Report Title: The Analysis of Trace Forensic Evidence Using Isotope Ratio Mass Spectrometry: Differentiating Fibers

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Abstract

Fibers are often found at crime scenes and current methods can determine the chemical composition and color of the fiber as well as physical characteristics. Unless the fiber contains DNA, there is no way to compare a fiber found at a crime scene with fibers found in a suspect's possession. This study aims to determine if isotope ratio mass spectrometry (IRMS) can be used to add an additional level of information about a fiber, potentially allowing two fibers to be compared for common characteristics. This could provide additional evidence that two fibers may have come from the same original source. Between 10 µg and 200 µg of fiber was used for each analysis with an elemental analyzer used to measure the ^{13}Carbon and ^{15}Nitrogen isotope ratios, or a high temperature conversion elemental analyzer for the analysis of ^{2}Hydrogen and ^{18}Oxygen isotope ratios.

A variety of types of non-colored fibers, including cotton, wool, silk, and a number of different synthetics were analyzed. Using a combination of all four isotopes, all fibers were able to be distinguished from other fibers of the same chemical composition but from different manufacturers, or from the same manufacturer but from different years. Homogeneity within a commercial shirt was studied to determine if sampling location is important when comparing two different fibers. All fibers from the same clothing panel were found to be isotopically homogenous, but depending on manufacturer some shirts were found to have different isotope ratios in the shirt body compared to shirt sleeves. This could be the result of different shirt panels being cut from different bolts of cloth and then sewn together. White and colored fibers were compared to determine if the coloring has an effect on the isotope ratio. The manner in which the shirt was constructed was found to determine the effect of color on the fiber isotope ratios. For shirts that were constructed of distinct fibers of different colors, differences in the isotope ratios of differently colored fibers were observed. For shirts woven from a single thread, with different regions colored differently, fibers of different colors exhibited consistent isotope ratio values. Thus, the fiber material itself, and not the coloring, appear to control the isotope ratios. Fibers were stained with blood, grass, or dirt to investigate whether common stains can change the isotope ratio values. Grass and dirt stains were not found to result in a significant change in isotope ratio values likely because these are surface stains. Blood stained fibers, however, did exhibit a change in isotope ratio values, most likely because blood can penetrate the fiber. Since fibers are routinely analyzed for DNA, pre- and post-DNA processed fibers were compared to determine if isotope ratio analysis can still be used after fibers have been treated to extract any DNA that might be present. Carbon and nitrogen isotope ratios were not found to change, but in one-third of the fiber samples, oxygen and/or hydrogen values were affected by the DNA processing protocol.

Based on the results of this study, isotope ratio mass spectrometry appears to be a potentially useful forensic technique for the analysis and differentiation of a wide range of fiber types, both natural and synthetic. Fibers of the same chemical composition but from different manufacturers or production batches can be differentiated using IRMS whereas fibers from the same clothing item have statistically indistinguishable isotope ratio values, provided that the fibers are sampled from the same region of cloth. Thus, fibers from a crime scene and those from a different source will likely be able to be distinguished from one another.

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Executive Summary

Synopsis of the Problem

Fibers are of special importance to forensic analysis due to their prevalence at crime scenes. Therefore, it is important to analyze these materials for any differentiating characteristics that could be used to connect a suspect with a fiber found at a crime scene, or to exclude potential suspects. In the past, various methods have been used to identify and analyze fibers. Infrared spectroscopy can discriminate different types of fibers (cotton, silk, polyester, etc.) with similar appearances. Further Infrared and Raman spectroscopy analysis can differentiate fibers with different dyes and contaminants. However, these techniques are unable to distinguish between different fibers of the same material and color (i.e. two different white cotton fibers). Therefore, without the differentiating chemical and visible characteristics (such as color, texture, etc.) that allow for the differentiation of fibers, different methods need to be used to distinguish between different fiber samples.

Purpose

IRMS can differentiate between these cotton samples because the percent abundances of given elements (i.e. the ratios of heavy to light isotopes) differ under various conditions.[8] Variations in isotope abundances of light elements are caused by processes that favor one isotope over another, a process called fractionation. Carbon isotope variations in plants reflect the isotope composition of the reactant (CO_2) in the ambient atmosphere around the plant, the diffusional isotope fractionation of CO_2 into the plant, and a large biochemical/enzymatic isotope fractionation. The oxygen and hydrogen isotope composition of plant tissues typically have strong geographic correlations, reflecting largely geographic patterns of isotope ratios of source water to the plant (typically in the form of precipitation in which the isotope composition is affected by fractionation associated with progressive condensation of water from the atmosphere). The plant tissue isotope composition is further modified by processes such as soil water evaporation, transpiration and the biochemical processes associated with biosynthesis, all of which respond to local environmental conditions. Furthermore, isotope ratios can change with time, due to shifting water supplies and other environmental factors. Fractionation may also occur as a result of manufacturing and processing, leading to even more variation of isotopic signatures.[8,9]

Many other types of substances have also been shown to have differing ratios of isotopes based on geographic location, ranging from olive oil and wine to human hair and illicit drugs.[10-14] More specifically, cotton has been shown to have differing isotope ratios based on growth stage and region of the plant, as described above.[15-17] Recently, manufactured and processed cotton has also been analyzed and shown to have different isotopic signatures based on geographic origin.[7]

In this study, this research on processed cotton was extended to investigate the isotope ratios of carbon, oxygen, nitrogen, and hydrogen in commercial items that had been previously worn or used such as cotton t-shirts, towels, and gauze. Cotton samples of various known and unknown origins were analyzed and compared. Three undershirts of the same manufacturing origin were also analyzed, as were the differences between cotton fiber and stitching from the same shirt to test for homogeneity of the shirts. In addition, the effect of coloring on cotton fibers was studied in order to determine if the added colors change the isotope ratios. Faded colors were included as were clothing items where the color had been worn away. Blood, grass and dirt stains were investigated to determine if these external sources affect the isotope ratios of the fibers. Other natural fibers, such as wool and silk, and synthetic fabrics were also investigated. In

order to extend beyond clothing fibers, carpets were included in the study. The final portion of this work involved studying if there is an effect on isotope ratios due to the chemicals used to extract DNA from fibers or exposure to environmental factors, such as snow, water, and sunshine.

Research Design

Cotton, wool, silk, and synthetic cloth was purchased from TestFabrics, Inc. In addition, t-shirts, jeans and carpets were obtained locally. For carbon and nitrogen analysis, samples (~0.200 mg) were weighed out and placed into 3.5 mm x 5 mm tin capsules. The samples were analyzed using a Costech ECS 4010 elemental analyzer (EA) in combination with a ThermoFisher Scientific Delta V Advantage isotope ratio mass spectrometer. The EA contained a combustion tube (1020˚C) that was filled with quartz wool, chromium oxide catalyst, and silvered cobaltous/cobaltic oxide, as well as a reduction tube (650˚C) filled with quartz wool and copper wire. To remove water from the post-combustion gases, a water trap filled with magnesium perchlorate was used. Three reference materials were used for normalization of isotope data with two measurements of each standard done at the beginning, middle and end of the sample batch run. For hydrogen and oxygen analysis, samples (~0.200 mg) were weighed into 3.5 mm x 5 mm silver capsules for ^{2}H and ^{18}O analysis, and loaded into a Thermo/Finnigan high temperature conversion elemental analyzer (TC/EA) coupled to a ThermoFisher Scientific Delta V Advantage isotope ratio mass spectrometer. The ceramic reactor tube in the TC/EA was held at 1450˚C, and was packed with a graphite tube, glassy carbon tube with glassy carbon granulate, and silver and quartz wool. The temperature of the GC column was set to 90˚C with a He flow rate of 95 ml/min. Again, standards were used at the beginning, middle and end of each run in order to normalize the results.

Four samples from the same t-shirt were collected with one sample kept as a control while the other three were dyed a different color with Rit dye in order to investigate the effect that dyes have on isotope ratio values. Portions of the dyed samples were also bleached with sodium hypochlorite in order to study the effect that bleach has on isotope ratio. In a separate study, three regions from the same t-shirt were obtained, with one serving as a control. The other two samples were stained by rubbing the sample against grass or with dirt. After sampling from the stained portions of the t-shirt, the samples were washed with detergents and sampled again from where the stains were present producing five groups of samples to be analyzed: original (control), grass stained, dirt stained, grass stained-washed, and dirt stained-washed.

Fiber samples also underwent a washing protocol used to extract DNA in order to determine if the reagents used in the DNA extraction result in a change in isotope ratio values. Finally, three different cloth samples were buried in snow, exposed to sunlight and rain, or submerged in a pond for one month to study the effects that environmental exposure has on the fiber isotope ratios.

Depending on the study, the isotope ratio for each sample type was measured 6-12 times. Data were collected across multiple days to ensure reproducibility of results. Each measurement was normalized to the certified reference standards using the three-point normalization function calculated for that batch sample run. A Grubb's test was performed by manual calculation (90% confidence level) on the normalized data to determine if any outliers were present. When comparing two sample types, a two-tailed F-test (95% confidence level) was performed in order to determine if the population variances were equal. The sample means were then compared using a two-tailed, two-sample t-test (95% confidence level) in order to determine if the two sample types were distinguishable using the isotope in question. For each study, all pair-wise combinations were analyzed in the same fashion.

Findings

Cotton from Different Sources

Eight different cotton samples were obtained. Three fibers from each of four different regions of each sample were taken to check for homogeneity and to ensure the data were representative of the whole sample. A total of three batches were run over the course of three days, for a total of twelve data points from each cotton sample.

A three-dimensional scatter plot shows the clear differentiability of the data (Figure E-1). The center of each ellipsoid is at the average isotope ratio values for that fiber, with the standard deviations used to provide the width, height, and depth of each ellipsoid. The space filled by each of the eight different types of cotton is isolated from all sides, demonstrating the unique isotopic signature of each sample. Such clear differentiability between each of the samples tested is of significant forensic importance, as a fiber with a given isotopic signature may be correlated to a source with a significant level of certainty in each of our samples.

