Application Note

Unraveling Structural Complexities of ADCs with Maurice Platforms

Introduction

Standalone monoclonal antibodies (mAbs) are already structurally complex, but their complexity increases significantly when conjugated with a linker and cytotoxic drug (payload) to form antibody-drug conjugates (ADCs). As a result, ADCs require stringent monitoring of their Critical Quality Attributes (CQAs) during various stages of development and manufacturing. The presence of charge species is a CQA and is subject to high variability due to the structure of ADCs. Ion exchange chromatography (IEX) is a common technique for analyzing charge variants, but it can be significantly influenced by surface charge distribution and hydrophobic interactions with the stationary phase of the column—both of which are affected by the presence and nature of the payload.[1] In contrast, imaged capillary isoelectric focusing (icIEF) separates analytes based solely on their isoelectric point (pI), making it less susceptible to effects from the linker-drug and thus better suited for ADC charge heterogeneity analysis.

This application note demonstrates how the **Maurice™ system** is uniquely positioned to address key challenges of ADC characterization, enabling high-resolution charge heterogeneity analysis with icIEF along with CE-SDS analysis for ADC size and purity.

Specifically, this study highlights how:

- pH labile linkers do not degrade with repeated analysis because of the Maurice system's On-Board Mixing (OBM) capability, which automatically mixes reagents and samples right before injection
- The 458 nm fluorescence filter on **Maurice** allows the detection of certain fluorescent ADC payloads
- Low fragmentation and high throughput are achieved with the Maurice CE-SDS method

Data are presented for Herceptin® (trastuzumab), a therapeutic mAb, and two trastuzumab-based ADCs: Kadcyla® (ado-trastuzumab emtansine), which connects trastuzumab and mertansine (DM1) via a non-cleavable thioether linker (attached to lysine residues of the mAb);[2,3] and Enhertu® (fam-trastuzumab deruxtecan), which uses a cleavable tetrapeptide linker to conjugate the topoisomerase I inhibitor deruxtecan to cysteines on the mAb.[4] These differences in conjugation between ADC types significantly influence their chemistry and stability, which can be monitored accurately with capillary electrophoresis.

Materials and Methods

TABLE // 1

All materials used in this study are listed in **Table 1.**

Material	Vendor	Catalog #
Herceptin (Trastuzumab)	Genentech	NA
Kadcyla	GlaxoSmithKline	NA
Enhertu	Bristol Myers Squibb	NA
Maurice OBM System	Bio-Techne	**090-000**
Maurice cIEF Cartridge		**PS-MC02-C**
Maurice cIEF Method Development Kit*		**PS-MDK01-C**
Maurice Turbo CE-SDS Application Kit*		**PS-MAK01-TS**
Maurice Turbo CE-SDS Cartridge		**PS-MC02-TS**
Sigma pI Markers	Sigma-Aldrich	77866, 89268
NDSB195		480001
Iminodiacetic Acid (IDA)		56781
Acetic Acid		A6283

Table 1. Materials and reagents used in this study.

*The Maurice cIEF Method Development and Turbo CE-SDS Application Kits contain all the necessary reagents for icIEF and CE-SDS analysis on Maurice, respectively.

Standard icIEF Method

The monoclonal antibody and ADC samples were prepared at a final concentration of 0.2 mg/mL in an ampholyte solution containing 4% Pharmalytes 8-10.5 and 3-10 (3:1), 400 mM NDSB195, 5 mM IDA, 5 mM arginine, and pI markers 5.5* and 9.5*. The samples were loaded onto a Maurice instrument along with the Maurice cIEF cartridge, with running conditions set at focusing for 1 minute at 1500 V, then 10 minutes at 3000 V. For data acquisition with native fluorescence (NF), a fluorescence exposure series of 5, 10, and 15 seconds was used and for detection with the 458 nm filter, a fluorescence exposure series of 15 and 25 seconds was used. All data were analyzed using Compass for iCE Software v4.4.

* The fluorescent pI markers from Sigma were used due to their ability to fluoresce in both the NF and 458nm filter channels.

OBM Method to Maintain Sample Stability

The OBM method on Maurice was used to analyze Enhertu, which is susceptible to pH-induced isoforms. In this workflow, 25 µL of Enhertu sample was mixed with 100 µL of pre-prepared ampholyte solution in the 6 mL vial (see above method for ampholyte components) directly within Maurice's autosampler. This approach was chosen to minimize sample handling and prevent degradation of Enhertu, a pH-labile ADC.

