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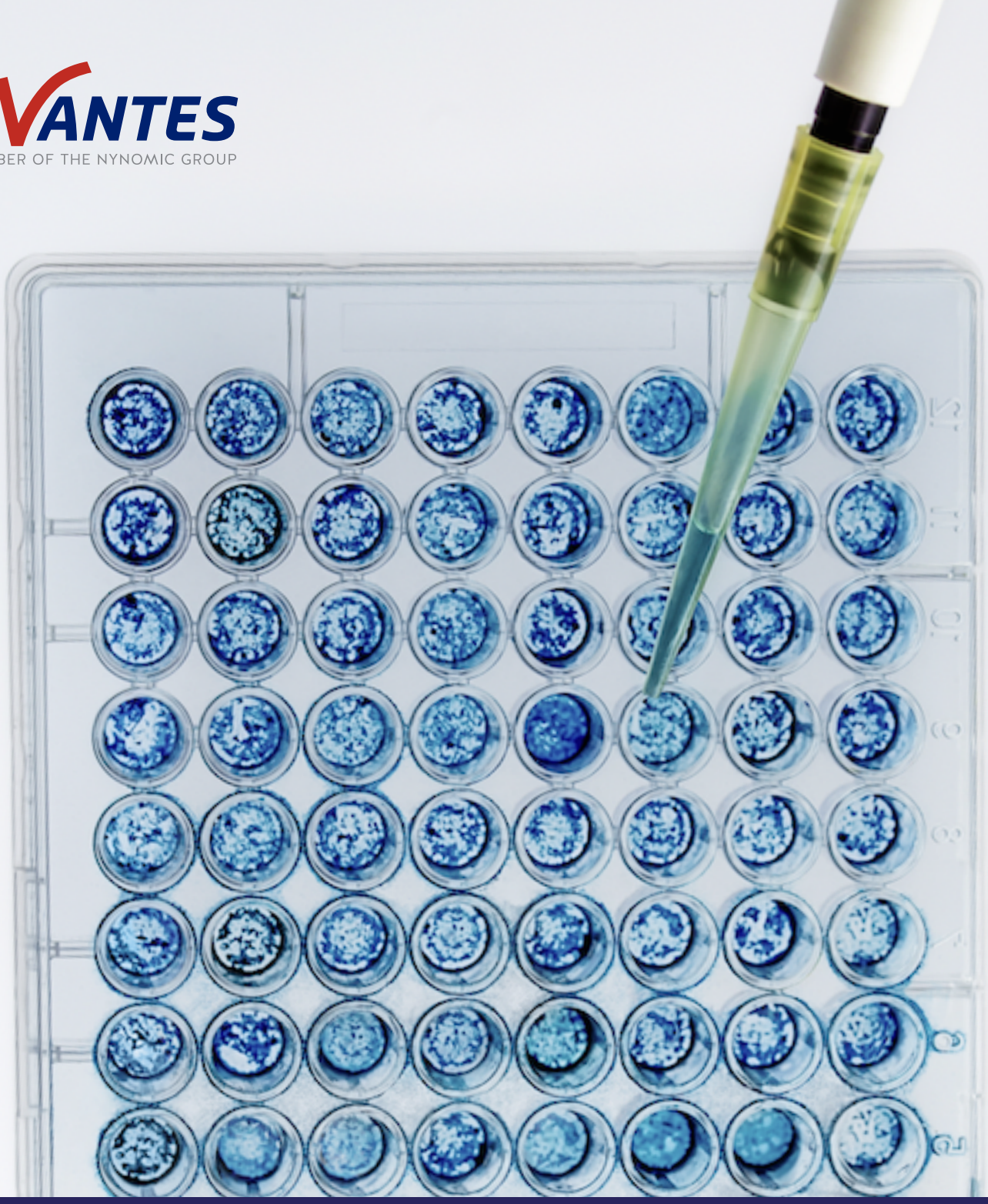
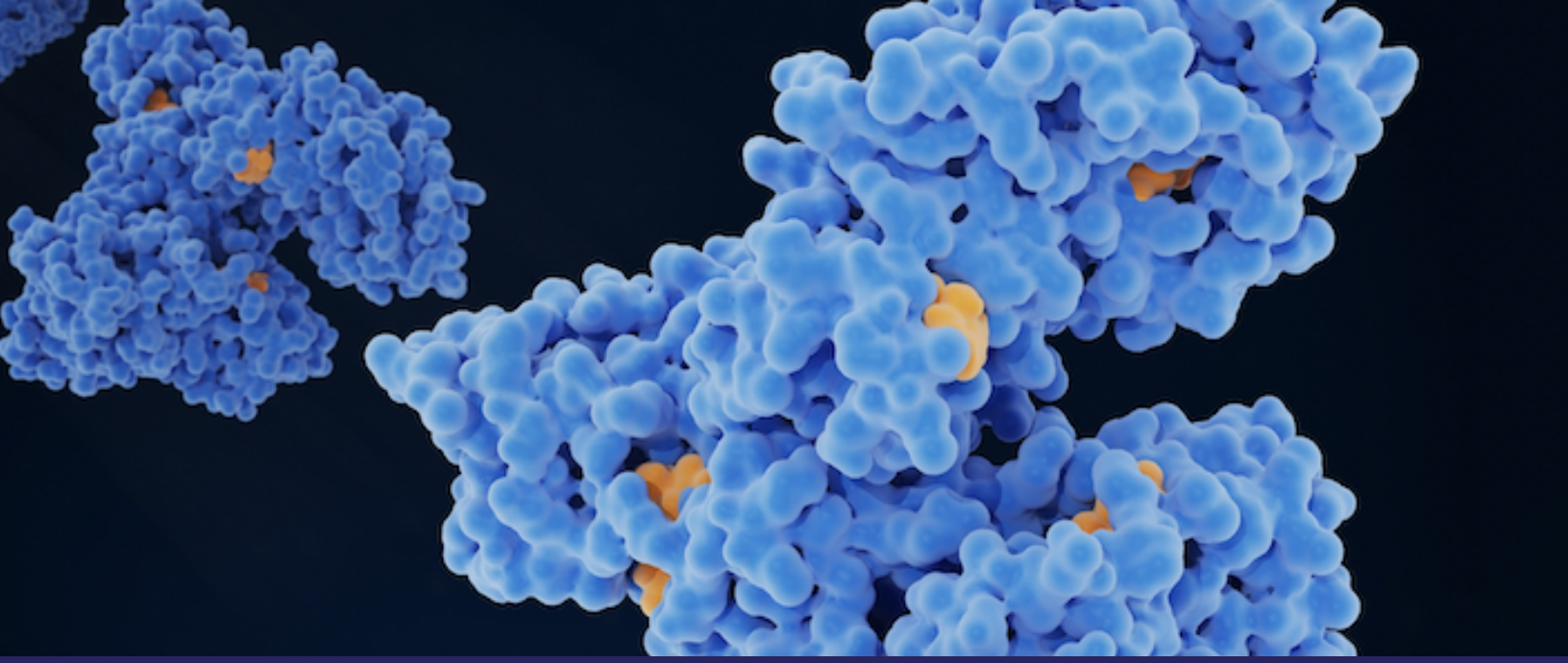


