

Insulin Structure and Stability Assessment

FLSS-42

ELEMENTAL ANALYSIS

FLUORESCENCE

GRATINGS & OEM SPECTROMETERS

OPTICAL COMPONENTS

FORENSICS

PARTICLE CHARACTERIZATION

R A M A N

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.)

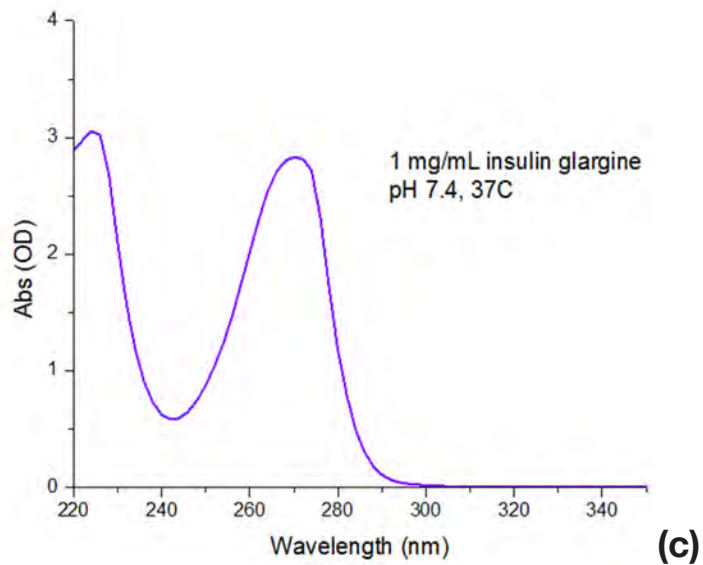
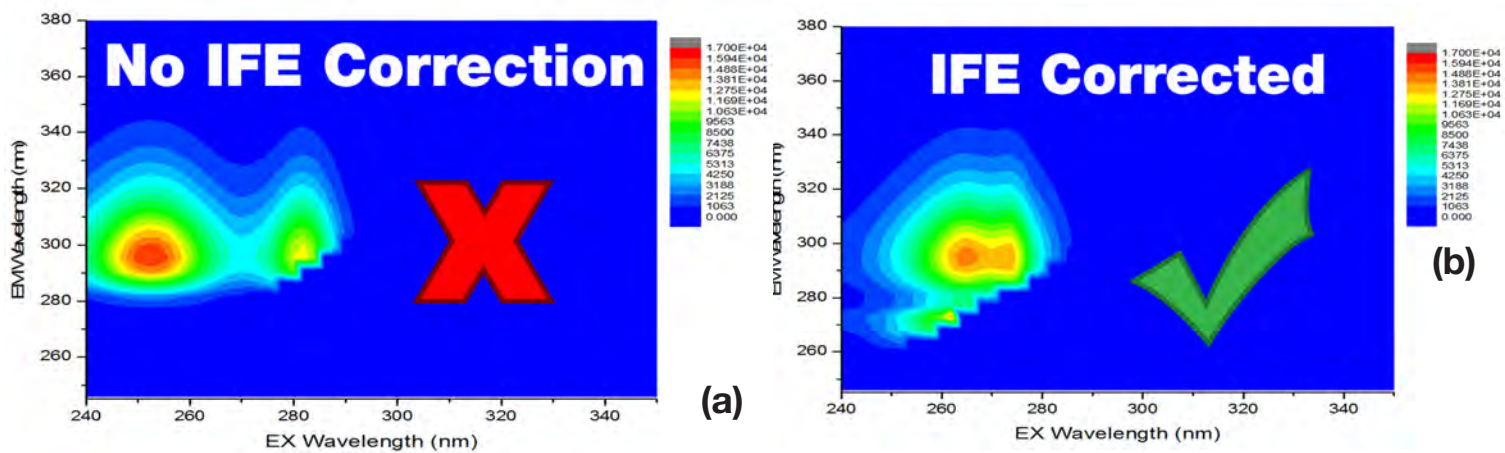
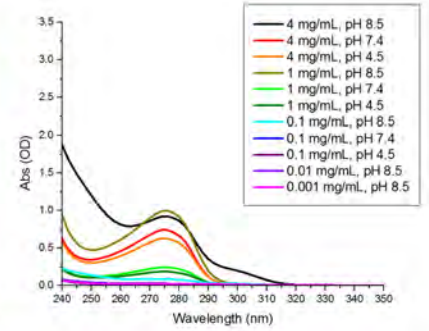
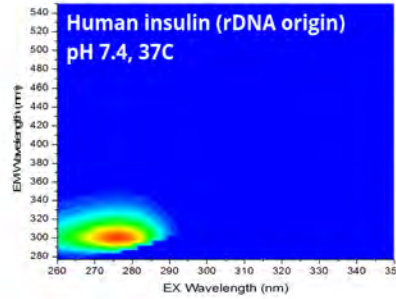
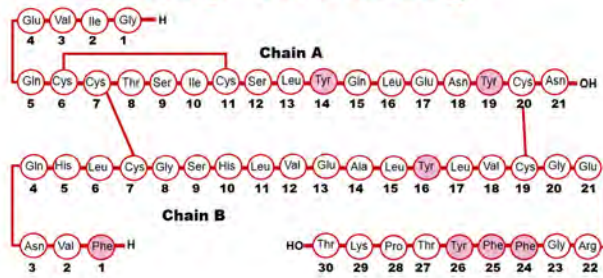


