Application Note

Biopharmaceuticals

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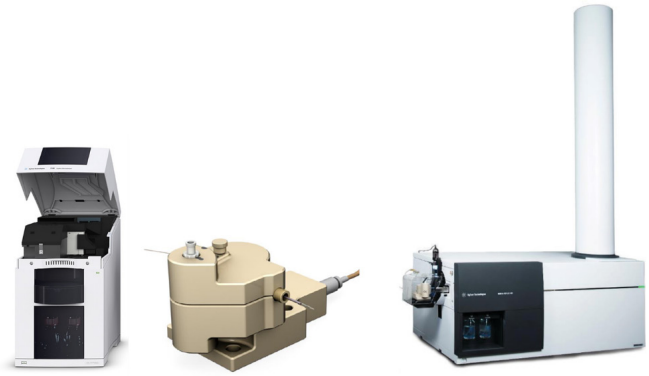
Charge Variant Analysis of Monoclonal Antibodies and ADCs Using CE/MS

Separation and qualitative analysis using CIEF/Q-TOF for characterizing charge variants in Humira and Kadcyla



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Abstract

This application note introduces a workflow for the charge variant analysis of protein-based therapeutics such as adalimumab (Humira) and trastuzumab emtansine (Kadcyla), using a capillary isoelectric focusing/mass spectrometry (CIEF/MS) system. The system consists of a CMP EMASS-II ion source, an Agilent 7100 capillary electrophoresis system (CE), and an Agilent 6545XT AdvanceBio quadrupole time-of-flight mass spectrometer (Q-TOF). This workflow provides an in-depth understanding of charge variant analysis through the separation and identification of various modifications in protein-based therapeutics.

Introduction

Charge variants occur during the manufacturing, transport, and storage of drugs due to chemical and structural modifications such as deamidation, glycosylation, and other changes in amino acid residues. Each modification, with its unique isoelectric point (pI), represents the heterogeneity of protein-based drugs.[1] Identification of these charge variants is crucial for quality control in the biopharmaceutical industry, as they can directly affect drug stability, efficacy, and immunogenicity.

CIEF is one of the separation technologies of charge variants. This technique generates a pH gradient within a capillary, allowing the separation of charge variants based on their pI values. Establishing an appropriate pH gradient is essential and depends on the expected pI range of the target drug.[2] In the separation process, the capillary is filled sequentially with buffer B (basic buffer) as the catholyte, sample buffer, and buffer A (acidic buffer) as the anolyte (Figure 1). The specific pH range of sample buffer is determined by the addition of zwitterionic electrolytes.

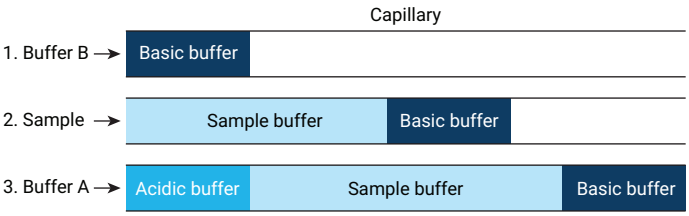

Figure 1. Sample injection sequence.

Applying voltage across the capillary ends generates an electric field, creating a pH gradient within the capillary. As the charge variants migrate along the electric field, they focus at the pH value where their pI matches the local pH. Under these conditions, charge variants with higher isoelectric points move closer to the catholyte, meaning that variants with faster migration times correspond to higher isoelectric points.

Monoclonal antibody (mAb) therapeutics typically show charge variants classified as acidic or basic. Acidic variants often result from modifications such as deamidation, lysine loss, and glycation, while basic variants stem from changes at the N- or C-terminus of the amino acid chain. Additionally, intermediates such as succinimide, pyroglutamate formation, or sialylation of glycans can also cause charge variants.[1]

Adalimumab (Humira) is a monoclonal antibody therapeutic used to treat inflammation by targeting the human immune system. Humira was used as an example for charge variant identification due to its various types of modifications.

Charge variant patterns based on pI can also be observed in antibody-drug conjugates (ADCs). ADCs have been developed as targeted biotherapeutics, especially for cancer treatment.

