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CASE STUDY
The Christian Doppler Laboratory
for Biosimilar Characterization

A Platform Method for Oxidation Monitoring in Monoclonal Antibodies and Fc-Fusion Proteins—a Biopharmaceutical Method Development Case Study

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The Christian Doppler Laboratory (CDL) for Innovative Tools for Biosimilar Characterization at the University of Salzburg, Austria, aims at accelerating innovations in biopharmaceutical characterization.

Accelerating innovations in biopharmaceutical characterization

Promoting the cooperation between science and business, researchers of the CDL develop workflows for biosimilar characterization with the goal of translating analytical methods into routine, transferable, and compliant solutions. Prof. Christian Huber and co-workers recently published a study entitled “A Generic HPLC Method for Absolute Quantification of Oxidation in Monoclonal Antibodies and Fc-Fusion Proteins Using UV and MS Detection” describing a middle-up approach for the characterization of oxidation variants in therapeutic monoclonal antibodies (mAbs) and Fc-fusion proteins[1].

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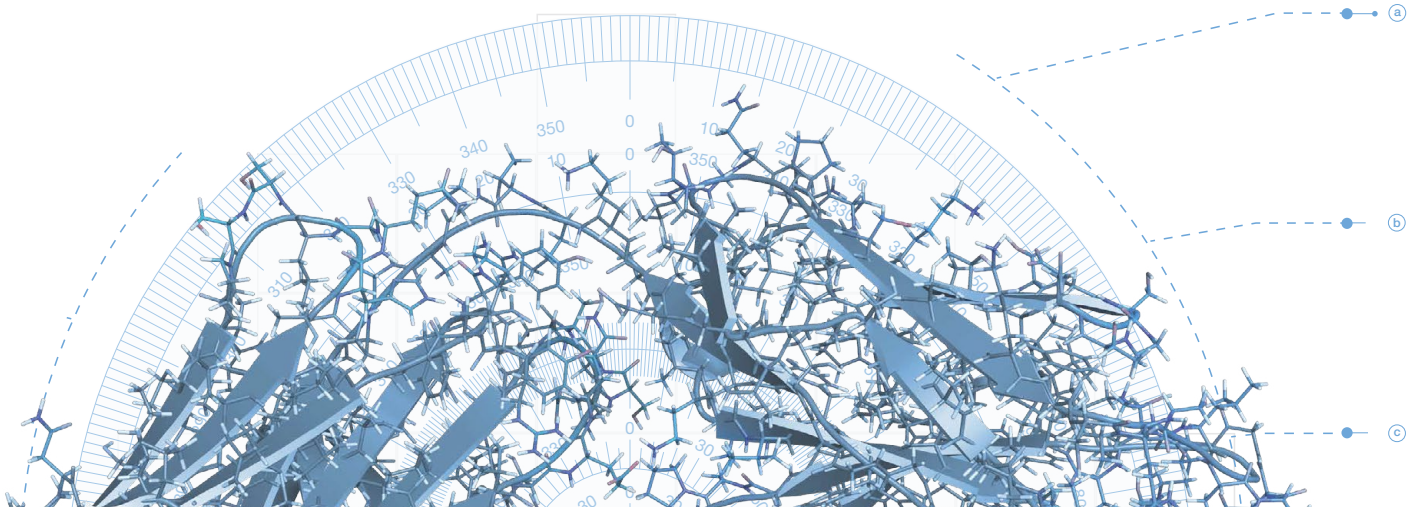
“Biosimilars are the fastest growing sector within the pharmaceutical industry. To ensure the effectiveness and safety of these drugs, an in-depth characterization at the molecular level is obligatory. Through the cooperation of academic research groups and industrial partners, the University of Salzburg is contributing to the development of safer and more affordable pharmaceuticals.”

—Prof. Dr. Christian Huber,
Chemist, Christian Doppler Laboratory Head



The CDL for Biosimilar Characterization comprises a multidisciplinary team of scientists focusing on method development and workflow optimization for the analysis of biosimilar drugs. Their aim is to accelerate innovations in biopharmaceutical characterization and to ensure that the developed methods can be translated into routine, compliant solutions for pharmaceutical companies. Collaboration with industrial partners within the CDL provides insights and alignment with regulatory requirements for biosimilars, whilst mentoring and developing the next generation of biopharmaceutical analytical scientists in an academic environment.

