

# Determination of Protein Unfolding Using UV-fluorometry

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## Overview

Intrinsic protein fluorescence at UV-range is well known phenomenon that has been used for a long time for protein research. It is mainly based on fluorescence of tryptophan that has the highest quantum yield of all amino acids. Tryptophan fluorescence is initiated when protein is excited with about 280 nm light and emission is happening at about 350 nm. This emission wavelength is anyhow dependent on the environment of the tryptophan residues; in polar environment emission is that about 350 nm when in non-polar environment emission is decreased to around 330 nm. The intrinsic protein fluorescence is always quite weak so using it as a research tool requires highly sensitive and well performing instrument.

## Introduction

In this work, measurement parameters for protein unfolding assay with Thermo Scientific™ Varioskan Flash spectral scanning multitechnology reader were optimized using lysozyme and BSA as example proteins. In addition, detection limit for pure tryptophan and BSA were determined to estimate the overall performance of the system in protein intrinsic fluorescence assays. Assays were performed in two formats: equilibrium type assay where protein samples were incubated with the denaturing agent until equilibrium and tryptophan fluorescence was measured by fluorometric spectral scanning where shifts of the emission peaks are revealed for each sample. Same samples were measured also using kinetic measurement where protein unfolding was followed kinetically using continuous detection of two emission wavelengths.

## Materials and Methods

### Instrumentation and consumables

All protein unfolding assays were performed with 384-well polystyrene solid black flat bottom microplate (Thermo Scientific NUNC, prod. code 262260) using fluorometric top reading. Actual fluorescence measurement were performed with Varioskan Flash spectral scanning multitechnology reader that has quadruple monochromator system (double excitation/double emission monochromators) for fluorometric measurement that ensures precise detection of fluorescence spectra.

### Assay performance and fluorescence spectra with Tryptophan and BSA

Concentration series of Tryptophan and BSA were made in distilled water. Then, 40 µl aliquots were transferred into a microplate with eight replicates. The plates were measured with 280 nm excitation and 350 nm emission wavelengths. Tryptophan spectra were measured using 20 pg/well concentration with 384-well plates and 40 µl volume.

FIGURE 1. Tryptophan and BSA fluorescence assays.

