ZipChip

Native charge variant analysis of biotherapeutics using ZipChip CE-ESI-MS: From comprehensive characterization to monitoring product degradation characteristics

INTRODUCTION

Charge heterogeneity of biotherapeutics is an important product quality attribute (PQA) that is monitored to ensure safety and efficacy of drug products. Assessing the charge variant profile is an essential step in characterization of biotherapeutics during development and production, as well as lot release testing. Traditional methods for charge variant analysis (CVA) involve the use of capillary electrophoresis (CZE or cIEF) or ion exchange liquid chromatography coupled with optical detection. The major drawback of these methods is that optical detectors provide peak profiles without structural information of the charge variants. While ion exchange chromatography can be coupled with mass spectrometry, optimizing separation of charge variants can be a time-consuming process.

ZipChip is a powerful technology that integrates capillary zone electrophoresis and ESI into a single microfluidic device. This device can be seamlessly coupled with commercial high resolution mass spectrometers to obtain high quality MS data for a variety of analytes. Analytes are separated based on differences in their electrophoretic mobility that is determined by the overall charge and size of analytes in a given background electrolyte (BGE) solution. Large proteins such as mAbs can be transported through the microfluidic device and sprayed into the mass spectrometer in a near-native state by using optimized BGE conditions. As the native mAbs migrate through the separation channel under the influence of the electric field, differences in the proteoforms' electrophoretic mobility result in the separation of charge variants, which are then introduced into the mass spectrometer upon electrospray ionization. The ZipChip method does not require optimization of separation and the same charge variants method can be applied to variety of different protein samples.

The Thermo Scientific™ Orbitrap Exploris™ 240, a hybrid quadrupole Orbitrap mass spectrometer, is an ideal instrument for performing MS analysis of intact biopharmaceuticals under native conditions. Sensitivity and dynamic range under both denaturing and native conditions, provides exceptionally low limits of detection. With the option to extend the mass range up to m/z 8000, native MS can be used for studies even where sample is limited. The high-quality MS data with excellent mass accuracy allows confident detection of small mass change modifications.

In this work, we have demonstrated the capabilities of ZipChip coupled with the Orbitrap Exploris 240 mass spectrometer for comprehensive analysis of mAb charge variants using the NIST mAb reference material. The same high resolution separation method was also applied for monitoring product stability of trastuzumab after prolonged storage.

METHODS

Instruments. This work was performed using a commercially available microfluidic CE-ESI system (ZipChip®, 908 Devices Inc.). All data were acquired on an Orbitrap Exploris 240 mass spectrometer enabled

ZipChip mounted to a Thermo Scientific Orbitrap mass spectrometer.

with BioPharma option (Thermo Fisher Scientific).

Consumables. Background electrolyte (BGE) from the commercially available Native Antibodies Kit was used. The microfluidic chip of the "high resolution native" (HRN) variety was used to analyze the samples.

Samples. NIST Monoclonal Antibody Reference Material 8671 and trastuzumab samples were diluted directly from formulation buffer using the ZipChip Native Antibodies BGE to a concentration of 0.5 mg/ mL.

ZipChip Method Settings. Following method settings were used on the ZipChip:

ZipChip protocol: Intact Charge Variant Analysis[1]
Chip type: HRN
BGE type: Native Antibodies. One bottle of BGE from the Native Antibodies Kit was spiked with 5mL DMSO purchased from Alfa Aesar (p/n 22914).
Field Strength: 500 V/cm
Injection volume: 1nL
Pressure Assist Start Time: 0.5 min
Analysis time: 10 min

MS Method Settings. All data were acquired in positive mode ESI using the following settings:

Scan Range (m/z): 2,500-8,000
Orbitrap Resolution: 30,000
Sheath gas: 2
In-source CID (V): 125
Normalized AGC Target (%): 300
RF Lens (%): 60
Microscans: 5

Data Processing. Charge variant separations were visualized using Thermo Scientific™ Xcalibur Qual Browser software. Data were processed using Thermo Scientific™ Biopharma Finder™ 4.0 software.

RESULTS AND DISCUSSION

In-depth characterization of mAbs. ZipChip separations for large intact proteins are predominantly based on differences in net positive charge on the proteoforms. Basic species have greater net positive charge whereas the acidic species have a lower net positive charge compared to the species belonging to the main variant. This determines the migration order of the mAb charge variants in ZipChip analysis.