With this research focus in mind, the team embarked on the development of a fast and generic method to quantitate protein oxidation in biotherapeutics. Oxidation of methionine is a post-translational modification (PTM) which is considered a potential critical quality attribute (CQA) in biopharmaceutical production—this implies stringent monitoring all the way from drug development and manufacturing to lot release/quality control (QC). Methionine oxidation has been shown to affect the structure of the Fc region of mAbs[2] which may alter binding to the neonatal Fc-receptor (FcRn)[3], impacting pharmacokinetic properties, potentially leading to faster plasma clearance and altered clinical efficacy of the protein drug. Monitoring protein oxidation is typically performed using a conventional peptide-based bottom-up approach. One issue with bottom-up methods in this context is the risk of introducing artificial oxidation during tryptic digestion.

Characterization at the intact protein or subunit level, on the other hand, provides valuable information on a protein's structure, as the context of modifications is maintained. The CDL for Biosimilar Characterization therefore prioritizes the application of intact protein and subunit analysis over peptide mapping approaches, which commonly involve lengthy, variable sample preparation and a loss of information on the context of the detected modifications.

The authors of the study initially determined three main requirements to be fulfilled by the method in development:

1. Rather than being tailored to one specific molecule, the method should be **applicable for a range of molecules sharing one common property**—the Fc domain of IgG1 antibodies.
2. The method should **ideally be UV-based, with the possibility of coupling chromatographic separation directly to mass spectrometry (MS)**. This analytical setup facilitates easy method transfer from the discovery and development laboratory into a QC environment.
3. The approach should **provide insights into protein oxidation while maintaining the context of other modifications** such as glycosylation, applying a so called "middle-up" approach. Additionally, this strategy minimizes artificial oxidations which may be introduced during proteolytic digestion.

Selecting the parts for biosimilar method development success

The middle-up workflow for protein oxidation quantitation developed within the CDL comprises:

- **Thermo Scientific™ MAbPac™ RP column**
- **Thermo Scientific™ UltiMate™ 3000 BioRS UHPLC system**
- **Thermo Scientific™ Q Exactive™ Hybrid Quadrupole-Orbitrap™ mass spectrometer**
- **Thermo Scientific™ Chromeleon™ 7.2 Chromatography Data System (CDS) software**

Other studies have shown protein oxidation analysis by hydrophobic interaction chromatography (HIC) or ion exchange chromatography (IEC)[4, 5]. HIC and IEC methods tend to be HPLC-UV-based only, and usually do not offer the opportunity to hyphenate to MS for further characterization. In order to overcome this limitation, the method developed by Regl *et al*. involved an ion-pair reversed phase (IP-RP) separation, which could readily be coupled to MS in order to deliver further structural information[1].

Initial attempts provided evidence that oxidized and non-oxidized mAb variants could not be baseline resolved by HPLC and MS at the intact protein level: On the one hand, mass spectra revealed overlap of isotopic envelopes, making separation by IP-RP-HPLC increasingly important. On the other hand, baseline chromatographic separation at the intact protein level—a prerequisite for quantitation—could not be achieved. Because of these limitations at the intact protein level, a middle-up approach involving the analysis of Fc/2 subunits was pursued as described in Figure 1.

"Protein oxidation is a PTM which is considered a potential CQA in biopharmaceutical production—this implies stringent monitoring all the way from drug development and manufacturing to lot release/QC."

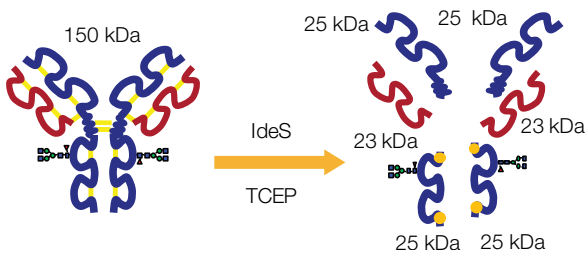
—Dr. Therese Wohlschlager,
Biotechnologist, Christian Doppler Laboratory

1 Biopharmaceuticals containing oxidized variants

A generic workflow developed for characterization of oxidation in biopharmaceuticals involving:

2 Proteolysis with IdeS & reduction

Digestion of the mAb with IdeS (FabRICATOR®, Genovis AB) and reduction of disulfide bonds with tris(2-carboxyethyl)phosphine (TCEP).



