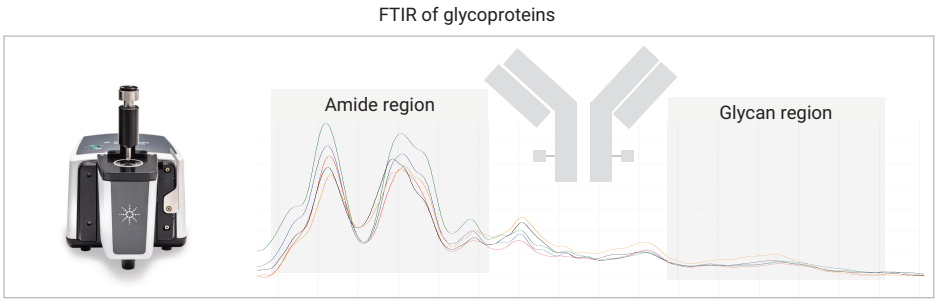
Application Note

Biopharma/Pharma

Protein Glycosylation Analysis Using the Agilent Cary 630 FTIR Spectrometer



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Abstract

Fourier transform infrared (FTIR) spectroscopy is a well-established tool in biopharmaceuticals for characterizing protein structure and assessing the integrity of therapeutic proteins. Glycosylation is a critical quality attribute (CQA) of protein biopharmaceuticals. This application note demonstrates the use of an Agilent Cary 630 FTIR spectrometer to identify and compare glycosylation in monoclonal antibodies (mAbs) and glycoproteins. The results suggest that FTIR is a simple and informative technique that provides a unique glycosylation FTIR signal to study protein samples.

Introduction

Protein glycosylation is a crucial post-translational modification (PTM) impacting the efficacy, stability, and integrity of therapeutic proteins, including monoclonal antibodies (mAbs) and other biopharmaceuticals. This process involves attaching sugar molecules, or glycans, to specific amino acid residues on proteins. Glycosylation is classified as a critical quality attribute (CQA) and has important functions in biological processes, impacting protein structure, solubility, and ability to resist proteolytic degradation.[1] Therefore, monitoring and controlling glycosylation is essential in developing and producing therapeutic proteins.

Various analytical techniques are employed to monitor glycosylation in biopharmaceutical production. These techniques include FTIR spectroscopy, mass spectrometry, high-performance liquid chromatography, capillary electrophoresis, nuclear magnetic resonance spectroscopy, and lectin arrays. However, most of these techniques require time-consuming complex sample preparation steps. Alternatively, FTIR is a robust analytical technique that provides a fast and easy tool for protein research. It is a nondestructive technique used to study protein molecules under different experimental conditions. The building blocks of glycans (monosaccharide/sugars), amide groups of protein, and water molecules produce distinguishable fingerprint absorption bands in FTIR. It has also been used to monitor the carbohydrate content and glycosylation profiles in glycoproteins.[2,3]

In this study, we used an Agilent Cary 630 FTIR spectrometer to demonstrate an FTIR spectroscopy method for protein glycosylation analysis. The results show that FTIR spectra of glycoproteins provide an FTIR signal corresponding to the glycosylation profile, allowing a rapid, nondestructive analysis method with minimal sample preparation.

Experimental

Instrumentation

The Cary 630 FTIR spectrometer was fitted with a single reflection diamond attenuated total reflection (ATR) module. Data acquisition was carried out with an Agilent MicroLab software using the parameters shown in Table 1.

Table 1. Agilent Cary 630 FTIR spectrometer with ATR operating parameters.

Parameter	Setting
Spectral Range	4,000 to 650 cm^{-1}
Sample/Background Scans	140/128
Resolution	2 cm^{-1}
Zero Fill Factor	None
Apodization	Triangular
Phase Correct	Mertz
Sampling Technology	ATR

Materials

Fetuin, alpha1-acid glycoprotein, ribonuclease B, and cytochrome c were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ristova, Reditux, and antibody-drug conjugate (ADC)/Kadcyla were purchased from Alliance Pharm (Singapore). NIST mAb monoclonal antibody reference standard was from Agilent Technologies (Santa Clara, CA, USA). PNGase F was obtained from New England BioLabs. Zeba Spin Desalting columns (7 K MWCO) were obtained from Thermo Fisher Scientific (Rockford, IL, USA). Ultrapure water was collected from an in-house MilliporeSigma Milli-Q system (Burlington, MA, USA).