Trastuzumab emtansine, also known as Kadcyla, has a form of conjugation with a covalently linked cytotoxic reagent DM-1. Kadcyla is a non-site-specific ADC, meaning it comprises a mixture of ADCs with different drug-antibody ratios (DARs), rather than a single DAR value. The conjugation with lysine results in differences in isoelectric points of variants.

Agilent-CMP CE/MS system

Charge variants separated by CIEF are monitored using UV detection or laser-induced fluorescence detection (LIF) in quality control and research phases. In this application note, a CIEF/MS system (Figure 2) was applied to the identification of individual charge variants.

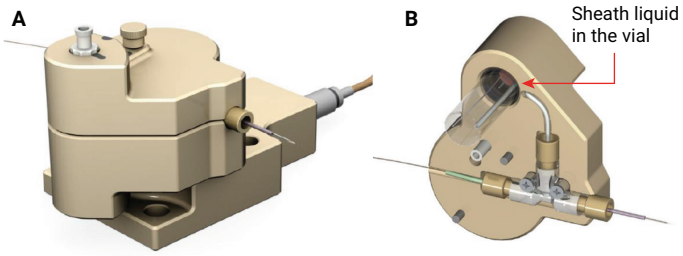

Figure 2. EMASS-II CE/MS interface overall view (A) and internal view (B).

The EMASS-II ion source connects the CIEF and MS systems, providing efficient ionization through a sheath liquid and emitter tip system. The capillary end is positioned inside the emitter tip, where the sheath liquid mixes with the sample buffer, facilitating the ionization of separated variants (Figure 3). The distance between the capillary end and the emitter tip, as well as the distance from the emitter tip to the MS inlet, can influence ionization efficiency. The external power supply delivers a low flow rate of sheath liquid and sample buffer mixture, ranging from tens to hundreds of nanoliters per minute, enhancing the sensitivity of the mass spectrometer.[3]

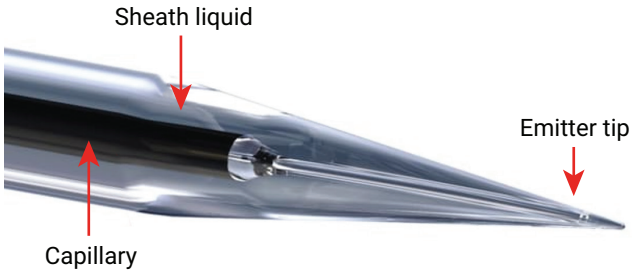

Figure 3. Arrangement of capillary and emitter tip.

In this analysis, the pH gradient within the capillary was formed by the 7100 CE system using the CIEF/MS reagent kit and ampholyte. The protein charge variants were separated from high pH to low pH and moved toward the capillary outlet. At the capillary end, the sample mixed with the sheath liquid inside the emitter tip and was sprayed into the mass spectrometer in small amounts, driven by the external power source.

The charge variant mass values were calculated using data obtained from the 6545XT AdvanceBio Q-TOF. Additionally, qualitative analysis of each variant was conducted by estimating potential modifications based on mass shifts relative to the main component.

Experimental

Materials

The types of antibodies and ADCs used in the analysis were as follows:

- Humira (adalimumab), 0.1 mg/mL
- Kadcyla (trastuzumab emtansine), 0.5 mg/mL

Sample preparation

Ampholytes (Pharmalyte) in the sample buffer play a crucial role in separating charge variants. The pH range, concentration, and composition of Pharmalyte are determined by the pI of the target protein.[4]

Three types of Pharmalyte with different pH ranges (3 to 10, 8 to 10.5, and 5 to 8) were dissolved in S35 CIEF/MS reagent kit, (CMP Scientific Corp.) and used as sample buffers.

- **Sample buffer for Humira:** 0.2% Pharmalyte 3 to 10, 0.8% Pharmalyte 8 to 10.5 in S35
- **Sample buffer for Kadcyla:** 0.5% Pharmalyte 5 to 8, 0.5% Pharmalyte 8 to 10.5 in S35

The stock solutions of each sample were diluted with their respective sample buffer to final concentrations of 0.1 mg/mL (Humira) and 0.5 mg/mL (Kadcyla).

Analysis

The CE, CE/MS ion source, and MS analysis conditions are summarized in Table 1. The CIEF/MS reagent kit (CMP Scientific Corp.) included buffer A (anolyte), buffer B (catholyte), buffer C (sample diluent), S35, and sheath liquid (SL), and they did not require any further dilution before use.

