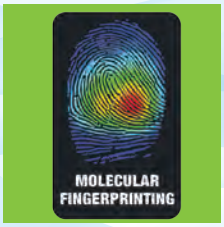
HORIBA Scientific

Insulin Structure and Stability Assessment

FLSS-42

ELEMENTAL ANALYSIS
FLUORESCENCE
GRATINGS & OEM SPECTROMETERS
OPTICAL COMPONENTS
FORENSICS
PARTICLE CHARACTERIZATION
RAMAN
SPECTROSCOPIC ELLIPSOMETRY
SPR IMAGING



Assessing Insulin structure and stability with patent-pending A-TEEM™ Technology and UV-Vis absorbance spectroscopy

Introduction

Stability and aggregation of insulin are studied using simultaneous fluorescence excitation emission matrices (EEMs) and UV-Vis absorbance spectroscopy **[1]**. Insulin is a protein-hormone, produced by the pancreas and is necessary for basic metabolic processes. The different types of commercial insulin therapeutics generally fall into two categories: short-acting and long-acting insulin. The difference between some short-acting and long-acting insulin is, in some cases, only one to three residues in the protein sequence. This difference in sequence, along with controlled pH of storage and delivery, is used to either trigger or prevent the formation of insulin dimers and hexamers in the blood stream. The formation of these aggregates lets the body absorb insulin more slowly and the absence of aggregates makes it absorb more quickly. **[7]** Changes in protein stability and structure, such as those important to the pharmacokinetics of insulin, can potentially be measured using fluorescence emission spectra, UV-Vis absorbance spectra or sometimes both, using intrinsically fluorescent amino acids. Furthermore, UV-Vis spectrophotometers and fluorometers are typically separate instruments. Here we present a new and fast method for simultaneously generating the individual excitation and emission spectra for all fluorescent sample components providing information needed to correct the fluorescence spectra for the sample concentration-dependent inner filter effects (IFE), as already described **[2]**.

Experimental Method

This study employs the method of simultaneous absorbance-transmission and fluorescence excitation emission matrix (A-TEEM™) spectroscopy with the dedicated patented Aqualog® instrument **[3]**.

The Aqualog®-coupled software is equipped with a built-in tool for normalization to water Raman scattering or quinine sulfate units for the defined emission conditions, correction for the influence of inner filter effects and Rayleigh masking. The analysis of the fully corrected EEM data involves the multivariate routine known as PARAFAC (Solo™ package from Eigenvector Research, Inc.).

Because commercial insulin formulations are high in concentration (4 mg/mL), the IFE correction is very important for measuring the EEM fingerprint, as shown in Figure 1. Using the reagent-free Aqualog® method, absorbance spectra are simultaneously collected with each EEM to correct for non-linearity in fluorescence intensity, specifically resulting from IFE at high concentration/high absorbance solutions.

Results and discussion

Three different insulin proteins have been analyzed at varying concentrations (0.001 mg/mL-4 mg/mL), pH (4.5–8.5) and temperatures (5°C–37°C): recombinant human insulin **[4]**, insulin-aspart (commercially available, fast acting), and insulin-glargine (commercially available, long-acting) as shown in Figure 2. The duration of the data acquisition was a full 30 minutes, during which about 125 EEMs and simultaneous Absorbance Spectra were recorded (15 seconds each). (Note, temperature equilibration of each sample made the total experiment time longer.)

