5-12-2021

Amino Acid Composition Analysis as a Means to Differentiate Hair Samples from Individuals of Similar Demographics and the Effect of Hair Treatments

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UNIVERSITY OF NEW HAVEN
HONORS PROGRAM

2020-2021 Honors Thesis

Amino Acid Composition Analysis as a Means to Differentiate Hair Samples from Individuals of Similar Demographics and the Effect of Hair Treatments

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A thesis presented in partial fulfillment of the requirements of the Undergraduate Honors Program at the University of New Haven.

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12 May 2021

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Table of Contents

ACKNOWLDEGMENTS

Sincere thanks to Dr. Alyssa Marsico for her patience and support throughout this entire process.

Special thanks to my classmates for their encouragement and Sandra Hartman-Neumann for her support and ideas.

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Hair is a forensically-relevant exhibit due to its ability to be shed, torn, or transferred during a crime. Current forensic hair analysis includes microscopic hair comparison and mitochondrial or nuclear DNA analyses, each with limitations. Novel methods, such as those evaluated herein, have potential to complement conventional techniques. These include evaluating functional groups with infrared (IR) spectroscopy, elements with laser induced breakdown spectroscopy (LIBS), and amino acid ratios with gas chromatography- mass spectrometry (GC-MS). Hair from two demographically similar individuals was divided into three sample groups per individual: untreated, bleached, and dyed. Spectral differences between individuals and treatments were evaluated with IR. Five of eleven bands differed between individuals, and seven bands differed after treatment. LIBS analysis revealed calcium, potassium, and sodium to be significantly different among individuals. Decreases in carbon, nitrogen, oxygen, and hydrogen were observed after treatments. Six derivatized amino acids were identified with GC-MS to produce fifteen amino acid ratios. One ratio was found to be significantly different among individuals. Additional significant differences would likely surface with retesting. Complications with GC-MS made comparisons between individuals and treatments difficult. individual 2 displayed lower variances, so comparisons between treatments were based on their data. For individual 2, three amino acid ratios were found to be significantly different after bleaching, while none were after dyeing. Variances in GC-MS analysis made the amino acid ratio stability after treatments difficult to establish.

Further research is required to better understand the effects of chemical treatments on elements, functional groups, and amino acids.

KEYWORDS: Forensic science, hair, amino acids, cosmetic hair treatments, permanent oxidative hair dye, bleaching, laser-induced breakdown spectroscopy and LIBS, attenuated total reflection-Fourier transform infrared spectroscopy and ATR-FTIR, gas chromatography-mass spectrometry and GC-MS

Introduction

Structure and Composition of Human Hair

Hair possesses a physical structure that is conducive to its role in Forensic Science as a common form of evidence. Each strand of hair can be divided into three different regions: the root, the shaft, and the tip. The general features of these regions are visible at the macroscopic level, although magnification is required to observe any finer or internal characteristics. When viewing a strand of hair, the tip and root reside at the two ends, with the shaft in between them. The visual appearance of the tip end of the hair can be helpful to examiners, as it can indicate whether the hair has been cut, split, or broken.[1] This can act as another point of comparison for examiners to make between unknown and known hair samples.[1]

At the opposite end of the hair, the root is embedded in the skin at the site of the hair follicle. This section resides in the area of the scalp where new growth occurs. There are three phases of growth that can be observed in the root area of each hair strand. The first of these is the anagen phase, which is an initial growth period where there is active production of hair by the follicle, which is made possible by a connection between the root of the hair and the follicle itself.[2] As a consequence of this connection, if hair is torn out during the anagen phase, a follicular tag will be present. This describes a segment of tissue near the root of hair that contains a rich source of nuclear DNA. Cases such as this allow for nuclear DNA analysis to be performed, providing information as to the individual source of the hair.[2, 1] During the anagen phase, the hair is able to grow beyond the follicle via repeated cellular division at the root. Specialized cells at the base of the follicle, an area called the dermal papilla, are heavily involved in the process of hair

growth, and trigger the cycle through hormonal signaling. This prompts a series of cell divisions in which a membrane-bound stem cell, called a mother cell, separates itself to become two daughter cells, and these daughter cells subsequently separate themselves. The division of these cells stops when differentiation of cell lines starts. This differentiation includes changes in their cell-line specific cytoplasmic expression of keratin, cell position, and shape. The nuclei of the cells stop functioning as they reach the cuticle and cortex layers of the hair after traveling up the bulb. During this stage, keratin and keratin associated proteins (KAPs) are being synthesized in the hair. These proteins, along with keratin itself, play a role in the strength and structure of the hair shaft.[3, 4] Gradually, the loss of functional nuclei in cells causes a buildup of dead and hardened cells starting in the cortex, which is known as keratinization. This is also followed by a slow breakdown of the non-keratin components in the cells.[1,2,4,5]

This is followed by the catagen phase, which is an adjustment period between the first and third phases during which the growth of hair is slowed. The final stage is the telogen phase, which occurs when there is a discontinuation of the hair growth, followed by eventual release of the hair from the follicle. The cycle can then be repeated as the hair matrix forms a new hair starting again in the anagen phase.[2, 1]

In between the root and tip regions is the shaft of the hair. While the length of this section depends upon how long an individual maintains their hair, this section is generally responsible for most of the overall length. The size and structure of hair, often owed to the shaft region, make it macroscopically visible and easily collected, and its layers allow for a level of protection from the environment. There are 3 main layers of hair, arranged roughly as concentric cylinders (Figure 1). The central-most cylinder is the

medulla. This layer is composed of air, and appears dark under a microscope. The medulla may be absent in certain finer hair types and can be easier to find in coarser types.[6] The pattern of the medulla may be expressed as continuous, interrupted, fragmented, or absent. A sampling of hairs from a single human individual may contain pieces with each of these patterns, as the medulla will vary even across one individual's head.[6, 2] The adjoining layer is the cortex, which is generally the largest of the three layers. The hair's pigments and proteins, including keratin specifically, are found in this section. This layer is responsible for providing many of the visual characteristics that we associate with a piece of hair, including its color, thickness, and overall shape.[2] The outermost layer, the cuticle, serves as a protective shell to shield the biological materials within the hair shaft. This layer is commonly expressed as having an arrangement similar to that of shingles on a roof, with row upon row of overlapping flattened and keratinized cells.[7] These hardened cells help to preserve hair's structure, and can safeguard the hair against forms of chemical damage.[2,6,7,8]

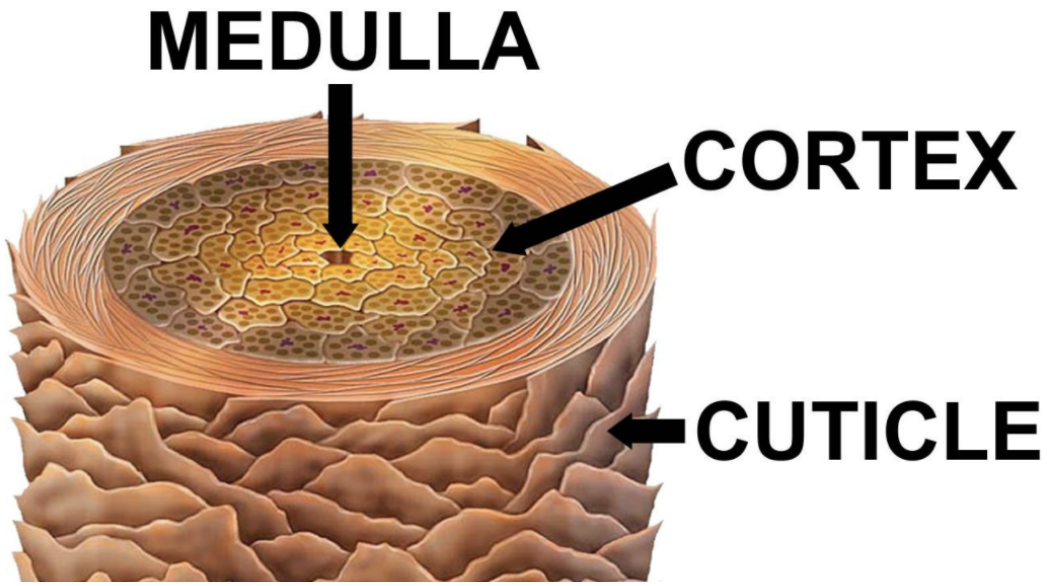


Figure 1: Three main layers of hair. [9]

The physiological response of the cuticle to different chemical conditions may be of significance to the information stored within the hair. In alkaline conditions, for example, the flattened cells of the cuticle swell and become softer. In acidic conditions, however, the cells decrease in size and harden. This knowledge has been used in the development of many cosmetic hair treatments, which often use alkaline conditions to manipulate the cuticle into adding or removing pigment.[7]

Significance of Human Hair as Forensic Evidence

In the context of Forensic Science, hair is a common exhibit type due to its ability to be easily shed or torn out during the commission of a crime, and its capability of transferring easily once shed. The macroscopic, microscopic, biological, and chemical information in the hair can potentially provide the identification of that hair's donor. As a result, the presence of hair at a scene can be utilized to develop linkages between victims, suspects, objects, and locations. Due to the fact that hair has the potential to create such probative linkages, analysts need to perform examinations that provide the most potential useful information investigatively. Current analysis of hair samples routinely involves microscopic hair comparison (MHC), mitochondrial DNA (mtDNA) analysis, and nuclear DNA analysis.[10]

Current Methods

Microscopic Hair Comparison (MHC)

One of today's conventional methods for forensic analysis is microscopic hair comparison (MHC). In the MHC technique, the examiner uses comparison microscopy to

view two hair samples simultaneously. The examiner should start by evaluating the species of the sample donor, and confirming if the unknown from the scene is human hair. Different features of the two samples can then be assessed, including hair color, length, diameter, cuticle thickness, overall appearance, chemical treatments, shaft form, and the attributes of any medulla present. The more features that the unknown and known samples share, the more likely they are to have originated from the same source.[11]

Unfortunately, because this analysis consists largely of subjective observations made by the analyst, there is always a possibility of error. The method is also limited by the sample size and variation, as there is known to be a wide range of hair colors and types originating from a singular individual. Damaged samples can be additionally difficult to accurately compare, as the damage may impact certain visual or physical characteristics. Due to the fact that many of the features of MHC are subject to external influences, statistics cannot be utilized to support the results of the examination.[11]

In the 2015 MHC Analysis Review done by the Innocence Project, the Federal Bureau of Investigation (FBI), the National Association of Criminal Defense Lawyers (NACDL), and the United States Department of Justice (DOJ) 90% of trial transcripts reviewed held unacceptable errors in the form of laboratory reports or testimonies with erroneous statements or overstatements of the findings.[12] This particular review concentrated on cases starting before the year 2000. After this, in acknowledgment of the method's subjectivity, mitochondrial DNA testing became a routine step after MHC analysis.[12]

Mitochondrial DNA analysis

Mitochondrial DNA (mtDNA) analysis is now routinely used to supplement MHC examinations. This form of DNA is present in the hair shaft, which is protected by the cuticle. This makes mtDNA less susceptible to degradation than potential nuclear DNA found. Additionally, mtDNA can be found throughout the entire hair shaft, while nuclear DNA can be present in the shaft, but is difficult to obtain a profile from as a result of the damage caused by the keratinization process.[13] As such, there is a greater likelihood of having mtDNA present, and within adequate levels. Unfortunately, it is expensive to complete a profile using mtDNA. This form of biological material is also passed through maternal lines, meaning it is only capable of determining whether someone has originated from the material line rather than providing the same individualizing power as nuclear DNA. For example, two brothers with the same mother would share the same indistinguishable mtDNA profile. Not only is mtDNA analysis only able to confirm or eliminate someone's relation within a maternal line, but it is also expensive and the most time-consuming of forensic DNA analyses.[13-16]

Nuclear DNA analysis

In scenarios where the hair is capable of enduring the context of its environment, it can be analyzed for nuclear DNA. This form of DNA will be found in nucleated cells, which are richly present in the follicular tag, but are present in a damaged state in the hair shaft due to the keratinization process.[13] The follicular tag consists of tissue still partially attached to the root that originates from the hair follicle. Nuclear DNA analysis relies on the fact that the follicular tag is present and intact. If this is the case, extraction and

identification can be performed on the DNA present after amplification by Polymerase Chain Reaction (PCR). For the extraction and identification that follow, techniques such as the QIAmp DNA Mini Kit or the Chelex method can be applied.[17] With nuclear DNA analysis, however, there is greater risk for degradation, leading to small amounts of DNA or only damaged DNA available. In a sample with an inadequate amount of intact DNA, the conditions may not be conducive to producing helpful typing results.[13-14, 17, 19]

Novel Methods and the Central Dogma

Conventional methods of forensic hair analysis can be extremely useful, and techniques such as nuclear DNA analysis are even capable of providing strong discriminating power under the right circumstances. However, this is only possible when the hair possesses an adequate source of undamaged DNA, such as from a follicular tag. Additionally, the hair found on scene may not always be in the most ideal form as a result of persistent UV, heat, or weather damage. However, other information provided by DNA can still be examined in hair to provide investigators with information about the source. Following the Central Dogma of biology, DNA is transcribed into RNA, which is translated into proteins.[20] During the process of transcription, RNA polymerase attaches to the start of a gene, moving along the DNA to produce a strand of messenger RNA (mRNA), out of free bases in the nucleus. This is followed by translation, where the sequence of mRNA is used to produce a sequence of amino acids in protein synthesis. The mRNA is read three bases at a time. Each three base segment is referred to as a codon, and corresponds to a particular amino acid, or the start or stop of the chain.[20]

As the DNA for each individual, except for monozygotic twins, is unique, it follows that the proteins and the amino acids they're composed of should reflect that same uniqueness. Additionally, these DNA products exist within the hair shaft, meaning no follicular tag is required for analysis, and they are more resistant to degradation than DNA itself. As a result, the analyses performed should strive to work with characteristics of the hair that are not as easily subjected to degradation, such as proteins, amino acids, and the elements that these are composed of.

Genetically Variant Peptides

One method of novel forensic hair analysis involves the investigation of genetically variant peptides (GVPs). A single nucleotide polymorphism (SNP), sometimes called a point mutation, describes a common type of genetic variation where individuals display a difference of a single nucleotide (T, A, G, or C). There are two main categories of SNPs: synonymous and nonsynonymous SNPs. Synonymous SNPs, which are also called silent mutations, result in a codon with the same meaning as the original (Figure 2). The outcome is the same as it would have been had the mutation not occurred, and the same amino acid is produced at that point in the sequence.[21]

Non-synonymous SNPs (nsSNPs), meanwhile, occur when the point mutation has resulted in a change in the amino acid sequence and protein expression. These can be further broken down into nonsense and missense mutations. A nonsense mutation occurs when the point mutation results in the modified codon acting as a stop codon, or putting an end to the peptide being constructed. A missense mutation refers to an instance where the point mutation has resulted in a different amino acid being produced at that location

in the peptide. This change is referred to as a single amino acid polymorphism (SAP). Genetically variable peptides (GVPs) occur when the SAP produced causes changes to the overall protein structure and function, leading to greater variation among individuals.[14,21] Proteomic analysis has observed GVPs within the proteins in hair and bone.[14, 22-25,] Despite the successes of proteomic hair analysis, the intricacies of technique can be inconvenient, especially in forensic science applications. For example, methods may require the proteins to remain whole throughout the procedure. As a result, new methods make use of the same concept as the proteomic analysis of these GVPs, but instead analyze the amino acid composition of the hair, bypassing the need to keep any proteins intact.[14,21-25]

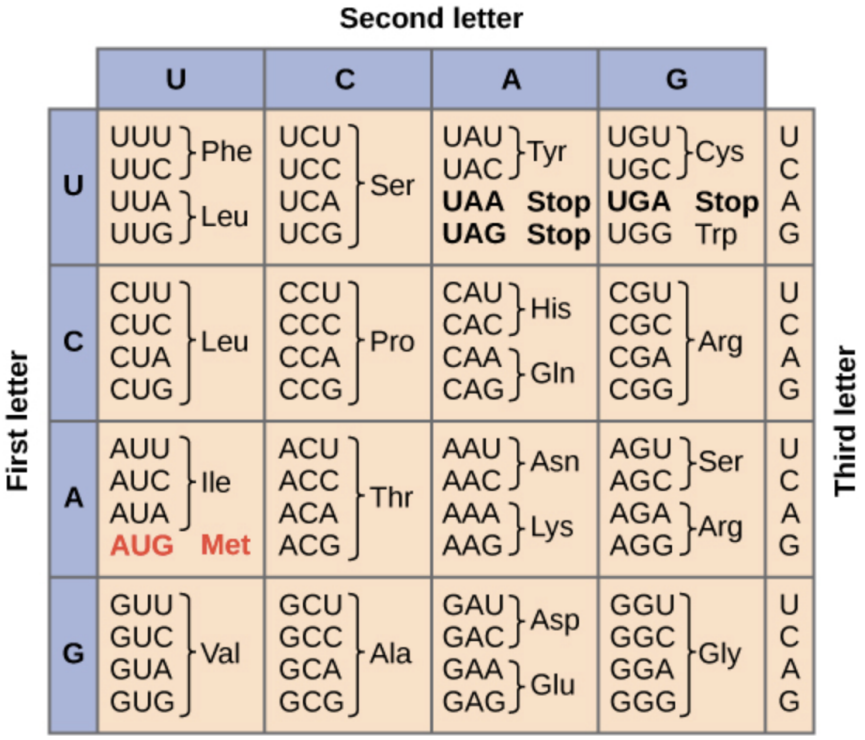


Figure 2: Codon chart showing the possibilities for synonymous and non-synonymous SNPs.[26]

GC-MS Analysis of Human Hair

Amino acid composition analysis was derived from GVP analysis as a simpler and more convenient method to observe the genetic differences among individuals. This method often utilizes gas chromatography- mass spectrometry (GC-MS). Gas chromatography is a separatory technique that utilizes a gaseous mobile phase and nonvolatile liquid or inert solid stationary phase to separate sample components. In GC, a heated injection port is utilized to convert samples to the gas state. The components of the gaseous sample are then separated based upon their interaction with the stationary phase, as well as their boiling point. The time that a specific compound requires to be eluted from the column is referred to as its retention time. Based on the quantity of each compound eluted at a given retention time, the GC can produce a total ion chromatogram (TIC). A TIC depicts the retention times of the various compounds as peaks, with the area under a given curve representing the quantity of that particular substance.

