

## Online IEX-MS of mAb Charge Variants Using a BioResolve SCX mAb Column, IonHance CX-MS pH Concentrates, and BioAccord System

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Samantha Ippoliti, Andrew Schmudlach, Matthew A. Lauber, Ying Qing Yu

Waters Corporation

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### Abstract

This application note demonstrates a direct IEX-MS method with broad utility for the successful detection of mAb charge variants. Platform methods can be easily developed using the BioResolve SCX mAb Column, IonHance CX-MS pH Concentrates, and BioAccord LC-MS System which provide robust and reproducible separations along with high quality mass spectra in the elucidation of various mAb-charge variant species.

### Benefits

- A novel salt mediated pH gradient ion exchange (IEX) method is demonstrated that employs volatile salts to enable direct coupling of mass spectrometry.
- The ability to directly couple IEX-MS reduces the dependency on traditional fractionation methods by facilitating the direct and simple identification of chromatographic peaks.

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## Introduction

Characterization of charge heterogeneity is critical for the development of biotherapeutic drugs, as many of these charge variants can have an impact on drug potency and efficacy.<sup>1,2</sup> Therefore, it is important to understand the possible impacts of charge variants and to monitor them throughout discovery, development, and manufacturing. Regarding charge variant characterization, options for analytical techniques include ion-exchange chromatography (IEX) or methods of capillary electrophoresis (CE) such as capillary zone electrophoresis (CZE) or isoelectric focusing (IEF). While all these methods are used to some degree for the analysis of charge variant heterogeneity, there are certain advantages and disadvantages to each of them.

The advantages of CE-based methods include less risk of non-specific interactions as there is no stationary phase<sup>3,4</sup> and increasing feasibility to couple to mass spectrometry (MS). The disadvantages of CE include the limitation in sample loading and poor reproducibility, both of which can complicate or limit fraction collection capabilities.<sup>5,6</sup> IEX, on the other hand, offers chromatographic reproducibility and considerably higher sample loading capacity. However, traditionally, IEX separations require high concentrations of salts that are not compatible with mass spectrometry (MS) analysis, which has left a gap in the characterization of charge variants.

Recently, it has been shown<sup>7,8</sup> that direct IEX-MS characterization of these charge variants is possible, if volatile salts are employed. Here we present a novel, direct IEX-MS method using ammonium-based mobile phases which is applicable to a wide range of monoclonal antibody (mAb) species. The analysis is carried out on a BioResolve SCX mAb Column using certified IonHance CX-MS pH Concentrates on the BioAccord LC-MS System. The BioAccord (Figure 1) is a user-accessible system comprised of the ACQUITY UPLC I-Class PLUS System, TUV detector, and ACQUITY RDa Detector, controlled with UNIFI, a compliance- ready software.