Figure 1. EEM (a), (b) and Absorbance (c) data of 1 mg/ml Insulin Glargine pH 7.4, Temp = 37°C

Human insulin, (rDNA origin)

pH 7.4-9 and 37°C

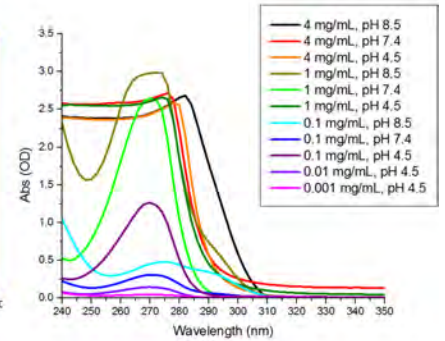
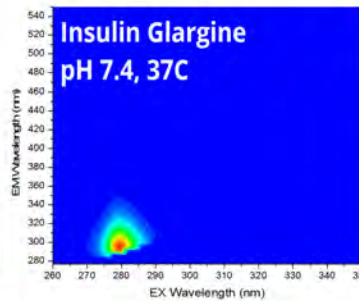
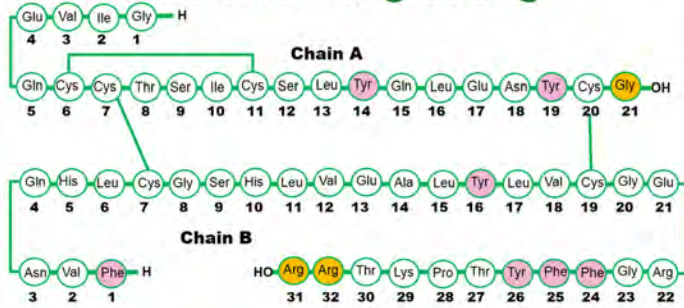
Naturally "fast acting"



Insulin Glargine Sequence (long acting)

Hexamer formation at pH 7.4 and 37°C

Commercial "long acting"



Insulin Aspart (fast acting)

Monomers at pH 7.4 and 37°C

Commercial "fast acting"

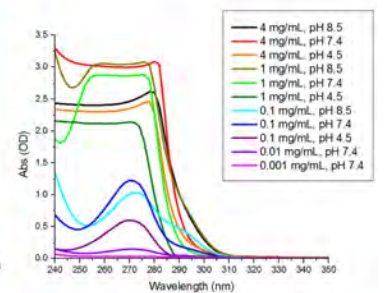
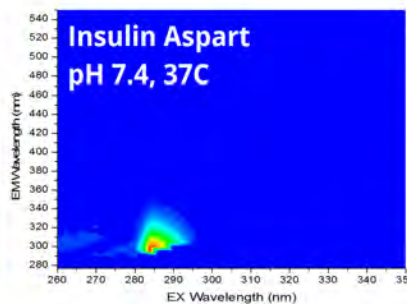
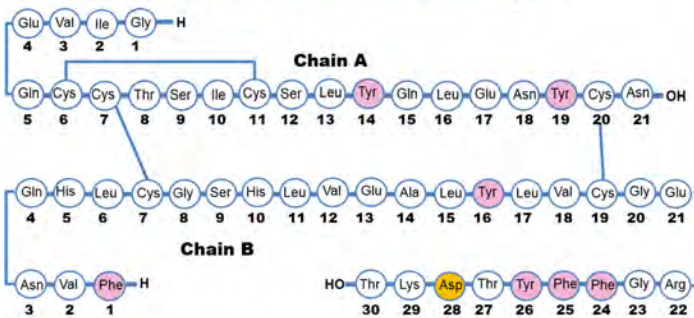


Figure 2. Fully corrected EEMs and Absorbance spectra of human insulin (rDNA origin), Insulin Glargine and Insulin Aspart. Highlighted in yellow are amino acids that differ from human insulin sequence and in pink are intrinsically fluorescent amino acids.

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).

