BRUKER

CONFOCHECK

- Infrared Protein Analysis

FTIR

Innovation with Integrity

CONFOCHECK: FTIR System for Protein Analytics

FTIR Protein Analysis

Infrared spectroscopy measures molecular vibrations caused by the specific absorption of infrared (IR) radiation by chemical bonds. For over 35 years, it has been known that the shape and frequency of the amide I band, which is assigned to the C=O stretching vibration within peptide bonds, is characteristic for the structure of the studied protein. From this single band the presence and relative percentage of secondary structure elements (α-helix, β-strand) in a protein can be determined and structural changes detected with high sensitivity.

The CONFOCHECK is a dedicated Fourier transform infrared (FTIR) spectrometer system for protein analysis in aqueous solution.

The main applications are the detection of conformational changes, concentration analysis and determination of the secondary structure. Its specific design facilitates a fast data acquisition with a high sample throughput and includes calibrations for protein concentration and secondary structure analysis.

FTIR Protein Analysis

Information Content of IR Protein Spectra

The information content of the IR protein spectrum is not limited to the amide I band. Since IR spectroscopic data follows Lambert-Beer´s law, the protein spectrum can be utilized for concentration determination. Also, side chains of various amino acids can be monitored and directly compared under varying environmental conditions (pH, salts, ligands), or in mutant proteins with altered primary sequence. Moreover, all other chemical components used in protein samples like certain pH buffers or protein-stabilizing agents are also represented in IR spectra by characteristic bands. Therefore, this method is also applied to reveal any changes of the chemical environment within the aqueous protein solution. In general the concentration of any molecular analyte dissolved in water can be determined by the CONFOCHECK, often down to the ppm level.

Fig. 1A: Secondary Structure information

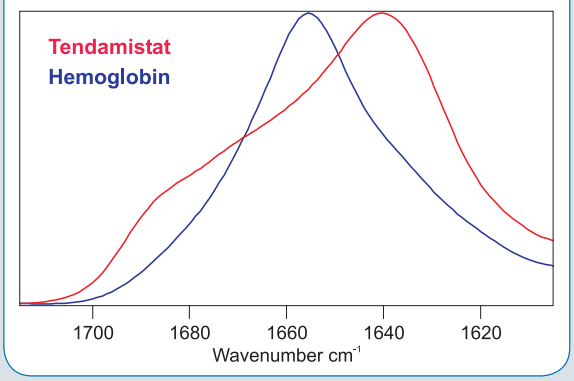
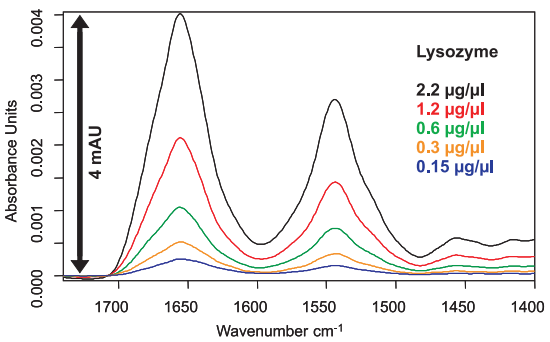


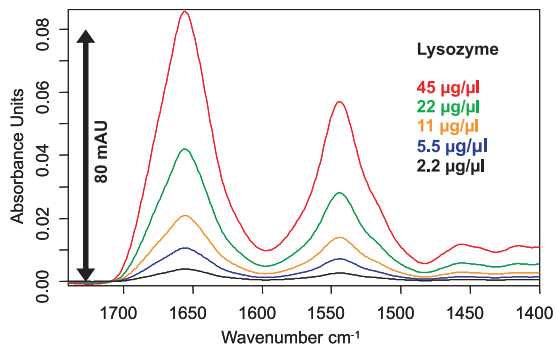
Figure 1A: The amide I band of the IR spectrum of a protein reflects its secondary structure. Here, IR spectra of Hemoglobin and Tendamistat are shown. The Hemoglobin amide I band has a position (~1652 cm^{-1}) and shape clearly indicative of a protein with a high α-helical content. The Tendamistat amide I band is characteristic (broad peak at ~1635 cm^{-1}) of a protein with a high β-sheet content.

