Optimizing Fluorescence PMT Gain and Excitation Wavelength for HPLC DL-Amino Acid Detection

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Abstract

Amino acids exist as enantiomers that can be separated and detected using HPLC-FLD. An optimal PMT (photomultiplier tube) gain, and wavelength of excitation must be determined to lower the limit of detection (LOD) and quantitation (LOQ) as increasing the PMT gain amplifies the signal on the HPLC-Spectra. The HPLC-FLD spectra of OPA-IBLC derivatized histidine was measured at PMT gain 5 and 7 for the excitation wavelengths of 340- , 260-, and 240- nm. Hence, the optimal excitation wavelength and PMT gain can be determined by comparing the fluorescence spectra.

Introduction

Amino acids are chiral and exist as their L-enantiomer in biological systems. When an organism dies, its amino acids interchange between the L and D form until it reaches a 50:50 ratio which can be used as a dating method in paleontology. However, this racemization process is affected by environmental factors[1] While studies on mollusk shells on HPLC has been conducted[2,3], there has been no precedent study done on avian eggshells. One of the environmental factors that affects the eggshell's racemization is the exposure to fire. The ratio between the enantiomers can be quantified through the integration of the HPLC-FLD spectra peaks. To be detectable by the HPLC-FLD, the amino acids must first be derivatized by o-phathaldehyde (OPA) and N-isobutyryl-L-cysteine (IBLC) as OPA reacts with primary amines and thiols of amino acids while IBLC allows for the separation of the amino acid diastereomers.

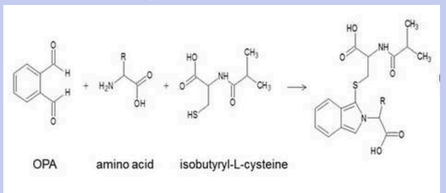

Figure 1. shows the derivatization process of an amino acid using OPA and IBLC

A method developed by Agilent for the enantioseparation of amino acids using HPLC-FLD used excitation wavelengths of 230- and 450- nm[4] while another research experiment done on a fluorometric spectrometry showed optimal excitation wavelength of histidine at 340- and 260- nm[5]. The two wavelengths as well as 230 nm from the Agilent method were set as excitation wavelengths on the HPLC-FLD method for comparison. The PMT is useful for very weak signals when the amino acid concentration is low, its signal amplifying effects increases with the voltage gain applied to it.

Methodology

Using the injection program and solvent gradient found in the Agilent amino acid enantioseparation method[4], 5mM DL-Histidine solution in pH 10 borate buffer was prepared as the sample which was derivatized using excess 260 mM IBLC and 170 mM OPA in the borate buffer. The derivatized sample is then mixed with 0.1% acetic acid solution before being injected into the column. The solvents used in this procedure were 45:45:10 solution of acetonitrile (ACN), methanol (MeOH), and water with a 50 mM sodium acetate buffer at pH 6. Instead of using the 440-nm excitation wavelength, 340-, 260-, and 240- nm excitation wavelengths were used instead. Two runs were done with two different PMT gains, 5 and 7, to compare and determine an optimal PMT gain value.