Optimized ADC-Specific icIEF Method

For Maurice instruments without OBM, an icIEF method was optimized specifically for unstable ADC molecules. While largely similar to the standard icIEF method, modifications included the addition of 20 mM acetic acid to the ampholyte solution containing 3% Pharmalytes 8-10.5 and 3-10 (3:1), 400 mM NDSB195, 5 mM IDA, 5 mM arginine, and pI markers 5.5 and 9.5. The autosampler temperature was reduced to 4°C to minimize degradation.

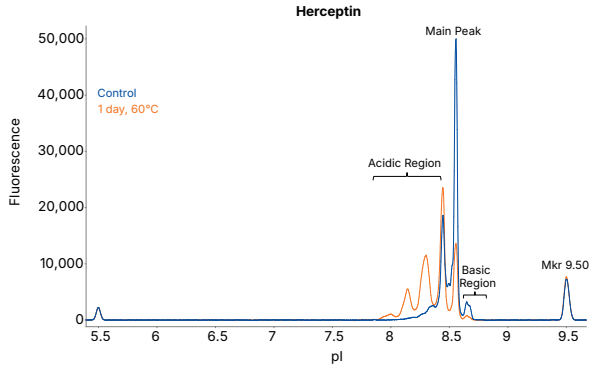
Results

In addition to being the gold-standard for charge heterogeneity analysis, icIEF has long been known to be a stability-indicating method for a variety of molecules.[5] **Figure 1** demonstrates the power of icIEF by comparing profiles of native samples with stressed samples for each of the three molecules (Herceptin, Kadcyla, and Enhertu). Marked differences between the charge heterogeneities of native and stressed sample are observed, with degradation confirmed after samples were incubated at 60°C for one day. An increase in acidic species is observed in all three degraded molecules, with bar graphs quantifying this difference (see **Figure 1 in the Appendix**).

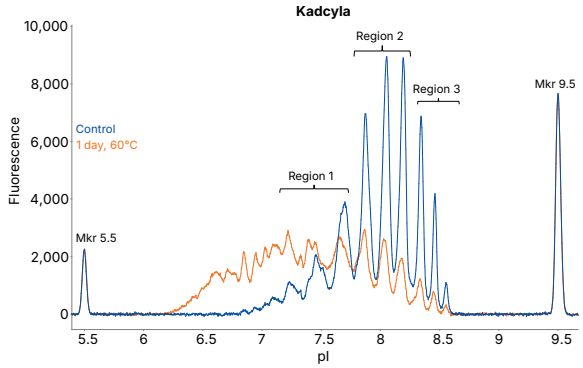
Reproducibility of the icIEF method was also assessed for all three molecules. **Table 2** shows quantitative data for the percent peak area (%PA) values for Herceptin, Kadcyla, and Enhertu under native conditions. While both Herceptin and Kadcyla showed excellent reproducibility with %RSD values ≤1.72, Enhertu showed far more variability, as evidenced by the high %RSD values. These results were unsurprising, however, considering Enhertu has a pH-labile linker that likely underwent degradation in the ampholyte mixture.

FIGURE // 1

1A



1B



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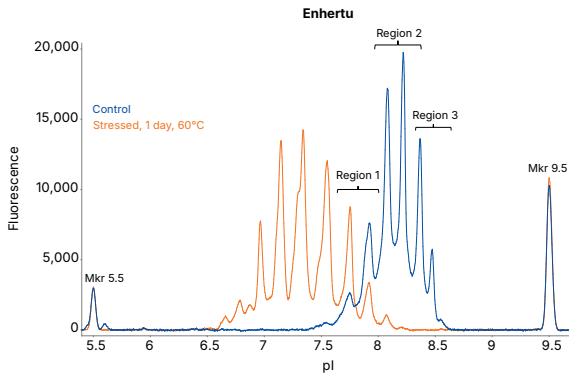


Figure 1. Analysis of (A) Herceptin, (B) Kadcyla, using the standard icIEF method and (C) Enhertu using the ADC icIEF method on Maurice. Heat-stressed variants of all three molecules show degradation (increase in acidic species) after incubation at 60°C for one day.

