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WHITE PAPER

USE OF MAURICE CE-SDS IN ICH Q6B BASED BIOSIMILAR COMPARABILITY EXERCISES

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INTRODUCTION

The biosimilars market is a hugely significant sector of the biopharmaceutical industry with long-term, significant growth projections. The main attraction for manufacturers to produce biosimilars is the decreased time to market, owing to a more abbreviated pathway to regulatory approval. The route to market places strong emphasis on the analytical and characterization techniques used to investigate the structure of the molecule and, most importantly, side-by-side comparability exercises between the biosimilar and innovator products.

Both the EMA and FDA have set out clear guidelines for companies aiming to produce biosimilars, including the expectations for analytical investigation into structure[1-3]. In these documents, the EMA and FDA both cite ICH Q6B as the guideline for biosimilar testing[4] and highlight the need for using orthogonal techniques that enable cross-verification of data and conclusions from different methodologies. ICH Q6B specifies the areas that are to be investigated for assessing molecular structure at the level of primary (amino acid sequence, post translational modifications, structural characterization) and physicochemical structures (including charge profiles, molecular weight, spectral properties, secondary and tertiary structure, amongst others). ICH Q6B also details the need for impurity assessment of the product from both a product and process standpoint and covers this in the requirement for electrophoretic patterns, using gel electrophoresis as an example.

While no specific methods for analytical assessment of electrophoretic patterns are noted in the document, recommendations of analytical approaches are suggested to fulfil each expectation and are caveated by phrases such as "or other suitable procedures." A newer technique that improves upon the standard gel electrophoresis approach is capillary gel electrophoresis. In this technique, proteins within a sample are separated in a gel-filled capillary based on their electrophoretic mobility, which in turn is determined by each protein's charge and size. When subjected to an electric field, proteins of different sizes migrate at different velocities, and this migration is detected by methods such as UV absorbance, fluorescence, and mass spectrometry[5]. When compared with conventional slab gel electrophoresis, capillary gel electrophoresis offers several advantages; the run times are shorter, smaller proteins can be more easily separated, and analysis is done in real time. With the current pace of biotherapeutic development and manufacturing, considerable efforts are geared towards making such analytical methods more sophisticated and advantageous than ever before.

Maurice™ and Maurice S. are two of the platforms available for fully automated and highly quantitative capillary electrophoresis-sodium dodecyl sulphate (CE-SDS) analysis. The Maurice S. offers a fully automated CE-SDS platform, while Maurice allows for both CE-SDS and icIEF (imaged capillary isoelectric focusing) analyses. These instruments are designed to significantly reduce run times (as short as 25-35 minutes) while providing high-quality and reproducible results. Through UV absorbance (220 nm), high-resolution peaks are obtained for proteins ranging from 10 kDa to 270 kDa. Equipped with pre-assembled cartridges, the Maurice platform eliminates the cumbersome step of cutting capillaries. Data is rapidly generated once the samples are loaded into the system and the batch is set up, allowing method development within a day.

Importantly, in addition to the Compass for iCE software, Maurice and Maurice S. can be used with Waters™ Empower® 3 Chromatography Data Software, using the Maurice Empower® Control Kit. Such software versatility ensures the instruments' ease of use and enables an easy transfer into GMP QC laboratory environments. The studies presented here demonstrate the utility of Maurice platforms in biosimilar development through the characterization of certain monoclonal antibodies, heavily glycosylated species, and PEGylated species.

MATERIALS AND METHODS

The following materials were obtained from Bio-Techne: Maurice CE-SDS PLUS Cartridge (PN PS-MC02-SP), CE-SDS Plus Sample Buffer (PN 046-567), 25X Internal Standard (PN 046-144), Conditioning Solution 1 (PN 046-014), Conditioning Solution 2 (PN 046-015), Separation Matrix (PN 046-386), Wash Solution (PN 046-569), Top Running Buffer (046-384), Bottom Running Buffer (PN 046-385). The following materials were obtained from Millipore Sigma: 250 mM Iodoacetamide (PN I1149-5G), 14.2 M β-mercaptoethanol (PN 35602BID), PNGase F (PN G5166). Sialidase was procured from Ludger (PN E-S001).

For CE-SDS analysis, all samples were diluted to between 0.25-1 mg/mL with the 1X CE-SDS PLUS Sample Buffer in at least a 1:1 ratio, to a volume of 50 µL in 0.5 mL Eppendorf tubes. A vial of lyophilized 25X Internal Standard was dissolved in 240 µL 1X Sample buffer. 2 µL of this solution was added to each sample as a 10 kDa internal standard marker. For non-reduced samples, a 250 mM solution of iodoacetamide was prepared in ultra-pure water and 2.5 µL of this solution was added to each non-reduced sample. For reduced samples, 2.5 µL of β-mercaptoethanol was added to each sample.

