Compendium

BioPharmaceutical approach with vibrational spectroscopy

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Summary

Heavily-regulated biopharmaceutical manufacturers are increasing their use of the vibrational spectroscopy techniques mid-infrared (MIR), near infrared (NIR), and Raman spectroscopy because of these techniques' rapid, accurate analysis capabilities and their complementary nature. MIR spectroscopy is the analytical tool of choice for material verification in small molecule manufacturing due to its simplicity of implementation and its reliability and specificity. Recently, Raman spectroscopy has gained popularity in large molecule manufacturing, since it has increased sensitivity because of the resonance enhancement caused by the large size of molecules, as well as sensitivity to polymorphism. For certain applications like positive raw material verification of protein purification resins, the use of photoacoustic spectroscopy with Fourier-transform infrared (FTIR) offers unique selectivity and sensitivity. Vibrational spectroscopy plays a major role for analysis in upstream, downstream, and fill-finish processes. To support upstream processes, MIR, NIR, and Raman spectroscopy can be utilized for multi-attribute raw material testing. Further, in downstream processing, critical quality attributes (CQAs) like glycosylation, aggregation, and degradation can be determined at line or inline using Raman spectroscopy. Recently, NIR spectroscopy was demonstrated to have a wide variety of potential applications to improve speed and efficiency in different downstream unit operations, including capture chromatography, protein PEGylation reactions, and tangential flow ultrafiltration.

Real-time release testing (RTRT) in biopharmaceutical manufacturing has increasing importance. In this regard, macro-Raman measurements through primary packaging offer faster alternative tests for CQAs like pH, osmolality, and potency (strength), along with the positive identification of the drug product.

In addition, identification can be accomplished with macro- and micro-Raman spectroscopy in lyophilized biopharmaceutical analysis of cake morphology, siliconization, distribution of the drugs along with foreign particulate.

Currently, CQAs such as moisture and potency are commonly determined using destructive and time-intensive techniques like Karl Fischer titration. Since NIR spectroscopy is very sensitive to moisture, employing NIR for such analysis allows accurate and repeatable analysis of lyophilized cake through glass vials or containers in less than a minute.

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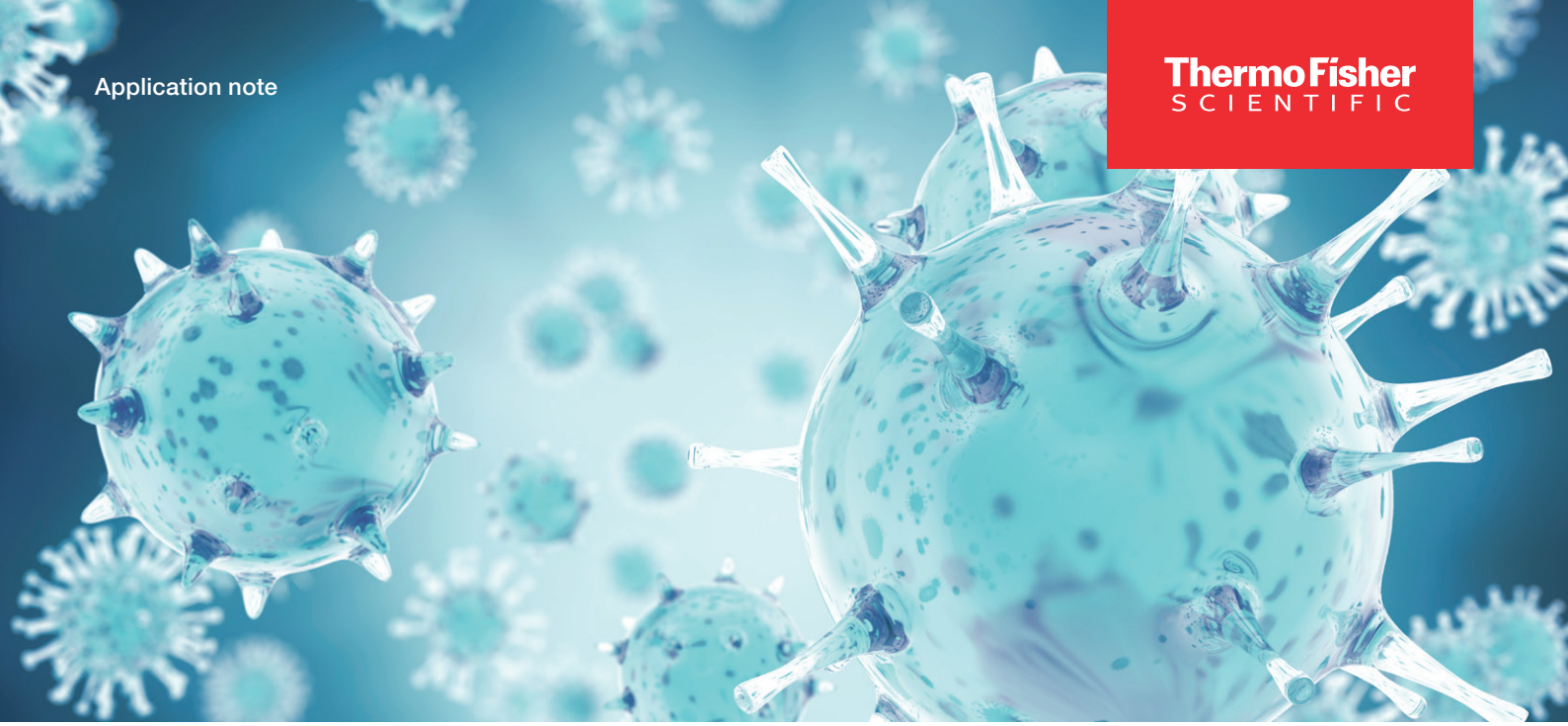

Application note

Protein secondary structure elucidation using FTIR spectroscopy

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Keywords

FTIR, ATR, protein structure elucidation, Biocell calcium fluoride cell, ConcentrateIR2 ATR, transmission

Abstract

Fourier-transform infrared (FTIR) spectroscopy is one of the most versatile analytical tools used across various disciplines. In this study, the Thermo Scientific™ Nicolet™ iS10 and Nicolet iS50 FTIR Spectrometers, equipped with attenuated total reflection (ATR) FTIR and transmission FTIR, were used for the determination of protein secondary structures. Structure calculations based on a protein database as well as spectral deconvolution are discussed. The analyses were quick and easy.

Introduction

Protein secondary structure describes the repetitive conformations of proteins and peptides. There are two major forms of secondary structure, the α-helix and β-sheet, so named for the patterns of hydrogen bonds between amine hydrogen and carbonyl oxygen atoms that create the peptide backbone of a protein.[1] Understanding protein secondary structure is important to gain insight into protein conformation and stability. For example, temperature dependent analysis of the secondary structure is critical in determining storage conditions for maintaining active therapeutic proteins.[2] Protein secondary structure is also crucial in understanding the structure–function relationship and enzyme kinetics of various proteins.[3]

FTIR has long been established as a powerful analytical technique to investigate protein secondary structure and local conformational changes.[1, 4] A typical protein infrared (IR) spectrum often contains nine amide bands, with vibrational contributions from both protein backbone and amino acid side chains. Among which, of particular pertinence to protein secondary structure are amide I and amide II bands. The absorptions associated with C=O stretching are denoted as amide I, whereas those associated with N–H bending are amide II. Since both C=O and N–H bonds are involved in the hydrogen bonding between different moieties of secondary structure, the positions of both amide I and amide II bands are sensitive to the secondary structure composition of a protein,[3, 4] although the amide II band is widely viewed as a less useful predictor for quantifying the secondary structure of proteins.

The shifts in the amide I band are often small compared to the intrinsic width of the band, resulting in one broad peak instead of a series of resolved peaks for each type of the secondary structure. Mathematical procedures such as Fourier self-deconvolution and second derivatives can be used to resolve the overlapping bands for the quantitative analysis of protein secondary structure.[3] Table 1 shows the secondary structure band assignments for proteins in water. Note that all assignments are depicted as a range, as the exact position of each peak varies from protein to protein due to the differences in hydrogen bonding interactions and the environment of the proteins.

Secondary structure	Band assignment in water
α-Helix	1,648–1,657 cm^{-1}
β-Sheet (high-frequency component)	1,623–1,641 cm^{-1} 1,674–1,695 cm^{-1}
Random	1,642–1,657 cm^{-1}
Coils	1,662–1,686 cm^{-1}

Table 1. Secondary structure band assignments for protein in water.[2]

With a range of sampling techniques, including transmission, ATR, and infrared reflection absorption spectroscopy (IRRAS), FTIR is particularly advantageous in terms of its versatility and general applicability compared to other analytical techniques for protein secondary structure analysis. Protein sample forms suitable for FTIR analysis include lyophilized powders, water solution, and colloids, to name a few. We report herein two examples of protein secondary structure determination using transmission FTIR and ATR, respectively. Both methods are fast, consume a minute amount of sample, and require minimal sample preparation.