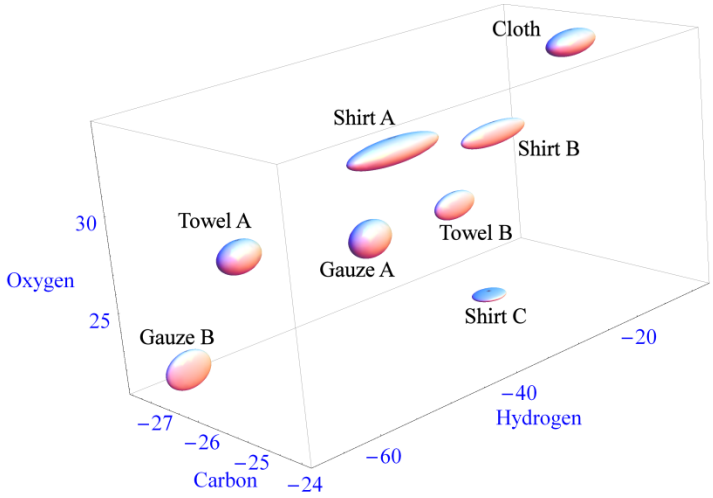

Figure E-1. Three-dimensional scatter plot showing differentiability of eight cotton types.

T-Shirt Homogeneity

Three samples were taken from four different regions of each t-shirt (front and back panels, left and right sleeves) for a total of twelve samples per shirt. This process was repeated for all three t-shirts to test for homogeneity of different regions of the shirts. Two of the t-shirts showed no statistical difference in isotope ratio between any of the four regions for any of the three isotopes. This demonstrates the homogeneity of the cotton material used in the shirt manufacturing process, which is important for the matching of a fiber found at a crime scene to a shirt in the possession of a suspect. However, one shirt exhibited isotopic differences between the back panel and the two sleeves. This discrepancy between different parts of the shirt could arise from different bolts of cloth being used for each part of the shirt, with the various parts later stitched together. This implies that in an IRMS forensic investigation, fiber samples from each separate part of the shirt (front panel, back panel, sleeves, pocket, etc.) should be analyzed and the isotope ratios of each part compared to the evidence in question.

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Cotton from Different Years/Manufacturing Processes

In order to investigate whether isotope ratios are affected by manufacturing process and/or the year of manufacture, four bolts of cotton cloth that underwent different factory processing from two different years were purchased. Three of the four cloth samples were purchased in 2011 and 2013 from the same manufacturer to study if the year of manufacture affected the isotope ratio values.

All seven cotton sources were found to be differentiable, which means temporal differences as well as factory processing can have significant effects on the isotope signatures as can be seen in Figure E-2.

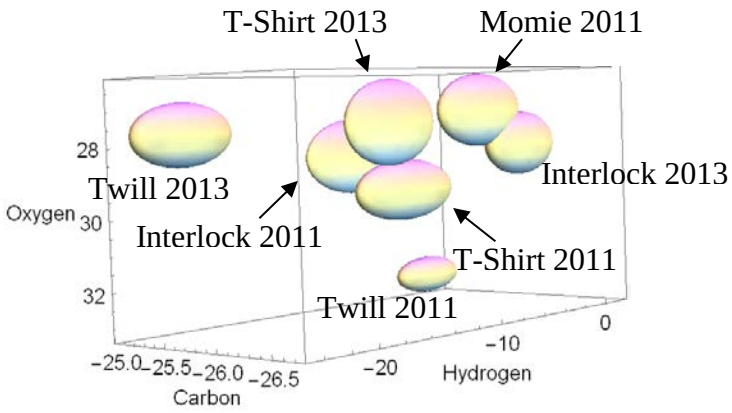

Figure E-2. Three dimensional representations of δ values for carbon, hydrogen, and oxygen for different types of cotton cloth. The ellipses themselves represent the standard deviations, while the centers of the ellipses are the averages.

Thus, fabrics that were made by the same manufacturer using the same process but from two different years were distinguishable, as were fabrics made in the same year but with different manufacturing processes, although different fabrics made in the same year still could have come from different cotton sources.

Colored Shirt

A cotton knit polo shirt consisting of aqua, blue, green, red, and white coloring was analyzed to determine if coloring affects the isotope ratio values. The corresponding tri-variate plot is shown in Figure E-3.

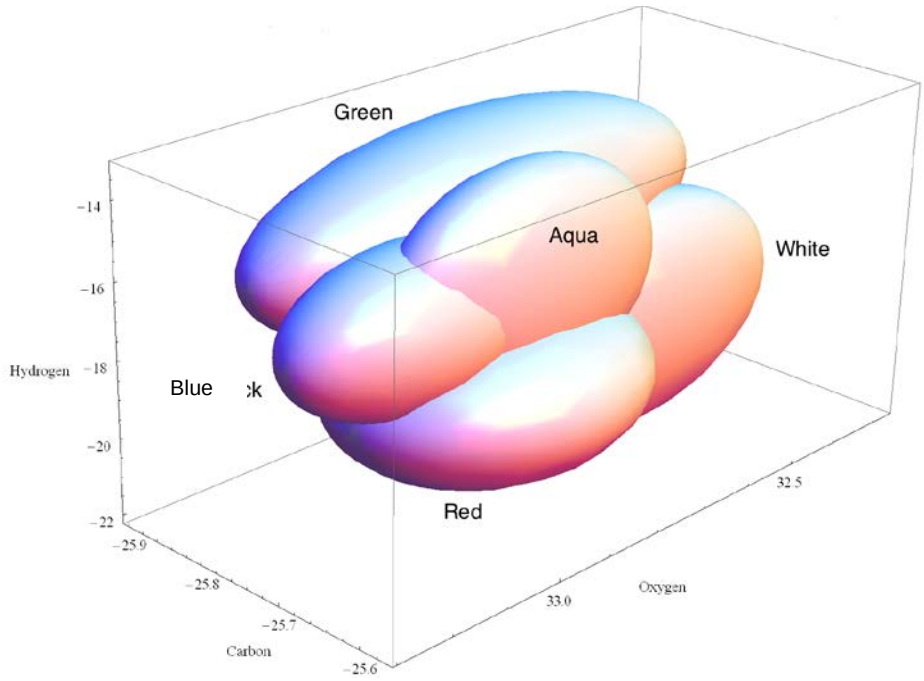

Figure E-3. Carbon, hydrogen, and oxygen isotope ratios (‰) of five different colors from polo shirt (Aqua; Blue; Green; Red; White).

This shirt showed virtually no isotope distinction between different colors. The polo shirt body was constructed from a single thread with distinct color regions. Thus, all fibers, regardless of color, were from the same cotton thread, with coloring added.

Dyed Shirts

The results of the polo-shirt analysis seemed to indicate that for fibers from the same cotton source the addition of color didn't significantly change the isotope ratio values. If this is the case, it would not be critical to match the exact color when comparing fibers from the same source. Since colors fade or are worn away at inconsistent rates, this would make fiber analysis by IRMS much easier. A follow-up study started with cotton from a single source and added different coloring to confirm whether or not the addition of dye changed the isotope ratio values. The results of t-test analysis indicated that the addition of dye, regardless of which color, did not significantly change the isotope ratio values for any of the three elements. This correlates what was found for the dyed polo. Thus, the addition of dye to cotton fibers does not change the isotope ratio values. This is likely due to the relatively small amount of dye added to a comparatively large mass of cotton. Any isotope ratio differences in the dye contributes such a small amount to the total isotopes measured that there is no significant change in the overall measured value.

Bleached Shirts

After the t-shirt materials were dyed, bleach was added to visibly remove the color and the isotope ratios were again measured. Statistical analysis showed that bleaching did result in a significant change in isotope ratios. This is especially true for both oxygen and hydrogen isotopes, which is not surprising since bleach is an oxidizing agent and results in chemical changes to oxygen and hydrogen atoms. Even non-dyed fibers experienced a change in isotope ratio after exposure to bleach. To investigate whether bleaching had a similar effect on

commercially colored shirts, bleach was added to the multi-colored button-down shirt and the isotope values were measured and compared to pre-bleached values. Figure E-4 shows the tri-variate plot of the isotope ratio values before and after bleaching.

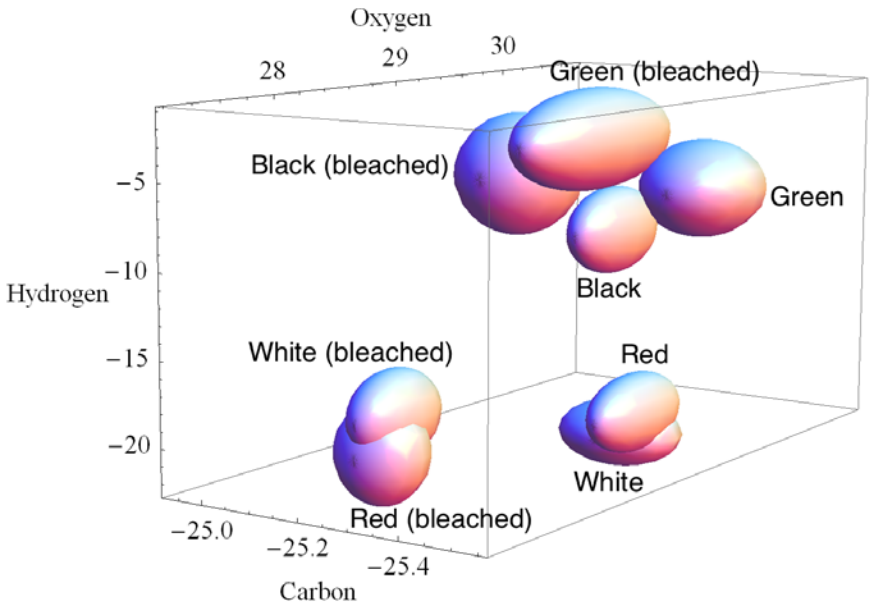

Figure E-4. Carbon, hydrogen, and oxygen isotope ratios (‰) of samples taken of four different colors from body of button-down shirt pre- and post-bleaching.

Consistent with what was observed with the pre- and post-bleaching of the t-shirt, significant isotope ratio differences were observed for all four colored fibers. Therefore, bleached fibers should not be compared to non-bleached fibers, as the isotope ratios will be different due to the bleaching action itself, regardless of whether the cotton fibers initially had similar isotope ratio values.

