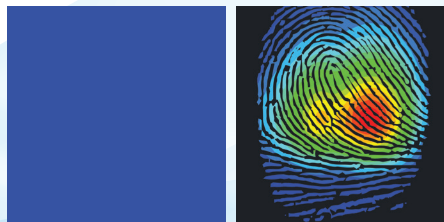
HORIBA

Fluorescence

Simultaneous A-TEEM™ and UV-Vis Analysis for Insulin Structure and Stability Assessment



Application Note

FL-2025-1-28

Introduction

Stability and aggregation of insulin are studied using simultaneous fluorescence excitation-emission matrices (EEMs) and UV-Vis absorbance spectroscopy. Insulin is a protein-hormone produced by the pancreas and is necessary for basic metabolic processes. Commercial insulin therapeutics generally fall into two categories: short-acting and long-acting insulin. The difference between some short-acting and long-acting insulin lies, in some cases, in only one to three residues in the protein sequence. This variation, along with the controlled pH of storage and delivery, is used to either trigger or prevent the formation of insulin dimers and hexamers in the bloodstream. The formation of these aggregates allows the body to absorb insulin more slowly, while their absence leads to faster absorption.

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 both, by leveraging intrinsically fluorescent amino acids. Traditionally, UV-Vis spectrophotometers and fluorometers are separate instruments. This application note presents a new and fast method for simultaneously generating individual excitation and emission spectra for all fluorescent sample components, providing information needed to correct the fluorescence spectra for sample concentration-dependent inner filter effects (IFE).

Experimental Method

This study employs the method of simultaneous absorbance-transmission and fluorescence excitation-emission matrix (A-TEEM™) spectroscopy using the Veloci BioPharma Analyzer. The Veloci BioPharma Analyzer is equipped with software tools for normalization to water Raman scattering or quinine sulfate units under defined emission conditions, correction for the influence of inner filter effects, and Rayleigh masking. Fully corrected EEM data were analyzed using the multivariate routine known as PARAFAC (Solo™ package from Eigenvector Research, Inc.).

Because commercial insulin formulations are highly concentrated (4 mg/mL), IFE correction is critical for measuring the EEM fingerprint. Using the reagent-free Veloci BioPharma Analyzer method, absorbance spectra are simultaneously collected with each EEM to correct for non-linearity in fluorescence intensity caused by IFE in high-concentration/high-absorbance solutions.

Results and Discussion

Three different insulin proteins were 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, insulin aspart (fast-acting, commercially available), and insulin glargine (long-acting, commercially available). Data acquisition lasted 30 minutes, during which approximately 125 EEMs and simultaneous absorbance spectra were recorded (15 seconds each). Temperature equilibration extended the total experiment time.

Figures:

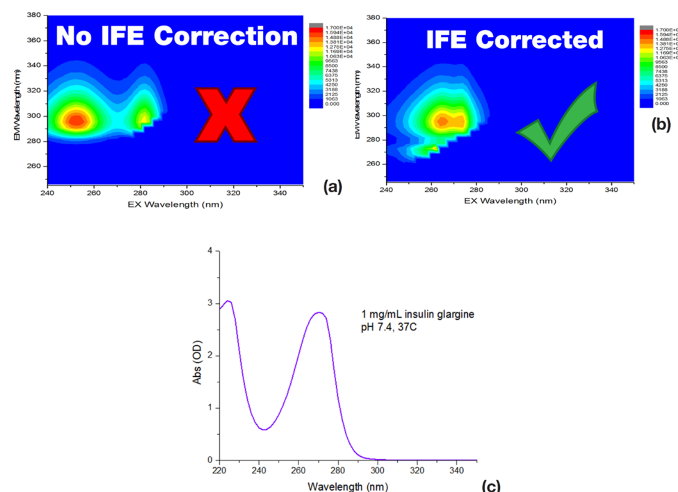
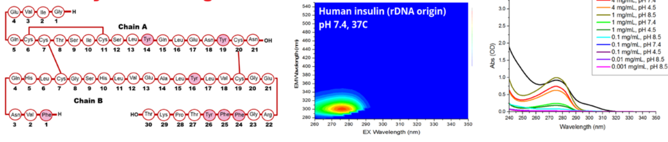


Figure 1: EEM and Absorbance data of 1 mg/mL insulin glargine, pH 7.4, Temp = 37°C.

Human insulin, (rDNA origin)

pH 7.4-9 and 37°C

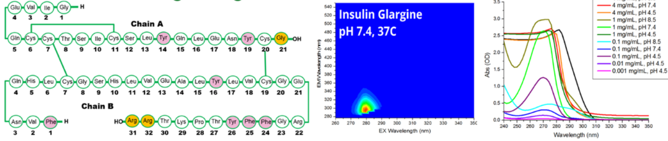
Naturally "fast acting"



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"