FTIR workflow

The protein glycosylation workflow using the Cary 630 FTIR is shown in Figure 1. mAb samples were desalted using spin columns and the concentration was estimated to be 50 mg/mL. Protein samples were dissolved to 5 mg/mL without any further treatment. A sample of 15 µL was loaded on the diamond crystal of the ATR module and the absorption spectrum was recorded quickly (Table 1). After each measurement, the surface of the crystal was cleaned with ultrapure water. A background was recorded with the parameters used for the sample spectrum before every new sample. Agilent MicroLab Expert software, version 1.1.0.1, was used for the data analysis.

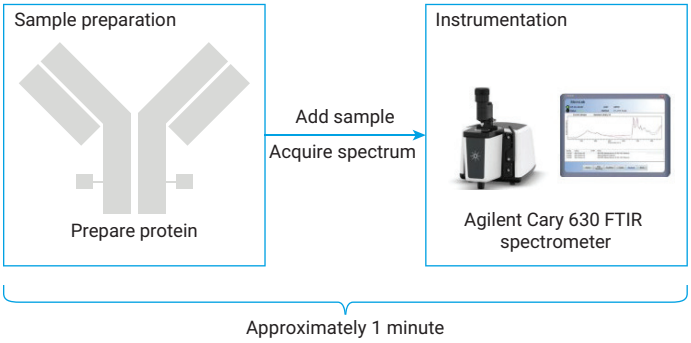

Figure 1. Protein glycosylation analysis workflow using an Agilent Cary 630 FTIR spectrometer.

Deglycosylation

The desalted mAbs were prepared in 20 mM Tris buffer pH 8.0. PNGase F was added to mAb solution containing the 5x reaction buffer. The digestion was carried out at 37 °C overnight.

Data analysis

The averaged spectra of five measurements were used for data processing. A blank average spectrum of water was subtracted from the averaged spectra of protein using the "Subtract Spectra" function of the Spectrum Arithmetic under the Mathematics tab. The blank subtracted protein spectra were merged using "Merge View" of the 2D View tab. Further processing and data analysis were done using baseline correction; the spectrum was normalized to equal peak area between 1,718 and 1,476 cm^{-1}. Savitzky-Golay smoothing algorithm (smoothing window 9) was applied using the Mathematics tab. Peak areas were calculated using the "Peak Picking" feature under the Mathematics tab. The data analysis steps are depicted in Figure 2.

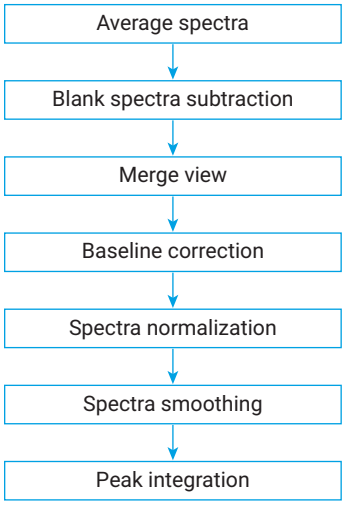

Figure 2. Data analysis procedure in Agilent MicroLab Expert software.

Results and discussion

Proteins exhibit characteristic amide absorption bands in FTIR spectroscopy, which are related to secondary structure and confirmation. The amide I band (1,600 to 1,800 cm^{-1}) and amide II band (1,470 to 1,570 cm^{-1}) result from the absorption of the carbonyl and N-H group.

Glycosylation is one of the common PTMs that occur in biopharmaceuticals. Carbohydrates linked to glycoproteins have an absorption band distinct from the protein amide region. The infrared (IR) absorption of glycan spans broadly in the 1,200 to 950 cm^{-1} region where proteins have very low signal intensity. It has been demonstrated that the amount of carbohydrates in the glycoprotein correlates with the intensity of these spectral bands.[3]

In this study, various glycosylated proteins with different levels of glycosylation were selected to evaluate the FTIR method. The formulated mAbs contain excipients that can exhibit absorption bands, and these signals may interfere with glycan analysis in the 1,200 to 950 cm^{-1} region. Therefore, mAb samples were buffer-exchanged with water using spin columns. Figure 3 shows the comparison of FTIR spectra of formulated and buffer-exchanged mAbs. The desalting step is necessary to eliminate buffer interference with the glycosylation measurement.