4 mg/mL: 4 Components

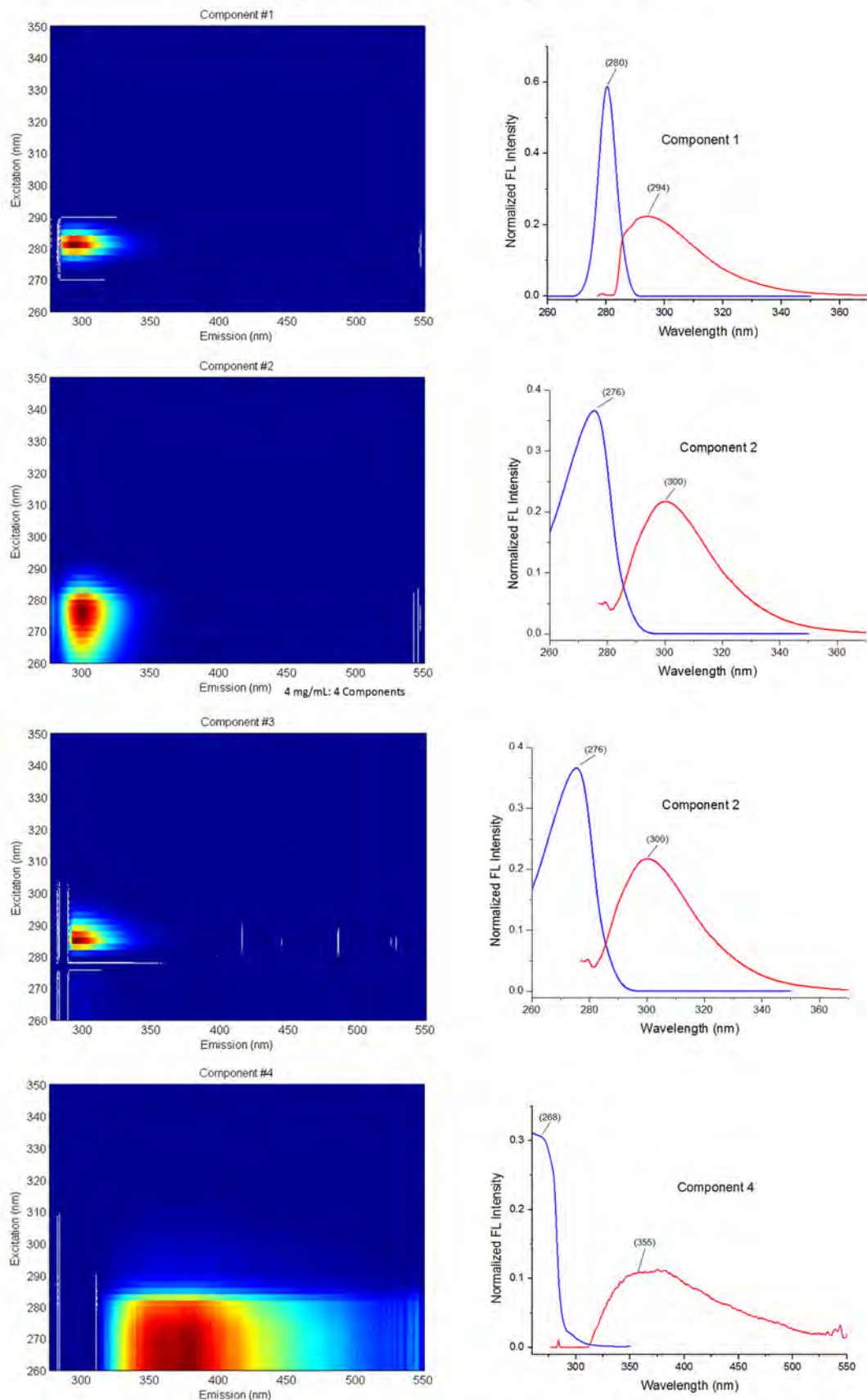


Figure 3. Component EEMs from PARAFAC multivariate chemometric analysis identified in the commercial formulations.

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™.

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 has successfully been used to characterize other complex biological solutions such as vaccines, enzymes, and 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>

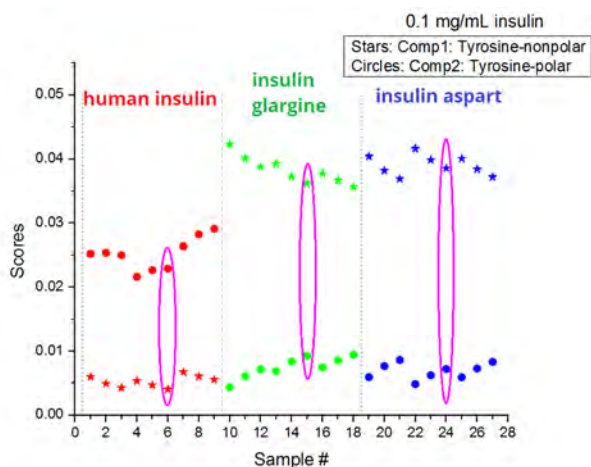


Figure 4. Component EEMs from PARAFAC Multivariate Chemometric Analysis for human insulin (red), insulin glargine (green) and insulin aspart using commercial formulations (4 mg/mL insulin concentration). Circled in PINK are all data points measured at pH 7.4 and 37°C.

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