Received: 11 November 2022 | Revised: 16 November 2022 | Accepted: 17 November 2022

DOI: 10.1002/sscp.202200142

RESEARCH ARTICLE

High-efficient characterization of complex protein drugs by imaged capillary isoelectric focusing with high-resolution ampholytes

Teresa Kwok | **She Lin Chan** | **Mike Zhou** | **Anna Schaefer** | **Tao Bo** | **Tiemin Huang** | **Victor Li** | **Tong Chen**

Advanced Electrophoresis Solution Ltd, Cambridge, Canada

Correspondence
Tong Chen, Advanced Electrophoresis Solution Ltd, Cambridge, Canada.
Email: tony.chen@aeslifesciences.com

A carrier ampholyte is a molecule containing both acid and base functionality that is critical for imaged capillary isoelectric focusing. The quality of an imaged capillary isoelectric focusing separation for protein charge variants' characterization is highly dependent on attributes of the carrier ampholytes used including baseline signal, linearity of the pH gradient, pI discrimination, and consistency between manufactured lots. AESlytes are a high-resolution carrier ampholyte series that have been developed for the high-resolution and selective characterization of diverse and complex protein drugs including diverse fusion proteins, antibody-drug-conjugate, bi-specific antibodies, and viral proteins. While routine commercial ampholytes usually cannot solve such challenges, AESlytes demonstrate a reduction in baseline noise and distinguishably increased consistency between lots as compared to other commercial ampholytes. Here we apply AESlytes for the imaged capillary isoelectric focusing separation of several commercial fusion proteins and biosimilars with excellent repeatability. In addition, AESlytes with narrow-range pH were employed directly coupled to a mass spectrometer for optimizing the separation resolutions allowing more reliable and accurate protein charge variant identification. Our study demonstrates that innovative high-resolution carrier ampholytes as critical reagents play an essential role in high-performance imaged capillary isoelectric focussing and tandem mass spectrometry analysis the routinely commercial ampholytes cannot achieve, especially for extremely complex protein drugs.

KEYWORDS
antibody-drug-conjugate, bi-specific antibody, carrier ampholytes, fusion protein, imaged capillary isoelectric focusing, mass spectrometry, monoclonal antibody

Article Related Abbreviations: AD, acrylamide derivative; ADC, antibody-drug-conjugate; CA, carrier ampholyte; FC, fluorocarbon; icIEF, imaged capillary isoelectric focusing; mAb, monoclonal antibody; MC, methylcellulose; QC, quality control.

1 | INTRODUCTION

Charge variants of recombinant proteins can arise due to post-translational modifications, and degradation reactions such as deamidation, C-terminal lysine clipping, and glycation. Comprehensive characterization of charge variants is critical both for generic drugs and biosimilar drugs. This is because the variants can result in altered product efficacy and pharmacokinetics or even complete product inactivation, especially when these modifications are located in the complementarity-determining regions [1–4]. Thus, there is an essential need for reliable characterization of charge variants for the assessment of critical quality attributes and to ensure consistency during the manufacturing of therapeutic monoclonal antibodies (mAbs) for clinical and commercial development [5–6].

The most common methods for charge variant monitoring in the biotechnology industry include IEC, traditional IEF, and imaged capillary IEF (icIEF) [7–16]. Specifically, icIEF technology based on pI differentiation is becoming the gold standard across the pharmaceutical industry for protein charge variant characterization. An additional step requires the determination of intact charge variant mass. High-resolution MS is a powerful tool for the characterization of molecular weight and associated post-translational modifications [17, 18]. However, commonly used mobile phases of IEC, cIEF, and icIEF are incompatible with MS. In recent years, some researchers have developed an IEC-MS method with a volatile mobile phase for charge variant analysis of mAbs [19, 20], human growth hormone [21], bispecific and impaired IgGs [22, 23]. cIEF and icIEF are alternatives to IEC for charge variant analysis, and recently icIEF-MS has attracted much attention to utilizing for protein charge variant analysis [24–28]. Compared to IEC, charge variant analysis by icIEF as a front separation method is faster and can provide a higher separation resolution of protein charge variants.

A carrier ampholyte (CA) is a molecule containing both acid and base functionality and the quality of an icIEF separation is highly dependent on several attributes of the CAs used including baseline signal, linearity of the pH gradient, pI discrimination, and consistency between manufactured lots. Recently, antibody-drug conjugates (ADCs), bi-specific Abs, and fusion proteins have regained the special attention of scientists due to their unique therapeutic effects [29–31]. However, routine commercial ampholytes usually do not have enough selectivity and pI resolution to effectively separate these extremely complex proteins.

AESlytes introduced in this study have been developed as HR ampholytes that demonstrate a reduction in baseline noise and distinguishably increased consistency between lots. Their HR contributes largely to their effective characterization of diverse protein therapeutics meanwhile the routine commercial ampholytes usually cannot solve such challenges based on our comparable experiments. This study demonstrates the success of AESlytes in the study of fusion proteins created through the joining of two or more genes that originally coded for separate proteins. In addition, AESlytes were employed to icIEF-MS for optimizing the separation resolutions with more reliable and accurate protein identification. Our study indicates that innovatively HR ampholytes as critical reagents play an essential role in high-performance icIEF and icIEF-MS analysis that routine ampholytes cannot achieve, especially for extremely complex protein drugs.

2 | METHODS AND MATERIAL

2.1 | Chemicals

All CAs (AESlytes, a commercial name from Advanced Electrophoresis Solutions Ltd supplies) used in this study including HR and ultra-high (UH) ampholytes were obtained from Advanced Electrophoresis Solutions Ltd (AES, Cambridge, ON, Canada). Fusion proteins, ADC, bi-specific antibody, viral protein, recombinant protein, and mAb-AT-1 studied were kindly donated from Thermo Fisher Scientific (China). Mass spectrometry grade ACN, formic acid, urea, and methylcellulose (MC) were purchased from Fisher Scientific (Hampton, NH).

2.2 | Solutions for icIEF

For icIEF analysis, the sample solutions for studied protein drugs employed in this study were listed in Table 1. Urea solution (480 mg/ml) was added to protein samples with the final concentration of 4 mol/L for improved icIEF repeatability.

