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WHITE PAPER

Intact Mass Analysis for Accelerated Development and Approval of Biologics

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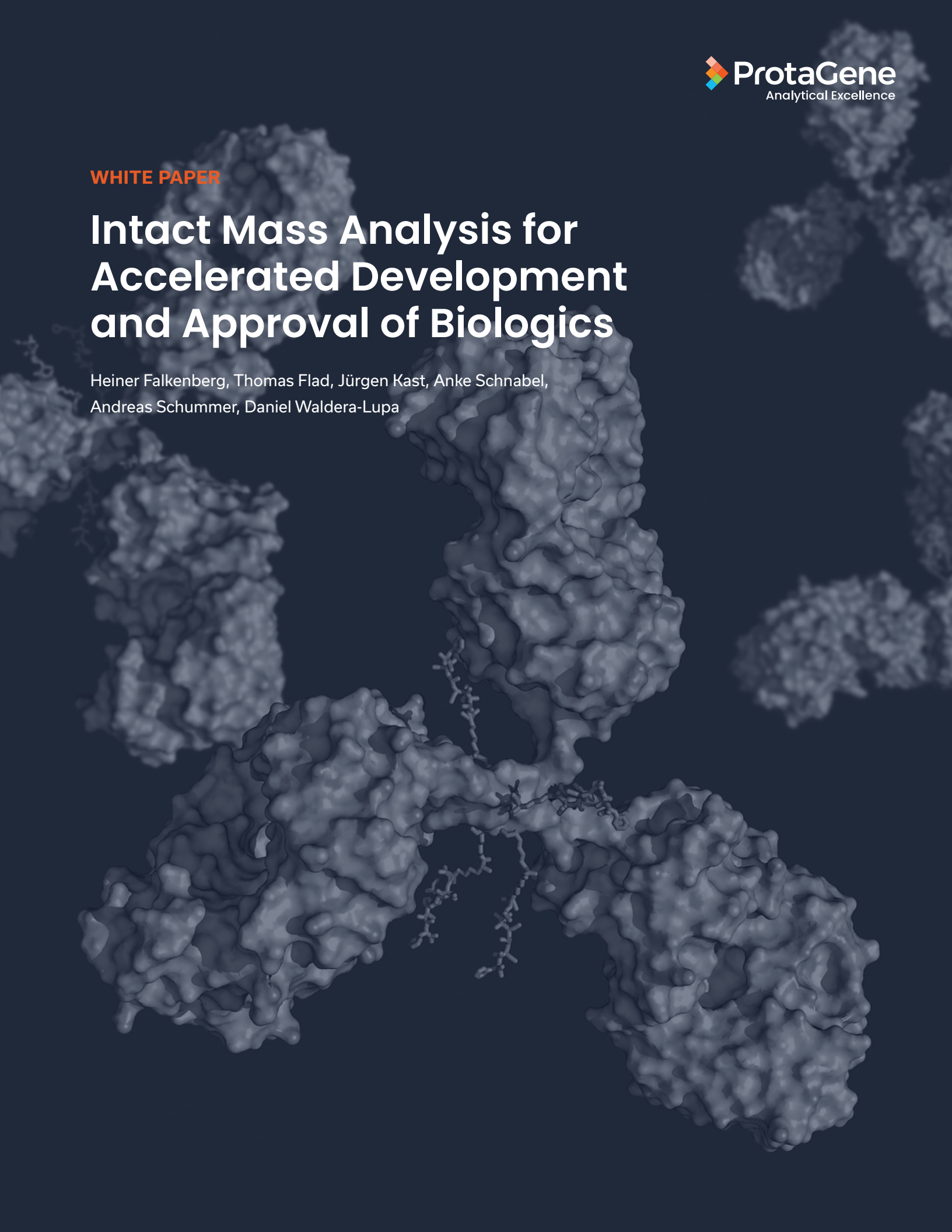

Introduction

Biologics is a rapidly growing sector of the pharmaceutical industry. They hold great promise as potent and effective therapies. Many new biological entity (NBE) candidates comprise complex new protein formats with multiple specificities as a basis for novel modes of action (MOAs) for breakthrough therapies in oncology, autoimmunity, and other severe disease areas.

- Intact mass analysis as a strategy for early-phase CQA evaluation of biologics
- MS-coupled LC methods available and their advantages over LC-only methods
- Examples for the characterization of purity, size/aggregates, and charge heterogeneity as CQAs
- Introduction of CE-type separations as new and cutting-edge MS-coupled methods
- Complementarity with other, more traditional approaches for CQA evaluation

Analytical monitoring of critical quality attributes (CQAs) of a rapidly growing pipeline of these new-format NBE candidates has become an increasing challenge for the pharmaceutical industry with regards to both throughput and specificity. Traditional protein analytical methods such as liquid chromatography or electrophoresis are often limited in their ability to provide sufficient information on the CQA situation of development candidates. Moreover, in-depth analysis of development candidates, which delivers an understanding of the structure-function relationship, has traditionally been applied only during extended characterization in later development stages – typically phase 3 clinical trials. As a result of this back-loaded analytical strategy, pitfalls associated with CMC (chemistry, manufacturing, and controls), such as unexpected amino acid variants or site-specific modifications directly compromising the MOA, occasionally go undetected until the very late stages. Undoubtedly, such a situation is rather disastrous, as the regulatory authorities may stop the approval process, and the development company may revert to much earlier stages, with significant modifications to the production process.