The charge variant profile for the NISTmAb generated on ZipChip-MS is shown in Figure 1. The profile shows excellent separation resolution of basic and acidic variants from the main variant. Four discernible features are observed in the base peak electropherogram for the NISTmAb. As seen from Figure 1, basic variants migrate first, followed by the main variant, which in turn is followed by the acidic variant with the least net positive charge. The raw mass spectra generated by averaging the spectra across each of the four main peaks in the electropherogram along with their deconvoluted mass spectra are shown in Figure 2. A zoom-in of the most intense charge state (+27) shows the glycosylation profile of the molecule. Each charge state shows an intrinsic heterogeneity resulting from N-glycosylation of the Fc region. The glycans in the Fc region of the NISTmAb are unable to shift the isoelectric point of the proteoforms and therefore have the same electrophoretic mobilities. This results in the co-migration of the glycoforms.

The migration order and a mass difference of +128 and

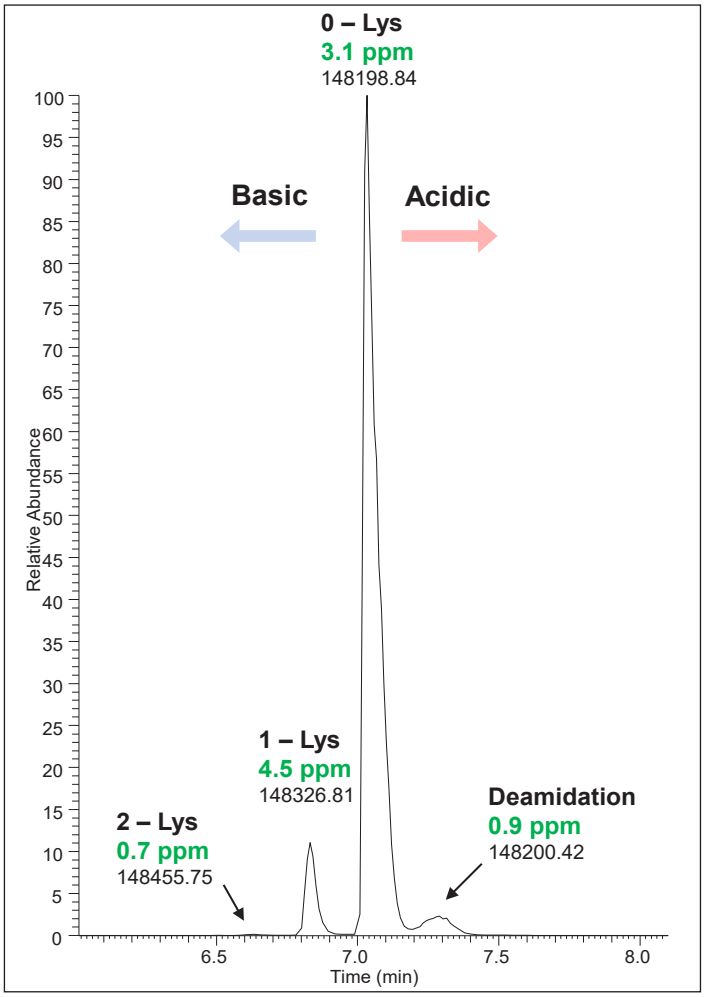

Figure 1: Base peak electropherogram showing NISTmAb charge variants

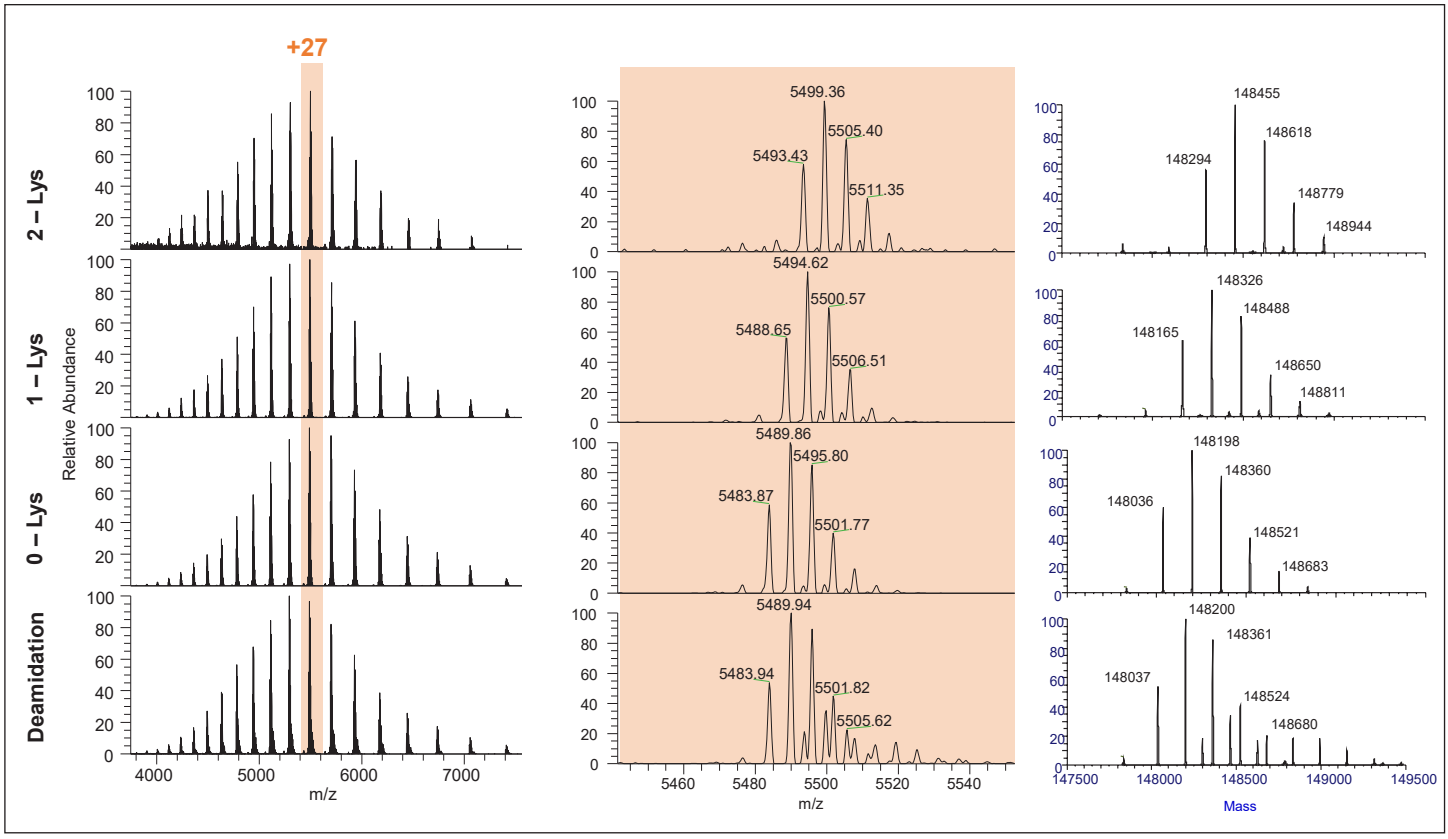

Figure 2: Raw mass spectra for NISTmAb charge variants (left), zoomed-in spectra showing prominent charge state (+27) for charge variants (middle) and deconvoluted mass spectra (right).

+256 Da observed for the two basic variants relative to the main variant together indicate that these peaks are consistent with one and two C-terminal lysine modifications respectively. The presence of a basic lysine residue increases the net positive charge on the protein thereby increasing its electrophoretic mobility as shown in the electropherogram in Figure 1. A mass shift of ~1Da observed between the acidic and main variants suggests that the acidic variant is a deamidated version of the main variant. Deamidation manifests as a separate peak from the main variant in the acidic region in ZipChip-MS data. Near isobaric species such as deamidations cannot be readily identified at the intact level without the use of this method. Each molecule contains an N-glycosylation site on both of its heavy chains, so glycan assignments are made as pairs of glycans which yield the masses observed. Differentiating G1F/G1F from G0F/G2F is not possible at the intact molecular level. ZipChip separation coupled with HRAM capability of the Orbitrap Exploris 240 mass spectrometer allows for confident identification of several different proteoforms of the NIST mAb. The results are summarized in Table 1. As shown, glycoforms of the main, acidic and basic charge variants can be easily identified despite large differences in relative abundances of these species.