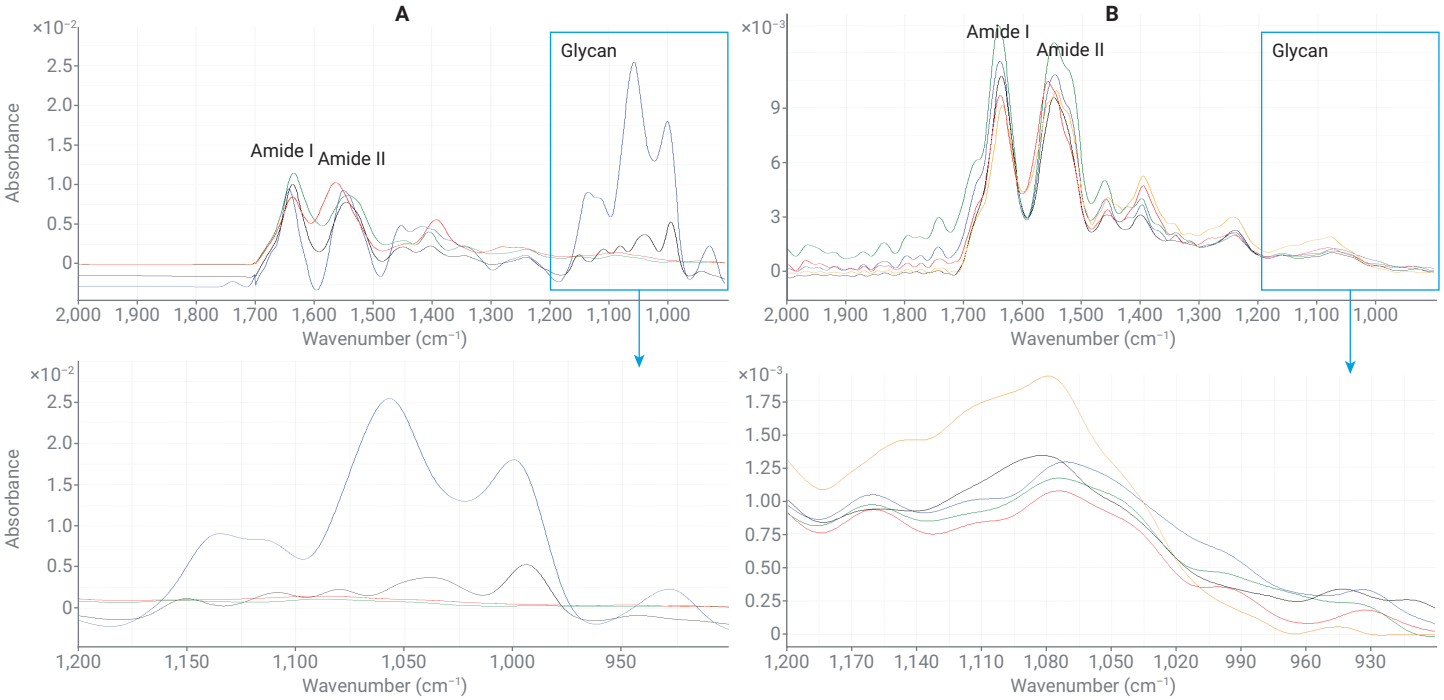

Figure 3. Processed FTIR spectra of (A) formulated and (B) desalted mAbs. The top panel shows the 2,000 to 900 cm^{-1} region covering the protein and glycosylation absorption bands. The bottom panel shows the zoom view of glycosylation spectral region (1,200 to 900 cm^{-1}). Samples: ADC (blue), Herceptin (black), NIST mAb (green), Innovator mAb (yellow), and Biosimilar mAb (red).

Five spectra for each protein were collected, taking an average of 140 scans. Figure 4 shows the mean spectra of each protein scaled to amide bond regions. The zoom spectra in the 1,200 to 950 cm^{-1} region reveals the variation in glycosylation levels of the glycoproteins. The nonglycosylated protein cytochrome c did not exhibit any IR absorbance band in this region. To access the global glycosylation levels, the area under the curve was calculated by integrating the 1,179 to 965 cm^{-1} spectrum region. Figure 5 shows the spectral band area related to glycosylation for each protein. The peak area clearly shows the comparable global glycosylation level of each protein to the reported values.[4,5]