To overcome these challenges, there has recently been a paradigm shift in the analytical strategy of the biopharmaceutical industry. This new approach, in which CQAs are elucidated at much earlier phases, typically in the form of development feasibility assessments in late research, delivers a solid foundation of knowledge on structure and function, which then serves as a basis for producing and controlling high-quality biologics.

A core technology of this in-depth analytical strategy is mass spectrometry, which, because it delivers very concise information on multiple characteristics, is also known as the multi-attribute method (MAM).

Here, we provide an overview of the use and advantages of mass spectrometry, in particular of intact mass analysis for CQA evaluation of biologics.

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Advantage of MS-coupled methods over LC-only

Most CQAs result in a change in the molecular mass, making MS-coupled techniques the methods of choice for identifying modifications. Most methods used in QC labs are based on the intact molecule. Why not couple all routinely used LC-based methods directly to the mass spectrometer?

The combination of liquid chromatography and mass spectrometry (LC-MS) can be used to separate compounds within a sample while reaping the benefits of mass spectrometry as a detector for structural identification of the individual compounds: high sensitivity, selectivity, and molecular specificity. The most important advantage is the identity information. Based on the molecular mass, most chromatographically separated peaks can be assigned unambiguously. A few requirements must be met for successfully coupling the methods. Not all solvent components are MS-compatible and must be replaced with volatile components. If the solvent changes, the column and gradient must be adapted in parallel. Because mass spectrometric detection of the different sample components requires efficient ionization, ion-suppressing components from the formulation buffer have to be removed prior to analysis.

Sequence verification or identification of low-molecular-weight (LMW) and high-molecular-weight (HMW) species requires considerable mass accuracy. High-resolution mass spectrometry offers a high degree of spectral quality, which leads to an accurate and precise molecular weight determination. To determine post-translational modifications (PTMs), such as deamidation, succinimide formation, or truncation, it can be helpful to use middle-down approaches. The reduction or specific cleavage of the biopharmaceutical reduces its size, which is advantageous, as it improves resolution in the mass spectrometry experiment, delivering sub-ppm mass accuracy and leading to unambiguous identification of the PTM.

LC-MS is widely applied for regulatory compliance purposes in the pharmaceutical industry, as the CQA risk assessment will be included in the biologics license application (BLA) submission.

Here we present some common LC-MS methods that can add a great deal of information to classic LC-only workflows **(Table 1)** — even as a one-time characterization.

RPLC-MS

Reversed-phase liquid chromatography (RPLC) in combination with MS is one of the most widely used chromatographic approaches for the characterization of therapeutic proteins. It is able to answer several queries related to therapeutic protein product development, such as assessment of identity, purity, and integrity. It is able to easily measure the molecular weight and primary structure, as well as identify post-translation modifications such as glycosylation or oxidation of the intact protein, subunits, and peptides. RP-MS enables fast, easy, and exact intact mass detection for identification studies within the framework of GMP-release testing.

The separation mechanism in reversed-phase chromatography depends on the hydrophobic binding interaction between

TABLE 1: The advantage of MS-coupling vs. classic LC-only methods

LC-MS Method	Separation Principle	Primary CQA	Advantage of MS Coupling	Additional cQA	Orthogonal LC Method
RP-MS	Hydrophobicity	Purity	Sequence verification	Glycoform distribution, oxidation, Gln/pGlu, thioether, glycation, fragmentation	RP-UV
SEC-MS	Size: large molecules elute first	Monomer/ aggregates/ fragments	Identification of LMW species, determination of fragmentation sites	Glycoform distribution	SEC-UV
IEX-MS	Charge	Charge heterogeneity (Gln/pGlu, Asn deamidation, Asp/ isoAsp)	Identification of charge variants (Lys truncations, succinimide intermediates)	Glycoform distribution	IEX-UV
CE-MS	Charge/size/ molecule-specific behavior	Charge heterogeneity (Gln/pGlu, Asn deamidation, Asp/ isoAsp)	Identification of variants, determination of fragmentation sites	Glycoform distribution	CZE, cIEF
HILIC-MS	Size: small molecules elute first	Size	Identification of LMW species, determination of fragmentation sites	—	CGE

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the soluble compound in the mobile phase and the immobilized hydrophobic ligand in the stationary phase. The compounds are separated based on their hydrophobic character.

In feasibility studies, an intact **(Figure.1)** and reduced **(Figure. 2)** IgG1 antibody (trastuzumab) was analyzed to identify the exact mass of the main form and to detect and identify the mayor glycosylation isoforms. In **(Figure 3)**, RP-MS was used to identify the oxidative state of an N-deglycosylated antibody under different stress conditions. An increase in the oxidative level was identified using longer incubation times and higher hydrogen peroxide concentrations.

FIGURE 1: RP-MS analysis of an IgG1 antibody. Total ion chromatogram (upper), mass spectrum (middle), and deconvoluted mass spectrum of trastuzumab with annotated glycan forms.