2.3 | Imaged cIEF

CEInfinite icIEF (AES) was employed in this study. Note that, 100 μm ID fluorocarbon coated (AES, cat. no. CP00201) is utilized for icIEF-UV analysis. Note that, 200 μm ID acrylamide-coated (AD) capillary cartridges (AES, cat. no. CP00303) and micro-tee integrated (AES, cat. no. CP00303M) are used for icIEF-MS. All these whole-column imaging detection cartridges have a 5 cm long separation capillary, and a 50 μm ID transfer capillary is assembled for both icIEF-MS. The 200 μm AD coated icIEF-MS cartridge used for icIEF-MS includes a quartz union (works as a micro-tee), connecting the make-up

TABLE 1 Sample preparation of studied fusion proteins

No.	Final concentration (mg/ml)	Volume (μl)	1% MC (μl)	H_2O (μl)	Ampholyte	Sample name
1	0.5	10	35	46	UH (pH 3–10)	Commercial Etanercept
2	5.3	10	35	46	UH (pH 3–10)	Biosimilar Enbrel
3	5	10	35	46	UH (pH 3–10)	Enbrel analogue-1
4	3.2	20	35	36	UH (pH 3–10)	Enbrel analogue-2
5	4	10	35	46	HR (pH 3–10)	rhVEGFR-Fc
6	1.2	60	35	0	HR (pH 3–10)	EPO-HAS fusion protein
7	7.8	10	35	46	HR (pH 3–10)	rhGLP-1-Fc fusion protein

All sample solutions contained 4 M urea.

Abbreviations: HR, high-resolution; UH, ultra-high.

solution and transfer capillary to ESI of MS. Both the make-up solution capillary and transfer capillary have a 100 μm ID.

As for icIEF-UV analysis, the focus was 1 min at 1000 V, 1 min at 2000 V, and 10 min at 3000 V. Note that 0.35% MC solution was utilized for pre-rinse before each run. As for icIEF-MS, the focus was 1 min at 1000 V, 1 min at 2000 V and 10 min at 3000 V, and 3000 V during mobilization; the mobilization speed was 50 or 100 nl/min with water containing 0.1% (v/v) formic acid, across the separation capillary, and 5 μl/min make up solution (water: ACN = 1: 1, v/v, containing 0.5% formic acid, v/v) added through a micro tee. Mobilisation time was 15 min.

2.4 | High-resolution MS

A Thermo Q-Exactive Plus mass spectrometer with a Biopharma option equipped with an Ion Max ESI Ion Source with a 34-gauge needle (Thermo Fisher Scientific, Bremen, Germany) was used for mass measurement. The spray voltage: 3.6 kV, sheath gas: 20 L/min, auxiliary gas: 5 L/min, S-lens RF 70 eV, capillary temp: 275°C, resolution 35,000@*m/z* 200, scan range of precursor ion 2000–8000 *m/z*, and the maximum injection time 200 ms.

Biopharma Finder (BPF 5.0) from Thermo Fisher was used for the data analysis including intact protein deconvolution.

3 | RESULTS AND DISCUSSION

3.1 | ASElyte UH and HR CAs for high-performance icIEF

The quality of an icIEF separation is highly dependent on several attributes of the CAs used including baseline signal, linearity of the pH gradient, and consistency between manufactured lots. Currently, commercial brands of ampholytes have been frustrating the biopharmaceutical industry due to spectral artifacts which have extremely negative impacts on accurate pI measurement and quantitation of protein charge. These artifacts include an unexpected "dip" of the baseline (pH 6–7) due to histidine contained in routine CAs and a "shift" of both peak and pI during icIEF separation. A "dip" is commonly observed in the acidic range when using pH 3–10 and mixed ampholytes 5–8 and 8–10.5 provided by routine commercial brands, and occurs because of variable lots and sample excipients. Similarly, a "shift" of both peak and pI results from unstable lots of ampholytes and is disastrous in quality control (QC) and product release. Moreover, no amount of method optimization can overcome the above troubles with the use of routine commercial ampholytes

AESlytes HR and UH CA series developed in our study were composed of mixtures of small molecules with specific pKa for forming pH gradient, which demonstrates a reduction in baseline noise and distinguishably increased consistency between lots when compared to other CAs brands of the same pH range. AESLytes CAs are commercial products and can be customized according to application requests by adjusting the compositions and corresponding concentrations with desirable resolutions. This unparallel lot-to-lot stability of AESlyte (Figure 1A) mitigates "Dip" occurrence and "shift" of pI and peak in icIEF separation, which guarantees consistency in QC method development. A hemoglobin isomers (A, S, F, and C) mixture was focused with three different lots of 3–10 HR AESlytes, as well as three lots of other 3–10 CAs, as the pI of hemoglobin A, which is standard for ampholyte manufacturing. Figure 1A shows that in comparison to the three lots of AESlytes, the other commercial brands show an increase in hemoglobin isomer variability.

Another concern is that while icIEF uses UV light at 280 nm to detect proteins, the CAs themselves can

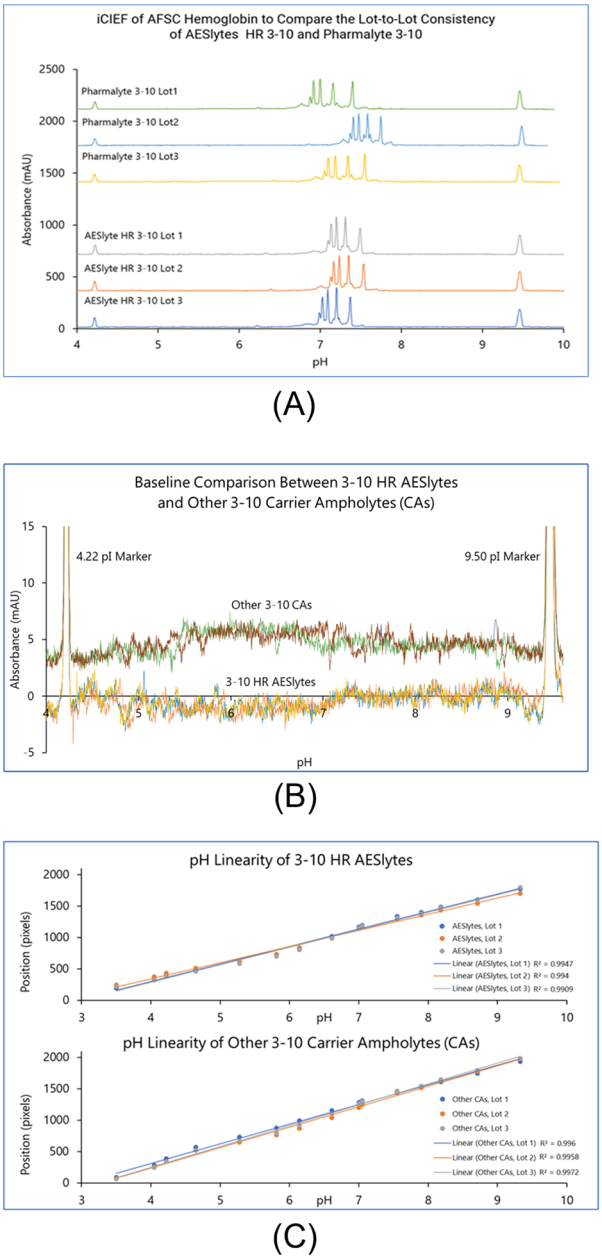

FIGURE 1 AESlytes ampholytes' advantages and its comparison with other commercial carrier ampholytes (CAs) at 4% concentrations

