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Finding Optimal OPA-IBLC Derivatized Amino Acid Analysis Parameters with Three-Dimensional Fluorometric Scans for HPLC Method Validation

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Abstract

Three-dimensional fluorometric scans of OPA-IBLC derivatized glutamic acid and histidine solutions revealed that decreasing concentration maintained maximum λ_{em} but produced different maximum λ_{ex}. In pH 0.4 M sodium borate buffer (pH 10.4), 50 μM derivatized amino acid solutions had maxima around 350- to 450-nm λ_{ex}-λ_{em} where 12.5 μM solutions had maxima around 230- to 450-nm λ_{ex}-λ_{em}. In the lower concentration, the λ_{ex} peak at 230 nm was about 80% higher than 350 nm. Thus, collecting spectra with 230 nm λ_{ex} would provide better LOD and LOQ. Results inform HPLC standard concentrations since differential quenching effects may produce non-linear calibration curves.

Introduction

Amino acid racemization

Amino acids are monomeric units of protein. Amino acids exist in two stereoisomeric forms: D and L. These isomers are nonsuperimposable mirror images of each other. That is, the molecular composition between two amino acids is the same, but the spatial orientation differs. In biological systems, amino acids are almost exclusively found in the L-form — a phenomenon known as homochirality.[1] When an organism dies, the L amino acids racemize at a known rate into the D form until they are found in equal quantities by the mechanism shown in **Figure 1**.[2] If the D- to L-amino acid ratio is known, one can estimate the post-mortem interval (PMI) up to a theoretical 200,000 years in warm climates and 1,000,000 years in cold ones[3], a technique called amino acid racemization dating.[4]

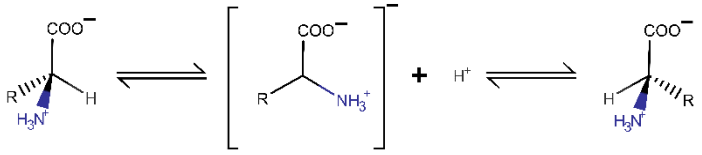

Figure 1: Amino acid racemization reaction. The left and right amino acids are the L- and R- forms, respectively.

HPLC separation

Such ratios can be determined by high performance liquid chromatography (HPLC). HPLC is an analytical technique that pumps solvent, or mobile phase, through a column packed tightly with beads, the stationary phase. The mobile and stationary phases can be polar or nonpolar. Reverse phase HPLC (RP-HPLC) uses a nonpolar stationary phase and polar mobile phase. Polar molecules are carried through the column as they are dissolved by the mobile phase and elute first. Nonpolar molecules are attracted to the stationary phase and are retained in the column, meaning they elute later. This differential interaction causes separation, as seen in **Figure 2**.

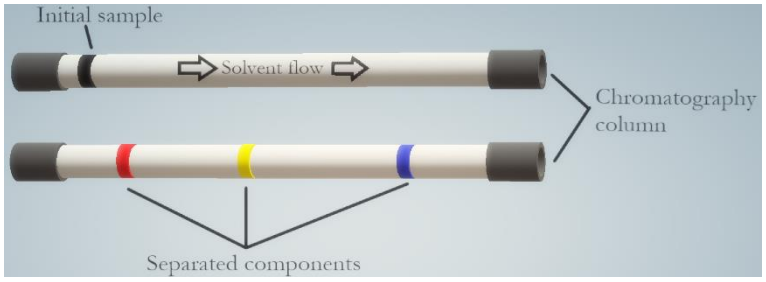

Figure 2: High performance liquid chromatography (HPLC) separation. The initial sample (black) is separated into three components (blue, yellow, red) based on their attraction to the stationary and mobile phases.

Derivatization

D- and L- amino acids are separated and identified with HPLC because of OPA-IBLC derivatization. O-phthalaldehyde (OPA) is a fluorescing molecule that reacts with primary amines and tags amino acids for fluorescence detection. N-isobutyryl-L-cystine (IBLC) is a chiral thiol that reacts with the amino acid enantiomers to form diastereomers which allows separation. The reaction of OPA and IBLC with amino acids is shown in **Figure 3**.[5]

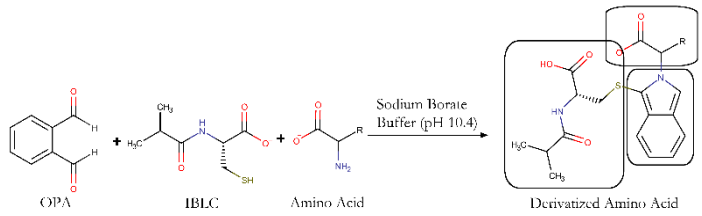

Figure 3: OPA-IBLC derivatization of amino acids.

