Technical Note

Guide to choosing the right protein/peptide analysis method for Direct Detect™ quantitation

Spectroscopic Principles of Direct Detect™ Quantitation

Infrared (IR) spectroscopy exploits the fact that molecules absorb specific frequencies characteristic of their structure. To form a protein or a peptide, amino acids are covalently linked via amide (peptide) bonds. Amide bonds absorb electromagnetic radiation in multiple regions of the mid-IR spectrum, including the strong band at 1600-1690 cm^{-1} ("Amide I"; Figure 1). In order to determine protein and peptide concentration, the Direct Detect™ Spectrometer measures the intensity (peak height) of the Amide I band, which is assigned to C=O stretching vibration of the peptide bond (about 80%) with a minor contribution from C-N stretching vibration (about 20%).

Three Methods, Three Sets of Standard Curves

The instrument is equipped with three analysis methods, each of which calculates analyte concentration in a distinct way depending on sample condition and spectral features in the analysis region (area of the spectrum between 1702 and 1602 cm^{-1}). Direct Detect™ software includes standard curves for each of the available methods. The incorporated calibration curves have been developed using a series of ten concentration points (each in triplicate), where the concentration value of each point was verified by amino acid analysis. All three curves have been developed using National Institute of Standards and Technology (NIST) bovine serum albumin (BSA) in phosphate-buffered saline (PBS). Spectra used to generate the calibration curves are not supplied with the software.

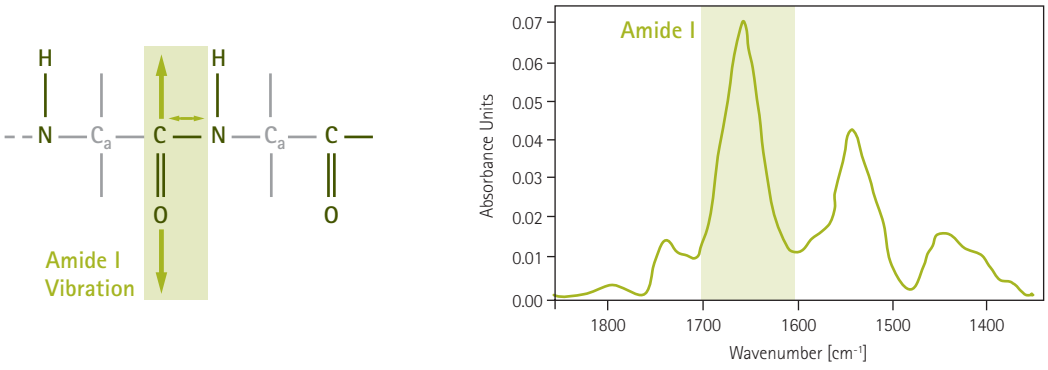

Figure 1.

Vibrations responsible for the Amide I band in the mid-infrared spectra of peptides and proteins (left panel). Amide I and Amide II bands are characteristic of proteins and peptides; analysis region is shaded (right panel).

Choosing Between the Three Methods

When determining the intensity of the Amide I band using the analysis methods, there are two important spectral features to consider: (1) Height of Amide I band and (2) Baseline. The intensity of the band is determined by using its height, which can be fixed or variable, as shown in Figures 3 and 8. In addition, it is important to consider the point from which the height of the Amide I band is determined by drawing an appropriate baseline. Again, there are multiple options for achieving this, which are provided in the following methods.

Analysis Method 1

Analysis Method 1 ("AM1") has been developed for analysis of protein and peptide solutions in a non-interfering environment (buffers or solvents that do not contain any chemical structures absorbing in the Amide I region of the spectra). The method should be applied to the measurements delivering spectral profiles such as that shown in Figure 1 (processed spectra) and Figure 2 (screenshot of raw data displayed by Direct Detect™ software), in which interfering components do not encroach into the Amide I band and the width of the band does not extend beyond the analysis region (1702 – 1602 cm^{-1}).

In practice, the full mid-IR spectrum of analyzed samples is collected by the Direct Detect™ spectrometer, and then, the software subtracts the buffer signal from only a part of the spectrum (1850 – 1350 cm^{-1}), resulting in a partially processed spectrum covering only the region where subtraction has been applied, including the Amide I and II regions. The Amide I band is used to calculate protein or peptide concentration by comparing the intensity of the analyzed signal to that of the known reference (NIST BSA in case of preloaded standard curves). The Amide II band is used for spectral reference only. Amide I signal from proteins and peptides is, in general, approximately two times stronger than the Amide II signal.

