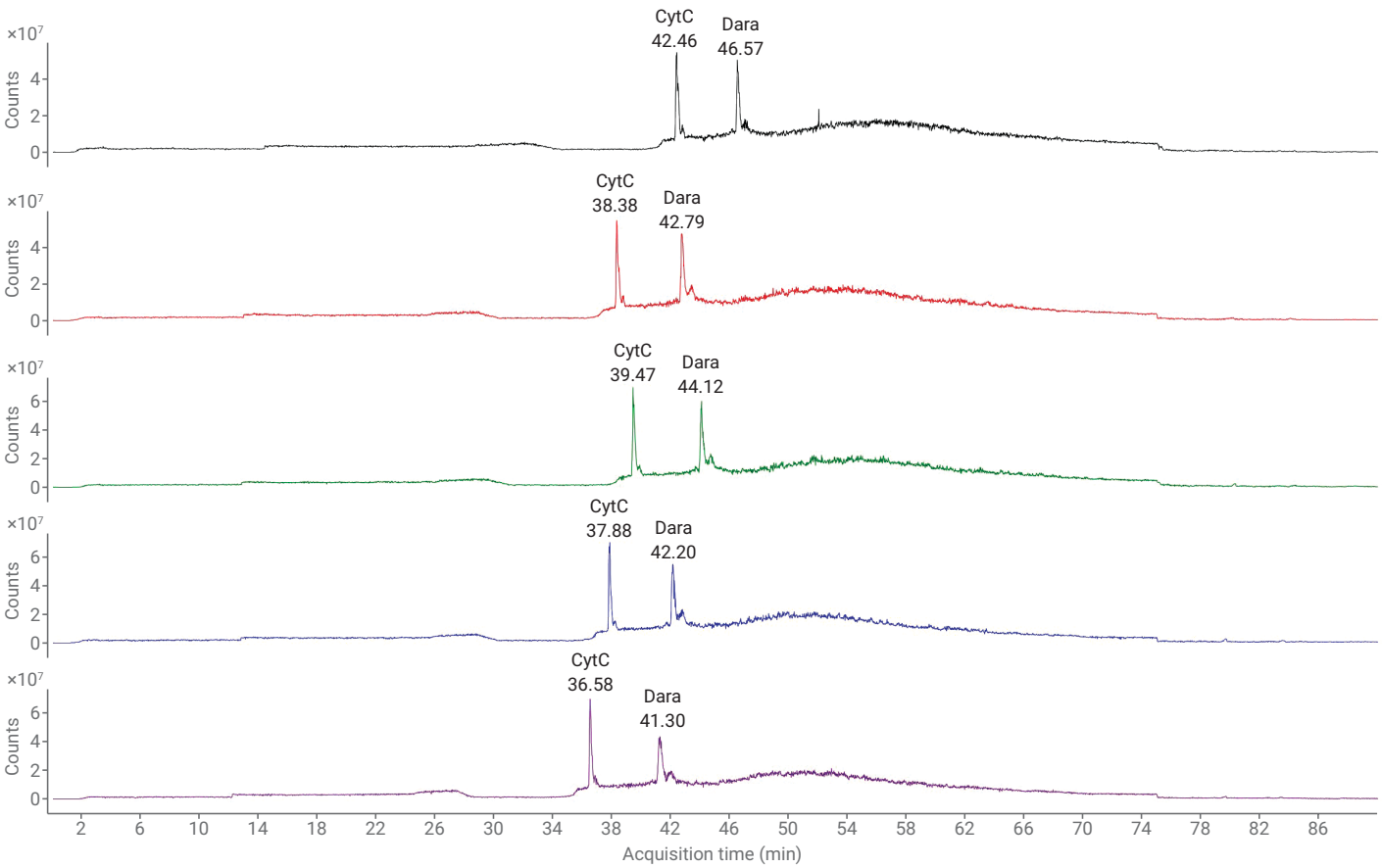

Figure 7. Reproducibility of five CIEF-MS injections of daratumumab.

References

1. Zhu, G.; Sun, L.; Dovichi, N. J. Simplified Capillary Isoelectric Focusing with Chemical Mobilization for Intact Protein *Analysis. J. Sep. Sci.* **2017**, *40(4)*, 948–953.
2. Dai, J.; *et al.* Capillary Isoelectric Focusing-Mass Spectrometry Method for the Separation and Online Characterization of Intact Monoclonal Antibody Charge Variants. *Anal. Chem.* **2018**, *90(3)*.
3. Dai, J.; Zhang, Y. A Middle-Up Approach with Online Capillary Isoelectric Focusing/Mass Spectrometry for In-Depth Characterization of Cetuximab Charge Heterogeneity. *Anal. Chem.* **2018**, *90(24)*, 14527–14534.
4. Ouyang, Y.; *et al.* Negative-Ion Mode Capillary Isoelectric Focusing Mass Spectrometry for Charge-Based Separation of Acidic Oligosaccharides. *Anal. Chem.* **2019**, *91(1)*, 846–853.

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