Table 1. CIEF/MS analytical method.

	Parameter	Value
Instrument	Capillary Electrophoresis	Agilent 7100 CE
	CE/MS Interface	EMASS-II ion source (CMP Scientific Corp.)
	Mass Spectrometer	Agilent 6545XT Q-TOF
CE	Separation Capillary	PS1 capillary, 100 cm (CMP Scientific Corp.)
	Anolyte	Buffer A, CIEF/MS reagent kit (CMP Scientific Corp.)
	Catholyte	Buffer B, CIEF/MS reagent kit (CMP Scientific Corp.)
	Sheath Liquid	CIEF/MS reagent kit (CMP Scientific Corp.)
	Sample Buffer	S35, CIEF/MS reagent kit (CMP Scientific Corp.)
	Sample Introduction	Flush 30 s (buffer B), Flush 90 s (sample buffer)
	CE Capillary Voltage	27.0 kV, 15 mbar
CE/MS Ion Source	Electrospray Emitter	20 µm tip size (CMP Scientific Corp.)
	Electrospray Voltage	2.4 kV
	Distance from End of Capillary to End of Emitter Tip	1.1 mm
	Distance from Emitter Tip to Front End of MS	60 mm
MS	Polarity	Positive
	Drying Gas Temperature	365 °C
	Drying Gas Flow	2 L/min
	Nebulizer	0 psi
	Capillary Voltage	2,500 V
	Fragmentor	370 V
	Skimmer	140 V
	Mass Range	1,000 to ~ 6,000 *m/z*
	Acquisition Rate	0.5 spectra/sec
	Acquisition Mode	Positive, extended mass range (*m/z* 10,000)

Results and discussion

The results were processed using Agilent MassHunter BioConfirm 12.0 software. Total ion chromatogram (TIC) and base peak chromatogram (BPC) results, shown in Figures 4 and 5, reveal the separated peaks of each charge variant for Humira and Kadcyla.

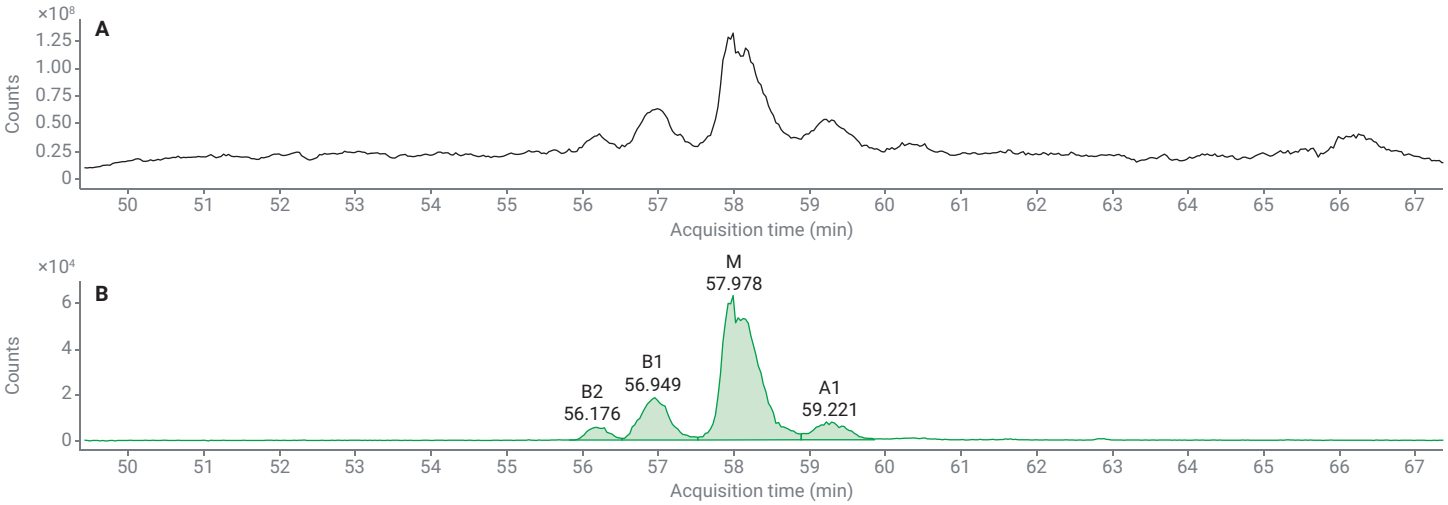

Figure 4. (A) TIC and (B) BPC of Humira.