Samples were vortexed, briefly centrifuged, and incubated at 70° C for 10 minutes. After incubation, samples were left at room temperature to cool for 5 minutes, before being transferred to 2 mL vials equipped with inserts and centrifuged at 1,000 RCF for 10 minutes. A Maurice CE-SDS PLUS Cartridge) equipped with the Top Running Buffer was used with the following reagents: Conditioning Solution 1, Conditioning Solution 2, Separation Matrix, Wash Solution, and the Bottom Running Buffer.

For de-N-glycosylation, samples were buffer exchanged into ammonium bicarbonate (50 mM), pH 8.4. PNGase F was added, and samples were incubated at 37° C overnight. Samples were then acidified to pH 3-4 and analyzed with CE-SDS following the method described above as appropriate for reduced or non-reduced samples. For de-sialylation, samples were diluted in 5X reaction buffer. To this solution, 1 µL of Sialidase solution was added and incubated at 37° C overnight. Samples were then acidified to pH 3-4 and analyzed via CE-SDS following the CE-SDS methodology as appropriate for reduced and non-reduced samples. All samples were analysed under default injection conditions using a Maurice S CE-SDS system. Default separation voltage used and separation time varied between 25 minutes and 45 minutes.

RESULTS

MONOCLONAL ANTIBODIES

Monoclonal antibodies (mAbs) make up the majority of biosimilars currently under development and on the market. They are multichain glycoproteins, composed of 2 heavy (H) and 2 light (L) chains linked via 16 disulfide bridges (for an IgG1). This complex bridging pattern across four separate protein chains leaves open the possibility of mispairing during biosynthesis. This can result in the formation of molecules composed of a lesser number of bridged chains, for example, 2 heavy chains linked to a single light chain. These malformed species constitute product-related impurities and therefore must be evaluated for their presence and quantity, which can be measured with CE-SDS.

FIGURE 1 shows results obtained from the CE-SDS analysis of Denosumab, a biologic commonly used for the treatment of bone loss. The data clearly indicate the presence of several minor peaks in the samples. These peaks have run positions consistent with light-heavy (LH), light-light-heavy (LLH) and heavy-heavy-light (HHL) mis-paired forms of the intact monoclonal antibody.

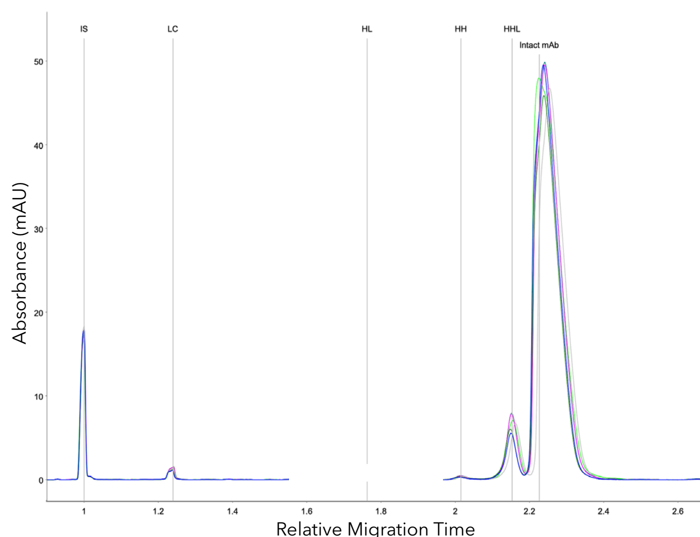

FIGURE 1: Stacked profiles of 6 non-reduced samples of the monoclonal antibody Denosumab. Data shows six overlaid samples with peaks for HHL, LLH, and LH forms.

Reduction of these samples prior to CE-SDS analysis results in these signals disappearing (**FIGURE 2**), consistent with their assignment as mismatched combinations of light and heavy chains. Two major peaks, identified as the light and heavy chains, are detected after reduction, along with a small signal of the non-glycosylated heavy chain. **FIGURE 3A** shows the same three peaks in the stacked traces for three reduced Denosumab samples. Treatment of these reduced samples with the enzyme PNGase F, which releases N-glycans from the heavy chain, results in a shift in profile and shows the deglycosylated heavy chain now running at the same position as the non-glycosylated heavy chain as seen in **FIGURE 3B**, thus confirming the original assignment in **FIGURES 2 and 3A**. Data from repeat analyses of a single sample demonstrated high reproducibility with rapid analysis times.