The separate constituents then enter the mass spectrometer (MS), which is a technique that generates, separates and detects gas phase ions. These ions are created by exposing the sample to a beam of high energy electrons. The resulting ions are allowed to travel through the mass analyzer, which makes use of an electric field to separate the ions based on their mass-to-charge (*m/z*) ratio. The ions are then detected in the order in which they exit the mass analyzer. This process involves both qualification and quantification of the ions detected. The resulting mass spectrum can be utilized in the identification of a substance, and is said to be a chemical fingerprint. A mass spectrum depicts the abundance for ions of each mass-to-charge ratio detected, and can be interpreted to provide information regarding a substance's structure, molecular weight, and

composition. Mass spectra collected from unknowns can also be compared to those in existing databases.[27]

Amino acid composition analysis using GC-MS resolves the issue of fragility previously discussed regarding the GVP analysis. When looking at the amino acids specifically, the analyst no longer needs to worry about keeping certain fragments intact, and can instead digest the entire hair sample to get the individual residues. This is beneficial because if protein degradation has previously occurred, it will not impact the analysis since they will be broken down into their constituent amino acids regardless.[28-29] As this technique would not suffer from the negative impacts of degradation, it has the potential to be a useful forensic hair examination method in cases where degradation prevents MHC or the successful production of a DNA profile.[28-29]

ATR-FTIR Analysis of Human Hair

Novel methods of forensic hair analysis can even look past the protein products of the DNA, and focus on the functional groups of these proteins, which is possible and convenient with attenuated total reflectance- Fourier transform infrared spectroscopy (ATR-FTIR). IR analyses involve the stimulation of sample molecules with infrared radiation. The resulting spectrum depicts the % transmittance versus the wavenumber. Peak locations, intensities, and shapes can provide information as to the functional groups present in the samples. For example, NH, OH, CH, C=C, or C=N, which are all types of bonds present in amino acids, will appear at different wavenumbers and have slightly different appearances from one another (Figure 3).[31]

Table 13.4 Frequencies of Important IR Stretching Vibrations

Type of bond	Wavenumber (cm^{-1})	Intensity
C≡N	2260–2220	medium
C≡C	2260–2100	medium to weak
C=C	1680–1600	medium
C=N	1650–1550	medium
	~1600 and ~1500–1430	strong to weak
C=O	1780–1650	strong
C—O	1250–1050	strong
C—N	1230–1020	medium
O—H (alcohol)	3650–3200	strong, broad
O—H (carboxylic acid)	3300–2500	strong, very broad
N—H	3500–3300	medium, broad
C—H	3300–2700	medium

Figure 3: Various functional groups seen with infrared spectroscopy and the wavenumbers that they are denoted by.[30]

In an ATR-FTIR analysis, the sample doesn't require additional sample preparation, and can be examined directly in its liquid or solid state. This is possible due to the use of an optically dense crystal, which has a greater refractive index than the sample. The instrument directs a beam of IR radiation at the crystal, and the resulting internal reflectance produces a wave that surpasses the crystal's surface to enter the sample. At wavenumbers where the sample absorbs the IR radiation the wave will be attenuated, or reduced in intensity as a result of its path.[31] This altered beam then returns to the crystal, exits through the opposite side, and is directed to the detector to be recorded.[31] This is a convenient form of analysis for hair because there is so little sample preparation required, and spectra can be obtained quickly. As will be further discussed in the literature review section, current research suggests that the functional groups observed with ATR-FTIR can aid in amino acid analysis and ion analysis, which may

help differentiate between cosmetic treatments and individuals.[32] As such, the analysis of the functional groups in the hair can be used to complement current methods based on the chemical components of their hair.[31-32]

LIBS Analysis of Human Hair

New analysis techniques can even venture one step further, examining the individual elements that compose the functional groups of the amino acids within the proteins created by the unique DNA. This can be accomplished using laser induced breakdown spectroscopy (LIBS), which is an elemental analysis technique. As implied by the name, LIBS creates a microplasma at the sample surface by subjecting it to repeated laser pulses.[33-34] After the final pulse of the laser, the plasma is allowed to cool. Simultaneously, light is emitted from the plasma and corresponds to electrons of the atoms and ions at the excited electronic states falling back down to their ground states.[33-34] The detector identifies the wavelengths of light emitted during this process, and records them. A library search function can be used to identify the various peaks within the spectrum, which is possible because different elements produce different spectral peaks based on the unique wavelength of light emitted when going through these energy state transitions. Thus, the chemical composition of a sample can be rapidly discerned via LIBS analysis. Studies suggest changes to elements such as C, H, N, O, Ca, Na, and K can be detected in hair after cosmetic treatments.[34] The analysis of the elements in the hair can be used to complement current identification methods based on the chemical components of their hair.[33-34]

Chemical treatments

Bleaching

Bleach is a common cosmetic treatment utilized to lighten the color of the hair. During the process of bleaching, the hair is lightened by destroying the pigment's chromophore, which is the actual group responsible for the coloring of the hair.[2] The coloring of a hair strand arises from a multitude of factors, including the transparency of the cuticle, whether air is present in the medulla, and the pigment granule density and makeup.[2] Species referred to as melanin reside within the hair's pigment granules, which exist in the cortex of the hair.[2] Bleach is typically at a pH of between 9 to 11, as the alkaline pH softens the cuticle, allowing for access into the cortex.[2,35] This is typically accomplished by a powder mixture of ammonia or a type of metasilicate, as well as persulfate salts.[2,35] Bleach products formulated for hair also contain hydrogen peroxide to destroy these melanin granules, resulting in lightened hair. Bleaching has also been found to alter the amino acid residues in hair, including decreases in levels of cysteine, tyrosine, histidine, threonine, methionine, and lysine.[2,35] The impact on cysteine has been found to be the most significant, as bleaching causes the disulfide bonds associated with these residues to be cleaved oxidatively.[2,35] Analysis of the hair's amino acids, functional groups, or elements may therefore detect differences between an individual's untreated and bleached hair.[2, 35-36]

Dyeing

Although there are many types of hair dyes, there are four main categories; temporary, semi-permanent, demi-permanent, and permanent dyes.[37] These groupings are designated based upon the longevity of the dye imparted. Hair dyes can also be broadly labeled as either non-oxidative or oxidative. Oxidative hair dyes utilize an oxidizing agent such as hydrogen peroxide to yield chemical changes to the hair shaft. Non-oxidative hair dyes only deposit color with the use of preformed dyes onto the hair, and thus do not require oxidation to occur.[37, 7, 35]

Temporary non-oxidative hair dyes have a low lifespan, and generally depart the surface of hair after a single wash. These types of dyes are typically acidic with a high molar mass. As mentioned, acidic conditions cause hardening of the cuticle. This response, combined with the use of high molar mass dyes aids in keeping the dye only on the outside surface of the hair rather than allowing penetration into the hair shaft. The characteristics of these preformed dyes are chosen to allow for the highest possible solubility in water while also having the lowest level possible penetration into the hair shaft.[37, 7, 35]

Semi-permanent non-oxidative hair dyes provide the next level of color lifespan. These products are formulated with cationic or basic dyes of a low molar mass. The use of lower molar mass dyes, coupled with alkaline pH products, is designed to enable a small amount of penetration into the cortex.[37] As previously mentioned, elevated pH levels cause swelling and softening of the cuticle, causing a more open conformation which enables the dye to access the cortex region. The chosen dyes are also selected for

their affinity to the keratin proteins within the hair shaft.[37] This enables the dyes to remain consistent for up to six washes.[37, 7, 35]

Demi-permanent hair products provide a level of color durability between those provided by semi-permanent and permanent dyes. This amounts to a color lifespan of approximately 20 washes, which is made possible due to the use of hydrogen peroxide.[37] This oxidizing agent is required because these dyes make use of oxidation dye precursors in formulation with semi-permanent molecules to produce the desired color, so a reaction is necessary to produce the final dye molecules.[37]

Permanent oxidative hair dyes, which are the most popular of the four types, are often desired for their ability to provide wash- and fade-resistant color. These products involve pairing ammonia or ammonium hydroxide with an oxidizing agent like hydrogen peroxide, along with other compounds called coupling bases (dye precursors) and reaction modifiers (dye couplers) (Figure 4).[37, 35] The ammonia or ammonium hydroxide is necessary to create an alkaline environment, which again encourages the cuticle to open, allowing the dye into the cortex region. The formation of color begins quickly after combining the given products, and is the result of reactions between the dye precursors and the oxidizing agent to produce the colored active intermediates.[37, 7, 35]

Once the product has entered the cortex, made possible by the alkalizing agent, the oxidizing agent is then necessary again to ensure that the active intermediates react with the dye couplers. The dye couplers alone do not produce much color, but when added to the oxidizing agent and precursors, they serve to modify the reaction and subsequently the resulting color produced by the dye precursors.[35] Essentially, the reactions between small precursor molecules with one another, and between active

intermediates and couplers, result in large dye molecules of a high molar mass and of the chosen color. This prevents the color from easily departing the hair shaft after the reaction has occurred, making the style wash-resistant.[37, 7, 35] Analysis of the hair's amino acids, functional groups, or elements may therefore detect differences between an individual's untreated and dyed hair as a result of these dye molecules and any oxidation that has occurred.

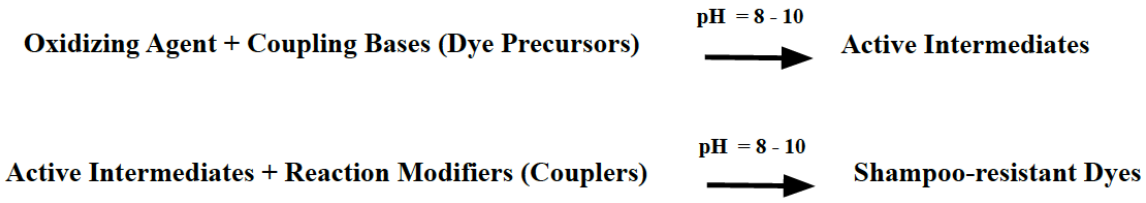


Figure 4: General process of imparting permanent hair dye into the cortex.

Literature Review

Novel Methods

The fragility of nuclear DNA, cost and lack of specificity with mtDNA, and subjectivity in MHC analysis have driven the field to a different source of genetic information within human hair: proteins, and the amino acids that they are composed of. As demonstrated by Parker et al., this can be done through proteomics. This study evaluated the proteome of the human hair shaft to assess if proteomics would be practical and feasible in hair analysis. As mentioned, proteins are less subjected to degradation than DNA is, which makes it makes them an appealing genetic target for forensic purposes.[14] Single nucleotide polymorphisms (SNPs) can cause variations in individuals' DNA, which can subsequently result in single amino acid polymorphism (SAP). These SAPs have the potential to affect protein structure and function, leading to genetically

variable peptides (GVPs). The GVPs caused by these SAPs can be detected in the hair, and can thus be used to differentiate between hair samples from individuals of different ancestries. Parker et al. obtained hair from African and European-American ancestry. GVPs were detected via mass spectrometry-based shotgun proteomics, and the analysts were able to detect differences in the nsSNPs present based on the subjects' ancestry.[14]

In a similar study, by Wu et al., mass spectrometry-based shotgun proteomics was also utilized for the detection and classification of GVPs to uncover the variations in individual's nsSNPs. Rather than volunteers from various ancestral backgrounds, as was done by Parker et al., Wu et al. obtained samples from monozygotic twins. One finding was that the expression levels of the various hair proteins arose mostly as a result of genetic factors. Essentially, people who are not related are more likely to be easily individualized. On this same basis, even dizygotic twins are more easily differentiated than monozygotic twins are. Wu et al. also found that the proteins analyzed contained GVPs as anticipated, which allowed for the detection of nsSNPs. Ten pairs of twins were effectively sorted and differentiated from each other through the evaluation of the analyzed peptides, demonstrating the potential usefulness of proteomics for distinguishing between people. Wu et al. recommended that additional investigation be conducted into the GVP yields based on environmental changes, age, or cosmetic treatments.[22] The success by Wu et al. in differentiating between monozygotic twins has shown the power of this novel method for the forensic individualization of hair evidence.[22]

Despite the successes seen with GVPs thus far, a study by Carlson et al. chose to review the obstacles that come with this form of analysis in order to improve it. One of

the drawbacks assessed by this study was the magnitude of the sample size required in proteomics analysis, which is about 40 to 800 cm of hair. This can be problematic for applications in forensic science, where sample sizes are often limited. As such, Carlson et al. focused on the development of a method requiring only 1 mm hair sample sections. In this study, 63 proteins were able to be identified via nano-flow liquid chromatography tandem-mass spectrometry. Out of the identified peptides, 1,507 out of 3,409 were classified as unique.[18] The potential for peptides in hair to be unique indicated the likelihood for amino acid ratios, such as those within this study, to also be unique, as they are used to compose the peptides.[18]

In a study by Jackson et al., individuals were placed into categories based on both dietary and non-dietary factors. The isotope ratios of fourteen of the hair's amino acids were assessed via liquid chromatography- isotope ratio mass spectrometry (LC–IRMS). Out of the fourteen amino acids included in the study, they were grouped as nonessential and essential, as the two groups are not influenced by one another. They found that both the dietary and nondietary factors can independently affect hair's amino acid isotope ratios. The nondietary factors that they included as areas of interest in affecting the carbon isotope value were sex, body mass index (BMI), genetic disorders, and diseases. Therefore, Jackson et al. succeeded in showing that factors outside of simply an individual's geographic origin can impact their amino acid isotope ratios. This indicated that the factors included in this study, such as cosmetic treatments, may also have the potential to impact an individual's amino acid ratios.[38]

The study by Rashaid et al. also looked at additional factors beyond geographic origin, including age and sex. GC-MS was utilized to observe the quantities of amino

acids present after digestion and derivatization of the hair samples. Based on these results, classification rates were then calculated for age, region of origin, and sex, which were respectively 83%, 61%, and 94%. The findings also suggested a connection between the composition and other factors, including hair color, race, and diet, but did not test for these specifically. This further indicated the potential for the cosmetic treatments analyzed in the current study to impact an individual's amino acid ratios. Overall, Rashaid et al. displayed the capability of amino acid composition analysis with GC-MS to be successful in distinguishing between individuals based on a variety of factors, including those selected in the current study.[28]

The connection between aging and amino acid concentrations was investigated by Rieck. This study utilized HPLC to measure the amino acid concentrations of 17 amino acids in samples from three female volunteers across 270 months. Each of the 17 amino acids were able to be detected throughout the analyses. The results revealed that the amino acid concentrations had a divergent age-association, with varying results for each of the three volunteers. Based on the findings, Rieck suggested that factors such as hormonal and dietary variations were potentially related to these variations. This study showed other areas for variation in individual's amino acid composition, as well as the possibility for differentiation of individuals from one another.[39]

Novel methods of forensic hair analysis can even look past the protein products of the DNA, and focus on the functional groups of these proteins, which is possible with infrared spectroscopy. In the study by Mujeeb and Zafar, Fourier transform infrared (FTIR) spectroscopy was utilized to examine samples of human hair from female individuals from a range of 0.3 to 75 years old. The hair was cut into small fragments and

combined with potassium bromide (KBr) to produce a KBr pellet for analysis. For data analysis, the researchers divided the resulting spectra into five regions; Region I (4000 to 3200 cm^{-1}), Region II (3200 to 1400 cm^{-1}), Region III (1400 to 900 cm^{-1}), Region IV (900 to 800 cm^{-1}), Region V (800 to 600 cm^{-1}). The regions were selected as such as a way to categorize the information that could be obtained from them. Specifically, Region I was chosen to show information related to carboxylic acids and hydrogen bonds, along with the water within the hair. Region II was devised to display bands associated with functional groups, including asymmetric stretching in proteins and hydrogen stretching. Region III was of interest due to the information it provided related to the biological minerals within the samples. The researchers specifically noted indications of glucose, carbon ions, phosphate ions, and certain functional groups associated with keratin. Region IV displayed out-of-plane bending vibrations of NO^{2-}, NO^{3-} and CO^{2-}_3, and indications of S-O bond esters. The absorption of CO_2, NH_2, and SO^{4-}_2 ions were visible in Region V, as well as N-H wagging and cis-double bonds (=C-H). In each of the spectra, there was a prominent band at approximately 1636 cm^{-1}, which was representative of the Amide I groups in the hair proteins, and the β pleated conformation that they hold. Two other bands were consistently observed at about 1532 cm^{-1} and 1529 cm^{-1}, which they felt corresponded respectively to the C=C stretching and N-H stretches in the proteins. The Amide III band constituents were visible at about 1241 cm^{-1}, resulting from N-H bending and C-N stretching. Bands related to carbohydrates, glucose in particular, included stretching vibrations of C-O, C-O-H, and C-C ring vibrations which were visible at 1176 cm^{-1} , 1174 cm^{-1} , 1044 cm^{-1}, 1034 cm^{-1} , 921 cm^{-1}, and 918 cm^{-1}.