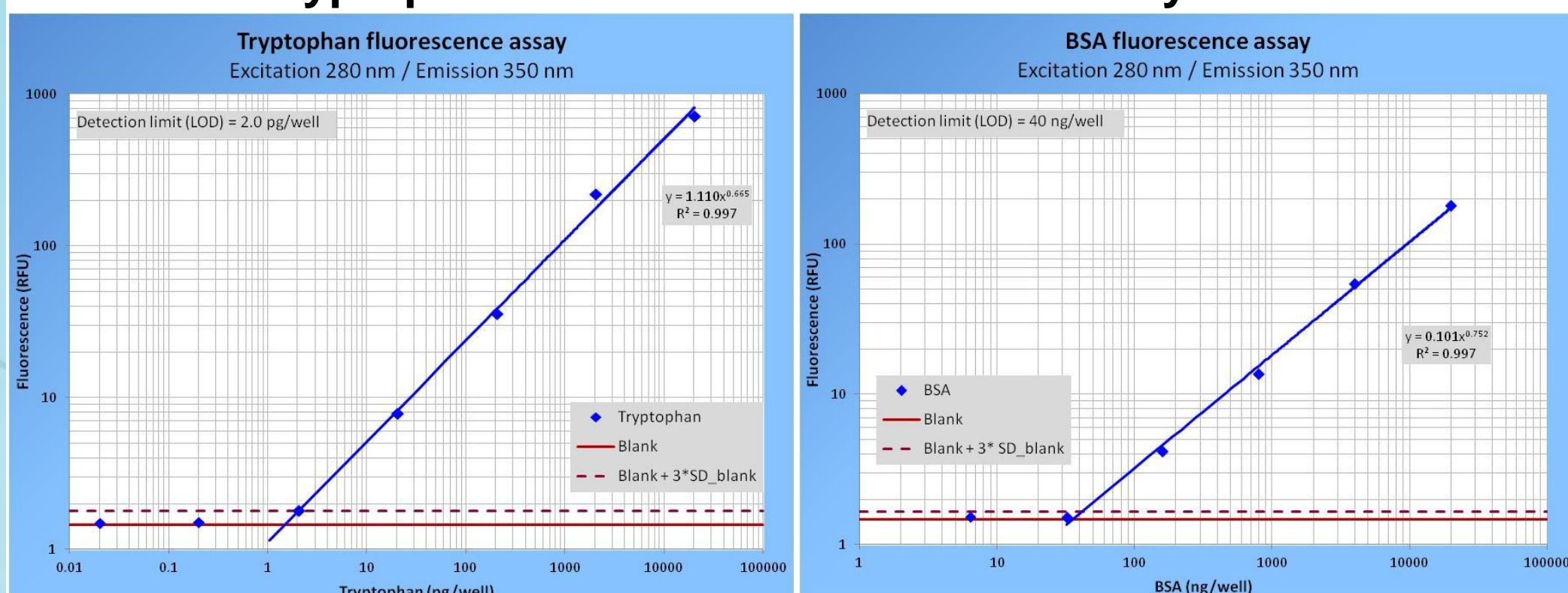
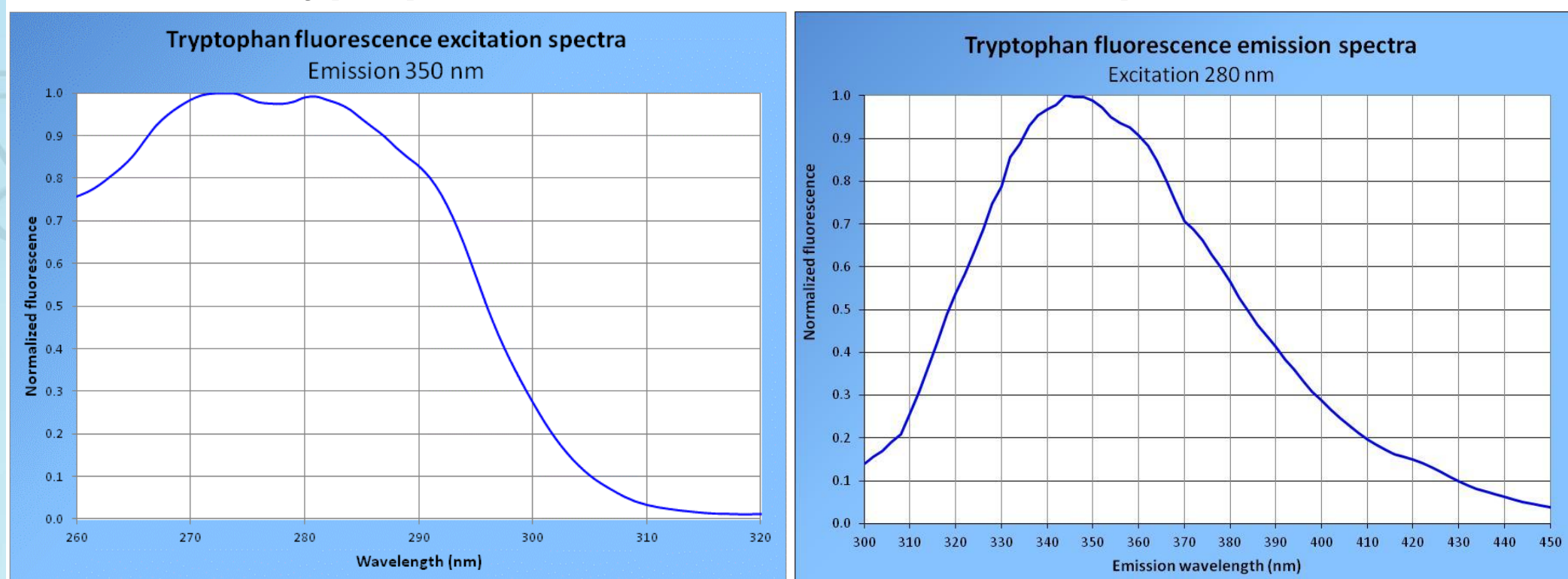


