Assessment of Active Biopharmaceutical Ingredients Prior To and Following Removal of Interfering Excipients

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The EMA guideline on Biosimilars covering analytical considerations recommends the application of extensive state-of-the-art characterisation studies to the biosimilar and reference medicinal products in parallel, to demonstrate with a high level of assurance that the quality of the biosimilar is comparable to the reference medicinal product.

The EMA recognises that “for some analytical techniques, a direct or side-by-side analysis of the biosimilar and reference medicinal product may not be feasible or give limited information (e.g. due to the low concentration of active substance and/or the presence of interfering excipients such as albumin). Thus samples could be prepared from the finished product (e.g. extraction, concentration, and/or other suitable techniques). In such cases, the techniques used to prepare the samples should be outlined, and their impact on the samples should be appropriately documented and discussed (e.g. comparison of active substances before and after formulation/ deformulation preparation).”

This is the first significant hurdle that has to be overcome in terms of providing analytical data to support a claim of biosimilarity. A number of innovator products are formulated at low concentration with relatively high concentrations of protein, amino acid, mono- and di-saccharide and/or surfactant stabilisers. All of these common excipients can and will interfere with the analytical data obtained. For example, protein and amino acid excipients interfere with amino acid composition analysis and potentially peptide mapping, whereas saccharide and Tween surfactant based excipients similarly interfere with monosaccharide composition analysis. A number of surfactant based excipients can also interfere with mass spectrometric intact molecular weight analyses and with peptide mapping. It is therefore necessary to remove these excipients to a level where any interference is at least minimised.

A number of techniques are available for this purification including but not limited to chromatography (based on affinity, hydrophobicity, size or charge), size/molecular weight based filtration and dialysis. It is important to try a variety of purification techniques to allow the most appropriate method for the product in question to be chosen.

The recovery of the product through the process should always be checked (a purification technique only recovering the minority of a product is obviously not desirable). Once a satisfactory purification regime has been developed a more thorough assessment of the product(s) prior to and post purification should be performed. These techniques should demonstrate that the Active Biological Ingredient has not changed significantly in a structural/ physico-chemical sense and is suitable for the side-by-side comparison to assess comparability.

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The assessment should include tests to assess where possible:

1. Intactness
2. The protein backbone (including analysis of disulphide bridges and Post Translational Modifications (PTMs)
3. Oligosaccharide profile
4. Secondary and Tertiary structure

1. Intactness — Assessment of molecular weight or size

Assessing the intact molecular weight profile of a biopharmaceutical product prior to and following purification to remove interfering excipients, can be performed relatively quickly and provides significant data to assess any impact on the product during or following purification. On-line LC/ES-MS analysis of proteins and glycoproteins using Quadrupole-Time of Flight (Q-TOF) instrumentation has revolutionised the measurement of intact molecular weight. As an example, the type of data that can be obtained from intact molecular weight analysis of a monoclonal antibody, intact and following reduction is shown below.

The major peaks observed are 162 mass units apart which is consistent with variation in galactosylation of the N-linked oligosaccharides present on the heavy chains of the molecule. The mass spectrometric data obtained from analysis of the monoclonal antibody following reduction are shown in Figure 2.