Finally, symmetric P-O and symmetric P=O stretches, associated with the ion calcium phosphate, were also represented by the bands around 1238- 1241 cm^{-1}.[32]

Overall, the study by Mujeeb and Zafar provided helpful insight into the bands to consistently expect in human hair, as well as their associations to certain ions or protein groups. The researchers also commented that IR spectroscopy may be helpful in analyzing the amino acids and ions of hair based on their observations, and mentioned the reproducibility of the technique. They did cite several cases of successful analysis of oxidatively damaged hairs, such as by bleaching, but warned that IR may not always be suitable for this form of analysis as it only penetrates the outer layers of the hair.[32]

New analysis techniques can even venture one step further by examining the elements that compose the functional groups of the amino acids within the proteins created by the unique DNA. In the study by Santos and Periera, laser induced breakdown spectroscopy (LIBS) was utilized to examine a total of 127 untreated and bleached hair samples from 63 individuals. A hydraulic press was used to create pellets from each sample, without the addition of any chemicals. For the LIBS analysis, a laser pulse energy of 80 mJ was used, along with a 125 μm spot size, and a 0.5 μs gate delay. Using the pellets, 924 total spectra were obtained per sample by mapping an area within 11 designated lines spaced 4 mm apart. The TruLIBSTM database software from Applied Spectra, as well as the NIST database were utilized in the data analysis. Elements included in the analysis were C, H, Na, Mg, K, and O. Levels of potassium and sodium were noted to show correlation with the bleached samples. The researchers suggested that this was due to residues of products included in bleaching formulations, including potassium and ammonium persulfate ($K_2S_2O_8$ and $(NH_4)_2S_2O_8$), sodium silicate

(Na_2SiO_3), ethylenediaminetetraacetic acid disodium salt (EDTA, $C_{10}H_{14}N_2Na_2O_8$), and sodium lauryl sulfate ($CH_3(CH_2)_{10}CH_2(OCH_2CH_2)_nOSO_3Na$). Overall, Santos and Periera displayed the possibility of attributing a hair sample, whether untreated or bleached to the same individual, which is indicative of the potential success for the current project evaluating both bleaching and dyeing treatments.[34]

Chemical Treatments

Bleaching

Cosmetic treatments, such as bleaching, have the potential to affect an individual's amino acid ratios. An investigation by Kuzuhara analyzed black and white hairs before and after bleaching. This was done via Raman microscopy, which was able to examine various hair's cross-sections at different depths. Hydrolysis and amino acid analysis were then performed with a Hitachi Amino Acid Analyzer. The resulting amino acid compositions were compared between untreated and treated groups, as well as between white and black hair colors. The bleaching cream utilized was a 5.9 wt % hydrogen peroxide concentration, which equates to a 20-volume developer. As mentioned, hydrogen peroxide is generally the main oxidizing agent. Other ingredients, such as persulfate salts are commonly included in formulations as "accelerators".[40] For the virgin white hair after the bleaching treatment, the Raman examination showed the Sulfur–Sulfur (S-S) and Carbon–Sulfur (C-S) band intensities declined in the cuticle, while the Sulfur–Oxygen (S-O) band of cysteic acid increased.[40] The increase in the band intensity for S-O was indicative of disulfide cleavage in the cuticle to produce cysteic acid.[40] Interestingly, the same findings were intensified for virgin black hair after

treatment. This indicates that the black hair was damaged to a greater extent by the bleaching than the white hair.[40] One potential reason for the provided by Kuzuhara was that a cation-exchange resin may have been produced by the virgin black hair's melanin granules. This resin could have promoted decomposition of the oxidizing agent, thereby producing increased disulfide cleavage in black hair samples compared to the levels seen in white hair samples.[40]

Kuzuhara also did amino acid analysis in which estimations were made regarding the 1/2‑cystine and cysteic acid levels. Cystine describes a molecule created through the linking of two individual cysteine molecules via a disulfide bond, and a 1/2 cystine refers only to one half of it. For both the black and white virgin hair samples, the 1/2‑cystine levels were observed to decline. Meanwhile, the levels of cysteic acid were amplified in both samples after the bleaching treatment, with this observation magnified in the black hair samples.[40] There were other changes noted to occur only in the dark hair; lysine, methionine, and histidine decreased in the virgin black hair after treatment with bleach, but were left unaffected in the virgin white hair.[40] This observation indicates that black and white hair are not impacted evenly by bleaching treatments, and there is decomposition in the lysine, methionine, and histidine of virgin black hair during treatment.[40]

Dyeing

Dyeing is another form of cosmetic treatment with the potential to affect an individual's amino acid ratios. In the study by Gama et al., the impact of permanent oxidative hair dye on Caucasian hair samples was assessed in cases where conditioners

were and were not present as a follow-up treatment. This study was of particular interest due to type of dye chosen, which is also the class of dye selected for this current study. To analyze the effects of the dyes in both sample groups, Gama et al. made use of three analysis methods; differential scanning calorimetry (DSC), thermogravimetric analysis (TG), and derivative thermogravimetric (DTG) analysis. For the permanent oxidative hair dye used in this study, as well as the one applied in the current research project, the two main constituents are the oxidant and the alkaline agent, which need to be combined directly before application.[41] As described in the literature review, the resulting reaction produces the desired coloration within the cortex of the hair, as well as on the surface. When using DSC to analyze dyed hair samples, either with or without conditioners, three endothermic events and two endothermic events were observed.[41] The evaporation of water within the hair was associated with the first event.[41] Endothermic reactions related to the denaturation of keratin polypeptide due to disulfide cleavages in cysteines was observed in the third and fourth events.[41] Exothermic reactions related to keratin proteins were noted in both the second and fifth events. In the second event, keratin's a-form crystallites were modified to become the amorphous b-form. The fifth event was complex, but was believed to occur as a result of oxidative degradation.[41] Interestingly, the use of a conditioner after the hair dyeing treatment did not appear to lessen the damages seen by the oxidative coloring process. However, the use of a conditioner concurrently with the hair dye was actually shown to result in less overall damage. When looking at the DSC curves for these specific samples, the presence of the conditioner appeared to not only dislocate the start of the third event, but also prevent the occurrence of the fourth event.[41] Gama et al.'s findings generally indicate the level of harm to hair

keratins during treatment with permanent oxidative dyes, as well as displaying the potential for further oxidative damage to the other organic materials within the hair.[41] This suggests the chance for the amino acid ratios within an individual's hair to be altered after a treatment with permanent oxidative hair dye.[41]

Materials and Methods

Materials

The bleach, hair dye, and infrared heat lamp were each purchased through Amazon. The bleach was Professional BW 2 Dedusted Extra Strength Powder Lightener and Pure White 30 Creme Developer Maximum Lift from Clairol. L'Oreal Paris Superior Preference Fade-Defying and Shine Permanent Hair Color, in the shade 2BL Black Sapphire was utilized for the hair dye. A Coospider HC-100 Ceramic Infrared Clamp Lamp was used for heating during the dyeing procedure. HPLC Grade Methanol was purchased from MilliporeSigma (Burlington, MA). The following eleven amino acids were obtained from Fisher Scientific (Hampton, NH): L-valine, L-proline, L-leucine, L-serine, L-threonine, L-phenylalanine, L-cysteine, L-tyrosine, L-aspartic acid, L-glutamic acid, and L-norvaline. Hydrochloric acid (6 M) was obtained from Fisher Scientific (Hampton, NH). Ethyl acetate was also obtained from Fisher Scientific (Hampton, NH). The N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) derivatizing agent was purchased from Sigma-Aldrich (St. Louis, MO).

A Leica Stereomicroscope and a Leica DM EP microscope were both used in the microscopic analysis of the hair samples. A Nicolet™ iS10 ATR FTIR Spectrometer, J200 Laser-Induced Breakdown Spectroscopy (LIBS), and an Agilent Technologies

6890N/GC-MSD equipped with a 30.0 m x 250 μm x 0.25 μm DB-5 column were used for the instrumental analysis.

Hair Samples

Following IRB approval (IRB #2020-083), hair samples were obtained from both of the two individuals via brushing or cutting. The two individuals were both Caucasian, female, between twenty to thirty-five years of age, displayed similar natural hair colors with no recent cosmetic treatment, and were currently living on the northeastern coastline of the United States when the hair was collected. The hair samples from both individuals were separated into three groups (untreated, bleached, and dyed), and each group was placed into its own scintillation vial. The untreated groups for both individuals underwent a two-step washing procedure. First, the samples were washed with ~3-4 mL of deionized water and shaken vigorously. The samples were then removed, placed into a cleaned vial, and washed with ~3-4 mL of HPLC grade methanol and shaken again before being allowed to dry.

Cosmetic Processing

For the bleach mixture, 25 mL of Clairol Pure White 30 Creme Developer Maximum Lift was mixed with ~14 g of Clairol Professional BW 2 Dedusted Extra Strength Powder Lightener. This mixture was thoroughly applied to both hair groups assigned for bleaching, and left to sit on tin foil for 30 minutes. Both sample groups were thoroughly washed with water to remove the bleach mixture. The samples then underwent the two-step washing procedure previously described.

For the dye mixture, L'Oreal Paris Superior Preference Fade-Defying and Shine Permanent Hair Color, in the shade 2BL Black Sapphire was utilized. The Color Gel was mixed with the Color Optimizing Creme and the resulting dye was applied thoroughly to coat the designated hair samples. Both hair samples were wrapped in tin foil and placed approximately 10 cm away from the light source of a Coospider HC-100 Ceramic Infrared Clamp Lamp. The ambient temperature was 34.2 ± 0.8°C in this area, which was done to imitate the temperature radiated by a typical human head during the dyeing process, along with the infrared heat lamps used in many salons to accelerate the dyeing process. The samples were turned after 10 minutes to ensure even heating. After an additional 15 minutes, the samples were removed and rinsed with warm water until the water ran clear. The L'Oreal Paris Color and Shine Conditioner was used to coat the hair and was allowed to sit for 3 minutes before rinsing. These samples then underwent the same two-step washing procedure described previously.

Sample Analysis

Microscopy

Each sample group was analyzed at 40X with top lighting only using a Leica Stereomicroscope, and at 100X, 200X, and 400X with a Leica DM EP microscope. Observations were made related to pigment granule size, pigment density, pigment distribution, cuticle thickness, medulla continuity, overall hair color, shaft form, cuticle scale pattern, shaft width, and medullary index.

After calibration of the ocular reticle with a 1 mm/0.01 mm stage micrometer at both 100X and 200X on the Leica DM EP microscope, nine total measurements were

made for the shaft width. In general, the measurements were made presumptively and photographed at 100X, and then reviewed at 200X and photographed again. An average shaft width was calculated for each group, along with the standard deviation. An F-test Two-Sample for Variances was applied to determine whether variances were considered equal or unequal. Based on this, either a Two-Sample Assuming Equal Variances t-test or a Two-Sample Assuming Unequal Variances t-test was performed to produce p-values. These resulting p-values would indicate if the change in shaft width was statistically significant after the bleaching and dyeing procedures, and if there was a significant difference in the shaft widths of the untreated hair of individuals 1 and 2.

ATR-FTIR

For each of the samples, three separate areas of a clump of hair were analyzed with the Nicolet™ iS10 ATR FTIR Spectrometer and the Omnic Software. Each of the sample spectra collected were produced using 32 scans at a resolution of 4.000. A library search was done for each of the spectra using the Omnic Specta Software in order to identify possible chemical components.

LIBS

Samples were analyzed using the J200 LIBS from Applied Spectra and the Axiom software. Parameters including the gate delay, spot size, % laser energy, and number of shots were varied and optimized to produce acceptable spectra. Library searches were performed for each spectrum using the Clarity software to identify elements present. Each sample was analyzed in 3 different runs using two sets of parameters; Parameters 7

and 14, which are outlined in Table 1 below. The resulting intensities for the chosen elements were averaged, and the average element intensities were compared between the two individuals and after both forms of chemical treatment. To distinguish between cases of equal and unequal variance, an F-test Two-Sample for Variances. Based on the results of the F-test, either a Two-Sample Assuming Equal Variances t-test or a Two-Sample Assuming Unequal Variances t-test was performed. This was done to produce p-values, which would indicate which element intensities were significantly different between individuals 1 and 2, and after the two chemical treatments. The standard deviations and standard errors of the means were also calculated for each element intensity average produced for the untreated, bleached, and dyed sample groups for both individuals. These were necessary to evaluate the repeatability and reproducibility attained.

Table 1: Parameters 7 and 14 used for LIBS analysis.

	Parameters 7	**Parameters 14**
Gate Delay (μs)	0.5	2.5
% Laser Energy	60	60
# of Shots	15	15
Spot Size (μm)	40	40
Rep Rate (Hz)	10	10
Warmup Energy	100	100
Warmup Shots	50	50
Warmup Rep Rate (Hz)	10	10

GC-MS

The single and mixed amino acid standard solutions were prepared using the following eleven amino acids: L-valine, L-proline, L-leucine, L-serine, L-threonine, L-phenylalanine, L-cysteine, L-tyrosine, L-aspartic acid, L-glutamic acid, and L-norvaline. Norvaline was used as the internal standard because it is an amino acid not produced by humans. To prepare the single amino acid standard solutions, 100 μL of ethyl acetate was mixed with ~2 mg of amino acid and vortexed for thirty seconds. This was followed by 100 μL of N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA), the derivatizing agent, and an additional thirty seconds of vortexing. To derivatize the amino acids, each of the eleven vials was then placed in a 100°C sand bath for thirty minutes. To prepare the mixed amino acid standard solution, 1.1 mL of ethyl acetate was added to a new autosampler vial. Then, 1 μL of each of the derivatized single amino acid standard solutions was added to the vial. The mixed amino acid standard was then vortexed for thirty seconds.

To prepare the samples, the two-step washing procedure previously described was repeated, and the samples were allowed to dry completely before placing them in a -20°C freezer for a minimum of eighteen hours. Scissors were used to cut the frozen hair samples into pieces of approximately 1 cm in length, which was necessary to increase the surface area available for digestion. About 3 mg of each cut-up hair sample was added to their own new test tubes with screw caps. The samples were hydrolyzed with 1200 μL of 6 M hydrochloric acid and a 110°C sand bath for twenty-two to twenty-four hours. Each of the digested hair sample solutions was then transferred to a 1.5 mL Eppendorf tube. The tubes were all centrifuged at 3200 RPM for multiple five- to ten-minute intervals.

This was repeated until a hair pellet formed at the bottom of the tube. Once adequately centrifuged, 100 µL of the supernatant was added to a new autosampler vial, and nitrogen gas was used to evaporate the samples to dryness. As a derivatization control and an internal standard, approximately 0.3 mg of L-norvaline was added to the autosampler vials. To re-dissolve the samples, 100 µL of ethyl acetate was added to the autosampler vials and they were vortexed for thirty seconds. This was followed by the addition of 100 µL of N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) and another thirty seconds of vortexing. The vials were then placed into a 100°C sand bath for thirty minutes. Directly before the gas chromatography-mass spectrometry (GC-MS) analysis, 75 µL of the derivatized hair sample was transferred to a new autosampler vial containing 1.5 mL of ethyl acetate and was vortexed for thirty seconds.