FIGURE 2. Tryptophan excitation and emission spectra



### Protein unfolding with equilibrium assay

Lysozyme and BSA were used as model protein for unfolding studies. Lysozyme stock solution (7mg/ml) was prepared in Tris-HCl buffer (pH 7.4) and 70 µg aliquots were transferred into 384-well microplate. Guanidine hydrochloride was added into 0-6 M concentrations and wells were filled up to 100µl with Tris buffer. The samples were incubated for two hours and the fluorescence emission spectra between 310–400 nm were determined. Different reading times were used to reveal the effect of the reading speed to the result quality. For BSA denaturation assay BSA stock solution (30 mg/ml in distilled water) was added into 384-well microplate as final BSA concentration of 10 µg/well. Then guanidine hydrochloride was added with different concentrations from 0 to 3 M final concentration in the well. The plate was incubated at RT for 1.5 hours and fluorescence emission spectra was measured.

### Protein unfolding with kinetic assay

Kinetic protein unfolding was measured with lysozyme only. Similar guanidine hydrochloride concentration series was prepared as in equilibrium assay but fluorescence was measured kinetically for 90 min starting immediately after the addition of the denaturing agent. Measurement was performed as dual emission kinetic format, where two emission wavelengths (330 nm and 350 nm) were measured using fixed 280 nm excitation.

## Results and Discussion

### Assay performance and fluorescence spectra with Tryptophan and BSA

Lysozyme and BSA calibration curves with calculated detection limits (IUPAC 3\*SD) are shown in Figure 1. With 384-well plates it is possible to detect 2 pg/well tryptophan and 40 ng/well BSA.

Tryptophan excitation and emission spectra measured in water are shown in Figure 2. and it is clearly seen that emission maximum close to 350 nm is typical for polar environment. Excitation maximum is somewhat wider so that about the same excitation efficiency is reached with the wavelength range around 270–285 nm.