Fig. 1B: Lysozyme in water, low concentrations



Figures 1B and 1C: IR protein spectra follow Lambert-Beer's law and are informative of concentration. A dilution series of Lysozyme in phosphate buffer saline (PBS) was generated and the IR spectrum at each concentration was acquired. Figure 1B contains Lysozyme IR spectra from a low concentration (0.15 µg/µl - 2.2 µg/µl), 100 sec acquisition time. Figure 1C shows Lysozyme IR spectra from high concentrations (2.2 µg/µl - 45 µg/µl), 25 sec acquisition time. The spectra clearly demonstrate the correlation between IR signal intensity and protein amount over a large dynamic range.

Fig. 1C: Lysozyme in water, high concentrations



FTIR Protein Determination and Structure Analysis

The demanding research lab and biopharmaceutical environments necessitate that an IR system provides accurate and reproducible protein measurements, rapid analysis (< 1 minute), and ease of use.

The CONFOCHECK system satisfies these demands. Unique in that it combines the necessary hardware with software for precise spectral measurement and interpretation using internal calibration models.

The stability of the system permits high sensitivity (dynamic range: 0.03 to >200 µg/µl) and direct analysis of proteins in any biological buffer with a spectrum acquisition time of under 1 minute. Due to its large dynamic range dilution of protein samples is not required for infrared protein analysis which makes it superior to CD spectroscopy and biochemical staining techniques. IR spectroscopy can also be applied to quantify, in parallel, other components of aqueous samples like detergents, sugars, buffer components, and contaminants (e.g. urea, acetonitrile, etc).

The robustness of the system enables the resolution of small differences (<1 %) in protein structure, critical to assessing protein stability to differing stresses, formulations, and conjugations. These structural changes include conversion form α-helices to β-sheets; from intra- to intermolecular β-sheets; and unfolding of α-helix and β-sheet. Also, the degree of protein aggregation can be monitored by quantifying intermolecular beta sheet formation. The inclusion of internal concentration and secondary structure calibrations minimizes user setup time and simplifies spectra interpretation.

Even structural effects on a protein upon binding to drugs or interacting with substrates can be measured with FTIR spectroscopy. The CONFOCHECK enables also to study the formation of multimeric structures like aggregates or fibrills, often in real time.

Typical applications in Pharma:

- Pre-formulation and formulation studies: determine the effects of differing formulations on protein stability, quantify protein aggregation, analyze protein conjugates
- Forced degradation studies: examine protein stability and aggregation, perform accelerated stability tests (ph, temperature, mutations, etc…)
- Quality control: test protein concentration, structure, stability, and purity in the production environment
- Binding studies: analyze the effects of protein ligand binding on protein structure

Stability Study

Stability studies of an antibody using the AquaSpec transmission accessory.

Fig. 2A: Native and stressed antibody

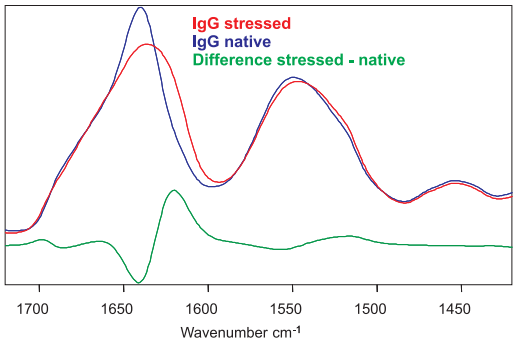


Figure 2A: Examination of antibody stability by difference spectroscopy. The difference spectrum (green) of the stressed antibody (red IR spectrum) minus the unstressed antibody (blue IR spectrum) reveals a loss of intramolecular β-sheets (negative band at 1640 cm^{-1}) and formation of intermolecular β-sheet (positive band at 1622 cm^{-1}). These secondary structure changes are quantifiable.

Fig. 2B: Antibody stability in different formulations

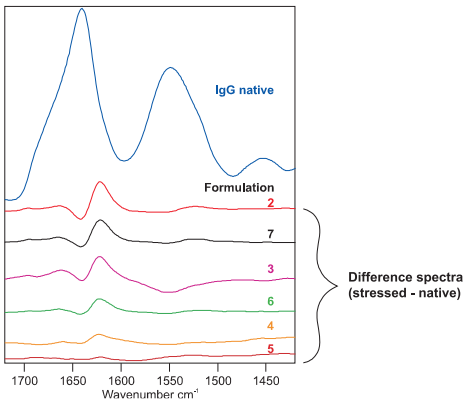


Figure 2B: Identification of the optimum antibody stabilizing formulation. The blue spectrum is the initial protein spectrum of the unstressed reference sample against which the difference spectra were calculated. The difference spectra originate from six similarly stressed samples containing the same antibody but being formulated differently. More stabilizing formulations will generate difference spectra with lower intensity. Therefore, the most stable protein formulation is formulation number 5. The ratio of each difference spectrum with the amide I band of the native protein correlates to the amount of structurally changed protein. Therefore, conformational changes of proteins are not only detected but also quantified by IR spectroscopy.