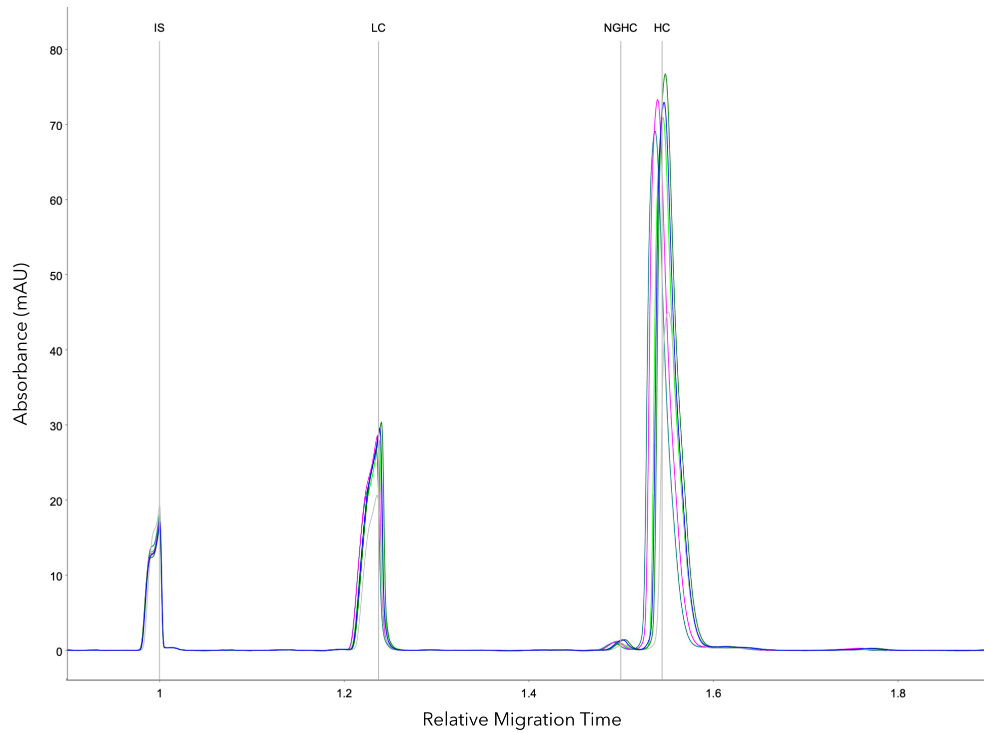

FIGURE 2: **Stacked profiles of the same six Denosumab samples in FIGURE 1 following reduction.** Peaks of the light chain, heavy chain, non-glycosylated heavy chain are shown.

