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Assessing Amino Acid Racemization in Avian Eggshells through Reverse Phase Liquid Chromatography

Nikitha Nelapudi
Andrews University, nikitha@andrews.edu

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Nikitha Nelapudi

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Advisor: Dr. Lisa Ahlberg

Primary Advisor Signature: 

Department: Chemistry and Biochemistry

Abstract

The extent of amino acid racemization (AAR) observed in organic material offers insight into geochronology. Within the scope of AAR research, Avian eggshells are reliable sources of data due to retention of indigenous amino acids through calcification. This project develops a robust quantification methodology for up to 9 D/L amino acids using reverse-phase HPLC (RP-HPLC) with precolumn derivatization. Reliable quantitation is established via statistical assessment of detectability and calibration curves. Progress has focused on obtaining adequate enantioseparation and reproducible data of single D/L amino acids.

Introduction

Between a pair of chiral molecules, termed enantiomers, living organisms distinguish and selectively favor one form of a chiral molecule over its enantiomer. This phenomenon, called biological homochirality, is exhibited by amino acids, the foundational structural unit of proteins. In all biological functions, the "left-handed" form of an amino acid, termed L-amino acid, is selectively favored over the "right-handed" form, termed D-amino acid. Upon death, amino acids present in the organic material undergo racemization, the interconversion of L-amino acids into a racemic mixture of L- and D-amino acids (Hendy, E. et al. 2012). The process of amino acid racemization (AAR), kinetically described as a first order reaction, depends on time and environmental factors such as temperature, pH, and climate. Therefore, determining D/L amino acid ratios present in organic material can provide geochronologic data, offer insight into paleoenvironmental conditions, and aid climate reconstruction. While there are diverse organic systems that can provide said information, Miller et al. (2000) found avian eggshells to be a reliable source of AAR data due to their ability to retain indigenous amino acids through calcification, thus protecting these molecules from the decomposition process.

To interpret the amino acid content held within the matrices of an eggshell, this project aims to develop a methodology for determining D/L amino acid ratios in emu and ostrich eggshells through chromatography. Among laboratories focusing on geochronology, gas chromatography (GC) or high-performance liquid chromatography by ion-exchange (IEC-HPLC) were the primary forms of chromatography used for D/L amino acid separation. In 1998, Kaufman and Manley developed an analytical procedure involving reverse-phase HPLC (RP-HPLC) to complete stereoisomeric separation in mollusk shells. When examined through interlaboratory analysis, RP-HPLC preformed comparably similar to GC and IEC-HPLC. Their method combines the advantages of both GC and IEC-HPLC, allowing for simple sample preparation and the ability to run several amino acids in one sample with higher sensitivity. This project fills a gap in available research by providing an alternative and effective way to examine enantiomeric separation of amino acids in avian eggshells through RP-HPLC with precolumn derivatization.

Previous student researchers have achieved distinct retention times and narrow gaussian peaks for many of the standard D/L amino acids seen in Figure 1 below. Utilizing the same HPLC system, current endeavors of the project focus on replicating previous data through reliable quantitation of standard amino acids, via analysis of detectability and calibration curves. Thus, establishing a foundation for which the reliability and repeatability of future work will be ensured.

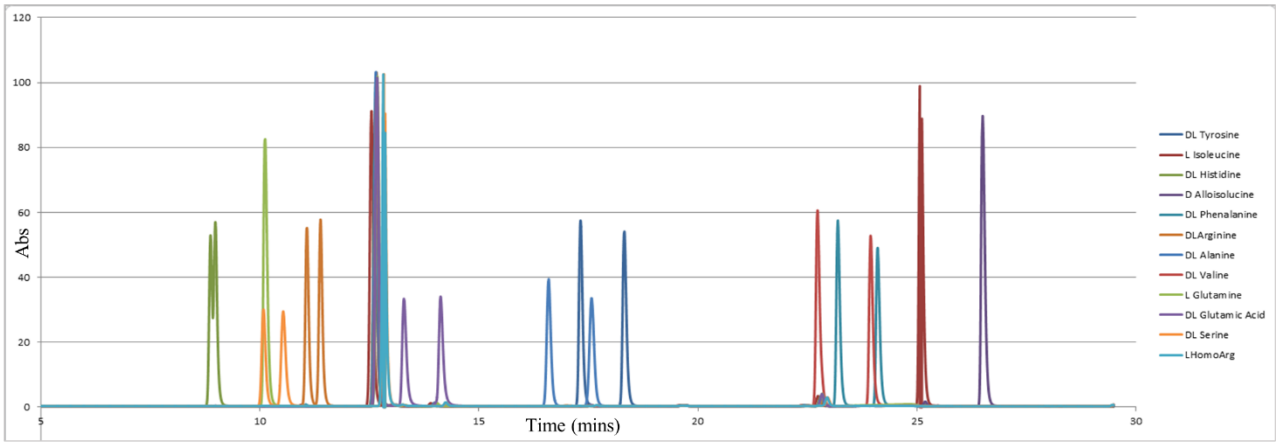

Figure 1. Past student researchers have constructed this chromatogram containing overlapping absorbances for several amino acids over time (mins).