TABLE // 2

Percent Peak Area (n=3)			
Herceptin	Acidic Region	Main Peak	Basic Region
Average	36.2	58.3	5.5
%RSD	**0.78**	**0.43**	**1.72**
Kadcyla	Region 1	Region 2	Region 3
Average	30.2	53.1	16.6
%RSD	**0.16**	**0.09**	**0.28**
Enhertu	Region 1	Region 2	Region 3
Average	11.2	71.3	17.5
%RSD	**27.00**	**1.84**	**24.45**

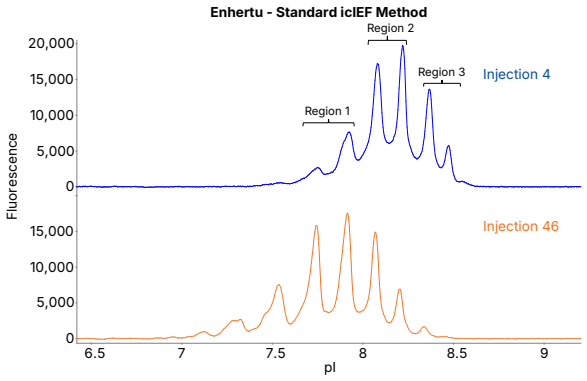
Table 2. Reproducibility assessment of the standard icIEF method for the analysis of Herceptin, Kadcyla, and Enhertu. Results were highly reproducible for Herceptin and Kadcyla but were poor for Enhertu because of its pH labile linker.

The OBM capability of Maurice is specifically designed to allow for sample-ampholyte mixing right before injection into the system. This is an automated process that minimizes the duration of the sample's interaction with the ampholyte. It is particularly useful for unstable samples but provides added advantages of reduced hands-on time and user-to-user-variability.[6] In this study, OBM was leveraged for the analysis of all three molecules. While results for Herceptin and Kadcyla continued to be highly reproducible (data not shown), the most notable difference was observed for Enhertu. **Figures 2A** and **2B** show a comparison of Enhertu analyzed with the standard icIEF method and the Maurice OBM method, respectively. In turn, each figure shows charge profiles of the sample resulting from analysis over a batch of 49 injections. Later injections in the batch are prone to pH-induced degradation with the standard icIEF method, as shown in **Figure 2A**, while they remain relatively stable when analyzed with the Maurice OBM method, as shown in Figure **2B**. **Table 3** shows the quantitative analysis of these results (%PA), further corroborating the significant improvement afforded by the OBM method.

Figure 2. A comparison of the standard icIEF method (A) and the Maurice OBM method (B) for the analysis of Enhertu. The standard icIEF method involved mixing the sample and ampholyte mixture manually, resulting in longer interaction between the two, ultimately causing pH-induced degradation as evidenced by later injections. In contrast, later injections in the batch remained stable without any indication of pH-induced degradation when analyzed with on-board mixing.

FIGURE // 2

2A



2B

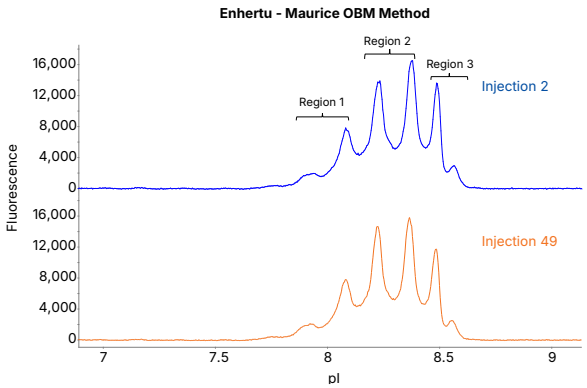


TABLE // 3

Enhertu Percent Peak Area (n=49)						
	Standard icIEF Method			Maurice OBM Method		
	Region 1	Region 2	Region 3	Region 1	Region 2	Region 3
Average	38.9	47.5	13.6	22.5	57.5	20.0
%RSD	**43.12**	**22.05**	**49.58**	**4.59**	**0.98**	**6.29**

Table 3. Quantitative results of icIEF analysis with Enhertu using the standard and OBM methods. Data reproducibility improved dramatically for Enhertu when analyzed with the Maurice OBM method, as suggested by the %RSD values of ≤6.29, brought down from **24.45** when analyzed with the standard icIEF method.

The ADC-optimized icIEF method was also successful in curbing pH-induced degradation for later injections over a batch. **Figure 3** shows charge profiles of Enhertu over a batch of 49 injections, captured earlier and later in the run. The profiles remained consistent throughout injections; a conclusion further supported by quantitative results in **Table 4**.