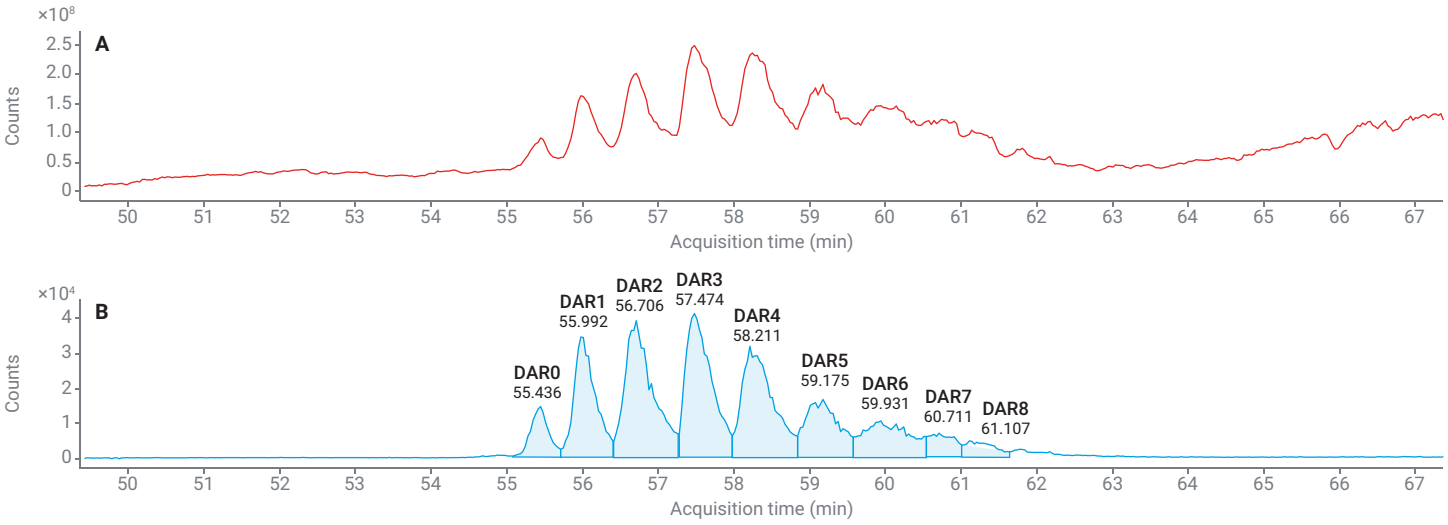

Figure 5. (A) TIC and (B) BPC of Kadcyla.

The Humira charge variants are categorized as basic variant 2 (B2), basic variant 1 (B1), and acidic variant 1 (A1) based on the main peak (M). The MS spectrum results for each variant are shown in Figure 6, where multiple charged ion patterns and the specific precursor ion ($[M+46H]^{46+}$) can be observed. In addition, the mass differences between the three peaks and the main peak were used to estimate the nature of the modifications. For more accurate mass identification of the variants, the raw spectra were processed using the deconvolution method in the BioConfirm software (Figure 7).