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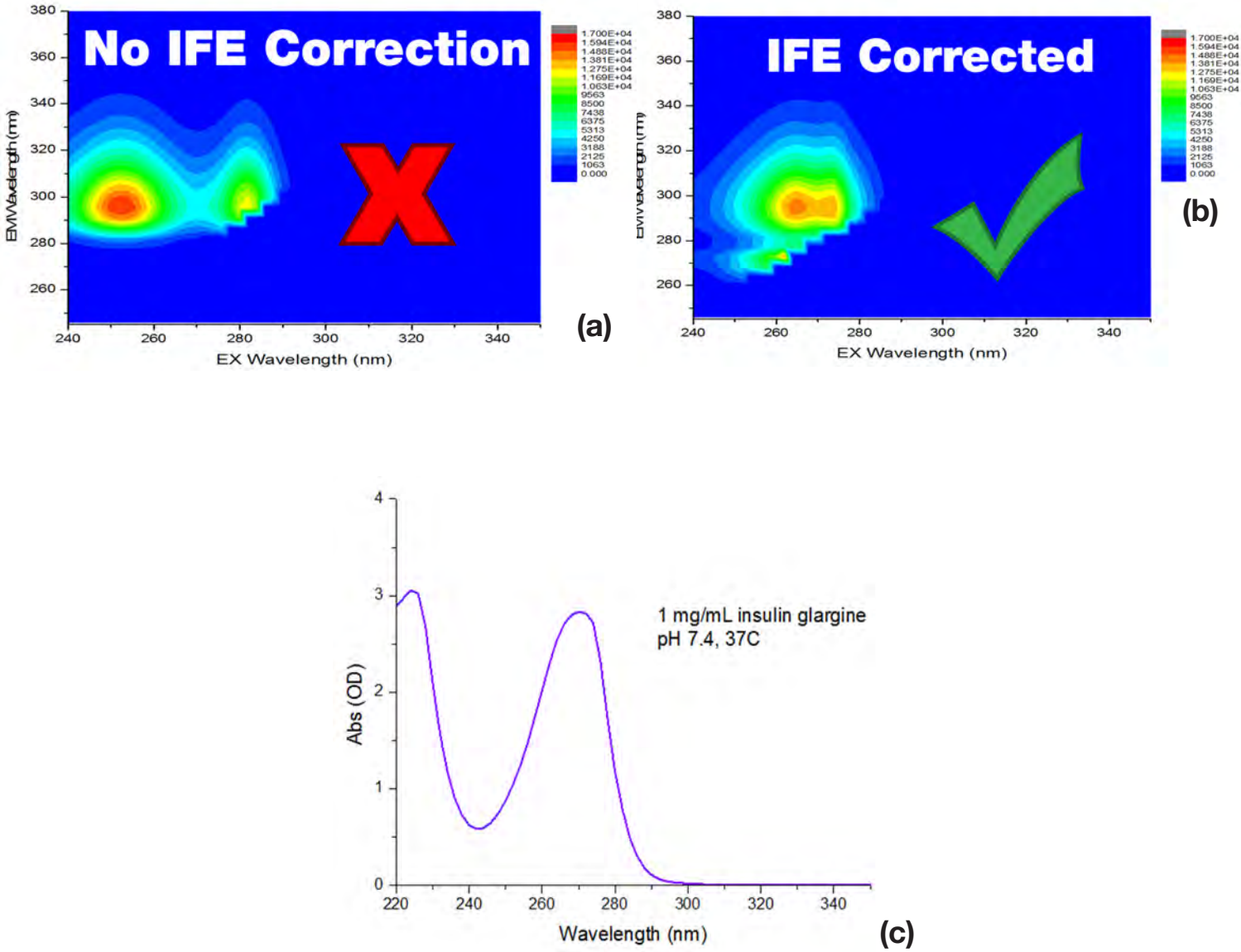

Figure 1. EEM (a), (b) and Absorbance (c) data of 1 mg/ml Insulin Glargine pH 7.4, Temp = 37°C