An Agilent Technologies 6890N/GC-MSD equipped with a 30.0 m x 250 µm x 0.25 µm DB-5 column was used for analysis with the parameters listed in Table 2. The particular derivatized amino acid retention times were determined through the analysis of the derivatized pure standards. A total of two replicates for each of the three sample groups for both individuals 1 and 2 were analyzed. An ethyl acetate solution was run on the GC-MS as a negative control at the start of each automated run.

Table 2: Parameters used for GC-MS analysis.

Parameter	Value
Initial Temperature	80℃
Hold Time	1 min
Ramp Rate	25℃/min
Final Temperature	280℃
MSD Solvent Delay	3 min
Total Run Time	10 min

Each hair sample, as well as any standards analyzed, produced its own total ion chromatogram (TIC), which can be seen in Appendix I. Any amino acids present in the hair samples were identified using the NIST/NIH/EPA Mass Spectral Library on the GC-MS.[46] The corresponding tables showing the areas under the curve provided for each amino acid derivative peak are also given in Appendix I. The resulting areas for each amino acid derivative were divided by the area of the detected and identified derivatized internal standard peak, L-norvaline 2TMS derivative. To produce the total of fifteen amino acid ratios, the areas of amino acid's derivative peaks relative to the internal standard present in their respective sample were compared to the areas of the other amino acids derivative peaks relative to the internal standard present in their respective samples. A list of the fifteen amino acid ratios constructed is provided in Table 9.

To distinguish between cases of equal and unequal variance, an F-test Two-Sample for Variances was performed for each comparison made between individuals and treatment types. Based on the results of the F-test, either a Two-Sample Assuming Equal Variances t-test or a Two-Sample Assuming Unequal Variances t-test was performed. This was done to produce p-values, which would indicate which amino acid ratios were significantly different between individuals 1 and 2, and after the two chemical treatments. The standard deviations and standard errors of the means were also calculated for each amino acid ratio produced for the untreated, bleached, and dyed sample groups for both individuals. These were necessary to evaluate the repeatability and reproducibility attained.

Results and Discussion

Microscopy

Untreated, bleached, and dyed hair samples from both individuals 1 and 2 were analyzed at 40X, 100X, 200X, and 400X. Hair samples from individuals 1 and 2 were compared at these magnifications to assess their morphology. When observing individuals 1 and 2 prior to collection, there is a macroscopic difference in that individual 1 possesses a slightly curlier hair type than individual 2, who had straight hair. However, when observing hair samples collected from both individuals, it was difficult to tell that two individuals possessed somewhat different overall hair patterns, especially when the other observed morphological characteristics were similar across both samples. As shown in Figure 5, the images at 40X showed a similar range in hair coloring, and the images at 100X showed similar overall width, color, and medulla pattern.

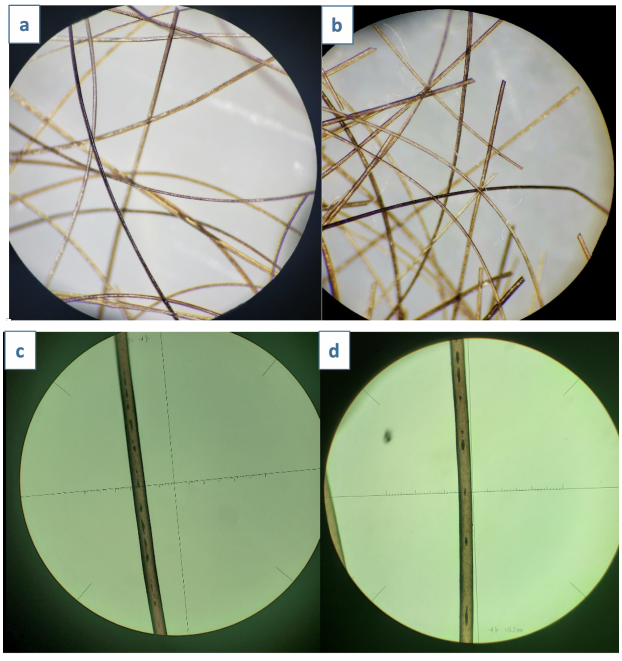


Figure 5: Microscopic images of untreated hair samples from individual 1 and 2 at (a,b) 40X magnification and (c,d) 100X magnification. Hair from individual 1 appears in (a) and (c), while hair from individual 2 appears in (b) and (d).

In general, the untreated hairs of individual 1 and 2 were found to be morphologically similar in terms of color, cuticle thickness, pigment distribution, and pigment granule size. Additional comparison between the morphological characteristics of the untreated samples for both individuals, as well the changes in these characteristics after treatment with bleach or dye, is shown in Table 3. Due to the similarities, as well the potential for subjectivity, this MHC was considered unable to fully distinguish between the untreated hairs from individuals 1 and 2, especially in cases where sample size was limited by only mounting several hairs. If these hairs were analyzed in a traditional forensic setting, they would be sent for mitochondrial or nuclear DNA analyses. This is why IR bands, element intensities, and amino acid ratios will be used as a point of analysis in hair in this thesis. These methods will be investigated as a potentially useful method for differentiating between individuals of a similar background or even for differentiating between individuals of similar demographics.

Table 3: Morphological characteristics of the untreated, bleached, and dyed hair samples from individuals 1 and 2, including the pigment granule size, density, and distribution; cuticle thickness; medulla patterns; overall coloring; and shaft forms.

Feature	Ind 1 Untreated	Ind 1 Bleached	Ind 1 Dyed	Ind 2 Untreated	Ind 2 Bleached	Ind 2 Dyed
Pigment Granule Size	Medium	Some pieces have no pigment, others have fine pigment granules remaining	Artificial and uniform appearance, but medium when still observed	Medium	Some pieces have no pigment, others have fine/medium pigment granules remaining	Artificial and uniform appearance, but medium when still observed
Pigment Density	Medium			Medium		
Pigment Distribution	Peripheral	Peripheral when visible	Peripheral	Peripheral	Peripheral when visible	Peripheral
Cuticle Thickness	Thin - Lightly serrated	Thin - Heavily serrated with a broken, rougher surface and some areas hanging off	Thin - Dye in Cuticle, some serrations and breakages	Thin - Lightly serrated	Thin - Heavily serrated with a broken, rougher surface and some areas hanging off	Thin - Dye in Cuticle, some serrations and breakages
Medulla	Fragmented, trace, and absent patterns observed	Less prominent, trace when present - less common in lightest pieces with no pigment granules, more common in pigmented pieces	Fragmented, trace, and absent patterns observed. Hard to see in heavily pigmented pieces	Discontinuous, fragmented, trace, and absent patterns observed	Less prominent, trace when present - less common in lightest pieces with no pigment granules, more common in pigmented pieces	Discontinuous, fragmented, trace, and absent patterns observed. Hard to see in heavily pigmented pieces
Overall Color	Medium brown, but several strands of light brown and few strands of dark brown also noted	Range: few medium brown, some light brown, blonde, and white	Black, artificial in appearance, some pieces display dye lines or color changes	Medium brown, but several strands of light brown and few strands of dark brown also noted	Range: few medium Brown, some light brown, blonde, and white	Black, almost blue on some pieces, very artificial, some pieces display dye lines or color changes
Shaft Form	Arched, twisted, wavy, and straight	Mostly arched, some twisted forms present	Twisted, arched, wavy forms present	Twisted, wavy, and straight forms present	Mostly straight, some twisted forms present	Twisted and straight forms present

Finally, the shaft widths were obtained for each sample group at 100X and 200X magnification (Table 4). After averaging the value for each group, it was found that individuals 1 and 2 did not display significantly different shaft widths based on the measurements obtained. This serves as another point of morphological similarities between the two groups, and highlights the potential benefits to be yielded in investigating novel techniques for differentiation of the hair samples. In general, the shaft width was observed to decrease after both treatments, but was most noticeable after bleaching. Based on the t-testing, the difference in shaft width after bleaching was indicated to be statistically significant for both individuals, but was not considered to be statistically significant after dyeing for either individual. This highlighted the difference

in the severity between bleaching and permanent dyeing treatments. In general, the loss of volume after both treatments, with the bleaching in particular, was likely indicative of the loss of proteins from within the hair shaft, which has also been seen in previous works. A larger relative decrease in shaft width was observed for individual 1 after bleaching, pointing to a potentially increased susceptibility to this treatment [42]

Table 4: Average shaft widths and corresponding standard deviations for the untreated, bleached, and dyed hair samples from individuals 1 and 2 obtained through measurements at 100X and 200X magnification are displayed in (a); P-values from two-sample t testing are shown in (b), where significantly different element intensities (p-value ≤ 0.05) are depicted in blue.

(a)

	Average Shaft Width (um)	Standard Deviation
Ind 1 Untreated	74.2	22.3
Ind 1 Bleached	45.4	15.2
Ind 1 Dyed	68.8	17.5
Ind 2 Untreated	85.2	18.5
Ind 2 Bleached	61.6	6.0
Ind 2 Dyed	79.4	15.9

(b)

Two Sample t Test P Values				
Ind 1 Untreated vs. Ind 1 Bleached	**Ind 1 Untreated vs. Ind 1 Dyed**	**Ind 2 Untreated vs. Ind 2 Bleached**	**Ind 2 Untreated vs. Ind 2 Dyed**	**Ind 1 Untreated vs. Ind 2 Untreated**
0.006	0.576	**0.005**	0.492	0.271

ATR-FTIR Analysis

There were several differences in the observed bands for the ATR-FTIR spectra of individuals 1 and 2, as well as several shifts after the bleaching and dyeing treatments associated with protein deformations or cysteine oxidations. Example spectra from the untreated, bleached, and dyed samples from both individuals are included in Appendix I. A summary of the bands observed in the ATR-FTIR spectra of the untreated, bleached, and dyed hair samples from both individuals is given in Table 5. This table also provides

the characteristic functional groups associated with a band at that wavenumber, based on reports and spectra tables from Millipore Sigma, Mujeeb and Zafar, and Barton.[43, 32, 44] For convenience, bands appearing around certain wavenumbers have been assigned numbers 1-11, and these assignments are also outlined in Table 5.

Table 5: Observed bands in the ATR-FTIR spectra for the untreated, bleached, and dyed hair samples from individuals 1 and 2. All general wavenumbers and the corresponding functional groups associated are also provided and were obtained from Millipore Sigma and the study by Mujeeb and Zafar.[43,,32]

#	General Wave number[43, 32] (cm^{-1})	Observed Wavenumbers (cm^{-1})						Characteristic Functional Groups Associated[43, 32]
		Ind 1 Untreated	Ind 1 Bleached	Ind 1 Dyed	Ind 2 Untreated	Ind 2 Bleached	Ind 2 Dyed	
1	3283	3276.11	3281.00	3275.58	3278.65	3274.83	3280.44	O-H carboxylic acids (and derivatives), alcohols, & phenols
2	2925	2927.41	2920.20	2924.98	2919.29	2928.06	2918.39	C-H (symmetric stretching of CH_2)
3	2850	--	~2850	--	2849.81	--	2849.81	C-H symmetric stretching of CH_2 in fatty acids & symmetric stretching of lipid acyl CH_2 groups
4	1639	1641.32	1641.68	1634.41	1643.85	1643.53	1640.37	H-OH bending of water, NO_2 of nitro groups, amide I band components (β pleated protein structures), C=C, C=N, NH_3
5	--	--	--	--	1582.64	--	--	--
6	1529	1515.00	1529.77	1527.74	1525.42	1522.69	1536.70	Amide II bands: protein C-N, N-H, NO_2 of nitro groups, carboxylic acids & derivatives

7	1454, 1455	1450.41	1450.89	1447.25	1439.91	1448.96	1451.59	CH_2, CH_3 asymmetric bending modes of proteins & lipids
8	1400 (w)	1408.85	1401.96	1403.66	~1410	~1410	~1410	–OH bending,–CH3 out-of-plane bending, –C–O–H in-plane bending, –CH2– twisting and wagging
9	1241	1229.66	1233.66	1234.10	1232.76	1229.46	1228.75	Amide III band: proteins (C-N), C-N stretching of amines within free amino acids
10	1071	~1075	1080.76	1076.79	~1075	~1080	~1080	Stretch of cysteine monoxide
11	1020, 1034 - 1040	1039.23	1024.54	1022.28	1022.11	1040.82	1040.35	C-O amide-I band, C-N, C-C, glucose, sulfonates, cysteic acid

Bands #1, 4, 8, and 10 (~ 3283, 1639, 1400, and 1075 cm^{-1}, respectively) were observed at similar wavenumbers for individuals 1 and 2 (Table 5). Bands #1 and 4 also remained fairly consistent after bleaching and dyeing treatments, and did not display large shifts or changes in % transmittance for either individual. For individual 1 after bleaching and dyeing, band #8 shifted to a lower wavenumber. For individual 2 after bleaching and dyeing, band #8 remained at a relatively consistent wavenumber. Bands at this wavenumber are associated with –OH bending, $–CH_3$ out-of-plane bending, –C–O–H in-plane bending, $–CO_2$ stretching, and $–CH_2–$ twisting/wagging. Barton suggested that basic treatments, including bleaching and dyeing, would likely result in the carboxylic acid groups being in their ionized forms. This would result in bands closer to 1400 cm^{-1}, as was observed with the bleached and dyed hair of individual 1. It is possible that differences in the structural integrity of the untreated hair belonging to individuals 1 and

2 resulted in the difference in susceptibility to display certain IR shifts after chemical treatment.

For bands #2, 7, and 11 (~ 2925, 1455, and 1034 cm^{-1}, respectively) untreated hair from individual 1 possessed a band at a higher wavenumber than individual 2. For bands #6 and 9 (~ 1529 and 1241 cm^{-1}, respectively) untreated hair from individual 2 displayed a band at a higher wavenumber than individual 1. These were representative of potential differences in the untreated hair of individuals 1 and 2, specifically potential differences in amino acid side chains that are more prominent on the outer portion of the hair shaft.

Band #3 (~2850 cm^{-1}) was interesting because it was present in only individual 1's bleached hair but was present in the untreated and dyed hair for individual 2 (Table 5). A band at this wavenumber is generally associated with the C-H symmetric stretching of CH_2 in fatty acids, as well as the symmetric stretching of lipid acyl CH_2 groups.[32] This was another case where differences in the content of the untreated hair belonging to both individuals may have contributed to their respective responses after specific chemical treatments.

Band #5 (~ 2850 cm^{-1}) was distinctive in that it was only observed in the untreated hair of individual 2, but not the untreated hair of individual 1 (Table 5). A band associated with this wavenumber in hair was not noted in the work by Barton, but was shown to have similar trends in the study on untreated hair by Mujeeb and Zafar.[44,32] In their research, some individuals displayed a band at this wavenumber while other individuals did not. The presence of this band aided in distinguishing the ATR-FTIR spectra from individual 1 from those from individual 2. This peak may disappear in the

bleached spectra of individual 2 due to the deformation of certain organic components of the hair.[44,32]

Band #9, which appears around 1241 cm^{-1} , showed opposite trends for individuals 1 and 2 (Table 5). For individual 1, the band appeared at 1229.66 cm^{-1} in the untreated hair, then shifted to higher wavenumbers (1233.66 cm^{-1} and 1234.10 cm^{-1}) in the bleached and dyed hair, respectively. For individual 2 the band appeared at 1232.76 cm^{-1} in the untreated hair, then shifted to lower wavenumbers (1229.76 cm^{-1} and 1228.75 cm^{-1}) in the bleached and dyed hair, respectively. This was an interesting observation because bands around this wavenumber are associated with amide III band proteins, such as C-N, and the C-N stretching of amines within free amino acids. This is possibly indicative of a protein-related difference between the untreated hair samples of individuals 1 and 2, and thus a subsequent difference in the resulting response post-exposure to chemical treatment. In the work by Barton, it was suggested that changes at 1241 cm^{-1} were associated with deformations to the proteins caused by bleaching treatments.[44] Additionally, a band at this wavenumber was seen in previous work by Silverstein et al and Barton, and was suggested to be the result of the oxidation of cysteine yielding cysteine dioxide.[45,44] This suggests why individual 2's untreated hair shows a shift from a higher wavenumber to a lower one, closer to the one observed in previous works, after the bleaching and dyeing treatments. It is still unclear why individual 1's hair already possesses a band at a lower wavenumber and shows a shift to higher wavenumbers.