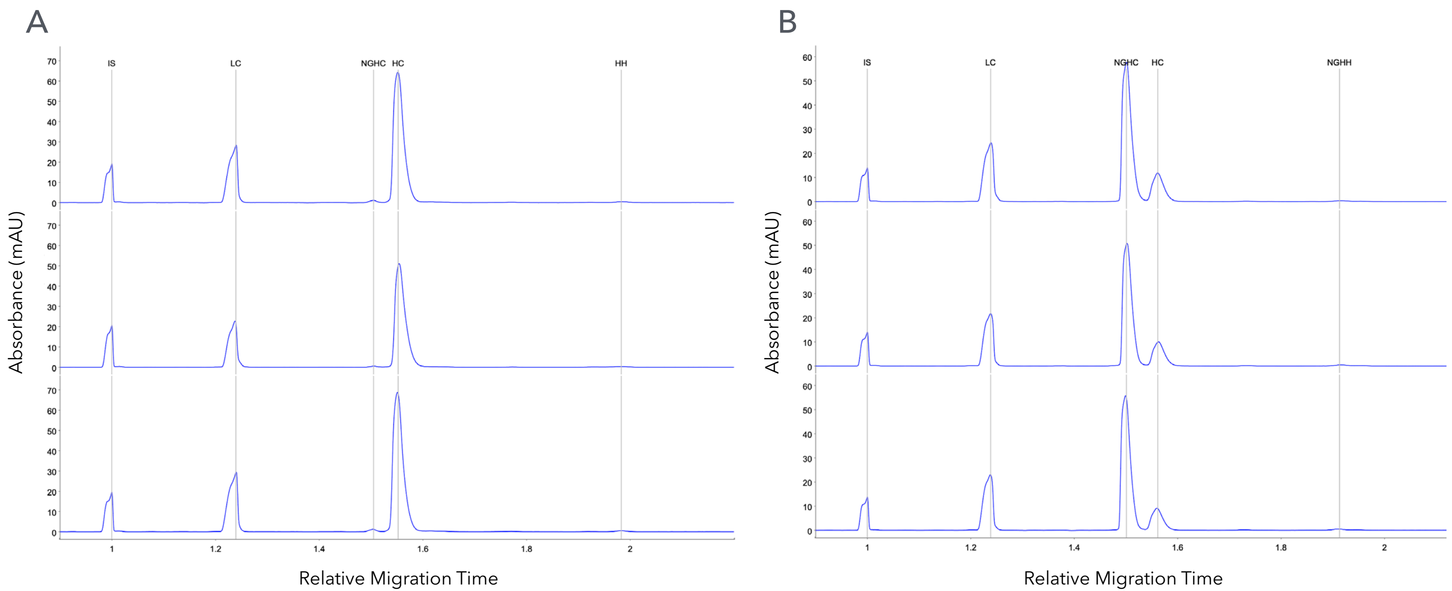

FIGURE 3: **Stacked traces for three reduced Denosumab samples. (A)** Peaks for the heavy, light, and non-glycosylated heavy chains are observed. **(B)** Treatment of the same three reduced samples with the PNGase F enzyme results in the de-glycosylation of the heavy chain, which shifts to the position typically seen for non-glycosylated heavy chains.

HEAVILY GLYCOSYLATED SPECIES

Heavily glycosylated molecules are structurally quite complex due to the nature and extent of glycosylation on the molecule. Glycan species also confer specific physico-chemical properties to the molecule due to their polyhydroxylated nature, large size, and the negative charges they carry due to the presence of sialic acid.

A good example of a heavily glycosylated molecule is Etanercept, which is used to treat certain autoimmune conditions like rheumatoid arthritis and psoriatic arthritis. Etanercept is a dimeric fusion protein composed of a human IgG1 Fc region linked to the extracellular domain of the human TNF receptor. The two monomeric subunits are linked via the Fc region disulfide bridges. Etanercept is heavily glycosylated, carrying 3 N-glycans and up to 14 O-glycans on each monomer unit. Because the presence of monomeric species (rather than homodimer) is likely during synthesis, samples need to be assessed and CE-SDS can be used to investigate this possibility.

Heavily glycosylated molecules will naturally have a high degree of heterogeneity because of varying glycosylation profiles. Differences in the degree of sialylation commonly seen for N-and O-glycans as well as lactosamine repeat units, fucosylation, and number of antennae are commonly observed. This heterogeneity does not preclude the use of CE-SDS but does result in a relatively broad peak that runs at an anomalously high mass position (because of the heterogeneity and the increased spatial volume of the molecule due to the relatively large hydrodynamic volume of the glycans).

FIGURE 4A shows the CE-SDS profile for three batches of intact Etanercept, where a single, broad peak can be seen in each case. There is no evidence of monomeric species in these data. This is confirmed by analysis of the same samples following reduction, which clearly shows the run position of the monomer units (**FIGURE 4B**). The reduction from dimer to monomer results in a sharpening of the peak due to the decrease in complexity in terms of glycosylation (from 6 N-glycosylation sites and up to 28 O-glycosylation sites per dimer to 3 N-glycosylation sites and up to 14 O-glycosylation sites per monomer) and a decrease in the overall heterogeneity of the sample.

In comparison, Matrix Assisted Laser Desorption Ionisation (MALDI) mass spectrometric analysis produces a clear dimer peak approximately at 121 kDa (**FIGURE 5A**) but also demonstrates the presence of a doubly charged signal approximately at 61 kDa, the same mass expected for the educed monomer. The monomer can be detected by MALDI-MS after reduction (**FIGURE 5B**). Similar to the CE-SDS data, the intact dimeric species is relatively broad due to the glycan heterogeneity and the reduced monomeric peak is somewhat sharper since this heterogeneity is now decreased due to the monomerization step. Treatment of Etanercept with PNGase F gives rise to both a lower mass and a sharper peak due to the loss of significant heterogeneity via the removal of N-glycans.