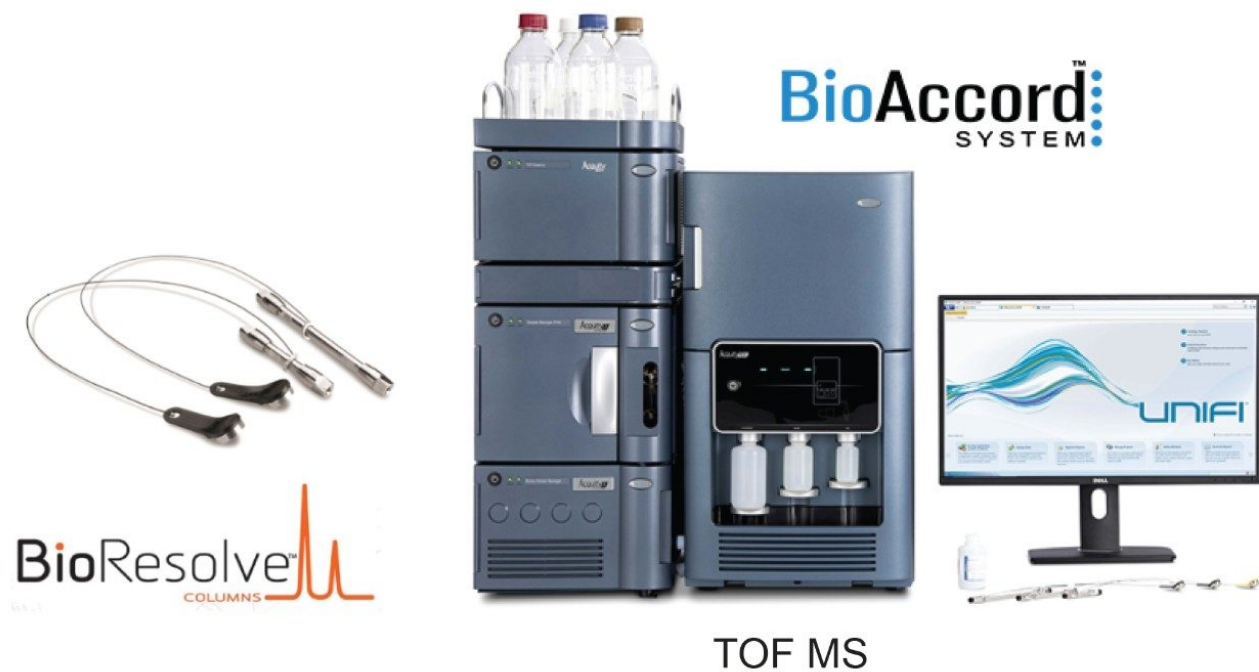


Figure 1. BioResolve SCX mAb Column and BioAccord System (ACQUITY UPLC I-Class with TUV System and RDa Detector, controlled by UNIFI Software for acquisition and data processing).

The I-Class provides robust chromatographic separation, and the RDa with SmartMS Technology delivers accurate mass information with a simplified user experience.

This application note will show the broad applicability of the method on the BioAccord LC-MS System, as well as its value for identifying charge variants formed upon forced degradation of mAbs. The ability to directly couple IEX separations to MS reduces the dependency on fractionation for simple and peak identification.

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## Experimental

### Sample preparation

#### Forced degradation of trastuzumab

A sample of trastuzumab (50  $\mu$ L @ 20 mg/mL) was buffer exchanged into 100 mM sodium phosphate, pH 8.0

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using BioRad Micro Bio-Spin chromatography columns (#732-6221), according to manufacturer protocol. The buffer exchanged trastuzumab sample was further diluted to 2 mg/mL in 100 mM sodium phosphate, pH 8.0, and then split into two aliquots. One aliquot was frozen at -80 °C until analysis, and the other was incubated at 25 °C for one week.

## IdeS digestion of mAb samples

A 50 µg quantity of each antibody sample (NIST mAb, rituximab, infliximab, and trastuzumab [T0 and one week stressed]) was digested by incubating at 37 °C for 30 minutes with 50 units of FabRICATOR enzyme (Genovis, A0-FR1-008) in 25 mM NaCl, 25 mM Tris, 1 mM EDTA, pH 8.0 (with a final sample concentration of 1 mg/mL). A 1 mg/mL aliquot of each nonreduced antibody sample was also prepared for analysis, and 10 µg of each sample was then injected for IEX-MS analysis.

## LC conditions

LC system:	ACQUITY UPLC I-Class PLUS
Detectors:	ACQUITY TUV Detector, ACQUITY RDa MS Detector
LC column:	BioResolve SCX mAb, 3 µm, 2.1 × 50 mm with mAb Charge Variant Standard (p/n: 176004342)
Column temp.:	30 °C
Sample vial:	12 × 32 mm Glass Vial, Total Recovery (p/n: 600000750CV)
Mobile phase A:	10-fold dilution of IonHance CX-MS pH Concentrate A (p/n: 186009280) (50 mM ammonium acetate, pH 5.0, 2% acetonitrile)
Mobile phase B:	10-fold dilution of IonHance CX-MS pH Concentrate B (p/n: 186009281) (160 mM

ammonium acetate, pH 8.5, 2% acetonitrile)

### Gradient table (Intact mAb):

Time (min)	Flow rate (min)*	%A	%B
Initial	0.100	60.0	40.0
1.00	0.100	60.0	40.0
21.00	0.100	2.0	98.0
22.00	0.100	2.0	98.0
23.00	0.100	60.0	40.0
30.00	0.100	60.0	40.0