Monitoring stability of therapeutic proteins. The same generic ZipChip-MS charge variant analysis methodology can also be used to monitor the extent of sample degradation over time. As described earlier, the selectivity offered by the ZipChip separation and the HRAM capabilities of the Orbitrap Exploris 240 together provide a quick and easy way to monitor critical quality attributes such as deamidations.

The stability of trastuzumab stored at -80°C for an extended period of time can be easily assessed by comparing its charge variants profile with that of the original drug product. Figure 3 shows the base peak electropherogram of trastuzumab drug product (left) and comparison of the deconvoluted mass spectra of the main variant and acidic variant 1 (right). A ~1 Da shift in masses of the acidic variant 1 with respect to the main variant, together with the migration order of the peaks suggests that acidic variant 1 is consistent with deamidation.

	Modification	Average Mass (Da)	Theoretical Mass (Da)	Mass Error (ppm)	Intensity	Relative Abundance
Main (0 – Lys)	1xA2G0F,1xA2G1F	148198.84	148199.30	3.1	6.10E+08	100.00%
	1xA2G0F,1xA2G2F	148360.44	148361.44	6.8	5.00E+08	82.01%
	2xA2G0F	148036.66	148037.16	3.4	3.61E+08	59.19%
	1xA2G1F,1xA2G2F	148522.14	148523.58	9.7	2.33E+08	38.19%
	1xM5,1xA2S1G1F,2xLys,1xGlycation	148844.45	148842.94	10.2	2.85E+07	4.67%
	1xA2G0F,1xA1G0F	147833.27	147833.97	4.7	1.84E+07	3.02%
Basic 2 (1 – Lys)	1xA2G0F,1xA2G1F,1xLys	148326.81	148327.47	4.5	6.26E+07	10.27%
	1xA2G0F,1xA2G2F,1xLys	148488.42	148489.61	8.0	4.96E+07	8.13%
	2xA2G0F,1xLys	148165.14	148165.33	1.3	3.78E+07	6.21%
	1xA2G1F,1xA2G2F,1xLys	148650.42	148651.76	9.0	2.16E+07	3.54%
	1xA2G0F,1xA1G0F,1xLys	147961.45	147962.14	4.6	3.30E+06	0.54%
Basic 1 (2 – Lys)	1xA2G0F,1xA2G1F,2xLys	148455.75	148455.65	0.7	9.23E+05	0.15%
	1xA2G0F,1xA2G2F,2xLys	148618.44	148617.79	4.4	7.37E+05	0.12%
	2xA2G0F,2xLys	148294.50	148293.51	6.7	5.43E+05	0.09%
	1xA2G1F,1xA2G2F,2xLys	148780.58	148779.93	4.4	3.34E+05	0.05%
	2xA2G2F,2xLys	148944.11	148942.07	13.7	9.86E+04	0.02%
	1xA2G0F,1xA1G0F,2xLys	148091.31	148090.31	6.8	3.59E+04	0.01%
Acidic (Deamidation)	1xA2G0F,1xA2G1F,1xDeamidation (N)	148200.42	148200.29	0.9	2.14E+07	3.51%
	1xA2G0F,1xA2G2F,1xDeamidation (N)	148361.33	148362.43	7.4	1.82E+07	2.98%
	2xA2G0F,1xDeamidation (N)	148037.73	148038.14	2.8	1.14E+07	1.87%
	1xA2G1F,1xA2G2F,1xDeamidation (N)	148524.02	148524.57	3.7	8.72E+06	1.43%
	1xA2G0F,1xA1G0F,1xDeamidation (N)	147835.23	147834.95	1.9	9.08E+05	0.15%

Table 1: Identification of various NISTmAb proteoforms along with their abundance relative to the most abundant proteoform.