Worn Jeans Material

Since the addition of clothing dye didn't lead to significant changes in the isotope, the opposite experiment was undertaken. Three pair of blue jeans were sampled with the material subsequently rubbed on a rough surface until most of the color was visibly worn away. When pulled apart, jean material consists of a dyed thread along with a non-dyed thread. Both of these threads were sampled, with the dyed thread rubbed in order to remove the dye. The worn sections were then resampled. Consistent results were observed from all of the jeans; threads from the white cross-stitch were shown to be differentiable from the blue threads or rubbed blue threads as shown in Figure E-5 for one representative pair of jeans. The blue threads were statistically indifferentiable from the rubbed blue threads, which further supports the results from the dyeing experiment that dyes do not significantly alters that isotope ratios of cotton threads.

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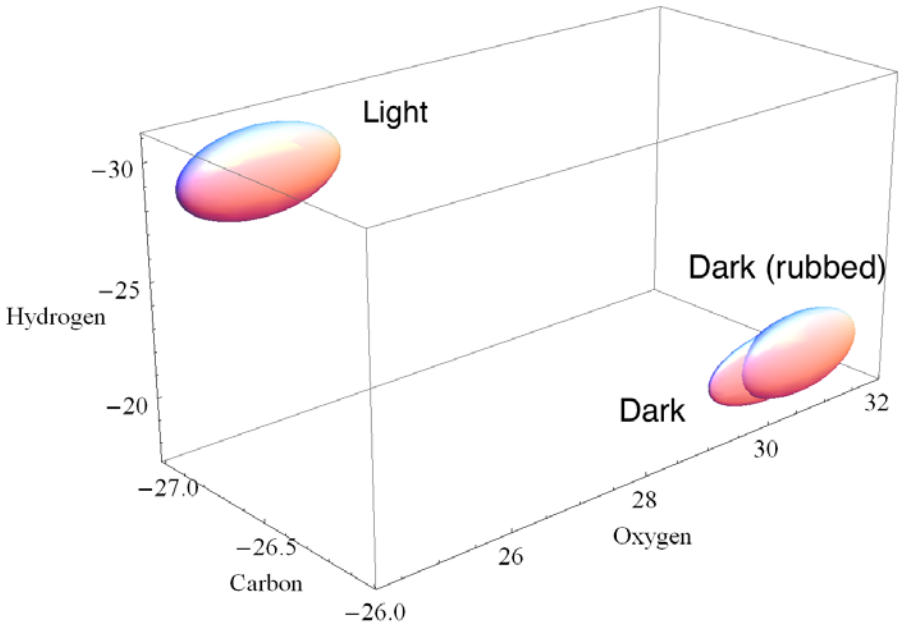

Figure E-5. Carbon, hydrogen, and oxygen isotope ratios (‰) of threads from a pair of jeans, including cross-stitch (Light), blue thread (Dark), and blue thread with dye physically rubbed-off (Dark (rubbed)).

Wool and Silk

Five non-colored wool samples were purchased from two different years. These samples included two different types of wool (worsted and jersey) that were each purchased in both years in order to determine if there were isotope ratio differences between temporally distinct samples made by the same manufacturer. Using nitrogen, oxygen and hydrogen isotope ratios, all five of these wool fibers were differentiable. Eight different silk samples were purchased, with the same four fabric types purchased in 2011 and again in 2013. Using a combination of isotope ratio measurements, all eight silk samples were differentiable from one another.

Since they are natural fibers, the same environmental factors that affect cotton from different locations and/or years would be expected to have similar effects on both silk and wool. Silk and wool from different years could be differentiated using isotope ratios as could different fabric types, which could either be the result of different manufacturing processes or of raw material from different initial sources. It therefore appears that naturally-derived fibers in general may be able to be distinguished from one another using isotope ratio analysis.

Synthetics

Six different synthetic fabrics were purchased in 2013 and again in 2014. As fibers from these fabrics are chemically different from one another, other simpler methods can be used to differentiate the fibers from the same year from one another. In order to determine if synthetic fibers of the same chemical composition can be distinguished using isotope ratios, the $\delta^{13}C$ and $\delta^{15}N$ ratios were measured for each fiber and statistically compared to the same fiber type from the other year. The combination of carbon and nitrogen isotope ratios allowed for all fiber combinations to be distinguished. Therefore, hydrogen and oxygen isotope analysis was not done, but adding these elements could add further discrimination power. As was found with cotton fibers, chemically consistent synthetic fibers can likely be differentiated using isotope ratio analysis.

Carpet

Since synthetic clothing fibers were found to be differentiable, six different nylon carpet samples were obtained and eight replicate samples were analyzed for each. These six carpet samples included two each of three different colors. Using carbon, oxygen and hydrogen isotope ratios, all six carpet samples could be differentiated, even when the color was visibly identical, as shown in Figure E-6.

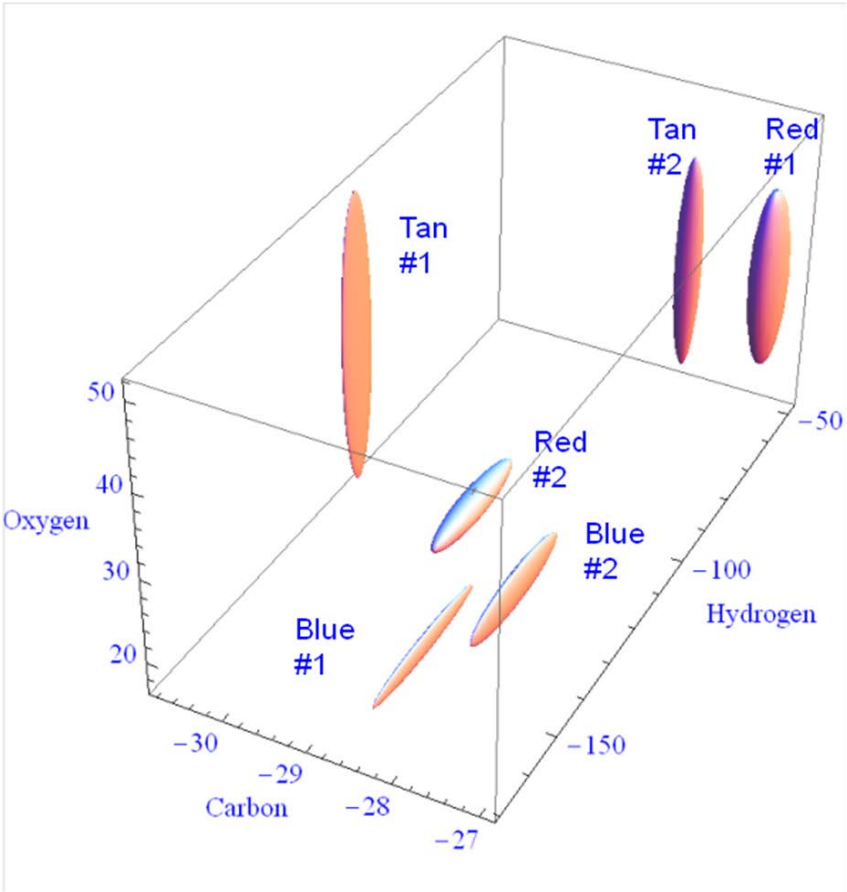

Figure E-6. Carbon, oxygen, and hydrogen isotope ratios from a variety of carpet samples.

Thus, as would be expected based on the synthetic fiber analysis discussed above, different carpet fibers could be differentiated, even when they had similar coloring patterns. Thus, isotope ratio analysis can be applied to non-clothing fibers, such as carpet from a crime scene or from a car trunk, in order to potentially show similarity between two fibers or to exclude two fibers as having originated from the same source.

Effect of Surface Stains

Different samples from a t-shirt were stained with grass and dirt. Since all samples came from the same t-shirt, the only changes to the isotope ratios would occur through its stain treatment and preparation. When the five groups of t-shirt samples: original (control), grass stained, dirt stained, grass stained-washed, and dirt stained-washed, were analyzed, all five samples were found to be indistinguishable from each other. A three-dimensional trivariate plot, shown in Figure E-7, allows for easy visualization of this. The overlap between the ‘bubbles’ in

the plot is consistent with the lack of differentiability between analytes. Thus, the introduction of stains, at least surface stains such as grass and dirt, does not appear to significantly change the isotope ratio values for the fibers. This is most likely due to the relatively small mass of the stain material compared to the mass of the cotton fibers. Since the stain material makes up such a small percentage of the overall total mass, it does not affect the overall measurement, even though the stain material most likely has a different isotope ratio value than does the cotton fiber itself.

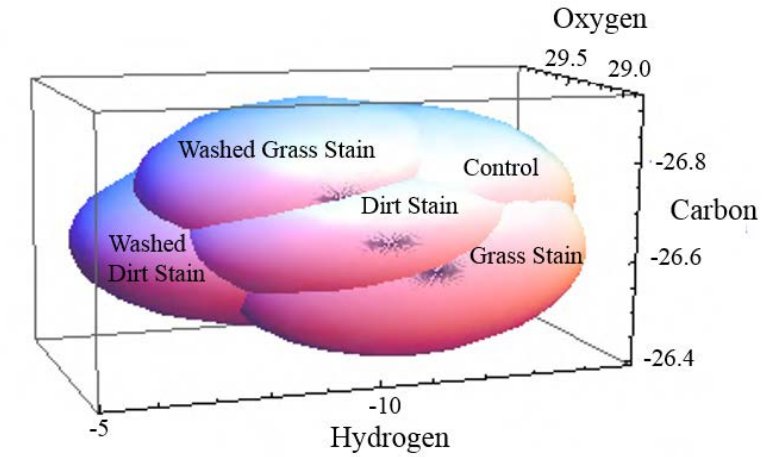

Figure E-7: Trivariate plot of grass- and dirt-stained fibers

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Effect of Blood stains

Another type of stain that could be expected at a crime scene is blood. Cow blood was introduced to sampled white cotton, allowed to soak into the fabric, dry and was then washed off with cold running water. The blood stained fabric was resampled and the isotope ratios analyzed along with the pre-stained fibers. In this case, all three isotope values can be used to distinguish the blood-stained fibers from the non-blood stained fibers. Thus, blood stained fibers show a difference in their isotope ratio values. In contrast to grass and dirt stains, the blood stains can penetrate the entire fiber, and may also deposit cells which can be retained in the fibers, even after washing with water. Therefore, blood stained fibers found at a crime scene should not be used to compare to non-blood stained fibers, unless care is taken to ensure all traces of blood have been removed.