Temperature Ramp

Thermal stability studies of a therapeutic antibody using the BioATR II accessory.

The CONFOCHECK software permits the user designated configuration of temperature ramps. When the temperature range (maximum from 0° - 95° C), increment and equilibration time are set, the system starts to acquire automatically a spectrum at each predefined temperature.

The IR spectra of an antibody at different temperatures (25-95° C) are shown (Figure 3A). As indicated by the unchanged amide I band shape the protein maintains its native-sheet structure up to ~60° C. Further heating causes the amide I band maximum at 1636 cm^{-1} to decrease in intensity indicative of intramolecular-β-sheets unfolding. Unfolding of the native secondary structure leads to intermolecular-β-sheet formation reflected by an increasing IR signal at 1623 cm^{-1}.

The percentages of the structural changes (Figure 3B) at each temperature were determined by calculating the relative signal change in the amide I band (difference spectroscopy as shown in Fig. 2B).

Using the derivative of the curves of Figure 3B, two melting points of different structural domains of the antibody can be distinguished; one at ~66° C and one at ~79° C (Figure 3C).

Fig. 3A: Antibody spectra in temperature ramp

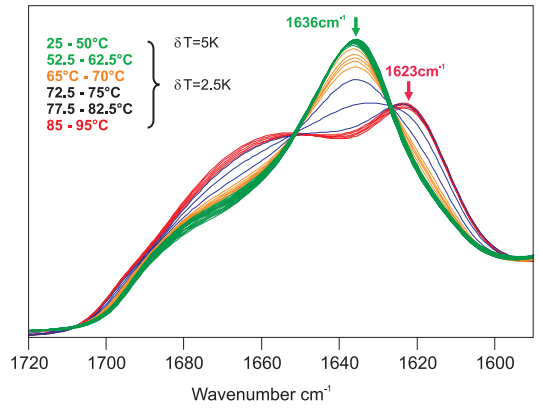
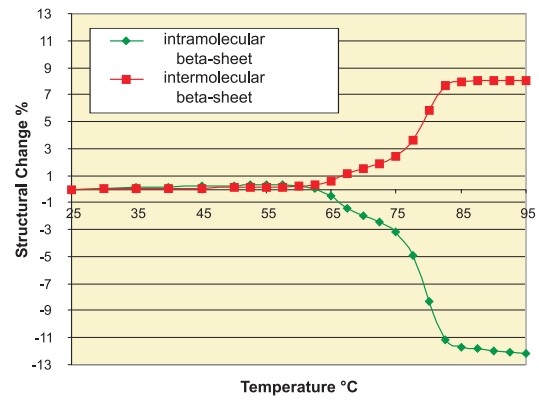