APPLICATION NOTE

SPECTROSCOPIC TECHNIQUES FOR PROTEIN ANALYSIS



INTRO

PROTEIN ANALYSIS & SPECTROSCOPY

Quick and accurate quantification of protein content is critical for modern biology, biochemistry, and biophysics labs worldwide. Not only are researchers interested in detecting the total protein content, but they are also interested in protein classification/identification and proteomics. Although researchers have many tools at their disposal for understanding the complexities of protein, the cornerstone of any protein laboratory is absorbance and fluorescence spectroscopy. We have discussed both methodologies in a previous application note "Biomedical Applications for Spectroscopy" which is a great resource of the method fundamentals and therefore are not covered in this app note. Instead, this application note will provide a high-level overview of the specific ways these spectroscopic techniques are utilized for protein analysis

To demonstrate how powerful these techniques are for protein analysis we have decided to focus our attention on one particularly interesting classification of pronates known as albumin. Human serum albumin is the most common protein in the body. It is responsible for regulating osmotic pressure and plays a crucial role in maintaining overall blood pressure. Human serum albumin also serves as a carrier for a wide range of hydrophobic molecules like steroids in the bloodstream. In addition to the medical importance of human serum albumin, bovine serum albumin (BSA) is also extremely important because of its wide range of uses in the lab. BSA is used in applications ranging from stabilizing enzymes and antibodies to preventing other proteins from adhering to the inner walls of microcentrifuge tubes. But the primary reason why BSA is so popular is its use as the standard by which all other proteins are measured.

ABSORBANCE SPECTROSCOPY

The Bradford Test

Currently, the most popular way of quantifying unknown protein concentration is through a methodology known as the Bradford test. This assay-based approach measures the change in the absorption spectrum of a reagent in the presence of proteins. The Bradford assay utilizes an acidified solution, Coomassie brilliant blue dye (also known as the Bradford reagent). In this highly unstable cationic state, Coomassie brilliant blue has a peak absorption in the blue at ~465 nm resulting in a reddish-brown coloration. When protein is introduced to the assay, there is an electron exchange that destabilizes the protein, allowing it to bind with the Coomassie brilliant blue, producing the more stable anionic form. Once in the stable state, the peak absorbance of the molecule shifts to ~595 nm producing the blue coloration for which the dye is named (see figure 1).