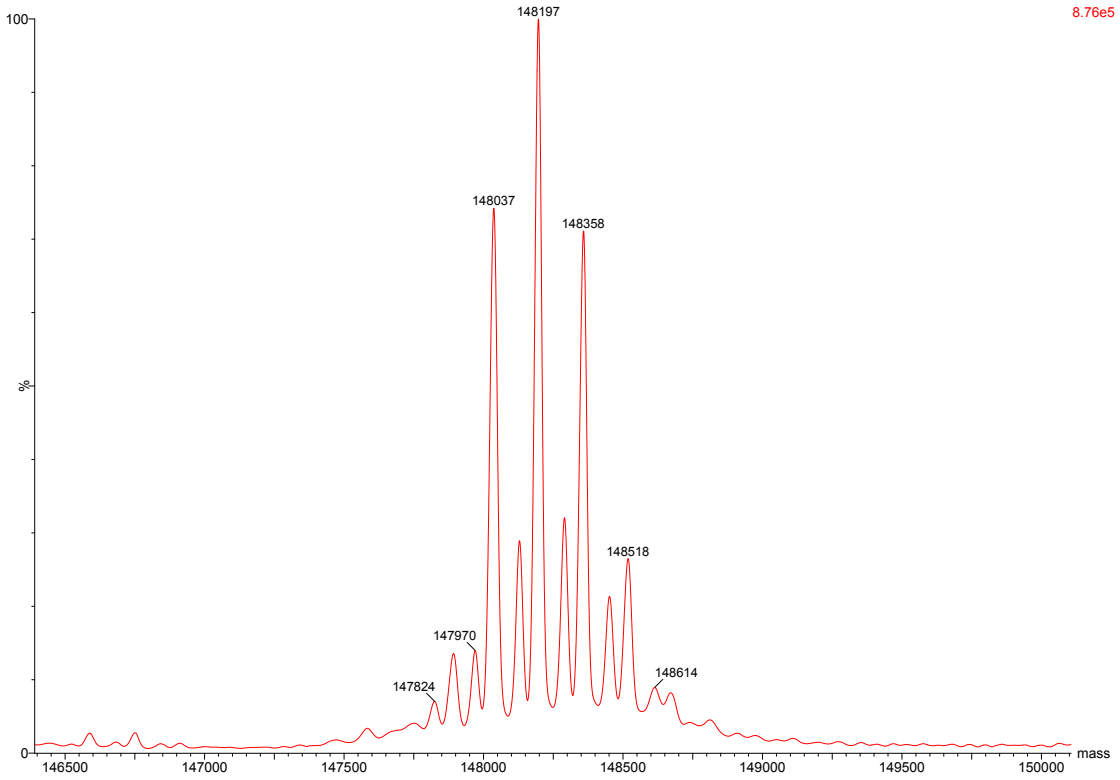

Figure 1: The deconvoluted mass spectrum obtained from on-line LC/ES-MS (Q-TOF) analysis of an intact monoclonal antibody.

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The signal shown in Figure 2a is consistent with that expected for the light chain and the signals observed in Figure 2b are consistent with the heavy chain including the expected major N-linked oligosaccharides.

Figure 2a: The deconvoluted mass spectrum obtained from on-line LC/ES-MS (Q-TOF) analysis of a monoclonal antibody following reduction. Average of the spectra acquired during elution of the released light chain.