FIGURE // 3

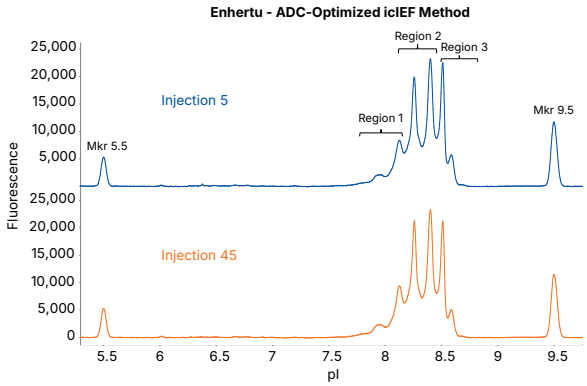


Figure 3. Evaluation of sample stability across multiple injections using the ADC-optimized icIEF method. Charge profiles for Enhertu remained consistent between early (injection #5) and late (injection #45) runs, demonstrating that the ADC-optimized icIEF method preserves sample integrity throughout the batch. The absence of degradation in the bottom electropherogram indicates that the method effectively minimizes pH-induced instability during analysis.

TABLE // 4

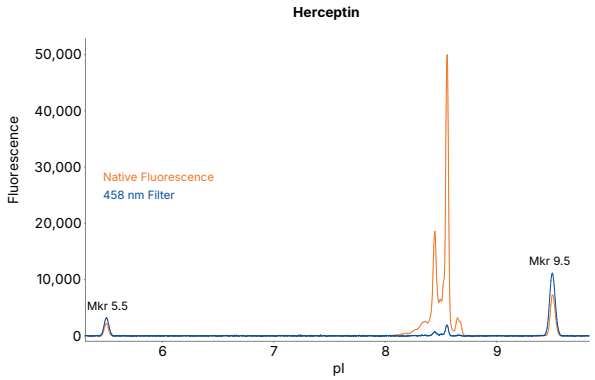
Enhertu Percent Peak Area (ADC-Optimized Method, n=49)			
	Region 1	Region 2	Region 3
Average	20.1	55.6	24.3
%RSD	2.49	0.64	2.53

Table 4. Quantitative results of icIEF analysis with Enhertu using the ADC-optimized icIEF method. Results were consistent over a batch of 49 injections, as shown by the %RSD values of ≤2.53. These findings suggest that the optimized method was successful in maintaining stability of the pH labile linker found in Enhertu.

Leveraging the 458 nm filter in the Maurice system proved to be another useful tool to understand the charge distribution of Enhertu. The 458 nm filter enables selective fluorescence detection of species that absorb light near that wavelength, particularly useful for ADCs like Enhertu where the linker or payload exhibits fluorescence in this range.
The fluorescence mode enables the detection of unknown peaks, as demonstrated in a study by Leng *et al.*[7] **Figures 4A** and **4B** offer a contrast between the profiles of Herceptin and Enhertu, respectively, when analyzed with the 458 nm filter. Herceptin does not emit fluorescence at 458 nm, whereas the profile of Enhertu closely mirrors its native fluorescence trace, thus suggesting that the payload or linker is fluorescently active at 458 nm. Furthermore, Enhertu has a homogenous DAR (drug-antibody ratio) of 8 around all the cysteines of the hinge region.

FIGURE // 4

4A



4B

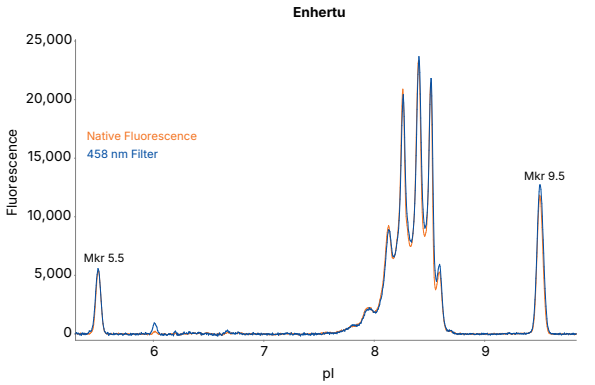


Figure 4. icIEF analysis of Herceptin (A) and Enhertu (B) using the 458 nm filter in Maurice. An overlay of profiles of each molecule analyzed with native fluorescence (280 nm) and the 458 nm filter are shown. Herceptin does not emit fluorescence at 458 nm, whereas Enhertu shows highs fluorescence at that wavelength.