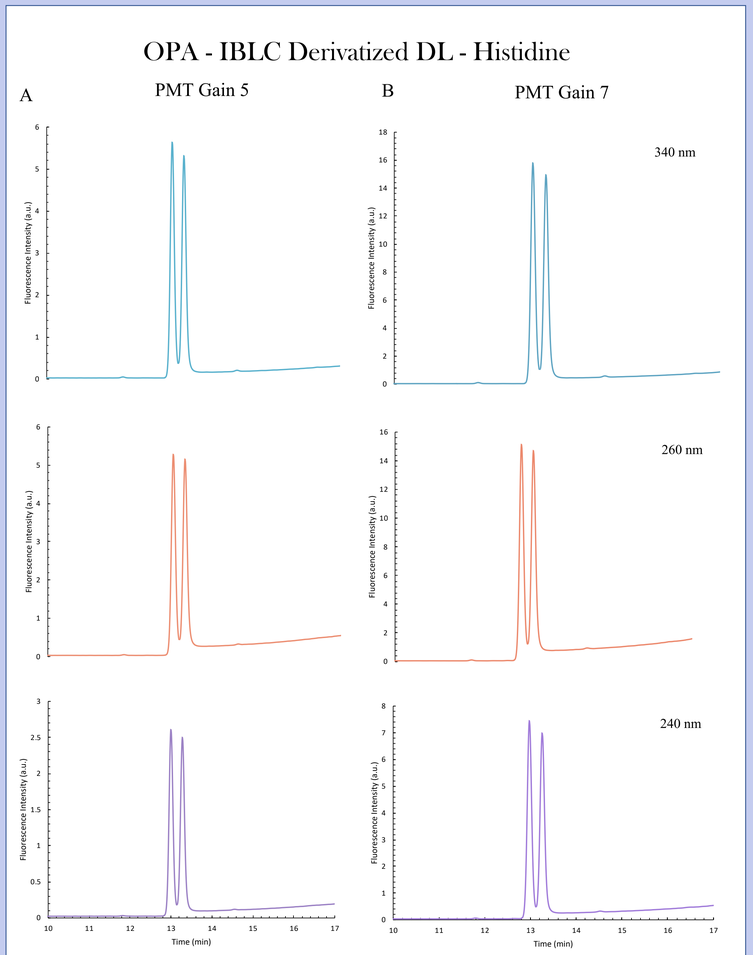

Figure 2. Shows the fluorescence intensities of the separated derivatized DL-histidine diastereomer at 360- , 260- , and 240- nm with a (A) PMT gain of 5 and (B) PMT gain of 7

Results and Analysis

Figure 2. (A) and (B) shows separation of the derivatized DL-histidine and its fluorescence intensity at 340-, 260-, and 240- nm. (A) shows the PMT gain set at 5 while (B) shows the PMT gain set at 7. Comparing the integrations at the different wavelengths seen in Table 1. the 340- and 260- nm has the highest signal integration at PMT gain 5 with 1.31067 (peak 1) and 0.06846 (peak 2) for 340 nm and 1.28518 (peak 1) and 0.09686 (peak 2). Again the 340- and 260- nm displays the highest signal integration at PMT gain 7 with 1.89792 (peak 1) and 1.86063 (peak 2) for 340 nm and 1.86802 (peak 1) and 1.90375 (peak 2) for 260 nm. This shows that the Agilent procedure excitation wavelength of 240 nm is not the optimal wavelength that should be used in the fluorometric HPLC DL-amino acid detection as it has the lowest integration values compared to the other two.

The PMT gain voltage change did increase the integration of the fluorescence intensity signals in this experiment. However, a gradient of PMT gain might be needed to determine an optimal PMT gain as increasing it does not only increase the signal but also the noise that is acquired by the machine during detection which could affect the LOD and LOQ by decreasing the signal to noise ratio.

Wavelength/ nm	Peak	PMT 5 Integration	PMT 7 Integration
340	1	1.31067	1.89792
	2	0.06846	1.86063
260	1	1.28518	1.86802
	2	0.09686	1.90375
240	1	0.61278	0.89361
	2	0.03642	0.87098

Table 1. Shows the integration values calculated using the trapezium method of the fluorescence intensities of DL-histidine at 340-, 260, and 240- nm at PMT gain of 5 and 7. Peak 1 is the peak that appeared at 12.8 min while peak 2 appears at 13.4 min.

Conclusion

When using Fluorescence as a method of detection on the enantioseparation of amino acids on the HPLC, the optimal excitation wavelengths that should be used are 340- and 260- nm as they showed the highest integrations of the peaks regardless of PMT gain change. While increasing the PMT gain voltage increased the integration and signal height in the fluorescence spectra, adding a smaller PMT gain than 5 and a higher PMT gain than 7 could help determine whether increasing the PMT will alter the signal to noise ratio too much that it will influence the LOD and LOQ.

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