Methods

The following procedure is a modification of the research completed by Kaufman *et al.* (1998) and Brückner *et al.* (1991). Analysis is performed using the Agilent 1260 Infinity II LC System comprising of a binary pump, standard degasser, autosampler, diode array detector and fluorescence detector (Figure 2). The column employed is an Agilent Poroshell 120 EC-C18 (2.7 µm 4.6 x 150 mm). Proper column function was tested by the routine run of the column standard. For the column in use, the standard was made of 10 µg/mL Uracil, 400 µg/mL Phenol, 50 µg/mL 4-Chloro Nitrobenzene and 80 µg/mL Naphthalene in a mobile phase that is 70% acetonitrile and 30% water.

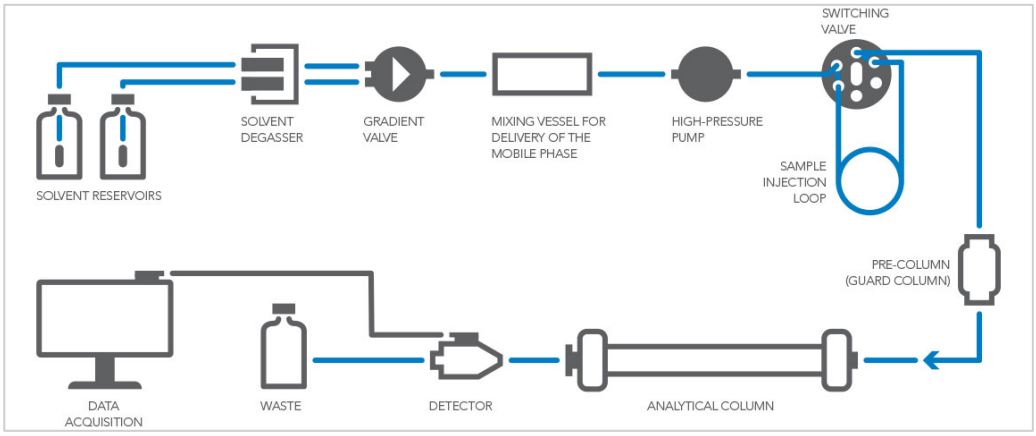

Figure 2. A representation of the HPLC system used. From the solvent reservoirs to the waste, the diagram depicts the flow of the mobile phase through the components of the instrument. The sample becomes incorporated into this flow after the injection loop.

To construct calibration curves, the D/L amino acids valine and alanine were selected due to their structural simplicity and resolution of peaks as seen from Figure 1 and research conducted by Penkman *et al.* (2008). It was found that concentrations in the micro and nano molar range were easily detectable (Penkman *et al,* 2008; Kaufman *et al,* 1998). Thus, starting from 0.001 M, standard amino acid concentrations were incrementally chosen until 0.01 mM. These standard solutions are made through serial dilutions with 0.05 M HCL. Prior to dilution, 0.03 mM of L-*homo*-arginine is added to the 0.001 M stock solution of D/L amino acid, acting as an internal standard.

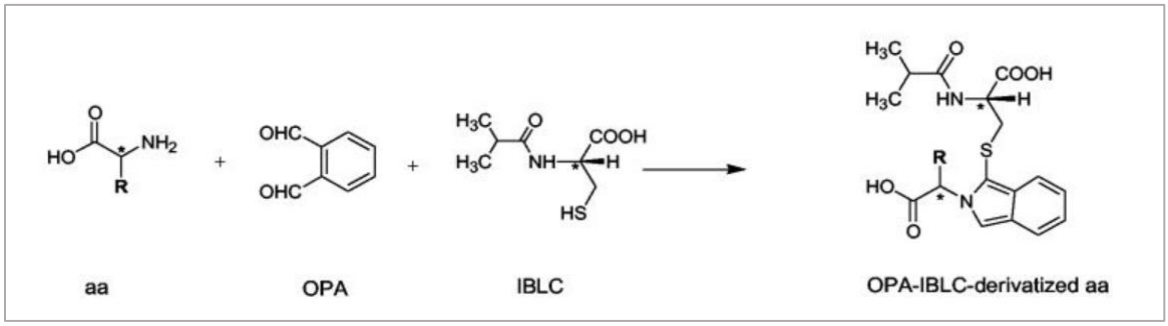

Figure 3. A generic amino acid is derivatized with 0.17 M o-phthaldialdehyde (OPA) and 0.26 M N-isobutyryl-L-cysteine (IBLC) in a potassium borate buffer (1 M, pH 10.4). The resulting diastereomeric derivative aids separation and detection through fluorescence.