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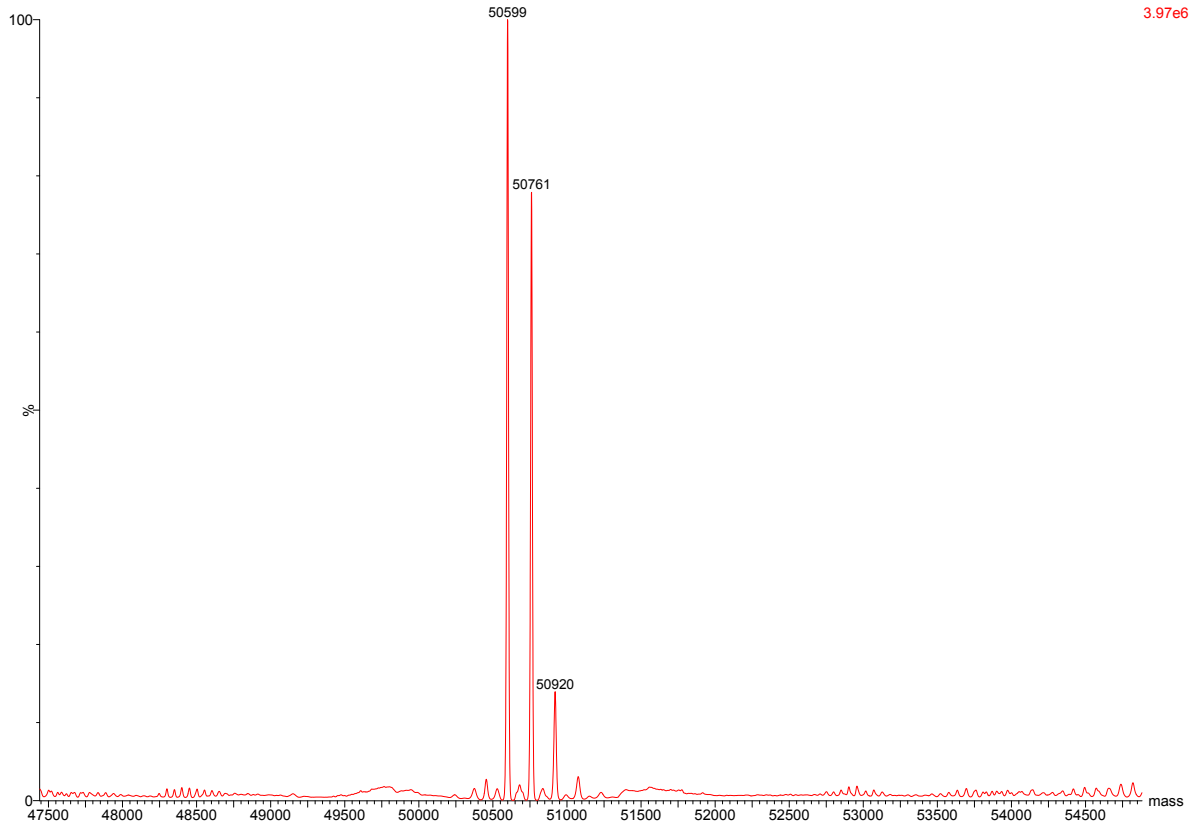

Figure 2b: The deconvoluted mass spectrum obtained from on-line LC/ES-MS (Q-TOF) analysis of a monoclonal antibody following reduction. Average of the spectra acquired during elution of the released heavy chain.

Monoclonal antibody products are often formulated at relatively high concentration with a mixture of amino acid, sugar and surfactant type excipients. It is therefore usually possible to obtain the above quality of data for samples analysed prior to and following purification. This is not necessarily the case for blood circulating hormones e.g. EPO and FSH (which are formulated at relatively low µg amounts/mL with significant levels of interfering excipients).

2. The protein backbone — Peptide mapping

Peptide mapping (i.e. MS analysis of specific protease digests of the Biopharmaceutical usually utilising on-line Reverse Phase-High Performance Liquid Chromatography separation with Ultraviolet (UV) and Electrospray-Mass Spectrometric detection (LC/ES-MS)) provides molecular weight information for the peptides released from a protein or glycoprotein by the protease of choice. The data obtained are able to provide a good assessment of the protein backbone prior to and following purification. It should be noted that peptide mapping allows the observation of the presence or absence of predicted peptide masses using a template protein (or nucleotide) sequence; therefore allowing a first order confirmation of the expected protein primary structure, together with the

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detection of post-translational modification (PTM) events leading to a shift in mass, but it does not provide confirmation of the amino acid sequence itself which must be obtained by further experiment including MS/MS fragmentation analysis. Where possible LC profiles with UV and MS detection for material prior to and following purification should be obtained and compared. Surfactant stabilisers, where present, may well interfere with mass spectrometric ionisation of the eluting peptides and in such cases a comparison of the UV profiles obtained from analysis of digests of the product before and after purification should be used for the assessment.

During the comparison, an assessment of free sulphydryl groups and disulphide bridges should be considered. Analysis of each digest before and after reduction, can provide the data necessary for an assessment of disulphide bridges and free thiols.

