**Part I: Preliminary Information**

**Title:** Quantification of the Chemical Markers of Melanin to Enhance Early Diagnosis of Melanoma

**Abstract:**

Melanoma diagnosis is clinically challenging and subjective. High discordance rates among experienced pathologists demonstrate the need for more chemically robust diagnosis methods. Melanoma is characterized by overproduction of the pigment melanin, which is present in two forms in human skin – eumelanin and pheomelanin. Although recent research suggests the incidence of melanoma is related to the relative concentrations of eumelanin and pheomelanin, no available technique precisely and accurately quantifies the chemical markers of melanin. This research proposes to improve the sensitivity and specificity of the diagnostic tests for melanoma by introducing solid phase extraction (SPE) and high performance liquid chromatography (HPLC) analysis with mass spectrometric (MS) detection. Separating, quantifying, and identifying the chemical markers of melanin using these techniques will be used to test the hypothesis that a high eumelanin to pheomelanin ratio corresponds to malignant melanoma.

**Personal Statement:**

There is a short poem by Walt Whitman titled “When I Heard the Learn’d Astronomer” that I was introduced to in, of all places, my high school biology class. It is written from the perspective of a student in a large astronomy lecture, seemingly overwhelmed as “the proofs, the figures, were ranged in columns before me.”1 The student quickly exits the lecture hall into the crisp night air, “and from time to time, look’d up in perfect silence at the stars.”1 I felt a special connection to the student of the Learn’d Astronomer when I first read this poem. The student, who is intrigued by the beauty of the night sky, has difficulty sitting through a lecture on the subject. While I find reading textbooks and participating in class lectures important, I believe hands-on, experiential learning outside of the classroom is truly invaluable. It was not until I traveled to Spain with Spanish language students in my high school that I began to comprehend the importance of cultural awareness and immersion. It was not until my high school anatomy labs that I became fascinated with the biochemical processes of the body, many of which are topics of exciting scientific inquiry and research. And it was not until my seventh grade bridge building project that I realized, despite countless hours of planning and meticulous placing of toothpicks, not all bridges are built to last. Similar to the student of the Learn’d Astronomer, my intellectual curiosity is best explored in firsthand experiences.

Throughout my education, I have never been satisfied with memorizing theories or lists of equations. I am constantly asking questions to further understand how and why something works. I believe that obtaining this extra level of understanding is vital for critical thinking and full exploration of unanswered academic questions. I understand that my ability to learn outside the classroom is somewhat dependent upon the knowledge obtained through lectures and reading. However, I believe applying knowledge in creative ways is not only an opportunity to learn, but also a scholarly responsibility to help expand and improve a field of study and ultimately have a positive impact on society.

My desire to learn outside of the classroom through experiential learning is the primary reason I decided to attend Elon. The ability to study abroad, participate in service-based learning, gain hands-on experience through internships and advance a field of study through research are the opportunities I believe will define my undergraduate education. My interest in the biochemical functions of the human body and the implications for diseases such as melanoma is what first sparked my interest in conducting research with Dr. Glass. I am excited for the opportunity to explore the intriguing chemical processes of melanin and to apply my findings to the diagnosis of melanoma. After graduation, I intend to continue my education in graduate school, specifically studying the biochemical basis of disease. Knowledge gained in the classroom and laboratory has inspired my interest in medicinal chemistry, and conducting undergraduate research in this field will greatly aid my postgraduate endeavors. The Lumen Prize will help expand the depth of my undergraduate project, provide the funds to obtain important chemicals and instrumentation, and allow me to share my findings and learn from experts at national conferences. The prize will also aid my personal academic growth, provide the opportunity to take responsibility for a project, work independently while questioning assumptions and testing hypotheses, and collect results that help advance an intriguing discipline of study.

**Part II: Project Description**

**Focus:**

Over the past two decades, the fatality rate for melanoma increased more than 5% in the United States, while the overall cancer fatality rate decreased significantly in both men (19%) and women (11%).2 During the same period the total diagnosis of melanoma increased 140%, faster than any other cancer.3 Melanoma is a cancerous tissue characterized by overproduction of the pigment melanin, a complex polymer that is responsible for determining hair and skin color. Two forms of melanin are present in human skin – eumelanin (black/brown in color) and pheomelanin (orange/red in color). Granules of melanin fill specialized skin cells and, in healthy humans, protect from harmful UV rays. Although optical imaging qualitatively suggests that a high ratio of eumelanin to pheomelanin corresponds to the uncontrolled production of melanin, characteristic of melanoma, no chemical analysis to date has successfully quantified this ratio.4-6