In the next step, the partially processed spectrum is integrated. In the integration method called Analysis Method 1, the software anchors the baseline at fixed points, between 1702 and 1602 cm^{-1} and determines the Amide I signal value at the highest point between these wavenumbers (see Figure 3). The Amide I signal of any given protein will appear within the analysis region (again 1702 – 1602 cm^{-1}); however, the exact location of its highest point depends on protein secondary structure. Analysis Method 1 accounts for possible shift in the location of highest point of Amide I band.

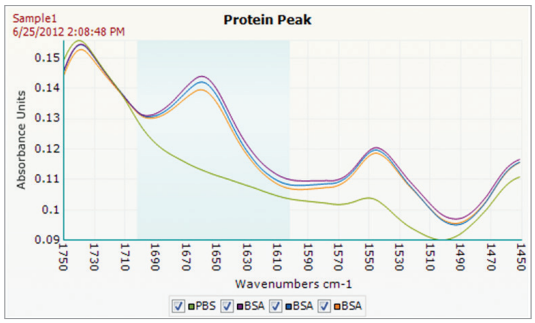

Figure 2.

Screenshot of protein and buffer spectra (displayed by the Direct Detect™ instrument) that were correctly analyzed using Analysis Method 1. "b" = buffer (PBS), "1, 2 and 3" = protein samples.

Note: Amide I band max = ~ 1660 cm^{-1}.

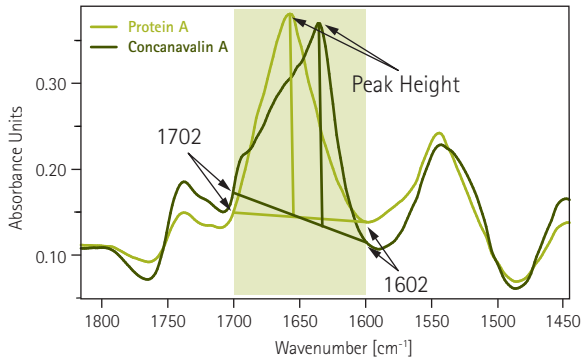

Figure 3.

Visual explanation of Analysis Method 1 (AM1).

Note: The "Analysis Region" is shaded.

Analysis Method 2

In cases where the analyzed protein/peptide is solubilized in a buffer that can interfere with IR signal of Amide I band (e.g., Tris), Analysis Method 2 might allow quantitation, regardless of the interference. The accuracy of the quantitation depends on whether the absorbance of the interfering agent overwhelms the protein's signal. All buffers containing chemical bonds that absorb mid-IR radiation within or close to analysis region (1702 – 1602 cm^{-1}) should be considered as potentially interfering. There are two ways to determine whether a given buffer/buffer component will interfere with measurement:

A. Non-empirical approach includes analysis of chemical structures of all buffer components. If bonds like C=O or C-N are present, the compound will most likely absorb in the analysis region and should be considered as interfering. For example, examining the chemical structure of monocitrate, the main component of commonly used citrate buffer, easily allows its designation as an "interfering buffer" (Figure 4).

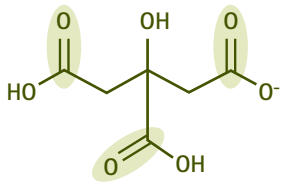

Figure 4.

Structure of monocitrate showing presence of three C=O bonds.

Similarly, analyzing the chemical composition of Tris buffer (Figure 5) reveals presence of a C-N bond that will, by definition, absorb in the Amide I region.

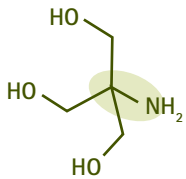

Figure 5.

Structure of Tris showing presence of C-N bond.

B. Unknown buffers can be empirically tested for interference using the Direct Detect™ instrument to measure spectral interference in the analysis region (Figure 6). All cases in which buffer shows absorbance in Amide I region should be considered as interference. In most cases, when it is possible to distinguish protein signal from the signal of the buffer, Analysis Method 2 might be used for accurate quantitation.