As mentioned above, these data should also be used to look for differences in Post-Translational Modifications (PTMs). Extensive purification could have an impact on the deamidation and oxidation state of the product in particular, and these data should be used, for example, to assess any changes in Asparagine, Glutamine and Methionine residues.

Isoform profiling can also be used to provide an overall full molecule assessment of deamidation and other charge based changes occurring during purification. Imaging capillary IsoElectric Focusing (cIEF) has revolutionised analysis of the isoforms of Biopharmaceuticals. cIEF is free solution IsoElectric Focusing (IEF) in a capillary column which detects focused protein zones using a whole column UV absorption detector that avoids disturbing these focused zones. This technology has the comparable resolution of traditional gel IEF, but incorporates the advantages of a column-based separation technology, including quantitation (using UV at 280nm) and automation.

The raw data obtained from cIEF profiling of a monoclonal antibody is shown in Figure 3.

(a) *(b)*

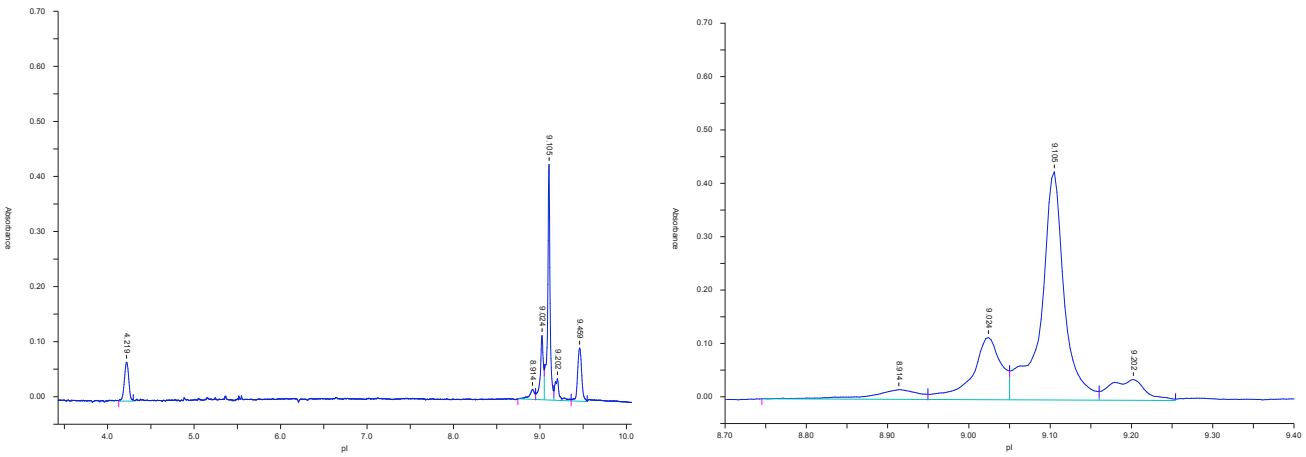

Figure 3: The UV (280nm) response across the capillary obtained following capillary isoelectric focusing of a monoclonal antibody by iCE3 (a) full electrophoretogram (the peaks around pI 4.2 and 9.5 are internal pI markers) (b) zoom of region of interest

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The data obtained allows an accurate determination of the pI of each isoform and quantitation of each isoform based on the UV peak area. An increase in acidic isoforms following purification could for example suggest that the purification process is inducing deamidation.

3. Oligosaccharide profile

Oligosaccharides present on glycoproteins are prone to degradation and particularly to losses of sialic acid content (desialylation). It is therefore important to demonstrate that no significant changes in oligosaccharide profile have been induced by the purification process. Products such as monoclonal antibodies have one N-linked glycan consensus sequence (Asparagine-Xxx-Serine or Threonine, where Xxx can be any amino acid except Proline) within each heavy chain located in the hinge region. Erythropoietin (EPO) has 3 N-linked and one O-linked glycosylation site. In most cases the intact molecular weight data and information obtained from analysis of the glycopeptide(s) in the mass spectrometric peptide mapping experiments outlined above will allow assessment of the comparability of oligosaccharide profiles prior to and following purification. The glycans must however be released and analysed to allow an assessment of the full population including minor species. The data obtained from analysis of the N-Glycans released from a monoclonal antibody are shown in Figure 4 below.

