

## Application Note AN B401

# An insight into the (un)stable protein formulation

The Fourier-Transform Infrared (FTIR) spectroscopy is a very sensitive method in the field of protein-biochemistry. It allows profound statements about the secondary structure of proteins in aqueous solutions as well as the identification and quantification of conformational changes. Especially in the field of pharmaceutical formulation of proteins like antibodies, this method has proved to be very useful. Here, the routine determination of the protein stability under several varying conditions which have to be evaluated is a challenging task.

Under what conditions does the protein remain stable? How long does this stability continue? In the process of optimizing formulations, these and other questions call for an answer. Classical protein-biochemical methods use, for example, analytical size-exclusion chromatography to detect multimeric aggregates in the presence of intact monomers. In most cases, however, the denaturation observed in this way begins mechanistically already at an earlier point of time. Often, it starts in with the conformational change of the protein and is typically accompanied by the formation of an antiparallel  $\beta$ -sheet structure. These conformational changes can be detected very sensitively using FTIR spectroscopy. So, this technique enables the identification of unstable formulations already at an very early stage. From every peptide bond that links the individual amino

### Typical issues in the field of protein formulation

- Protein stability in dependence of pH-value, temperature, salt concentration and stabilizing agents
- Characterization of protein unfolding and denaturation
- Determination of the secondary structure (content of  $\alpha$ -helix,  $\beta$ -sheet) of proteins

acids of the protein results a contribution to the typical amide bands (A and B, as well as amide I to VII) in the FTIR spectrum. The shape of the amide I band (C=O stretching vibration of the peptide bond) depends sensitively on the conformational structure of a protein and reflects the combination of the specific spectral properties of the secondary structure elements ( $\alpha$ -helix,  $\beta$ -sheet and random coil). Changes of the amide I band shape are an direct indication for changes of the protein structure. By comparing the shape of the amide I band of an unknown protein with those of proteins with structures already analyzed by x-ray diffraction or NMR, its secondary structure can be derived by using multivariate statistical methods. As an illustration, figure 1 shows the IR absorption spectra of two proteins

that differ greatly in the secondary structure. The secondary structure of most other proteins is between these two extremes, and consequently, the corresponding amide I band shape takes an intermediate position as well. So, there are significant, but relatively small differences in the absorption that are characteristic for the specific protein secondary structure. To make these spectral features in the amide I band more obvious, the second derivation of the spectrum can be used (fig.1, insert). On the basis of these spectral differences, the secondary structure of an unknown protein can be determined by the use of chemometric methods (e.g. Partial Least Square (PLS)).

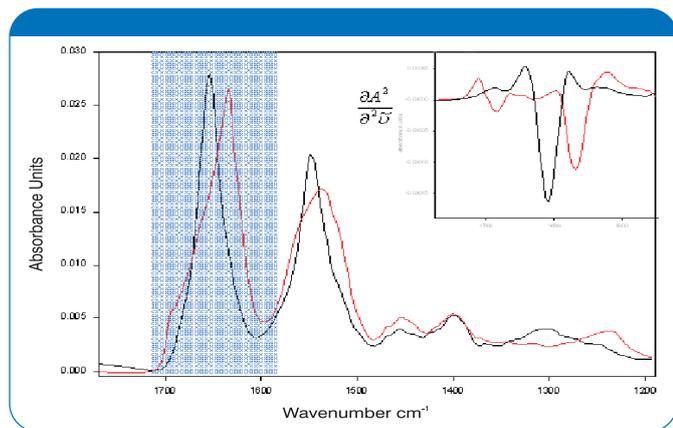


Figure 1: FTIR spectra of proteins with extreme ratios in their secondary structure composition: myoglobin (—, 73.9%  $\alpha$ -helix, 0%  $\beta$ -sheet) and concanavalin A (---, 0.9%  $\alpha$ -helix, 64.2%  $\beta$ -sheet), concentration 20 $\mu$ g/ $\mu$ l in phosphate buffer. These spectra have been measured using the FTIR system CONFOCHECK.