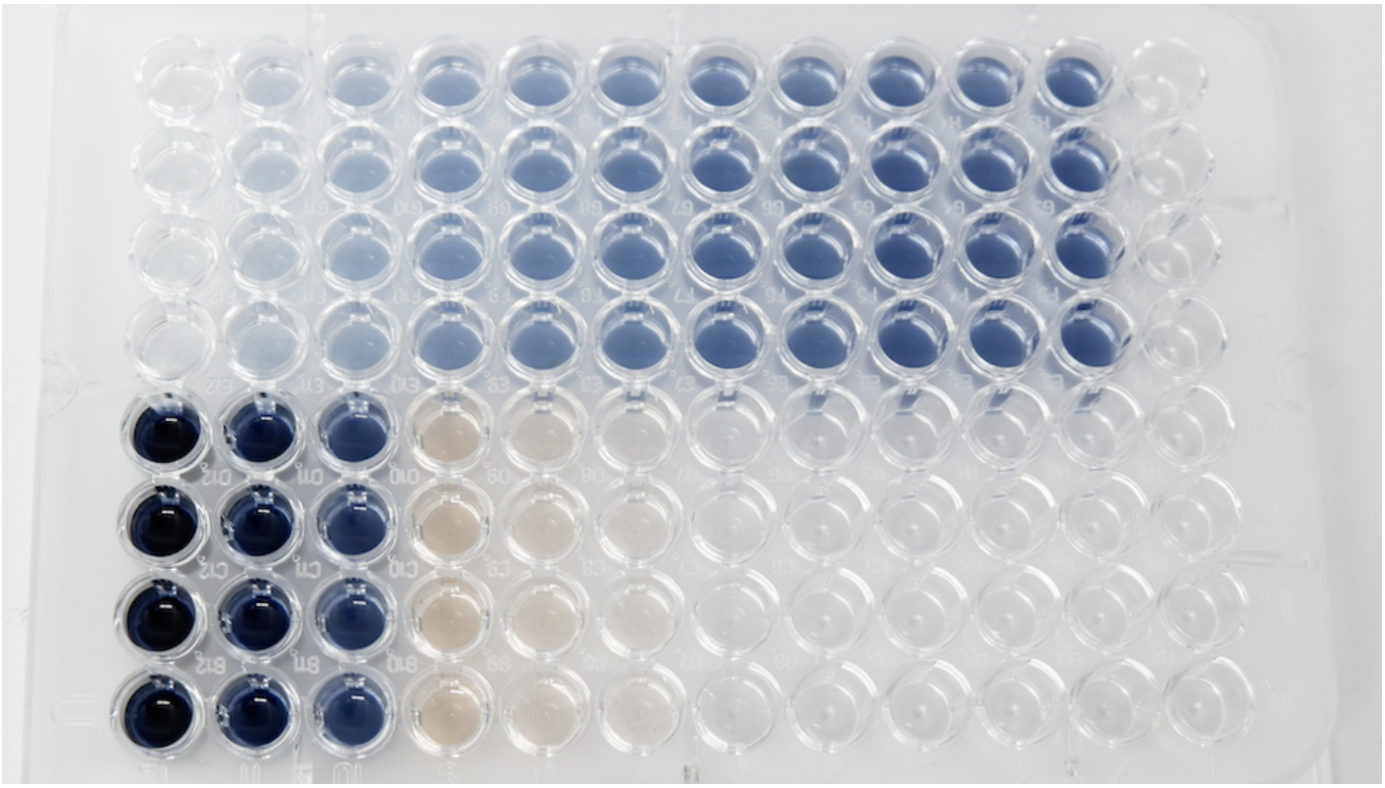


FIGURE 1: Typical Bradford Protein Assay

To accurately use the Bradford test to determine the concentration of the analyte, one must first prepare a set of calibration standards. This is where BSA comes into the picture. The standard Bradford test protocol requires a serial dilution of BSA to be prepared and then assayed with the reagent producing a set of colorimetric standards. The next step in the protocol is to measure the absorption spectra of each concentration of BSA to build up a calibration curve. Fitting this curve via linear regression provides a simple linear equation by which you can determine the concentration of an unknown protein.

ABSORBANCE SPECTROSCOPY

Direct UV/Vis Absorbance

While the Bradford test is without a doubt the most straightforward way of determining the concentration of an unknown protein, the heavy dependence on the use of a reagent makes it less desirable for the quantification of know proteins. As a rule of thumb, the higher the number of double bonds (π-electrons) in a molecule, the smaller the gap between the HOMO and LUMO bands, resulting in absorbance in the UV and visible regions of the spectrum. Since proteins are formed from amino acids containing at a minimum one carboxyl group (and often several other double bonds), it should be no surprise that most proteins are highly absorptive in the UV, typically around 280nm.

Therefore, when researchers want to identify specific protein concentrations, as opposed to unknown protein concentrations) it is often far easier to build calibration curves in the UV since there is no need for reagents. For example, figure 2 shows the UV absorbance spectrum for three different concentrations of BSA in water (1 mg/ml, 0.5 mg/ml, and 0.25 mg/ml). Using the absorbance value at 277 nm, a linear calibration curve was fitted with an R-squared of 0.9836.