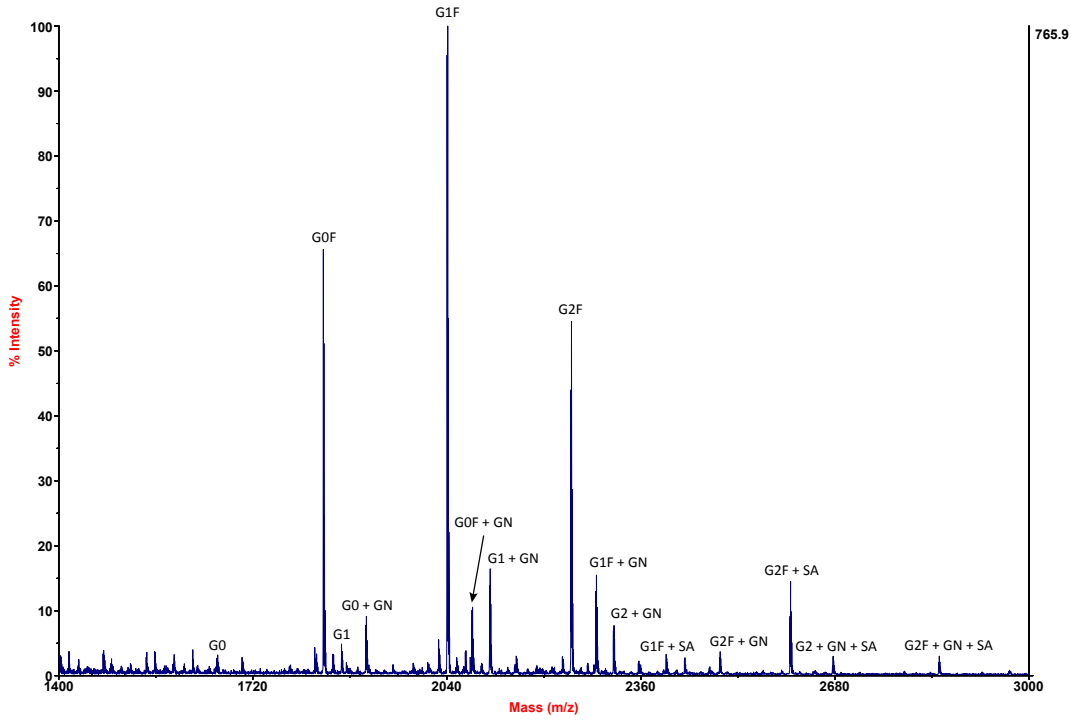

Figure 4: The raw data obtained from MALDI-TOF mass spectrometric analysis of the N-glycans released from a monoclonal antibody (the N-glycans were permethylated prior to analysis).

The major signals observed are consistent with the commonly encountered monoclonal antibody glycans G0F, G1F and G2F (see Figure 4).

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The HILIC-FLR trace obtained from analysis of the N-glycans released from a monoclonal antibody product (following 2-AB labelling) is shown in Figure 5.