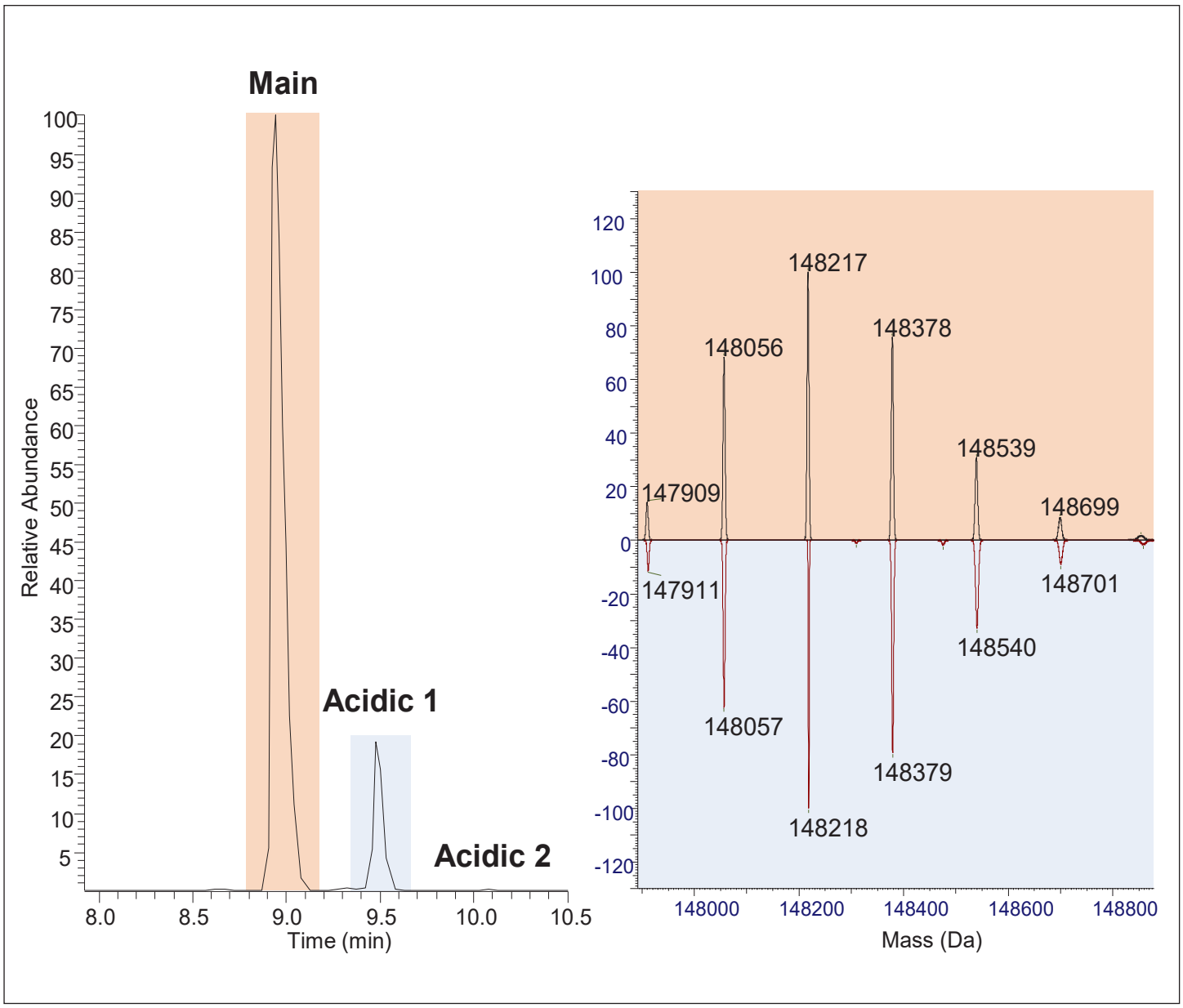

Figure 3: Base peak electropherogram showing charge variants for trastuzumab (left) and comparison of the deconvoluted mass spectra of the main variant and acidic variant 1 (right)

As seen from Figure 4, the older sample shows a much higher relative abundance for acidic variants compared to the original drug product. For example, relative abundance of acidic variant 1 is 24% in the older sample and only 13% in the drug product. The same trend is observed in case of acidic variant 2 (0.7% for older sample v/s 0.2% for the drug product). The MS data for acidic variants provide confirmation that the acidic species observed in both samples are identical and are consistent with deamidation. This suggests that deamidation is one of the major degradation pathways for trastuzumab upon prolonged storage at low temperatures.