absorb UV light as well. To combat this, AESlytes have a greater proportion of "working" CA molecules so that sufficient resolution is maintained at lower concentrations. This allows a lower and more uniform baseline for pH 3–10 HR AESlytes compared to the commercial brands most notably between pH 6 and 9 (Figure 1B). Furthermore, in our recent study, AESlytes with ranges as narrow as one pH (using HR AESlyte 7–8) unit were created for increased resolution, specifically contributing to the highly-efficient separation of charge variants of extremely complex proteins [32].

A linear pH gradient is another critical factor of effective icIEF separation. To calculate the linearity of the pH gradient for AESlytes compared to other commercial ampholytes, 15 pI markers between pH 3 and 10 were focused. As illustrated in Figure 1C, the AESlytes showed comparable linearity to the other 3–10 CAs.

3.2 | icIEF of fusion proteins with the use of ASElyte UH and HR ampholytes

Fusion proteins contain multiple protein domains to combine multiple therapeutic functions [30]. At present, current technologies usually cannot efficiently characterize the charge variants of such a complex protein. Typically, the fusion protein is separated as peak groups with poor resolution and peak shape under routine icIEF conditions. In our study, AESlyte UH 3–10 ampholytes were used for profiling Etanercept, a commercially available fusion protein. As shown in Figure 2, the multiple peaks observed were located in an acidic pH range and the major peaks had much better resolutions than those from recently reported results utilizing commercial Serva ampholytes [33]. In addition, UH 3–10 ampholytes contributed to the outstanding repeatability of Etanercept's characterization in terms of peak shape, pI, and profiles of multiple peaks observed. Moreover, AESlyte UH 3–10 ampholytes were utilized for characterizing the Etanercept (Enbrel) biosimilar and its two analogs as illustrated in Figures 3 and 4, respectively. The separation profile of fusion proteins is often complex, with several groupings of peaks that correspond to varying degrees of modifications. Here, the biosimilar fusion protein was divided into four groupings of protein charge variants (G1: pH 5.10–5.30; G2: pH 5.30–5.65; G3: pH 5.65–5.85; and G4: pH 5.85–6.30) where Group 1 was the most acidic and the region where Group 4 was the most basic. In Table 2, the peak areas of four groups of Enbrel biosimilar in the icIEF electropherogram were listed with satisfactory repeatability, indicating a greater percentage of acidic components in the sample solution. As seen in Figure 4, Enbrel biosimilar and its two analogs showed different peak patterns in the icIEF and analogue-2 hinted charge variants with more acidic modifications. This knowledge is critical when evaluating the critical quality attributes of a biosimilar.

Another fusion protein, rhVEGFR-Fc that treats chronic neuropathic diabetic foot ulcers, was analyzed with the use of AESlyte HR 3–10 ampholytes and also showed 4 peak groupings from acidic to basic regions (Figure 5). Table 3 listed the relative peak areas of four groups and Group 2

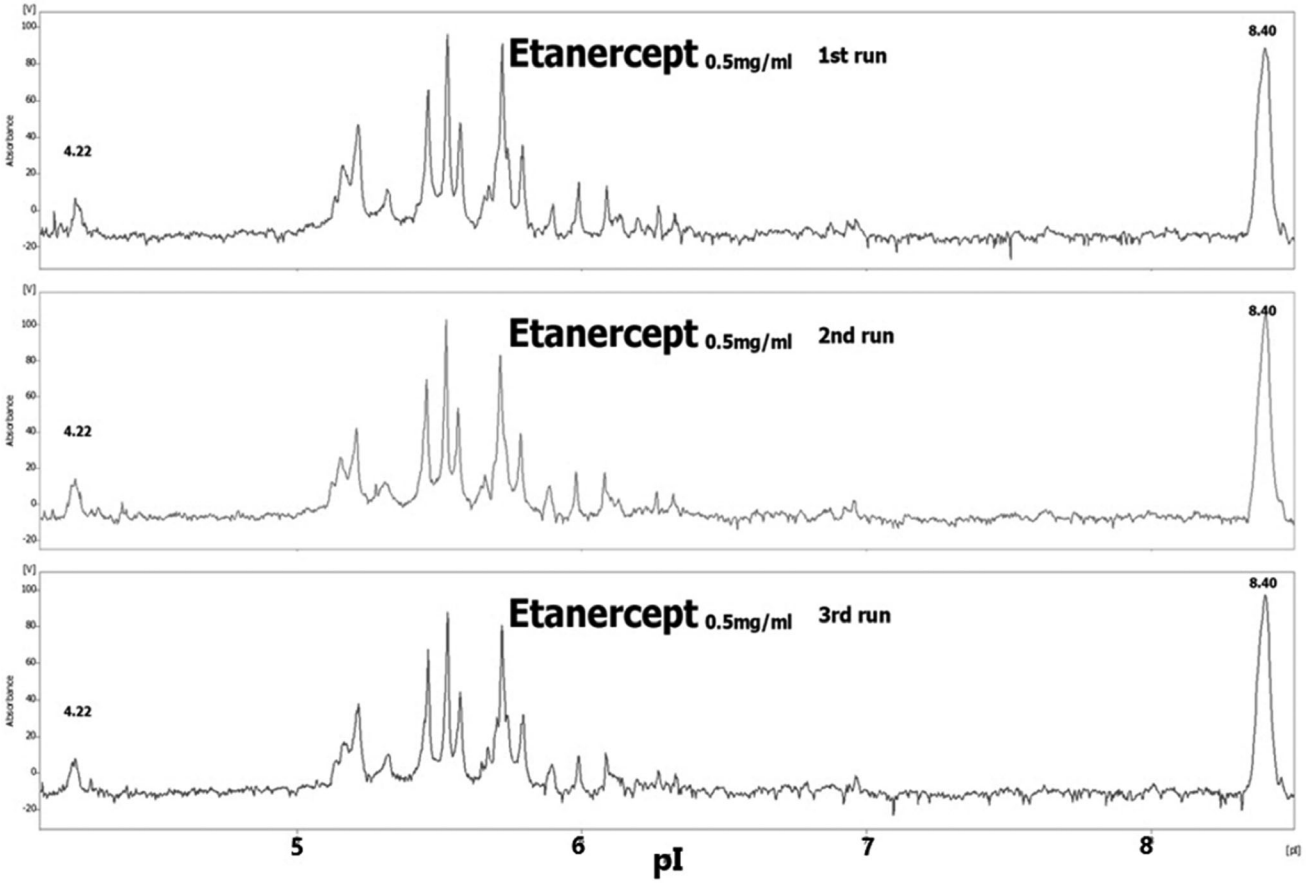

FIGURE 2 Imaged capillary isoelectric focusing (icIEF) separation of commercial Etanercept ($n = 3$) with the use of AESlyte ultra-high (UH) 3–10