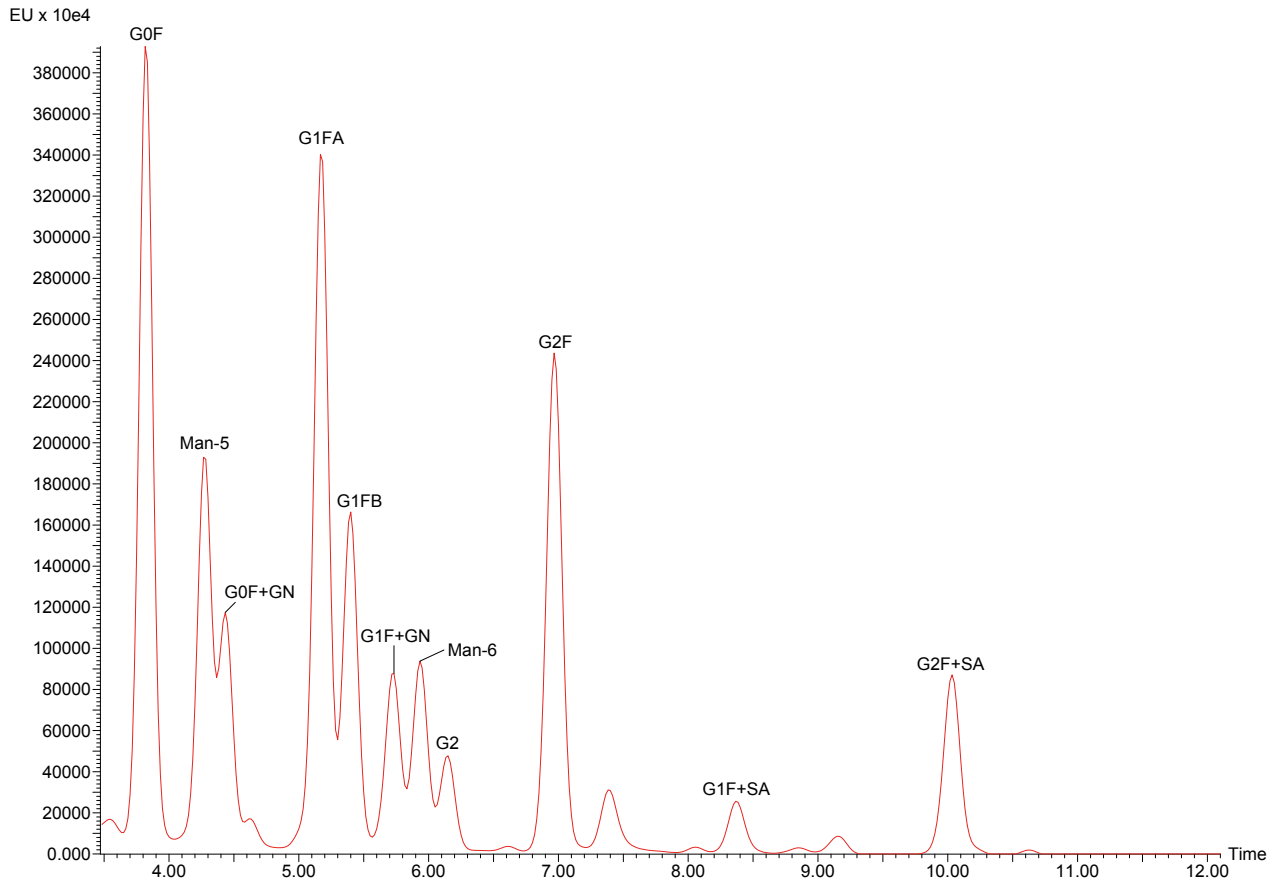

Figure 5: The raw data obtained from HILIC-FLR analysis of the N-glycans released from a monoclonal antibody.

Both these sets of data can be used to provide a relative quantitation of the N-linked oligosaccharides observed and suggest the structures of the N-linked glycans present and therefore allow an assessment of oligosaccharide population pre- and post-purification.