Experiment

All proteins were procured from Sigma-Aldrich (MO, USA) and used as received. For the transmission studies, a BioCell™ Calcium Fluoride Cell (Biotools, Jupiter, FL) was used, and all measurements were carried out at ambient temperature. A 10 µL protein solution was placed at the center of the window, and the protein solution was sandwiched between the two CaF_2 windows, and placed in the holder. The concentration of protein tested was between 6 and 12 mg/mL. A 6 µm path length was created by sandwiching the two CaF_2 windows. CaF_2 windows are suited for water-based sample analysis. As water has a significant absorption peak at 1,645 cm^{-1} region, a small path length of 6 µm can effectively avoid saturated water peaks.

A purged Nicolet iS10 FTIR Spectrometer, equipped with a DTGS detector, was used for transmission analysis. The scan parameters used were 256 scans with a resolution of 4 cm^{-1}. The Thermo Scientific Smart OMNI-Transmission™ Accessory allows for a quick purge of the chamber, eliminating the need for water vapor subtraction in most analyses. Secondary structure analysis of the buffer-subtracted spectra was carried out using the built-in feature of the PROTA-3S™ FT-IR Protein Structure Analysis Software. Secondary structure calculation in PROTA-3S software is based on a database of 47 secondary structures (for more information visit **www.btools.com**).

For ATR analysis, a ConcentratIR2™ Multiple Reflection ATR Accessory (Harrick Scientific Products, Inc. Pleasantville, NY) with diamond crystal was used in a Nicolet iS50 FTIR spectrometer equipped with a mercuric cadmium telluride (MCT) detector. The diamond ATR has ten internal reflections with a nominal angle of incidence of 45 degrees. A 10 µL protein solution in phosphate buffer was dried on the surface of the ATR crystal under a stream of nitrogen. Scan parameters used were 256 scans and a resolution of 4 cm^{-1}. Secondary structure determination was carried out using the peak resolve feature of the OMNIC™ Software.

Results and discussion

Transmission-FTIR with Bio Cell

Figure 1 shows the overlay of three FTIR spectra: phosphate buffer, cytochrome C at 6 mg/mL and 12 mg/mL in phosphate buffer, respectively. At first glance, the spectra are predominantly water bands. The three spectra show little difference, even at a high protein concentration of 12 mg/mL.

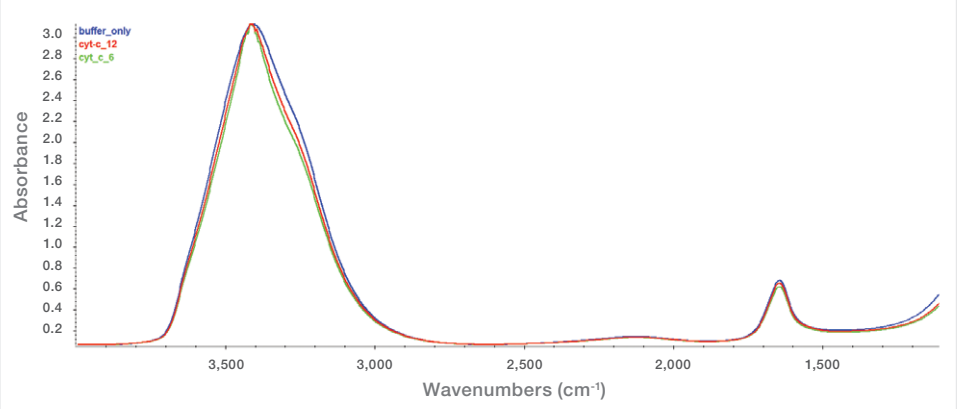

Figure 1. Transmission-FTIR spectra for cytochrome C in phosphate buffer (cytc_12) at 12 mg/mL and 6 mg/mL (cytc_6), and phosphate buffer blank.

Next, the buffer spectrum was subtracted from the raw protein spectra using the PROTA-3S software, and the results are shown in Figures 2A (cytochrome C) and 2B (concanavalin). The amide I and II peaks are clearly discernible in both spectra. The amide I peak position for cytochrome C spectra is 1,654 cm^{-1}, suggesting an α-helix dominant secondary structure. For concanavalin A, the amide I peak centers at 1,633 cm^{-1}, and there is also a noticeable shoulder peak at 1,690 cm^{-1} (red circle), indicative of the β-sheet component and its associated high-frequency component.[2]

Table 2 summarizes the secondary structure prediction using the PROTA-3S software. The cytochrome C has 45% α-helix and 5% β-sheet, whereas concanavalin A has 42% β-sheet and 4% α-helix. Differences in secondary structure composition between X-ray and FTIR data are likely due to the physicochemical state of the protein samples such as crystalline versus solution, temperature, pH, buffer conditions, etc. Furthermore, different prediction algorithms could have slightly varying outputs.[7] Notwithstanding the differences in analytical technique, sample state, and prediction algorithms, the secondary structure elucidation by FTIR using PROTA-3S software is largely in line with that from X-ray. Transmission-FTIR measurements combined with PROTA-3S software offer a facile and fast means to analyze the secondary structure of proteins in solution[2, 3] with minimal sample prep.

ATR-FTIR with ConcentratIR2 Accessory

When the quantity and concentration of protein are limited, FTIR measurements with the ConcentratIR2 Multiple Reflection ATR offer a better alternative than transmission-FTIR spectroscopy. The unique design of this ATR accessory allows for the direct measurement of protein powders, gels, solutions as well as proteins dried on the ATR surface. When concentrating proteins on the crystal surface, caution should be exercised in buffer selection since buffer will also concentrate on the surface of the crystal.

Only those buffers with minimum or no peaks in the amide I and II region should be selected. Figure 3 shows the ATR-FTIR spectra of BSA in phosphate buffer, dried on the crystal from a 1 mg/mL solution. In addition to the amide I and II bands, there are spectral features of the side chain, such as 1,515 cm^{-1} from tyrosine and 1,498 cm^{-1} from aspartic acid. Side chain peaks are critical for the elucidation of protonation and de-protonation states of various amino acids.[2]

	α-Helix (%)		β-Sheet (%)		Random (%)	
Protein	FTIR	X-ray	FTIR	X-ray	FTIR	X-ray
Cytochrome C	45	41	5	0	50	59
Concanavalin A	4	0	42	48	54	52

Table 2. Comparison of secondary structure calculation from FTIR (PROTA-3S) and X-ray data.

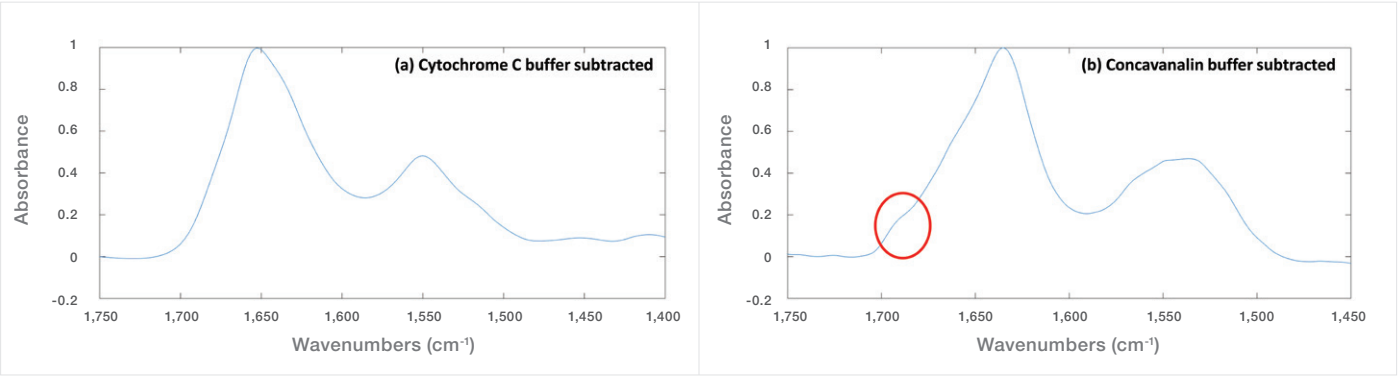

Figure 2. FTIR spectra of (a) cytochrome C and (b) concanavalin A after the buffer spectrum was subtracted using PROTA-3S software.