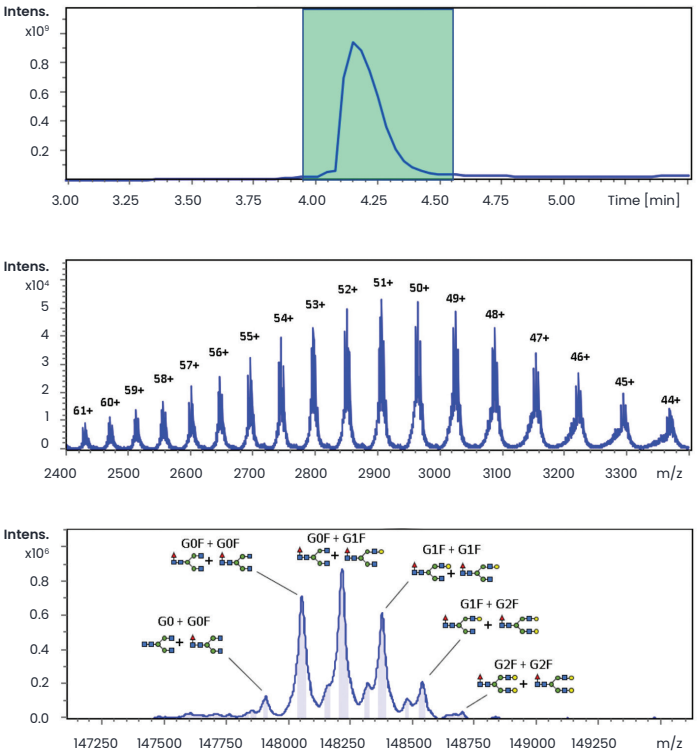


FIGURE 3: RP-MS analysis of an N-deglycosylated IgG1 antibody under different oxidative stress conditions. Total ion chromatogram (upper) and deconvoluted mass spectrum (lower) of samples under different stress conditions with annotated oxidation levels.

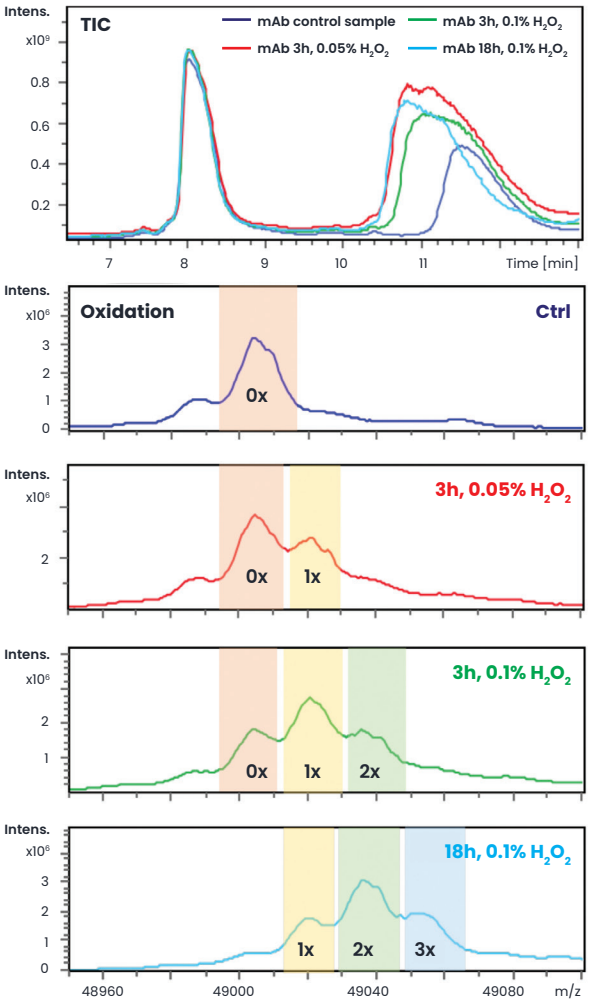
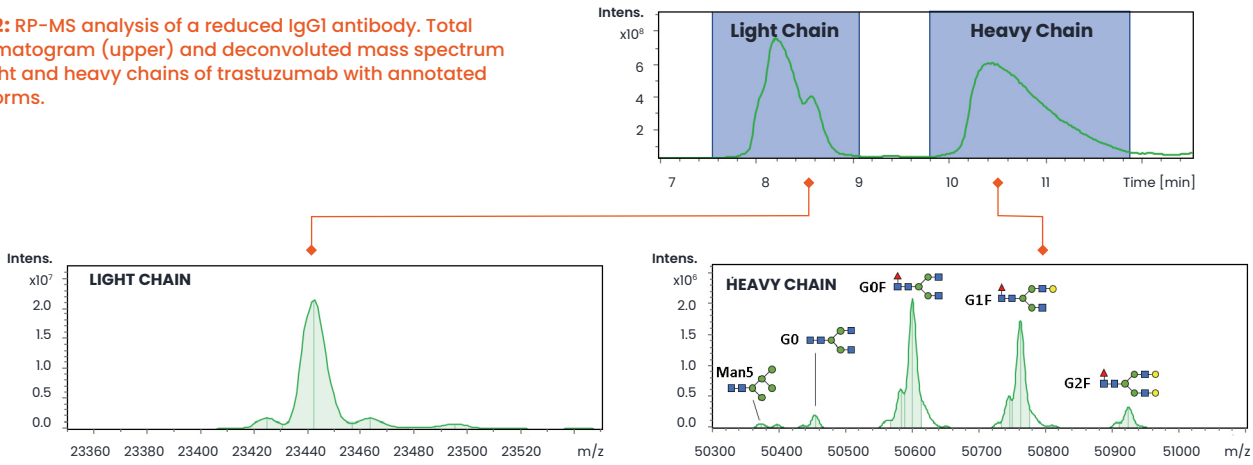


FIGURE 2: RP-MS analysis of a reduced IgG1 antibody. Total ion chromatogram (upper) and deconvoluted mass spectrum of the light and heavy chains of trastuzumab with annotated glycan forms.