ADC Analysis with Maurice CE-SDS

Analyzing ADCs with CE-SDS allowed for the resolution of individual subunits based on size, enabling the detection of variants such as fragments and conjugated species. With the Maurice Turbo CE-SDS cartridge, results per injection were available in 6 or 10 minutes, depending on whether the samples were reduced or intact.

Figure 5A shows stacked profiles of Herceptin and Kadcyla under non-reduced conditions, with the expected intact peaks.

Figure 5B shows stacked profiles of the two molecules under reduced conditions, where well-resolved light chain (LC) and heavy chain (HC) peaks are seen along with non-glycosylated heavy chain (NGHC).

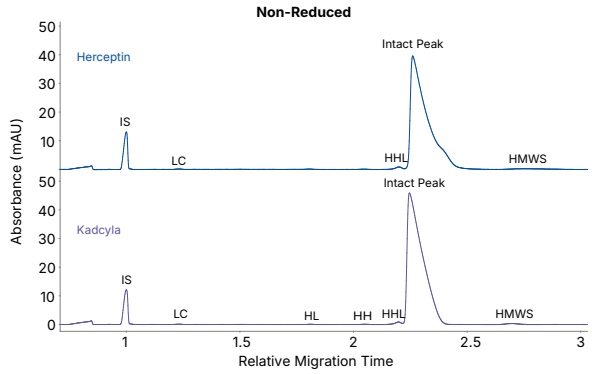
Figure 5C shows overlaid profiles of Enhertu, under non-reduced and reduced conditions.

The only notable difference between the two Enhertu profiles was the presence of two high molecular species detected under non-reduced conditions that align with the heavy-light (HL), heavy-heavy (HH) and (HHL) variants of Herceptin. The high degree of comparability in CE-SDS profiles for Enhertu under reduced and non-reduced conditions is likely due to its site-specific conjugation, where drug-linker molecules are attached to the hinge-region cysteine groups. The four hinge region disulfide bonds are replaced with eight drug conjugation sites, and this new intact structure is not covalently bonded like the original antibody. Though Enhertu is highly homogeneous with an average DAR of 8, some literature reports a DAR of 7.7, indicating remaining disulfide variants.[8] This offers a plausible explanation for the comparable migration behavior observed in both CE-SDS modes but with 3% more high molecular weight species (HMWS) for non-reduced CE-SDS.

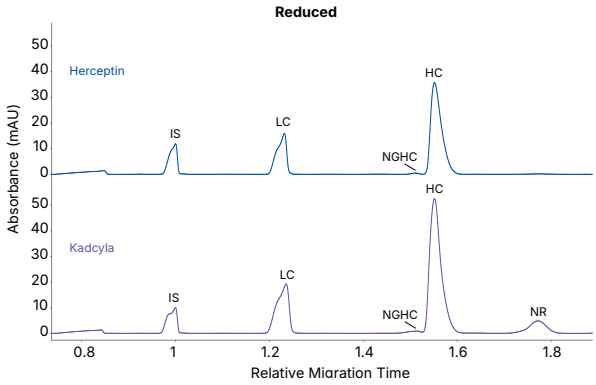
Tables 5, 6, and **7** show quantitative results of CE-SDS analysis. For analysis of stressed samples of Kadcyla and Enhertu, see **Figure 2** in the Appendix.

FIGURE // 5

5A



5B



5C

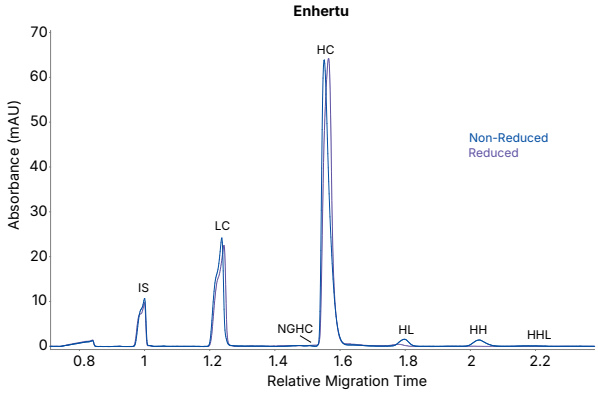


Figure 5. CE-SDS analysis of Herceptin and Kadcyla under non-reduced (A) and reduced conditions (B), and analysis of Enhertu under non-reduced and reduced conditions (C) on Maurice. For Herceptin and Kadcyla, the intact peak is seen under non-reduced conditions along with other minor peaks, while the LC, HC, and NGHC are detected under reduced conditions. In contrast, Enhertu has similar profiles under both non-reduced and reduced conditions. (HMWS: high molecular weight species; NR: non-reduced peak).