TABLE 2 Peak areas of separated groups by imaged capillary isoelectric focusing (icIEF) for biosimilar Enbrel

No.	Final concentration (mg/ml)	Volume (μl)	1% MC (μl)	H_2O (μl)	Ampholyte	Sample name
1	0.5	10	35	46	UH (pH 3–10)	Commercial Etanercept
2	5.3	10	35	46	UH (pH 3–10)	Biosimilar Enbrel
3	5	10	35	46	UH (pH 3–10)	Enbrel analogue-1
4	3.2	20	35	36	UH (pH 3–10)	Enbrel analogue-2
5	4	10	35	46	HR (pH 3–10)	rhVEGFR-Fc
6	1.2	60	35	0	HR (pH 3–10)	EPO-HAS fusion protein
7	7.8	10	35	46	HR (pH 3–10)	rhGLP-1-Fc fusion protein

Abbreviations: HR, high-resolution; UH, ultra-high.

TABLE 3 Relative peak areas of separated groups by imaged capillary isoelectric focusing (icIEF) for rhVEGFR-Fc

No.	Group-1	Group-2	Group-3	Group-4
Relative peak area	14.0%	48.5%	16.6%	20.9%
RSD ($n = 3$)	12.6%	4.3%	8.5%	9.4%

demonstrated the highest relative concentration close to 50%.

Finally, fusion proteins, EPO-HAS, and rhGLP-1-Fc were analyzed using AESlyte HR 3–10 ampholytes as illustrated in Figure 6. EOP-HAS indicated Groups 1–3 from acidic to basic regions and more acidic Groups 1 and 2 had higher peak area ratios. As for GLP-Fc, two strong peaks

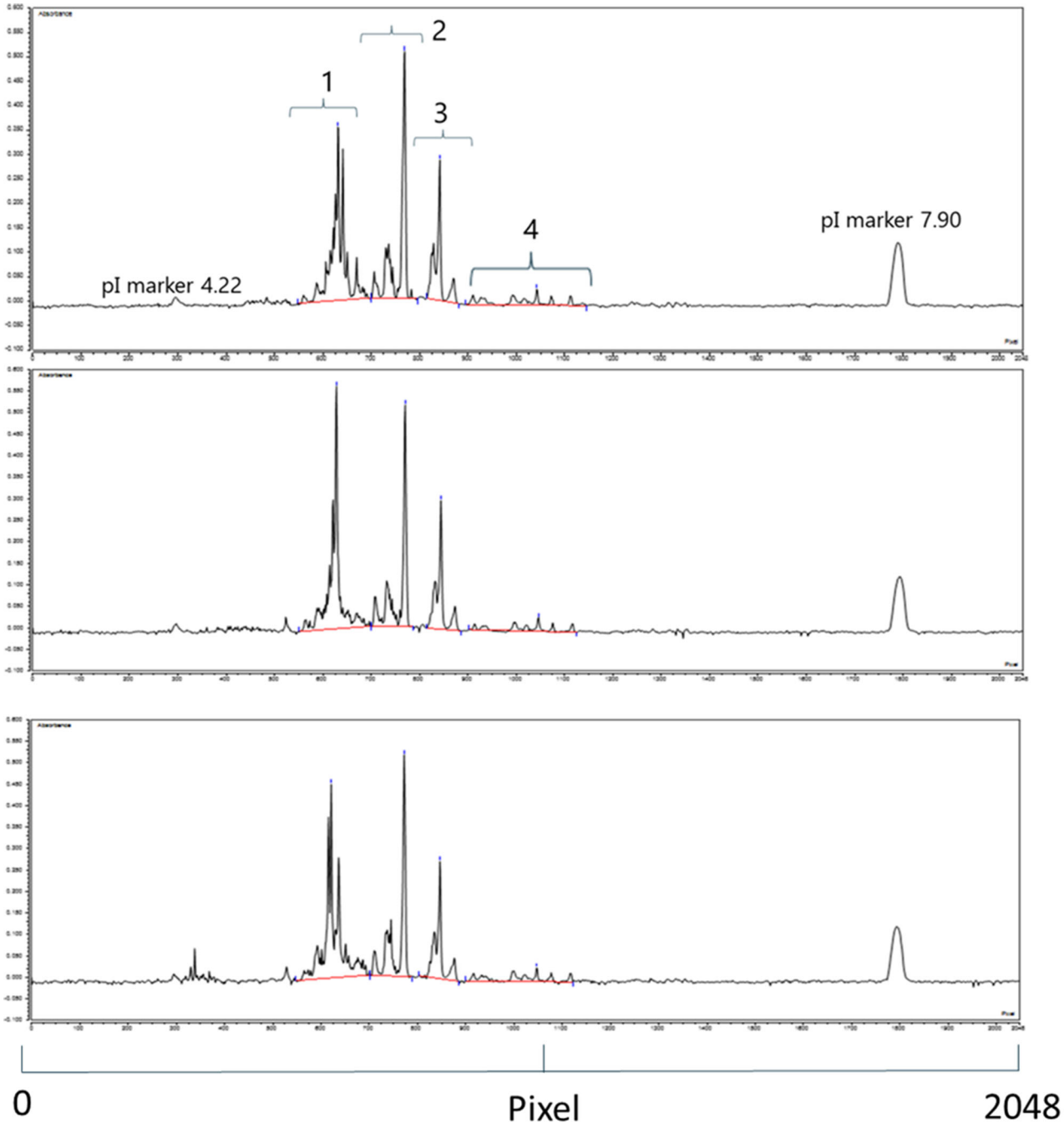

FIGURE 3 Imaged capillary isoelectric focusing (icIEF) separation repeatability of Enbrel biosimilar

with good peak shape were baseline separated, indicating two major charge protein isomers.