Geochronology

Amino acid racemization rates within mollusk,[6] coral,[7] and ostrich eggshells[3] protein matrices are well established and have been applied to geochronological studies. Additionally, the effect of environmental conditions on mollusk shells has been explored — including heat, pH, and ionic strength.[8, 9] Comparable research on eggshells has not been done. This research aims to develop an RP-HPLC method to separate OPA-IBLC derivatized DL-amino acids to determine the effect of such conditions on racemization rate.

Method validation enhances experimental rigor by optimizing method settings. Limits of detection (LOD) and quantitation (LOQ) are validation parameters that establish the lowest concentrations that can be reliably detected and quantified, respectively. Choosing optimal fluorescence excitation (λ_{ex}) and emission (λ_{em}) wavelengths for derivatized amino acids maximizes detector response and consequently lowers LOD and LOQ, meaning smaller amounts of sample can be detected. Such optimization allows smaller protein fraction analysis, widens eggshell sample selection, and expands viable sample preparation techniques. Previous research has used both 240-[10, 11] and 340-nm[12, 13] λ_{ex} for OPA-IBLC amino acid analysis. This research uses three-dimensional fluorometric scans of OPA-IBLC derivatized histidine and glutamate to determine optimal fluorescence analysis parameters. Fluorimeter results are verified with an HPLC fluorescence detector (FLD) post-column separation.

Methodology

Part A – Fluorimeter

Borate buffer

A 0.4 M sodium borate buffer was prepared by dissolving about 2.47 g boric acid in distilled water with a few drops of 6 M sodium hydroxide. After stirring to dissolve the boric acid powder, the solution was diluted to 100 mL and pH adjusted to 10.4 with 6 M sodium hydroxide (solution is hereafter referred to as "borate buffer"). [*]

Stock solutions

Four stock solutions were required for fluorescence analysis: OPA, IBLC, histidine, and glutamate. All four solids were purchased from Sigma Aldrich. OPA solutions were prepared by dissolving a precisely known amount of OPA in pure methanol in a pre-weighed vial using a vortexer. The vial was re-massed and an aliquot immediately transferred to a 10 mL volumetric flask with a 1000 µL micropipette. The remaining volumetric flask volume was filled with borate buffer. To find solution concentration, the methanol density was determined by averaging the mass of 1 mL methanol volumes dispensed with a 1000 µL micropipette. This was used to determine the OPA methanol solution volume and, accordingly, 10 mL OPA stock concentration. The IBLC and amino acid stock solutions were

[*]A 1 M sodium borate buffer was previously used but precipitated since it was close to the solubility limit. Such high borate buffer concentrations may similarly precipitate in the LC system or on the column and should be avoided in favor of lower concentrations, like 0.4 M.

prepared by massing precisely known amounts of solid into separate 10 mL volumetric flasks and diluting with borate buffer.

Derivatization & dilution

IBLC and histidine or glutamate were mixed with 1 mM OPA in ten-fold excess to create 1 mM derivatized amino acid solutions. The excess amino acid and IBLC drive the derivatization reaction in **Figure 3** to completion to ensure no unreacted OPA fluoresce contributes to derivatized amino acid spectra. Solutions were allowed to derivatize for longer than 5 minutes but shorter than 4 hours. Aliquots of the 1 mM derivatized solutions were transferred to a 10 mL volumetric flask, acidified to pH 4 with 1 mL glacial acetic acid, and the remaining volume filled with borate buffer to create 50 μM solutions.

To narrow dilution intervals, 2.500 mL of the 50 μM derivatized amino acid solutions were added to respective quartz Starna cell cuvette with a 1000 μL micropipette and their two-dimensional excitation spectra collected with 450 nm λ_{em}. Then, three 0.5 mL borate buffer volumes were added to the cuvettes and their excitation spectra recorded after each addition. This was repeated for 25 μM, 12.5 μM, and 3.75 μM derivatized histidine and glutamate solutions for a total of 12 dilutions. Histidine-IBLC and glutamate-IBLC blanks were prepared analogously to the IBLC and amino acids procedure above to make 500 μM solutions (ten-fold excess)