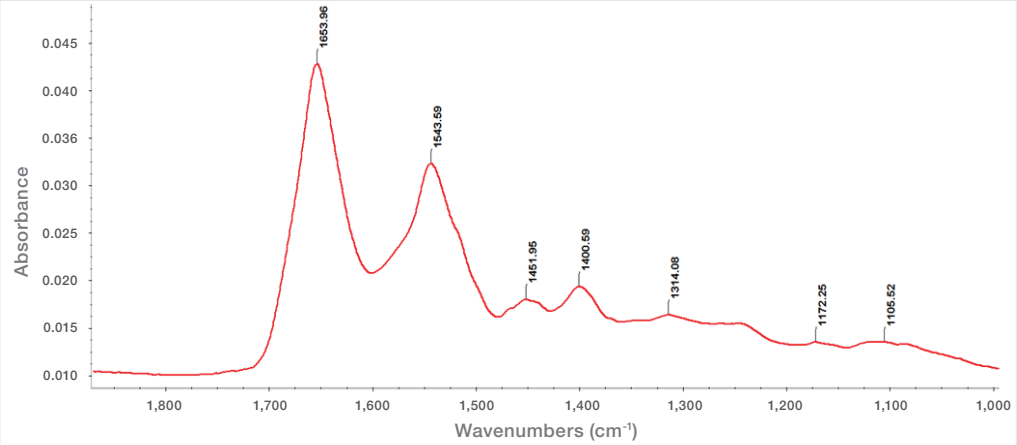

Figure 3. Amide I and II for 1 mg/mL BSA analyzed using ConcentratIR2 ATR on the Nicolet iS50 FTIR Spectrometer equipped with an MCT detector.

Peak deconvolution of the amide I peak (Figure 4) of BSA was carried out using the OMNIC software. It is important to note that second derivative analysis is often performed prior to deconvolution to clearly identify the peaks required for peak fitting.[2] In the current study, the second derivative peaks obtained (results not shown) are well correlated to the secondary structure peak assignments in Table 1. In order to obtain a good peak shape for peak fitting, a baseline correction on the amide I region was also performed. Baseline correction also effectively excluded the contributions from the amide II region. The deconvolution of amide I resulted in 5 peaks, and the area under each peak was then evaluated against the total area. Amide I peak deconvolution shows a secondary structure composition of 47% α-helix, 3% β-sheet, 24% coils, and 26% random, which is to published FTIR5 and X-ray data.

Conclusion

In this note, we have demonstrated two examples of protein secondary structure elucidation using FTIR spectroscopy. Transmission-FTIR measurements combined with PROTA-3S software provides a facile means to analyze secondary structure of proteins in solution with minimal sample preparation. When the quantity and concentration of protein are limited, ATR-FTIR offers a better alternative by drying the proteins in ATR crystals directly. The data were collected using an older model, the Nicolet iS10 Spectrometer. An improved model, the Nicolet iS20 Spectrometer, offers superior speed and performance over this predecessor model.

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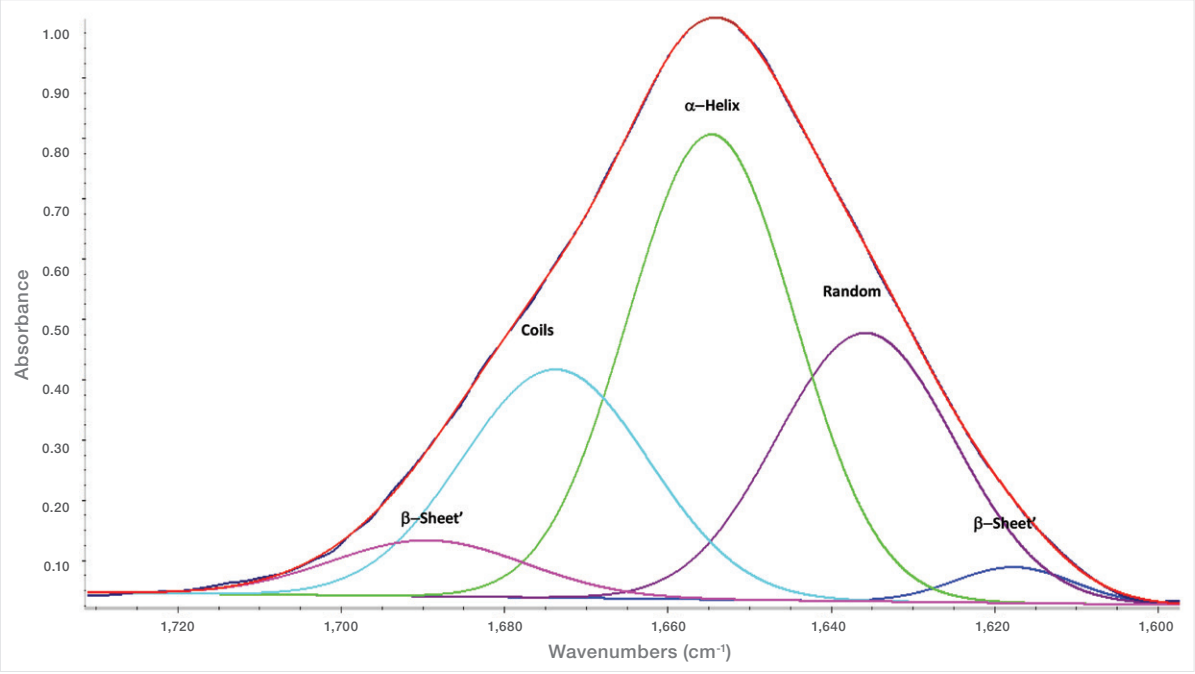
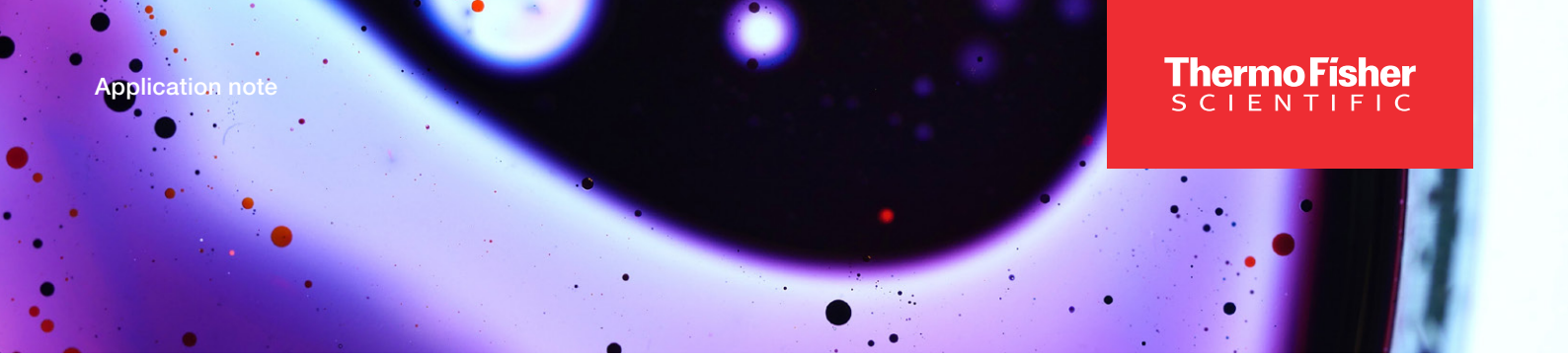


Figure 4: Peak deconvolution of amide I peak of BSA using Peak Resolve function of OMNIC software.



Application note

Protein concentration prediction in cell cultures

The next stage in NIR bioprocess analysis

Author

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Figure 1. Antaris MX FT-NIR Process Analyzer used for collecting thespectroscopic information from the cell cultures.

Introduction

Biologically produced materials are an increasingly important aspect in many industrial processes including those related to pharmaceuticals, food, diagnostics, and fuels. Most of these biologicals are produced in fermentors and bioreactors in which specialized cell cultures grow and manufacture the molecule of interest. Many different types of cells are used in culturing and producing biopharmaceutical products including genetically engineered bacterial and yeast cells. However a majority of the products are proteins cultured from mammalian systems such as Chinese hamster ovary (CHO), green monkey (VERO), or human embryonic kidney (HEK) cell lines. Many of these products are large complex proteins, hormones or polysaccharides that are impossible or difficult to manufacture in large quantities any other way. A recent survey of the US Food and Drug Administration noted that there are over 350 biologicals approved for various uses, including vaccines and diagnostic and therapeutically important antibodies.

Bioprocesses that produce the desired materials by nature rely on complex biological systems to synthesize their useful products. While typical chemical manufacturing processes have relatively little variability, the inherent complexity of biological systems makes a great deal of variability from batch to batch inevitable. As a consequence of the complexity and variability of the processes, it has been estimated that 30% of the production batches need to be reprocessed for quality reasons, which results in a tenfold loss in profit. Industries that rely on these complex biological systems benefit greatly from closely monitoring the growth of their cell cultures and production of the target molecule. Process analytical technology (PAT) initiatives in bioprocesses improve the overall product quality, reducing waste by accounting for this inherent variability.

Monitoring and controlling cell culture conditions greatly reduces this variability and results in improved target protein production. Fourier transform near-infrared (FT-NIR) spectroscopy has proven to be a useful technology for monitoring and controlling manufacturing processes including more specific bioprocess applications. It is also part of PAT initiatives across many industries including bioprocessing. Previous work performed on cell cultures using NIR spectroscopy has usually focused on monitoring and controlling nutrients, waste products, cell densities and other parameters related to the health of the cell culture. While these parameters are useful for determining the relative health of the cell culture, the more important parameter of interest is the actual production and concentration of the target molecule. Very few NIR studies have determined and measured protein concentrations in actual cell culture conditions. This application note demonstrates the feasibility of using the Thermo Scientific™ Antaris™ MX FT-NIR Process Analyzer (Figure 1) to predict protein concentrations at biologically relevant concentrations in dynamic cell cultures.