3.3 | icIEF of diverse complex proteins with the use of ASElyte UH ampholytes

The success of novel AESlytes is not limited to analyzing fusion proteins. The novel UH ampholytes described here were also used for the effective separation of multiple protein isomers for other categories of complex protein drugs including an ADC, recombinant protein, viral protein, and a bi-specific antibody (Figure 7). Notably, the eight different drug loadings of the analyzed ADC have displayed separation thanks to the subtle pI discrimination from ASElyte UH CAs. The ability to analyze drug-antibody ratio with icIEF analysis is an extreme advantage as it can affect a drug's therapeutic index and therefore is a critical parameter in drug design and QC [34].

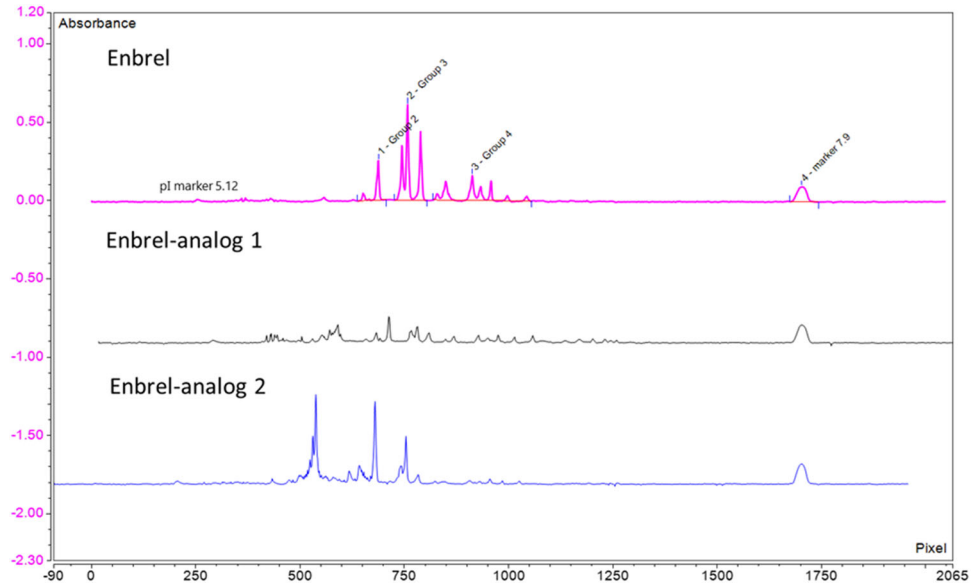

FIGURE 4 Imaged capillary isoelectric focusing (icIEF) separation of biosimilar Enbrel and its two analogs

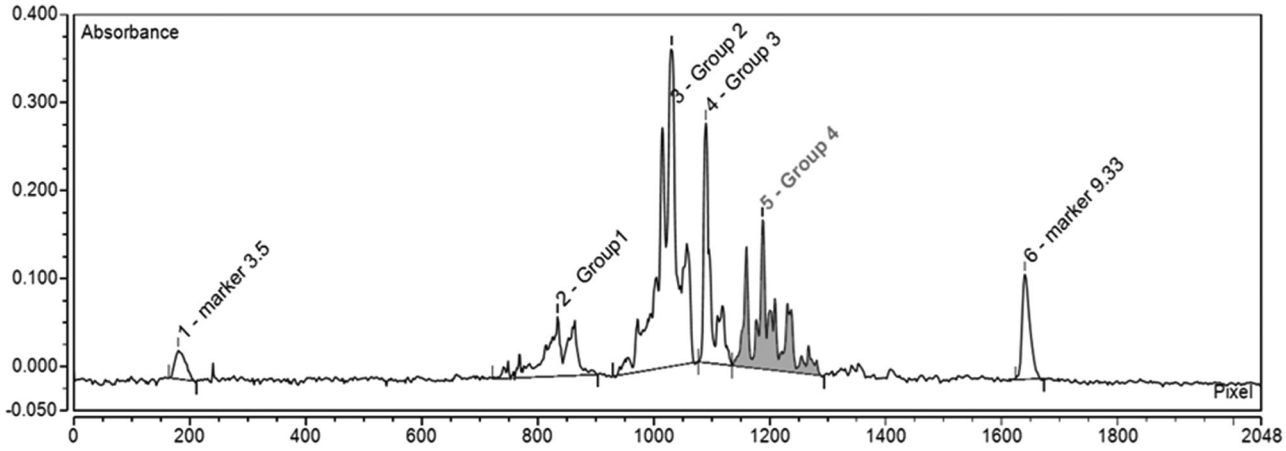

FIGURE 5 Imaged capillary isoelectric focusing (icIEF) separation of rhVEGFR-Fc

3.4 | AESlyte UH and HR narrow pH ampholytes for optimal icIEF MS/MS

A unique directly coupled icIEF-MS system was developed in our laboratory and patented capillary cartridges were employed to eliminate the need for chemical migration when coupled to online MS [35]. The use of only proprietary capillary-coated cartridges and separation solvents during icIEF separations greatly reduces the need for polymers and urea. These features combined enable the isolated protein-charged variants to be directly used for high-sensitivity MS characterization, thus retaining the excellent separation resolution of icIEF for MS analysis.

Additionally, the constructed system requires no special modifications to the ionization source and can be directly connected to the mass spectrometer from the different leading mass spectrometer brands. After proteins' focusing is completed along the separation capillary, water containing 0.1% (v/v) formic acid as mobilization solvent from the syringe pump drives the focused protein bands out of the separation capillary toward the MS ion source (ESI) at 50 nl/min flowing rate using 200 μm ID. AD-coated capillary cartridge. Sheath liquid or make-up solution (water:ACN = 1:1 v/v, containing 0.5% v/v formic acid) helps the effluents direct into ESI. The seamless interface to MS based on micro-fluidity prevents sample loss enhancing the sensitivity of MS detection of proteins. The whole process is automatic and highly user-friendly.