Band #11, which appears around 1040 cm^{-1}, was also seen in previous work by Silverstein et al and Barton.[45,44] This band was suggested to be due to sulfonates and

cysteic acid formed through the oxidation of cysteine.[40] This is likely why the band appears at 1040 cm^{-1} for individual 2 after the oxidative bleaching and dyeing treatments. Individual 1 already possessed the band at 1040 cm^{-1} prior to the chemical treatments, and then displayed a shift to lower wavenumbers after bleaching and dyeing (Table 5). In the study by Santos et al. it was explained that cysteic acid is present in α-keratin as one of its constituent amino acids. However, it's intensity can be increased by exposing the hair to sunlight, which can cause an oxidation effect photochemically, or through the use of decolorants, such as bleach. This is because the bleaching agent converts residues of cysteine to cysteic acid, which occurs through the oxidation reaction within the hair shaft, starting first at the disulfide bond.[47] Additionally, one of the eight individuals in the study by Mujeeb and Zafar did possess a band at a similar wavenumber despite having untreated hair, so it's possible that the band at 1040 cm^{-1} associated with cysteic acid is simply not present intensely enough to be observed in all hair types when untreated. This is especially true considering individual 1 possessed curly hair, and cysteine is typically present in greater amounts in curly hair types.[6] These bands shifted to 1022- 1024 cm^{-1}, another area associated with SO_2 groups, like the one in cysteic acid, after bleaching and dyeing before increasing in intensity as expected for individual 1.[48] Individual 2's untreated hair displayed a stronger band at 1022 cm^{-1}, and then showed a shift to 1040 cm^{-1} after the bleaching and dyeing treatments. As mentioned, although both ~1020 cm^{-1} and ~1020 cm^{-1} have been previously associated with S-O related groups, such as those in cysteic acid, it's unclear why individuals 1 and 2 display opposite trends for the observed shifts. In the study by Kuzuhara et al. it was found that black and white hair types were not impacted evenly by the bleaching treatment, especially in the region regarding the

S-O of cysteic acid, so it's possible that a similar sentiment is true for curly versus straight hair.[45,44, 47-48]

LIBS Analysis

A total of 16 sets of parameters were explored on LIBS by adjusting the shot size, gate delay, % laser energy, and number of shots. These are referred to as Parameters 1-16, and an overview of these parameter sets is provided in Table 6. In summary, Parameters 1-4 looked at increasing the % laser energy; Parameters 2, 5, and 6 investigated the optimal number of shots; Parameters 6 and 7 explored the shot size; Parameters 7-15 looked at various gate delays; and Parameters 14 and 16 looked at increasing the % laser energy at a new gate delay.

In Parameters 1-4 looking at laser energy, it was thought that the 50% laser energy was too low to obtain adequate intensity peaks from the hair samples. Meanwhile, the 65% and 70% seemed to be too aggressive, as they often went all the way through the hair and into the slide underneath. Thus, to avoid too much interference from the slide, 60% laser was thought to be the optimal level while still providing adequate intensity peaks from the hair samples. In Parameters 2, 5, and 6 investigating the optimal number of shots, it was found that the 10 shots produced fair results, but could be improved upon. Using 20 shots often resulted in the hair moving around and the laser firing at the slide rather than the intended target. As a result, 15 shots was chosen as the optimal number of shots for this analysis. Parameters 6 and 7 explored the shot size. The starting size was 50 μm, but was thought to be too large as the laser often hit the hair partially, while also hitting some of the slide. The 40 μm spot size in Parameters was preferred as it was easier to ensure that a larger proportion of the area fired on was hair rather than the slide

underneath. Parameters 7-15 were devised to look at various gate delays from 0.5 µs to 3.0 µs. With the lowest gate delay, there was observed to be a peak at approximately 266 nm that was present due to the glass slide. This peak was no longer present when reaching the 2.5 µs gate delay. However, this also resulted in the loss of other relevant peaks around 250 nm to 290 nm. The lower gate delay Parameter Sets also displayed a slight elevation of the baseline from approximately 580 nm to 680 nm. This area was gradually flattened as the gate delay increased. However, the peak intensities were also decreasing slowly as the gate delay increased. When using the 3.0 µs gate delay in Parameters 15, many of the peak intensities were beginning to decrease. Parameters 14 and 16 looked at increasing the % laser energy at the 2.5 µs gate delay to see if there was any improvement to the peak intensities. There was a slight improvement noted, and many peaks did display improved intensities. However, for the sake of comparison between the two sets of parameters selected, it was decided that Parameter Sets 7 and 14 would be utilized for subsequent sample analyses. This was because the only difference between these two sets was the gate delay, so any differences between the two sets could be related to that change. After the sample analyses, it was determined that based on the parameters modified in this study, the optimal parameters on the LIBS for hair are Parameter Set 7.

Table 6: Summary of LIBS Parameter Sets 1 through 16. The optimal set, Parameters Set 7, is given in blue.

PARAMETERS	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Gate Delay (us)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.7	0.9	1.1	1.3	1.5	1.7	2.5	3	2.5
% Laser Energy	50	60	65	70	60	60	60	60	60	60	60	60	60	60	60	70
# Shots	10	10	10	10	20	15	15	15	15	15	15	15	15	15	15	15
Shot size (um)	50	50	50	50	50	50	40	40	40	40	40	40	40	40	40	40

Three spectra were obtained for the untreated, bleached, and dyed hair from each individual with both sets of parameters. This was also done for the slide that the hair was mounted on during the LIBS analysis. Example spectra from analysis of individual 1's untreated hair with Parameters 7 and 14 are shown in Figure 6.

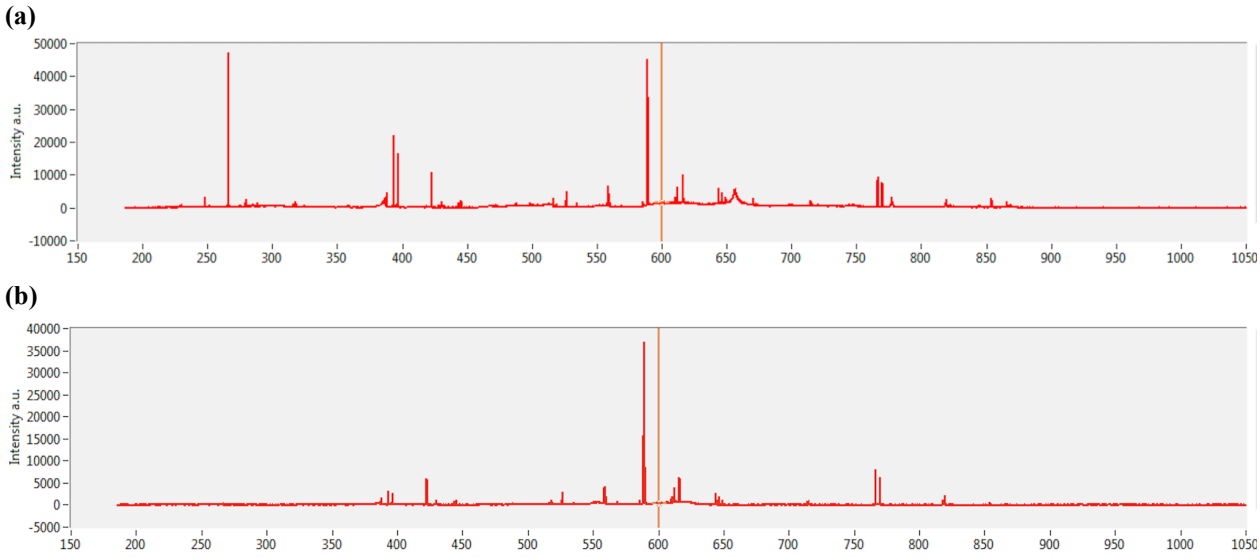


Figure 6: Example LIBS spectra from individual 1's untreated hair with (a) Parameters Set 7; and (b) Parameters Set 14. The x-axis displays the wavelength in nm.

During the method optimization analyses, it was noted that there were fluctuations in the laser energy being applied. It was then decided that the power meter would be monitored for consistency. After a successful run of quality assurance and quality control (QA/QC) protocols, the power meter was monitored at the desired % laser energy to gauge the expected output values. It appeared that the fluctuations of the laser on this particular LIBS instrument at 60% laser energy were in the range 9.85 ± 0.3 mJ. As such, if the laser energy was out of the range of 9.85 ± 0.3 mJ, the run was rejected and repeated. This was done to ensure that variations in samples were more likely to be due to the samples themselves rather than variations in the laser energy applied to the samples.

Finally, comparisons were made between the average element intensities for the selected elements to evaluate any potential differences between the two individuals and the effects of the chemical treatments. The average element intensities and standard errors of the means obtained for the untreated, bleached, and dyed hair samples from individuals 1 and 2 using Parameters Set 7 are shown as bar graphs in Figure 7, and are condensed in Table 7.

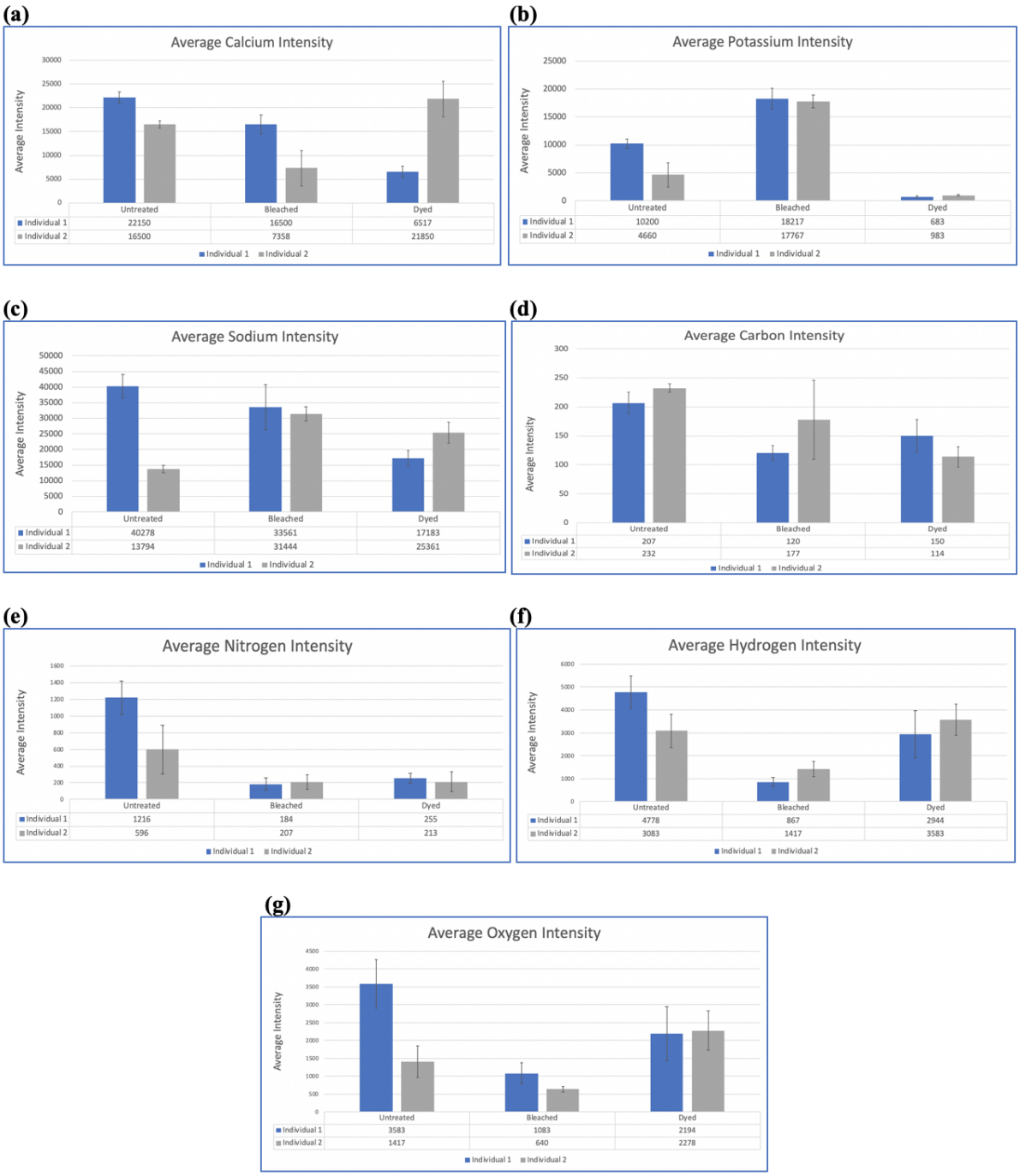


Figure 7: Bar graphs of average intensity from the three Parameter 7 runs for the untreated, bleached, and dyed sample groups from individuals 1 and 2, including (a) calcium, (b) potassium, (c) sodium, (d) carbon, (e) nitrogen, (f) hydrogen, and (g) oxygen.

Using Parameters Set 7, there was found to be three elements with significant differences in average element intensity between the untreated hair of individuals 1 and 2,

which were in calcium, potassium, and sodium. This was interesting as these are associated with amino acid side chains, potentially corroborating the fact that individuals 1 and 2 would display differences in their amino acid ratios. After bleaching, there was a statistically significant increase in potassium. This was likely the result of the several persulfate salts used as accelerators in the bleach that was applied. The statistically significant increase in sodium in individual 2's bleached samples may be the result of the sodium silicates and EDTA (Ethylenediaminetetraacetic acid) in the bleach. In general, there was observed to be a correlation between the potassium and the sodium and the bleached samples, which was in agreement with previous studies.[34] It was also noted that individual 1's hair, which was curly, appeared to be more susceptible to significant changes after chemical treatments. This aligns with research showing that curlier hair types generally have lower strength and stress break loads.[34, 49]

For the elements O, H C, and N, no significant differences were observed between individuals 1 and 2. For oxygen specifically, there was a noticeable difference in the average oxygen intensities for individuals 1 and 2, but it was not considered to be statistically significant with a p-value of 0.056. There was also observed to be a decrease in the average oxygen intensity after bleaching and dyeing for both individuals 1 and 2. However, this trend was shown to be more severe after the bleaching treatment. A statistically significant difference in the average oxygen intensity after a chemical treatment was only found for individual 1 after the bleaching procedure. This potentially suggests that individual 1's curly hair type may have been more susceptible to the damages caused by chemical treatments, which was previously indicated by individual 1's greater relative loss in shaft width after bleaching compared to individual 2. A very

similar overall trend was observed for hydrogen; there was an observed difference in the average oxygen intensities for individuals 1 and 2, but it was not considered to be statistically significant due to the variations observed for this element in both samples. Similar to oxygen, there was also observed to be a decrease in the average hydrogen intensity after bleaching and dyeing for both individuals 1 and 2. However, this trend was shown to be more severe after the bleaching treatment. This further indicates the strength of bleaching treatments and their potential ability to deform proteins and lead to overall protein loss, as seen in previous works.[42] A statistically significant difference in the average hydrogen intensity after a chemical treatment was only found for individual 1 after the bleaching procedure. This reinforced the idea that individual 1's curly hair type may have been more susceptible to the damages caused by chemical treatments. Nitrogen also displayed very similar trends to both the oxygen and the hydrogen; the average intensity decreased after bleaching and dyeing for both individuals, but was slightly more severe after the bleaching treatment. This again pointed to the potential ability of bleaching treatments to deform proteins and lead to protein loss.[42] A statistically significant difference in the average nitrogen intensity after a chemical treatment was only found for individual 1 after the bleaching procedure, again indicating a potentially increased vulnerability in the hair type of individual 1. Finally, carbon aligned with the trends of the oxygen, hydrogen, and nitrogen; the average intensity decreased after bleaching and dyeing for both individuals, but was observed to a greater extent after bleaching. This observed decrease in carbon again indicated the ability of chemical treatments, bleaching especially, to deform proteins and cause loss of proteins.[42] A statistically significant difference in the average carbon intensity after a chemical

treatment was only found for individual 1 after the bleaching procedure, which again pointed to a different reaction to the chemical treatments due to the slight difference in hair types of individuals 1 and 2.[42]

Table 7: Average element intensities, along with the standard errors of the means for the untreated, bleached, and dyed hair for individuals 1 and 2 obtained with Parameters Set 7 are shown in (a). P-values from two-sample t testing for the elements analyzed using Parameters Set 7 are shown in (b), where significantly different element intensities (p-value ≤ 0.05) are depicted in blue.