NIR spectroscopy uses light between 10,000 and 4,000 cm^{-1} to determine the identity and quantity of a variety of materials. Most molecules of interest absorb light in this region through combination or overtone vibrations. The advantage of performing spectral analysis on these absorption bands is that the light is able to penetrate more deeply into the material under analysis and does not require dilution or manipulation of the sample. Therefore NIR analyzers can be coupled directly into a process stream or tank where spectral analysis can be performed without human intervention. FT-NIR has been implemented in many different industrial, pharmaceutical and other process settings for many years and has proven to be extremely valuable in collecting real-time analytical data automatically. When used in process environments, the Antaris MX FT-NIR Process Analyzer is easily coupled to process control computers where it is an integral part of maintaining optimal manufacturing conditions. Because of these advantages and the need to control the inherently variable biological systems found in cell culture technologies, NIR is an excellent choice for analyzing different components in bioreactors including proteins.

Methods

Chinese hamster ovary (CHO) cell cultures were grown at optimal conditions until the cell concentrations reached approximately one million per millemeter, representing a typical cell density for a young and growing culture. Samples of the cell culture were tested on a Nova BioProfile® analyzer to determine concentrations of glucose, glutamine, lactate, and ammonia. The concentrations of these materials changed throughout the experiment and accounted for some variability that might be encountered across multiple cultures. The concentrations were variously and singularly altered by spiking the samples with nutrients or waste products or diluting the samples with unaltered cell culture. Each of those four components was altered so that two or three different concentrations were represented for each. Table 1 lists the concentration ranges for the various nutrient, waste, and protein components of the tested samples. This methodology also has the effect of removing covariance between the different components and protein present.

Component	Range (g/L)
Protein	0.16–5.00
Glucose	7.98–8.12
Glutamine	0.28–0.58
Lactate	0.45–0.90
Ammonia	0.05–2.39

Table 1. Concentration ranges of various components. The solutions represent over 35 different protein concentrations that also vary in concentrations of nutrient and waste components.

Ultrapure bovine albumin protein was added to the solutions to represent target protein synthesized by the cells. Genetically modified cell cultures are designed to produce the target protein in large quantities almost exclusively to all other cellular proteins. As a result, the protein concentrations in the cell culture media will often approach and exceed 5.0 g/L and consist almost entirely of the target molecule. Albumin protein is an excellent mimic for recombinant proteins because it is available in extremely pure form and contains NIR active groups essentially identical to a typical target protein from a cell culture. In this case, purity is extremely important because any extraneous material present will also have a NIR signal and would lead to confounding results. The albumin protein material was carefully weighed and added to the cell cultures in concentrations ranging from 0.16 to 5.0 g/L. Over 35 different solutions were produced that had a range of nutrient and waste as well as protein concentrations. These varied solutions resulted in 54 spectra that were used to build the chemometric method and 20 spectra that were used to validate that method.

The cell culture samples were scanned with an Antaris MX FT-NIR Process Analyzer in the range between 10,000 and 4,000 cm^{-1}. The analyzer was coupled to a transflectance probe with an adjustable path length. The gap distance was set to 1.25 mm for a total path length of 2.5 mm. Sixteen scans were averaged per spectrum and were collected using eight wavenumber resolution with a gain of 0.1. Sample time took approximately 15 seconds. Two spectra were collected per sample. Figure 2 shows images of the probe before insertion into a cell culture sample and during spectral collection.

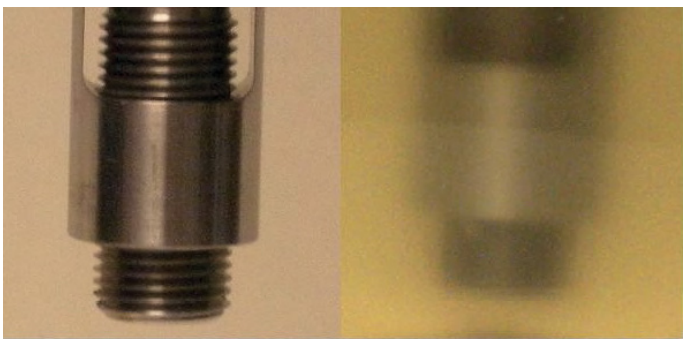


Figure 2. Transflectance probe used for data collection. Left panel shows the design of the probe with the adjustable pathlength. Right panel shows probe inserted into cell culture during data collection.

The sample spectra were loaded into the Thermo Scientific TQ Analyst™ Pro Edition Software for chemometric analysis using a partial least squares (PLS) method with a constant pathlength. The spectra were analyzed in the first derivative using a Norris smoothing filter. Two regions were used for the analysis: 8,910 to 5,340 cm^{-1} and 4,830 to 4,340 cm^{-1} These two regions collected information across a wide range of data points while avoiding the totally attenuating water peak centered around 5,100 cm^{-1}. Figure 3 shows representative raw spectra and the first derivative spectra of the samples.

Results

PLS analysis of the protein concentrations in the various cell culture samples revealed excellent predictive capabilities within the range of materials tested. The 54 spectra used to develop the PLS method are shown on a calibration plot (Figure 4) that compares the calculated protein concentrations versus the actual concentrations.

The calibration plot can be used to determine how well the method predicts the actual protein concentrations in the samples. The plot developed by the chemometric method resulted in a correlation coefficient of 0.977. Root mean square error of calibration (RMSEC) was 0.33 g/L and the Root mean square error of prediction (RMSEP) calculated from the 20 validation samples was 0.31 g/L. Additionally, the Root mean square error of cross validation (RMSECV) was 0.51 g/L. These errors indicate that the protein concentration in the cell culture samples can be predicted to 0.5 g/L or less. Approximately 1/3 of this error was attributed to the balance used to weigh the protein material.

Conclusions

Measuring protein concentrations in living dynamic cell cultures was successfully performed with the Antaris MX FT-NIR Process Analyzer. Protein concentration is a critical parameter in determining the success and quality of a cell culture in manufacturing a viable end product. This NIR technique successfully demonstrates the ability to measure and monitor protein concentrations in real time at relevant concentrations. The developed method shows excellent correlation with actual protein concentrations between 0.16 and 5.0 g/L and with errors of less than 0.5 g/L.

This application demonstrates the continued capability of the Antaris MX FT-NIR Process Analyzer to be successfully used in bioprocess environments where it can safely, accurately and automatically monitor and control cell cultures. While previous NIR studies have monitored cell culture conditions to promote optimal protein production, few have actually monitored and predicted protein concentrations. This feasibility study shows the power of the Antaris MX FT-NIR Process Analyzer to correctly predict target protein concentrations in a live and dynamic cell culture.

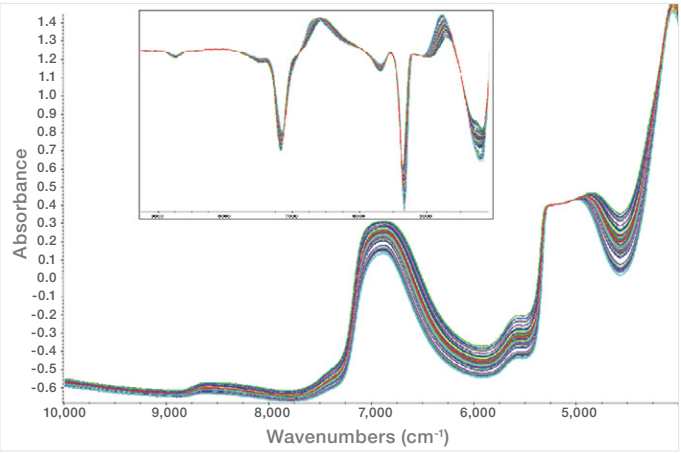


Figure 3. Representative raw spectra showing the variability present in the cell culture samples. Regions of analysis avoided the attenuated water peak at 5,100 cm^{-1}. Inset shows the first derivative spectra used for the PLS chemometric method.