TABLE // 5

Percent Peak Area (Non-Reduced)							
Herceptin (n=3)							
	LC	HC	HL	HH	HHL	Intact	HWMS
Average	0.3	0.0	0.2	0.1	2.0	96.7	0.6
%RSD	-	-	-	-	2.40	0.01	-
Kadcyla (n=3)							
	0.1	0.0	0.1	0.1	0.8	98.3	0.6
	-	-	-	-	-	0.10	-

Table 5. Percent peak area (%PA) values for Herceptin and Kadcyla resulting from non-reduced CE-SDS analysis. %RSD values are calculated for peaks with %PA >1 and demonstrate the reproducibility of this method.

TABLE // 6

Percent Peak Area (Reduced)					
Herceptin (n=3)					
	LC	HC Fragments	NGHC	HC	NR
Average	32.33	0.00	0.60	66.63	0.47
%RSD	0.15	-	-	0.07	10.10
Kadcyla (n=3)					
	29.5	0.0	1.2	61.6	7.8
	0.16	-	4.04	0.08	0.61

Table 6. %PA results for Herceptin and Kadcyla obtained from reduced CE-SDS analysis. %RSD values are reported for peaks with %PA>1%, highlighting the method's reproducibility.

TABLE // 7

CESDS Turbo	Percent Peak Area – Enhertu (n=3)					
Non-Reduced						
Enhertu NR	LC	NGHC	HC	HL	HH	HHL
Average (3N)	30.9	0.4	65.0	1.8	1.8	0.2
%RSD	0.15	-	0.13	0.00	2.67	-
Reduced						
Average (3N)	30.63	0.47	68.20	-	-	-
%RSD	0.15	-	0.12	-	-	-

Table 7. %PA results for Enhertu and non-reduced and reduced conditions.

Conclusion

Certain ADC molecules can be especially challenging to analyze due to the inherent instability of their structural components. Using Trastuzumab and Trastuzumab-based ADC as examples, this app note demonstrated how icIEF and CE-SDS methods on Maurice provide critical information on these complex biotherapeutic molecules. Maurice icIEF's native fluorescent mode enabled the detection of charge variant changes in stressed samples, while its on-board mixing (OBM) capability proved critical in preserving sample integrity by minimizing pH-induced degradation during analysis. The 458 nm fluorescence filter on Maurice offered an additional layer of information, confirming the presence of fluorescent payload-linked species in Enhertu.

Complementary CE-SDS data further validated the structure and expected subunit profiles of the ADCs. By combining high-resolution charge and size-based separation with customizable detection modes, Maurice delivers a comprehensive, streamlined platform for detailed ADC analysis across a range of attributes. Additionally, the newer MauriceFlex platform enables charge variant fraction collection alongside icIEF and CE-SDS capabilities. This added capability allows for the downstream characterization of unknown ADC charge peaks using mass spectrometry methods. To learn more about Maurice and MauriceFlex systems, visit **bio-techne.com/maurice.**

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6. Application Note – Improving Charge Variant Analysis with Maurice Native Fluorescence

7. Leng, C., Sun, S., Lin, W., Pavon, J. A., Gennaro, L., Gunawan, R. C., Bu, X., Yang, T., & Li, S. (2024). Imaged capillary isoelectric focusing method development for charge variants of high DAR ADCs. *Analytica Chimica Acta*, 1251, 343176. https://doi.org/10.1016/j.aca.2024.343176