3 Separation & detection

This middle-up approach enables mass spectrometric resolution of the resulting Fc/2 subunits for accurate quantitation both of oxidized and non-oxidized forms of the mAb. Since the IdeS digestion step is very short and is performed at moderate temperatures, the risk of artificial oxidation is minimized. The obtained mAb subunits are subsequently separated by IP-RP-HPLC on a MAbPac RP column before detection in the Thermo Scientific™ Dionex™ UltiMate™ 3400RS VWD Variable Wavelength Detector and the Q Exactive MS.

4 Quantification of oxidation

Oxidation is identified based on the chromatographically separated peaks as well as extracted ion chromatograms revealing mono- or double oxidation, as indicated by +16 and +32 Da mass shifts with respect to the non-oxidized variant. Calibration and quantification of UV- and MS data were all performed using Chromeleon 7.2 CDS.

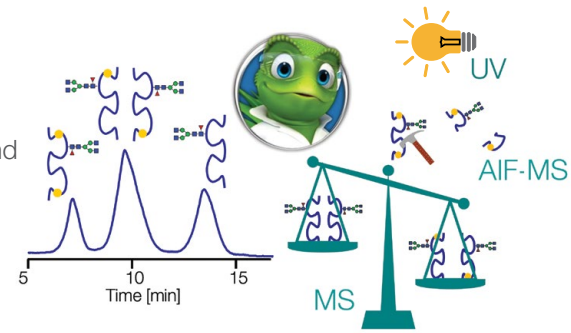


Figure 1. Typical workflow for quantification of oxidation in the Fc/2 subunits of monoclonal antibodies and fusion proteins.

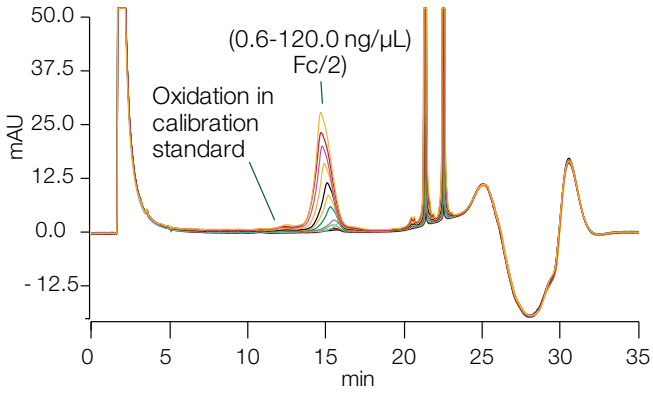


Figure 2. Overlay of ten representative chromatograms of MabThera® (Rituximab, Roche) originator at different concentrations, as used for external calibration both of the UV-spectroscopic and mass spectrometric detection systems. Conditions: Sample: 6.0–1200 ng Fc/2 of MabThera on column. Proteolysis with IdeS for 1 h at 22 °C; Reduction: 5 mM TCEP for 30 min at 60 °C; HPLC: MAbPac RP, 4 µm, 1500 Å, 150.0 × 2.1 mm i.d., 200 µL/min; 80 °C; 28.9% acetonitrile in 0.1% trifluoroactic acid for 5 min, 28.9–29% acetonitrile in 5 min 29–30% acetonitrile in 9 min, 30–45% acetonitrile in 5 min; Detection: UV @ 214 nm, Q Exactive MS; HESI (200 °C) at +4.0 kV; Full Scan *m/z* 1000–2500; R = 140,000 @ *m/z* 200.