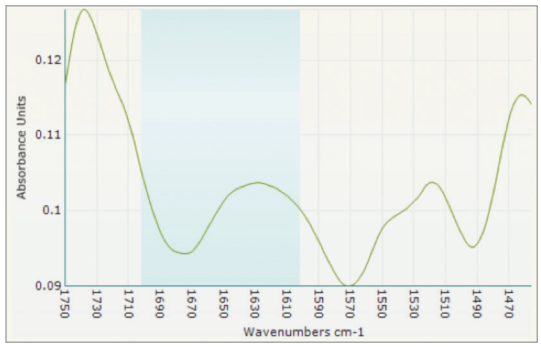

Figure 6.

Example of a buffer of unknown composition that shows absorbance in the Amide I region (shaded) of mid-IR spectra (screenshots of the spectrum displayed by the Direct Detect™ instrument; analysis region is shaded).

Analysis Method 2 has been developed to account for buffer interference appearing to the right of Amide I band (Figure 7). In Analysis Method 2, similarly to other two analysis methods, the software subtracts buffer signal from only part of the spectrum (1850 – 1350 cm^{-1}), resulting in a partially processed spectrum covering the region where subtraction has been applied. In the next step, the partially processed spectrum is integrated. In the integration method called Analysis Method 2, the software anchors the baseline between 1702 – 1602 cm^{-1} (same as in AM1) and then determines strength of the amide signal at the predetermined wavenumber, 1658 cm^{-1} (see Figure 8). The Amide I signal of any given protein will appear within analysis region (again 1702 – 1602 cm^{-1}); however the signal can be obstructed by the interfering buffer. Measurement of protein signals at predetermined wavenumber allows Analysis Method 2 to compensate for the buffer interference.