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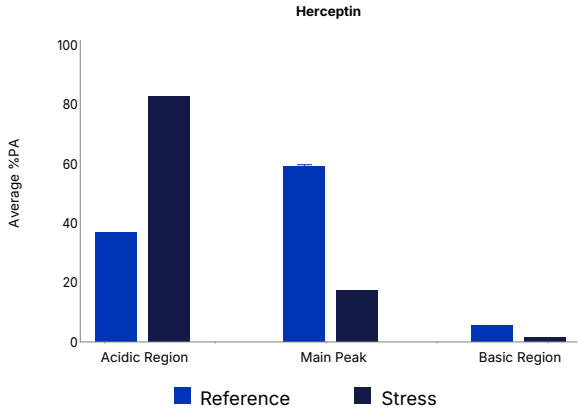
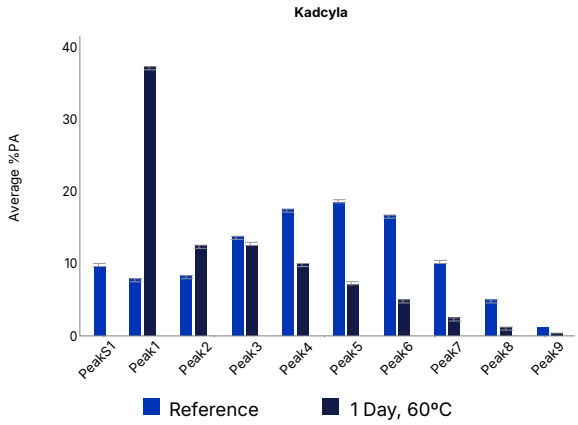
APPENDIX

Figure 1

1A



1B



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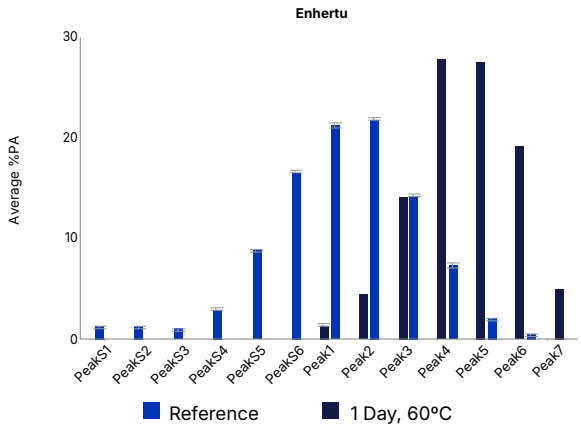
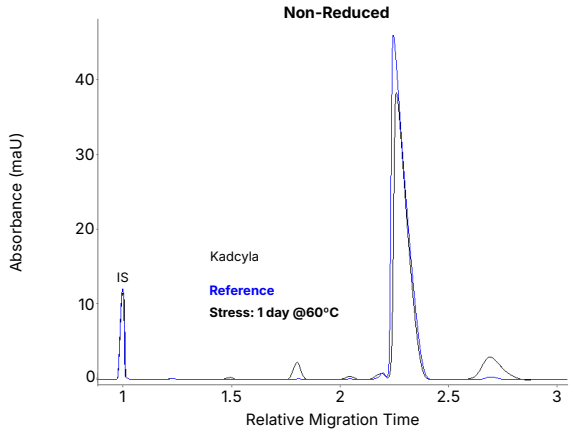


Figure 1. A graphical comparison of charge variants found in native and stressed samples of Herceptin (A), Kadcyla (B), and Enhertu (C). An increase in acidic peaks is observed for each molecule when subjected to temperature stress.

Figure 2

2A



2B

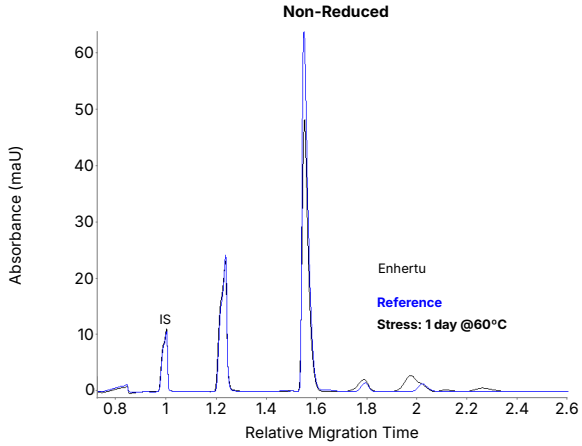


Figure 2. CE-SDS analysis of stressed samples of Kadcyla (A) and Enhertu (B) under non-reduced conditions. When subjected to temperature stress, new low molecular weight and high molecular weight (HMW) species are observed for Kadcyla, while an increase in HMW species are observed for Enhertu.