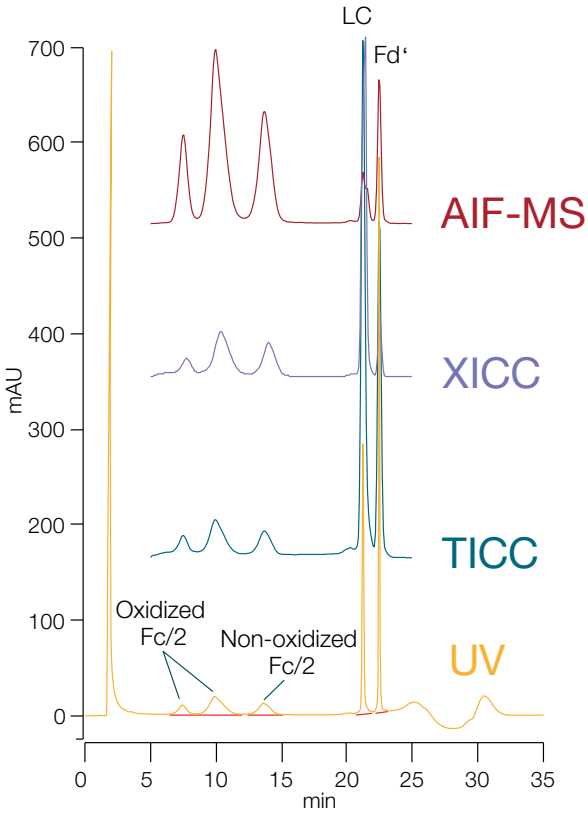
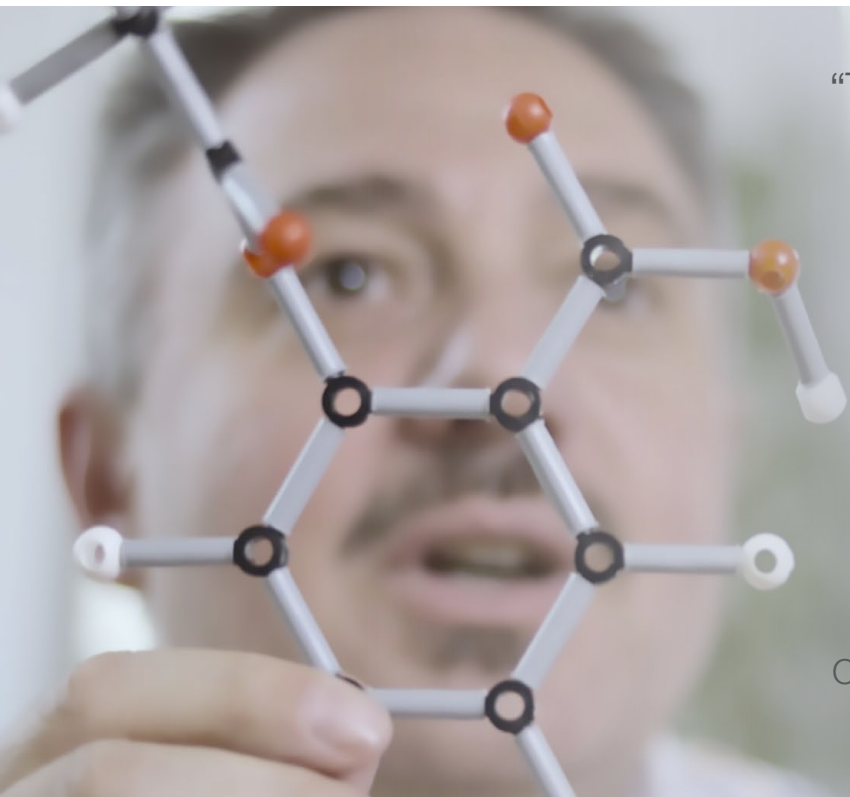


Figure 3. Chromatograms illustrating the different detection modes evaluated using UV- and MS detection, specifically UV detection at 214 nm (UV), full-scan total ion current detection (TICC), extracted ion current chromatogram detection of the seven most abundant charge state signals (XICC), and extracted ion current chromatograms of two fragments (b34 and y45) obtained upon all ion fragmentation (AIF-MS). Experimental conditions as in Figure 2.



"This method is unique in its ability to monitor oxidation; it is generic for any IgG 1 mAb, and it can be used in research and QC environments—where UV is preferred for detection and cGMP compliance is required—it even allows the site of oxidation to be determined via top- or middle-down MS!"

—Prof. Dr. Christian Huber,
Chemist, Christian Doppler Laboratory Head

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"We found the MAbPac RP column to be very robust and well-resolving. Although we used a very shallow gradient for the separation, we observed variability below 1% when we evaluated the intra- and inter-day standard deviations for retention times. Additional peak parameters were very robust, as corroborated by the RSD values for peak area, peak height, and peak width at half height."

—Christof Regl, MSc,
Biologist, Christian Doppler Laboratory

Christof Regl and his colleagues believe that this middle-up workflow of Fc/2 subunits could be developed into a 'multi-attribute monitoring' (MAM) method. Moreover, the approach enables absolute quantification of any IgG1 mAb at the Fc/2 level. This provides a broader picture of the influence of oxidation and other PTMs (e.g., lysine clipping, pyroglutamate formation, degradation, glycosylation) on the mAb. The method is unique in its ability to monitor oxidation; many other studies offer structural analysis and assignment of modifications, but absolute quantification of the oxidation on these samples is rarely performed. The method developed within the CDL can be applied in both research (top- or middle-down MS for site-specific determination of oxidation) and QC environments (UV-only for detection and quantitation in a cGMP environment).