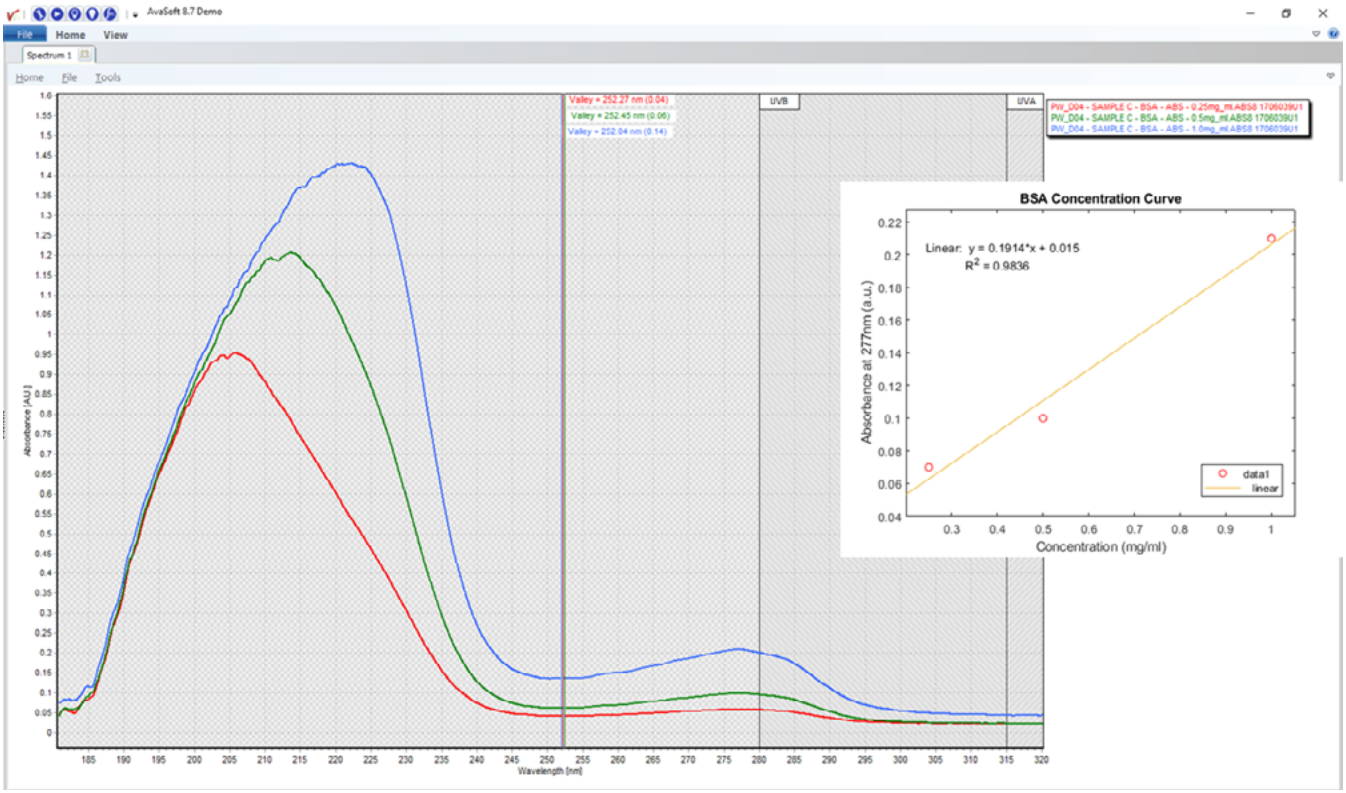


FIGURE 2: UV absorbance spectra of BSA at three difference concentrations; 1mg/ml, 0.5mg/ml, and 0.25mg/ml.

ABSORBANCE SYSTEM REQUIREMENTS

The most important considerations are stability and dynamic range when performing absorption spectroscopy. If the light source is unstable or the spectrometer's baseline fluctuates, it will result in measurement-to-measurement variations, making quantitative analysis impossible. Therefore, it is vital to use a constant current light source. For broad-band UV/Vis absorption spectroscopy, the AvaLight-DHc full range compact light source is the ideal choice. This light source integrates a deuterium and tungsten halogen lamp into one fiber-coupled package with Avantes' proprietary supply electronics to provide a 200 nm to 2500 nm spectrum with less than 1mAU (0.01%) of fluctuation. If you are only concerned about narrow region of interest a LED light sources are generally preferred. These sources are extremely stable, but they are also lower power consumption and offer longer lifetimes. Avantes offers a wide range of high-powered LED illumination sources such as the AvaLight-HPLED-285, the AvaLight-HPLED-470, and AvaLight-HPLED-625, which can be used for Bradford assay measurement.

The spectrometer's dynamic range is crucial when looking to differentiate small concentrations since it will manifest as a slight change in the absorbance spectrum. As a result, CMOS-based spectrometers, such as the AvaSpec-ULS2048CL-EVO, are preferable due to their larger dynamic ranges than CCD detectors. These spectrometers can be easily optimized for the UV (200-400nm), visible (350-750nm), and UV/Vis (250-850nm) detection depending on the grating of choice. Figure 3 shows a typical absorbance spectroscopy setup using the AvaSpec-ULS2048CL-EVO spectrometer, AvaLight-DHc light source, and CUV-UV/VIS sample holder from Avantes. Given the highly absorptive nature of proteins, specialized cuvettes with 1 or 1 mm pathlengths are recommended. Another challenge associated with protein measurements may be volumetric limitations on samples and/or the desire to measure in-line during an assay. Given this, Avantes offers a variety of microliter volume flow cells (see Figure 4) which can replace the cuvette holder shown in Figure 3. Such flow cells are available in pathlengths as small as 1 mm.