To prepare each standard amino acid sample for passage through the column, pre-column derivatization of the amino acid is completed to ensure improved signal response and detection by RP-HPLC. The pre-column derivatizing reagent contains 0.17 M o-phthaldialdehyde (OPA) and

0.26 M N-isobutyryl-L-cysteine (IBLC) in a potassium borate buffer (1 M, pH 10.4). OPA is known to increases the fluorescence response of primary amines while IBLC enhances enantioselectivity and stabilizes the OPA-IBLC derivatized amino acid (Hess, Sonja et al., 2004). The sulfhydryl group of IBLC reacts with OPA and the amino group of an amino acid to produce diastereomeric isoindole derivatives seen in Figure 3. This reaction proceeds as directed by the injection program in Table 1. Optimization of this derivatization procedure would prove useful for better resolution but also for attaining lower limits of detection and quantitation. To optimize this precolumn derivatization, aspects of signal detection were explored and manipulated. Originally discussed by Kaufman et al. (1998), there are three components of the injector program that were varied in order to impact the derivatization procedure: reagent volumes, mixing time, and the order of reagents mixed. Mixing time is an important consideration since too short would result in an incomplete reaction and too long would impact the stability of the derivative product. The volumes of the derivatizing reagent are important to understand the point at which increasing OPA/IBLC solution no longer increase fluorescence response. The order of reagents mixed would influence how uniformly derivatization is carried out.

Table 1. This injector program displays the steps that instruct the autosampler to draw and derivatize the sample. A needle wash, before and after the sample is drawn, is not shown.

Function	Parameter
Draw	Draw 2.5 µl from location vial "__" (pH 10.4 borate buffer) with default speed and default offset
Draw	Draw 1 µl from sample with default speed and default offset
Mix	Mix 3.5 µl from air with default speed for 5 times
Wait	Wait 0.2 minutes
Draw	Draw 0. 5 µl from location Vial "__" OPA solution with default speed and default offset
Mix	Mix 4 µl from air with default speed for 10 times
Draw	Draw 0. 5 µl from location Vial "__" IBLC solution with default speed and default offset
Mix	Mix 4.5 µl from air with default speed for 10 times
Inject	Inject
Wait	Wait 0.1 minutes
Valve	Switch valve to "Bypass"

To carry the sample through the HPLC, the mobile phase varied through the experiment. The two solvent systems used were methanol and 23 mM sodium acetate buffer (pH 6) or acetonitrile and 23 mM sodium acetate buffer. The reasons for why both these solvent systems are needed is elaborated on in the results section. To prevent contamination such as bacterial growth, 1.5 mM sodium azide was added to the sodium acetate buffer. Prior to each run, the column was equilibrated with the mobile phase for at least 30 mins at a flow rate of 1 mL/min. After each run the column and the needle are washed with methanol to prevent interference through carryover.

After derivatization and advancement through the column, the analyte is detected by the diode array detector through UV absorption. This was completed at wavelengths of 338 nm and 280 nm with a 10 nm bandwidth. Fluorescence detection was also employed. Fluorescence was measured at an excitation wavelength of 230 nm and an emission wavelength of 445 nm (Buckner et al, 1991). Integration of peaks detected is completed by the computer software, ChemStation. To

ensure repeatability, the peak areas and retention times of samples run several times are compared through relative standard deviation. This process also allows for simple identification of laboratory errors such as sample contamination or an inaccurate fluorescence response. In addition, analysis of peaks includes a set statistical analysis method for assessing noise, drifting baselines, distortions in peak shape such as tailing or fronting, etc. All the parameters mentioned thus far need to be understood before constructing calibration curves, graphs of peak area versus concentration. Calibration curves offer the ability to understand the limit of detection and quantitation which exposes the extent of reliable data the instrumentation is able to provide. In addition, the linear range can help identify when the derivatization procedure begins to deviate from expected proportionality between peak area and analyte concentration.

