Application Note

Quantitation of Peptides and Amino Acids with a Synergy™HT using UV Fluorescence

Introduction

Eukaryotic and prokaryotic cells contain a number of compounds that are fluorescent with UV light excitation. Proteins and peptides, with aromatic amino acids are intrinsically fluorescent when excited with UV light. Many enzymatic cofactors, such as FMN, FAD, NAD and porphyrins, which are also intrinsically fluorescent, add to the protein fluorescence. These moieties have a common trait in that they all contain aromatic ring structures that absorb UV light for excitation. There are also special proteins such as Green Fluorescent Protein, which has an internal serine-tyrosine-glycine sequence that is modified post-translationally and is fluorescent in the visible light region.

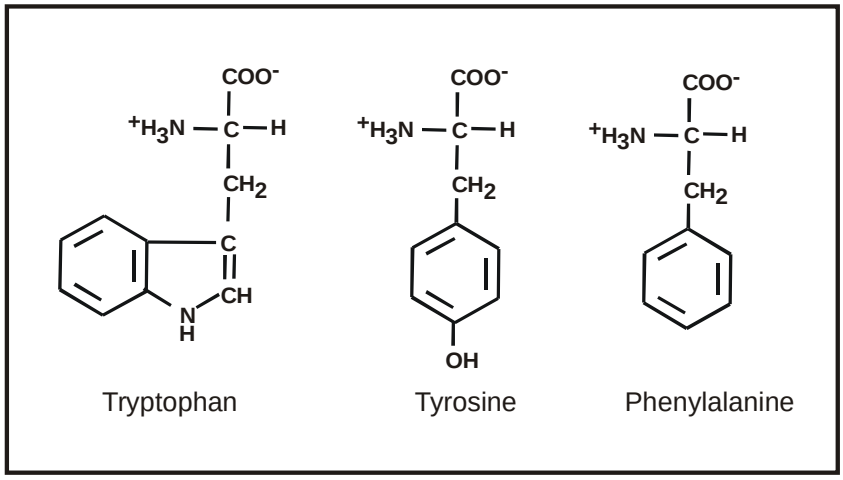

Figure 1. Chemical Structure of the Aromatic Amino Acids.

The three amino acid residues that are primarily responsible for the inherent fluorescence of proteins are tryptophan, tyrosine and phenylalanine (Figure 1). These residues have distinct absorption and emission wavelengths and differ in the quantum yields (Table 1). Tryptophan is much more fluorescent than either tyrosine or phenylalanine. However, the fluorescent properties of tryptophan are solvent dependent. As the polarity of the solvent decreases, the spectrum shifts to shorter wavelengths and increases in intensity. For this reason, tryptophan residues buried in hydrophobic domains of folded proteins exhibit a spectral shift of 10 to 20 nm. This phenomenon has been utilized to study protein denaturation [2]. Tyrosine can be excited at wavelengths similar to that of tryptophan, but emits at a distinctly different wavelength. While tyrosine is less fluorescent than tryptophan, it can provide significant signal, as it is often present in large numbers in many proteins. Tyrosine fluorescence has been observed to be quenched by the presence of nearby tryptophan moieties via resonance energy transfer, as well as by ionization of its aromatic hydroxyl group. Phenylalanine is very weakly fluorescent and can only

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be observed in the absence of both tryptophan and tyrosine. Due to tryptophan's greater absorptivity, higher quantum yield, and resonance energy transfer, the fluorescence spectrum of a protein containing the three amino acids usually resembles that of tryptophan.

Table 1. Fluorescent Characteristics of the Aromatic Amino Acids.