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IEX-MS

Biopharmaceutical therapeutics such as monoclonal antibodies are complex proteins and heterogeneous in their biochemical and biophysical properties due to multiple post-translational modifications and degradations that may induce charge heterogeneity. These charge variants can impact the safety and efficacy of the therapeutic products, and monitoring these charge heterogeneities during development is an important quality requirement for regulatory authorities.

Ion exchange chromatography (IEX) is the gold standard method for the characterization of protein charge variants and is used to separate and isolate protein charge variants for subsequent characterizations. IEX coupled online to MS combines the benefits of the non-invasive separation and quantification of charge variants (ion exchange chromatography) with precise determination of molecular weight and identification of PTMs (high-resolution mass spectrometry). Furthermore, it allows for high throughput monitoring of charge heterogeneity without a time- and material-consuming fractionation step.

Separation in IEX chromatography depends on the reversible adsorption of charged molecules to immobilized groups of opposite charge on the column. Because conventional, nonvolatile IEX buffers with high salt concentrations cannot be used for IEX coupled online to MS, an MS-compatible buffer system with a combined pH and volatile salt gradient is used instead.

In a feasibility study, IEX-MS was used for separation and identification of mAb charge variants. In **Figure 4**, an unstressed IgG antibody was analyzed. The prominent charged variants are related to the presence of C-terminal lysines. The C-terminal lysine additions are easily separated and confirmed by MS detection. For a stability test, a commercially available IgG1 mAb was treated with elevated temperature and high pH for one week and compared to an unstressed sample. A considerable increase in acidic variants is observed in the IEX-MS analysis of the native nonreduced antibody **(Figure 5)**.

CE-MS

A variety of post-translational modifications, such as glycosylation and lysine truncation, and chemical modifications, such as oxidation and deamidation, can occur during the manufacture of recombinant biopharmaceuticals. These charge variants are major CQAs due to their potential influence on biological activity and molecule stability. Analysis of charged variants is, therefore, a regulatory requirement for recombinant biopharmaceuticals. Several different approaches are widely used for monitoring and characterizing charge variants of recombinant mAb products; these approaches include ion exchange chromatography (IEX), capillary zone electrophoresis (CZE), and capillary isoelectric focusing (cIEF). However, electrophoretic methods do not elucidate the identity of a charged variant, and IEX gradients need to be developed individually for each mAb product.

Combining capillary electrophoresis (CE) with electrospray ionization (ESI) mass spectrometry offers the possibility of directly characterizing charged variants in mAb products in detail. Charge variant analysis using CE-ESI-MS can be performed with the ZipChip® system. The ZipChip® is a microfluidic zone electrophoresis chip with an integrated nano-ESI emitter. In CE-ESI-MS, the analyte is separated in an electric field based on differences in electrophoretic mobility, which is a function of the charge and size of the analyte. The analyte is sprayed directly into the mass spectrometer through the nano-ESI emitter in

FIGURE 4: UV chromatogram (upper left) and total ion chromatogram (upper right) of an IEX-MS analysis of an unstressed IgG1 antibody. Deconvoluted mass spectra (lower) of the main peak and of a basic variant for evaluation of C-terminal lysine clipping.

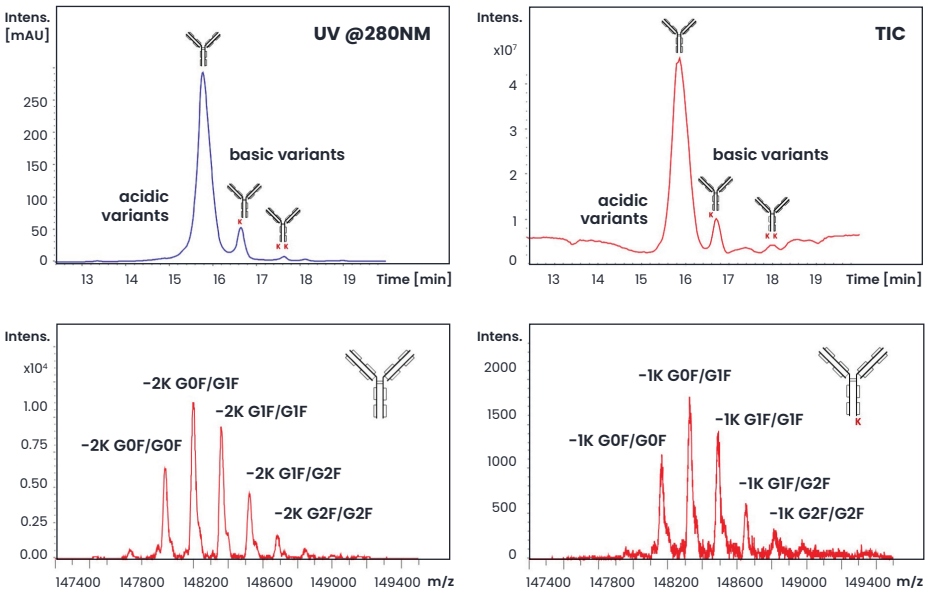
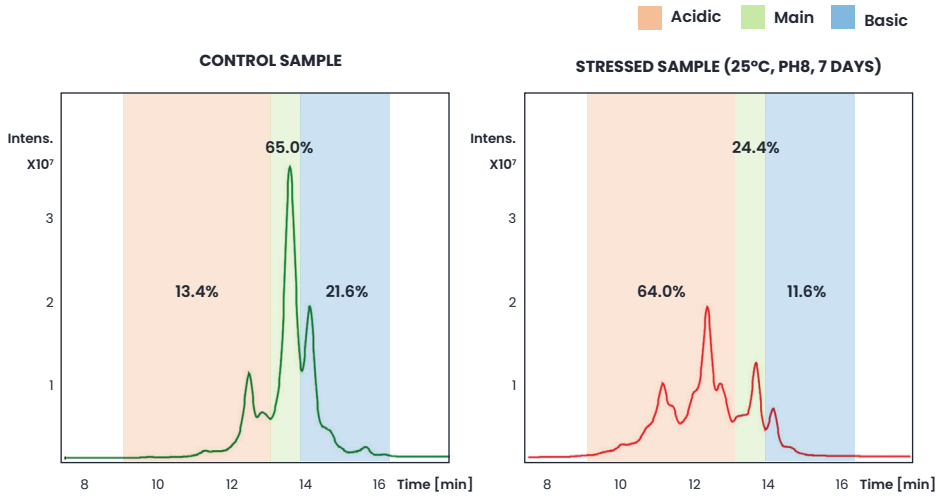