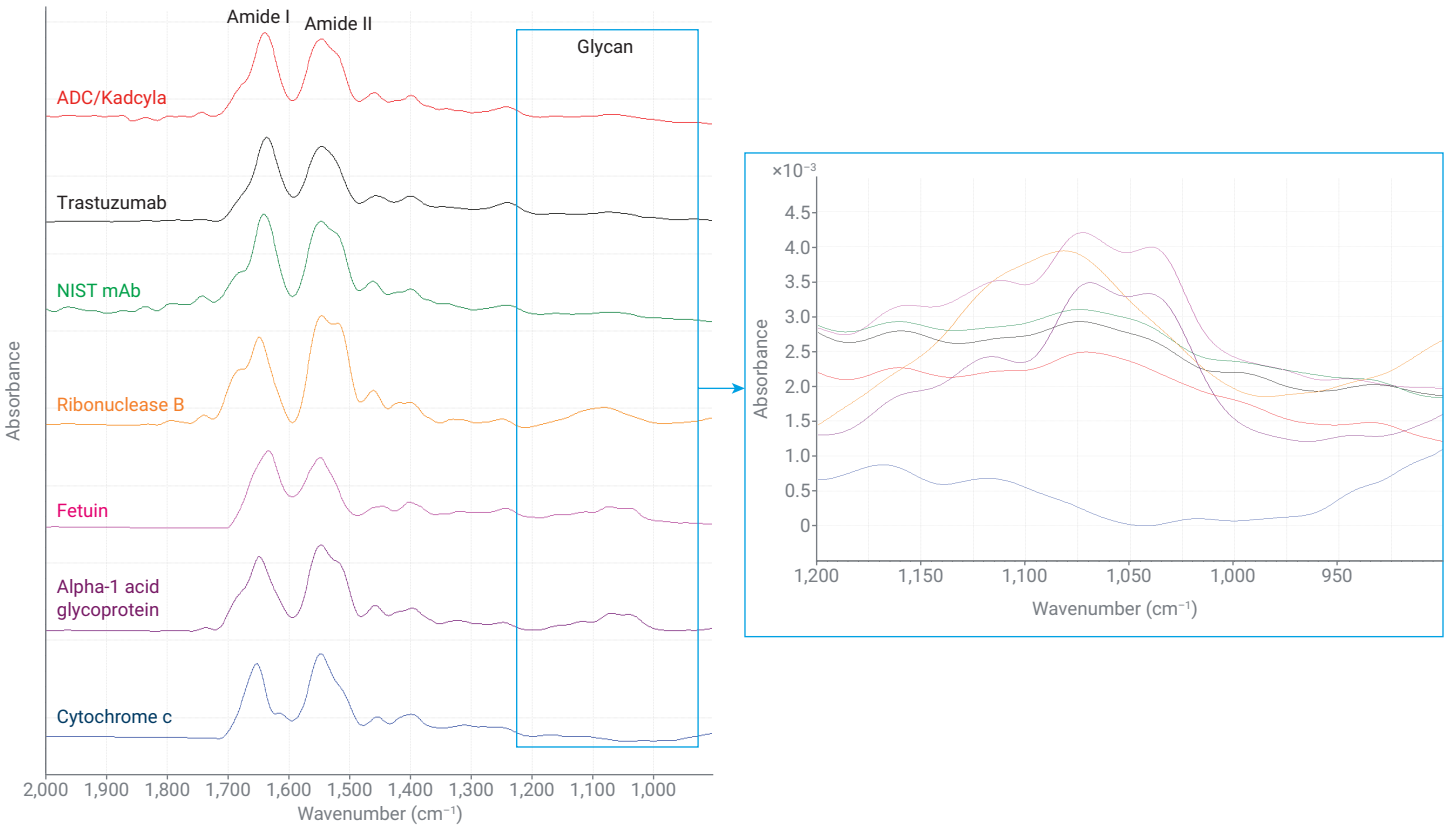

Figure 4. Processed FTIR spectra of proteins for the analysis of the global rate of glycosylation with zoom on the 1,200 to 900 cm^{-1} region, showing the different intensities of absorbance.

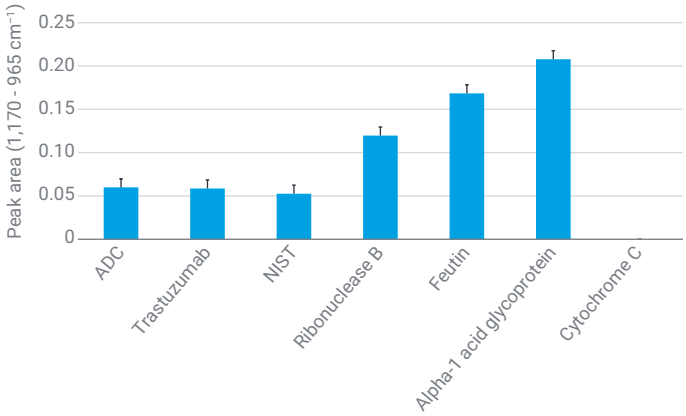

Figure 5. FTIR glycan spectra region 1,179 to 965 cm^{-1} peak area for the seven proteins.