	Absorption		**Fluorescence**	
Amino Acid	Wavelength (nm)	Absorbtivity	Wavelength (nm)	Quantum Yield
Tryptophan	280	5,600	348	0.20
Tyrosine	274	1,400	303	0.14
Phenylalanine	257	200	282	0.04

The Synergy HT multi-detection microplate reader is a robotic compatible microplate reader that can measure absorbance, fluorescence, and luminescence. The Synergy HT utilizes a unique dual optics design that has both a monochromator/xenon flash system with a silicone diode detector for absorbance and a tungsten halogen lamp with blocking interference filters and a PMT detector for fluorescence. A Synergy HT configured for time-resolved fluorescence is capable of measuring fluorescence in either the conventional mode where the fluorescent emission is measured with the excitation source still present, or in time-resolved mode where the fluorescence is measured at some point following the cessation of excitation. When the reader is in conventional fluorescence mode, it uses a tungsten-halogen lamp as a light source and band-pass filters in a filter wheel cartridge to provide wavelength specificity. When the reader is used in time-resolved mode it automatically switches to the xenon-flash lamp light source with a monochromator to select wavelength. The excitation filter cartridge is replaced with the TR-cassette that, in addition to directing the light from the different light source to the microplate, can also hold a single excitation filter if necessary. When in time-resolved mode, the user has the ability to control the time between the cessation of excitation and the initiation of fluorescence measurement (delay time), as well as the length of time the fluorescent signal is accumulated (collection time). In addition to a rapid cessation of light, the xenon-flash lamp has the added benefit of providing output in the UV wavelength range. Using a delay setting of zero allows us to take advantage of this ability to provide excitatory light in the UV range for conventional fluorescence measurements. All fluorescent measurements in this treatise were made using this capability of the reader.

Materials and Methods

L-Tryptophan (cat # T-8941); L-tyrosine (cat # P-2126); and L-phenylalanine (cat # T-3754), bovine serum albumin, BSA, (cat # A-0281), dibasic potassium phosphate (cat # P-3786) and sodium hydroxide (cat # S-5881) were purchased from Sigma-Aldrich Chemicals (St. Louis, MO). Several different microplates were used: for absorbance measurements, Costar UV transparent microplates (cat # 3635) and for fluorescence determinations, Costar opaque black microplates (cat # 3915). Several series of dilutions of amino acids were made using distilled water as the diluent. Following dilution, 200 ml aliquots were pipetted into microplate wells and absorbance scans from 200 nm to 350 nm in 1 nm increments were then made using a Synergy HT multi-detection microplate reader equipped with time resolved capable optics (BioTek Instruments, Winooski, VT). The absorbance at each wavelength of a well containing only 200 µl of water was subtracted from experimental samples and the data plotted using Microsoft© Excel. Using the same dilutions, fluorescence determinations of amino acids and proteins were made using a Synergy HT. All data was collected, analyzed and plotted with KC4 software (BioTek Instruments, Winooski, VT).

Several experiments investigating some of the unique properties of L-tyrosine fluorescence were performed. The effect of phosphate concentration on tyrosine fluorescence was assessed using dilutions of dibasic potassium phosphate ranging from 2.16M to 0.09M. Each dilution contained 0.1mg/ml of L-tyrosine and had a pH of 7.5. Using these dilutions, 200 µl aliquots were pipetted

into microplates in replicates of 8 and the fluorescence measured. The effect of pH on tyrosine fluorescence was determined by making two parallel dilution series of L-tyrosine. One was made starting with a concentration of 0.125 mg/ml L-tyrosine in water with water as the diluent, while the second started with 0.125 mg/ml of L-tyrosine in a 0.01N NaOH solution with 0.01N NaOH as the diluent. After the dilutions were prepared, 200 µl aliquots of each were pipetted in replicates of 4 to the same microplate and the fluorescence measured.

Results

As demonstrated in Figure 2, aromatic amino acids and proteins absorb UV light with two distinct peaks. The peak centered on 280 nm is the result of absorbance by the aromatic ring portion of their structure. The peak at lower wavelengths is caused by absorbance of peptide and carboxylic acid moieties in the compounds. On a molar basis tryptophan absorbs more light at 280nm than either tyrosine or phenylalanine. Note that BSA protein, which has an absorbance value at 230 nm similar to that of tryptophan, has less absorbance at 280 nm as a result of fewer aromatic rings on a molar basis.