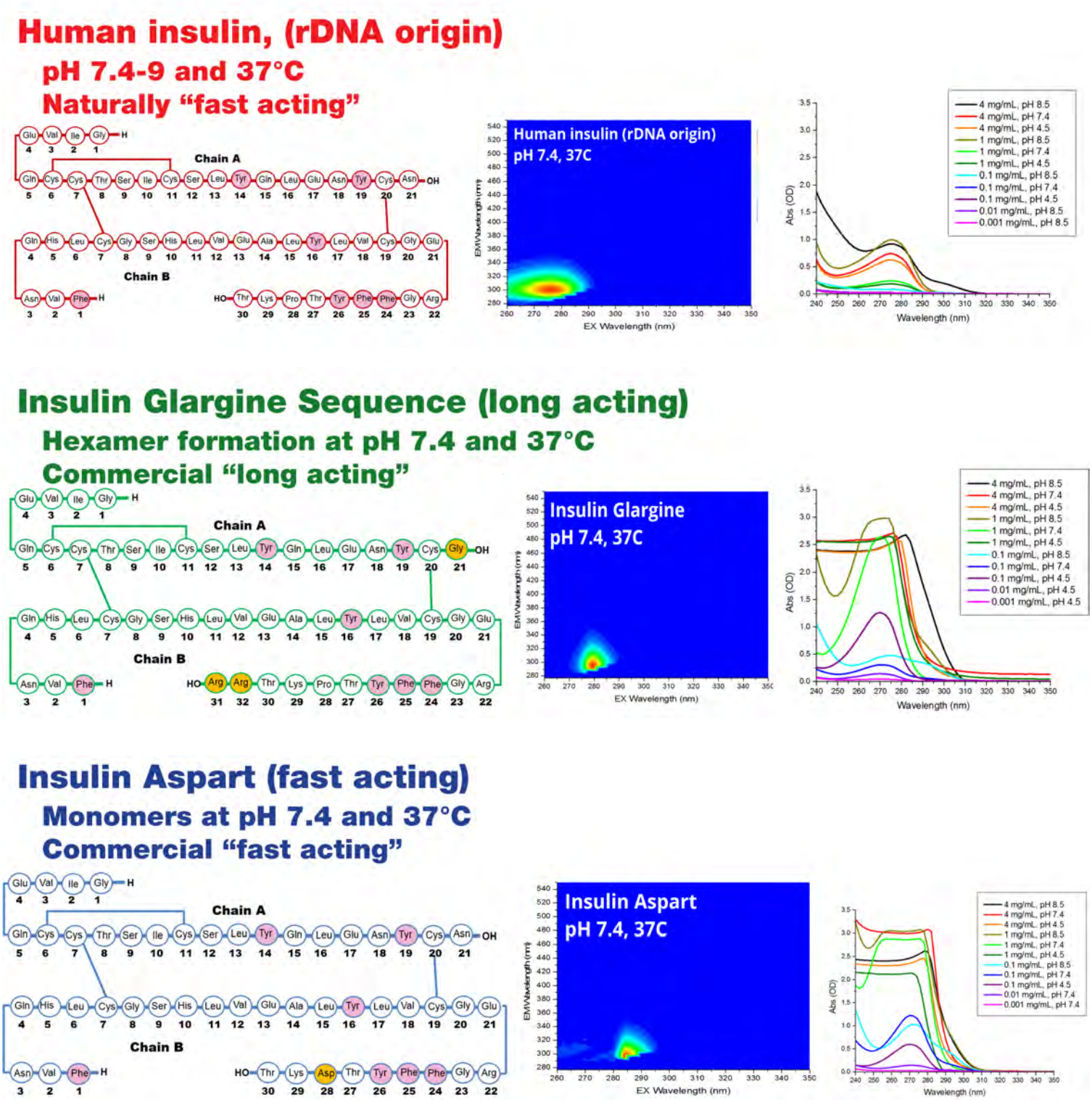

Figure 2. Fully corrected EEMs and Absorbance spectra of human insulin (rDNA origin), Insulin Glargine and Insulin Aspart. Highlighted in yellow are amino acids that differ from human insulin sequence and in pink are intrinsic fluorescent amino acids.

PARAFAC analysis of intrinsic fluorescence A-TEEM™ has been performed in order to characterize the aggregation state of the different types of insulin. Four components have been identified in the commercial formulations: Components 1, 2 and 3 (Comp1, Comp2 and Comp3) identified as Tyrosine **[5]** and Component 4 (Comp4) identified as m-cresol **[6]**, a preservative in insulin formulations **(Figure 3).**