As shown in Figure 8, icIEF-MS was utilized for characterizing charge variants of therapeutic mAb (mAb-AT-1). We demonstrate how various narrow pH ampholytes from AESlytes help in the optimization of separating charged

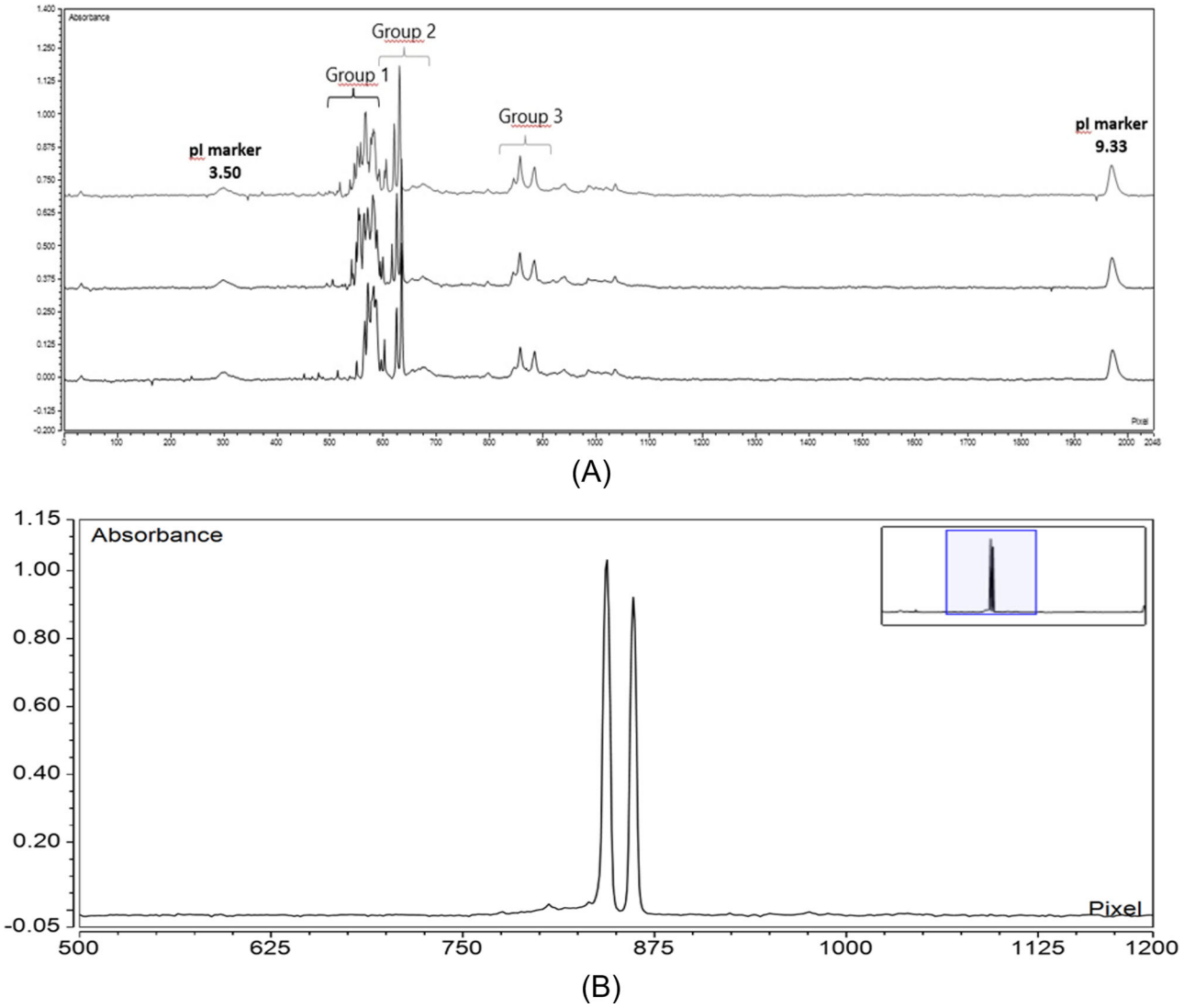

FIGURE 6 Imaged capillary isoelectric focusing (icIEF) separations of EPO-HAS (A) fusion protein and (B) rhGLP-1-Fc fusion protein

variants with small pIs discrimination in the icIEF-MS analysis. The use of 4% HR 8.5–9.5 could not achieve good separation of the main component and its two charge isomers, acidic variant (A1) and basic variant (B1), resulting in the poor discrimination of three protein peaks as illustrated in Figure 8A. However, the mixture of 2% HR 8.5–9.5 and 2% HR 8–10.5 improved the resolutions of the main component and its two charge variants, and ultimately three protein peaks could be separated in the total ion chromatogram despite full baseline separation being unachieved as shown (Figure 8B). Optimal separation resolution was achieved using 4% UH 8.5–9.5 and the basic charge variant achieved full baseline separation with the main component. This study demonstrates that HR ampholytes play a critical role in improved selectivity during the icIEF-MS analysis and our comprehensive selection of pH-range ampholytes can be used to customize an icIEF protocol for optimal performance.

4 | CONCLUDING REMARKS

AESlytes HR and UH CAs developed in this study offer a reduction in baseline noise and distinguishably increased consistency between lots when compared to other CAs brands of the same pH range in icIEF analysis. AESlytes CAs exhibit sufficient resolution maintained at lower concentrations and AESlytes with ranges as narrow as one pH unit was created for further increased resolution for the optimization in icIEF and icIEF-MS characterization of protein charge variants. Specifically, the high pI resolution rendered by AESlytes CAs contributes to the high-efficient separation of charge variants of diverse complex proteins including fusion proteins, ADC, bi-specific antibodies, viral proteins, and recombinant proteins. Our work validated the importance of high-quality CAs as critical reagents of icIEF and icIEF-MS technologies to solve the challenges in characterizing protein heterogeneity that