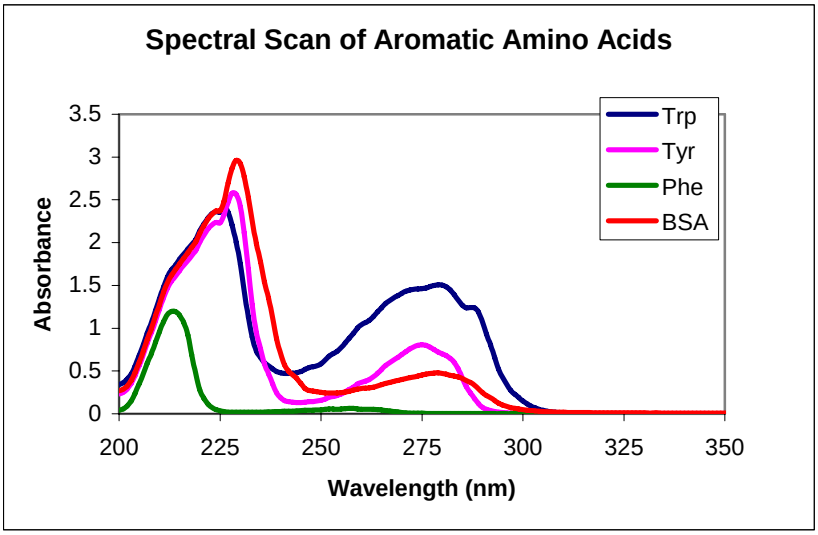

Figure 2. Absorbance Spectral scans of aromatic amino acids and bovine serum albumin (BSA). Spectral scans from 200 nm to 350 nm in 1 nm increments were performed on the amino acids, tryptophan, tyrosine and phenylalanine, as well as BSA protein in aqueous solution using a Synergy HT multi-detection microplate reader.

Using the monochromator to select excitation wavelength, precise tuning of the excitation wavelength is possible. As demonstrated in Figure 3, the fluorescent signal of tryptophan returned can vary as much as 20% over a 12 nm wavelength excitation range, despite using the same 340/30 emission filter for all determinations. Interestingly enough, the background fluorescence of the microplate was observed to vary quite dramatically over the same excitation wavelengths. Therefore it is important to examine the corrected signal (i.e. blank subtracted) as some raw signal values while greater may have a correspondingly larger background fluorescence signal resulting in a net lower signal (data not shown). Using the data from Figure 3, an excitation wavelength of 284 nm was used for subsequent determinations of L-tryptophan.