FIGURE 3. Spectral shift of lysozyme unfolding

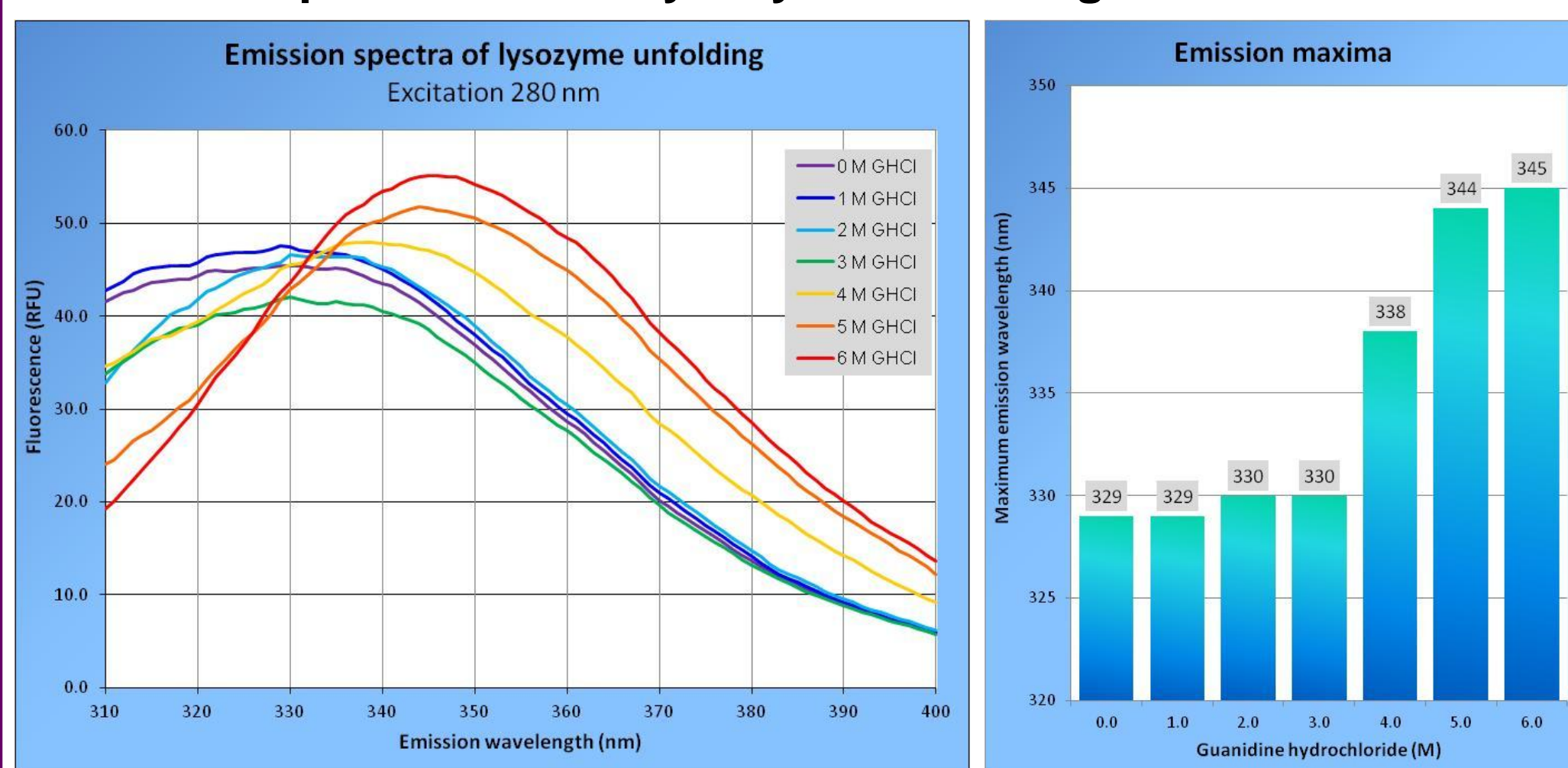
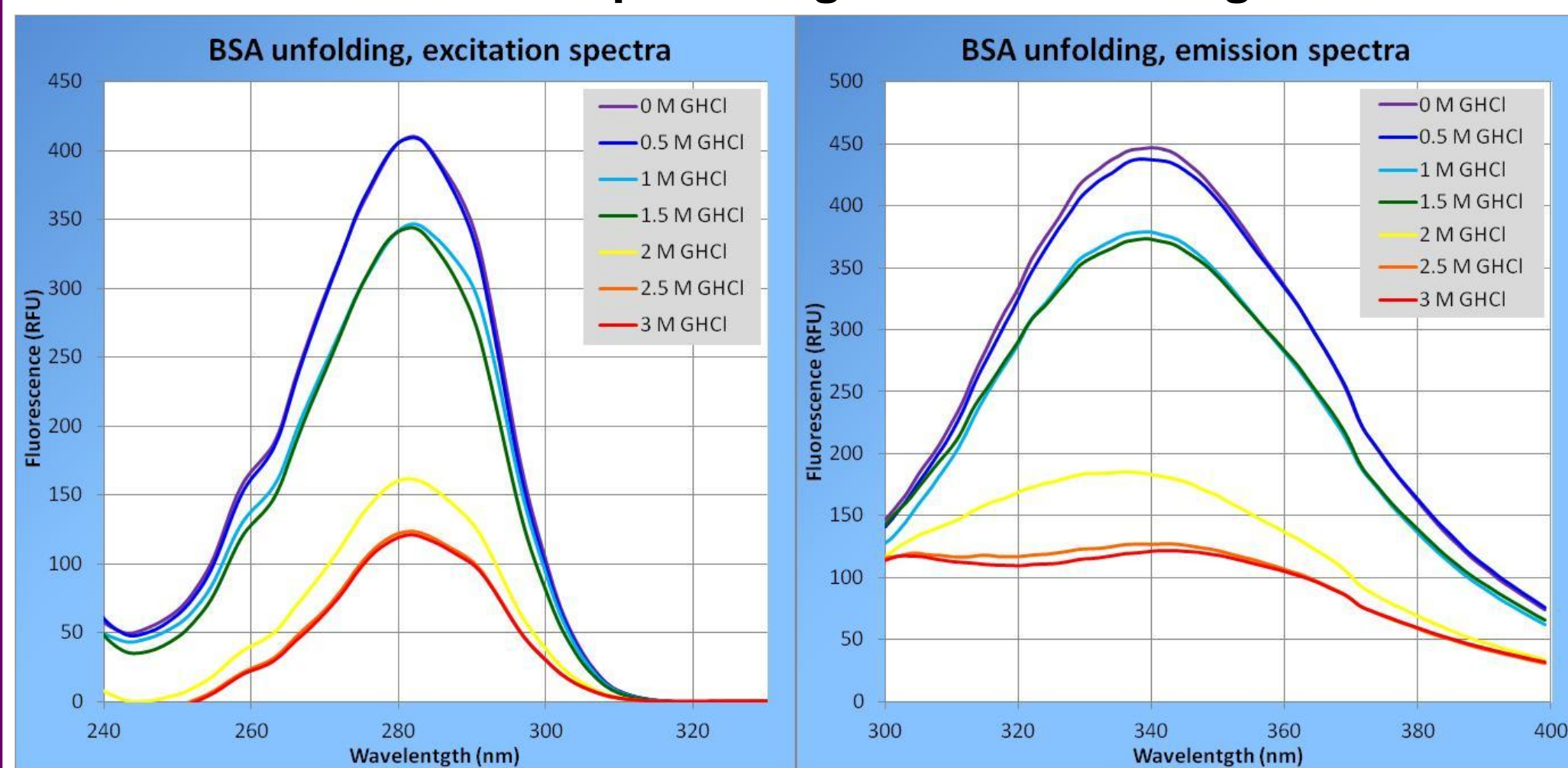