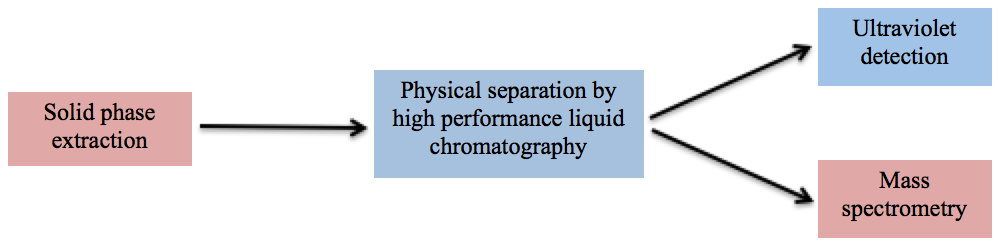
Current diagnostic tests for melanoma begin with inspection of suspicious moles followed by excision and examination by a pathologist. Pathologists look for irregularities in the symmetry, border, color, diameter and maturation of moles. Due to a limited understanding of the chemical structure of melanin, these tests for melanoma are largely subjective. Numerous studies have shown that pathologists examining the same skin biopsy samples often disagree on the diagnosis of melanoma.5,7-9 One recent study at USCF found a discordance rate of 14% among experienced pathologists, which suggests that approximately half a million specimens in the United States alone would be diagnosed differently by another pathologist.10 High discordance rates arise because the diagnostic tests for melanoma were developed to detect potentially fatal lesions greater than 3.0 millimeters in diameter and have not been altered or refined for the early-diagnosis of thin lesions less than 1.0 millimeter thick.11,12 Since early detection of thin lesions is essential to lowering melanoma fatality rates, the goal of this project is to develop robust chemical methods to objectively analyze and diagnose melanoma in thin lesions.

Although quantitative chemical methods are not available to diagnose melanoma in tissue samples, preliminary techniques have been developed to chemically analyze melanin pigments.13-17 Due to the insolubility and complexity of melanin, the most commonly used method to analyze melanin is alkaline hydrogen peroxide oxidation.13,18 This process breaks eumelanin and pheomelanin down to their unique chemical markers, which are then directly injected into a high performance liquid chromatography (HPLC) instrument and analyzed with ultraviolet (UV) detection.13,18 The HPLC system uses a column to physically separate a mixture of chemicals, including the chemical markers of melanin, based on different elution times in a nonpolar environment. Melanin markers have different levels of adhesion for the HPLC column coated with a nonpolar surface; markers with greater affinity for the column require a longer time to elute. UV detection provides limited information about the relative quantities of melanin markers separated through HPLC, but does not uniquely identify each chemical marker. Identification of the chemical markers requires mass spectrometry (MS) analysis. Overall, this project proposes to address the two primary problems in the existing technique for analyzing melanin that make it unsuitable for the chemical diagnosis of melanoma.

1. Skin tissue samples are highly complex. They contain many components that interfere with analysis of the chemical markers of melanin by eluting at the same time (coeluting). Peaks in HPLC-UV are additive, so coeluting components enhance measured signals and bias the observed ratio.

This project proposes to introduce solid phase extraction (SPE) of tissue samples before HPLC analysis. SPE effectively separates chemicals in a column based on electrostatic interactions between charged particles. Using a positively charged SPE column and designing experimental conditions such that the small carboxylic acid group(s) on melanin markers is (are) negatively charged, the markers will be isolated from largely uncharged proteins and lipids in skin tissue.

1. The potassium phosphate buffer system currently used to separate melanin markers using HPLC is incompatible with detection techniques other than UV detection, such as MS, which provides critical chemical data.

MS generates the molecular weight of a compound and provides insight on a compound’s chemical structure. Phosphate buffers currently used in HPLC analysis are incompatible with MS because the nonvolatile phosphate salts do not readily evaporate and accumulate on the mass spectrometer instrument.19 The build-up of salt rapidly diminishes the sensitivity and lifespan of the very expensive mass spectrometer. Phosphate buffers also quickly breakdown HPLC columns, necessitating high replacement costs.19 This project intends to replace the potassium phosphate buffer with ammonium acetate, a volatile buffer that evaporates readily and is compatible with HPLC columns utilizing UV and MS detection. The combination of HPLC with MS and UV detection enables separation, quantification, and identification of melanin markers in a single step, a clear advantage over current techniques. The proposed procedure for analyzing melanin is illustrated in Figure 1, where the blue boxes represent existing techniques for analyzing melanin and the red boxes represent proposed techniques.