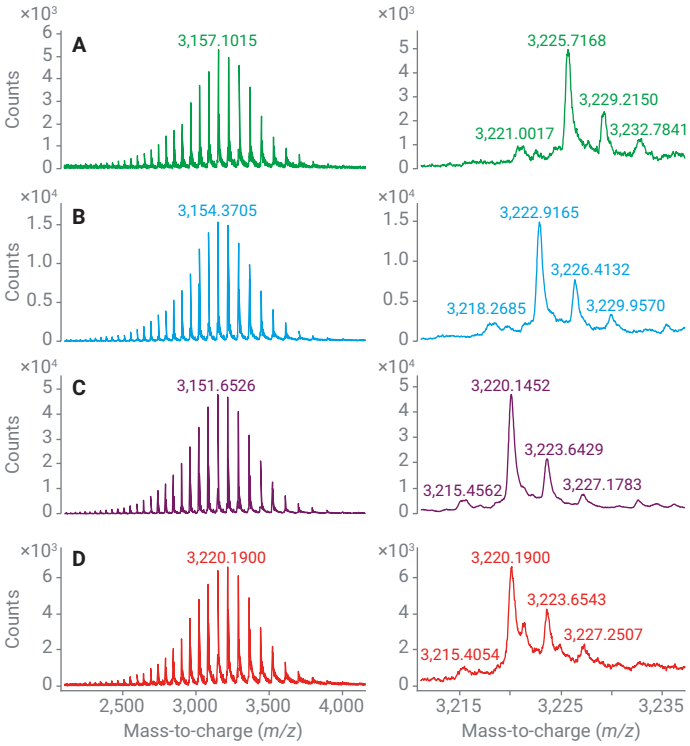

Figure 6. MS spectrum results of Humira; (A) B2, (B) B1, (C) M and (D) A1 (left: MS spectrum, right: zoomed spectrum for the $[M+46H]^{46+}$ charge state of each variant).

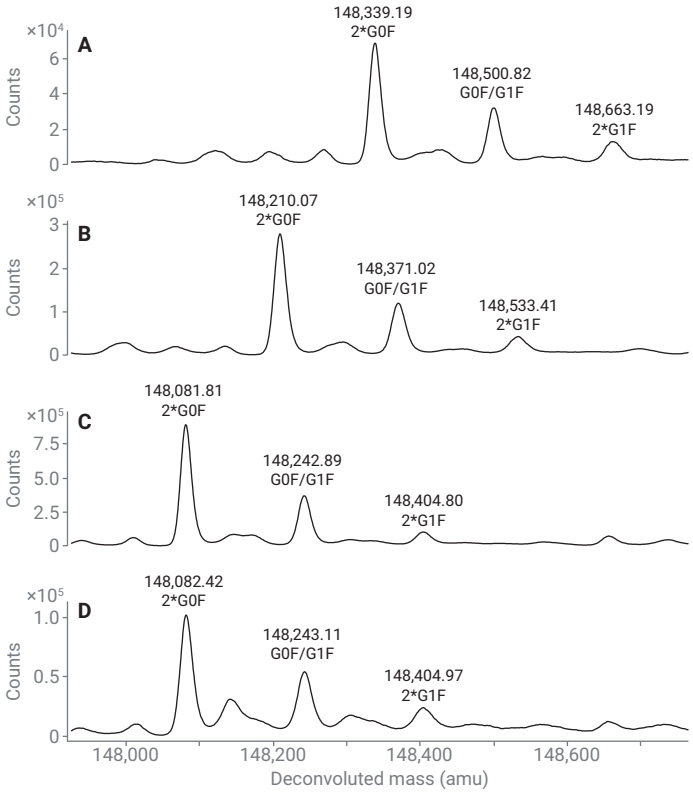

Figure 7. Deconvoluted spectrum results of Humira; (A) B2, (B) B1, (C) M and (D) A1.

Table 2 shows the retention time (minutes), calculated mass (Da), delta mass (Da) relative to the main peak, theoretical mass (Da), and mass accuracy (ppm) for the four separated peaks. In summary, B2 and B1 are related to lysine loss modifications, while A1 is estimated to be a charge variant resulting from deamidation.

The CIEF/MS system can easily be optimized for different sample analyses, such as Humira and Kadcyla, by adjusting the sample buffer preparation method without changing the analytical conditions. The sample preparation method, particularly the ratio of zwitterionic electrolytes, was optimized to achieve suitable separation conditions for Kadcyla.

Table 2. Identification results of Humira charge variants.

Classification	Retention Time (min)	Mass (Da)	Delta Mass (Da)	Theoretical Mass (Da)	Accuracy (ppm)
B2	56.187	148,339.1937	+256	148,339.1577	0.24
B1	56.954	148,210.0740	+128	148,210.9827	−6.13
M	57.987	148,081.8124	–	148,082.8077	−6.72
A1	59.220	148,082.4249	+1	148,083.7924	−9.23

Figure 5 shows the separated peak patterns of Kadcyla on the chromatograms. The separated peaks correspond to different DARs ranging from zero to eight. The MS and deconvoluted spectra for each peak are shown in Figures 8 and 9, confirming both the glycosylation patterns and DAR distributions of Kadcyla. Table 3 shows the expected masses based on the DAR distributions, confirming that Kadcyla is effectively separated in the CIEF/MS system. The average DAR is calculated from the deconvolution results using the mass of trastuzumab (DAR 0) and the delta mass of DM-1 (~ 956 Da).