Fig. 3B: Temperature induced structure changes IgG



Protein Dynamics software

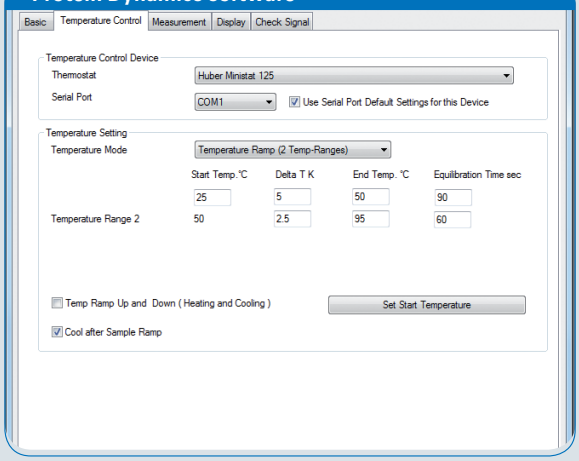
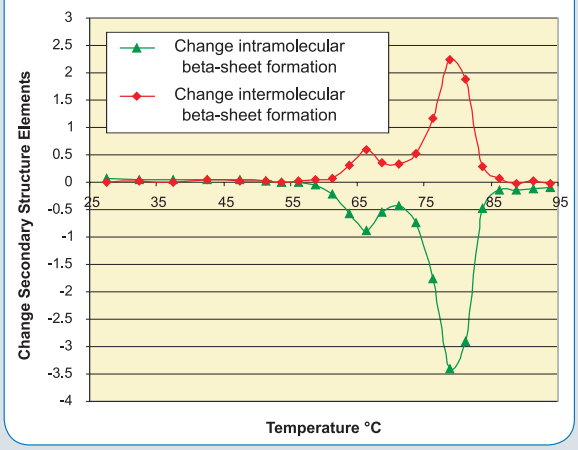
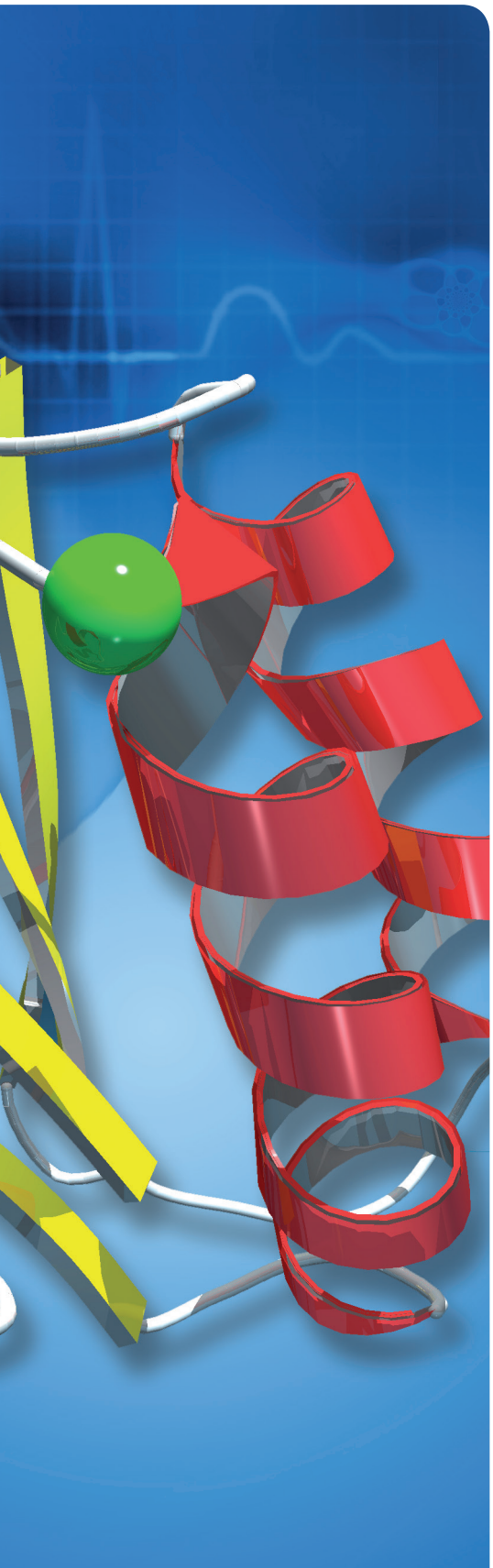


Fig. 3C: Denaturation temperature IgG



CONFOCHECK: A Dedicated FTIR System for Protein Analysis



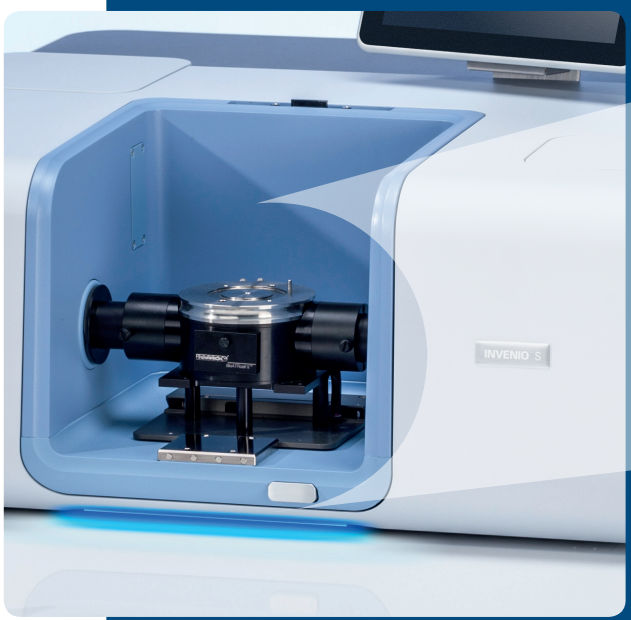
The CONFOCHECK is a compact IR spectrometer system dedicated to the analysis of proteins in aqueous solutions. Its robust design permits fast data acquisition while maintaining high sensitivity thus making high sample throughput possible. The system is controlled by a user friendly software interface that guides the user through the measurement procedure for spectral acquisition and temperature ramps. The system includes internal calibrations for protein concentration determination and secondary structure prediction, greatly simplifying experimental setup and spectral interpretation.