### Gradient table (IdeS Digest):

Time (min)	Flow rate (min)*	%A	%B
Initial	0.100	98.0	2.0
1.00	0.100	98.0	2.0
21.00	0.100	2.0	98.0

Time (min)	Flow rate (min)*	%A	%B
22.00	0.100	2.0	98.0
23.00	0.100	98.0	2.0
30.00	0.100	98.0	2.0

*\*It is recommended to use a low dispersion ACQUITY UPLC I-Class instrument for performing this type of gradient with a 0.1 mL/min flow rate. If another instrument is to be used, it might be of benefit to gradient fidelity to scale the method with an increase in flow rate to at least 0.15 mL/min.*

## ACQUITY RDa Detector settings

Mass range:	<i>m/z</i> 400–7000
Mode:	ESI+
Cone voltage:	150 V
Desolvation temp.:	350 °C
Capillary voltage:	1.5 kV
Lock mass:	Leu-enkephalin at 50 fmol/μL in 50/50 water/acetonitrile with 0.1% formic acid
Informatics:	UNIFI Scientific Information System

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## Results and Discussion

Until recently the investigation of charge variants required tedious fraction collection and buffer exchange or a complex 2D-LC instrument setup to acquire mass spectrometry data. Herein, a mobile-phase system based on IonHance CX-MS pH Concentrates has been devised. With these concentrates, optimized ammonium-based mobile phases can be quickly prepared for MS-compatible IEX separations. The certified IonHance CX-MS pH Concentrates are prepared as 10x strength buffers packaged in one-liter trace metal certified low-density polyethylene bottles to ensure uncompromised MS quality. Concentrate A is formulated to yield a pH 5.0 mobile phase, and Concentrate B is formulated to generate a higher ionic strength pH 8.5 mobile phase.

The resulting mobile-phase system, BioResolve SCX mAb Column, and BioAccord System make for a compelling new analytical approach for the biopharmaceutical industry, where charge variant analysis is widely employed for drug characterization and stability studies. Charge variant profiles change over time or stress conditions, making them a critical quality component. The ability to directly investigate new or increasing peaks in the IEX charge profile with MS-compatible mobile phases saves time and will reduce the necessity to send samples to specialized characterization labs. In addition, it eliminates potential artificial degradation due to sample manipulation during fraction collection.

This method was first established with NIST mAb, rituximab, and infliximab, as shown in Figure 2, respectively. The ToF settings were tuned for the optimal ionization of intact mAbs and subunits in native conditions. Source parameters were based on a balance of MS-signal intensity and mass resolution along with consideration of what conditions best preserve the native state of the antibody and subunits. For NIST mAb and infliximab, the prominent charge variants are related to the presence of C-terminal lysine. With this method, the C-terminal lysine variants are well resolved via IEX chromatography, and then their identity could be readily confirmed with MS detection. Accordingly, this IEX method can be applied to a variety of mAb samples without the need for extensive, individualized gradient optimization. A 10 µg mass load on a 2.1 mm I.D. column was sufficient for detecting variants down to at least a 1% relative abundance. In addition, the technique produces high quality mass spectra with minimal interference caused by formation of salt adducts.