FIGURE 5: Comparison of acidic, main, and basic fractions of total ion chromatograms of an IEX-MS analysis of a control sample (left) and a stressed sample (right) of an IgG1 antibody (trastuzumab). In order to generate the stressed sample, the antibody was incubated for 7 days at 25°C in a pH 8 buffer.



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the corner of the chip. The nanospray is stable and sensitive, ensuring highly sensitive, robust results. In addition, the short analysis time enables high-throughput measurements.

CE-ESI-MS for characterizing charged variants has some advantages over the typically used methods. First, charged variants can be separated by CE and identified and characterized directly via highly sensitive mass spectrometry. Second, CE parameters do not require optimization, and minimal sample preparation is necessary. This makes fast, high-throughput analysis of biopharmaceuticals possible. In addition, a large number of different molecules can be analyzed with CE-ESI-MS: amino acids, peptides, intact proteins, antibodies, and antibody-drug conjugates. Furthermore, proteins can be analyzed in their native or denatured state. However, if charge variants of a biopharmaceutical are insufficiently separated, CE-ESI-MS does

FIGURE 6: Base peak electropherogram of an IgG1 separated by CE-ESI-MS. The four different charge variants correspond to C-terminal lysine (2K, 1K, 0K) and acidic variants (e.g. deamidation).

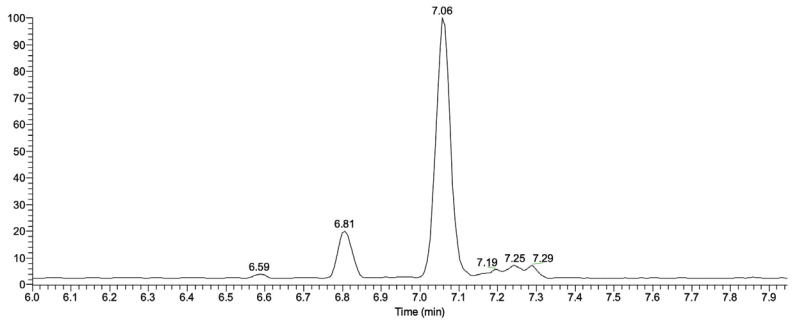
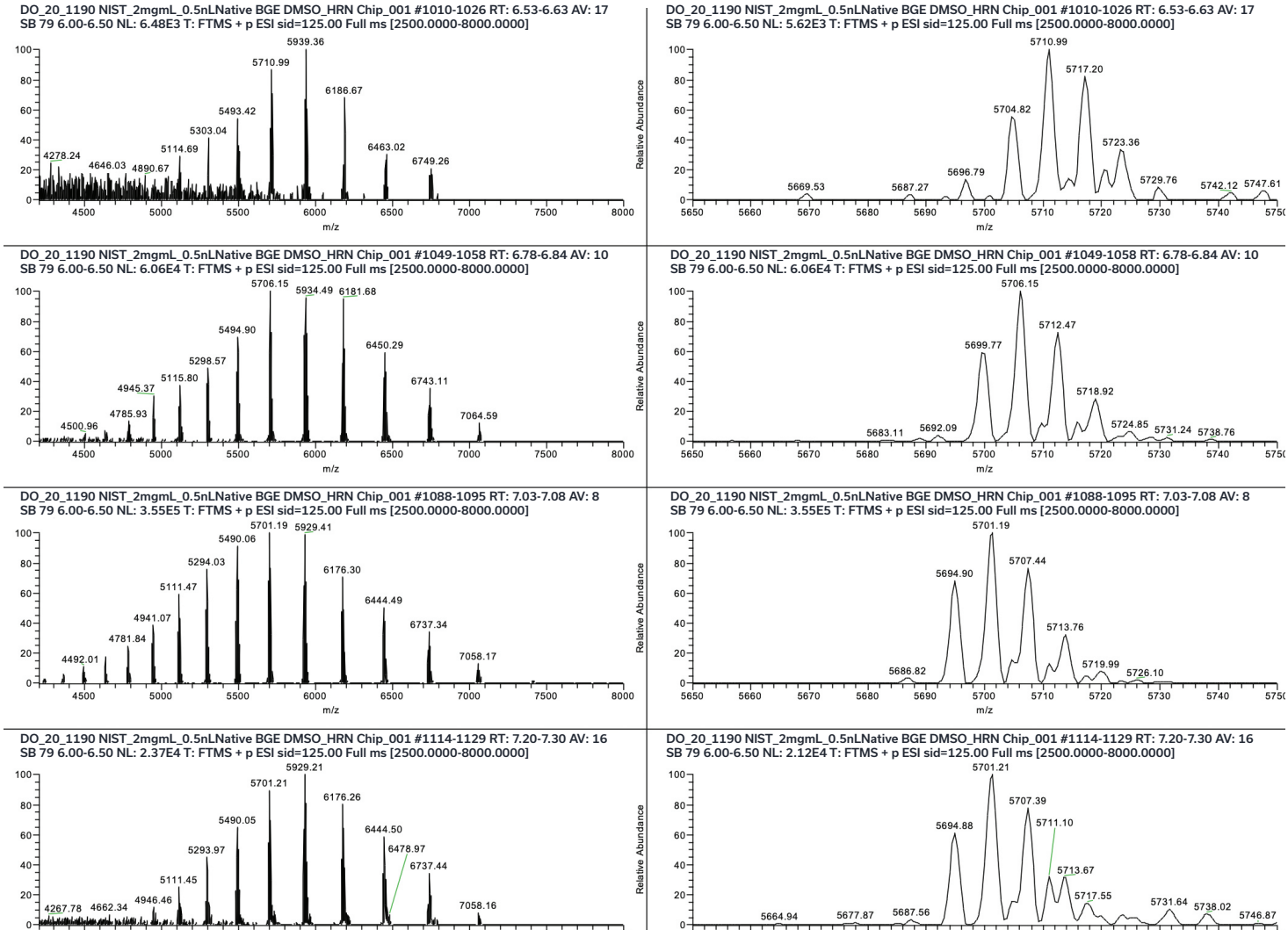