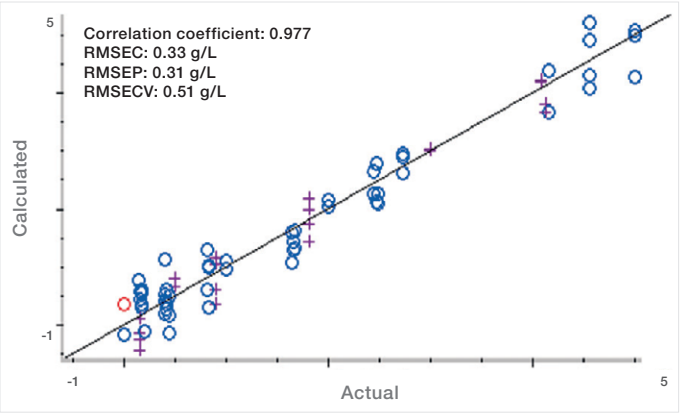
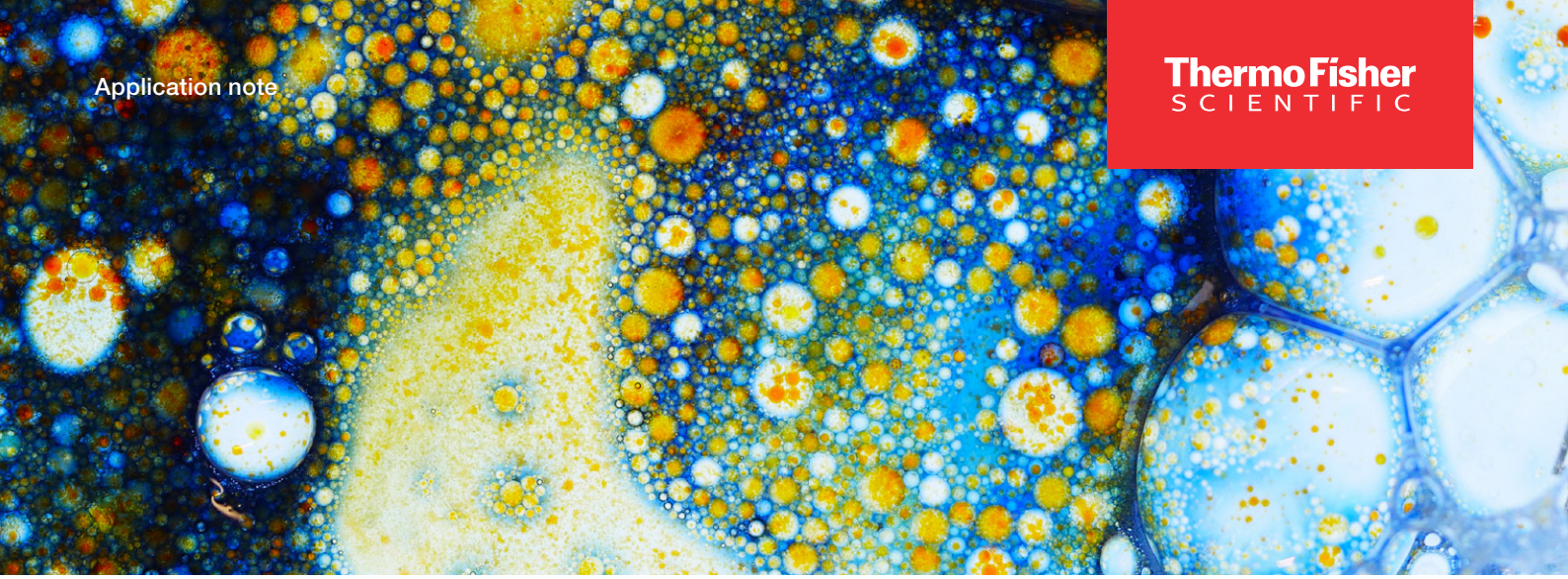


Figure 4. Calibration plot comparing the calculated protein concentrations to the actual concentrations from the PLS method. Root mean square errors are approximately 0.5 g/L or less. Blue circles (o) represent spectra used to create the method, purple crosses (+) are spectra used to validate the method.



Application note

Enabling real-time release of final products in manufacturing of biologics

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Keywords

DXR3 SmartRaman, spectrometer, biopharmaceutical, GMP, real-time release testing, QbD, RTRT, manufacturing, multi-attribute testing

Introduction

Biopharmaceuticals (or biologics) are manufactured using biological-expression systems (such as mammalian, bacterial, and insect cells) and have spawned a large and growing biopharmaceutical industry (BioPharmaceuticals). The structural and chemical complexity of biologics, combined with the intricacy of cell-based manufacturing, imposes a huge analytical burden to correctly characterize and quantify both processes (upstream) and products (downstream). In small-molecule manufacturing, advances in analytical and computational methods have been extensively exploited to generate process analytical technologies (PAT) that are now used for routine process control, leading to more efficient processes and safer medicines.

Raman spectroscopy is a vibrational spectroscopy technique with several useful properties (non-destructive, non-contact, high molecular-specificity, and robustness) that make it particularly suited for PAT applications in which molecular information (composition and variance) is required.

Typical good manufacturing practice (GMP) operations involve performing an extensive set of tests according to approved specifications before the material is released to the market or for further processing. Recent ICH guidelines (ICH Q8, Q9, Q10, and Q11), however, suggest an alternative real-time release strategy to provide assurance of product quality prior to release. Real-time release testing uses the principles of the pharmaceutical Quality by Design (QbD) to optimize release and stability testing. A combination of manufacturing process understanding, process control, and product knowledge can be used to demonstrate that the material was made according to GMP.

The exact approach to real-time release testing (RTRT) will vary depending on the process requirements. The RTRT strategy may be based on control of process parameters, monitoring of product attributes, or on a combination of both at appropriate steps throughout the process. Critically, the RTRT strategy should be based on a firm understanding of the process and the relationship between process parameters, in-process material attributes, and product attributes.

Quality, cost, and speed are the major drivers for implementing in-line monitoring, at-line monitoring, and real-time release.

Here, we review some of the most important applications of Raman spectroscopy to the manufacturing and analysis of biopharmaceuticals. This article covers two aspects of the biopharmaceutical-manufacturing process: identity/variance testing of raw materials and cell culture media; and multi-attribute product testing of a biologic drug product or final product testing of a biologic drug product.

Raw material characterization

Acceptance of raw materials today is often predicated on small-scale functional testing and/or limited analytical methods, which may not be representative of at-scale performance. This leads, in some cases, to fluctuating process outputs and, in extreme cases, not meeting predefined release criteria. Furthermore, many clinical products are developed using a small number of batches resulting in a narrow range of raw material variation and thus a limited process understanding. Especially in upstream cell culture, the unforeseen variability of various components of the cell culture media can impact a product's micro-heterogeneity and its critical quality attributes (CQA).

Multi-attribute tests for high-risk raw materials may include identity test, quantitative test for the concentration of key ingredients in a raw material, batch-to-batch variability test, and degradation tests.

One high-risk raw material encountered in biologics manufacturing is cell culture media. Identification of cell culture media samples by traditional liquid chromatography (LC) methods, such as amino acid or vitamin analysis, has high costs and requires significant analytical expertise and laboratory space. Raman spectroscopy offers many potential benefits, such as low cost, portability, and potentially limited skill required to operate the instruments.

Buffers are another set of critical raw materials used in downstream manufacturing. Osmolality is a measure of concentration and is considered a critical quality attribute and critical process parameter in bioprocessing. The yield and quality of a biologic are highly dependent on the optimization of the downstream process. Identity testing along with osmolality of buffers can be carried out using a multi-attribute method based on principal component analysis and partial list squares. Rapid testing of buffers through single-use flexi bags can be carried out using the fiber optics probe of the Thermo Scientific™ DXR3 SmartRaman Spectrometer at the point of use with no need for sample preparation.

Final product identity testing

Final product identification of biologics pre- and post-shipment is another regulatory requirement. Product testing for identity through different kinds of primary packaging (glass vials, syringes, glass bottles) poses a significant analytical challenge in the manufacturing of biologics. Fill finish sites may not have the necessary analytical expertise to carry out the tests and may have to send the samples to the parent site or external lab for testing, incurring time and money.

Moreover, biologics or small molecule drug products would also have to undergo retesting upon importation either from a third country in the EU member state or the USA when drug products have been sent to the USA from other countries. A full list of tests is typically carried out, including final product identity testing. For biopharma manufacturers, this involves either sending the samples back to the parent site for analysis or employing third-party labs in the country of import. This increases significant costs and delays in the delivery of highly needed drug products.

End product identity testing/final product identity testing of biologics after fill-finish or pre-shipping to the fill-finish line is carried out by a variety of analytical techniques depending on the molecule/registration dossier.

For example, the verification test for biologic proteins is peptide mapping—a long-established workflow for protein identification using LC/mass spectrography (MS). This complex separation technique requires protein extraction and clean-up, enzyme digestion, one or more stages of liquid chromatography, and two phases of mass spectrometry before the final spectrum is matched against protein databases. Although it is a standard methodology, peptide mapping necessitates an analytical lab with qualified technical resources, entails extensive time for preparation, and introduces significant costs in solvents, columns, and analytical equipment.

The DXR3 SmartRaman Spectrometer, with its high sensitivity and resolution, allows characterization of the drug product by evaluating the fingerprint region of the molecule. Therefore, the DXR3 SmartRaman Spectrometer's unique capability with sampling flexibility ensures repeatable measurements, and subsequent analysis allows rapid method development and deployment.

We ran a feasibility study for multinational drug manufacture whereby the primary goal was to set up a rapid multi-attribute end product test to differentiate 15 different types of drug products and determine the concentration of the two preservatives in the drug products.

For this feasibility test we were given 15 different types of biologic drug products that varied in concentration from 0.5 mg/mL to 6 mg/mL. Concentration of two preservatives A and B ranged from 0.85 mg/mL to 5.0 mg/mL and 0.42 mg/mL to 3.91 mg/mL respectively.