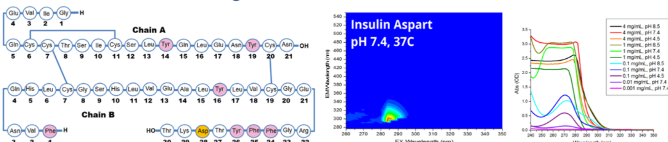
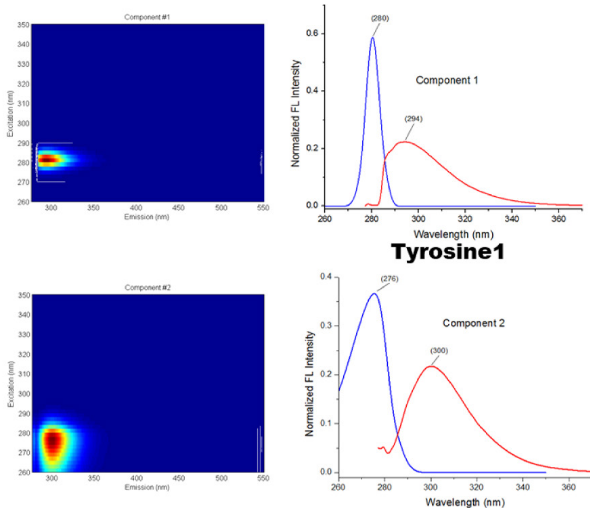


Figure 2: Fully corrected EEMs and absorbance spectra of human insulin (rDNA origin), insulin glargine, and insulin aspart. Highlighted in yellow are amino acids differing from the human insulin sequence; in pink are intrinsic fluorescent amino acids.

PARAFAC analysis of intrinsic fluorescence A-TEEM™ identified four components in the commercial formulations: Components 1, 2, and 3 (Comp1, Comp2, and Comp3) were identified as tyrosine, and Component 4 (Comp4) was identified as m-cresol, a preservative in insulin formulations (Figure 3).

4 Individual Components EEMs



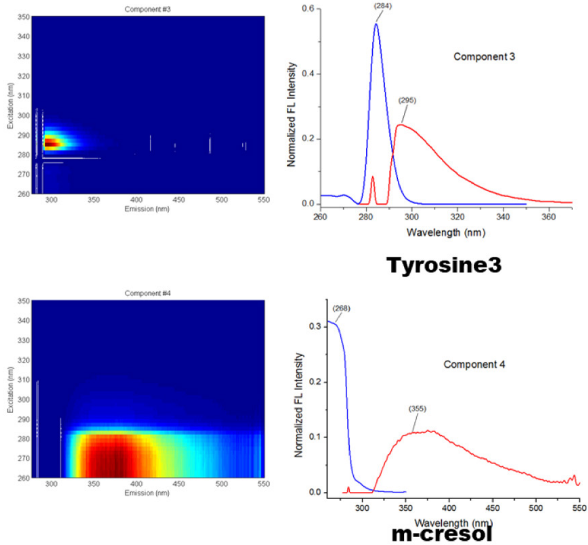


Figure 3: Component EEMs from PARAFAC multivariate chemometric analysis identified in the commercial formulations.

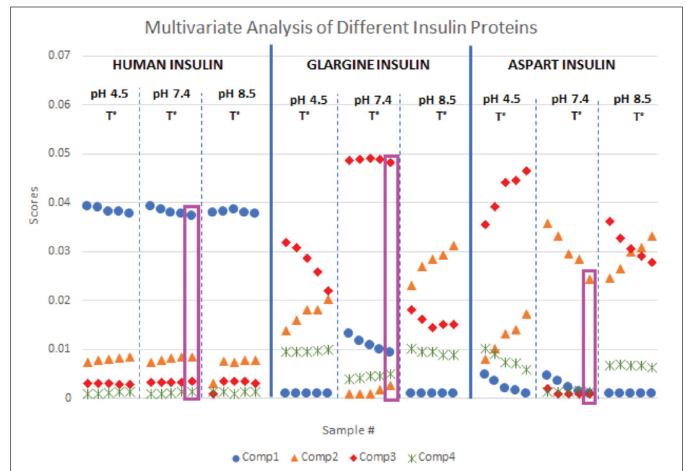


Figure 4: Score plots of component EEMs from PARAFAC multivariate analysis for human insulin, insulin glargine, and insulin aspart at 4 mg/mL concentration of protein at different pH (4.5, 7.4, 8.5) and temperature. Each group of 5 points represents the data measured at temperatures 5, 20, 25, 30, 37 oC in that order at each pH and insulin type.

Conclusions

The A-TEEM™ method, combined with the Veloci BioPharma Analyzer, is capable of characterizing protein therapeutic formulations for aggregation behavior in a matter of seconds, even under conditions where protein sequence differences are minimal. A-TEEM™ has been successfully used to characterize other complex biological solutions such as vaccines, enzymes, and cell culture media. This method provides valuable insights into protein aggregation behavior and structural stability.

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. 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/

3. 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)

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

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

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

7. 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)

info.sci@horiba.com **www.horiba.com/fluorescence**

USA: +1 732 494 8660
UK: +44 (0)1604 542 500
China: +86 (0)21 6289 6060
Taiwan: +886 3 5600606
France: +33 (0)1 69 74 72 00
Italy: +39 06 51 59 22 1
India: +91 80 41273637
Brazil: +55 (0)11 2923 5400
Germany: +49 (0) 6251 8475 0
Japan: +81(75)313-8121
Singapore: +65 (0)6 745 8300
Other: +33 (0)1 69 74 72 00