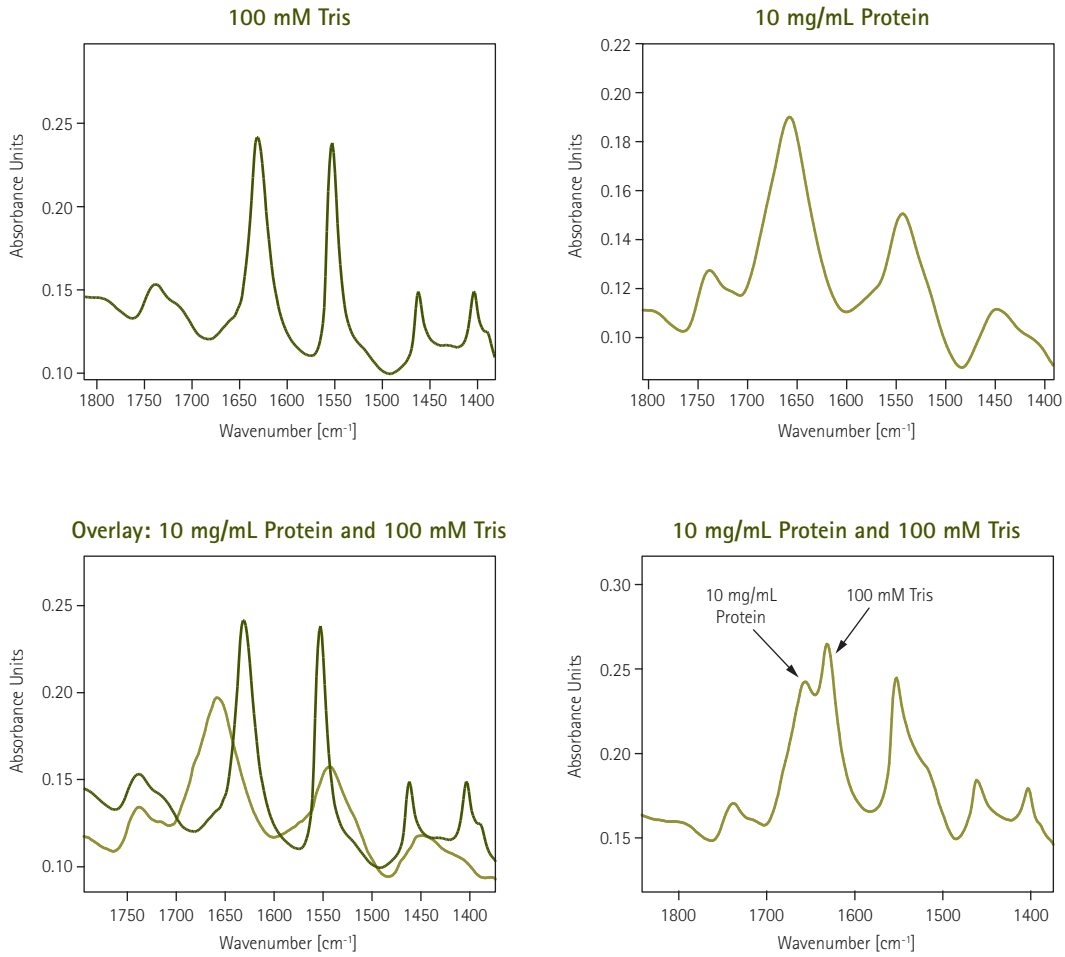

Figure 7.

Example of the mid-IR signal of the interfering buffer (top left), signal from pure protein (top right), the overlay of buffer spectrum with signal from pure protein (bottom left), and real (buffer subtracted) mid-IR spectrum of the protein in the interfering buffer (bottom right).

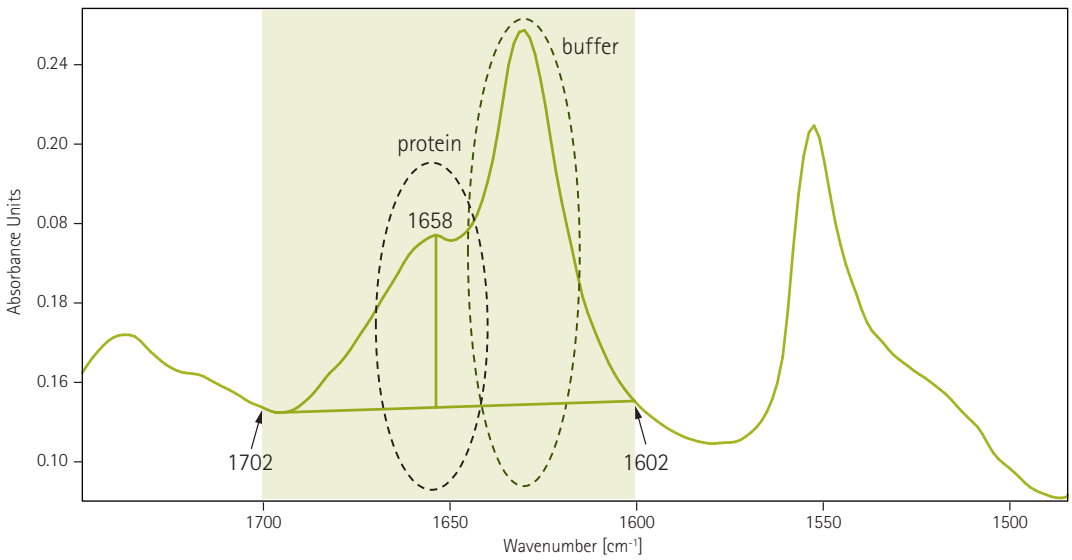

Figure 8.

Illustration of Analysis Method 2: processed spectrum of a protein in Tris buffer. Analysis region displays two signals—highest from the buffer and smaller (a shoulder) from the protein. Analysis Method 2 determines protein concentration based on the signal height at 1658 cm^{-1} with the baseline drawn between 1702 and 1602 cm^{-1}.

We emphasize that, at high concentrations of the interfering buffer, the Amide I "shoulder" will be completely obscured by the buffer signal, making Analysis Method 2 unusable. The concentration at which buffer signal starts to cover protein signal is completely buffer-dependent and has to be established experimentally for each and every interfering buffer. For example, 2 mg/mL BSA can be measured accurately in 50 mM Tris; however, the same 2 mg/mL BSA in 500 mM Tris cannot be accurately quantified by the Direct Detect™ spectrometer, because the BSA signal is undistinguishable from the Tris signal.