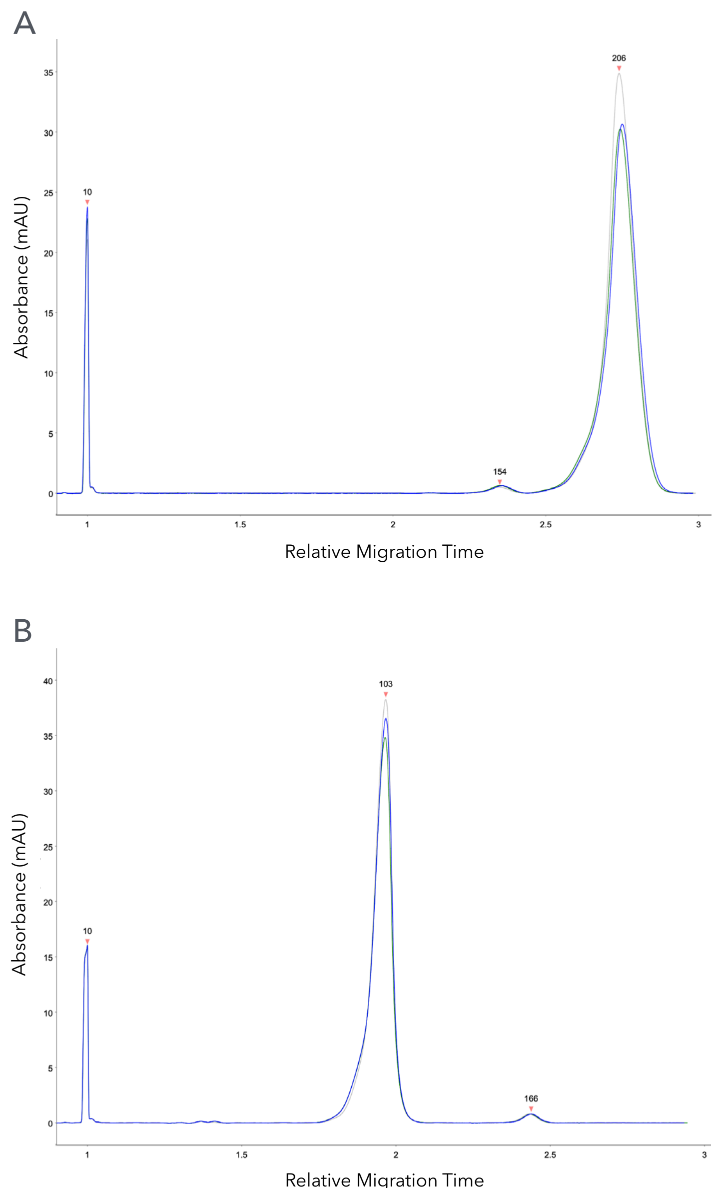

FIGURE 4: CE-SDS analysis of three batches of (A) non-reduced and (B) reduced Etanercept. Non-reduced Etanercept results in a single broad peak while no monomeric species are observed. For reduced Etanercept samples, a sharper peak is observed at the run position of monomeric species.

A few other components are also visible for both the reduced and non-reduced forms of Etanercept and some differences appear between the batches, information which is otherwise not visible in the untreated native state. This provides range information for peak profiles and, by extension, comparative sample properties for innovator and biosimilar samples, and allows an assessment of the degree of overlap between these ranges. The higher mass components are likely partially de-N-glycosylated Etanercept produced because of incomplete digestion by PNGase F. **(FIGURES 6A and 6B)**

A

121179

382.7

60876

% Intensity

Mass (m/z)

B

60852

6262.6

121448

% Intensity

Mass (m/z)

FIGURE 5: MALDI mass spectrum of one batch of (A) Etanercept in the native state and (B) reduced Etanercept. The intact dimer component can be seen at around 121 kDa. The signal at approximately 61 kDa is produced because of the dimeric component carrying 2 charges rather than 1, giving rise to a component of half the observed mass of the singly charged species. In the reduced species, the signal at around 61 kDa is the reduced monomeric mass.