Effect of DNA Extraction

Fibers are routinely analyzed for DNA content. This involves adding chemicals to extract the DNA. If these extraction chemicals do not affect the isotope ratio values, fibers could first be treated to remove any DNA present and then analyzed for isotope ratios. Ten fiber samples were obtained from six fabric types, including natural, synthetic, and blended fabrics. The DNA extraction protocol was then performed on each fabric and samples were again collected. The statistical analysis of the isotope ratio results showed that carbon and nitrogen isotopes are not affected by the DNA extraction process. Two fabrics showed statistically significant differences

in hydrogen isotope results and one fabric showed differences in oxygen isotope ratio values between the pre- and post-treated fibers. These results should be further studied but indicate that isotope ratio analysis should not be undertaken, at least for oxygen and hydrogen, for fibers that have underwent DNA extraction as the processing step may change the isotope ratio results.

Effect of Environmental Exposure

For fibers found outside, environmental exposure is a potential source of contamination and could lead to changes in isotope ratio values. In order to investigate the possible effect of environmental conditions, three fabrics were each exposed to different environmental conditions. One set was buried in the snow, a second set exposed to sunlight and rain, and a third submerged in a pond. After one month, the fabrics were washed to remove any large visible contaminants and sampled. The observed $\delta^{13}C$ isotope ratio values were compared to those obtained from control fibers that had not been exposed to the elements. Three of the nine comparisons with the control samples showed a difference in the carbon isotope ratio after environmental exposure. Upon closer inspection of the fibers, the exposed fibers were found to be stained, even after washing. These stains likely represent the inclusion of non-fabric matter that was not washed away. Therefore, if isotope ratio analysis is to be used for fibers exposed to the elements for relatively long periods of time, a better washing protocol must be developed. Otherwise, fibers exposed to the elements may not be able to be compared with fibers that were not exposed to the same conditions.

Conclusions

The research presented here indicates that isotope ratio mass spectrometry might be a valuable forensic analytical tool for the analysis of fibers. Both natural and synthetic fibers were shown to be differentiable from chemically similar fibers using a combination of isotope ratio measurements. This was true for fibers made by different companies as well as for those made by the same company but in two different years. Fibers from the same regions of the same garment, or from garments packaged together, were indistinguishable from one another by IRMS. Thus, fibers from the same source would be expected to have the same isotope ratio profile, while fibers from different sources can likely be differentiated even if they are made from the same chemical composition. While isotope ratios are not as unique as fingerprints or DNA, they are more distinctive than fiber color or chemical composition and thus, isotope ratio analysis would add an additional method for the comparison of fibers to determine if two fibers potentially have common origins. Coloring, surface stains or the wearing away of color from fibers does not appear to alter the isotope ratios to a significant extent. Exposure to other conditions, such as blood, bleach, DNA extraction chemicals and the elements can affect the isotope ratios, so these need to be considered if IRMS is to be used to analyze fibers.

While fiber analysis by IRMS cannot conclusively indicate that two fibers came from the same source, it can exclude a common source for two fibers. For fibers that are found to have the same isotope ratios, this result provides additional circumstantial evidence that they might be related. This analysis would enhance the ability to compare fibers over what is currently done and thus could find use as a forensic analysis technique in certain circumstances. While many instances may not require this technique, it could be useful in excluding or possibly including fibers from a common source.

I. Introduction:

Fibers are of special importance to forensic analysis due to their prevalence at crime scenes. Therefore, it is important to analyze these materials for any differentiating characteristics that could be used to connect a suspect with a fiber found at a crime scene, or to exclude potential suspects. In the past, various methods have been used to identify and analyze fibers. Infrared (IR) spectroscopy can discriminate different types of fibers (cotton, silk, polyester, etc.) with similar appearances.[1,2] Further IR and Raman spectroscopy analysis can differentiate fibers with different dyes and contaminants.[3] However, these techniques are unable to distinguish between different fibers of the same material and color (i.e. two different white cotton fibers). Therefore, without the differentiating chemical and visible characteristics (such as color, texture, etc.) that allow for the differentiation of fibers,[4,5] different methods need to be used to distinguish between different fiber samples. Oxygen isotope analysis has been shown to have potential for the analysis of currency.[6] Recent research suggests that by using isotope ratio mass spectrometry (IRMS) to analyze the ratios of isotopes in bleached cotton fibers it is possible to distinguish between samples based on their geographic origins.[7]

IRMS can differentiate between these cotton samples because the percent abundances of given elements (i.e. the ratios of heavy to light isotopes) differ under various conditions.[8] Variations in isotope abundances of light elements are caused by processes that favor one isotope over another, a process called fractionation. Carbon isotope variations in plants reflect the isotope composition of the reactant (CO_2) in the ambient atmosphere around the plant, the diffusional isotope fractionation of CO_2 into the plant, and a large biochemical/enzymatic isotope fractionation. The oxygen and hydrogen isotope composition of plant tissues typically have strong geographic correlations, reflecting largely geographic patterns of isotope ratios of source water to the plant (typically in the form of precipitation in which the isotope composition is affected by fractionation associated with progressive condensation of water from the atmosphere). The plant tissue isotope composition is further modified by processes such as soil water evaporation, transpiration and the biochemical processes associated with biosynthesis, all of which respond to local environmental conditions. Furthermore, isotope ratios can change with time, due to shifting water supplies and other environmental factors. Fractionation may also occur as a result of manufacturing and processing, leading to even more variation of isotopic signatures.[8.9]

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In this study, this research on processed cotton was extended to investigate the isotope ratios of carbon, oxygen, nitrogen, and hydrogen in commercial items that had been previously worn or used such as cotton t-shirts, towels, and gauze. Cotton samples of various known and unknown origins were analyzed and compared. Three undershirts of the same manufacturing origin were also analyzed, as was the differences between cotton fiber and stitching from the same shirt to test for homogeneity of the shirts. In addition, the effect of coloring on cotton fibers was studied in order to determine if the added colors change the isotope ratios. Faded colors were included as were clothing items where the color had been worn away. Blood, grass and dirt stains were investigated to determine if these external sources affect the isotope ratios of the fibers. Other natural fibers, such as wool and silk, and synthetic fabrics were also investigated. In

order to extend beyond clothing fibers, carpets were included in the study. The final portion of this work involved studying if there is an effect on isotope ratios due to the chemicals used to extract DNA from fibers or exposure to environmental factors, such as snow, water, and sunshine.

The initial hypotheses were that cotton fibers would continue to be differentiable based on isotope ratios once the cloth had been made into clothing items and that the addition of color to fibers would lead to significant changes in the overall isotope ratios for the fibers. While we hypothesized that isotope ratios from synthetic fibers would be measureable, it was unknown if there would be differences in these ratios since synthetic fibers are not grown and thus are not subject to the same isotope fractionation processes that are inherent in natural fibers.

II. Methods

2.A. Materials

The majority of the cotton, wool, silk, and synthetic cloth was purchased from TestFabrics, Inc. In addition, t-shirts were purchased locally and old, worn jeans were donated to the lab. Carpets were obtained as scrap from a local floor covering store. All consumable instrument reagents (copper wires, quartz wool, chromium oxide catalyst, silvered cobaltous/cobaltic oxide, glass reaction tubes, magnesium perchlorate, graphite tube, glassy carbon tube, glassy carbon granulate, and silver wool) were purchased from either Costech, or EA Consumables.

2.B. ^{13}C and ^{15}N isotope analysis

Samples (~0.200 mg) were weighed out and placed into 3.5 mm x 5 mm tin capsules for analysis of their nitrogen and/or carbon isotope ratios. The samples were introduced into an auto-sampler, which was sealed and purged with helium to avoid atmospheric contamination and analyzed using a Costech ECS 4010 elemental analyzer (EA) in combination with a ThermoFisher Scientific Delta V Advantage isotope ratio mass spectrometer. The EA contained a combustion tube (1020˚C) that was filled with quartz wool, chromium oxide catalyst, and silvered cobaltous/cobaltic oxide, as well as a reduction tube (650˚C) filled with quartz wool and copper wire. To remove water from the post-combustion gases, a water trap filled with magnesium perchlorate was used. Three reference materials were used for normalization of isotope data with two measurements of each standard done at the beginning, middle and end of the sample batch run. These six reference standard measurements allowed for instrumental drift to be monitored and were averaged in order to normalize the sample isotopic data. For carbon analysis, corn starch ($\delta^{13}C$ = -11.01‰)[18], asparagine ($\delta^{13}C$ = -24.45‰) and coumarin ($\delta^{13}C$ = -35.60‰)[19] were used in order to bracket the range of ^{13}C isotope values observed. For nitrogen analysis, Glutamic acid #41 ($\delta^{15}N$ = 47.57‰) [20], USGS34 Nitrate ($\delta^{15}N$ = -1.8‰) [21], and Glutamic acid #40 ($\delta^{15}N$ = -4.52‰) [20] were used.

2.C. ^{2}H and ^{18}O isotope analysis

Samples (~0.200 mg) were weighed into 3.5 mm x 5 mm silver capsules for ^{2}H and ^{18}O analysis, and loaded into an auto-sampler, which was sealed and purged with helium to avoid atmospheric contamination. A Thermo/Finnigan high temperature conversion elemental analyzer (TC/EA) coupled to a ThermoFisher Scientific Delta V Advantage isotope ratio mass

spectrometer was used to analyze the samples. The ceramic reactor tube in the TC/EA was held at 1450˚C, and was packed with a graphite tube, glassy carbon tube with glassy carbon granulate, and silver and quartz wool. The temperature of the GC column was set to 90˚C with a He flow rate of 95 ml/min. Two keratin standards, kudu horn (KHS, δ^2H = -54.1‰, $\delta^{18}O$ = 21.21‰)[22] and caribou hoof (CBS, δ^2H = -197‰, $\delta^{18}O$ = 2.39‰)[22], as well as coumarin (δ^2H = 82.3‰)[19] were used for normalization of the ^2H data, with benzoic acid 602 (BA602, $\delta^{18}O$ = 71.28‰)[23] replacing coumarin for normalization of the ^{18}O data. Two measurements of each standard were done at the beginning, middle and end of each sample batch run.