Fluorescence spectra collection

Fluorescence spectra were collected with a Cary Eclipse fluorimeter. All runs scanned with 2.5 nm λ_{ex} and 5 nm λ_{em} slit widths, 600 nm/min, and 550 V PMT voltage. The 50 μM derivatized amino acids, histidine-IBLC (blank, and glutamate-IBLC scans utilized a three-dimensional scan between 200–400 nm λ_{ex} with 400–500 nm λ_{em} with 2 nm excitation increments. The 12.5 μM solution spectra were collected with the same three-dimensional parameters and λ_{ex} and λ_{em} ranges, but with 1 nm emission increments. Data were exported as CSV files and processed in Microsoft Excel. Three-dimensional spectra of the blanks blank showed no fluorescence intensity and they therefore do not add fluorescence to the derivatized amino acid spectra.

Part B – HPLC

HPLC Instrumentation & Solvents

HPLC gradient grade acetonitrile and methanol were purchased from Sigma Aldrich and VWR analytical, respectively. Distilled water was produced from deionized water with Andrews University's water distillery facility. Two mobile phases were used for HPLC separation: (1) A 50 mM sodium acetate buffer (pH 6) with 50 mM sodium azide made by dissolving 1.9411 g sodium acetate, 0.075 mL of glacial acetic acid, and 1.6257 g sodium azide in 500 mL distilled water and pH correcting with 6 M NaOH (Solvent A) and (2) premixed 45:45:10 acetonitrile:methanol:water (Solvent B). All solvents were vacuum filtered through a 0.45 μm polyamide filter. Solvent A was prepared freshly biweekly to prevent bacterial contamination. Lower sodium azide concentration should be considered to limit salt precipitation in the column while maintaining levels for bacterial toxicity.

The Agilent LC system was equipped with an Agilent 1260 Infinity Fluorescence Detector (G1321B), Agilent 1200 Series Diode Array Detector (G1315C), Agilent 1260 Infinity Thermostatted

Column Compartment (G1316A), Agilent 1260 Infinity Autosampler (G1329B), Agilent 1260 Infinity Degasser (G1322A), and an Agilent 1200 Binary Pump System (G1312B). An Agilent Poroshell 120 EC-C18 2.7 μm 4.6 × 150 mm column (PN: 693975-902(T)) was used for amino acid enantiomeric separation. An Agilent InfinityLab Poroshell 120 EC-C18 4.6 × 5 mm 2.7 μm guard column was attached to the primary column to protect from contaminants. An Agilent Quick Connect with a 0.17 × 150 mm long stem capillary tube integrated the columns into the LC system.

Column Standard

To ensure proper column operation, a column standard containing 10 μg/mL uracil, 400 μg/mL phenol, 50 μg/mL 1,4-dinitrobenzene, and 80 μg/mL naphthalene was dissolved in 70:30 acetonitrile:water and run with a 3 μL injection volume in 70:30 acetonitrile:water mobile phase flowing at 1.8 mL/min. The standard was run without the column guard attached. Absorption spectra were collected with the DAD system at 254 nm with 4 nm slit widths and a 360 nm with 10 nm slit widths reference wavelength. The standards eluted at expected times and intensities. However, the backpressure reached about 340 bar where only 313 bar was expected. When the column standard was re-run with a new column under the same conditions, the pressure was 10 bar higher than expected. Standards were run after replacement of the precolumn inline filter, purge valve PTFE frit, piston seals, wash seals, seal wash gaskets. Since the pump maintenance and new, uncontaminated column did not reduce the pressure to expected levels, both the old column and instrument (possibly capillary tubing) contribute to elevated pressure.

Derivatizing reagents and amino acid

Solutions of 170 mM OPA and 260 mM IBLC were prepared freshly each week for pre-column amino acid derivatization by the method described in **Part A,** *Stock Solutions* section. The OPA solutions were stored in ambered vials for HPLC injection to prevent UV degradation. DL-histidine and DL-glutamate solutions were prepared in 50 mM hydrochloric acid to make 5 mM amino acid standards. All solutions were filtered through 0.2 μm PTFE syringe filters.

Parameter	Value
Mobile phase	A). 50 mM sodium acetate with 50 mM sodium azide (pH 6) B) 45:45:10 MeOH:ACN:Water
Flow rate	0.7 mL/min
Gradient pump	0 to 2 minutes, 4% B 2 to 4 minutes, 10% B 4 to 15 minutes, 20% B 15 to 27 minutes, 35% B 27 to 35 minutes, 50% B 35 to 37 minutes, 100 % B 37 to 42 minutes, 100% B
Post time	10 minutes at 4% B

Table 1: Gradient flow conditions for enantioselective HPLC method.