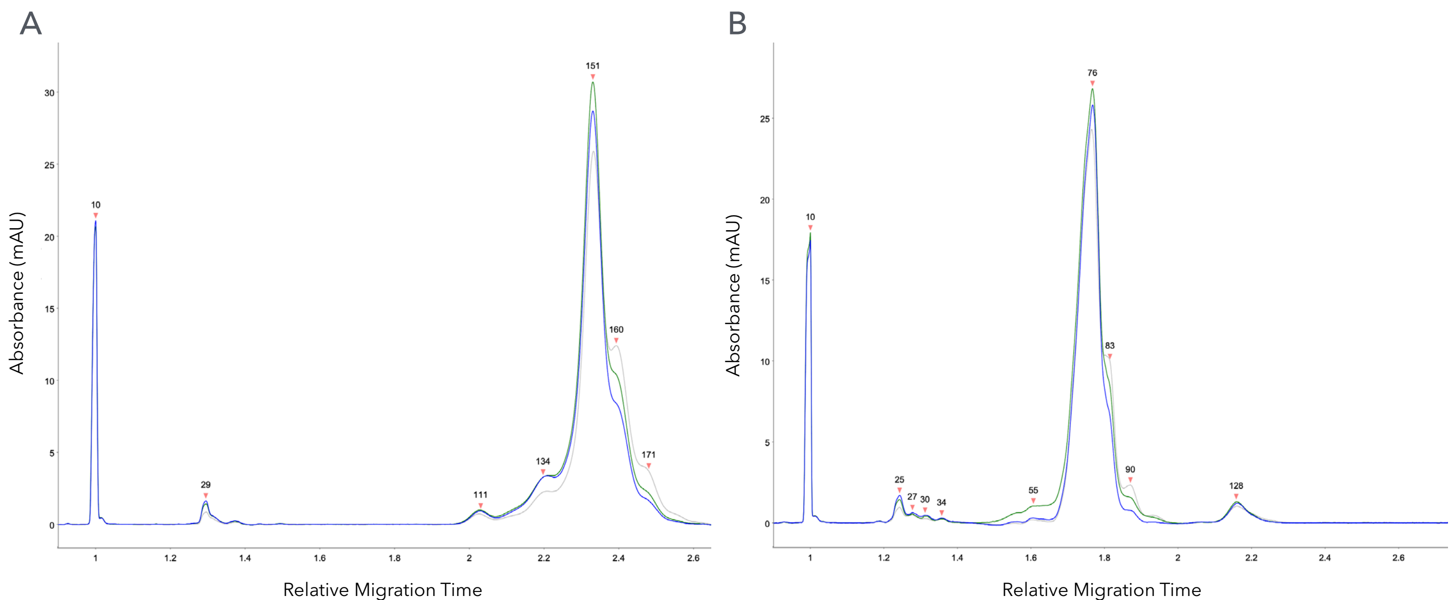

FIGURE 6: CE-SDS analysis of Etanercept following de-N-glycosylation of (A) non-reduced samples and (B) reduced samples using the enzyme PNGase F. The peaks of higher mass observed are attributed to partially de-N-glycosylated samples.

Treatment of de-N-glycosylated Etanercept with *Vibrio cholerae* neuraminidase (an enzyme that removes sialic acid residues that are in 2-3 or 2-6 linkage on both N-and O-glycans) decreases the peak broadening more as result of a further decrease in heterogeneity of the remaining glycans and gives more definition and clarity to differences that may exist between samples (**FIGURES 7A and 7B**). The slightly higher mass components on the edge of the main peak are now more resolved from the main peak because of decreased heterogeneity, supporting the idea that these represent partially de-N-glycosylated species. The decrease in peak profile heterogeneity by the action of *Vibrio cholerae* sialidase is mirrored in results obtained from icIEF of Etanercept (**Figure 8**). Here, the loss of sialic acid species carrying a negative charge causes a decrease in the overall net negative charge and decreases the heterogeneity seen. Separation is based on charge in icIEF rather than mass as with CE-SDS.