All samples and standards were equilibrated under the same atmospheric conditions for 72 hours prior to analysis to allow for equal deuterium exchange. This allowed for comparison of deuterium ratios for samples from the same batch even though absolute deuterium ratios were not calculated due to this exchange. For cotton fibers, 30% of the hydrogen atoms are exchangeable. Silk and Rayon also contain approximately 30% exchangeable H, while nylon contains 9%. Dacron, Creslan, Orlon, polyester and polypropylene fibers do not contain exchangeable hydrogen. In order to be able to directly compare hydrogen isotope data, all measurements were done in the same batch for all studies described below except for those in sections 3.A.1, 3.B.3, and 3.C.2.

2.D. Dyed and Bleached t-shirt Analysis

A T-shirt was selected from the pack of three Hanes white cotton T-shirts for this experiment. There were nine total samples types in this experiment taken from three different regions in the front panel of the T-shirt: regions A, B, and C. Ten replicate isotope ratio measurements were made from each sample region. The first three sample types were the original threads in these regions. The next three sample types were from the same regions after each was dyed a different color with Rit dye. The instructions on the Rit dye container were followed for application, rinsing, and drying in a commercial dryer. Location A was dyed green, B was dyed blue, and C was dyed red. The dyed fabrics of these locations were then bleached with sodium hypochlorite.

2.E. Stain Analysis

A single Hanes white cotton t-shirt was obtained from a 5-pack of shirts. After sampling from the body of the shirt, the sample source was cut laterally. The front half of the shirt was rubbed vigorously for 60 seconds against grass to produce a dark stain, while the back half of the shirt was treated similarly with dirt. After sampling from the stained portions of cotton, the shirt halves were washed with detergents and sampled again from where the stains were present. This procedure yielded five groups of samples to be analyzed: original (control), grass stained, dirt stained, grass stained-washed, and dirt stained-washed.

2.F. DNA Processing Analysis

DNA extraction buffer was made by making a solution of 0.01 M Tris base, 0.012 M sodium chloride, and 0.125 M disodium edetate buffered to pH 8. 320 µL of this solution was mixed with 40 µL of 20% SDS and 40 µL of 0.39 M DTT to make the final DNA Stain Extraction Buffer. Six different fiber types were processed as if they had DNA present that was to be extracted. Fibers were placed into a 1.5 mL microcentrifuge tube. 400 µL of DNA Stain Extraction Buffer was added followed by 10 µL of Proteinase K (20 mg/mL in 18 MΩ water). The tube was centrifuged at 10,000 g for 30 seconds to ensure all reagents were at the bottom of

the tube and then incubated at 56 ˚C for two hours. The tube was vortexed for 30 seconds and centrifuged at 10,000 g for 30 seconds to pool the reagents at the bottom of the tube. The fibers were removed, allowed to air dry and analyzed by IRMS.

2.G. Environmental Analysis

Four samples from each of three different fabric types (a cotton t-shirt, blue cotton denim jeans, and a 50:50 polyester:cotton blended jersey fabric) were obtained. Three wood holders were constructed by drilling three one inch holes through each holder. A sample of each of the three fabric types was then nailed over the three holes of each holder such that each of the three wooden holders had all three sample types attached to it. One set was buried in the snow, a second set was exposed to sunlight and rain, and a third set was submerged in a pond. After a one month exposure, the fabrics were removed from the boards and washed to remove any large visible contaminants. IRMS samples were taken from the center of each fabric in order to avoid sampling near the nail holes. The exposed fibers were then analyzed along with a control set of fibers that had not been exposed to the environment.

2.H. Statistical Analysis

The results of the measurements are expressed as δ-values, which are numerical representations of the ratio of the heavier isotope to the lighter isotope of a given element in comparison with international isotope standards: VPDB (Vienna Pee Dee Belemnite) for ^{13}C analysis, air for ^{15}N analysis, and VSMOW (Vienna Standard Mean Ocean Water) for ^{2}H and ^{18}O analysis. These values are derived from Equation 1, where R_{sample} is the ratio of heavier to lighter isotopes in the sample, and $R_{standard}$ is the same measured ratio of the corresponding reference material.[8]

$$\delta = \frac{1000 \times (R_{sample} - R_{standard})}{R_{standard}} \quad (1)$$

Depending on the study, the isotope ratio for each sample type was measured 6-12 times. Data were collected across multiple days to ensure reproducibility of results. Each measurement was normalized to the certified reference standards using the three-point normalization function calculated for that batch sample run. A Grubb's test was performed by manual calculation (90% confidence level) on the normalized data to determine if any outliers were present. When comparing two sample types, a two-tailed F-test (95% confidence level) was performed in order to determine if the population variances were equal. The sample means were finally compared in order to determine if the two sample types were distinguishable using the isotope in question. Two-tailed, two-sample t-tests (95% confidence level) were performed using either equal or unequal variances, depending on the results of the F-test. For each study, all pair-wise combinations were analyzed in the same fashion. The F-tests and t-tests were done using the Data Analysis add-on in Microsoft Excel.

Data were compiled into a three-dimensional trivariate plot in Wolfram Mathematica for visualization. Averages were treated as points with the standard deviations for each of the three

isotopes extended in the x, y, and z directions and covered in a surface, creating an ellipsoid around the central average.

III. Results

3.A. White Cotton

Since cotton does not contain nitrogen, only ^{13}C, ^{2}H, and ^{18}O isotope analyses were performed for cotton samples using the methods described previously.

3.A.1. Cotton from Different Sources

Eight different cotton samples were obtained. A men's Dockers t-shirt manufactured in Thailand was purchased, as was a men's Stafford t-shirt made in the United Arab Emirates and a boy's Hanes t-shirt that was made in Honduras. Two different cotton gauze pads, both made in China, were purchased from Dukal Corp. and Dynarex Corp. A sample of bleached mercerized cotton twill was purchased from TestFabrics, Inc. in the United States, and two different cotton towels of unknown origin were obtained.

Three fibers from each of four different regions of each sample were taken to check for homogeneity and to ensure the data were representative of the whole sample. A total of three batches were run over the course of three days, for a total of twelve data points from each cotton sample. The average and standard deviations for the normalized isotope ratios are shown in Table 1.

Table 1. Average ± standard deviations of $\delta^{13}C$, $\delta^{18}O$, and $\delta^{2}H$ values (‰) for eight cotton samples.

Sample	$\delta^{13}C$	$\delta^{18}O$	$\delta^{2}H$
Dockers	-26.2 ± 0.2	30.3 ± 0.7	-37 ± 7
Stafford	-25.0 ± 0.1	31.2 ± 0.6	-31 ± 5
Hanes	-24.1 ± 0.2	24.7 ± 0.2	-41 ± 2
Towel A	-27.2 ± 0.2	26.1 ± 0.9	-54 ± 3
Towel B	-25.1 ± 0.1	28.1 ± 0.7	-37 ± 3
Cloth	-25.6 ± 0.2	33.8 ± 0.7	-12 ± 4
Dukal	-26.3 ± 0.2	26 ± 1	-41 ± 3
Dynarex	-26.8 ± 0.1	22 ± 1	-65 ± 3

P-values, shown in Table 2, indicate that all eight types of cotton are statistically different in at least one of their $\delta^{13}C$, $\delta^{2}H$, or $\delta^{18}O$ values (Table 2). In many cases, $\delta^{2}H$ values seem to be the least useful due to their large standard deviation, but are still suitable for differentiation in the majority of cases. In addition, since the method of equal treatment was used by allowing the standards and samples to equilibrate with room air to ensure equal hydrogen exchange, the reported $\delta^{2}H$ values are not absolute values, but rather relative values. Thus, $\delta^{2}H$ comparison should be limited to sample batches that were collected and analyzed at the same time.

Table 2. P-values from ANOVA tests showing differentiability of eight cotton samples. The values at the top, middle, and bottom of each cell are for $\delta^{13}C$, $\delta^{18}O$, and $\delta^{2}H$ values, respectively. Bolded and highlighted values are higher than a p-value of 0.05 and are non-differentiable at the 95% confidence level.

	Dockers	**Stafford**	**Hanes**	**Towel A**	**Towel B**	**Cloth**	**Dukal**	**Dynarex**
Dockers	x	<0.001 <0.001 **0.17**	<0.001 <0.001 **0.09**	<0.001 <0.001 <0.001	<0.001 <0.001 **0.79**	<0.001 <0.001 <0.001	**0.30** <0.001 **0.11**	<0.001 <0.001 <0.001
Stafford		x	<0.001 <0.001 <0.001	<0.001 <0.001 <0.001	**0.10** <0.001 <0.001	<0.001 <0.001 <0.001	<0.001 <0.001 <0.001	<0.001 <0.001 <0.001
Hanes			x	<0.001 <0.001 <0.001	<0.001 <0.001 0.01	<0.001 <0.001 <0.001	<0.001 <0.001 **0.79**	<0.001 <0.001 <0.001
Towel A				x	<0.001 <0.001 <0.001	<0.001 <0.001 <0.001	<0.001 **0.27** <0.001	<0.001 <0.001 <0.001
Towel B					x	<0.001 <0.001 <0.001	<0.001 <0.001 <0.001	<0.001 <0.001 <0.001
Cloth						x	<0.001 <0.001 <0.001	<0.001 <0.001 <0.001
Dukal							x	<0.001 <0.001 <0.001
Dynarex								x

A three-dimensional scatter plot shows the clear differentiability of the data (Figure 1). The space filled by each of the eight different types of cotton is isolated from all sides, demonstrating the unique isotopic signature of each sample. Such clear differentiability between each of the samples tested is of significant forensic importance, as a fiber with a given isotopic signature may be correlated to a source with a significant level of certainty in each of our samples.