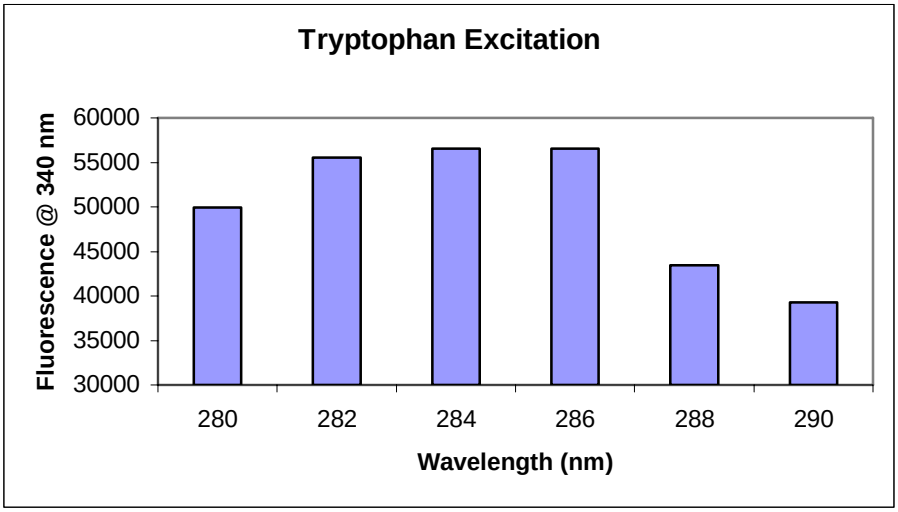

Figure 3 Excitation peak determination for L-tryptophan in solution. L-tryptophan in solution was excited with UV light at various wavelengths and the fluorescence was measured using a BioTek Synergy HT TR with a 340/30 filter. All readings were obtained using the same sensitivity setting of 100 and the blank value at that wavelength subtracted.

Using the optimized excitation wavelength, several dilutions of L-tryptophan were aliquoted into a microplate and the fluorescence measured. The resultant concentration curve plotted in KC4 software demonstrates a linear relationship between L-tryptophan concentration and fluorescence (Figure 4). Concentration determinations can be made with a high degree of confidence, as the correlation coefficient (r2) for the linear regression analysis was calculated to be 0.9989. Using fluorescence, the limit of detection of L-tryptophan was found to be 62.5 ng/ml, which converts to 12.5 ng/well.

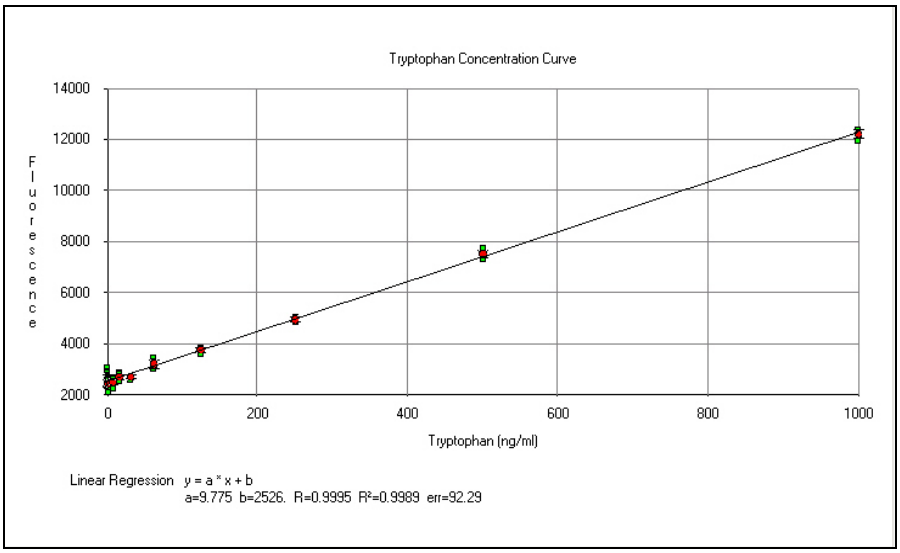

Figure 4. Tryptophan Concentration Curve. A series of dilutions of L-tryptophan were made using distilled water as the diluent. Aliquots of each concentration (200 µl) were pipetted into microplates in replicates of 8. The fluorescence was determined using an excitation wavelength of 284 nm in conjunction with a 340/30-emission filter. Readings were made from the top using a sensitivity setting of 95.

The peak excitation wavelength for L-tyrosine was determined in a similar fashion as described for tryptophan. Excitation wavelengths from 268 nm to 278 nm in 2 nm increments were tested for their ability to generate L-tyrosine fluorescence when measured with a 310/20-emission filter. Figure 5 demonstrates that wavelengths between 270 nm and 276 nm produce equivalent blank-

subtracted fluorescent signals. Subsequent experiments used 274 nm as the excitation wavelength. Several dilutions of L-tyrosine were then aliquoted into a microplate and the fluorescence measured. As seen in Figure 6, the fluorescent signal of L-tyrosine with an excitation wavelength of 274 nm and a 310/20-emission filter is directly related to amino acid concentration. Using a least means squared linear regression analysis, the correlation coefficient (r2) was greater than 0.998. Under these conditions, samples with as little as 800 ng/ml of L-tyrosine (160 ng/well) can be determined.