These commercial drug products were supplied in their native glass vials varying in size and volume. A picture of such glass vials is shown below (Figure 1).

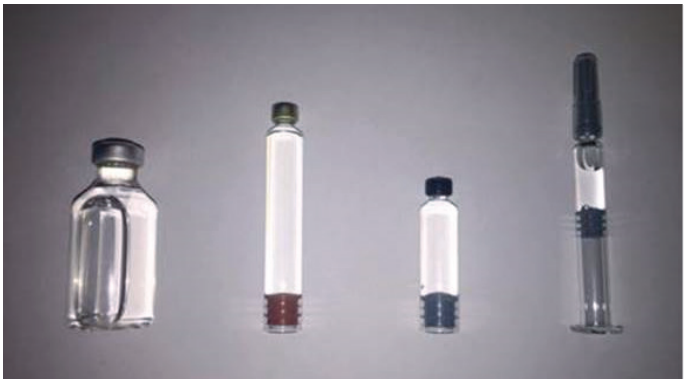

Figure 1. Typical native glass vials.

Reversed-phase high-performance liquid chromatography (HPLC) is currently used for the final product identity test and quantitative measurement of two preservatives in the final drug product.

DXR3 SmartRaman Spectrometer with universal sampling plate and 180-degree sampling module was used to acquire spectra of 15 drug products. To acquire each spectrum, a 532 nm laser with 40 mW power and 1 minute of scanning time was used. Ten spectra were acquired for each sample to accommodate the variability of glass vials and scattering effects.

DXR3 SmartRaman Spectrotometer offers excellent selectivity, repeatability, and full wavelength range to characterize biologics based on the characteristic band assignment (Table 1 and Figure 2).

Band frequency (cm^{-1})	Region	Vibrational mode	Protein structure assignments
870–1,150	Backbone, skeletal stretch	C_α-C, C_α-C_β, C_α-N	Secondary structure elements: α-helix, β-sheets, less-ordered structure
1,200–1,340	Amide III	N-H in-plane, Cα-N stretch	Hydrogen bonding, secondary structure
1,400–1,480	Side chain deformations	CH_2 and CH_3 deformations	Local environments, intermolecular interactions of side chains
1,510–1,580	Amide II	N-H deformations and C-N stretch (observed in UVRR and not conventional Raman spectra)	Local environments, intermolecular interactions of side chains
1,630–1,700	Amide I	C=0 stretch N-H in-plane bending	Secondary structure elements: α-helix, β-sheet, less-ordered structure

Table 1. Characteristic Raman band assignment.

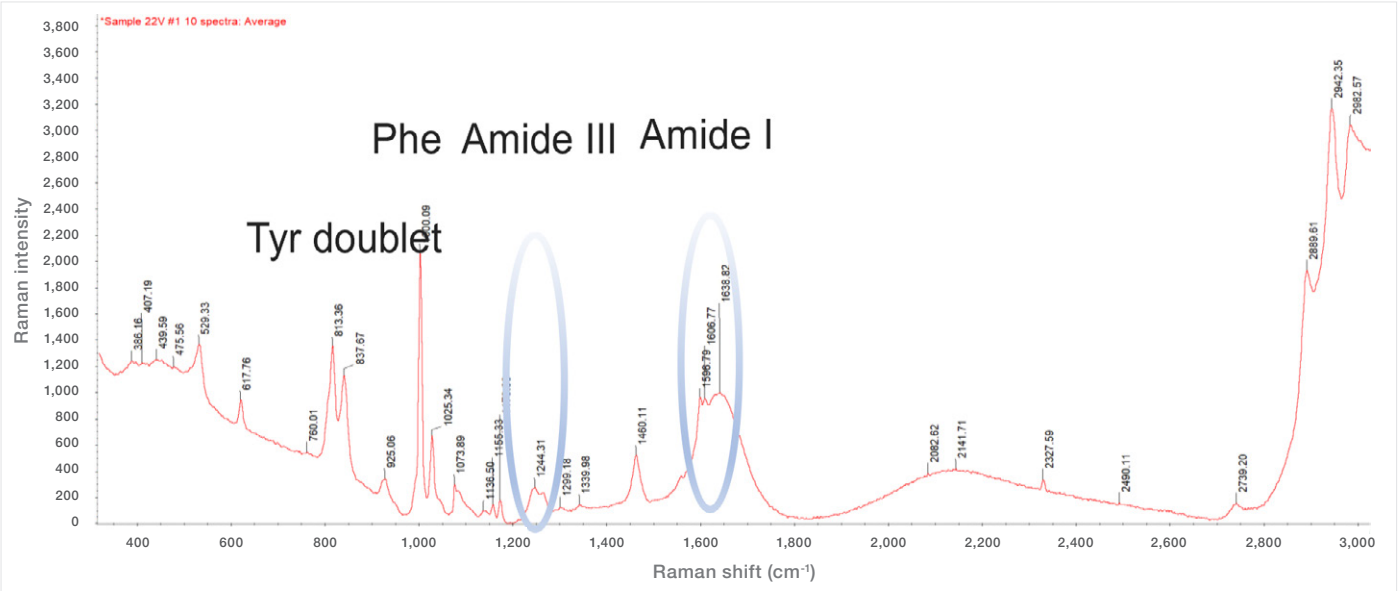

Figure 2. DXR3 SmartRaman spectrum showing characteristic bands of a biologic drug product.

Figure 3 shows the spectra of a sample containing a drug product against its placebo. It is imperative to establish that technique chosen for a feasibility study. In this case, Raman spectroscopy is sensitive enough to detect the differences between the drug product and its placebo. DXR3 SmartRaman Spectrometer offers high sensitivity to determine the significant differences between placebo and actual drug products.

Figure 4 is showing spectra of different classes of drug products. These spectra were utilized to build the discriminant analysis method on the Thermo Scientific™ TQ Analyst™ Software. TQ Analyst Software is a validated qualitative and quantitative method building software offering full compliance for pharmaceutical applications.

The discriminant analysis classification technique can be used to determine the class or classes of known materials that are most similar to an unknown material by computing the unknown's distance from each class center in Mahalanobis distance units. The discriminant analysis technique is typically used to screen incoming materials or final products to determine if they are compound/molecule a, b, or c.

Discriminant analysis methods typically specify at least two classes of known materials, but the method also works with only one class. Multiple standards may be used to describe each class (at least one class must contain two or more standards). Multiple regions of the spectrum may be used for the analysis.

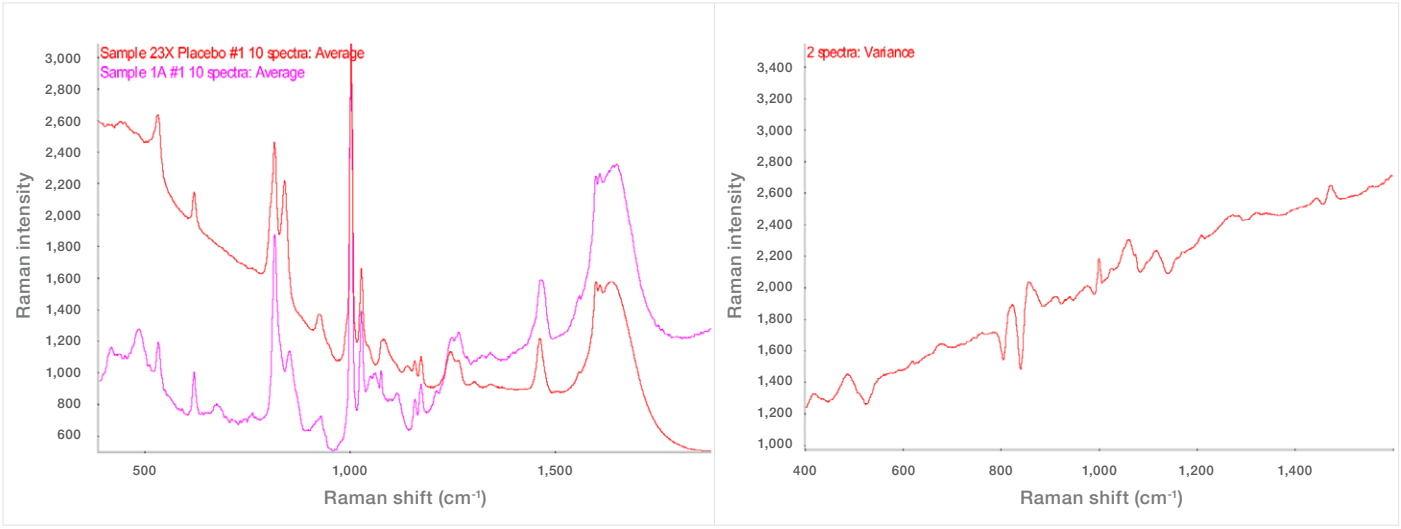

Figure 3. Raman spectra of drug product and its placebo and variance spectrum.