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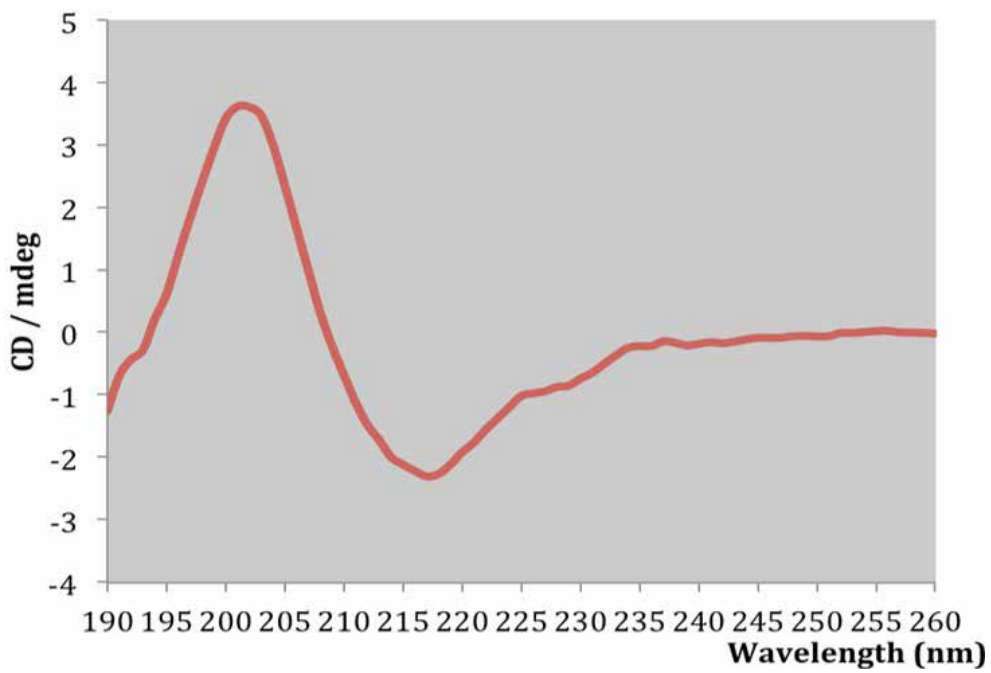

Figure 6: The raw data obtained from near-UV analysis of a monoclonal antibody

4. Secondary and Tertiary Structure assessment

It is also important to assess products pre- and post-purification for secondary and tertiary structure. A significant change in tertiary structure can suggest degradation of the product during or following the purification process.

Circular Dichroism (CD) analysis is commonly used to assess the secondary structure of Biopharmaceuticals. The data obtained from CD analysis of a monoclonal antibody is shown in Figure 6.

Assessment of the raw data using CDSSTR software allows the relative percentages of the various secondary structures to be estimated as shown in Table 1.

In summary, a battery of analytical techniques should be used to show that the integrity of the Active Biopharmaceutical Ingredient has been retained to the extent possible following purification. In our opinion, these analyses should assess the intactness, protein backbone, PTMs, oligosaccharide profile and secondary/ tertiary structure of the product pre- and post-purification.

% α-helix	% other helix	% β sheet	% turn	% other
0	10	36	10	42

Table 1: The result of CDSSTR algorithm assessment of the near UV CD data shown in Figure 6.

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Once the purification process is optimised and qualified, batches of the innovator should be analysed alongside batches of biosimilar. We recommend side-by-side analysis of the tests within ICH Topic Q6b and any other regulatory documents specific for the product being developed. This includes the following analyses:

Structural Characterisation	Physico-Chemical Analysis
Amino acid sequence	Molecular weight or size
Amino acid composition	Isoform pattern
Terminal amino acid sequence	Extinction coefficient (or molar absorptivity)
Peptide map	Electrophoretic patterns
Sulphydryl group(s) and disulphide bridges	Liquid Chromatographic patterns
Carbohydrate structure	Spectroscopic profiles

Where possible batches should be analysed as received. Analysis of purified product should only be performed where significant interference from excipients is observed. Full structural and physico-chemical analysis of a number of batches of biosimilar alongside innovator will be required to provide the required assessment of structural comparability.

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