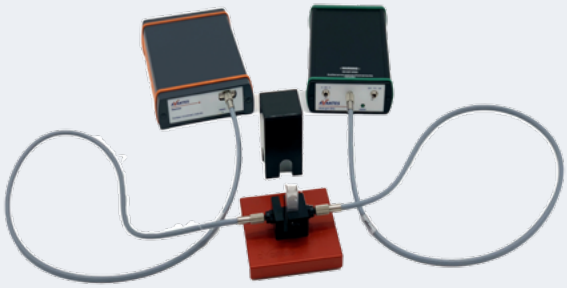


FIGURE 3
Typical absorption spectroscopy setup

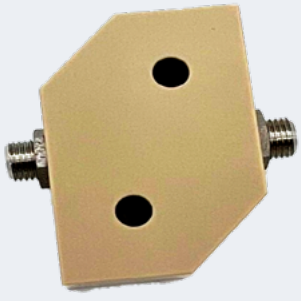


FIGURE 4
Microfluidic flow cell with SMA fittings

FLUORESCENCE SPECTROSCOPY

Absorption spectroscopy is an excellent tool for quantifying proteins, but neither Bradford assays nor directed UV absorption is effective for identification and classification of unknown proteins. Fortunately, while most amino acids (and therefore proteins) have similar absorption properties due to the similarities in the HOMO/LUMO bandgap, the differences in molecular structure (functional groups) lead to very different vibrational energy levels, producing significant differences in fluorescence emission spectra.

One way to better understand this effect is to look at the differences between the absorption and fluorescence properties of tryptophan and tyrosine, key amino acids both present in BSA. From the data shown in Table 1, we can see that, as expected, the two amino acids both have incredibly similar absorption peaks at 280 nm for tryptophan and 274 nm for tyrosine. But the two amino acids have different peak fluorescence wavelengths at 348 nm for tryptophan and 303 nm for tyrosine. Comparatively, we see an absorption peak in the BSA spectra shown in figure 2 at 277 nm and a fluorescence peak at 339 nm (see figure 5).

	Absorption		Fluorescence	
	Peak Wavelength	Absorptivity	Peak Wavelength	Quantum Yield
Tryptophan	280 nm	5,600	348 nm	0.20
Tyrosine	274 nm	1,400	303 nm	0.14