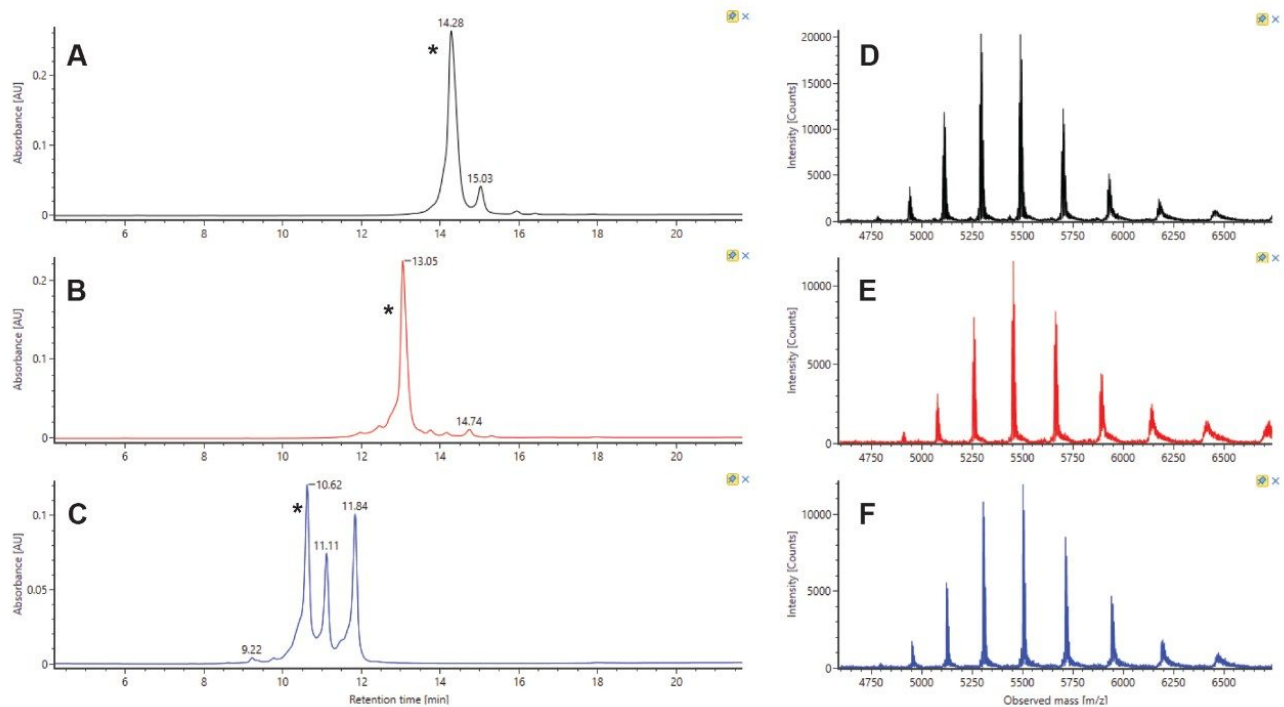


Figure 2. IEX-MS of intact mAbs. Left panel shows UV trace at 280 nm for (A) NIST mAb, (B) rituximab, and (C) infliximab. Right panel (D, E, and F) displays combined native MS spectra for main peaks (denoted with “\*”) for NIST mAb, rituximab, and infliximab, respectively.

As such, this IEX-MS method example is well suited to drug stability monitoring. To showcase this, we chose to investigate the forced degradation study of trastuzumab, a mAb which is well known to easily deamidate under elevated pH and temperature. Figure 3 shows the IdeS digest of unstressed (T0) versus stressed trastuzumab. With the stressed condition, there is an increase of 14.1% in acidic variants of the Fab region (Peaks C and D), and 4.5% of the Fc region (Peak A). The increase in acidic variants for IdeS digests corresponds well with the increase in acidic variants observed in nonreduced analysis (18.7%), as shown in Figure 4.