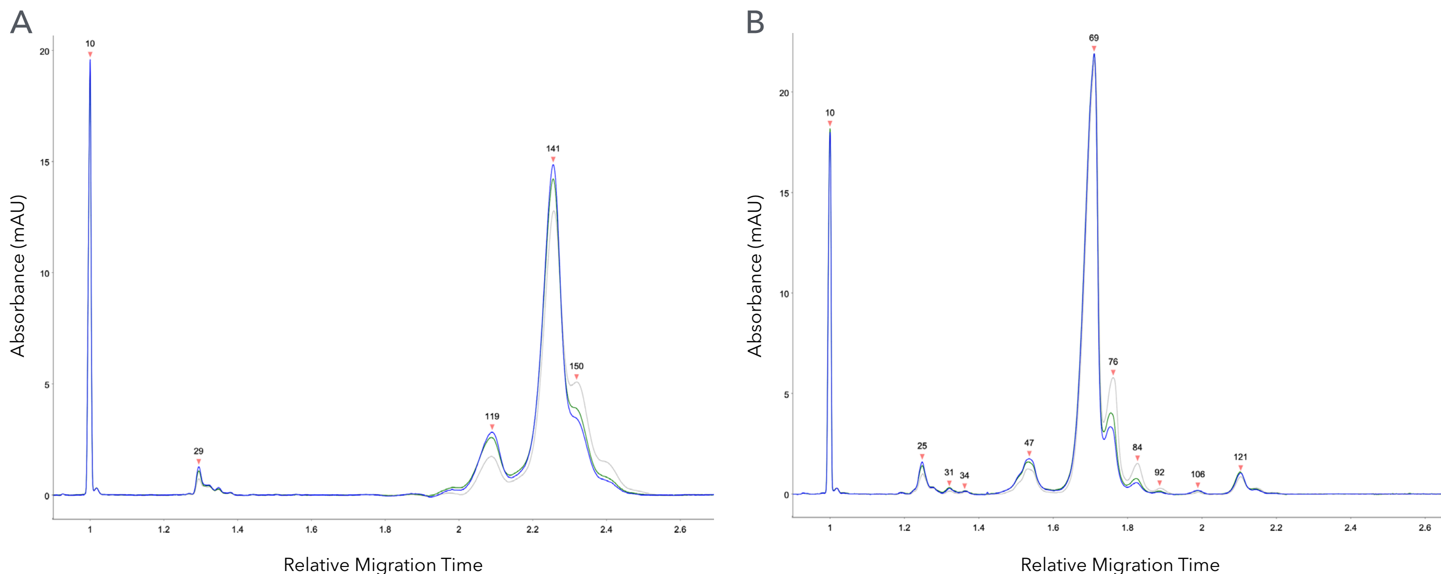

FIGURE 7: CE-SDS analysis of (A) non-reduced and (B) reduced Etanercept samples when subjected to de-N-glycosylation by PNGase F and desialylation using ***Vibrio cholerae*** neuraminidase. Desialylation resulted in a decrease in peak broadening and better separation, likely due to a decrease in glycan heterogeneity.

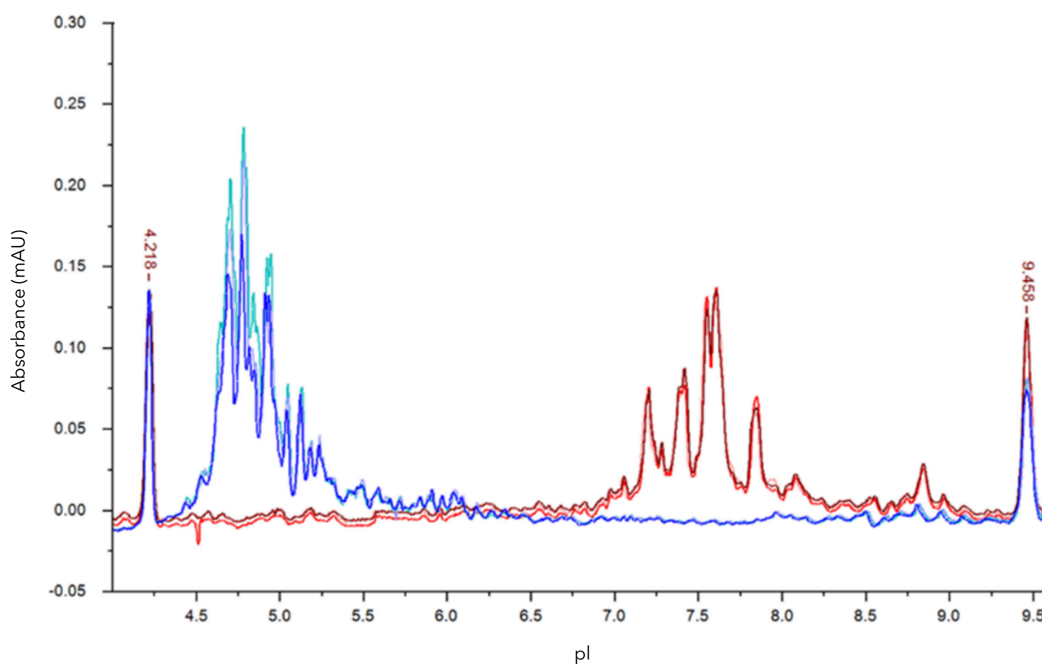

FIGURE 8: icIEF electropherogram of three batches of Etanercept. Etanercept prior to (blue) and following (red) treatment with Vibrio cholerae sialidase, where a decrease in the net negative charge is observed.

PEGYLATED SPECIES

Many biopharmaceutical drugs are manufactured as PEGylated species, where one or more PEG (polyethylene glycol) units of several kDa are attached to the protein chain (e.g. at the N-terminus). Common examples of PEGylated species are granulocyte colony stimulating factor (G-CSF) e.g. Neulasta® and human growth hormone (hGH) e.g. Somavert®. CE-SDS analysis of PEGylated species can be used to reveal the presence of under-PEGylated and/or non-PEGylated forms. **FIGURE 9** shows the CE-SDS analysis of a protein carrying a single PEG chain. The data demonstrate that for this sample, there is a clear signal for the PEGylated sample at 26 kDa and a signal at 17 kDa representing some non-PEGylated species. Data shown in **FIGURES 10 and 11** are from a different PEGylated sample where three separate batches of samples were analysed as received. The overlaid data demonstrate that the samples gave reproducible profiles. The small signal detected at ~52 kDa (**FIGURE 11**) can be highlighted for further analytical investigations and may represent over-PEGylation of a small percentage of the product.