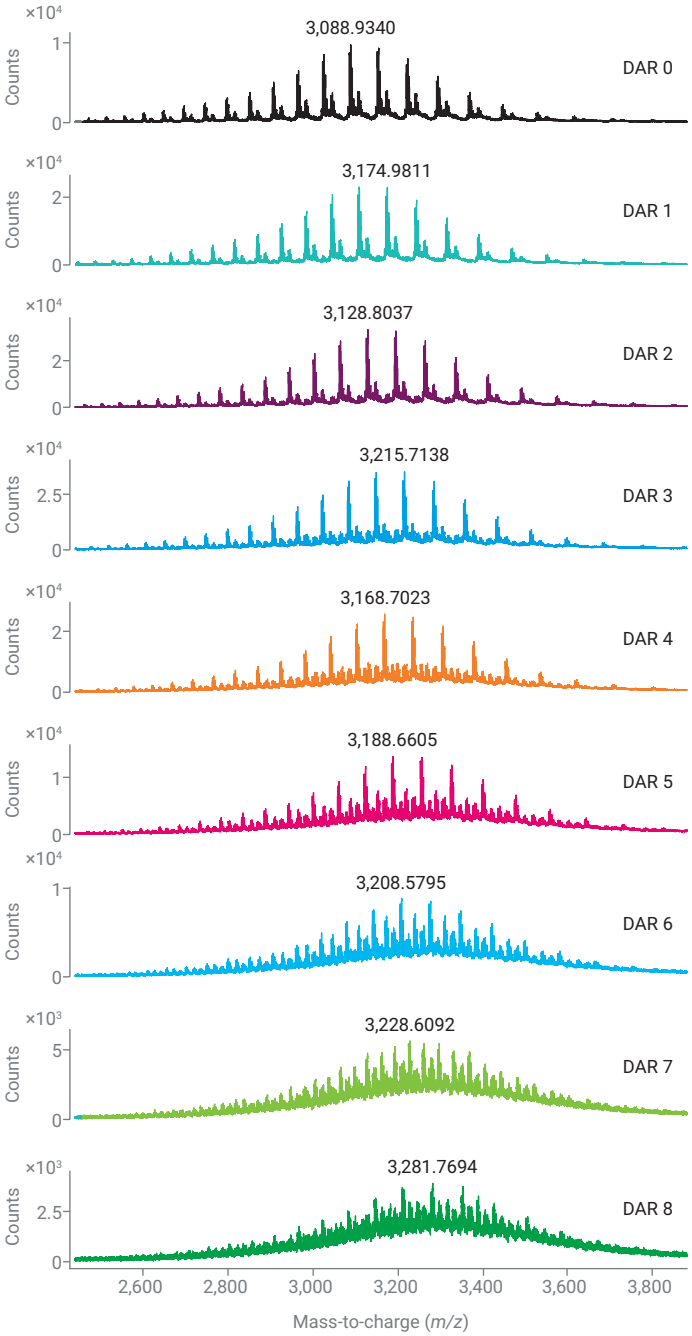

Figure 8. MS spectrum results of Kadcyla

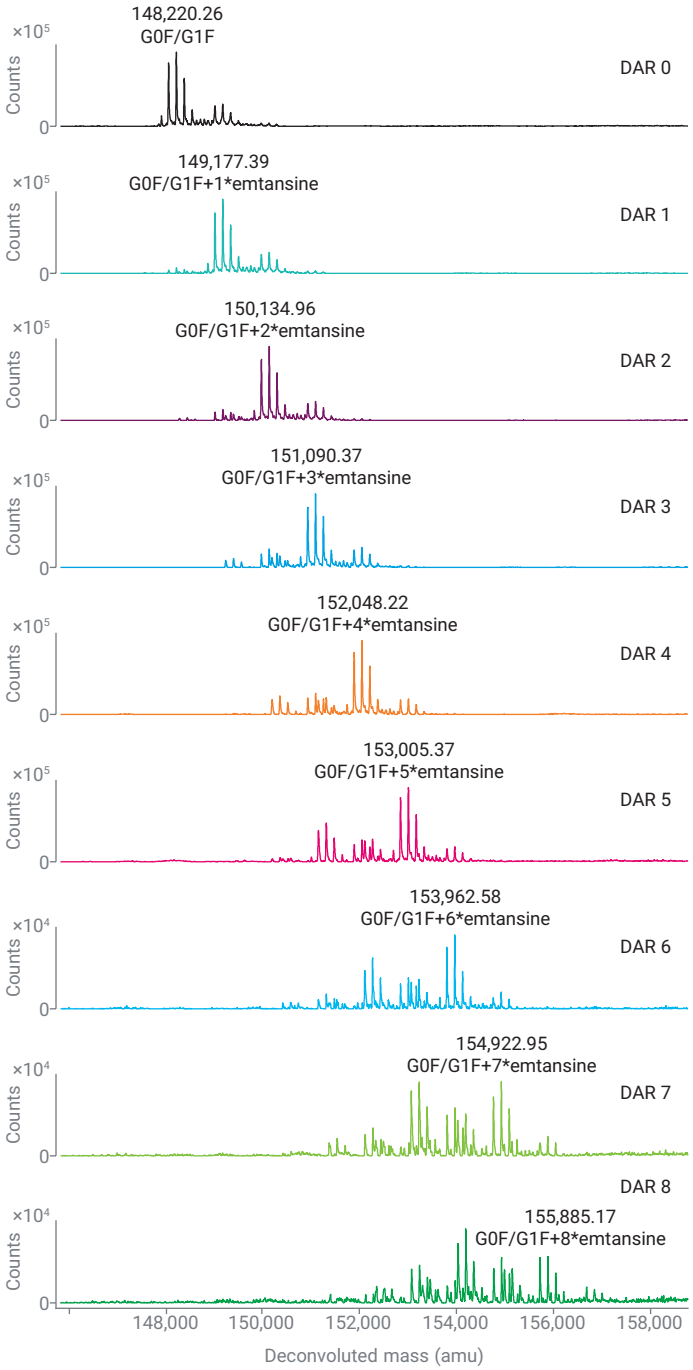

Figure 9. Deconvoluted spectrum results of Kadcyla.

Table 3. Identification results of charge variants of Kadcyla.

Classification	Retention Time (min)	Theoretical Mass (Da)	Theoretical Delta Mass (Da)	Delta Mass (Da)
DAR 0	55.443	148,220	–	–
DAR 1	55.976	149,177	+956	+957
DAR 2	56.709	150,134	+956	+957
DAR 3	57.475	151,090	+956	+956
DAR 4	58.208	152,048	+956	+958
DAR 5	59.174	153,005	+956	+957
DAR 6	59.941	153,962	+956	+957
DAR 7	60.707	154,923	+956	+961
DAR 8	61.107	155,885	+956	+962

Conclusion

To establish the CIEF/MS analysis conditions, it is essential to consider the composition of the anolyte, catholyte, and zwitterionic electrolyte. Additionally, selecting a suitable sheath liquid for the ionization of charge variants is crucial. In this analysis, a CMP CIEF reagent kit was used to minimize sample and reagent preparation, allowing easy adaptation of the analysis method to various proteins and charge variants. Enhanced sensitivity in mass analysis results was achieved due to the nanoflow controlled by an EMASS-II electrospray system.

Using the CIEF/MS system, the charge variants of Humira and Kadcyla were analyzed. The results indicated that three charge variants of Humira were identified, attributed to lysine loss and deamidation modifications. For Kadcyla, approximately nine charge variants were identified, and their effective separation based on molecular weight differences demonstrated that the system successfully resolved the ADC drug-antibody ratio (DAR) distribution under the given conditions.

As discussed, this application proposes the CMP EMASS-II ion source, Agilent 7100 capillary electrophoresis system, and Agilent 6545XT AdvanceBio Q-TOF system for the separation and qualitative analysis of charge variants with varying isoelectric points in their native state. This system is expected to play a significant role in elucidating the structures of various charge variants in future protein therapeutics.

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DE-005414

Printed in the USA, April 8, 2025
5994-7668EN

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