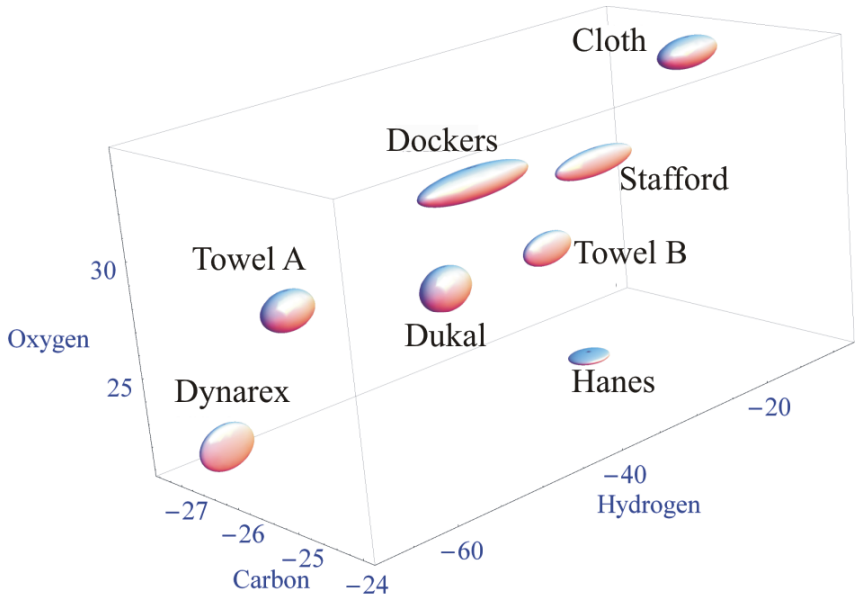

Figure 2. Three-dimensional scatter plot showing differentiability of eight cotton types.

3.A.2 T-Shirt Homogeneity

Three samples were taken from four different regions of each t-shirt (front and back panels, left and right sleeves) for a total of twelve samples per shirt. This process was repeated for all three t-shirts to test for homogeneity of different regions of the shirts. Two of the t-shirts showed no statistical difference in isotope ratio between any of the four regions for any of the three isotopes. This demonstrates the homogeneity of the cotton material used in the shirt manufacturing process, which is important for the matching of a fiber found at a crime scene to a shirt in the possession of a suspect. However, one shirt exhibited isotopic differences between the back panel and the two sleeves (Table 3). This discrepancy between different parts of the shirt could arise from different bolts of cloth being used for each part of the shirt, with the various parts later stitched together. This implies that in an IRMS forensic investigation, fiber samples from each separate part of the shirt (front panel, back panel, sleeves, pocket, etc.) should be analyzed and the isotope ratios of each part compared to the evidence in question.

Table 3. P-values for δ^2H from the Stafford shirt showing statistical differences between the back panel and two sleeve portions of the shirt. Bolded and highlighted values are higher than a p-value of 0.5, and are therefore statistically indistinguishable at the 95% confidence level

Stafford Shirt	Left Sleeve	Right Sleeve	Front	Back
Left Sleeve	x	**0.13**	**0.43**	<0.001
Right Sleeve		x	**0.49**	<0.001
Front			x	**0.67**
Back				x

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3.A.3 T-shirts of Identical Origins

When three Hanes t-shirts from the same package were analyzed, the $\delta^{13}C$, $\delta^{2}H$, and $\delta^{18}O$ values for all three shirts were statistically indistinguishable (Table 4). Standard deviations were also extremely low, indicating the homogeneity within shirts of identical origins. Presumably, shirts that are packaged together were manufactured by the same company in the same location at the same time, with cotton from the same source, leading to the observed similarities. From a forensic standpoint, these results could allow for the testing of any shirt that comes from the same suspected batch as the crime scene sample. This ability could prove to be a useful tool in the event a suspect has disposed of the original shirt in an effort to conceal evidence, since other shirts from the same packaging could equally demonstrate a potential link between suspect and crime.

Table 4. Average δ-values of three Hanes undershirts from the same package showing homogeneity.

		Average ± St. Dev.
Shirt 1	**$\delta^{13}C$**	-24.2 ± 0.2
	$\delta^{18}O$	24.6 ± 0.2
	$\delta^{2}H$	-40.3 ± 1.8
Shirt 2	**$\delta^{13}C$**	-24.1 ± 0.2
	$\delta^{18}O$	24.7 ± 0.1
	$\delta^{2}H$	-40.4 ± 2.3
Shirt 3	**$\delta^{13}C$**	-24.1 ± 0.3
	$\delta^{18}O$	24.7 ± 0.2
	$\delta^{2}H$	-41.5 ± 1.2

3.A.4 Stitching Versus Cloth

Fibers from t-shirt cloth and the stitching used to join different cloth pieces are visibly different, so their isotope ratios were also compared. Stitching fibers and cloth fibers were differentiable for all three shirts tested (Table 5). This presumably arises from the use of different cotton sources for cotton thread and cotton cloth. Such distinct variation between the two parts of the shirt illustrates the importance of testing both stitching and fiber when carrying out forensic analysis. Careful analysis of both types of fiber would help to avoid a false-negative correlation originating from a δ-value from the wrong component of the shirt.

Table 5. δH values of stitching and cloth fibers from three t-shirts. P-values show the differentiability between the two types of fiber.

	Dockers Shirt			Stafford Shirt			Hanes Shirt		
	$\delta^{13}C$	$\delta^{18}O$	$\delta^{2}H$	$\delta^{13}C$	$\delta^{18}O$	$\delta^{2}H$	$\delta^{13}C$	$\delta^{18}O$	$\delta^{2}H$
Stitching Fiber	-25.3 ± 0.1	11.3 ± 0.7	-71 ± 2	-24.7 ± 0.1	11.0 ± 0.4	-68 ± 4	-24.9 ± 0.1	21.9 ± 0.2	-76 ± 2
Cloth Fiber	-26.2 ± 0.2	30.3 ± 0.7	-37 ± 7	-25.0 ± 0.1	31.2 ± 0.6	-31 ± 5	-24.1 ± 0.2	24.7 ± 0.2	-41 ± 2
P-Value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

A three-dimensional scatter plot illustrates the differentiability of stitching and the cloth fibers from each shirt (Figure 2). It also reveals that the fibers are grouped tightly in very different regions of the plot, with the stitching fibers in the bottom left at low $\delta^{2}H$ and $\delta^{18}O$ values, and the cloth fibers grouped in the upper right at higher $\delta^{2}H$ and $\delta^{18}O$ values.

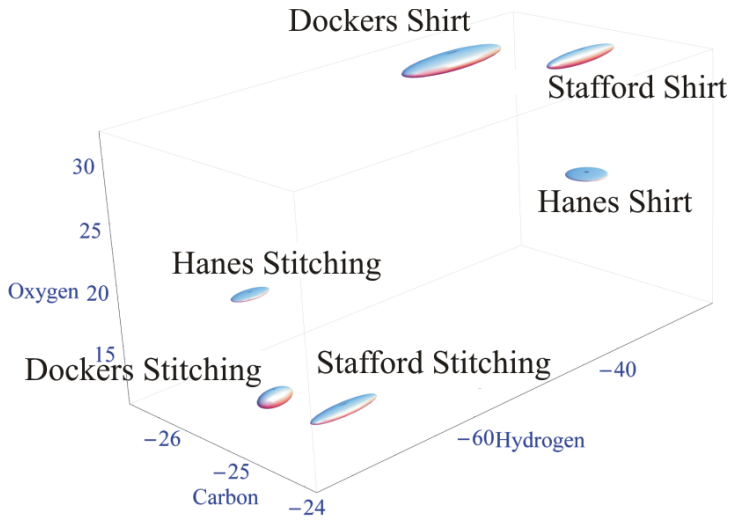

Figure 3. Three-dimensional scatter plot showing differentiability and grouping of stitching and cloth fibers.

3.A.5. Cotton from Different Years/Manufacturing Processes

In order to investigate whether isotope ratios are affected by manufacturing process and/or the year of manufacture, four bolts of cotton cloth that underwent different factory processing from two different years were purchased from TestFabrics, Inc. Samples of t-shirt material, interlock material, twill fabric, and momie cloth were purchased in 2011 and 2013 and examined, except for 2013 momie cloth which could not be obtained.

All seven cotton sources were found to be differentiable, which means temporal differences as well as factory processing can have significant effects on the isotope signatures. As can be seen in Table 6 and Figure 3, $\delta^{13}C$ values were fairly similar, ranging from -26.6 to -25.2, seemingly again not leaving much room for differentiability, but this time the standard deviation is so low (~0.1) that the carbon data are more useful. $\delta^{18}O$ values ranged from 27.3 to 32.7, and $\delta^{2}H$ values ranged from -1.1 to -21.2. The p-values for each isotope are given in Tables 7-9.

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Table 6. Averages ± standard deviations for carbon, hydrogen, and oxygen ratios for the bolts of analyzed cotton cloth, as well as their respective sample sizes (n) after Grubbs testing.

Cotton Type	n	$\delta^{13}C$	n	$\delta^{2}H$	n	$\delta^{18}O$
T-shirt 2011	12	-25.5 ± 0.1	9	-10.0 ± 4.1	12	28.5 ± 1.1
T-shirt 2013	12	-26.6 ± 0.1	9	-10.0 ± 3.5	12	27.3 ± 1.0
Interlock 2011	12	-25.2 ± 0.1	9	-21.2 ± 3.6	12	27.7 ± 0.9
Interlock 2013	12	-26.6 ± 0.1	8	-6.4 ± 3.0	12	28.3 ± 0.9
Twill 2011	12	-25.2 ± 0.1	8	-1.1 ± 2.8	12	32.7 ± 0.6
Twill 2013	12	-26.6 ± 0.1	8	-17.1 ± 3.4	12	27.5 ± 1.2
Momie 2011	12	-25.9 ± 0.1	9	-9.7 ± 4.1	12	29.6 ± 0.9

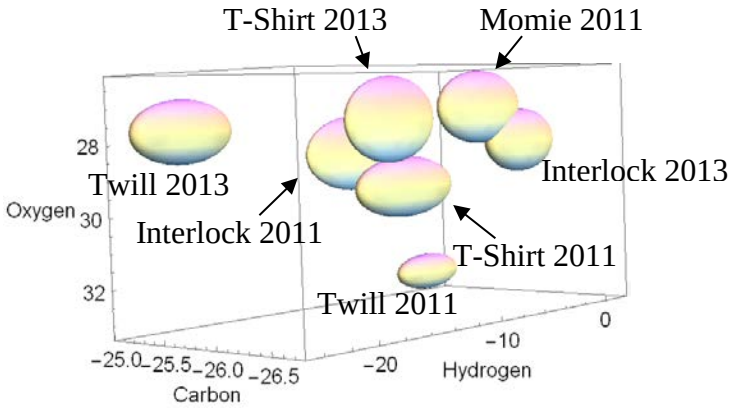

Figure 3. Three dimensional representation of δ values for carbon, hydrogen, and oxygen for different types of cotton cloth. The ellipses themselves represent the standard deviations, while the centers of the ellipses are the averages.