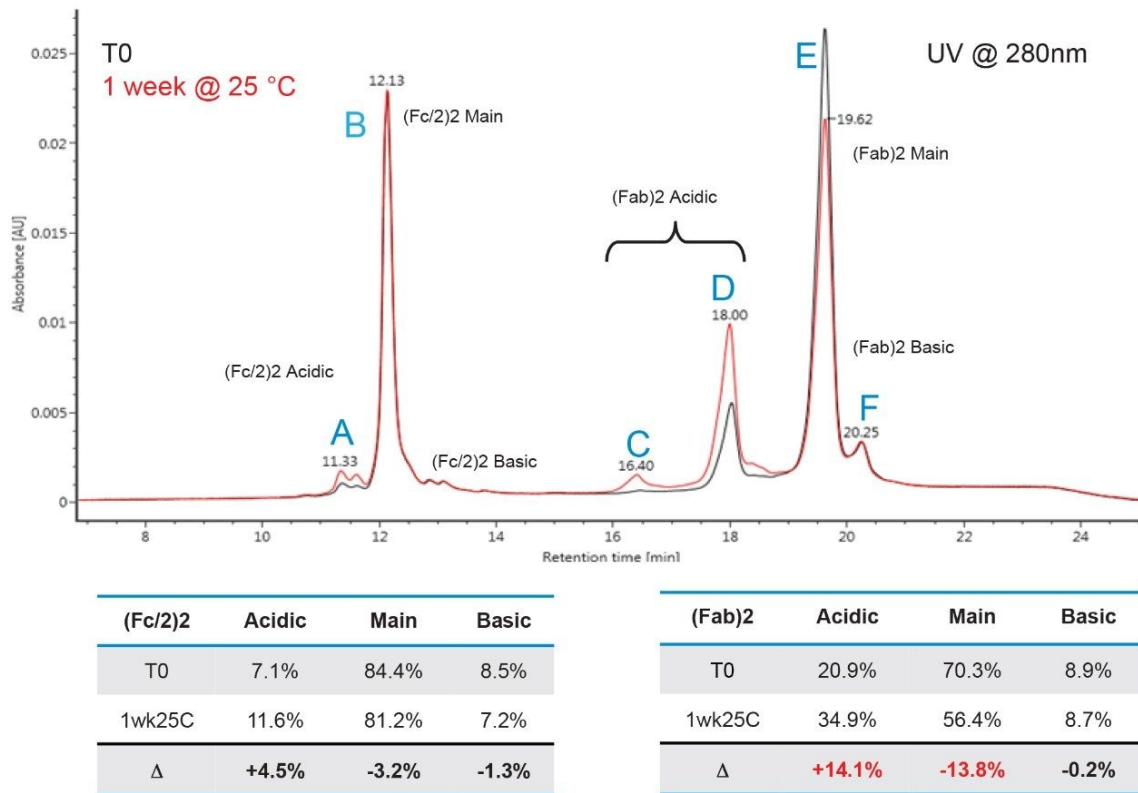


Figure 3. UV (280 nm) chromatogram overlay of T0 and one week stressed trastuzumab after IdeS digestion. Representative integrations are displayed for acidic and basic variants.

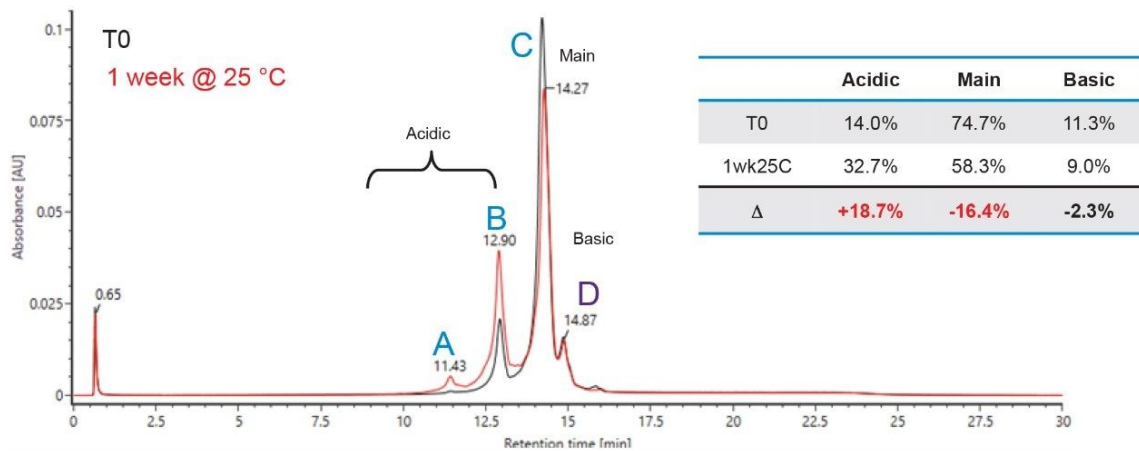


Figure 4. UV (280 nm) chromatogram overlay of T0 and one week stressed trastuzumab at the intact level of analysis. Representative peak integrations are displayed for acidic and basic variants.