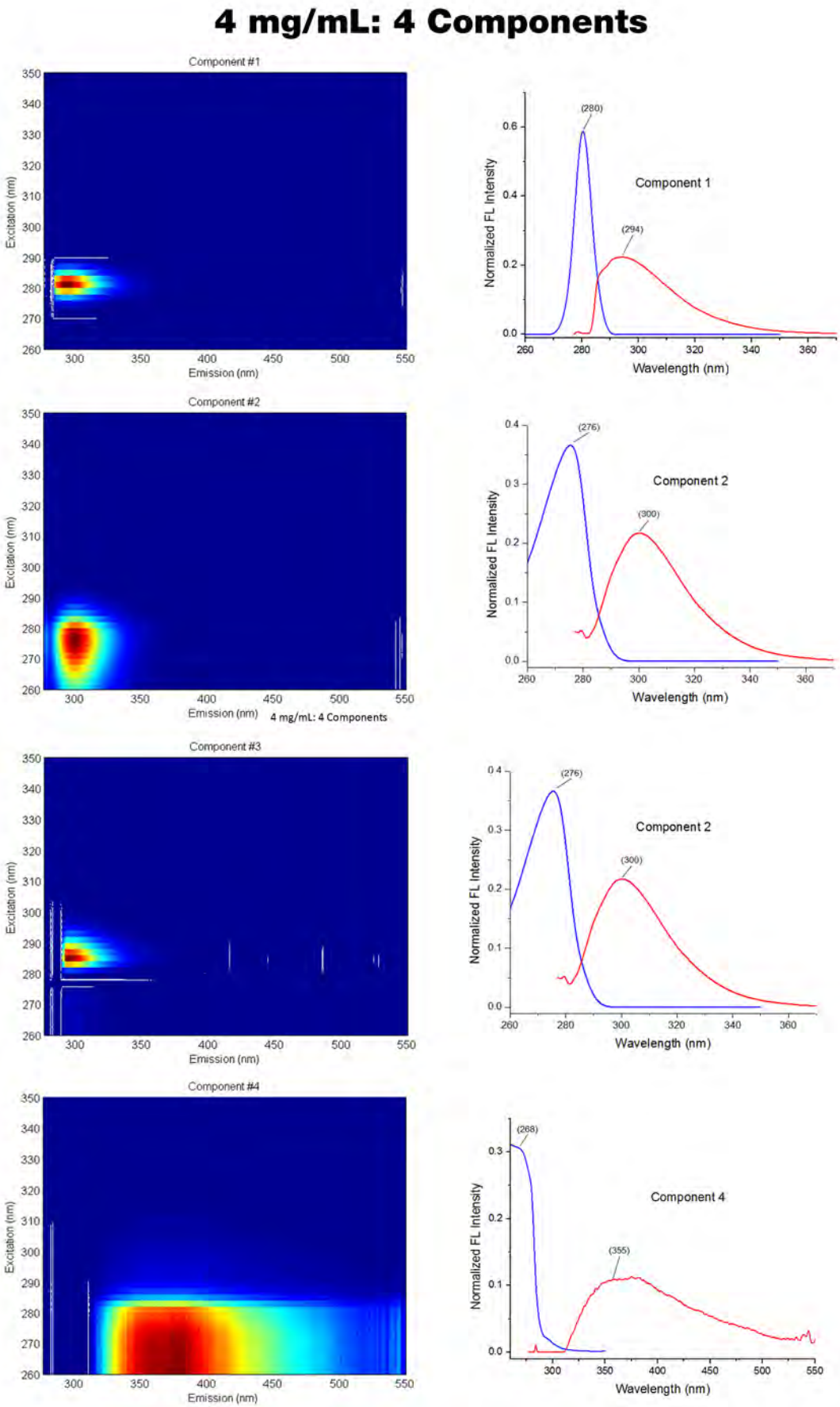

Figure 3. Component EEMs from PARAFAC multivariate chemometric analysis identified in the commercial formulations.

The red data points in **Figure 4** show the scores for a four-component PARAFAC model as fit to recombinant human insulin solutions at pH 4.5(first five data points), pH 7.4 (2nd five data points) and pH 8.5 (last five data points). Each of the five repeats is at temperatures 5°C, 20°C, 25°C, 30°C, and 37°C in that order. The models were repeated and plotted for insulin glargine (green data) and insulin aspart (blue data) under the same sequential conditions. Insulin glargine, which is sequenced to produce aggregates at biological pH and temperature, shows a much larger score for component 1(stars),which may be attributed to the blue-shifted spectrum of tyrosine when insulin is in aggregate form. Solutions of human insulin and insulin aspart, which are less likely to aggregate under the same conditions, show much lower scores for the same component 1. It is evident that insulin sequences that vary by only 1–3 amino acids can be differentiated by intrinsic fluorescence A-TEEM™.