Table 7: P values from the two sample T tests of the cotton carbon data.

(Two tailed)	T-Shirt 2011	Momie 2011	Interlock 2011	Twill 2011	T-Shirt 2013	Interlock 2013	Twill 2013
T-Shirt 2011		<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Momie 2011			<0.001	<0.001	<0.001	<0.001	<0.001
Interlock 2011				0.913	<0.001	<0.001	<0.001
Twill 2011					<0.001	<0.001	<0.001
T-Shirt 2013						0.951	0.426
Interlock 2013							0.420
Twill 2013							

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Table 8: P values from the two sample T tests of the cotton hydrogen data.

(Two tailed)	T-Shirt 2011	Momie 2011	Interlock 2011	Twill 2011	T-Shirt 2013	Interlock 2013	Twill 2013
T-Shirt 2011		0.883	<0.001	<0.001	0.990	0.063	0.002
Momie 2011			<0.001	<0.001	0.865	0.083	0.001
Interlock 2011				<0.001	<0.001	<0.001	0.028
Twill 2011					<0.001	0.002	<0.001
T-Shirt 2013						0.040	0.001
Interlock 2013							<0.001
Twill 2013							

Table 9: P values from the two sample T tests of the cotton oxygen data.

(Two tailed)	T-Shirt 2011	Momie 2011	Interlock 2011	Twill 2011	T-Shirt 2013	Interlock 2013	Twill 2013
T-Shirt 2011		0.022	0.056	<0.001	0.008	0.512	0.036
Momie 2011			<0.001	<0.001	<0.001	0.002	<0.001
Interlock 2011				<0.001	0.284	0.143	0.654
Twill 2011					<0.001	<0.001	<0.001
T-Shirt 2013						0.018	0.594
Interlock 2013							0.087
Twill 2013							

While no one isotope could be used to distinguish all 21 pair-wise combinations, the use of two isotopes, in particular carbon and hydrogen, allowed all fabrics to be distinguished from one another. Thus, fabrics that were made by the same manufacturer using the same process but from two different years were distinguishable, as were fabrics made in the same year but with different manufacturing processes, although different fabrics made in the same year still could have come from different cotton sources.

3.B. Colored Cotton

3.B.1. Colored Shirts

Two multi-colored cotton shirts were analyzed to determine if coloring affects the isotope ratio values. The first shirt was a button-down shirt with white, black, red, and green coloring. Fibers were obtained of each color from both the body of the shirt and from the sleeves, since previous studies (see section 3.A.2) had shown that some shirts present isotope differences between different shirt panels. Eight replicate measurements were made and compared using two-tailed t-testing. The p-values for all color/panel combinations are shown in Table 10 with the corresponding tri-variate plot shown in Figure 4.

Table 10. P-values from two-sample t-tests of threads taken of four different colors from body and sleeves of multicolored button-down shirt (White (body/sleeve), Black (body/sleeve), Green (body/sleeve), and Red (body/sleeve)). A p-value less than 0.05 indicates that the pair of colors are differentiable at 95% confidence level. A p-value greater than 0.05 indicates that they are undifferentiable based on the tested element. (n=8)

	Carbon	Hydrogen	Oxygen
Black (body)/Black(sleeve)	0.49	0.58	0.62
Black(body)/Green(body)	0.03	0.67	0.37
Black(body)/Green(sleeve)	0.12	0.03	0.19
Black(body)/Red(body)	<0.001	<0.001	<0.001
Black(body)/Red(sleeve)	0.009	<0.001	<0.001
Black(body)/White(body)	<0.001	<0.001	<0.001
Black(body)/White(sleeve)	0.04	<0.001	<0.001
Black(sleeve)/Green(body)	0.04	0.43	0.14
Black(sleeve)/Green(sleeve)	0.06	0.12	0.11
Black(sleeve)/Red(body)	<0.001	<0.001	<0.001
Black(sleeve)/Red(sleeve)	0.01	<0.001	0.003
Black(sleeve)/White(body)	<0.001	<0.001	<0.001
Black(sleeve)/White(sleeve)	0.18	<0.001	<0.001
Green(body)/Green(sleeve)	0.59	0.03	0.29
Green(body)/Red(body)	0.32	<0.001	<0.001
Green(body)/Red(sleeve)	0.69	<0.001	<0.001
Green(body)/White(body)	0.13	<0.001	<0.001
Green(body)/White(sleeve)	0.005	<0.001	<0.001
Green(sleeve)/Red(body)	0.06	<0.001	<0.001
Green(sleeve)/Red(sleeve)	0.33	<0.001	<0.001
Green(sleeve)/White(body)	0.03	<0.001	<0.001
Green(sleeve)/White(sleeve)	0.01	<0.001	0.001
Red(body)/Red(sleeve)	0.65	0.19	0.97
Red(body)/White(body)	0.41	0.06	0.94
Red(body)/White(sleeve)	<0.001	<0.001	<0.001
Red(sleeve)/White(body)	0.34	0.81	0.98
Red(sleeve)/White(sleeve)	0.002	<0.001	<0.001
White(body)/White(sleeve)	<0.001	<0.001	<0.001

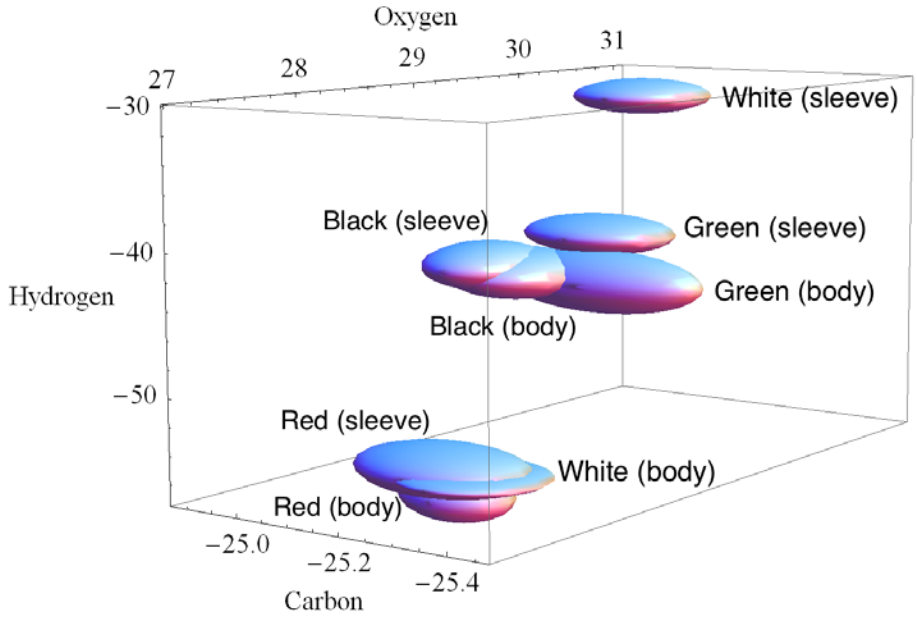

Figure 4. Carbon, hydrogen, and oxygen isotope ratios (‰) of samples taken of four different colors from body and sleeves of button-down shirt.

The results show that for some colors (black and red), there was no significant difference in the isotope ratios measured from the shirt body compared to the shirt sleeves. For other colors (white and green) isotope differences were observed for different shirt panels. Also, some colors (white and green, or red and black for example) were distinguishable from one another, while other color combinations (black and green from the shirt body for example) were statistically indistinguishable from one another. A closer look at the shirt construction revealed that each color was a distinct thread, with multiple threads woven together to create the shirt.

The second shirt was a knit polo shirt consisting of aqua, blue, green, red, and white coloring. Each color was sampled and analyzed (n=10). The p-values for all color combinations are shown in Table 11 with the corresponding tri-variate plot shown in Figure 5.