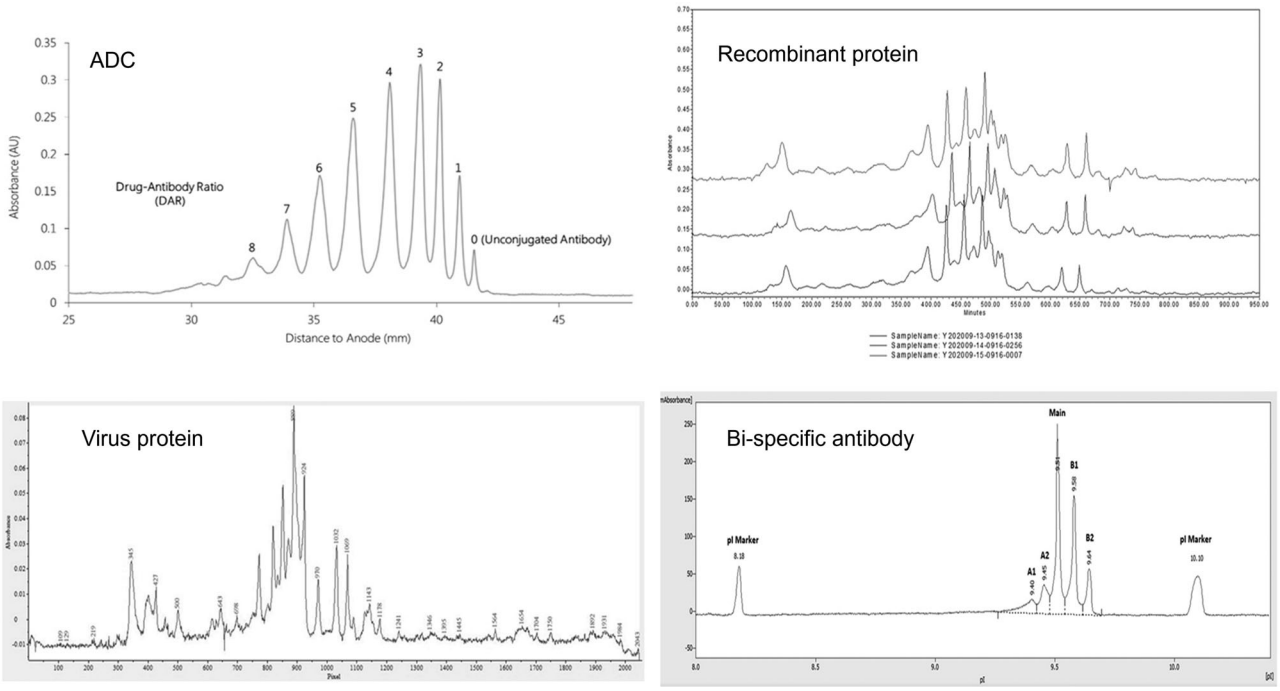

FIGURE 7 Imaged capillary isoelectric focusing (icIEF) for characterizing diverse complex proteins

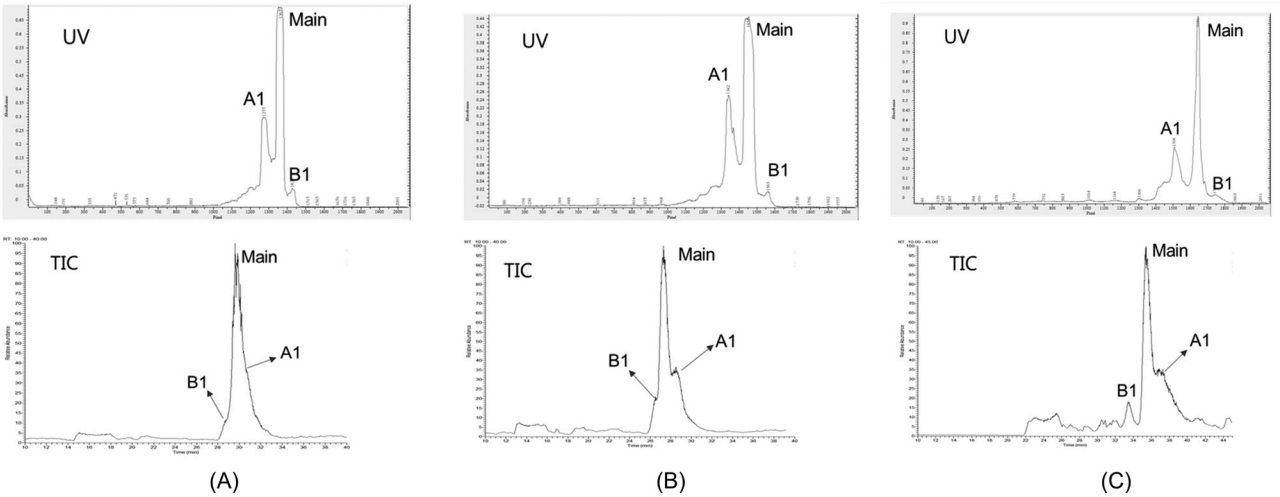

FIGURE 8 Ampholyte impacts on imaged capillary isoelectric focusing (icIEF)-MS separation of monoclonal antibody (mAb)-AT-1, using (A) 4% high-resolution (HR) 8.5–9.5; (B) 2% HR 8.5–9.5 and 2% HR 8–10.5; and (C) 4% ultra-high (UH) 8.5–9.5

routine commercial CAs cannot be achieved due to limited resolution. The features of AESlytes outlined here will be far-reaching in the design and QC of complex protein drugs.

ACKNOWLEDGMENTS

The authors acknowledge the financial support from Federal Economic Development Agency for Southern Ontario. And technical support from Thermo Fisher Scientific is highly appreciated.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Tong Chen https://orcid.org/0000-0002-4210-8082