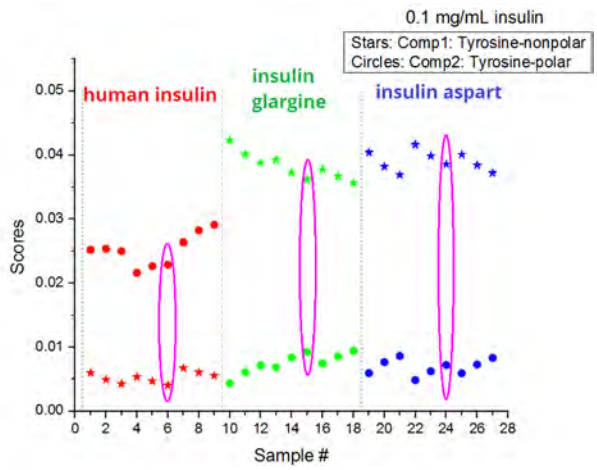

Figure 4. Component EEMs from PARAFAC Multivariate Chemometric Analysis for human insulin (red), insulin glargine (green) and insulin aspart using commercial formulations (4 mg/mL insulin concentration). Circled in PINK are all data points measured at pH 7.4 and 37°C.

Conclusions

The A-TEEM™ method, combined with the Aqualog® spectrofluorometer, is able to characterize protein therapeutic formulations for aggregation behavior in a matter of seconds, even under conditions where there are only small differences in protein sequence. A-TEEM™ is already routinely used to measure dissolved organic matter in water quality applications. This method could potentially be used to characterize other complex biological solutions such as vaccines, enzymes, and even cell culture media. Why not proteins?

Note

This Application Note is part of the article "*A-TEEM™, a new molecular fingerprinting technique: simultaneous absorbance-transmission and fluorescence excitation-emission matrix method,*" published in Methods and Applications in Fluorescence, Volume 6, Number 2 https://doi.org/10.1088/2050-6120/aaa818

References

[1] K. E. Steege Gall, M. Sandros, *Insulin Structure and Stability Assessed by Intrinsic Fluorescence and Simultaneous UV-vis Absorbance Spectroscopy Coupled with Chemometric Analysis,* Abstract in Biophys. J., 112 (3), Supplement 1, 197a (2017)

[2] A. M. Gilmore, *How to collect National Institute of Standards and Technology (NIST) traceable fluorescence excitation and emission spectra Fluorescence Spectroscopy and Microscopy (Methods and Protocols),* (Methods in Molecular Biology Vol 1076) Ed. Y. Engelborghs and A. Visser (Totowa,NJ: Humana Press)

[3] http://www.horiba.com/scientific/products/fluorescence-spectroscopy/steady-state/aqualog/aqualog-r-our-compact-benchtop-fluorometer-for-cdom-13031/

[4] R.G. Larkins, *Human insulin,* Aust. N. Z. J. Med., 13(6): 647-51 (1983)

[5] L. Ellis, J. M. Tavaré, B. A. Levine, *Insulin receptor tyrosine kinase structure and function,* Biochem. Soc. Trans., 19(2): 426-32 (1991)

[6] I. Van Faassen, A. M. Verweij-van Vught, M. Z. Lomecky-Janousek, P.P. Razenberg, E. A. van der Veen, *Preservatives in insulin preparations impair leukocyte function. In vitro study,* Diabetes Care, 13(1): 71-4 (1990)

[7] Donner T. Insulin – Pharmacology, Therapeutic Regimens And Principles Of Intensive Insulin Therapy. [Updated 2015 Oct 12]. In: De Groot LJ, Chrousos G, Dungan K, et al., editors. Endotext [Internet]. South Dartmouth (MA): MDText.com, Inc.; 2000-. Available from: https://www.ncbi.nlm.nih.gov/books/NBK278938/

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