(a)

Element	Individual 1 Untreated	Individual 1 Bleached	Individual 1 Dyed	Individual 2 Untreated	Individual 2 Bleached	Individual 2 Dyed
Calcium	22150 ± 1182	16500 ± 2021	6517 ± 1140	16500 ± 764	7358 ± 3745	21850 ± 3772
Potassium	10200 ± 869	18217 ± 1858	683 ± 205	4660 ± 2167	17767 ± 1144	983 ± 130
Sodium	40278 ± 3818	33561 ± 7138	17183 ± 2544	13794 ± 1163	31444 ± 2230	25361 ± 3275
Carbon	1352 ± 138	652 ± 61	1241 ± 240	1165 ± 188	990 ± 24	1154 ± 66
Nitrogen	1216 ± 200	184 ± 74	255 ± 58	596 ± 292	207 ± 86	213 ± 120
Hydrogen	4778 ± 722	867 ± 203	2944 ± 1029	3083 ± 741	1417 ± 333	3583 ± 682
Oxygen	3583 ± 682	1083 ± 300	2194 ± 761	1417 ± 441	640 ± 74	2278 ± 547

(b)

Parameters 7: Two Sample t Test P values					
Element	**Ind 1 Untreated v. Ind 1 Bleached**	**Ind 1 Untreated v. Ind 1 Dyed**	**Ind 2 Untreated v. Ind 2 Bleached**	**Ind 2 Untreated v. Ind 2 Dyed**	**Ind 1 untreated v. Ind 2 Untreated**
Ca	0.073	**0.001**	0.139	0.237	**0.016**
K	**0.017**	**0.000**	**0.006**	0.232	**0.077**
Na	0.453	**0.007**	**0.002**	**0.029**	**0.003**
H	**0.006**	0.218	0.109	0.646	0.177
O	**0.028**	0.246	0.224	0.288	0.056
C	**0.010**	0.709	0.407	0.960	0.466
N	**0.017**	**0.010**	0.271	0.291	0.155

The average element intensities and standard error of the means obtained for the untreated, bleached, and dyed hair samples from individuals 1 and 2 using Parameters Set 14 are shown in Figure 8, as well as condensed into Table 8.

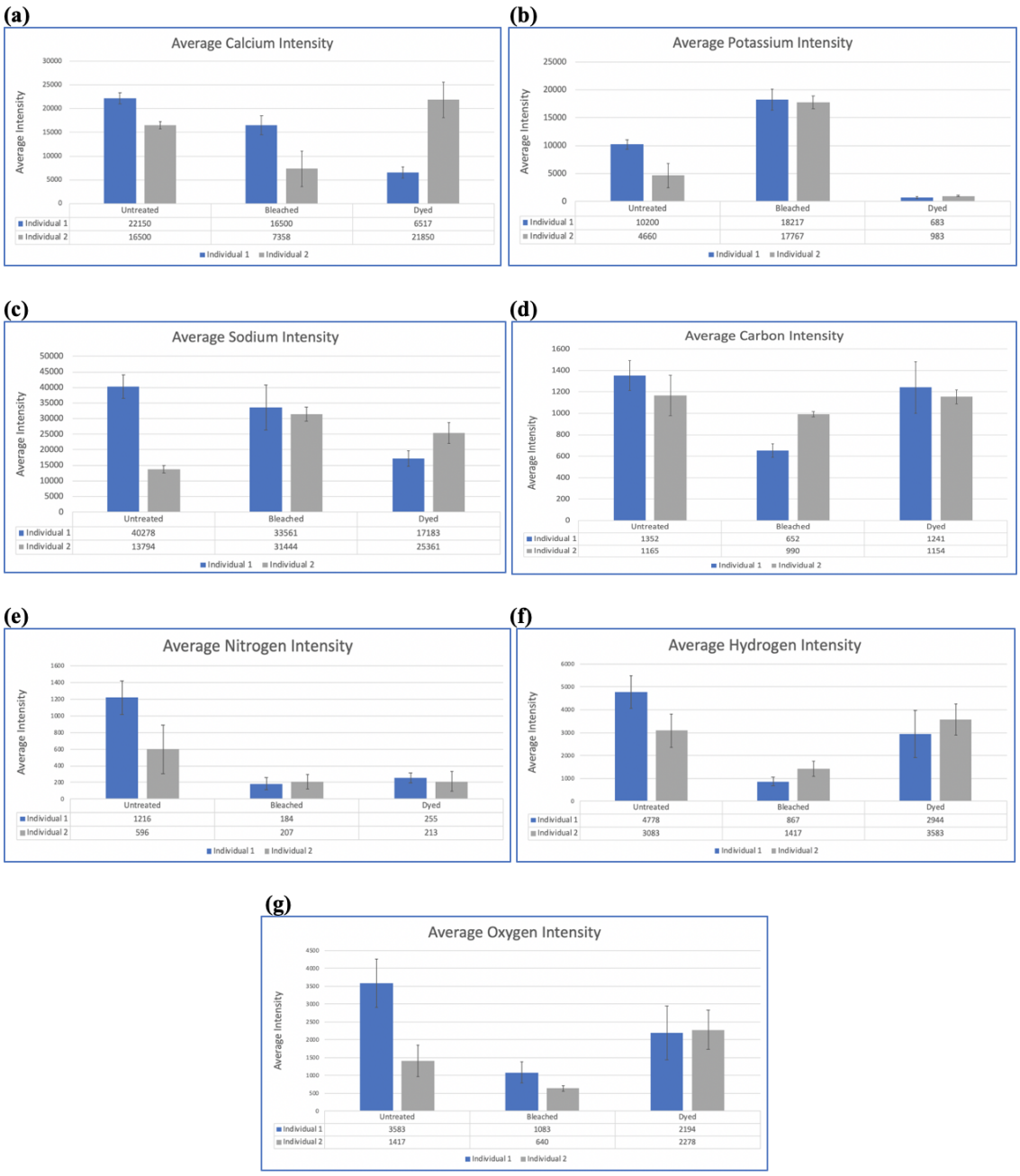


Figure 8: Bar graphs of average intensity from the three Parameter 14 runs for the untreated, bleached, and dyed sample groups from individuals 1 & 2, including (a) calcium, (b) potassium, (c) sodium, (d) carbon, (e) nitrogen, (f) hydrogen, and (g) oxygen.

With Parameters Set 14, many of the same general conclusions were drawn as were for Set 7. For example, both potassium and sodium were still in significantly different intensities for the untreated hair of the two individuals. Calcium was shown to

be significantly different between individuals 1 and 2 with Parameters 7. However, due to the change in gate delay, one of the peaks observed for calcium was no longer viable. Thus, when using Parameters 14, there appeared to potentially be a less complete and accurate view of the calcium present. This may have contributed to the inability to find a statistically significant difference between the calcium intensities for the untreated hair of individuals 1 and 2. One other issue observed when using Parameters 14 was that the intensities of peaks in general were decreased. This was especially magnified for the elements O, H, C, and N. This may have made it difficult for the peaks to be recognized by the software in some cases. This led to increased variation in the observed intensities for these elements and thus larger standard deviations and standard errors of the means. This made comparisons between the two individuals and the treatment types more difficult because the variances were so high. This served was another factor in the selection of Parameters Set 7 as the optimal set of Parameters over Parameters Set 14 for hair analysis.

Table 8: Average element intensities, along with the standard errors of the means for the untreated, bleached, and dyed hair for individuals 1 and 2 obtained with Parameters Set 14 are shown in (a). P-values from two-sample t testing for the elements analyzed using Parameters Set 14 are shown in (b), where significantly different element intensities (p-value ≤ 0.05) are depicted in blue.

(a)

Element	Individual 1 Untreated	Individual 1 Bleached	Individual 1 Dyed	Individual 2 Untreated	Individual 2 Bleached	Individual 2 Dyed
Calcium	5333 ± 441	7572 ± 1254	2278 ± 434	6805 ± 797	8917 ± 464	5028 ± 355
Potassium	6600 ± 1042	17678 ± 2173	1733 ± 633	2244 ± 571	22167 ± 1641	1667 ± 220
Sodium	15222 ± 1362	37878 ± 2273	14361 ± 1600	7567 ± 1507	38078 ± 3675	13611 ± 1020
Carbon	81 ± 24	71 ± 29	58 ± 6	53 ± 27	49 ± 21	52 ± 10
Nitrogen	22 ± 20	43 ± 12	62 ± 17	74 ± 5	54 ± 16	66 ± 9
Hydrogen	85 ± 12	98 ± 4	109 ± 17	141 ± 16	117 ± 14	172 ± 10
Oxygen	38 ± 5	54 ± 4	73 ± 5	53 ± 5	104 ± 12	60 ± 8

(b)

Parameters 14: Two Sample t Test P values					
Element	**Ind 1 Untreated v. Ind 1 Bleached**	**Ind 1 Untreated v. Ind 1 Dyed**	**Ind 2 Untreated v. Ind 2 Bleached**	**Ind 2 Untreated v. Ind 2 Dyed**	**Ind 1 untreated v. Ind 2 Untreated**
Ca	0.167	**0.008**	0.084	0.111	0.181
K	**0.013**	**0.034**	**<0.001**	0.399	**0.045**
Na	**0.001**	0.703	**0.002**	**0.029**	**0.020**
H	0.342	0.298	0.326	0.180	0.051
O	0.065	**0.009**	**0.015**	0.482	0.105
C	0.810	0.757	0.899	0.974	0.487
N	0.411	0.201	0.319	0.492	0.066

GC-MS Analysis

A total of two replicates for each of the three sample groups for both individuals 1 and 2 were analyzed using GC-MS. An example of the total ion chromatograms (TICs) produced for the untreated, bleached, and dyed hair samples from both individuals is included in Appendix I. There are twenty amino acids that are used to build human proteins, which include alanine, arginine, asparagine, aspartic acid, cysteine, glutamic acid, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine.[50] All of these appear in the

keratin protein found in human hair.[28,51] In the research conducted by Macri, eight total amino acids were successfully detected in the hair samples, which included valine, leucine, proline, serine, threonine, aspartic acid, phenylalanine, and tyrosine.[29] Meanwhile, Rashaid et al. were able to detect a total of fourteen amino acids in the hair samples, which consisted of the same eight found by Macri, as well as glutamic acid, cysteine, alanine, glycine, isoleucine, and lysine.[28,50-51]

In this research, six amino acids were successfully detected consistently in the hair samples of both individuals, including valine, leucine, serine, threonine, aspartic acid, and phenylalanine, provided in order from the shortest to longest retention time (Figure 9). Their identities were confirmed with the use of the NIST/NIH/EPA Mass Spectral Library database.[46] The overall order of retention, as well as the individual retention times were also visually compared to those obtained by Macri, and appeared to be consistent. Incomplete derivatization may be responsible for the absence of alanine, cysteine, glutamic acid, glycine, isoleucine, lysine, proline, and tyrosine in the majority of the samples analyzed.[46]

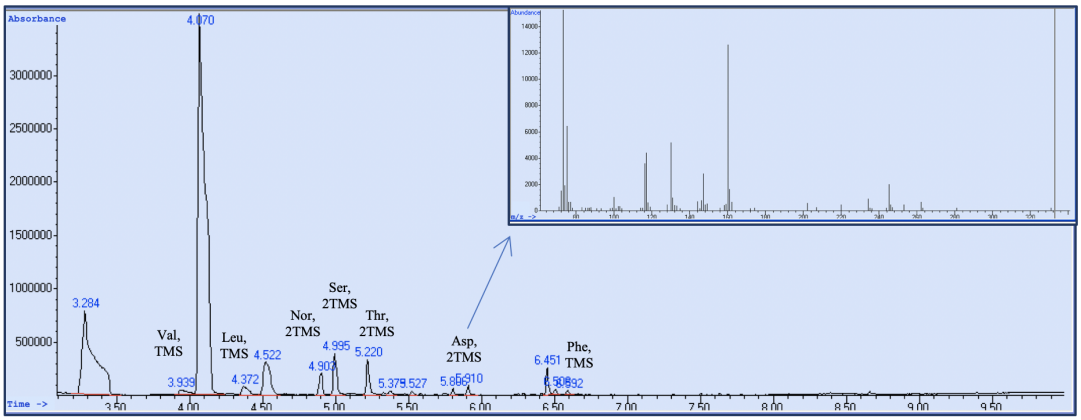


Figure 9: A total ion chromatogram (TIC) of a hair sample from individual 1 untreated; insert shows the mass spectrum of the L-Aspartic Acid 2TMS derivative.

Additionally, for the six amino acids detected, either the TMS and 2TMS derivative was detected. When BSTFA is used as a derivatizing agent, trimethylsilyl groups replace protons in the original structure of the amino acid, forming a trimethylsilyl (TMS) derivative. When two trimethylsilyl groups replace two protons in the amino acid structure, a 2TMS derivative is formed.[51] In cases where the TMS derivative was not detected, the 2TMS derivative was instead utilized. The same was true for the study by Macri, where TMS, 2TMS, and 3TMS derivatives were detected for some amino acids.[29] For the hair samples in this research, the following amino acid derivatives were used for data analysis: valine TMS derivative, leucine TMS derivative, serine 2TMS derivative, threonine 2TMS derivative, aspartic acid 2TMS derivative, and phenylalanine TMS derivative (Figure 9). Based on the work by Macri, the norvaline 2TMS derivative was used for the internal standard due to the fact that the norvaline TMS derivative (t_R= 4.070 min) was consistently seen at a high abundance.[51, 29]

Similar to the studies by Rashaid et al., Lam, and Macri, the observed abundances for serine and threonine were generally higher than the other amino acids.[29, 51, 52] Conversely, consistently lower abundances than the remaining amino acids was observed for both aspartic acid and phenylalanine. A trend of low phenylalanine abundances was also noted by Rashaid et al., Lam, and Macri, but only Macri observed a similar trend for aspartic acid.[29, 51, 52]

After the instrumental analyses, the amino acids were compared to the L-norvaline internal standard to produce a total of fifteen amino acid ratios to include each possible combination of the six amino acids detected. Unfortunately, there were several issues that prevented a fully quantitative analysis of the GC-MS results. The first

of these occurred when the digested samples were supposed to undergo centrifugation to form a pellet of hair, allowing the supernatant liquid to be easily retrieved. However, after an hour of centrifuging at increasing speeds, the hair still remained suspended in the samples. It is unclear why this step was unsuccessful in this project, as Macri was able to centrifuge all of the samples analyzed for several 5- to 10-minute intervals at 3200 rpm.[29] Ultimately, the samples had to be filtered using Kimwipes and Pasteur pipettes to adequately remove the hair, which may have influenced the quantity of the contents analyzed, as the supernatant was eventually collected in a different manner than achieved by Macri. Another issue arose when one set of samples was prepared for GC-MS and had to be left in the fridge overnight due to a motor error with the GC plunger. This can be problematic due to the fact that samples for this analysis are intended to be prepared within a relatively close window to the actual instrumental analysis. This is because the TMS group utilized for the derivatization of the amino acids can be displaced easily, which would result in the amino acids being undetectable by GC-MS due to their high boiling points. Thus, preparing derivatized samples for analysis with GC-MS and delaying their actual analysis may mean detecting a lower amount of derivatives. In this study, a second set of fresh samples was prepared the next morning and run alongside the first set. As a result, the averages produced for the subsequent analyses display high variance in some cases, likely due to the fact that the two sets were subjected to different conditions prior to GC-MS analysis. Additionally, the mixed amino acid standard was unsuccessful in detecting the desired amino acid derivatives. One potential reason for this is due to the age of the amino acids used to prepare this standard, coupled with the fact that the mixed amino acid standard only contained 1 μL of each derivatized amino acid

out of a total volume of 1111 μL. This method was successful for Macri, so it may be that due to the age of the amino acids, this concentration may have been too dilute for adequate detection. The mixed amino acid standard was originally going to be utilized for the quantification of the amino acids within the hair samples. Instead, for each of the six amino acids, the area of an observed amino acid derivative was divided by the area of the internal standard in that sample. The values yielded by this calculation were then compared to one another to produce amino acid ratios for a given sample.

Bar graphs of some of the resulting amino acid ratios are shown in Figure 10. Additional bar graphs are included in Appendix I. The amino acid ratios displayed in the bar graphs are also condensed to fit in Table 9, with their corresponding p-values produced after t-testing are included in Table 10. As can be seen in the figure, some amino acid ratios appear to be similar for the untreated hair of individuals 1 and 2, and others show more noticeable differences.