REFERENCES

1. Jin Y, Lin Z, Xu Q, Fu C, Zhang Z, Zhang Q, Pritts WA, Ge Y. Comprehensive characterization of monoclonal antibody by Fourier transform ion cyclotron resonance mass spectrometry. MAbs. 2019;11(1):106–15.
2. Higel F, Seidl A, Sörgel F, Friess W. N-glycosylation heterogeneity and the influence on structure, function and pharmacokinetics of monoclonal antibodies and Fc fusion proteins. Eur J Pharm Biopharm. 2016;100:94–100.
3. Wang W, Singh S, Zeng DL, King K, Nema S. Antibody structure, instability, and formulation. Pharm Sci. 2007;96(1):1–26.
4. William R, Strohl LMS, editors. Development issues: antibody stability, developability, immunogenicity, and comparability. In: Therapeutic antibody engineering. Sawston, UK: Woodhead Publishing; 2012:377–595.
5. Vlasak J, Ionescu R. Heterogeneity of monoclonal antibodies revealed by charge-sensitive methods. Curr Pharm Biotechnol. 2008;9(6):468–81.
6. Du Y, Walsh A, Ehrick R, Xu W, May K, Liu H. Chromatographic analysis of the acidic and basic species of recombinant monoclonal antibodies. MAbs. 2012;23:578–85.
7. Baek J; Schwahn AB, Lin S, Pohl CA, De Pra M, Tremintin SM. New insights into the chromatography mechanisms of ion-exchange charge variant analysis: dispelling myths and providing guidance for robust method optimization. Anal Chem. 2020;92:13411–9.
8. Farmerie L, Rustandia RR, Loughney JWM. Recent advances in isoelectric focusing of proteins and peptides. J Chromatogr A. 2021;1651(16):462274.
9. Creamer JS, Nathan JO, Lunte SM. Recent advances in the analysis of therapeutic proteins by capillary and microchip electrophoresis. Anal Methods 2014;6(15):5427–49.
10. Farmerie L, Rustandi RR, Loughney JW Dawod M. Recent advances in isoelectric focusing of proteins and peptides. J Chromatogr A. 2021;1651(16):462274.
11. Štěpánová S, Kašička V. Applications of capillary electromigration methods for separation and analysis of proteins (2017–mid 2021) – a review. Analytica Chimica Acta. 2022;1209:339447.
12. Wu J, McElroy W, Pawliszyn J, Heger CD. Imaged capillary isoelectric focusing: applications in the pharmaceutical industry and recent innovations of the technology. Trends Anal Chem. 2022;150:116567.
13. Navarro-Huerta JA, Murisier A, Nguyen JM, Lauber MA, Beck A, Guillarme D, Fekete S. Ultra-short ion-exchange columns for fast charge variants analysis of therapeutic proteins. J Chromatogr A. 2021;1657:462568.
14. Murisier A, Duivelshof BL, Fekete S, Bourquin J, Schmudlach A, Lauber MA, Nguyen JM, Beck A, Guillarme D, D'Atri V. Towards a simple on-line coupling of ion exchange chromatography and native mass spectrometry for the detailed characterization of monoclonal antibodies. J Chromatogr A. 2021;1655:462499.
15. Fekete S, Beck A, Veuthey JL, Guillarme D. Ion-exchange chromatography for the characterization of biopharmaceuticals. J Pharm Biomed Anal. 2015;113:43–55.
16. Di Marco F, Berger T, Esser-Skala W, Rapp E, Regl C, Huber CG. Simultaneous monitoring of monoclonal antibody variants by strong cation-exchange chromatography hyphenated to mass spectrometry to assess quality attributes of rituximab-based biotherapeutics. Int J Mol Sci. 2021;22:9072–89.
17. Yan YT, Xing T, Wang SH, Li N. Versatile, sensitive, and robust native LC–MS platform for intact mass analysis of protein drugs. J Am Soc Mass Spectrom. 2020;31:2171–9.
18. Tamara S, den Bo MA, Heck AJR. High-resolution native mass spectrometry. Chem Rev 2022;122(8):7269–326.
19. Zhang T, Bourret J, Cano T. Isolation and characterization of therapeutic antibody charge variants using cation exchange displacement chromatography. J Chromatogr A. 2011;1218(31):5079–86.
20. Rea JC, Moreno GT, Lou Y, Farnan D. Validation of a pH gradient-based ion-exchange chromatography method for high-resolution monoclonal antibody charge variant separations. J Pharm Biomed Anal. 2011;54(2):317–23.
21. Lapelosa M, Patapof TW Zarraga IE. Modeling of protein–anion exchange resin interaction for the human growth hormone charge variants. Biophys Chem. 2015;207:1–6.
22. Phung W, Han G, Polderdijk SGI, Dillon M, Shatz W, Liu P, Wei B, Suresh P, Fischer D, Spiess C, Bailey A, Carter PJ, Lill JR, Sandoval W.Characterization of bispecific and mispaired IgGs by native charge-variant mass spectrometry. Int J Mass Spectrom. 2019;446:116229.
23. Phung W, Han G, Polderdijk SGI, Dillon M, Shatz W, Liu P, Wei B, Suresh P, Fischer D, Spiess C, Bailey A, Carter PJ, Lill JR, Sandoval W. Data on charge separation of bispecific and mispaired IgGs using native charge-variant mass spectrometry. Data in Brief 2020;30:105435.
24. He XP, ElNaggar M, Ostrowski MA, Guttman A, Gentalen E, Sperry J. Evaluation of an icIEF-MS system for comparable charge variant analysis of biotherapeutics with rapid peak identification by mass spectrometry. Electrophoresis 2022;43:1215–22.
25. Mack S, Arnold D, Bogdan G, Bousse L, Danan L, Dolnik V, Ducusin M, Gwerder E, Herring C, Jensen M, Ji J, Lacy S, Richter C, Walton I, Gentalen E. A novel microchip-based imaged CIEF-MS system for comprehensive characterization and identification of biopharmaceutical charge variants. Electrophoresis 2019;40(23-24):3084–91.
26. Wang LY, Bo T, Zhang ZZ, Wang GB, Tong WJ, Chen DDY. High resolution capillary isoelectric focusing mass spectrometry analysis of peptides, proteins, and monoclonal antibodies with a flow-through microvial interface. Anal Chem. 2018;90:9495–503.
27. Montealegre C, Neusüß C. Coupling imaged capillary isoelectric focusing with mass spectrometry using a nanoliter valve. Electrophoresis 2018;39:1151–4.
28. Schlecht J, Moritz B, Kiessig S, Neusüß C. Characterization of therapeutic mAb charge heterogeneity by icIEF coupled to mass spectrometry (icIEF–MS). Electrophoresis Published online September 23, 2022.
29. Muñoz-Torrero D. Antibody-drug conjugates – a tutorial review. Molecules 2021;26(10):2943.
30. Pavlaki I. Fusion proteins drive cancer through phase separation. Nature 2021;595:591–5.
31. Salvaris R, Ong J, Gregory GP. Bispecific antibodies: a review of development, clinical efficacy and toxicity in B-cell lymphomas. J Pers Med. 2021;11(5):355
32. Zhang XZ, Tong C, Li V, Bo T, Du M, Huang TM. Cutting-edge mass spectrometry strategy based on imaged capillary isoelectric focusing (icIEF) technology for characterizing charge heterogeneity of monoclonal antibody. Anal Biochem. 2023;660:114961.

33. Wu G, Yu CF, Wang WB, Zhang RJ, Li M, Wang L. A platform method for charge heterogeneity characterization of fusion proteins by icIEF, Anal Biochem. 2022;638(1):114505.
34. Hamblett KJ, Senter PD, Chace DF, Sun MCC, Lenox J, Cerveny CG, Kissler KM, Bernhardt SX, Kopcha AK, Zabinski RF, Meyer DL, Fransisco JA. Effects of drug loading on the antitumor activity of a monoclonal antibody drug conjugate. Clin Cancer Res. 2004;10(20):7063–70.
35. Chen T, Kwok T, Esmin AR, Li V, Rozing G, Huang TM. High-efficiency preparative imaged capillary isoelectric focusing (icIEF) and icIEF-MS protein charge variant characterisation. Chromatogr Today 2021:14–18.

How to cite this article: Kwok T, Chan SL, Zhou M, Schaefer A, Bo T, Huang T, Li V, Chen T. High-efficient characterization of complex protein drugs by imaged capillary isoelectric focusing with high-resolution ampholytes. Sep Sci plus. 2022;1–11. https://doi.org/10.1002/sscp.202200142