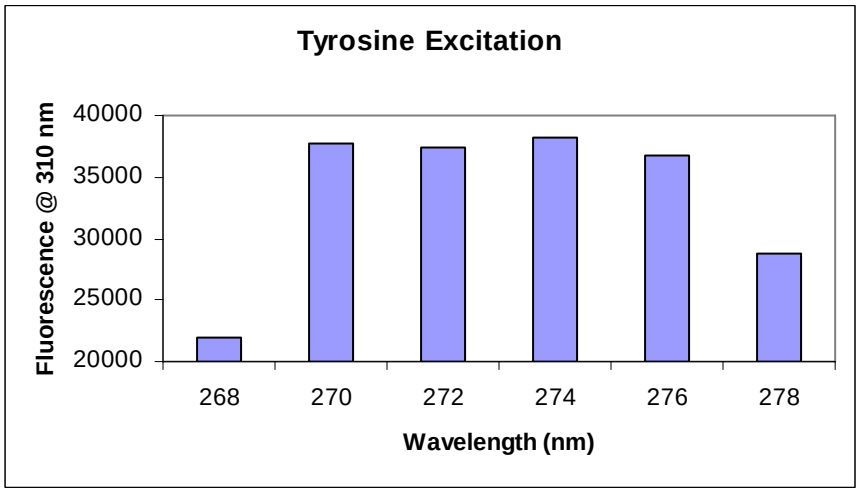

Figure 5. Excitation peak determination for L-Tyrosine in solution. L-tyrosine in aqueous solution was excited with UV-light at various wavelengths and the fluorescence was measured using a Synergy HT TR with a 310/20 filter. All readings were obtained using the same sensitivity setting of 150 and the value of the blank at that wavelength subtracted.

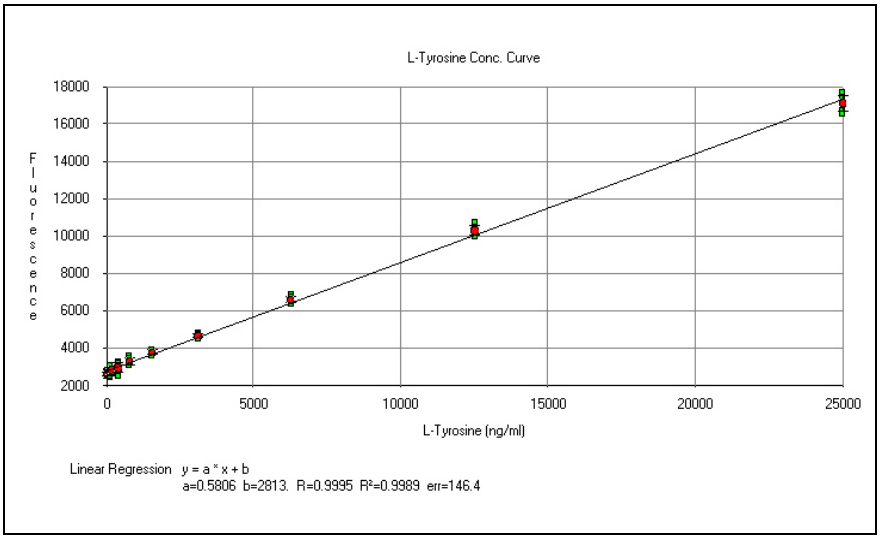

Figure 6. Tyrosine Concentration Curve. Several dilutions of L-tyrosine were prepared using deionized water as the diluent. Aliquots of each dilution (200 µl) were pipetted into microplates in replicates of eight and the fluorescence determined using a Synergy HT and KC4 Software. All determinations were made from the top with an excitation wavelength of 274 nm and a 310/20 nm emission filter, 50 reads per well and a PMT-sensitivity setting of 180.