FIGURE 4. Fluorescence quenching of BSA unfolding



### Protein unfolding with equilibrium assay

Lysozyme and BSA intrinsic fluorescence emission spectra with different concentrations of guanidine hydrochloride are shown in Figures 3. and 4. Intact lysozyme shows emission maximum at 330 nm when tryptophan residues are in polar environment inside the protein structure. Lysozyme stays intact up to 3 M guanidine hydrochloride concentration, with higher concentrations we can clearly see the spectral shift coming from protein unfolding and with 5 or 6 M guanidine hydrochloride we already see identical spectrum with pure tryptophan indicating total denaturation of the protein.

When BSA is denatured with guanidine hydrochloride similar spectral shift of the fluorescence emission is not seen. Instead, a strong fluorescence quenching effect is clearly seen, both in excitation and emission. This quenching of intrinsic fluorescence of albumins instead of spectral shifting is an old well know phenomenon (e.g. Zhao et. al., Spectrochimica Acta, vol 65, 2006, pp 811–817, Steiner et. al, J. Bio. Chem., vol 241, no. 3, 1966).

Figure 5. shows the effect of the measurement time used in the quality of the recorded spectra. Whewn spectra is read with very high speed the results is clearly more noisy than with the longer reading times. It therefore recommend to use long reading times, considering the stability of the samples and throughput requirements.