HPLC method

The enantioselective HPLC method shown in **Table 1**, developed by Agilent technologies,[11] was used to separate OPA-IBLC derivatized DL-histidine. Fluorescence spectra were collected with HPLC-FLD at 240, 260, and 340 nm λ_{ex} with 450 nm λ_{em} and a PMT gain of 7. All data were exported as a CSV file and processed in Microsoft Excel.

Step	Mode	Action
1	Draw	Draw 2.5 µL from borate buffer with default speed
2	Draw	Draw 0.5 µL from 5 mM DL-histidine
3	Mix	Mix 3.0 µL from air at maximum speed 10 times
4	Wait	Wait 0.5 minutes
5	Draw	Draw 0.13 µL from OPA with 100 µL/min speed
6	Draw	Draw 0.13 µL from IBLC with 100 µL/min speed
7	Mix	Mix 3.25 µL from air at maximum speed 20 times
8	Wait	Wait 0.5 minutes
9	Draw	Draw 15 µL from 0.1% acetic acid with default speed
10	Mix	Mix 20 µL from air at maximum speed 10 times
11	Wait	Wait 0.1 minutes
12	Inject	Injection
13	Wait	Wait 0.5 minutes
14	Valve	Switch valve to Bypass

Table 2: Injector program for enantioselective HPLC method.

Future research can combine steps 5 and 6 by creating one OPA-IBLC solution of 170 and 260 mM, respectively, and increasing the draw volume to 0.26 µL (per Agilent Technologies injection method).

Results

Part A - Fluorimeter

Fluorescence spectra results

Figure 4 and **Figure 5** show the three-dimensional fluorescence results for 50 µM derivatized histidine and glutamate, respectively. Two prominent peaks occur at 260 and 334 nm λ_{ex} with 440 nm λ_{em} for histidine and 260 and 336 nm λ_{ex} with 440 nm λ_{em} for glutamate.

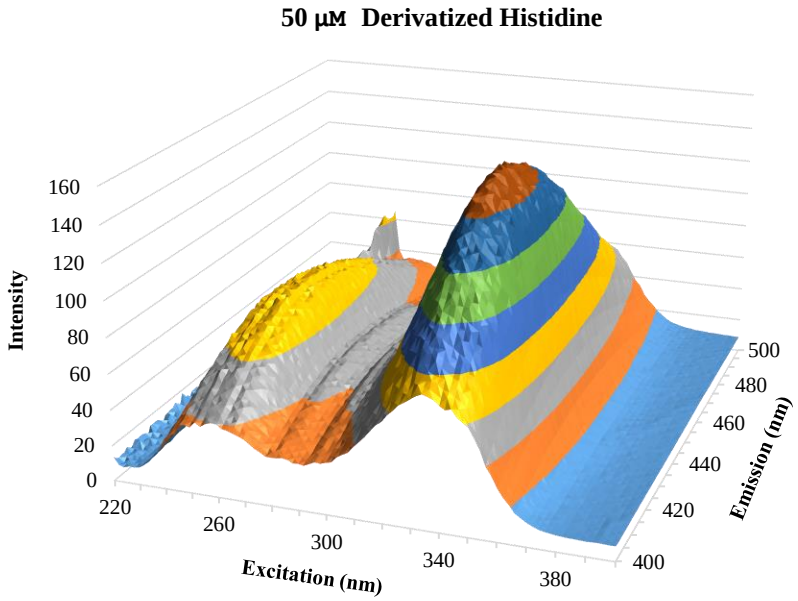

Figure 4: Fluorescence spectra of 50 µM OPA-IBLC derivatized histidine. Two prominent peaks maximize at 260 and 334 nm λ_{ex} with 440 nm λ_{em}.