Because L-tyrosine fluorescence is influenced by ionization, several experiments were performed to investigate this phenomenon. The phenolic hydroxyl group of tyrosine is capable of ionization under a number of different conditions (see Figure 7). The pKa of the group is reported to be 10.3; thus at high pH levels (e.g. pH 12) it would be expected to be primarily in the

ionized state. The fluorescence signal returned when tyrosine at pH 12 is excited at 274 nm is reduced when compared to the same concentration at pH 7 (Figure 8). It has been reported that the excitation peak of tyrosinate is 340 nm rather than near 274 nm [2].

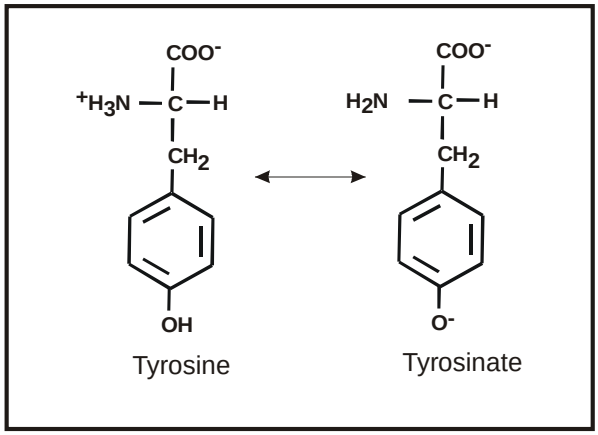

Figure 7. Ionization of tyrosine to tyrosinate. With increasing pH, protons are lost from tyrosine forming tyrosinate, which has markedly different fluorescent properties than the non-ionized form.

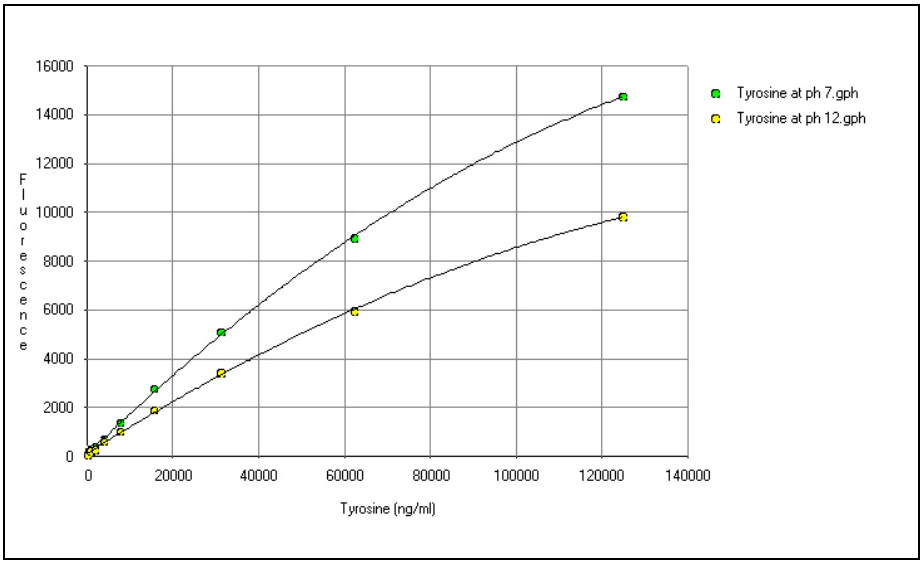

Figure 8. Effect of pH on Tyrosine Fluorescence. Several dilutions of L-tyrosine were made in either water (approximately pH 7) or 0.01N NaOH (pH 12). Samples (200 µl) of each dilution were aliquoted into microplates in replicates of four and the fluorescence determined using a Synergy HT with an excitation wavelength of 274 and a 310/20-emission filter. Samples were measured from the top at 50 reads per well and a PMT-sensitivity setting of 150.