For analysis, a protein sample is injected into the AquaSpec IR transmission cell in a flow-through manner. Typically, 20 µl of protein sample are sufficient to load the cell and to obtain a high quality protein spectrum within a minute. From the spectrum, the α-helix and β-sheet content within the protein and the protein's concentration are determined within seconds.

To follow temperature induced structural changes, 20 µl of a protein solution are pipetted onto the measurement crystal of the BioATR II accessory. The sampling area is sealed before the user defined ramping program is started. The sample chamber temperature is controlled by the user software via a thermostat: The measurements are performed automatically in the programmed temperature range (max. 0° C - 95° C) with defined increments and equilibration times. The resulting set of protein spectra allows the assignment of the type of structural changes exhibited over increasing temperatures and the calculation of the "melting point" of the protein secondary structures.

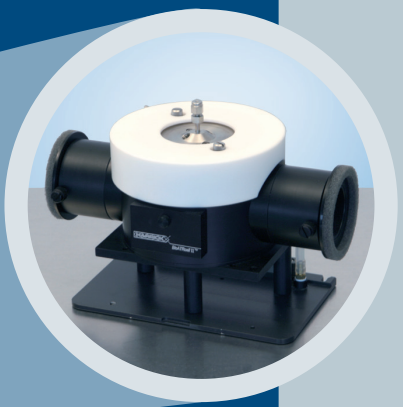
In addition to temperature ramping experiments, the BioATR II is also very suitable for analyzing insoluble (membrane bound) or precipitated proteins as well as monitoring protein aggregation or fibrillation processes. Protein lyophilizates can also be analyzed with the CONFOCHECK system, requiring low amounts of dried proteins (1 - 10 mg) for a typical measurement.

CONFOCHECK utilizes three FTIR sampling accessory cells to cover all protein applications.



AquaSpec cell

This flow through transmission cell is used to study proteins in aqueous solution. The analysis of conformational stability, concentration and secondary structure composition are typical applications. For biocompatibility, all tubes are made from PTFE. In-line filters are integrated to prevent contamination of the cell volume by aggregated and precipitated protein.



BioATR II

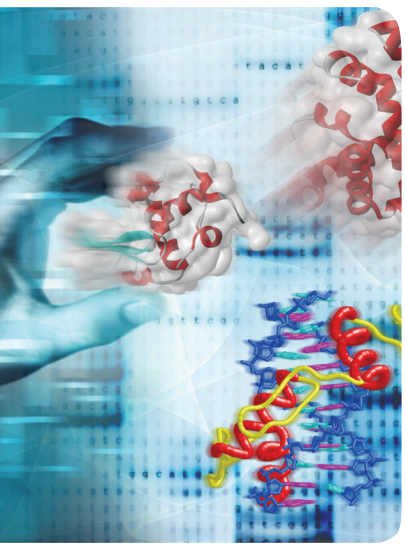
This ATR-accessory is dedicated to perform temperature ramp measurements for the investigation of thermally induced structural changes in proteins. Its optical path is optimized for the sensitive measurement of aqueous samples. Furthermore the BioATR II is suitable for analyzing aggregation and precipitation processes of water-soluble proteins and for the investigation of membrane proteins.

BioATR II with dialysis functionality

With this ATR-accessory dialysis experiments performed in the sampling compartment can be followed IR-spectroscopically. Low molecular weight compounds (ligands, salts, protons) can be applied to the protein of interest by dialysis in order to reveal structural alterations caused by the protein-ligand-interaction.

Applications for CONFOCHECK

- Protein quantification (down to 0.03 mg/ml in aqueous solution)
- Detection of conformational changes in proteins
- Protein dynamics: Unfolding, aggregation (temperature induced conformational changes)
- Determination of secondary structure
- Quantification of any molecular analyte in aqueous samples



Application Support

Bruker is staffed by expert life scientists and engineers that have in depth knowledge of protein analytics and instrumentation. Our product specialists are prepared to assist you with method development, selection and usage of sampling accessories, choices of optical components and software operation. We offer customized instruction and support packages to fit your needs.

Bruker systems are designed to provide many years of trouble-free operation, but should a problem occur a network of Bruker Optics offices and representatives throughout the world are ready to promptly respond to your needs. Professional installations and a high standard of post-delivery service are commitments Bruker makes to each of its customers.

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