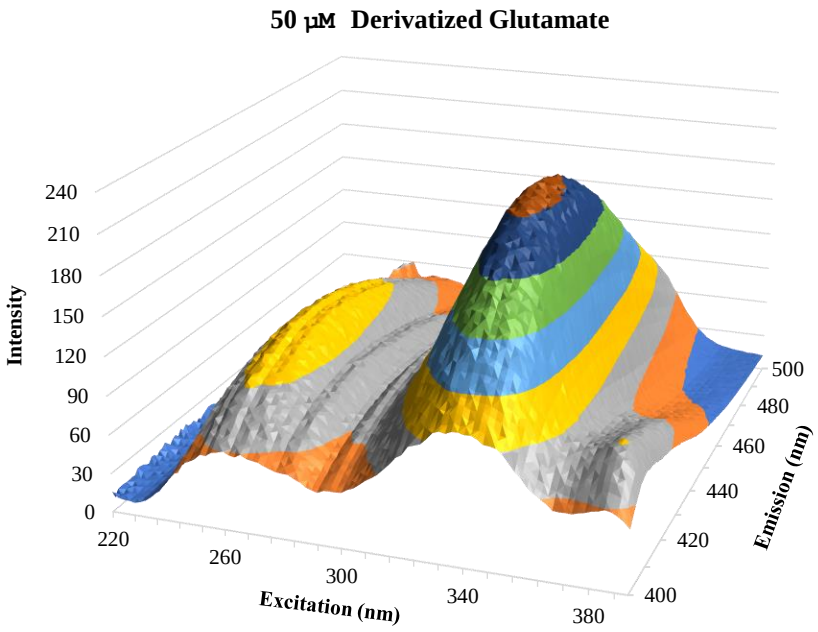

Figure 5: Fluorescence spectra of 50 µM OPA-IBLC derivatized glutamate. Two prominent peaks maximize at 260 and 336 nm λ_{ex} with 440 nm λ_{em}.

Figure 6 and **Figure 7** show three-dimensional fluorescence spectra for 12.5 µM derivatized histidine and glutamate, respectively. A pronounced third excitation peak around 230 nm emerged in the dilutions (also small peaks in the rear of **Figure 4** and **Figure 5**). The three peaks occur at 233, 259, and 336 nm λ_{ex} with 454 nm λ_{em} for histidine and 234, 359, and 338 nm λ_{ex} with 450 nm λ_{em} 260 and 336 nm λ_{ex} with 440 nm λ_{em} for glutamate.

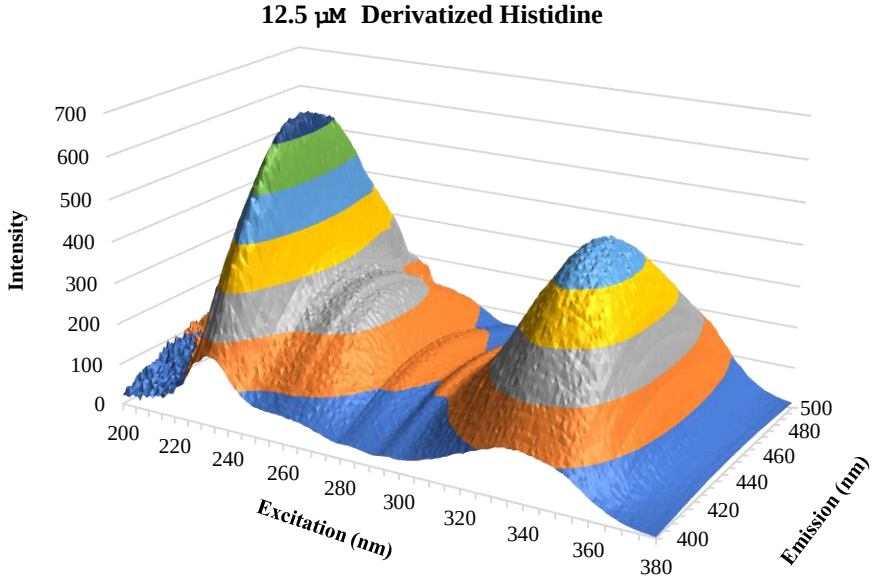

Figure 6: Fluorescence spectra of 12.5 µM OPA-IBLC derivatized histidine. Three prominent peaks maximize at 233, 259, and 336 nm λ_{ex} with 454 nm λ_{em}.