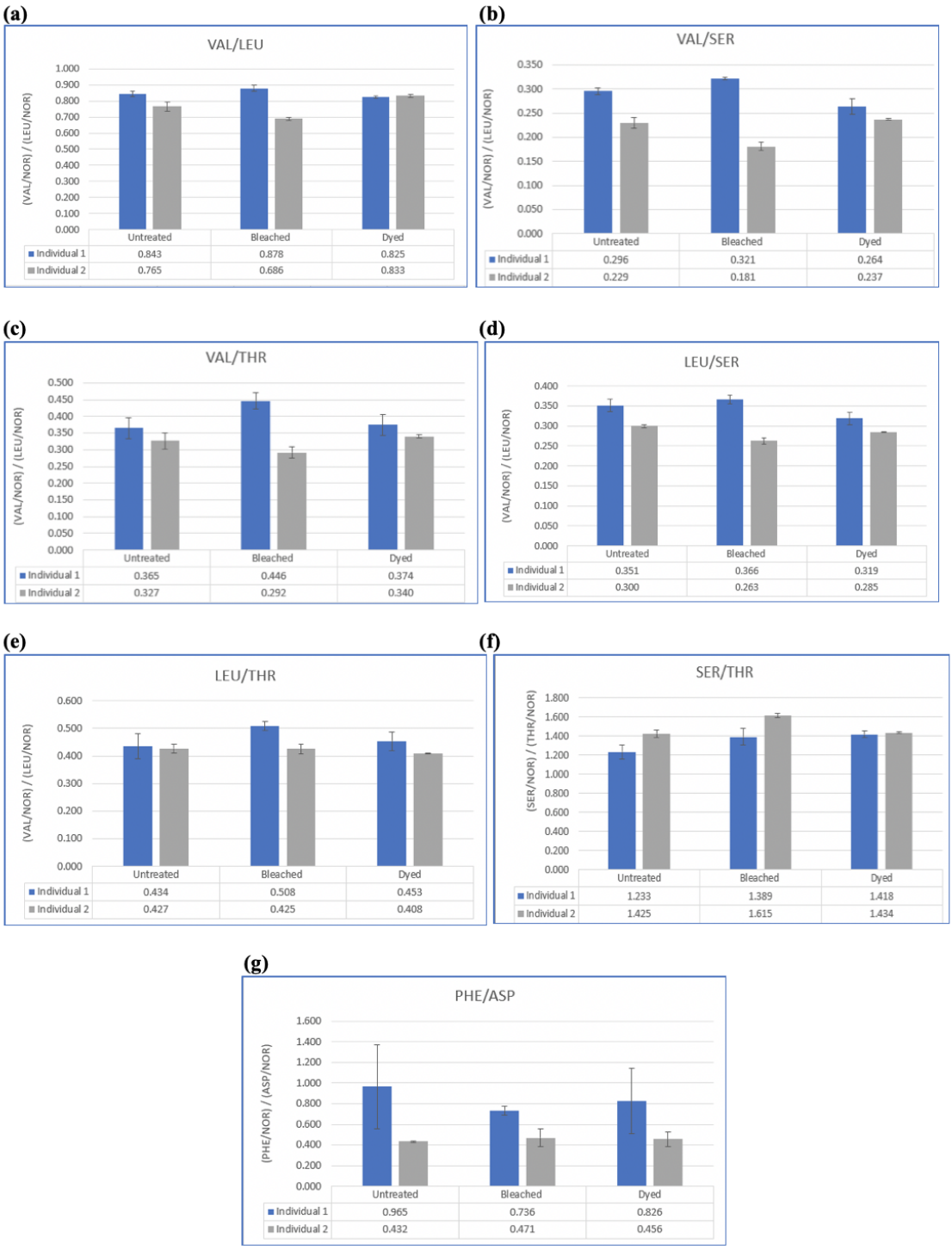


Figure 10: Bar graphs of the calculated amino acid ratios for the untreated, bleached, and dyed hair for individuals 1 and 2, including (a) Val/Leu, (b) Val/Ser, (c) Val/Thr, (d) Leu/Ser, (e) Leu/Thr, (f) Ser/Thr, and (g) Phe/Asp. The error bars are representative of the standard errors of the means.

Using a two-sample t-test (p-value ≤ 0.05), only one of the fifteen amino acid ratios (Val/Ser) was found to be significantly different between the untreated hair samples from individuals 1 and 2 when compared using a two-sample t-test (Table 10). Another amino acid ratio (Leu/Ser) was in the range of p-values from 0.05 to 0.10, with a value of 0.085. An increase in sample size, or less variation in the samples analyzed may have resulted in the detection of a significant difference for this amino acid ratio between individuals 1 and 2. One potential reason for the lack of significant differences between individuals 1 and 2 is the fact that one set of samples was stored in the fridge overnight while the second set that they were intended to be averaged with was prepared freshly for GC-MS analysis. The use of only two replicates for each sample group was also unideal, as a larger sample size would have provided a more complete reflection of the true data, and would have increased the likelihood of finding additional significant differences. Having only two replicates makes it impossible to recognize and address outliers, since in this situation it's impossible to tell which of the two runs could possibly be the outlier. Outliers may have been possible due to differences in derivatization among samples, incomplete derivatization, or differences in sample storage. The two sample sets were not derivatized on the same day, or subject to the same length of time prior to GC-MS analysis, which may have contributed to the observed variations. Another potential reason for the lack of significant differences uncovered was due to the fact that the amino acid ratios could not be calculated through a comparison to the mixed amino acid standard. The method applied in this research of comparing the amino acid derivatives to only the internal standard within each sample is most successful if the areas of all the internal standard peaks are consistent. These values were generally consistent for

bleached and dyed samples for both individuals, as well as the first untreated sample for individual 2. However, for both of the untreated samples of individual 1, and the second untreated sample from individual 2, the area of the internal standard peak was substantially higher than was observed for the other nine samples. This may be another reason for the difficulty in making any comparisons involving individual 1.

Additionally, there were especially high variances in ratios including the amino acids aspartic acid and phenylalanine, as can be seen in the bar graphs included in Appendix I. This was particularly true for the untreated hair of individual 1, which made it difficult to make any accurate comparisons between the untreated hair from both individuals. This also hindered the ability to discern whether the amino acid ratios were altered to a statistically significant level after the bleaching or dyeing treatments.

As individual 2 displayed much more reasonable variances overall, the t-testing comparing the untreated hair of this individual to their bleached and dyed hair will be discussed in greater detail as it may provide a more accurate reflection of the potential changes to amino acid ratios after chemical treatments. There were three amino acid ratios (Val/Phe, Leu/Phe, Ser/Thr) found to be significantly different between the untreated and bleached hair samples of individual 2. A significant difference in the Val/Phe ratio was also found in individual 1 after bleaching. There were no amino acid ratios found to be significantly different between the untreated and dyed hair samples of individual 2. This has the potential to be a positive finding because if dyed hair does not display significant differences in amino acid ratios compared to the original untreated form, there may still be the ability to connect an individual's untreated hairs after they've received a dyeing treatment. However, due to the circumstances surrounding the GC-MS

analysis in this study, further research will be required to additionally investigate the impacts on amino acid ratios after bleaching and dyeing.

Table 9: Fifteen amino acid ratios calculated for the untreated, bleached, and dyed hair for individuals 1 and 2. The average ratio values, as well as their corresponding standard errors of the means, are shown.

Amino Acid Ratio	individual 1 Untreated	individual 1 Bleached	individual 1 Dyed	individual 2 Untreated	individual 2 Bleached	individual 2 Dyed
VAL/LEU	0.842 ± 0.018	0.878 ± 0.018	0.825 ± 0.008	0.764 ± 0.027	0.686 ± 0.010	0.833 ± 0.009
VAL/SER	0.295 ± 0.007	0.321 ± 0.003	0.264 ± 0.016	0.229 ± 0.011	0.181 ± 0.008	0.237 ± 0.002
VAL/THR	0.364 ± 0.031	0.446 ± 0.024	0.374 ± 0.032	0.327 ± 0.024	0.292 ± 0.017	0.340 ± 0.004
VAL/PHE	42.2 ± 7.398	2.07 ± 1.926	3.10 ± 0.449	3.22 ± 0.082	2.11 ± 0.057	2.78 ± 0.122
VAL/ASP	43.7 ± 24.272	2.90 ± 0.217	2.70 ± 1.344	1.39 ± 0.014	0.997 ± 0.213	1.26 ± 0.145
LEU/SER	0.351 ± 0.016	0.366 ± 0.011	0.319 ± 0.016	0.299 ± 0.003	0.263 ± 0.008	0.285 ± 0.001
LEU/THR	0.434 ± 0.046	0.508 ± 0.017	0.453 ± 0.034	0.426 ± 0.017	0.425 ± 0.018	0.408 ± 0.001
LEU/PHE	49.9 ± 7.714	4.48 ± 0.162	3.75 ± 0.508	4.23 ± 0.258	3.07 ± 0.038	3.33 ± 0.183
LEU/ASP	51.3 ± 27.712	3.30 ± 0.314	3.26 ± 1.598	1.82 ± 0.084	1.45 ± 0.289	1.51 ± 0.157
SER/THR	1.23 ± 0.075	1.39 ± 0.087	1.42 ± 0.036	1.42 ± 0.039	1.62 ± 0.022	1.43 ± 0.007
SER/PHE	144 ± 28.426	12.2 ± 0.086	11.7 ± 1.009	14.1 ± 1.025	11.6 ± 0.203	11.7 ± 0.609
SER/ASP	150 ± 85.666	9.00 ± 0.595	9.97 ± 4.507	6.10 ± 0.352	5.47 ± 0.933	5.30 ± 0.567
THR/PHE	118 ± 30.231	8.84 ± 0.613	8.24 ± 0.500	9.94 ± 0.990	7.21 ± 0.222	8.16 ± 0.466
THR/ASP	126 ± 77.166	6.53 ± 0.836	6.96 ± 3.000	4.29 ± 0.364	3.38 ± 0.533	3.69 ± 0.377
PHE/ASP	0.965 ± 0.405	0.736 ± 0.044	0.826 ± 0.314	0.432 ± 0.006	0.471 ± 0.088	0.456 ± 0.072

Table 10: P-values from two-sample t testing for the amino acids analyzed in the hair are shown. Significant differences (p-value ≤ 0.05) are depicted in blue.

P-Values					
Amino Acid Ratio	**Ind 1 Untreated vs. Ind 1 Bleached**	**Ind 1 Untreated vs. Ind 1 Dyed**	**Ind 2 Untreated vs. Ind 2 Bleached**	**Ind 2 Untreated vs. Ind 2 Dyed**	**Ind 1 Untreated vs. Ind 2 Untreated**
VAL/LEU	0.293	0.469	0.115	0.142	0.141
VAL/SER	0.076	0.203	0.070	0.545	**0.035**
VAL/THR	0.171	0.849	0.361	0.649	0.435
VAL/PHE	**0.034**	0.119	**0.008**	0.091	0.119
VAL/ASP	0.341	0.340	0.312	0.441	0.331
LEU/SER	0.510	0.291	0.051	0.053	0.085
LEU/THR	0.268	0.767	0.960	0.464	0.897
LEU/PHE	0.107	0.105	**0.047**	0.105	0.106
LEU/ASP	0.333	0.333	0.334	0.215	0.324
SER/THR	0.304	0.156	**0.050**	0.839	0.149
SER/PHE	0.136	0.135	0.140	0.178	0.137
SER/ASP	0.347	0.350	0.591	0.349	0.341
THR/PHE	0.172	0.171	0.115	0.245	0.173
THR/ASP	0.364	0.365	0.292	0.369	0.358
PHE/ASP	0.630	0.811	0.737	0.773	0.414

Conclusion

Hair is a common exhibit type in the context of Forensic Science due to its ability to be easily shed or torn out during the commission of a crime, as well as its capability of transferring easily once shed. Current techniques for the forensic analysis of hair samples involve microscopic hair comparison (MHC), mitochondrial DNA (mtDNA) analysis, and nuclear DNA analysis.[10] However, there are a few limitations to each of these techniques: MHC can be subjective; mtDNA analysis is time-consuming, expensive, and not individualizing; and while nuclear DNA analysis is extremely powerful, it requires an adequate source of DNA. Thus, novel hair analysis methods would be a helpful complement to the current field. This thesis examined several novel methods for forensic hair analysis, including evaluating functional groups with ATR-FTIR, element intensities with LIBS, and amino acid ratios with GC-MS.

In this study, hair samples were obtained from two demographically similar individuals with morphologically similar hair. Microscopic analysis was utilized to further compare the untreated hair samples of these individuals and they appeared to be similar in many of the attributes examined.

After washing, the functional groups within the components of the hair were first analyzed directly with ATR-FTIR. This was done to evaluate any spectral differences between individuals and after treatment types. Areas of difference between individuals 1 and 2 were observed at 2925 cm^{-1}, 2850 cm^{-1}, 1529 cm^{-1}, 1455 cm^{-1}, and 1020 cm^{-1}/ 1040 cm^{-1} (5 of 11 total bands analyzed). Bleaching and dyeing treatments resulted in the shifting of select peaks, including the following: 1529 cm^{-1}, 1400 cm^{-1}, 1241 cm^{-1}, 1071 cm^{-1}, 1020 cm^{-1}/ 1040 cm^{-1} for both treatments; 3283 $cm^{-1}$and 2925 cm^{-1} for bleaching

specifically; and 1639 $cm^{-1}$and 1455 cm^{-1} for dyeing specifically (7 of 11 total bands analyzed for both treatment types). Several of the observed trends after chemical treatments were different in individuals 1 and 2, possibly indicating differences in the untreated hair of these individuals that would contribute to them responding differently to the same chemical treatments.

Analysis was also completed directly with LIBS to evaluate the potential for differences in element intensity to differentiate between individuals, as well as the effects of bleaching and dyeing and treatments on these intensities. After testing 16 sets of parameters, Parameters 7 was thought to be the optimal parameters for the LIBS analysis of hair. Elements such as calcium, potassium, and sodium were significantly different between individuals 1 and 2, which is interesting due to their association with protein side chains. Further investigation is required to continue the evaluation of the potential differentiating ability provided by the intensities of these specific elements. There were also observed to be decreases in N, O, C, and H after bleaching and dyeing, with a more noticeable effect after bleaching. These changes were found to be statistically significant after bleaching, and in some cases after dyeing, for individual 1 only. As the impacts were amplified for individual 1 in comparison to 2, this was thought to potentially be the result of the curlier, and thus more vulnerable, hair pattern observed in individual 1.

For the investigation into amino acid ratios with GC-MS , the hair samples had to be digested and derivatized prior to instrumental analysis. This analysis was used for the detection, identification, and quantification of the amino acid derivatives present in the hair samples. Six derivatized amino acids, including valine, leucine, serine, threonine, aspartic acid, and phenylalanine were successfully detected and identified consistently in

each sample group for both individuals. For each of the six amino acids, the area of an observed amino acid derivative was divided by the area of the L-norvaline internal standard in that sample. The values yielded by this calculation were then compared to one another to produce amino acid ratios for a given sample. A total of fifteen amino acid ratios were produced for the untreated, bleached, and dyed sample groups for both individuals. Two-sample t-testing was used to make comparisons between the average amino acid ratios for the untreated groups of individuals 1 and 2, and for comparisons among treatment types. Only one of the fifteen amino acid ratios (Val/Ser) was found to be significantly different between the untreated hair samples from individuals 1 and 2, and another amino acid ratio (Leu/Ser) was in the range of p-values from 0.05 to 0.10, with a value of 0.085. Comparison between individuals 1 and 2 was difficult due to the observed variance in the L-norvaline internal standard, variations in sample lifespan and storage prior to analysis, and the lack of a mixed amino acid standard to make comparisons to. As the variances in individual 2 were much smaller overall, the t-testing comparing this individual's untreated to treated hair was considered a more useful reflection. There were three amino acid ratios (Val/Phe, Leu/Phe, Ser/Thr) found to be significantly different between the untreated and bleached hair samples of individual 2. A significant difference in the Val/Phe ratio was also found in individual 1 after bleaching. There were no amino acid ratios found to be significantly different between the untreated and dyed hair samples of individual 2, which may be a positive finding because it may mean that ability to connect a dyed hair to the donor is retained despite the alteration. However, as a result of the complications surrounding the GC-MS analysis in this study,

further research is required to additionally investigate the impacts on amino acid ratios after bleaching and dyeing.

The data obtained from the ATR-FTIR and LIBS analyses indicated their potential ability to complement current forensic hair examination methods. Differences in several of the observed bands were observed for the two individuals with the use of ATR-FTIR. Of the seven elements analyzed with LIBS, there were found to be three elements with significant differences in average intensity between the untreated hair of individuals 1 and 2, which were in calcium, potassium, and sodium. Based on the differences between individuals 1 and 2 observed with the ATR-FTIR and LIBS analyses, as well Macri's and Lam's past successes, it is expected that additional significant differences would be found with the GC-MS analysis under more ideal circumstances if it was to be repeated.[29,52] These techniques may be especially useful in cases where the hair samples share similar morphology and originate from demographically similar individuals. Changes were observed in the ATR-FTIR analyses after both bleaching and dyeing for both individuals, and LIBS analysis also revealed significant changes for both individuals after chemical treatments. Due to the variances in the GC-MS analysis, the stability in amino acid ratios after chemical treatments was difficult to accurately establish. As such, further research will need to be conducted to better understand the effect that bleaching and dyeing treatments have on the elements, functional groups, and amino acids analyzed.[29,52]

Future Research

Novel methods of hair analysis, including evaluating functional groups with infrared (IR) spectroscopy, elements with laser induced breakdown spectroscopy (LIBS), and amino acid ratios with gas chromatography- mass spectrometry (GC-MS) show potential in complementing current forensic hair analysis methods. Additional work is still required to fully understand the observed changes in elements, functional groups, and amino acids after bleaching and dyeing, as well as the factors that impact the severity of these changes.