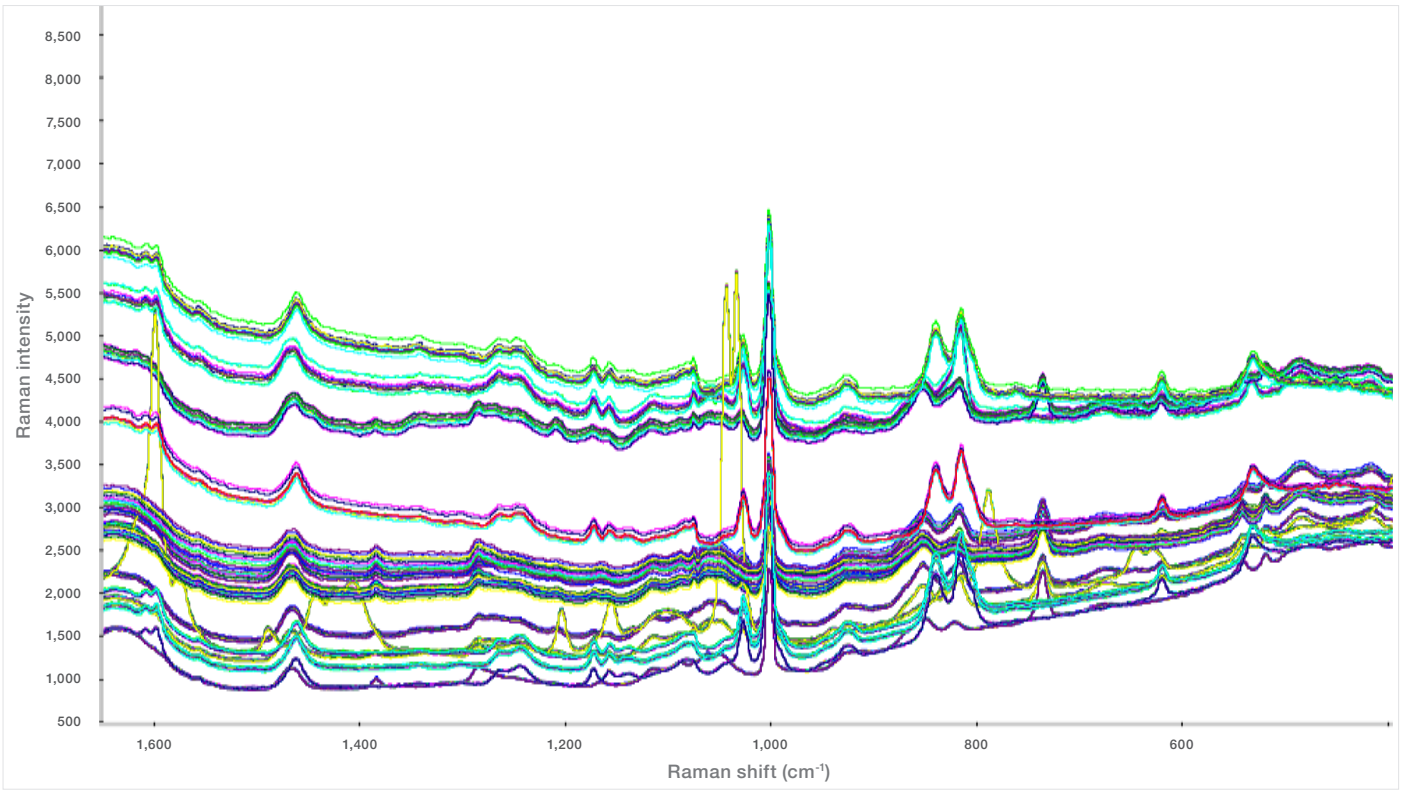

Figure 4. Raman spectra of different classes of drug products.

What does discriminant analysis do?

A discriminant analysis method applies the spectral information in the specified region or regions of an unknown sample spectrum to a stored calibration model to determine which class of standards is most similar to the unknown.

When the method is used to analyze an unknown sample or a class, the software performs a principal component analysis on the spectra of the standards and uses those results to determine score values for the unknown sample spectrum. The score plots are used to produce Mahalanobis distance values, which in turn are used to rank the classes.

The result of a discriminant analysis is the name of the class or classes that are most similar to the spectrum of the unknown sample. The Mahalanobis distance between the unknown sample and each reported class can also be reported. The closer each distance value is to zero, the better is the match.

After cross-validation, principal component scores plot revealed the class differentiation and the report indicated that all the classes of the different products were correctly identified with no mismatches to indicate false positives.

Quantitative analysis of biologics for preservative A and preservative B

As part of this feasibility study, our client also wanted to determine if the DXR3 SmartRaman Spectrometer test could be utilized to replace the HPLC test for measuring the concentration of two preservatives in their drug products. The level of preservative A was 0.85 mg/mL to 3.07 mg/mL and that of preservative B was 0.32 mg/mL to 2.57 mg/mL.

Pure samples of preservatives A and B were acquired as references, and to ascertain their presence in the final drug formulation.

Actual class	Mismatch	Calculated class	Calculated distance	Next class	Next distance
Product D		Product D	0.5809	C	4.5556
Product A		Product A	1.9869	I	12.9617
Product B		Product B	1.3796	E	25.1324
Product C		Product C	0.5417	D	3.8568
Product D		Product D	0.8466	M	9.0495
Product I		Product I	1.7709	A	13.9064
Product M		Product M	0.5284	S	3.3881
Product O		Product O	0.2244	X	17.3044
Product R		Product R	0.5419	C	4.4691
Product T		Product T	0.5944	X	2.3213
Product X		Product X	0.79	T	3.1646
Product S		Product S	1.1837	M	3.0829
Product N		Product N	1.0954	U	15.1798
Product U		Product U	0.1603	T	9.1738
Product S		Product S	1.8544	N	22.1624

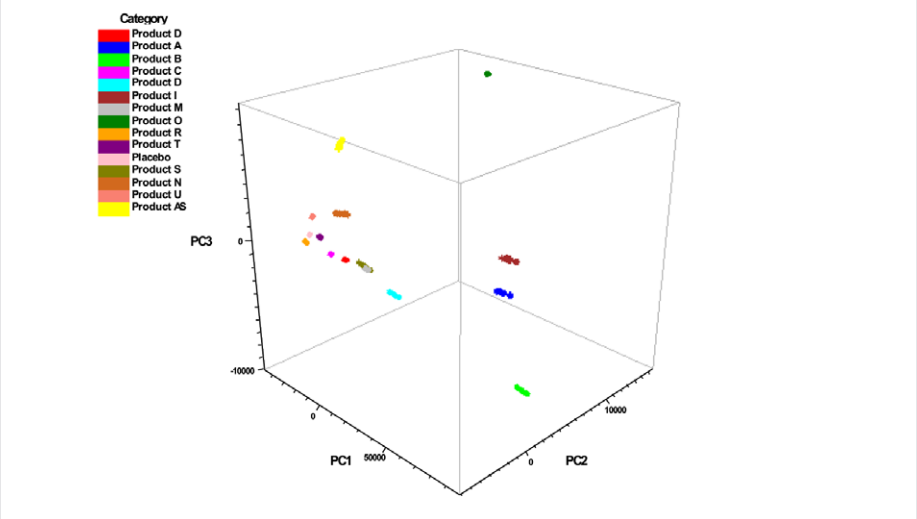

Figure 5. Analysis of preservative A and preservative B.

Samples of varying concentrations as per table 1 were acquired using the same parameters as of spectra acquired for identity test through 3 mL vial. Figure 6 is showing the spectra of the drug product with the two preservatives.

Four standards with the reference values were supplied in 3 mL and 10 mL vials and a validation sample to test the model for 3 mL and 10 mL vials.

Four spectra per standard were acquired and used to build the chemometric method. The final drug product samples were scanned with a DXR3 SmartRaman Spectrometer to acquire spectra in the range of 3500 to 50 cm-1 and captured with a single exposure of the CCD, avoiding stitching artifacts. The sample time took approximately 1 minute. Three spectra were collected per sample. The sample spectra were loaded into TQ Analyst Software for chemometric analysis using a partial least squares (PLS) method.

	Preservative A (mg/mL)	Preservative B (mg/mL)
Standard 1 3 mL and 10 mL	0.85	0.42
Standard 2 3 mL and 10 mL	1.27	1.12
Standard 3 3 mL and 10 mL	1.57	1.75
Standard 4 3 mL and 10 mL	3.07	2.57
Validation – 3 mL	1.57	1.75

Table 2. Calibration and validation sample.

	PLS results for 3 mL Cartridge	
	Preservative A (mg/mL)	Preservative B (mg/mL)
Validation sample: 3 mL	1.58 actual 1.57	1.71 actual 1.75
Real Sample in solution: 3 mL	1.56 actual 1.55	1.69 actual 1.77
Real sample in suspension: 3 mL	0.72 actual 0.69	1.23 actual 1.58

Table 3. Validation result for 3 mL sample.