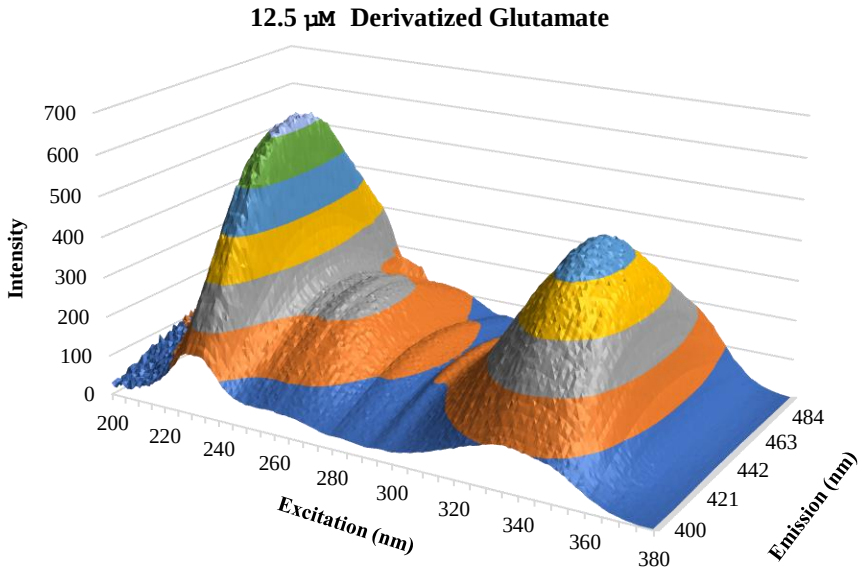

Figure 7: Fluorescence spectra of 12.5 µM OPA-IBLC derivatized glutamate. Three prominent peaks maximize at 234, 259, and 338 nm λ_{ex} with 450 nm λ_{em}.

The 50- and 12.5- µM three-dimensional spectra show that OPA-IBLC derivatized amino acid λ_{em} maxima are consistent within about 15 nm. The 260 and 340 nm λ_{ex} peaks appear in both the 50- and 12.5-µM spectra where the third 230 nm peak only appears in the 12.5 µM spectra. For the 50 µM solution, the 340 nm peak has greater intensity than the 260 nm peak and thus is preferred for DL-amino acid analysis at concentrations above 50 µM. Solutions below 12.5 µM derivatized amino acid produce maximum intensity at the 230 nm peak. Such solutions approach the LOD and LOQ and are therefore closer to the concentrations of interest — meaning the 230 nm λ_{ex} should be used for HPLC-FLD analysis. Additional three-dimensional scans should be taken to make spectra of comparable pH.[†] Though the pH is different between concentrations — which may influence spectral peak maxima wavelength — the degree of intensity increase at the 230 nm λ_{ex} should not be affected greatly by pH changes.

Quenching effects

Quenching occurs when emitted photons are absorbed by neighboring fluorescing molecules due to high concentration. Quenching effects can be determined if dilution causes an increase in fluorescence intensity (since photons can reach the detector instead of being absorbed by neighboring molecules). **Figure 8** and **Figure 9** show graphs of peak λ_{ex} fluorescence intensity at 450 nm λ_{em} versus serial dilution concentration to show the differential quenching effects between the three maximum peaks around 230, 260, and 340 nm. Higher determination coefficients (R^2) indicate less quenching. Determination coefficients were calculated with Microsoft Excel.

[†] Recall: the 50 µM spectra are pH 4 where the 12.5 µM spectra are pH 10.4.

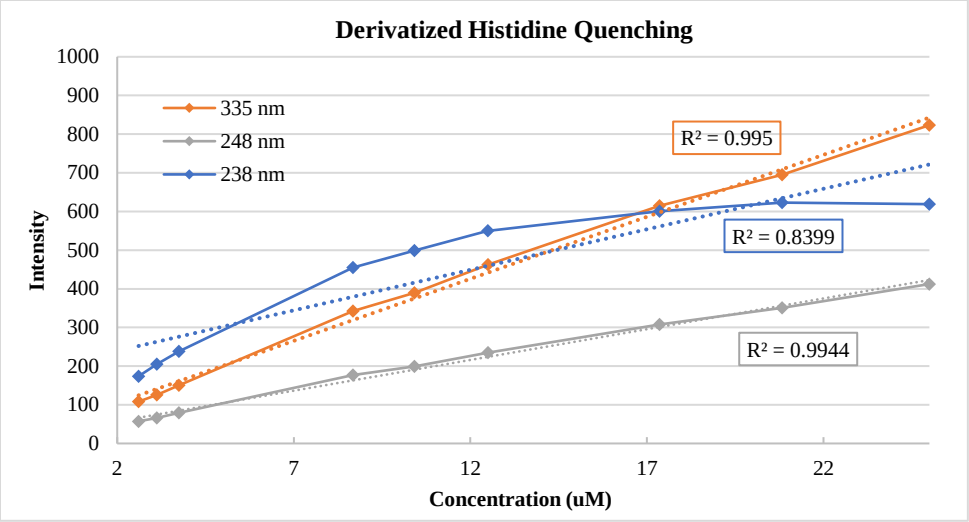

Figure 8: Intensity versus concentration charts for OPA-IBLC histidine between 2.6 and 25 µM to demonstrate quenching effects. Lower determination coefficients (R^2) indicate higher quenching.