Due to the complications that arose during the GC-MS analysis of this study, additional GC-MS analyses should be conducted to obtain a better understanding of how amino acid ratios are impacted with the use of bleach and permanent oxidative hair dyes.

Additional cosmetic hair treatments should also be investigated with each of the methods outlined in this study to gauge how different treatments alter the composition of an individual's untreated hair. This is important because if these techniques are ever to be implemented in forensics, it is inevitable that hair samples with other treatment types will be collected and involved in casework. As a result, an adequate understanding of the effects of each treatment on hair's element intensities, observed IR bands, and amino acid ratios is necessary. The different types of hair dyes, including temporary, semi-permanent, and demi-permanent dyes would likely produce different results than are seen with the oxidative permanent hair dye used in this study. As mentioned previously, temporary non-oxidative hair dyes have a short lifespan, and are easily washed out. An acidic environment is created to harden the cuticle and prevent dye from entering. Based on these characteristics, temporary hair dyes would not be expected to alter the actual

composition of the hair shaft, but it would be interesting to see if any dye residues remaining on the hair's surface after washing would impact the analyses. This is especially important for analyses such as ATR-FTIR and LIBS, where the area of analysis is largely focused on the outermost layers of the hair. Semi-permanent non-oxidative hair dyes use an elevated pH level to induce cuticle swelling and softening, allowing the dyes to access the cortex. Dyes chosen for these products are selected based on their affinity to the hair's keratin, so it would be interesting to see how intensely this would impact the various components of the hair. Demi-permanent hair dyes are of interest due to their use of hydrogen peroxide as an oxidizing agent. Oxidizing agents yield chemical changes within the shaft of the hair to produce the desired color effect. The results seen with demi-permanent oxidative hair dyes would be expected to be similar to those observed in this study with the use of a permanent oxidative hair dye, but of a lesser intensity. Chemical hair straighteners, also referred to as chemical relaxers, would be another interesting subject of study, as their method of action involves reducing and reforming disulfide bonds that occur within keratin. This treatment would also likely impact varying populations with varying degrees of intensity, as those with curly hair would possess more cysteine residues and disulfide bonds to be reduced and reformed compared to those with straight hair. Another cosmetic treatment suggested for inclusion in future study is hair glaze, which is an acidic treatment that closes the cuticle and coats the hair, rather than entering the cortex to impart semi-permanent color. While this treatment would not be expected to induce changes to the composition of the hair, it would be especially interesting to see how greatly the hardened coating on the hair shaft would

impact analysis like LIBS and ATR-FTIR since they are meant to directly analyze the outer layers of the hair sample.[37, 7, 35, 53, 54]

Another area that requires future attention is whether repetitive chemical treatment increases the likelihood of observing additional significant differences when compared to an individual's untreated hair. This may involve, for example, comparing an individual's untreated hair to hair that has been dyed once, five times, and ten times to evaluate the number of significant differences observed after each treatment plan. It would be beneficial to know whether the number of significant differences observed between an individual's untreated and dyed hair remains consistent regardless of the number of treatments sustained, or if the value increases before plateauing at a certain point.

It is also important that future studies review a range of hair types, ideally from straight hair to kinky hair. This is important due to the fact that certain amino acid residues, such as cysteine, are known to be present in greater amounts in curlier hair, and the disulfide bonds created by these cysteines are the targets of several cosmetic hair treatments. Based on the limited scope of this study, it appeared that Individual 1, who had curlier hair, was more susceptible to damage than Individual 2, who had straight hair. Thus, it would be expected that a greater number of significant differences may be observed after chemical treatments in those with coiled or kinky hair compared to those with straight hair.[6]

Additional research should also involve the inclusion of other hair colors. The study by Kuzuhara concluded through amino acid composition analysis that the black hair was damaged to a greater extent by the bleaching than the white hair.[40] It is

expected that blonde and brown hair would produce results somewhere between those observed for the white and black hair, but it would be helpful to know how closely related hair pigmentation and damaged sustained from bleaching actually are through the whole color range. It would also be interesting to evaluate this relationship in red hair and observe the extent of the damage imparted.

In conclusion, novel methods, such as element analysis through LIBS, evaluation of functional groups with ATR-FTIR, and amino acid analysis with GC-MS all have the potential to complement current forensic hair analysis techniques. These methods may provide analysts with the ability to deduce an individual's demographic information, as well as clues as to any chemical treatments applied. There is also potential to provide exclusionary information, which can aid in the investigative process and diversion of resources.

Appendix I: Example Spectra & Total Ion Chromatograms (TICs)

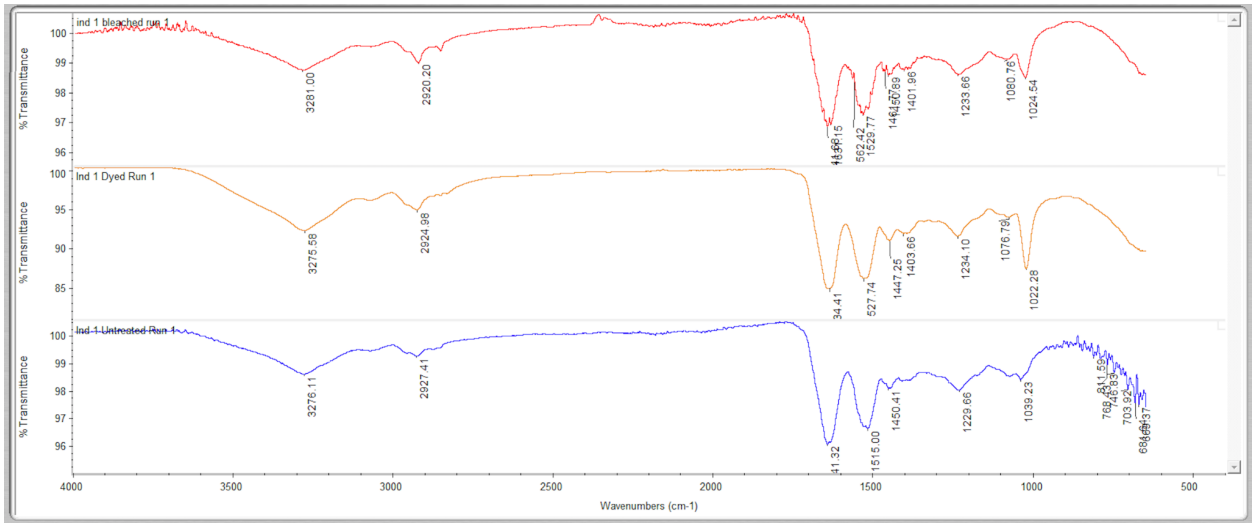


Figure A1: ATR-FTIR spectra from individual 1; (top) bleached sample, (middle) dyed sample, and (bottom) untreated sample.

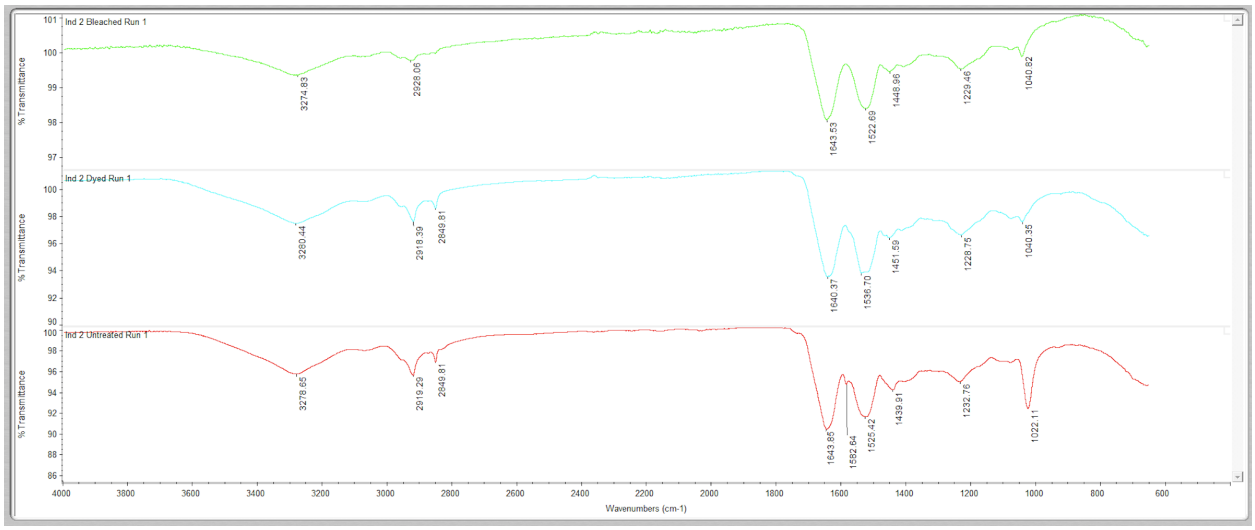
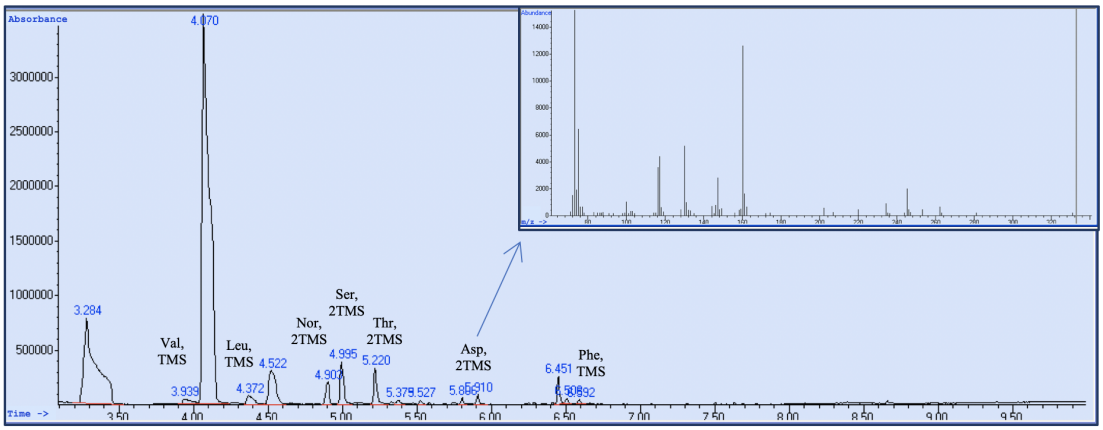


Figure A2: ATR-FTIR spectra from individual 2; (top) bleached sample, (middle) dyed sample, and (bottom) untreated sample.

(a)



(b)

Peak #	Ret Time	Type	Width	Area	Start Time	End Time
1	3.281	rVB	0.313	4541130	3.221	3.535
2	3.938	rBV	0.134	200814	3.898	4.032
3	4.067	rVV	0.209	12254480	4.032	4.241
4	4.371	rVB	0.129	237802	4.341	4.470
5	4.520	rBV	0.134	1010405	4.475	4.609
6	4.779	rBV	0.060	12357	4.744	4.803
7	4.903	rBV	0.065	324926	4.863	4.928
8	4.992	rVV	0.095	661312	4.968	5.062
9	5.082	rVV	0.040	29279	5.062	5.102
10	5.117	rVV	0.050	33157	5.102	5.152
11	5.167	rVV	0.040	16169	5.152	5.191
12	5.216	rVV	0.114	510680	5.191	5.306
13	5.331	rVV	0.045	37540	5.306	5.351
14	5.376	rVV	0.065	69043	5.351	5.415
15	5.430	rVV	0.040	18103	5.415	5.455
16	5.470	rVV	0.050	22100	5.455	5.505
17	5.525	rVV	0.060	41975	5.505	5.565
18	5.585	rVB	0.060	19304	5.565	5.624
19	5.754	rBV	0.065	36915	5.714	5.779
20	5.803	rBV	0.050	62088	5.779	5.828
21	5.908	rVB	0.095	102364	5.883	5.978
22	6.251	rBV	0.060	18763	6.206	6.266
23	6.286	rVB	0.055	24426	6.266	6.321
24	6.410	rBV	0.040	20483	6.386	6.425
25	6.450	rVV	0.060	298482	6.425	6.485
26	6.505	rVV	0.055	75686	6.485	6.540
27	6.590	rVV	0.065	57307	6.570	6.634
28	6.659	rVB	0.065	15019	6.634	6.699

Figure A3: (a) An example of a total ion chromatogram (TIC) of an untreated hair sample from individual 1 showing six detected amino acid peaks labeled with retention times; insert shows the mass spectrum of the L-Aspartic Acid 2TMS derivative. (b) An example of a chart providing the peak areas.

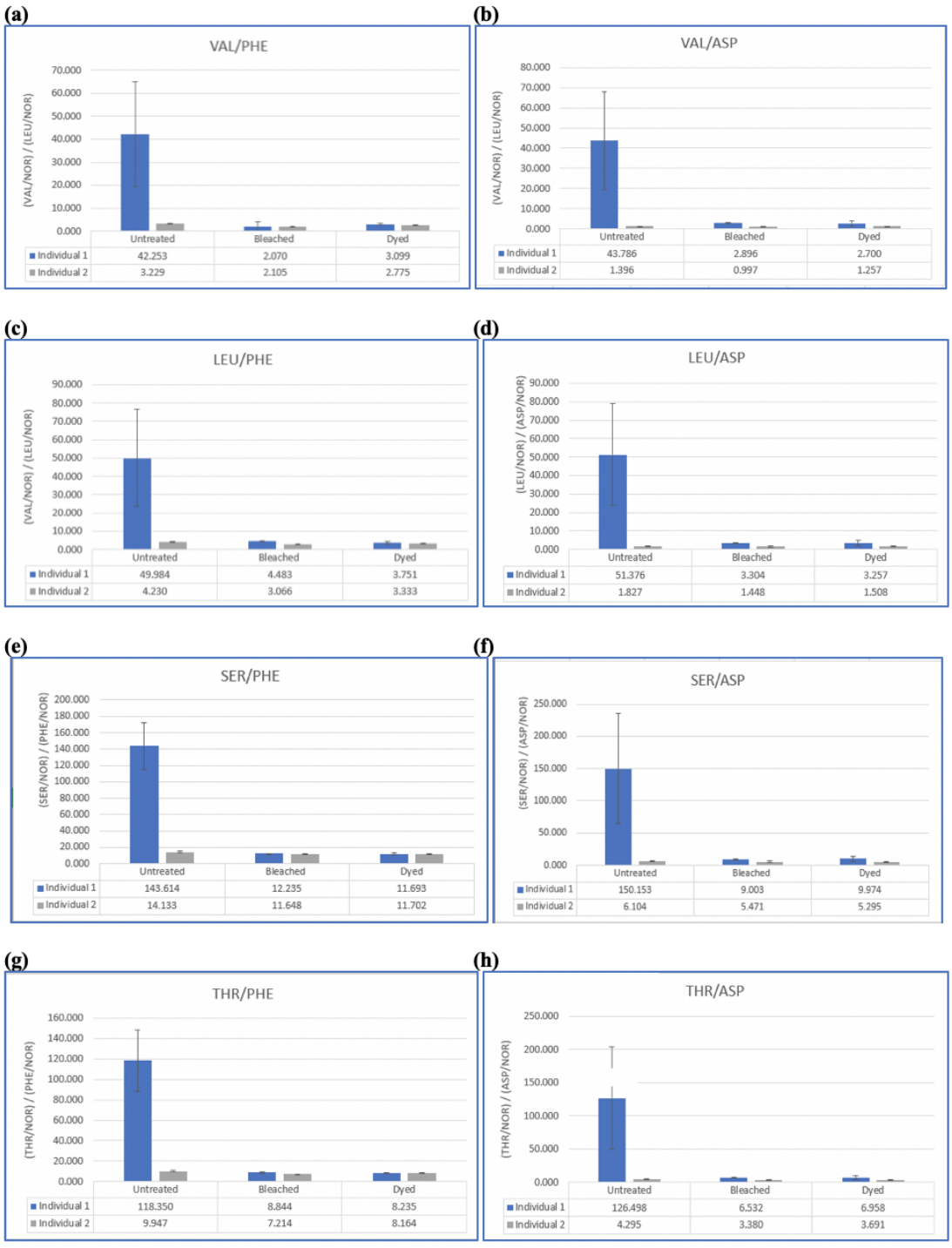


Figure A4: Bar graphs of the calculated amino acid ratios for the untreated, bleached, and dyed hair for individuals 1 and 2, including (a) Val/Phe, (b) Val/Asp, (c) Leu/Phe, (d) Leu/Asp, (e) Ser/Phe, (f) Ser/Asp, (g) Thr/Phe, and (h) Thr/Asp. The error bars are representative of the standard errors of the means.

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