TABLE 1: Absorption and fluorescence properties of tryptophan and tyrosine

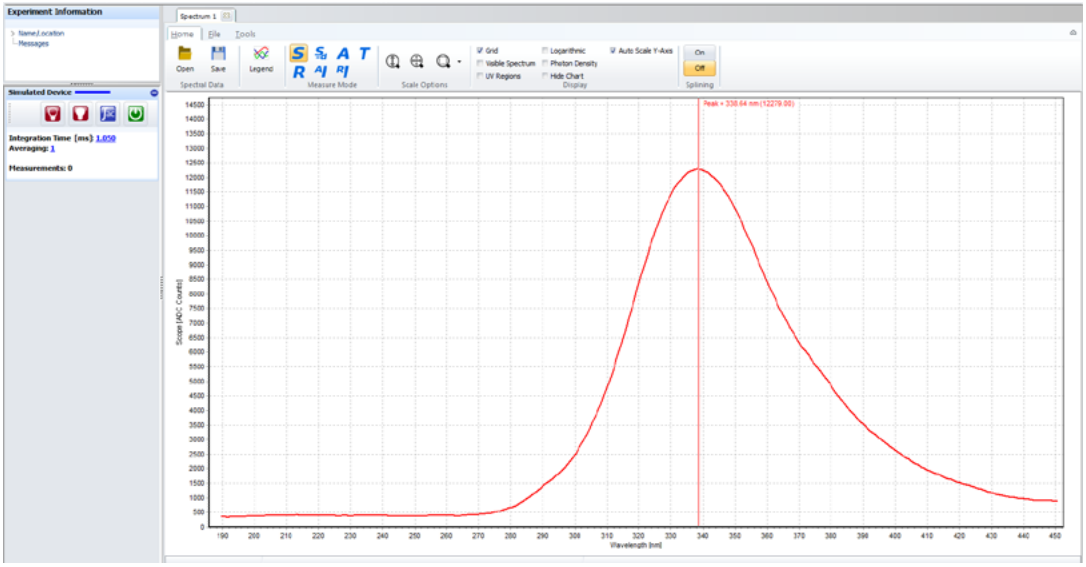


FIGURE 5: Fluorescence spectrum of BSA

Since different proteins are constructed from different combinations of amino acids, they will all have slightly different emission spectra based on their relative amino acid concentrations. Therefore, libraries can be created to identify specific proteins based on their emission spectra. Furthermore, a wide range of fluorophores can be functionalized to bond with specific proteins, making chemically sensitive fluorescence tags that significantly improve specificity.

FLUORESCENCE SYSTEM REQUIREMENTS

There are three main differences between the system requirements for absorption and fluorescence measurements, the most important of these being the measurement geometry. First, fluorescence spectroscopy requires the use of orthogonal excitation and collection ports to assure that the excitation source itself does not drown out the weaker fluorescence signal. When utilizing this setup, the port which is in line with the excitation source may be left open to prevent the transmitted light from bouncing around inside of the cuvette holder, capped with a mirror to create a double pass cell, or connected to a second spectrometer to measure the absorption spectrum simultaneously.

Due to the relatively weak nature of fluorescence compared to absorbance spectroscopy, it is generally preferable to utilize a high sensitivity spectrometer such as the AvaSpec-Hero or the AvaSpec-HS2048XL-EVO. Fortunately, though, when compared to other low light level spectroscopy applications, fluorescence has the advantage of relatively broad emission bands allowing for a spectroscopist to also utilizes a less sensitive spectrometer such as the AvaSpec-ULS2048CL-EVO or even the AvaSpec-Mini2048CL with a wide entrance slit to accept light into the spectrometer. Many of Avantes spectrometers come standard with interchangeable entrance slits or this can be added as an option (ULS Starline models) which allow the user to experimentally determine the optimal entrance slit width for each specific fluorescence application, further enhancing the flexibility afforded by fiber-coupled miniature spectrometers.

The final consideration is the spectral power of the excitation source. Since the intensity of the fluorescence signal will be directly proportional to the intensity of the excitation source, low spectral power sources such as deuterium and tungsten halogen lamps are typically not recommended. However, the AvaLight-HPLED-285 LED source is an excellent choice for most proteins since it is matched to an absorption band. For higher-end applications, the Avalight-XE-HP pulsed xenon light source offers much lower detection limits by producing maximum output energy of 39mJ per flash at a pulse repetition rate of up to 150 Hz. An additional advantage of xenon as an excitation source is the ability to select an excitation wavelength across its broad spectrum (200-1000 nm) and filter out the excitation band of interest using bandpass filters which can affixed to the source with Avantes direct attached filter holder (FH-DA).

FIGURE 6
Typical Fluorescence spectroscopy setups (left: micro low cell, right: cuvette cell holder)