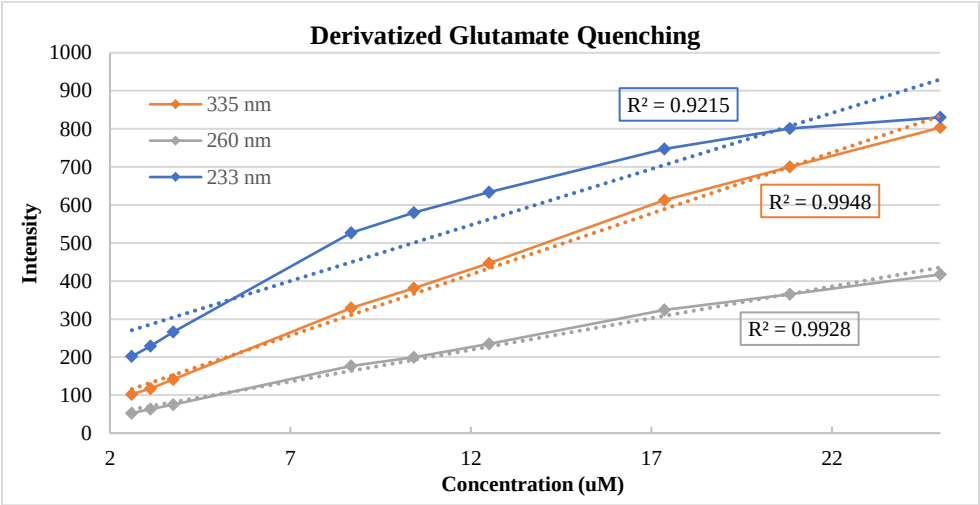

Figure 9: Intensity versus concentration charts for OPA-IBLC glutamate between 2.6 and 25 µM to demonstrate quenching effects. Lower determination coefficients (R^2) indicate higher quenching.

The ~230 nm peaks (blue) for both derivatized histidine and glutamate have determination coefficients below 0.95 which indicate poor linear fit. Such nonlinear relationship reveals quenching is occurring at this wavelength. The ~260 (grey) and ~340 nm peaks have determination coefficients above 0.992, which represent good linear fit. However, there is still a slight curve in charts which shows some quenching is occurring. Determination coefficients of the all three wavelengths below 3.75 µM are between 0.997 and 0.99991, meaning there is good linear relationship between peak intensity and concentration. Thus, all three wavelengths could be used for regression analysis at low concentrations — but the ~230 nm peak has the highest intensity and thus would produce lowest LOD and LOQ. Since the spectra and calibration are similar between glutamate and histidine, they are likely consistent for other amino acids. See *HPLC Separation & Analysis* section for analysis refinement suggestions for additional amino acids.

Part B – HPLC

HPLC Separation & Analysis[‡]

Derivatized DL-histidine was separated with HPLC by the method described in the **Experimental** section. HPLC-FLD recorded chromatographic spectra at 240, 260, and 340 nm λ_{ex} with 450 nm λ_{em}. Though the 240 nm peak did not show a maximum in the fluorimeter results, it should still be about 20% higher than the 340 nm peak at non-quenching concentrations. **Figure 10** shows the HPLC-FLD chromatogram for the three wavelengths. L- and D- histidine are the left and right peaks, respectively. **Table 3** summarizes the integration areas under each peak and the 340:240 nm peak area ratios. Peak area was calculated by manual trapezoidal integration from 12.8 min to 13.13 min (the minimum between the D- and L-peaks) and from 13.13 min to 13.45 min.