FIGURE 7: On the left side the MS spectrum of each of the four charge variants is shown. On the right side the deconvoluted MS spectrum is shown. The charged variants were detected with good resolution which allows to determine the glycosylation pattern of the IgG1.



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not allow for optimization and manipulation of run parameters such as buffer strength or gradient length. Direct coupling to the mass spectrometer also means ensuring that no buffer components are separated in the electric field and interfere with the nanospray. CE-ESI-MS is thus especially useful in the early stages of the biopharmaceutical development process, where a high degree of sensitivity is essential.

In a feasibility study, CE-ESI-MS was used for separating and identifying charged variants of an IgG1 mAb, which was therefore analyzed via CE-ESI-MS under native conditions. A total of four different charged variants — C-terminal lysine (2K, 1K, 0K) and acidic variants — were baseline separated in the electropherogram **(Figure 6)**. Resolution of the charged variants detected was good, making it possible to determine the glycosylation pattern of the IgG1 **(Figure 7)**. Detection resolution of even the 2K variant, which was present in only small amounts, was good, as was that of the acidic variants. The acidic charge variants revealed a very small mass shift as expected for deamidated species of an IgG1. The applied resolution yielded excellent mass accuracy (< 20 ppm), making identification unambiguous.

It was successfully demonstrated that CE-ESI-MS is capable of separating charged variants of native mAbs with excellent separation power and a high degree of mass accuracy. Moreover, the charge variant profile generated by CE separation using ZipChip® correlates very well with traditional approaches like cIEF and IEX.

SEC-MS

Size-exclusion chromatography (SEC) is a well-accepted technique for the detection and accurate quantification of aggregates, monomers, and fragments in biological drug products. The levels of aggregation and fragments are CQA that can significantly affect efficacy and lead to safety implications. These parameters have to be monitored during the development process for production lot comparisons, stability studies, and forced degradation analyses for quality control and assurance (Harberger et al., 2016).

SEC separates protein species according to their hydrodynamic volume (or size) in a porous stationary phase. Smaller protein species travel through the pores of the stationary phase, while bigger species cannot enter the pores and therefore elute first. This leads to a time-resolved chromatographic profile, allowing for relative quantification of protein species present in the sample.

SEC coupled with mass spectrometry can be used for accurately identifying the molecular mass of biomolecules and can serve as a complementary characterization method to low-resolution mass determination methods, such as multi-angle light scattering (MALS) or analytical ultracentrifugation (AUC), for confirming the identification of biomolecules. In the conventional SEC setup, UV is used for detection, due to high salt concentrations. SEC-MS using a volatile ammonium formate buffer, which is MS-compatible and preserves intact protein structures, enables the exact mass measurement of biomolecules in their native state without the need for desalting.

SEC-MS enables exact intact mass detection both of biomolecules, aggregates, and conjugates under non-denaturing conditions and of fragments (including heavy chains, light chains, Fab, and Fc) under denaturing conditions.