Introducing SPE and HPLC-MS techniques will allow greater sensitivity and specificity in the chemical analysis of melanin. These procedures will be used to analyze benign and malignant thin lesions, quantify the eumelanin to pheomelanin ratio, and explore the conjecture that a high ratio corresponds to malignant melanoma. While current research is exploring techniques to qualitatively estimate eumelanin and pheomelanin content, no studies to date provide a means to reliably quantify the ratio of these compounds. The ultimate goal of this research is to develop a quantitative and reproducible method for the diagnosis of melanoma, improving on the accuracy and accordance of current diagnostic tests. Such tests would allow for early treatment and lowered mortality rates of melanoma while also diminishing the high costs and stress of incorrect diagnoses.

**Proposed Experiences:**

*Spring 2015:* I will develop SPE methods to separate various chemical markers of melanin from non-melanin components. HPLC will be used to verify that separation proceeded with minimal sample losses. I will also read recent literature on melanin and collaborate with Dr. Glass to write a review paper*.*

*Summer 2015:* I will study abroad in the summer and fall at the University of Otago in New Zealand. While abroad, I will take unique upper-level biochemistry electives not offered at Elon. I will also maintain communication with Dr. Glass to analyze SPE data from the spring and submit the review paper.

*Fall 2015:* I will continue classes in New Zealand, maintain contact with Dr. Glass, and remain up-to-date on melanin literature. I will also propose my Honors thesis and apply for SURE.

*Winter 2016:* Upon returning from my semester abroad, I will meet with Dr. Glass regularly to familiarize myself with laboratory techniques and establish detailed plans for spring research.

*Spring 2016:* I will analyze known chemical markers and melanin-containing samples using HPLC-UV analysis to develop adequate separation and detection techniques. I will present my preliminary findings at SURF.

*Summer 2016:* I will continue my research through SURE. Tissue samples obtained from Duke University will be analyzed using the previously developed SPE and HPLC techniques. Multiple days will be spent at Duke University using their HPLC-MS instrumentation. I will be particularly interested in the correlation between malignant melanoma and eumelanin to pheomelanin ratio.

*Fall 2016:* I will continue collecting and analyzing data from the summer and begin corresponding with Dr. Shosuke Ito in Japan. Dr. Ito is capable of verifying our HPLC-UV results using standard techniques in the field. I will also draft a manuscript on my work to submit for publication in a peer-reviewed scientific journal. At this time, I hope to present my research at the Southeastern Regional Meeting of the American Chemical Society (SERMACS).

*Winter 2017:* I will continue drafting a manuscript for publication and will begin writing my Honors thesis.

*Spring 2017:* I will complete any final data collection, submit a manuscript to a scholarly journal, and defend my Honors thesis. I will also present my research at SURF and a national American Chemical Society (ACS) conference.

**Proposed Products:**

The ultimate goal of this two-year research project is to develop a robust chemical method for the diagnosis of melanoma. Specifically, I hope to quantify the concentrations of the chemical markers of melanin and draw conclusions about the relationship of eumelanin and pheomelanin concentrations to malignant melanoma. This research will hopefully culminate in one or more published articles, various oral and poster presentations at local and national conferences, and successful defense of my Honors thesis. I will submit a paper for publication on my completed research to a scientific journal such as *Pigment Cell and Melanoma Research*, *Journal of Chromatography*, or *Photochemistry and Photobiology*. I intend to present my research findings at SURE as well as national conferences such as SERMACS and ACS. Submitting articles for publication and presenting at conferences will enable me to actively contribute to chemistry and pigment research. It will also showcase the unique undergraduate research experience available to Lumen Scholars at Elon University.

**Part III: Feasibility**

**Feasibility Statement:**

The research experiences and products outlined above are feasible. Necessary laboratory equipment, including solid phase extraction (SPE) and high performance liquid chromatography (HPLC) with ultraviolet (UV) detection is available in Dr. Glass’s laboratory at Elon University. Mass spectrometry (MS) analysis will be completed during SURE using the instrumentation at Duke University. Use of facilities and transportation to Duke is costly, but is made possible through the stipend awarded by the Lumen Prize (budget below). Dr. Glass has obtained startup chemicals and materials, such as solvents and HPLC columns. Additional chemicals and materials required over the next two years are available for purchase with the Lumen award.

Research completed by Dr. Glass at Duke University has shown promise in SPE and HPLC separation of amino acids, and these techniques will be modified for the chemical analysis of melanin. Dr. Glass also has years of experience mentoring undergraduates at Duke University and will provide me with the necessary training and guidance throughout the next two years.