Figures 5 and 6 display the online MS data (combined spectra) collected for each of the charge-variant species in IdeS digested and non-reduced trastuzumab samples, respectively. The main peaks in the IdeS digested sample correspond to  $(Fc/2)_2$  and  $(Fab')_2$  species, and their mass spectra are shown in Figures 5B and 5E, respectively. Observed masses can be seen within 20 ppm of theoretical masses. Acidic species shown in Figure 5A are likely deamidated  $(Fc/2)_2$  species as well as the N-glycan species containing sialic acid. The acidic species represented by the spectra of Figures 5C and 5D are likely the well characterized  $(Fab')_2$  deamidation species, and species represented by Figure 5F is likely a conformational variant. In the non-reduced sample, Figure 6C corresponds to the main species (within 20 ppm of calculated mass of the trastuzumab G0F/G0F glycoform). Figures 6A–B and 6D likely correspond to deamidated species or conformational variants, respectively.

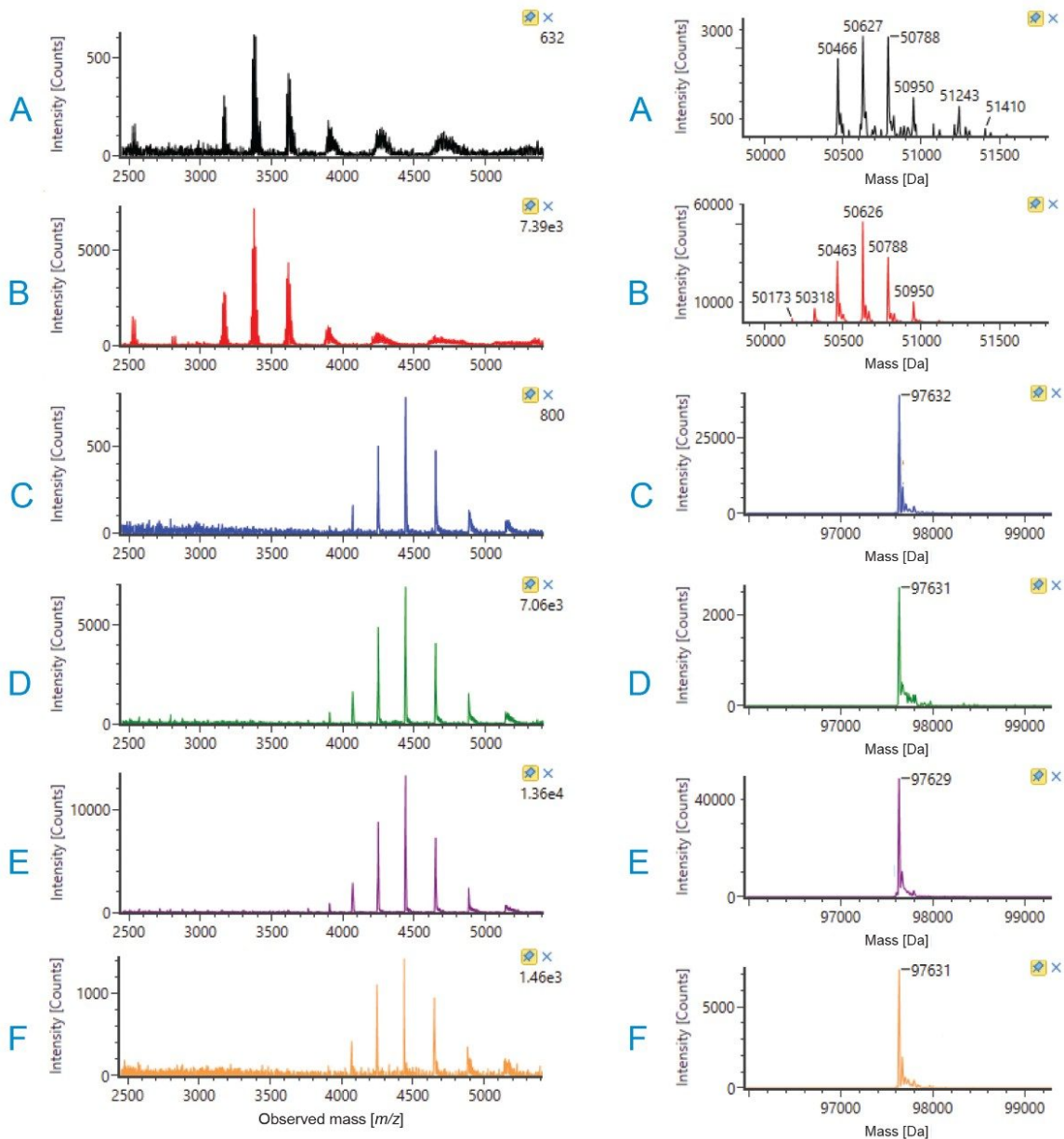


Figure 5. The panel on the left shows combined raw spectra for peaks A–F in Figure 3 (IdeS-digested trastuzumab); the panel on the right displays the corresponding MaxEnt1 deconvoluted spectra.

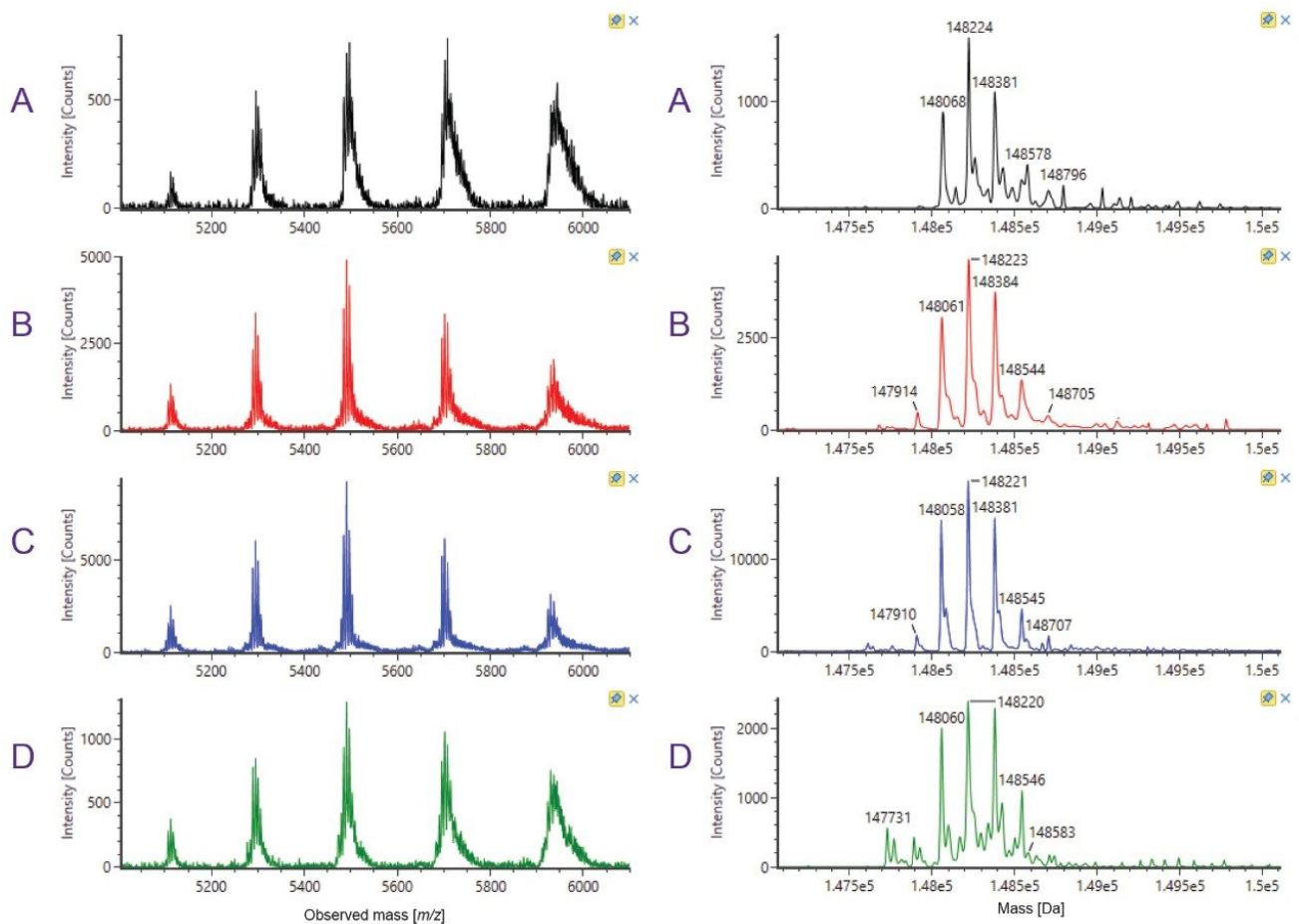


Figure 6. The panel on the left shows raw spectra for peaks A–D in Figure 4 (intact level of trastuzumab); the panel on the right displays the corresponding MaxEnt1 deconvoluted spectra.