As mentioned above, the the exact location of Amide I band maximum depends on the secondary structure. For proteins dominated by β-sheets, the Amide I signal peaks at around 1630 cm^{-1}; helical proteins show maxima at around 1660 cm^{-1} while proteins dominated by β-turns peak at around 1675 cm^{-1}. Because Analysis Method 2 measures the strength of Amide I signal at the predetermined wavenumber (1658 cm^{-1}) it is extremely important to develop calibration methods that account for the location of signal maximum. The pre-loaded NIST BSA method (NIST BSA AM2) can be useful for accurate analysis of all proteins displaying Amide I band maxima at around 1660 cm^{-1}.

Analysis Method 3

The integration method "Analysis Method 3" (AM3) has been developed to account for the situations in which the solution component (buffer or analyte) is absorbing between Amide I and Amide II bands (Figure 9). In these cases, spectral features of Amide I, Amide II and intervening signal blend together, preventing proper baseline anchoring within the analysis region (1702 – 1602 cm^{-1}). Analysis Method 3 has been developed in order to analyze samples for which no robust baseline can be established within analysis region. In this method, as in the other two analysis methods, the software subtracts buffer signal from only a part of the spectrum (1850 – 1350 cm^{-1}), resulting in partially processed spectrum covering the region where subtraction has been applied. In the next step, the partially processed spectrum is integrated. In Analysis Method 3, the software anchors a baseline, that runs parallel to the x axis, at a basepoint (1760 cm^{-1}) outside the analysis region (still 1702 – 1602 cm^{-1}) and determines strength of the amide signal at the predetermined wavenumber, 1658 cm^{-1} (Figure 10).

Again, because Analysis Method 3 measures strength of Amide I signal at the predetermined wavenumber (1658 cm^{-1}), it is important to develop calibration methods that account for the location of the signal maximum. Similarly to AM2, the pre-loaded NIST BSA method (NIST BSA AM3) can be useful for accurate analysis of all proteins displaying Amide I band maxima at around 1660 cm^{-1}.

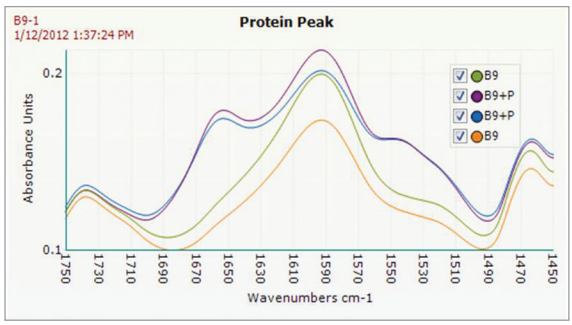

Figure 9.

Example of the buffer absorbing between Amide I and Amide II bands. Buffer spectra are displayed in green and orange. Protein in the buffer is shown in blue and violet.

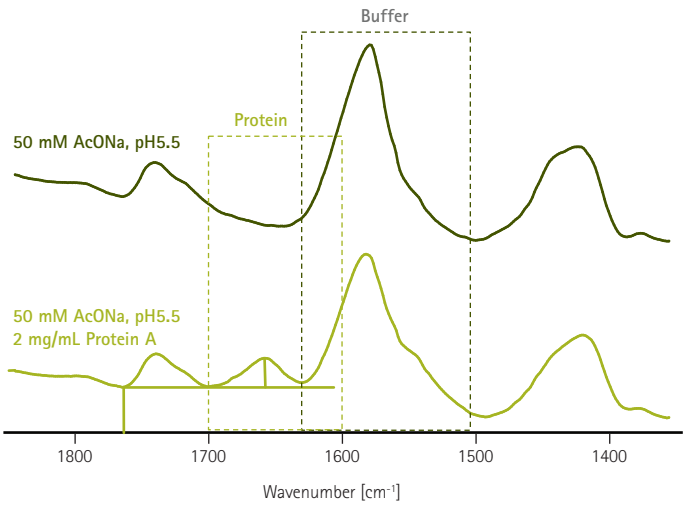

Figure 10.

Analysis Method 3. The baseline is anchored at 1760 cm^{-1} and the Amide I peak height is measured at predetermined wavenumber (1658 cm^{-1}).

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Direct Detect™ Spectrometer and Starter Kit	1	DDHW00010-WW
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Netbook Stand	1	
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Ethernet Cable	1	
Direct Detect™ Assay-free Cards (50/pk)	1	
Direct Detect™ Assay-free Cards (50/pk)	1	DDAC00010-GR
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