Factors other than pH can cause tyrosinate formation. As seen in Figure 9, the concentration of phosphate can influence the fluorescent signal of tyrosine even at neutral pH levels. Increasing levels of phosphate are capable of reducing the fluorescence seven fold. Phosphate is reported to form a ground-state complex with tyrosine that prepares tyrosine to ionize to tyrosinate as a result of excitation by light. The result is a decrease in emission with a 310/20 filter. Acetic acid is reported to cause a similar phenomenon, where the presence of high concentrations results in a loss of fluorescence signal (data not shown). In this example, it is caused by the acetate group removing the phenolic proton of tyrosine at neutral pH [2].

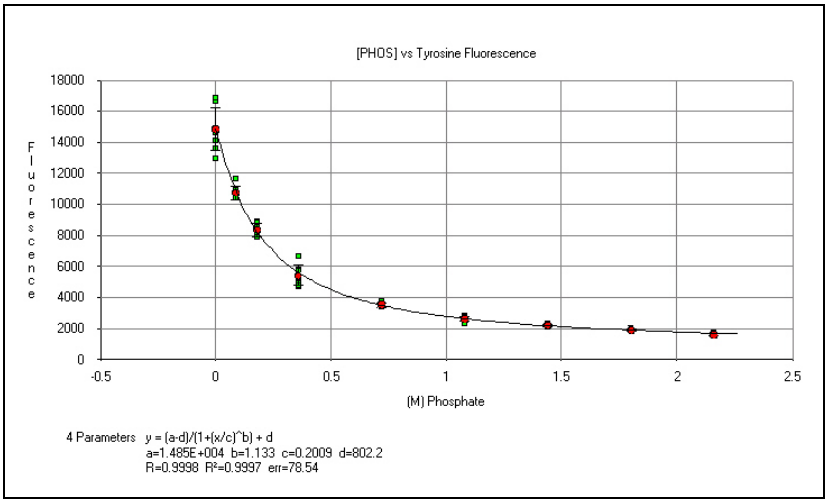

Figure 9. Effect of Phosphate Concentration on Tyrosine Fluorescence. Several dilutions of dibasic potassium phosphate (K2HPO4) were prepared with equal concentrations of L-tyrosine and at a pH of 7. Aliquots of each (200 µl) were pipetted into microplates in replicates of 8 and the fluorescence measured using a Synergy HT TR with an excitation wavelength of 274 nm and a 310/20 emission filter at a PMT-sensitivity setting of 150.

Using the same methodology, the fluorescence of several dilutions of bovine serum albumin (BSA) protein in aqueous solution were measured. Despite the presence of both tyrosine and tryptophan amino acids in the peptide, the maximal response resulted when the reading parameters for tryptophan were used. This agrees with previously reported findings that suggest that energy absorption by tyrosine is often transferred to tryptophan [2]. Figure 10 demonstrates the ability to use fluorescence to measure protein rather than specific aromatic amino acids. When dilutions of bovine serum albumin are prepared a linear relationship between fluorescence and protein concentration is observed. In this experiment, the wavelengths found to be optimal for tryptophan were used. Using these wavelengths a sample with a concentration as low as 400 ng/ml could be detected from the buffer only control. The output using the optimal wavelengths for tyrosine was considerably lower at the same sensitivity setting, yet demonstrated a similar linear relationship (data not shown).