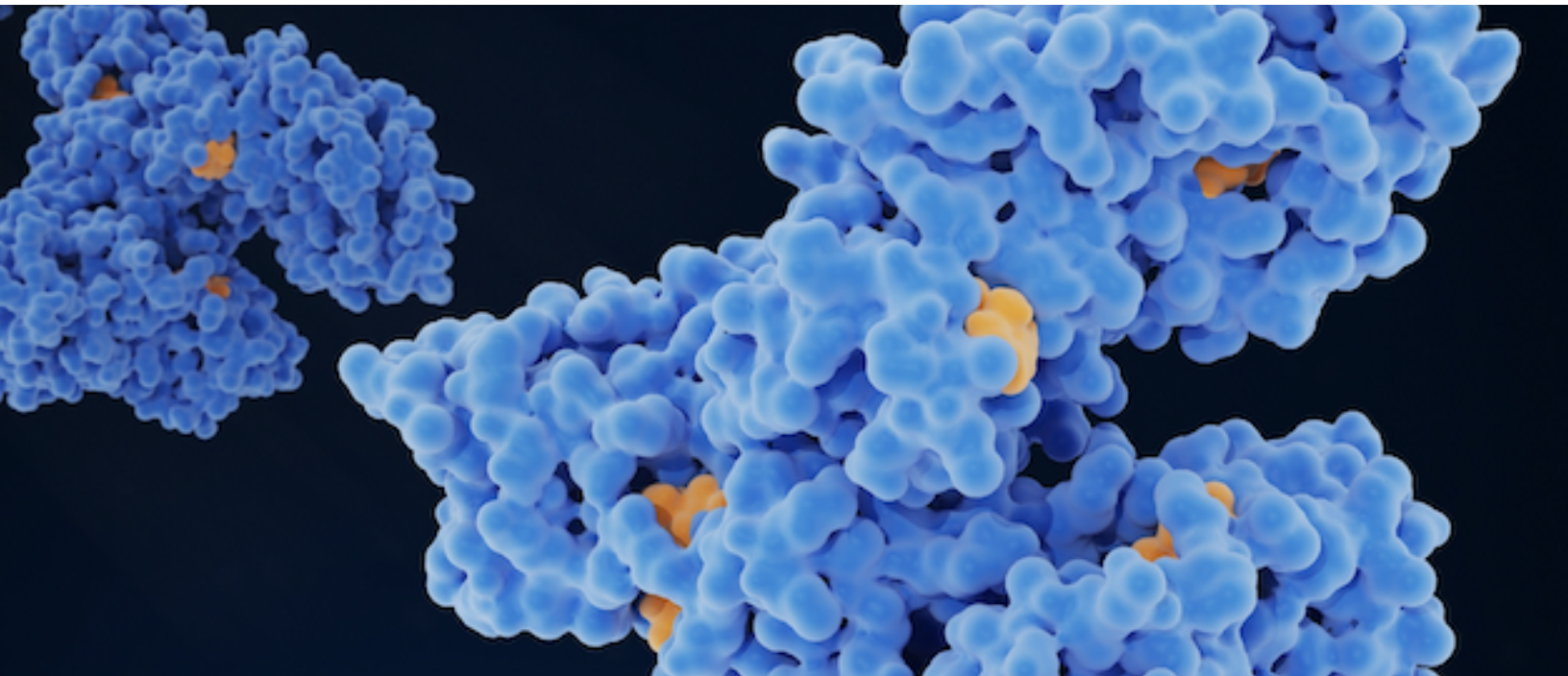
FINAL THOUGHTS

All Avantes spectrometers are available as stand-alone units OEM modules or can be integrated into multichannel rack mount systems that are ideally suited to inline protein analysis. For applications where size and cost are major drivers, the AvaSpec-Mini2048CL is an excellent alternative option.

These spectrometers can communicate via USB, Ethernet, and the native digital & analog input/output capabilities of the Avantes AS7010 electronics board provides for a superior interface with other devices. Additionally, the Avantes Avaspec DLL package, with samples programs in Delphi, Visual Basic, C#, C++, LabView, MatLab, and many other programming environments, enables users to develop their own code.

Contact Us

For more information about the full range of spectrometer options available from Avantes for fluorescence spectroscopy, please feel free to visit the website at www.avantes.com or give us a call at +1 (303)-410-8668 (USA) or +31 (0) 313 670 170 (Europe) where our knowledgeable applications specialists are standing by to help



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Curious how spectroscopy can help you reveal answers by measuring all kind of materials, in-line, at your production facility, in a lab or even in the field? Please visit our website or contact one of our technical experts, we're happy to help you.

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