One apparent challenge in this analysis is the assessment of isobaric and near-isobaric species such as deamidation or conformational differences. These charge variants have little or no mass difference in comparison to the full-size antibody, which makes it difficult to confidently assign these variants by intact mass. Peptide level characterization is required to confirm with high confidence, if these peaks are true isobaric or deamidated species. In the case of trastuzumab, the deamidation susceptibility is well characterized and expected at the levels observed here for acidic variants.<sup>4</sup> In practice, the direct IEX-MS method is useful to corroborate hypotheses about near-isobaric variants as well as to rule out other possible variants.

Furthermore, the use of a chromatographic method has a distinct advantage over other charge-based separations such as capillary electrophoresis or isoelectric focusing, because it lends itself more easily to fraction

collection and to performing other types of testing. The variant peaks separated by this IEX method can still be collected and analyzed via peptide mapping experiments to confirm the location of the modification, or they can be isolated for drug potency assays.

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## Conclusion

A direct IEX-MS method with broad utility has been successfully implemented and applied to a case study on the detection of mAb charge variants. Platform methods can be easily developed using the BioResolve SCX mAb Column, IonHance CX-MS pH Concentrates, and BioAccord LC-MS System which provide robust and reproducible separations along with high quality mass spectra in the elucidation of various mAb-charge variant species. The resulting workflow can increase efficiency for charge-variant characterization and monitoring, as it circumvents the need for fraction collection where MS-based peak assignment is sufficiently straightforward. The simplified buffer preparation, robust chromatographic separation, and clean MS spectra bring higher throughput and faster decision making in the development of biopharmaceuticals.

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## References

1. Schmid, I.; *et al.* Assessment of Susceptible Chemical Modification Sites of Trastuzumab and Endogenous Human Immunoglobulins at Physiological Conditions. *Communications Biology* 2018, 1 (28).
2. Diepold, K.; *et al.* Simultaneous Assessment of Asp Isomerization and Asn Deamidation in Recombinant Antibodies by LC-MS Following Incubation at Elevated Temperatures. *PLoS ONE* 2012, 7 (1): e30295.
3. Espinoza-de la Garza, C.; *et al.* Capillary Electrophoresis Separation of Monoclonal Antibody Isoforms Using a Neutral Capillary. *J. Vis. Exp.* 2017,(119): 55082.
4. Giorgetti, J.; *et al.* Intact Monoclonal Antibodies Separation and Analysis by Sheathless Capillary Electrophoresis-Mass Spectrometry. *European Journal of Mass Spectrometry* 2018, 25, 324–332.
5. Piešťanský, J.; *et al.* Two-Dimensional Capillary Electrophoresis with On-Line Sample Preparation and

- Cyclodextrin Separation Environment for Direct Determination of Serotonin in Human Urine. *Molecules* 2017, 22 (10), 1668.
6. Shaeper, J.; *et al.* Parameters Affecting Reproducibility in Capillary Electrophoresis. *Electrophoresis* 2000, 21 (7), 1421–1429.
7. Yan, Y.; *et al.* Ultrasensitive Characterization of Charge Heterogeneity of Therapeutic Monoclonal Antibodies Using Strong Cation Exchange Chromatography Coupled to Native Mass Spectrometry. *Anal. Chem.* 2018, 90 (21), 13013–13020.
8. Leblanc, Y.; *et al.* Charge Variants Characterization of a Monoclonal Antibody by Ion Exchange Chromatography Coupled On-Line to Native Mass Spectrometry: Case Study After a Long-Term Storage at +5 °C. *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.* 2017, 1048, 130–139.

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