FIGURE 5. Effect of fluorescence measurement time

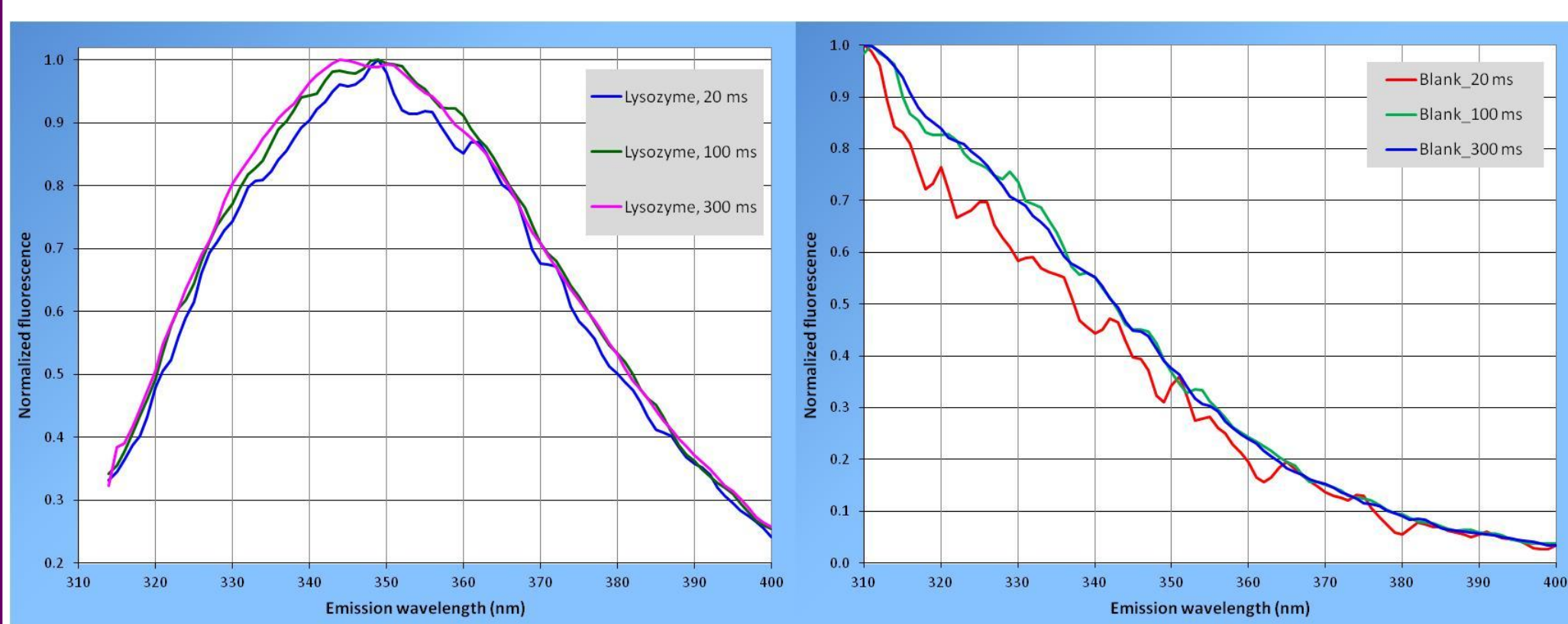
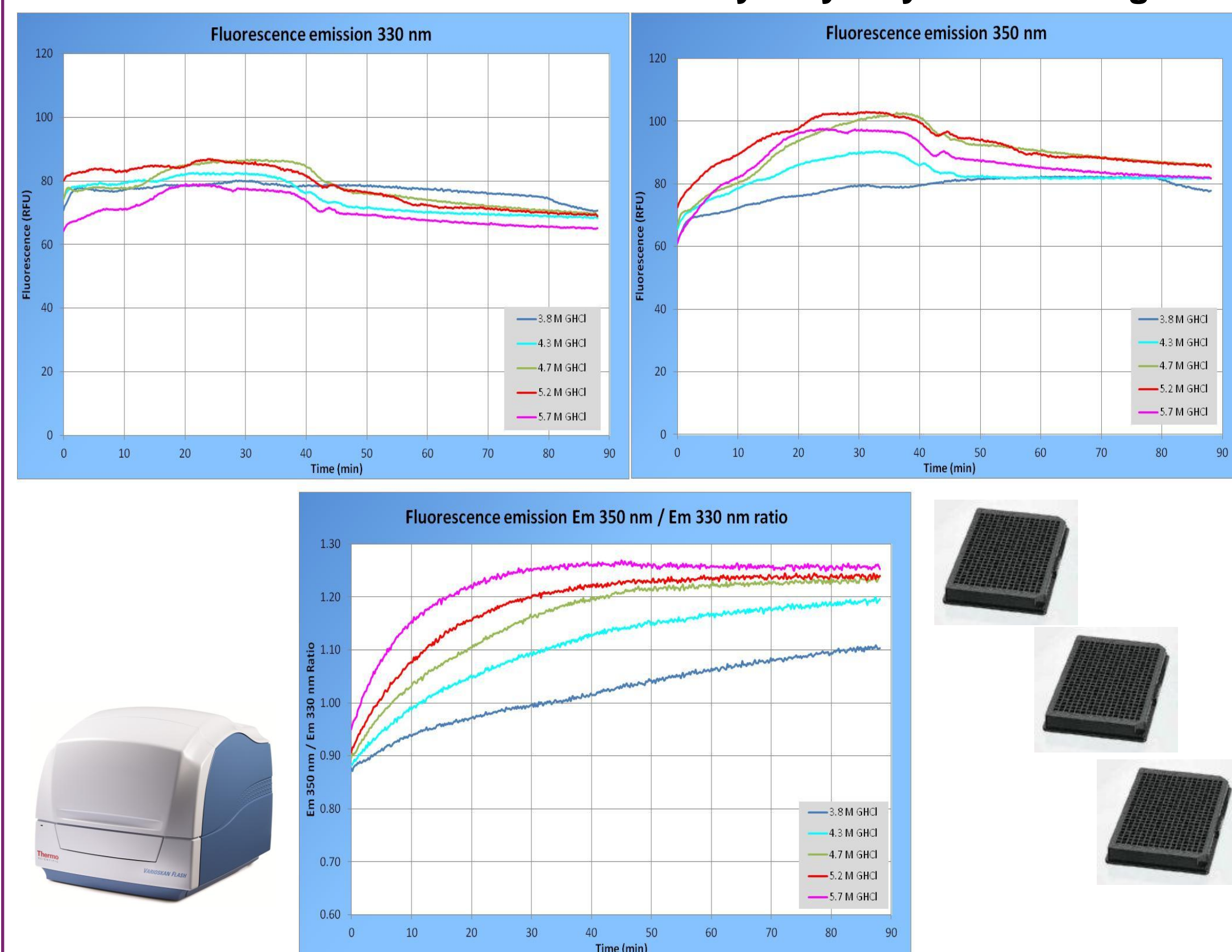