**Insert:** second derivation in the spectral range of the amide I band.

Generally, most proteins unfold when they are incubated at higher temperatures. Temperature increase can also be used to speed up denaturation processes that are induced by other effects in unstable formulations. Figure 2A shows the absorption spectra of antibody fragments acquired at a temperature of 70°C after three and sixty minutes incubation, respectively. The change in shape of the amide I band during the heating indicates a structural change that is resolved better in the 2nd derivations of the spectra (fig. 2A, insert). The difference of the spectra (fig. 2B) shows an increase in intensity at 1622 $\text{cm}^{-1}$  that points to the formation of intermolecular  $\beta$ -sheet structures. This peak can also be used for a qualitative and quantitative (!) characterization of protein formulations.

To test the stability of a formulation, proteins are often kept in complex buffers with a large number of additives like sugars, polyalcohols or amino acids. The proteins are stored either in dissolved state (liquid formulations), or in powder form (solid formulation) after lyophilization. Apart from the buffer composition and the lyophilization parameters (pressure, temperature profile and final moisture content), also the storage conditions (temperature, period of time) can have an influence on the integrity of the proteins. In the course of the formulation optimization, the analytics can reveal the effects of these conditions on the protein. If an effect on the protein structure is already detected at an early stage, the optimization process can be sped up and simplified considerably. Table 1 lists some typical issues that are relevant for the protein formulation. These questions can be answered using the FTIR spectroscopy.

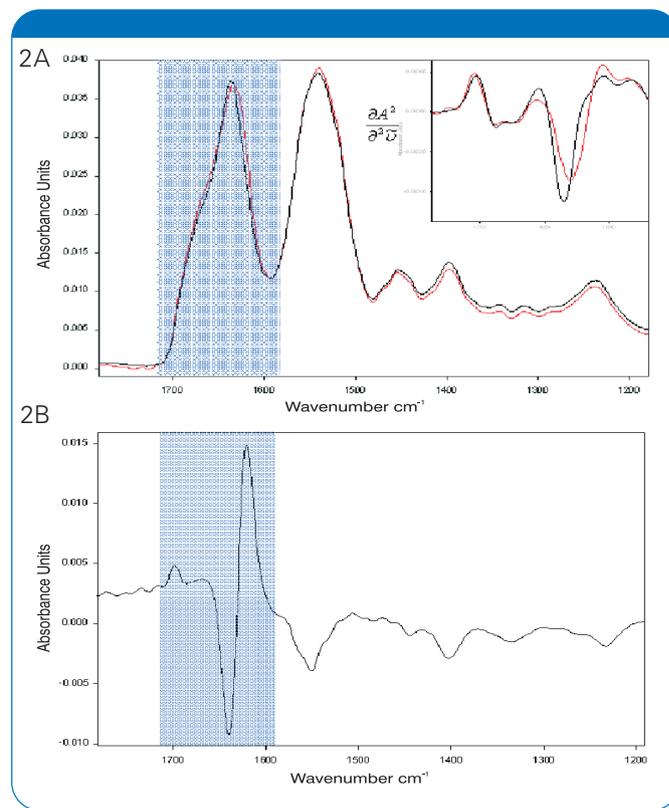


Figure 2: 2A: FTIR spectra of antibodies acquired during a heat treatment, incubation at 70°C for a period of 3 minutes (—) and 60 minutes (---).

**Insert:** second derivative in the spectral range of the amide I band. 2B: Difference of the spectra (60 minutes - 3 minutes, after a vector normalization). These spectra have been measured using the FTIR system CONFOCHECK.

**● Bruker Scientific LLC**

Billerica, MA · USA  
Phone +1 (978) 439-9899  
info.bopt.us@bruker.com

**Bruker Optics GmbH & Co. KG**

Ettlingen · Germany  
Phone +49 (7243) 504-2000  
info.bopt.de@bruker.com

**Bruker Shanghai Ltd.**

Shanghai · China  
Tel.: +86 21 51720-890  
info.bopt.cn@bruker.com

[www.bruker.com/optics](http://www.bruker.com/optics)