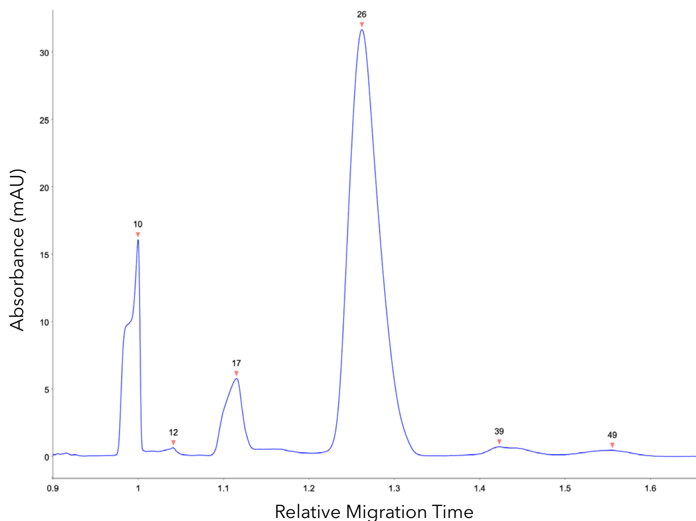

FIGURE 9: CE-SDS analysis of a PEGylated protein. A peak for the PEGylated sample is clearly observed at 26 kDa. A signal for non-PEGylated protein can also be seen.

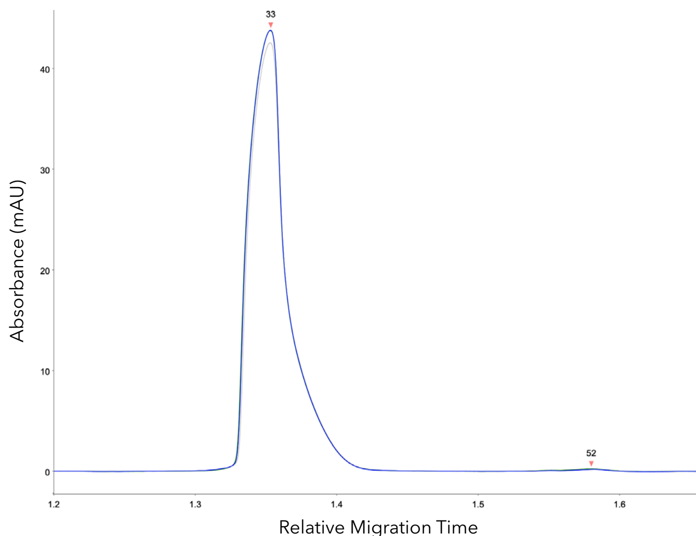

FIGURE 10: CE-SDS analysis of three separate batches of a PEGylated protein. The three batches overlay well, attesting to the method's reproducibility.

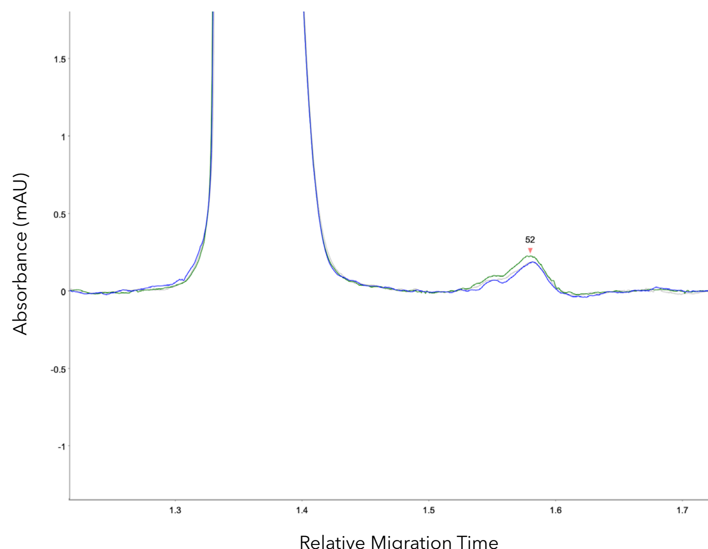

FIGURE 11: CE-SDS analysis of three separate batches of a PEGylated protein. This is an expansion of FIGURE 10 showing the peak at 52 kDa.

CONCLUSION

The results from the studies illustrated here show how the automated CE-SDS platform, Maurice, provides reliable insights into the structure of different types of biopharmaceuticals. Monoclonal antibodies, heavily glycosylated species, and PEGylated species were evaluated for size heterogeneity, producing results that were not only reproducible, but also consistent with those obtained from other analytical methods. Using a simple workflow, Maurice was used to screen multiple samples quickly for the presence of mismatched species and provided electrophoretic data on the impurity profiles of samples. The instrument's ease-of-use, efficiency, reproducibility, and high-quality data make it a useful platform for comparative analysis between biosimilar and innovator drugs. Because such analytical tools offer other key advantages like 21 CFR compliance, short run times and rapid method development, they can significantly accelerate the development of biosimilars.

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