CONCLUSION

The ZipChip-MS platform is a powerful tool for comprehensive characterization of biotherapeutics. The ZipChip native antibodies method can perform high-resolution charge variant separations coupled with native MS detection. The superb mass accuracy and high sensitivity of the Orbitrap Exploris 240 mass spectrometer enables detection and confident identification of proteoforms even with low relative abundance. The mass spectra of baseline resolved charge variants provide a means for in-depth characterization of biotherapeutics within short

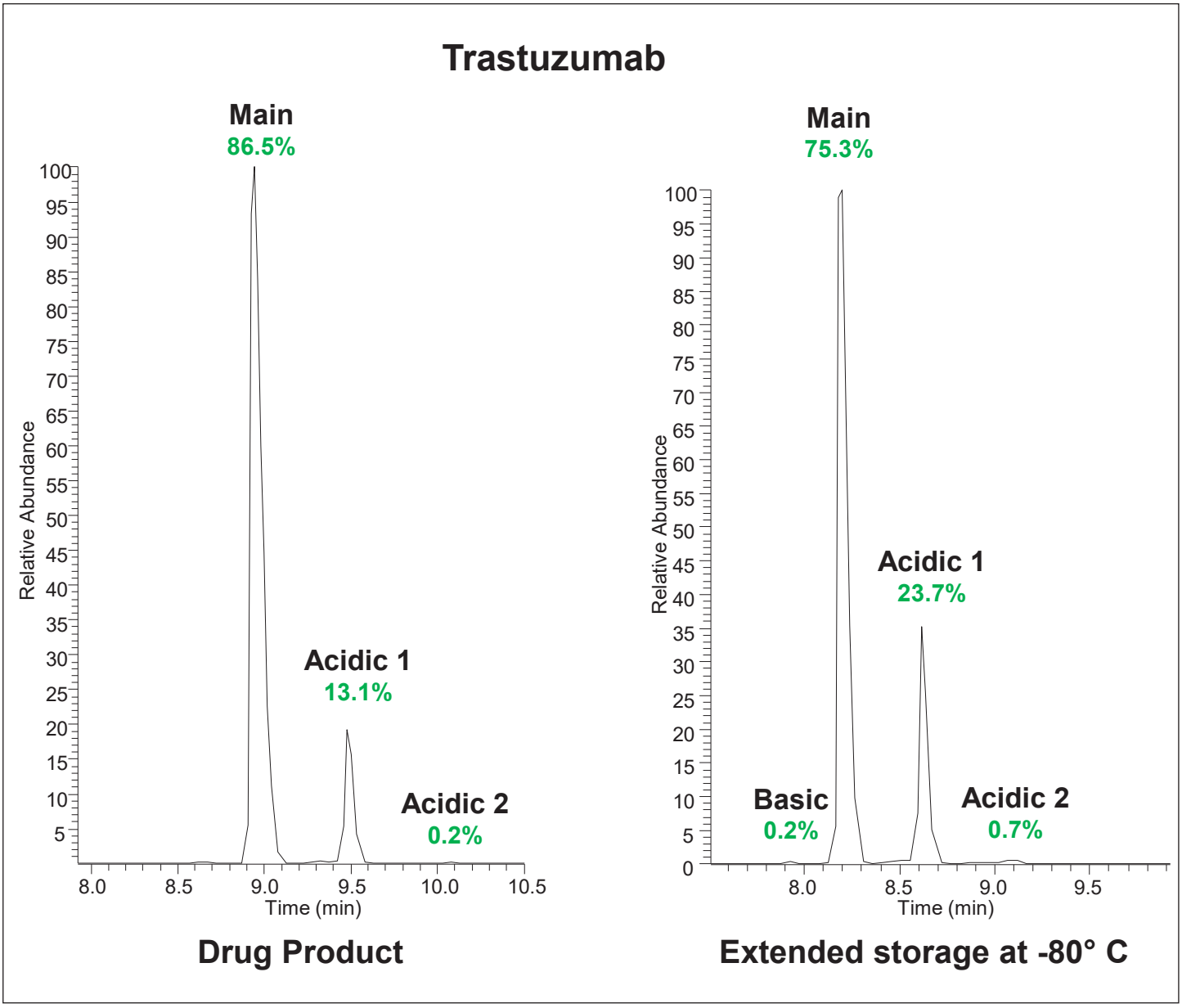

Figure 4: Base Peak Electropherograms showing trastuzumab drug product (left) and trastuzumab after extended storage at -80 °C (right)

analysis times. This generic method is applicable for analysis of several monoclonal antibodies without the need of laborious method development and can be readily adopted for different workflows involving mAb characterization such as monitoring product degradation characteristics.

REFERENCE: 1. 908devices. https://908devices.zendesk.com, 2021.

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