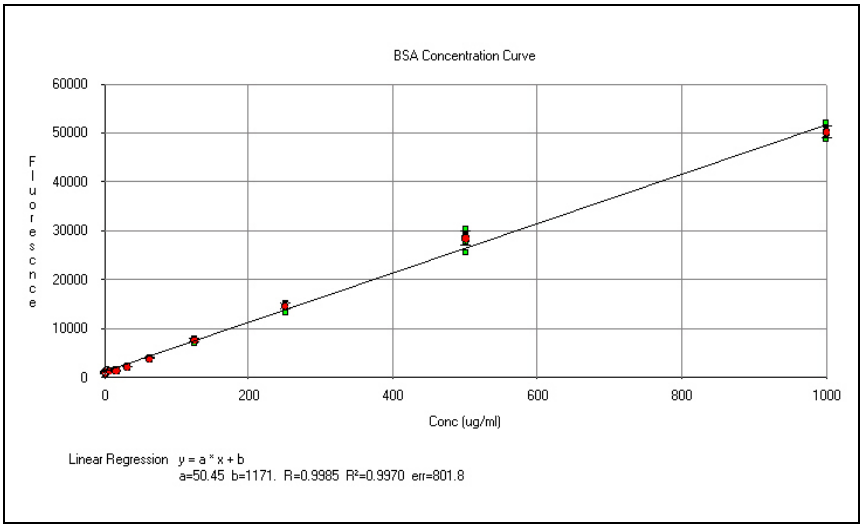

Figure 10. BSA Concentration Curve. A series of dilutions of bovine serum albumin (BSA) were prepared with water as the diluent. After dilution, 200 µl aliquots were pipetted into microplates in replicates of 8 and the fluorescence using an excitation wavelength of 285 nm and a 340/30-emission filter. Data was collected from the top at 100 reads per well with a PMT sensitivity setting of 100.

Discussion

These data presented demonstrate that the Synergy HT TR model is capable of UV-excitation fluorescence determinations. Time-resolved fluorescence requires a light source that can either be shuttered quickly or diminishes very rapidly. Because of its nature the xenon-flash lamp is often used for time-resolved measurements due to its rapid light decay. In regards to UV fluorescence, the xenon flash lamp also offers significantly more UV light output than the tungsten-halogen lamp usually used for excitatory light. Selecting "Time-resolved" fluorescence with a Synergy HT TR enables the xenon-flash lamp, but by selecting a time delay of 0 traditional fluorescence measurements are performed which utilize the xenon-flash lamp in conjunction with a monochromator to select wavelength. This combination allows the end-user to excite peptides in UV range with sufficient energy, while maximizing the excitatory wavelengths using the monochromator, yet still having the advantages of filter-based wavelength selection for emission wavelengths.

While most of the experiments performed were carried out using amino acids, peptides and proteins which contain aromatic amino acids, can be detected as well. When measuring peptides it is important to keep in mind that the local environment of the aromatic amino acids can have an effect on their spectra. Tryptophan, the most significant fluorescence emitter, will have an emission peak at lower wavelengths if it is buried within the hydrophobic inner regions of a protein [2]. Tyrosine moieties will often transfer their energy to adjacent tryptophan amino acids, while ionized tyrosinate also demonstrates wavelengths similar to tryptophan, suggesting that for many proteins a good starting point for excitation and emission wavelengths are those for tryptophan.

The Synergy HT TR reader is an ideal reader for protein measurements. Besides using UV fluorescence to detect proteins, the UV absorbance measurement capabilities of the Synergy HT TR can be utilized. Because it has two complete optical systems for absorbance as well as one for fluorescence, either can be used to detect protein without compromise. As with all of the BioTek readers, KC4 data reduction software provides reader control as well as exceptional data reduction capabilities. Several standard curves using different curve fits were produced as part of this treatise, demonstrating some of KC4's capabilities.

References

(1) Warburg, O. and W. Christian (1942) Biochem. Z. 310:384-421.
(2) Principles of Fluorescence Spectroscopy 2nd Edition (1999) Lakowicz, J.R. Editor, Kluwer Academic/Plenum Publishers, New York, New York.

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Rev. 4/18/03