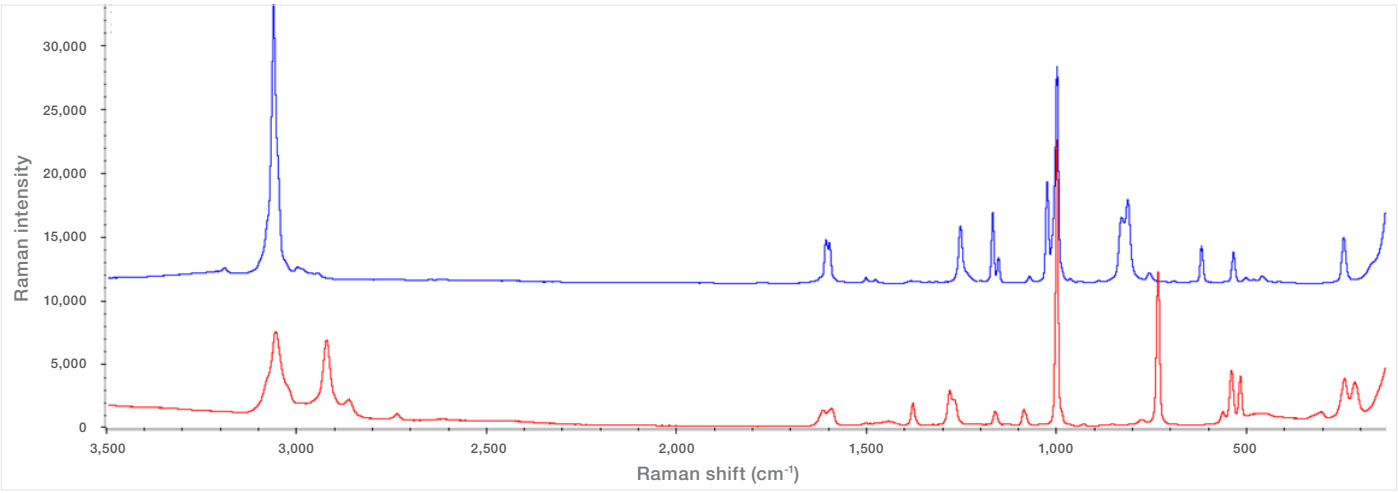

Figure 6. Spectrum in blue is from pure preservative A and spectrum in red is from pure preservative B.

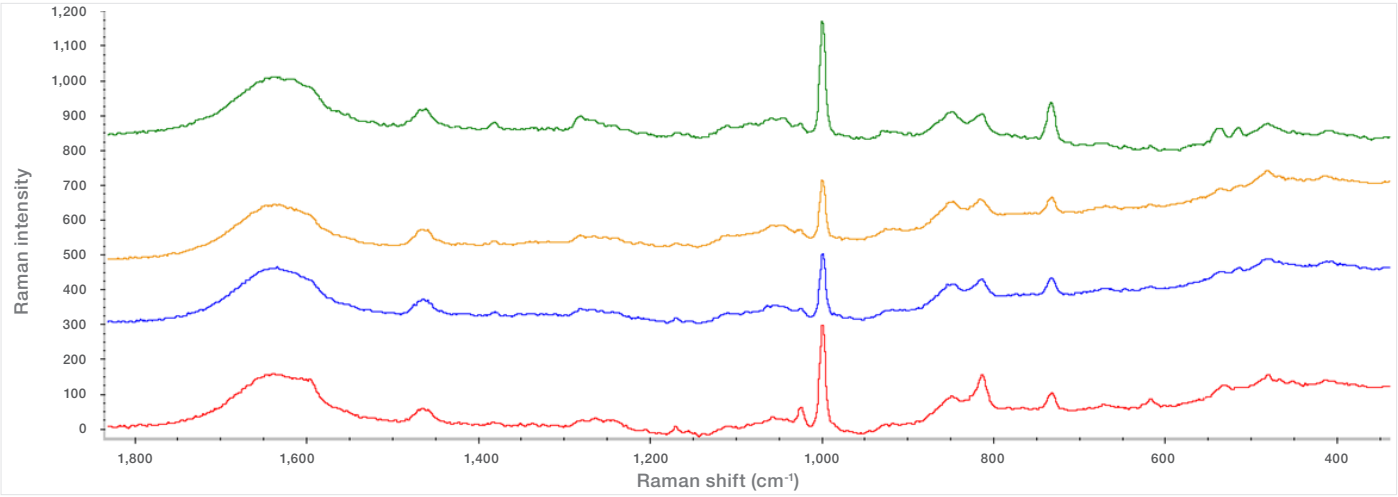

Figure 7. Spectra showing varying concentration of preservatives in final drug product.

Results

PLS analysis of the final drug product samples revealed excellent predictive capabilities within the range of materials tested. The spectra used to develop the PLS method for 3 mL cartridge are shown on calibration plots (Figure 8 and Figure 9) that compare the calculated preservative concentrations versus the actual concentrations. The calibration plot can be used to determine how well the method predicts the actual preservative concentrations in the samples. The plot developed by the chemometric method resulted in a correlation coefficient of 0.998 for preservative A. Root mean square error of calibration (RMSEC) was 0.0425 mg/mL, and the Root mean square error of prediction (RMSEP) calculated was 0.0372 for preservative A. The additional method for preservative B resulted in in a correlation coefficient of 0.999. The RMSEC was 0.0316 mg/mL, and the calculated RMSEP was 0.0496. The method was able to accurately predict the 3 mL validation sample and a real sample in solution (Table 3). The prediction can be improved when suspensions are allowed to settle and liquid phase is analyzed.

When 10 mL vial calibration samples were added to the above PLS method, method performance remained the same and was able to accurately predict the validation samples (Table 4).

Conclusions

A multi-attribute test to establish Final product identification and predicting concentrations of preservatives was done with the DXR3 SmartRaman Spectrometer by developing a discriminant analysis method and partial least square method. The final drug product identification test is part of release testing and current methods used are time-consuming and laborious. This Raman technique successfully demonstrates the ability to measure and monitor preservative concentrations either in the lab environment or at the line. The method developed shows excellent correlation with actual preservative concentrations with errors comparable to the reference analysis method. This application demonstrates the continued capability of the DXR3 Raman Spectrometer to be successfully used in bioprocess environments for implementing multi-attribute final product testing of biologics. Apart from the examples shown here, DXR3 SmartRaman Spectrometer can be used to implement at-line control strategies to monitor protein concentration, excipients concentration, and critical quality attributes like osmolality and pH. Many such examples are cited in the literature for Raman applications in biopharma manufacturing.

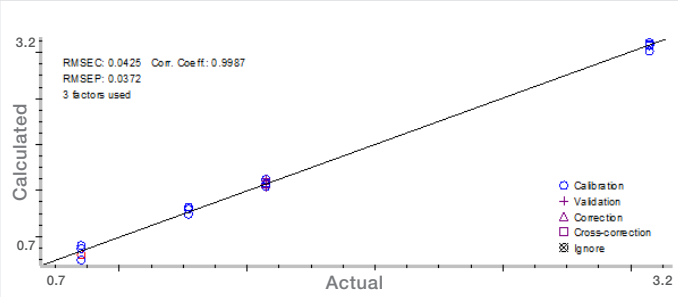

Figure 8. PLS model for preservative A —3 mL cartridge.

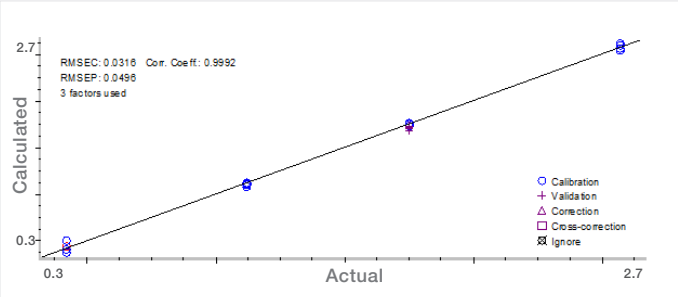

Figure 9. PLS model for preservative B —3 mL cartridge.

	PLS 3 mL cart and 10 mL vials	
	Preservative A (mg/mL)	Preservative B (mg/mL)
Validation sample: 3 mL	1.58 actual 1,57	1.71 actual 1,75
Real sample in solution: 3 mL	1.56 actual 1.55	1.65 actual 1.77
Real sample in suspension: 3 mL	0.80 actual 0.69	1.21 actual 1.58
Real sample in suspension: 10 mL	0.73 actual 0.68	1.32 actual 1.57

Table 4. Validation results for 3 mL 10 mL vials.

References:

1. European Medicines Agency. Guideline on Real Time Release Testing. https://www.ema.europa.eu/en/documents/scientific-guideline/guideline-real-time-release-testing-formerly-guideline-parametric-release-revision-1_en.pdf (accessed June 15, 2021).
2. Buckley K, Ryder AG. (2017). Applications of Raman spectroscopy in biopharmaceutical manufacturing: a short review. *Applied Spectroscopy*, 71(6): 1085-1116. https://aran.library.nuigalway.ie/handle/10379/7177 (accessed June 15, 2021).