FIGURE 6. Kinetic dual fluorescence assay of lysozyme unfolding



### Protein unfolding with kinetic assay

Kinetic assay is an efficient tool for protein stability testing with such proteins where spectral shift between 330 nm and 350 nm emissions happens during unfolding and denaturation. Kinetic unfolding curves of lysozyme are shown in Figure 6. Kinetic measurement was done using dual fluorescence mode where both 330 nm and 350 nm emissions were measured in every 10 seconds. Kinetic curves with single wavelength show quite notable variations over time, probably due to protein aggregations causing samples to become heterogeneous. When excitation beam of the reader is very narrow, more or less aggregations floating in the sample hit inside the beam. This causes fluctuations in fluorescence signals, but these fluctuations are identical in both 330 nm and 350 nm emissions. These fluctuations make using single emission quite complicated.

Using dual emission assay with two emission wavelengths and using their ratio as a result automatically eliminates these single wavelength fluctuations, as seen in Figure 6. Both single wavelength curves show remarkably fluctuations but their ratio shows very nice increasing curves with complete correlation with the concentration of denaturing agents.

## Conclusion

- The results show that Varioskan Flash spectrofluorometer is a powerful tool for protein unfolding studies using tryptophan fluorescence.
- Double excitation and emission monochromators offer sufficient spectral resolution to efficiently separate emissions from non-polar and polar environments
- High wavelength changing speed gives possibility to measure kinetic shifting from non-polar to polar environment with high accuracy.
- In kinetic assay it is possible to read whole 96 well plate with two emission wavelengths in about 20 seconds which guarantees reliable kinetic follow up of the protein unfolding.

## Acknowledgements

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For more information on Thermo Scientific microplate readers, see [www.thermoscientific.com/platereaders](http://www.thermoscientific.com/platereaders).

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