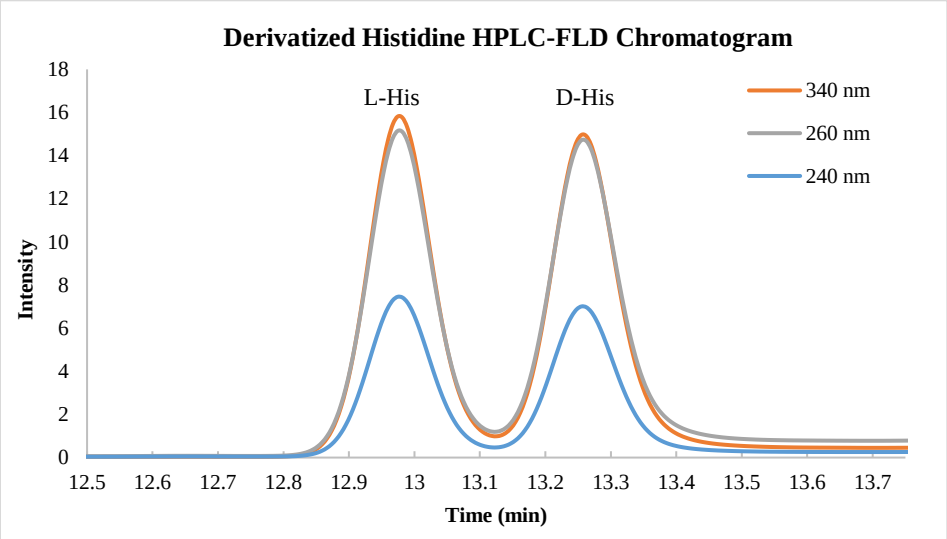

Figure 10: HPLC-FLD chromatogram for enantioseparated DL-histidine.

	Wavelength peak area			*Peak ratios*
	340 nm	260 nm	240 nm	340:240 nm
L-His	1.891	1.859	0.890	2.124
D-His	1.896	1.954	0.889	2.134

Table 3: Integration area for respective beaks and the integration ratio between the 340 and 240 nm peaks.

The 340:240 nm ratio is about 2 for both D- and L-histidine peaks, meaning the 340 nm wavelength's response was about twice that of the 240 nm. This result is contrary to the expectation that 240 nm λ_{ex} peak integration should be higher than 340 nm λ_{ex}. This evidences that quenching occurs in the HPLC-FLD with a 0.5 µL injection of 5 mM DL-histidine. Dilution should be analyzed with HPLC-FLD until the 240 nm peak integration to concentration relationship is linear. Amino-acid specific optimal λ_{ex} under can be found by systematically analyzing wavelengths between 230 and 240 nm for each amino acid with the HPLC-FLD multi-excitation feature and choosing the chromatogram with the highest

[‡] URS Researcher Daranthea Atmajda collected DL-histidine chromatographic data.

response. Additionally, the PMT gain can be increased to amplify the signal at lower concentrations to improve signal.

The Agilent enantioselective method produced near-baseline separation between the D- and L-histidine peaks. Separation may be improved by reducing the flow rate near amino acid elution times. This may also cause peak broadening or additional tailing and, consequently, peak overlap when multiple amino acids are separated simultaneously. The improved separation methodology can be applied to amino acids found in eggshell protein matrices, including: lysine, histidine, arginine, asparagine, glutamate, threonine, serine, glycine, alanine, valine, isoleucine, leucine, methionine, tyrosine, and phenylalanine.[3]

Conclusion

The λ_{ex}–λ_{em} parameters for optimal fluorescence for OPA-IBLC histidine and glutamate were investigated with three-dimensional fluorometric scans. Only ~260 and ~340 nm λ_{ex} peaks occurred in the 50 μM solutions where ~230, ~260, and ~340 nm λ_{ex} peaks occurred in the 12.5 μM solutions. The maximal signal was produced with ~230- to ~450 λ_{ex}-λ_{em} with the 12.5 μM DL-amino acid solutions. Two-dimensional fluorometric scans of serial dilution excitation spectra at 450 nm λ_{em} showed significant quenching effects occurred for the ~230 nm λ_{ex} peak where the ~260 and ~340 nm λ_{ex} peaks were mostly linear below 25 μM. Though the ~260 and ~340 nm λ_{ex} would have more linear calibration curves at higher concentrations, the ~240 nm λ_{ex} has higher intensity near the LOD and LOQ would be better for method optimization.

The Agilent enantioselective RP-HPLC method achieved near-baseline separation. With a 0.5 μL injection of 5 mM derivatized DL-histidine, the 340 nm λ_{ex} integration area was about twice that of the 240 nm λ_{ex} intensity. This indicates that quenching occurred in the HPLC-FLD and that standards lower than 5 mM should be used to produce linear calibration curves. Future research should dilute amino acid standards to determine calibration concentrations and estimate the LOD and LOQ. Then, the HPLC method should be improved to achieve baseline separation for all amino acids relevant to eggshell protein matrix analysis.

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