In a feasibility study, a stressed IgG1 antibody was analyzed to detect aggregation and fragments and to determine their masses and possible composition. In **Figure 8**, one HMW and two LMW species are detected beside the monomeric peak. The HMW species represents an aggregation of the antibody with a mass of 296.7kDa. The first LMW species (LMW1) has a mass of 100.85kDa, indicating two heavy chains; LMW2 was detected with a mass of 47.58kDa and 50.98kDa, representing antibody fragments.

HILIC-MS

Low-molecular-weight (LMW) and high-mo-

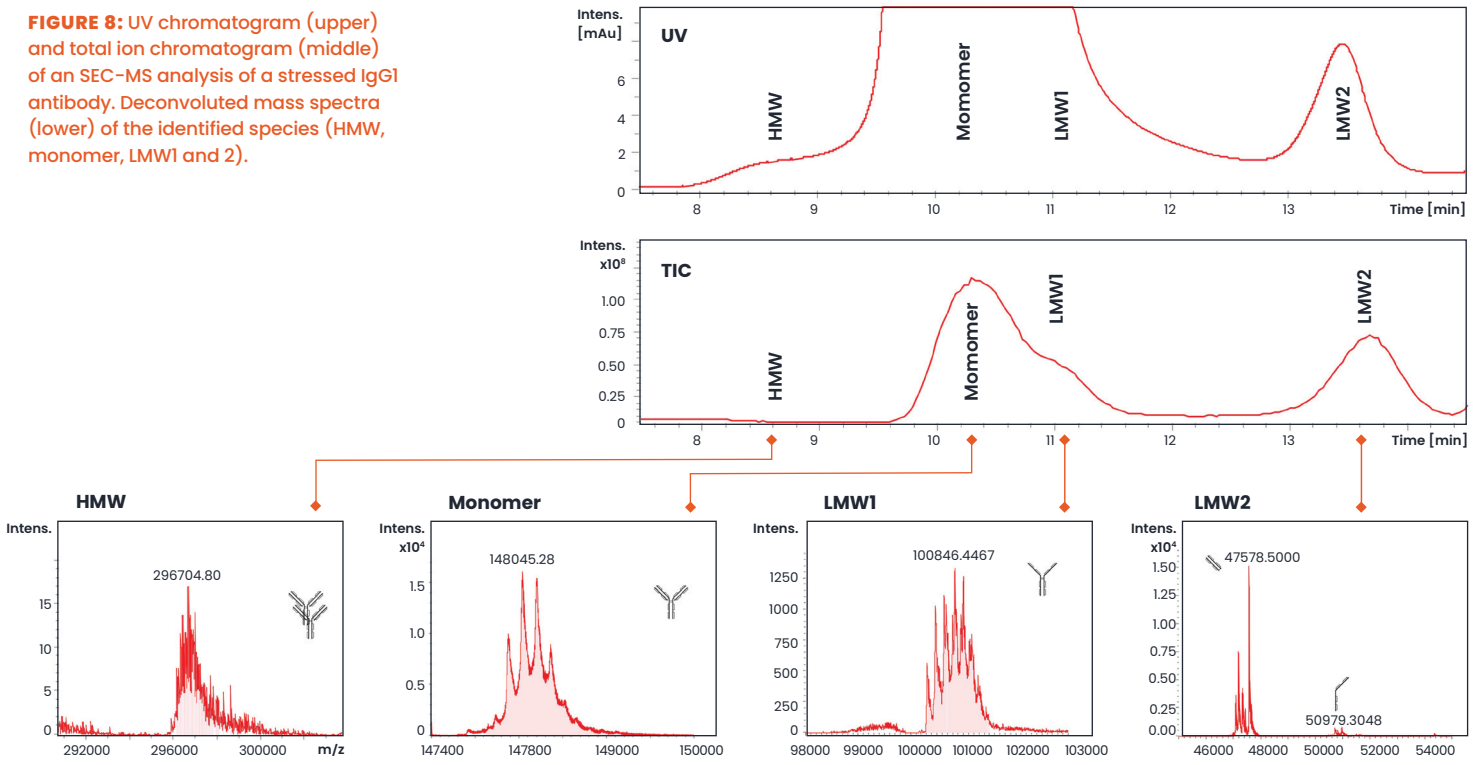

FIGURE 8: UV chromatogram (upper) and total ion chromatogram (middle) of an SEC-MS analysis of a stressed IgG1 antibody. Deconvoluted mass spectra (lower) of the identified species (HMW, monomer, LMW1 and 2).

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lecular-weight (HMW) species are examples of product-related impurities that contribute to the molecular weight heterogeneity of mAb products. Both species can potentially compromise both drug efficacy and patient safety due to unwanted immunogenicity. These product-related impurities are therefore considered to be CQAs that are routinely monitored during drug development and as part of release testing of a purified drug product. SDS-PAGE and CE-SDS under non-reducing conditions are widely used for the detection of LMW impurities in mAb products. However, the identification of LMW impurities has been challenging and based on empirical findings.

Combining chromatographic techniques with mass spectrometry offers the possibility of directly characterizing LMW impurities in mAb products in detail. Recently, it has been shown that hydrophilic interaction chromatography (HILIC) is very well suited for the separation of LMW species (Wang et al., 2018). In HILIC, analytes are separated based on their hydrophilicity/polarity. HILIC, therefore, uses hydrophilic stationary phases with reversed-phase type eluents. According to the proposed mechanism, the mobile phase forms a water-rich layer on the surface of the polar stationary phase alongside the water-deficient mobile phase, thereby generating a liquid/liquid extraction system. The analytes elute in the order of increasing polarity. In the application of HILIC to N-deglycosylated mAb, the elution of LMW impurities is mostly determined by the size of the MW species, making this similar to CE methods such as capillary gel electrophoresis (cGE).