To test the FTIR method, innovator and biosimilar mAbs were analyzed for glycosylation measurement. Figure 6 shows the comparison of FTIR analysis of glycosylated and deglycosylated Ristova and Redtiux mAb samples.

The absorption spectra of the mAbs before and after PNGase F treatment showed a reduction in band intensity. The glycosylation levels between these two mAbs were comparable, demonstrating the applicability of the FTIR method.

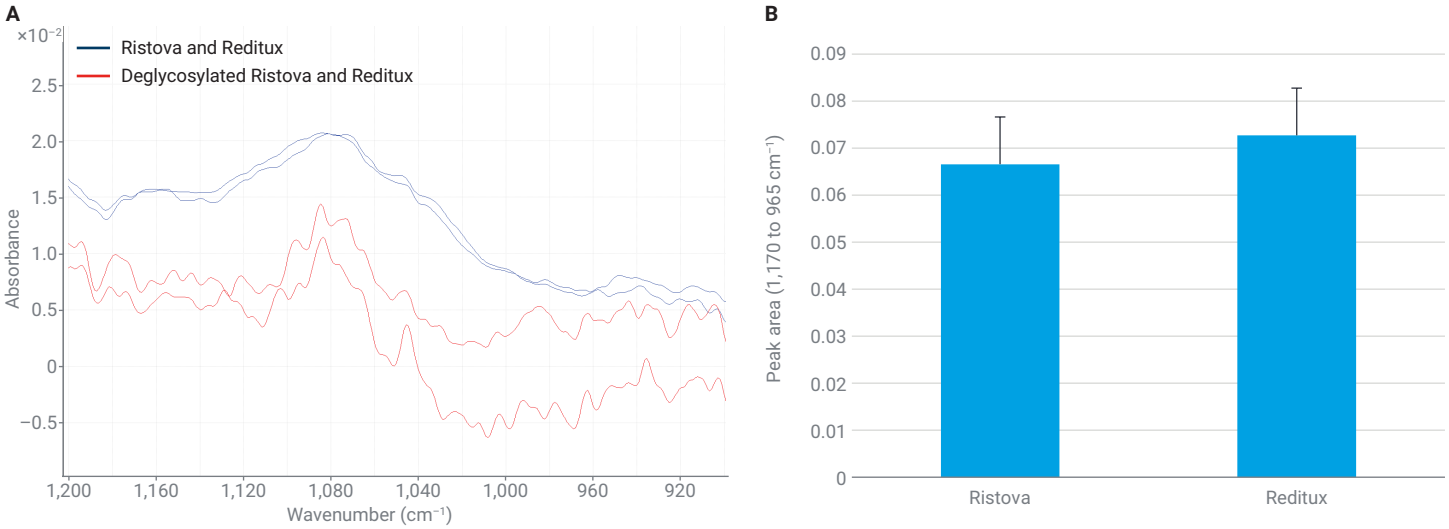

Figure 6. FTIR analysis of Ristova and Reditux mAbs. (A) processed FTIR spectra; glycosylated mAbs (blue), deglycosylated mAbs (red) and (B) glycan spectra region 1,179 to 965 cm^{-1} peak area.

Conclusion

This application note demonstrates the simplicity and usability of glycosylation analysis using an Agilent Cary 630 FTIR spectrometer. The FTIR method provides a spectral variation related to the global glycosylation level of protein, removing the necessity for extensive sample preparation or labeling. It provides a signal corresponding to the glycan profile that allows the identification of glycoproteins and enables rapid comparative glycosylation analysis between innovator and biosimilar therapeutic proteins. The user-friendly Agilent MicroLab Expert software streamlines data processing and shortens time to results. The technique may be used to verify batch consistency and product quality during the protein production process. Its capability for quick glycosylation estimation makes it an excellent fit for protein quality control workflows. In summary, the FTIR approach provides numerous advantages, including minimal sample preparation, reduced analysis time, and the capacity to analyze intact glycoproteins.

References

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