Results

The concentrations of standard amino acid solutions were 0.001 M, 0.0005 M, 0.0001 M, and 0.01 mM for DL-Valine. Two concentrations of 0.001 M and 0.0005 M were chosen for L-Alanine. Starting out with the injection program from Table 1, the standards of DL-Valine were run. The mobile phase gradient employed was run as follows: 0 min (75% 23 mM sodium acetate buffer, 25% acetonitrile) to 30 min (25% 23 mM sodium acetate buffer, 75% acetonitrile) with a flow rate of 1 mL/min. After several trials of varying concentrations, the sample chromatogram always resembled the blank chromatogram with UV detection. Another attempt was made that included fluorescence detection along with UV. The fluorescence detection produced a peak assumed to be DL-Valine that varied in area depending on the amino acid sample concentrations. Thus, even though previous analysis has been completed using UV detection, there may be a benefit to incorporate fluorescence detection in analysis.

In addition, consistent baseline separation has not occurred between D- and L- forms of amino acids. This is to be improved through alterations of the solvent gradient in a way that results in a less viscous solvent system and one that does not impede solubility of samples. Prior to the use of acetonitrile and sodium acetate buffer, methanol was used in place of acetonitrile. But this combination of methanol and sodium acetate buffer proved to be too viscous, so much so that back pressure arose above 400 bar consistently. It was assumed that there was a blockage at some point in the flow path. Thus, the guard column inline filter was replaced. Because pressure still remained, acetonitrile was replaced with methanol, but poor separation still occurred. Another avenue through which baseline separation may be achieved would be by examining column function. While running the column standard is a good measure of column function, future work could explore column efficiency by calculation of the number of theoretical plates and plate height. As the height of a theoretical plate decreases, the number of plates in a column increases, allowing for an increase in partitioning of a column (N=L/H), where N is the chromatographic efficiency or the number of theoretical plates.

In relation to optimization of the derivatization procedure, varying mixing time, order of reagents mixed, and derivatizing reagent volume did not change the signal response in a predictable manner. But this data is not conclusive since it was not replicated due to time constraints. Tables 2 and 3 provide examples of the variations possible in the injector program. Though results were inconclusive, optimization of the derivatization procedure should still be pursued due to the impact of signal response on quantitation.

Table 2. This is the original injector program found in Kaufman and Manley's paper. The most unique aspect of this derivatization procedure is that OPA and IBLC are in the same solution

Function	Parameter
Draw	Draw 1 µl from location vial "__" (pH 10.4 potassium borate buffer) with default speed and default offset
Draw	Draw 1 µl of OPA/IBLC derivatizing reagent (260 mM IBLC and 170 mM OPA in 1 M potassium borate buffer)
Draw	Draw 1-4 µl of sample (corresponding to about10 pM -10 nM of each amino acid isomer) in 0.01 M HCl and 0.03 mM *L-homo*-Arginine
Draw	Draw 1 µl of OPA/IBLC derivatizing reagent
Draw	Draw 1 µl of potassium borate buffer
	Mix in seat (3 mixing cycles; 12 µl mixing volume; 200 µl/min mixing speed)
Inject	Inject

Table 3. This program can be considered a hybrid of both the original and the current program. Many components of this program, such as volumes and length of time, were varied to more closely resemble either the original or current program. Perhaps including the bypass instruction from the current procedure would reduce broadening.

Function	Parameter
Draw	Draw 2.5 µl from location vial "__" (pH 10.4 borate buffer) with default speed and default offset
Draw	Draw 1 µl from sample with default speed and default offset
Mix	Mix 3.5 µl from air with default speed for 5 times
Wait	Wait 0.2 minutes
Draw	Draw 0. 5 µl from location Vial "__" OPA solution with default speed and default offset
Mix	Mix 4 µl from air with default speed for 10 times
Draw	Draw 0. 5 µl from location Vial "__" IBLC solution with default speed and default offset
Mix	Mix 4.5 µl from air with default speed for 10 times
Inject	Inject
Wait	Wait 0.1 minutes
Valve	Switch valve to "Bypass"

Conclusion

To determine the extent of racemization in organic material, this project introduces a methodology for quantifying D/L amino acid ratios in avian eggshells through reverse-phase chromatographic separation. To uncover the specific set of variables that lead to consistent baseline separation of D/L amino acids, standard amino acids are run, instrument limitations are understood and a set statistical analysis method for establishing repeatability is applied. Based on recent findings, analytical signal will be evaluated and optimized through derivatization and fluorescence detection. Ensuring validity of the method developed for standard amino acids guides the accurate analysis of multi-amino acid samples. Consequently, a comprehensive analytical method for gaining reproducible results is necessary.

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