Data analysis using Chromeleon 7.2 CDS gave calibration curves with correlation coefficients close to 1.0 and very narrow confidence intervals, as indicated by the orange lines in Figure 4. Calculation of the relative process standard deviations for the different quantification approaches yielded values in the range of 7.2–13.6%, which clearly indicates that the quantification method is robust and reliable. Compared to quantification *via* UV detection, the XICC method proved to give the most accurate quantitative results, showing a relative process standard deviation of 10.3% and a mean bias of only 7%. Raw data as well as data evaluations are freely available for download (DOI: 10.1021/acs.analchem.7b01755, and www.proteomexchange.org accession No. PXD006873) and may be utilized as benchmarking data.

Additionally, the study demonstrates that non-oxidized mAb can be used as a calibration standard, alleviating the need for isotopically-labelled standards or other molecules to be introduced into the analysis. This makes the entire protocol a 'platform approach' because you do not need to synthesize isotopically-labelled proteins or peptides. Even better, the standard sample does not need to be completely free of oxidized products, as this can be corrected for based on prior HPLC-UV analysis.

A generic 'platform method'—delivering specific results

Oxidation is a major tell-tale modification observed in mAb products that have passed their expiration date[6]. As oxidized variants of a therapeutic antibody may show altered clinical efficacy, reliable and accurate quantitation of oxidation is critical to ensure patient safety. With this middle-up technique, the CDL for Biosimilar Characterization has developed a fast, generic method for monitoring oxidation in Fc/2 subunits of any IgG1 mAb or Fc fusion proteins.

The benefits to this approach are multiple:

- Maintained context of the modification at the subunit level
- Detection by UV only, or coupled to MS for further characterization
- Easy transfer from an R&D environment to a QC laboratory
- Absolute quantitation of oxidized versus non-oxidized methionine for any IgG1 mAb or Fc fusion protein

"Even when I massively overloaded the column, it regenerated completely. It really is very robust. The MAbPac RP column did not have any problem with carryover and was stable for over 600 injections."

—Christof Regl, MSc,
Biologist, Christian Doppler Laboratory

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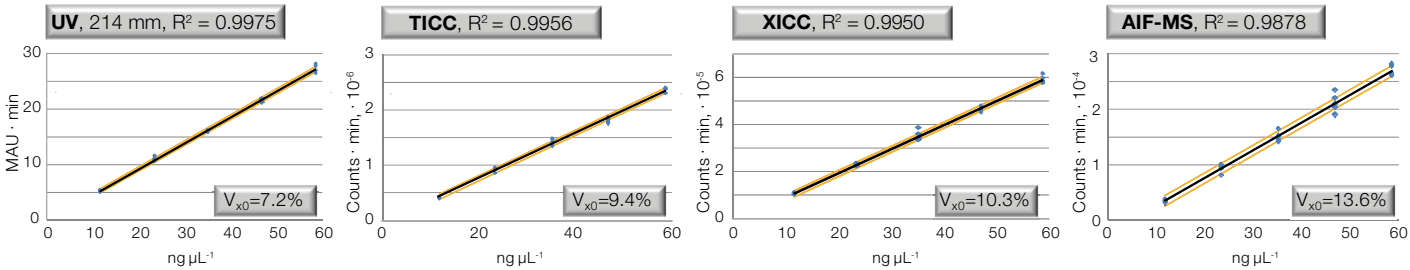

Figure 4. Calibration curves for the non-oxidized Fc/2 fragment of MabThera (Rituximab) using UV-spectroscopic and mass spectrometric detection. R^2 = correlation coefficient, V_{x0} = relative process standard deviation.

The method is very robust; digestion with IdeS minimizes the risk of inducing artificial oxidation and the MAbPac RP column offers outstanding separations, excellent resolution of oxidized species and demonstrated reproducibility over more than 600 injections. Moreover, protein carry-over between injections is minimal, which represents a basic requirement for reliable quantification. Prof. Huber and his co-workers believe that this workflow represents an appropriate standard method for any biopharmaceutical analytical lab which is challenged with the routine monitoring of methionine oxidation of an Fc-containing protein.

For more information on the Christian Doppler Laboratory for Innovative Tools for Biosimilar Characterization, please visit cdl-biosimilars.sbg.ac.at and www.thermofisher.com/CDL.

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