The timescale of this project is also within reason. A total of ten research credit hours (including two hours during the spring of 2015) will be dedicated to this project. While not all circumstances can be foreseen, the timeline and use of each research credit hour is within reason. This time commitment and my personal dedication and intellectual curiosity will ensure the research culminates in a complete and high quality project.

**Budget:**

* **$1550** – Chemical expenses for the various reagents and solvents acquired from Sigma-Aldrich and Fisher Scientific
  + $750 – various chemical markers of melanin
  + $75 – ethyl acetate (1L)
  + $230 – trifluoroacetic acid (100 ml)
  + $495 – Unforeseeable chemical expenses based on potential protocol modification
* **$750** – Purchase of HPLC column from Sigma-Aldrich
* **$6810** – Costs associated with use of mass spectrometry and high performance liquid chromatography instrumentation at Duke University
  + $6750 – total cost for use of HPLC-MS instrument
    - Reserved for four full (24 hour) days
  + $60 – transportation to and from Duke University
    - 8 days of round-trip travel (80 miles)
* **$1315** – Attending and presenting at various regional and national conferences
  + Southeastern Regional Meeting of the American Chemical Society (SERMACS) – Columbia, SC
    - $80 – registration fees
    - $375 – travel costs and hotel accommodation
  + National Meeting of the American Chemical Society (ACS) – San Francisco, CA
    - $110 – Registration fees
    - $750 – Travel costs and hotel accommodation
* **$4575** – Tuition and study abroad fees for the University of Otago

**Timeline:**

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|  | **Proposed Experiences** | **Proposed Products** |
| **Spring 2015** | * Complete two CHM499 research hours. * Become familiar with required laboratory techniques for the analysis of melanin. * Read scholarly literature and write a review paper on the physical and chemical properties of melanin. | * Develop techniques for separating mixtures using SPE and analyzing compounds using HPLC. * Establish the appropriate solvents for separating chemical markers of melanin. * Compose a review paper on melanin for publication. |
| **Summer 2015** | * Depart for New Zealand study abroad at the end of June. * Continue reading scholarly literature to remain up-to-date with melanin research. * Maintain contact with Dr. Glass to analyze SPE data collected in the spring. | * Further development of SPE protocols by analyzing preliminary SPE data collected in the spring. * Submit melanin review paper to scientific journal, such as *Photochemistry and Photobiology*. |
| **Fall 2015** | * Take Biostatistics, Proteins and Biotechnology, Epidemiology, and Population Biology at the University of Otago. * Complete 1 HNR498 hour while reviewing scholarly literature and proposing Honors thesis. * Maintain communication with Dr. Glass regarding advances in melanin literature and any changes to SPE protocol. | * Successful proposal of Honors thesis. * Development of reliable methods for isolating the chemical markers of melanin from skin tissue samples utilizing SPE. |
| **Winter 2016** | * Familiarize myself with laboratory techniques and establish detailed plans for spring research. |  |
| **Spring 2016** | * Conduct HPLC analysis with melanin-containing samples. * Take Quantitative Analysis, Genetics, and Neuroscience. * Complete 2 HNR498 hours. | * Develop protocols to separate melanin markers using HPLC. * Validate HPLC methods at Duke. * Present at SURF. |
| **Summer 2016** | * Continue research with SURE. * Obtain tissue samples from Duke University to analyze with SPE and HPLC-MS. | * Quantify the chemical markers of melanin in skin tissue samples. * Develop insight into the relationship between melanoma and the ratio of eumelanin to pheomelanin. |
| **Fall 2016** | * Analyze data from the summer and begin drafting a manuscript for journal publication. * Correspond with researcher in Japan to verify accuracy of HPLC-UV results. * Take Biochemistry, Seminar in Neuroscience, and Medicinal Chemistry. * Complete 2 HNR498 hours. | * Present at the Southeastern Regional Meeting of the American Chemical Society (SERMACS). * Develop further insight into the relationship between melanoma and the concentration of the chemical markers of melanin. |
| **Winter 2017** | * Collect final data needed to complete the project and finalize manuscript for publication. * Begin writing Honors thesis. * Complete 2 HNR498 hours. | * Submit manuscript to peer-reviewed scientific journals such as *Pigment Cell and Melanoma Research*, *Journal of Chromatography*, and *Photochemistry and Photobiology*. |
| **Spring 2017** | * Finish writing Honors Thesis. * Take Advanced Biochemistry and Seminar in Biochemistry. * Complete 1 HNR498 hour. | * Present at SURF. * Present at a national American Chemical Society (ACS) conference. * Successful defense of Honors Thesis. |

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