For characterizing LMW in mAb products, HILIC-MS has some advantages over the typically used methods. First, LMW impurities can be identified and characterized directly with the mass spectrometer, which is often required for the development of a molecule. Second, sample preparation for HILIC-MS needs to be milder than for PAGE or CE, reducing the likelihood of generating LMW artifacts. However, HILIC-MS requires N-deglycosylation of the mAb, which can reduce the stability of the molecule, resulting in precipitation. Furthermore, the resolution of HILIC separation is inferior to that of electrophoretic methods, preventing a baseline separation of the higher LMW (H2L) and the intact mAb. Nevertheless, HILIC-MS can be used as a semi-quantitative analysis for comparing LMW impurities between different samples or for identifying unknown LMW impurities in mAb products. HILIC-MS can thus make an important contribution to the development of biopharmaceuticals.

In a feasibility study, HILIC-MS was used for separating and identifying LMW species of an IgG1 mAb. A commercially available IgG1 mAb was, therefore, N-deglycosylated and partially reduced to generate a variety of different LMW species. Using HILIC-MS, six different species (five LMW) were chromatographically separated via HILIC and unambiguously identified via intact MS: LC (light chain), HC (heavy chain), H2 (HC-HC), HL (HC-LC), H2L (HC-HC-LC) and H2L2 (full mAb). The elution order was from low to high MW and corresponded to the MW of the different LMW species, except for the H2 and HL species **(Figure 9)**. No baseline separation was achieved for these two LMW species, and they do not follow the MW distribution. While the MS signal was used to identify the different LMW species, the UV signal allowed for quantitative evaluation. To check if separation by HILIC was similar to that achieved with cGE, each LMW species was fractionated, and the fractions were re-injected into cGE. A non-fractionated sample of the mAb was also measured with cGE as a control. The cGE profile of the non-fractionated mAb was highly comparable to the profile obtained from HILIC separation **(Figure 10)**. In addition, the migration times of the fractions collected by HILIC were in accordance with those of the non-fractionated sample. A slight shift in migration time was observed for all heavy chain fractions, due to the glycosylation.

It was successfully demonstrated that HILIC can be used for separating LMW species of mAbs with a MW-based elution order. Moreover, the chromatographically separated LMW species can be identified directly by MS. Additionally, the LMW species can be fractionated and re-injected into cGE to confirm the individual signals in the electropherogram.

FIGURE 9: HILIC-MS of N-deglycosylated IgG1.

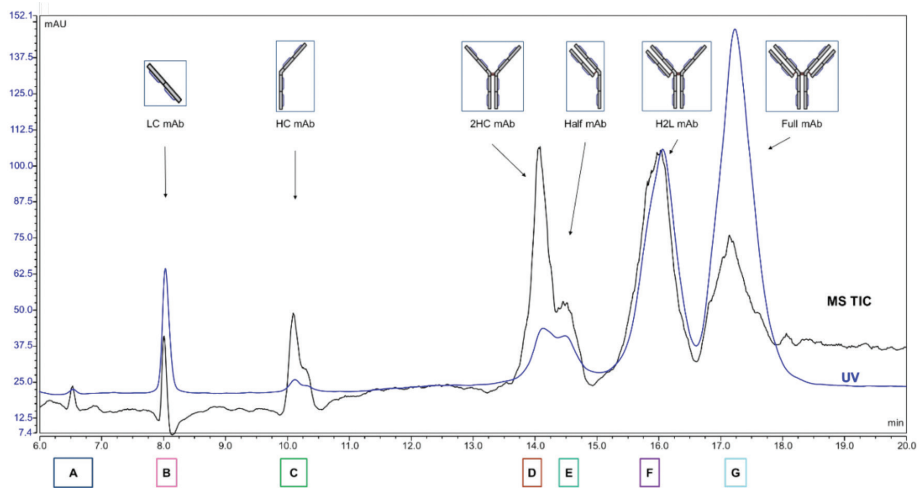
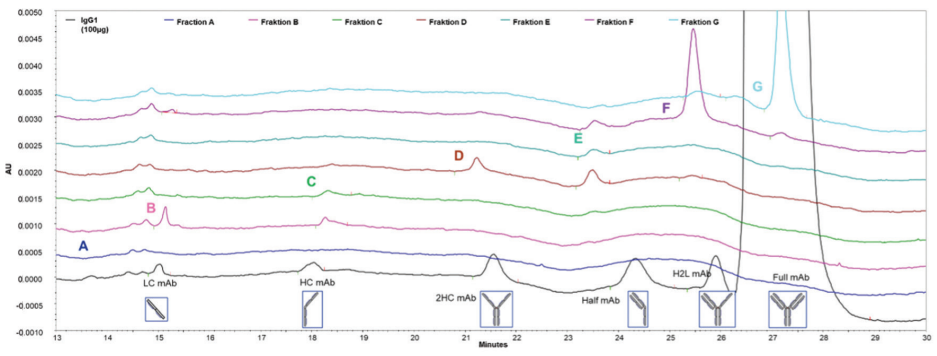


FIGURE 10: cGE of N-deglycosylated IgG1 LMW species fractionated with HILIC.



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