Table 11. P-values from two-sample t-tests of each pair of colors from polo shirt (Aqua, Blue, Green, Red, and White). A p-value less than 0.05 indicates that the pair of colors are differentiable at 95% confidence level. (n=10)

	Carbon	Hydrogen	Oxygen
Aqua/Blue	0.57	0.99	0.33
Aqua/Green	0.004	0.36	0.72
Aqua/Red	0.69	0.74	0.87
Aqua/White	0.1	0.11	0.18
Blue/Green	0.02	0.24	0.3
Blue/Red	0.97	0.68	0.39
Blue/White	0.32	0.05	0.04
Green/Red	0.08	0.21	0.61
Green/White	0.09	0.001	0.41
Red/White	0.53	0.26	0.12

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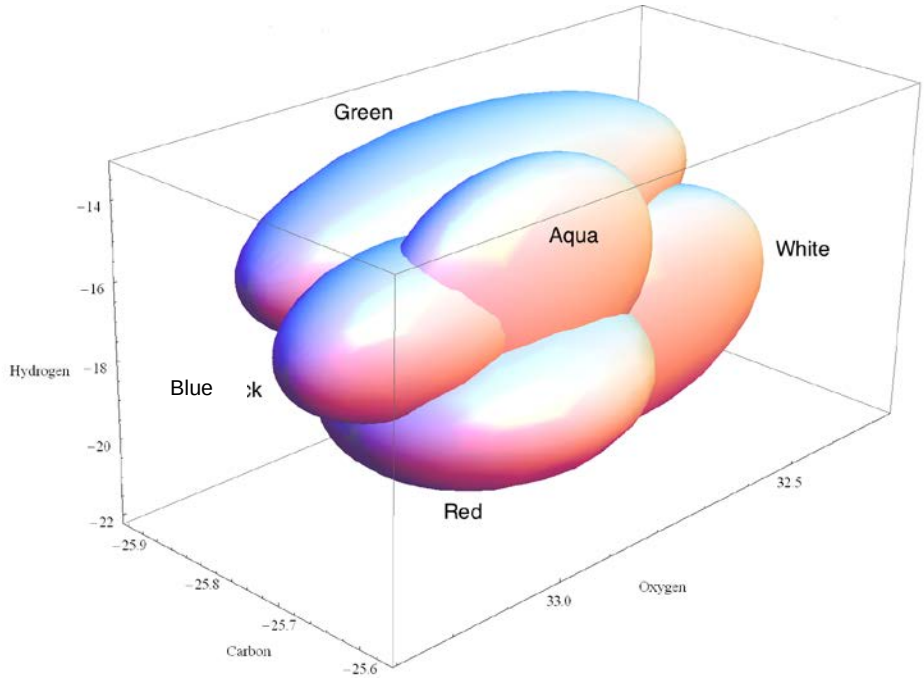

Figure 5. Carbon, hydrogen, and oxygen isotope ratios (‰) of five different colors from polo shirt (Aqua; Blue; Green; Red; White).

This shirt showed virtually no isotope distinction between different colors, as compared to the button-down shirt that did show partial color differentiation based on isotope ratios. Instead of each color being a distinct thread, as was the case with the button-down shirt, the polo shirt body was constructed from a single thread with distinct color regions. Thus, all fibers, regardless of color, were from the same cotton thread, with coloring added, as opposed to the button-down shirt that was comprised of different threads which could have originated from different cotton sources with distinct isotope ratios.

3.B.2. Dyed Shirts

The results of the polo-shirt analysis seemed to indicate that for fibers from the same cotton source the addition of color didn't significantly change the isotope ratio values. If this is the case, it would not be critical to match the exact color when comparing fibers from the same source. Since colors fade or are worn away at inconsistent rates, this would make fiber analysis by IRMS much easier. A follow-up study started with cotton from a single source and added different coloring to confirm whether or not the addition of dye changed the isotope ratio values. Consistent trends were seen in the isotope ratio data for the three regions tested with commercial dye. The δ-values for carbon, hydrogen and oxygen are reported in Table 12.

Table 12: δ-values (‰) $\pm$ standard deviation for carbon, hydrogen, and oxygen isotope ratios for regions A, B, and C; original and dyed threads

	Location A		Location B		Location C	
	Original shirt	Dyed Green	Original shirt	Dyed Blue	Original shirt	Dyed Red
$\delta^{13}C$	-26.56 ±0.24	-26.59 ±0.17	-26.62 ±0.10	-26.70 ±0.19	-26.65 ±0.17	-26.72 ±0.17
$\delta^{2}H$	-5.52 ±3.20	-6.40 ±5.59	-14.20 ±2.57	-13.05 ±3.32	-8.03 ±8.34	-9.94 ±8.56
$\delta^{18}O$	29.56 ±0.90	29.18 ±0.54	27.49 ±2.30	27.40 ±1.93	27.15 ±1.89	27.34 ±2.07

A t-test analysis, shown in Table 13, indicates that the addition of dye, regardless of which color, did not significantly change the isotope ratio values for any of the three elements. This correlates what was found for the dyed polo shirt presented in section 3.B.1. Thus, the addition of dye to cotton fibers does not change the isotope ratio values. This is likely due to the relatively small amount of dye added to a comparatively large mass of cotton. Any isotope ratio differences in the dye contributes such a small amount to the total isotopes measured that there is no significant change in the overall measured value.

Table 13: P-values from ANOVA tests showing non-differentiability between the original and dyed thread in all three regions using carbon, oxygen, and hydrogen isotope ratio data

	Carbon			Oxygen			Hydrogen		
Location	A	B	C	A	B	C	A	B	C
Non-dyed vs. Dyed shirt	0.75	0.21	0.24	0.058	0.81	0.89	0.43	0.44	0.25

3.B.3. Bleached Shirts

After the t-shirt materials were dyed, bleach was added to visibly remove the color and the isotope ratios were again measured. The post-bleach δ–values are shown in Table 14.

Table 14: δ-values (‰) $\pm$ standard deviation for carbon, hydrogen, and oxygen isotope ratios for regions A, B, and C after bleaching

	Location A	Location B	Location C
$\delta^{13}C$	-26.80 ±0.12	-26.76 ±0.10	-26.71 ±1.90
$\Delta^{2}H$	-14.02 ±3.40	-20.89 ±4.26	-17.18 ±2.07
$\delta^{18}O$	26.34 ±0.82	24.48 ±2.55	23.91 ±1.95

Statistical analysis, presented in Table 15, shows that bleaching did result in a significant change in isotope ratios. This is especially true for both oxygen and hydrogen isotopes, which is not surprising since bleach is an oxidizing agent and results in chemical changes to oxygen and hydrogen atoms.

Table 15: P-values from ANOVA tests showing differentiability between the original and dyed threads and bleached threads in all three regions using carbon, oxygen, and hydrogen isotope ratio data. P-values lower than 0.05 indicate a statistical difference between the two sample types.

	Carbon			Oxygen			Hydrogen		
Location	A	B	C	A	B	C	A	B	C
Non-dyed vs. Bleached shirt	0.0096	0.0037	0.28	<0.0001	<0.0001	<0.0001	<0.0001	0.0012	<0.0001
Dyed vs. Bleached shirt	0.0044	0.39	0.83	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

Even non-dyed fibers experienced a change in isotope ratio after exposure to bleach. To investigate whether bleaching had a similar effect on commercially colored shirts, bleach was added to the multi-colored button-down shirt and the isotope values were measured and compared to pre-bleached values. For each color combination, the deuterium isotope ratios for the pre- and post-bleached fibers were collected in the same batch allowing for direct comparison. Figure 6 shows the tri-variate plot of the isotope ratio values before and after bleaching.

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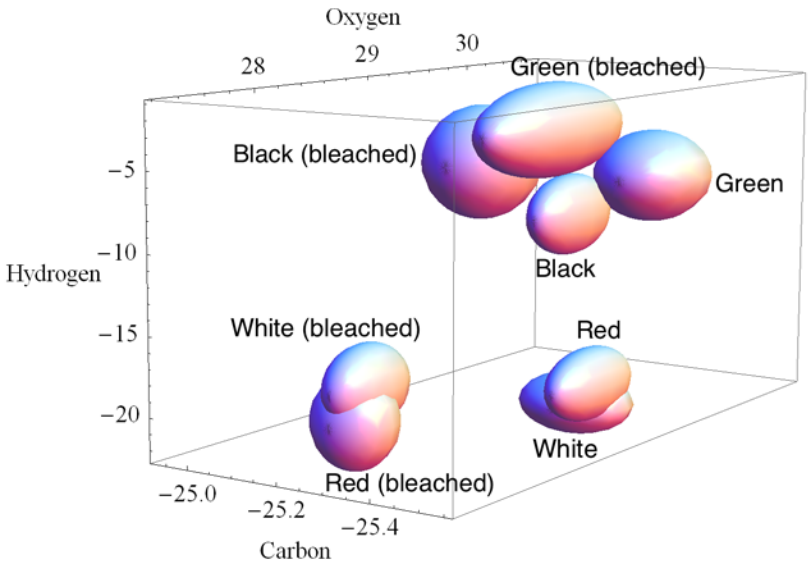

Figure 6. Carbon, hydrogen, and oxygen isotope ratios (‰) of samples taken of four different colors from body of button-down shirt pre- and post-bleaching. (n=11 for carbon, n=12 for hydrogen and oxygen)

Consistent with what was observed with the pre- and post-bleaching of the t-shirt, significant isotope ratio differences were observed for all four colored fibers. Therefore, bleached fibers should not be compared to non-bleached fibers, as the isotope ratios will be different due to the bleaching action itself, regardless of whether the cotton fibers initially had similar isotope ratio values.

3.B.4. Worn Jeans Material

Since the addition of clothing dye didn't lead to significant changes in the isotope ratios (Table 13), the opposite experiment was undertaken. Three pair of blue jeans were sampled with the material subsequently rubbed on a rough surface until most of the color was visibly worn away. When pulled apart, jean material consists of a dyed thread along with a non-dyed thread. Both of these threads were sampled, with the dyed thread rubbed in order to remove the dye. The worn sections were the resampled. Consistent results were observed from all of the jeans; threads from the white cross-stitch were shown to be differentiable from the blue threads or rubbed blue threads as shown in Table 17 and Figure 7 for one representative pair of jeans. The blue threads were statistically indifferentiable from the rubbed blue threads, which further supports the results from the dyeing experiment that dyes do not significantly alters that isotope ratios of cotton threads (Tables 12-13).

Table 16. δ-values (‰) ± standard deviation for jean experiment samples

Jeans	$\delta^{13}C$	$\delta^{18}O$	$\delta^{2}H$
Blue-dyed original	-26.09 ± 0.036	30.45 ± 1.09	-19.10 ± 1.61
Blue-dyed rubbed	-26.08 ± 0.065	30.81 ± 1.03	-19.30 ± 1.86
White cross-stich	-26.92 ± 0.082	25.41 ± 1.09	-29.03 ± 1.93

Table 17. P-values from two-sample t-tests of each pair of threads from a pair of jeans, including cross-stitch (Light), blue thread (Dark), and blue thread with dye physically rubbed-off (Dark

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(rubbed)). A p-value less than 0.05 indicates that the pair of colors are differentiable at 95% confidence level. (n=12)

	Carbon	Oxygen	Hydrogen
Dark/Light	<0.001	<0.001	<0.001
Dark/Dark (rubbed)	0.56	0.47	0.80
Light/Dark (rubbed)	<0.001	<0.001	<0.001

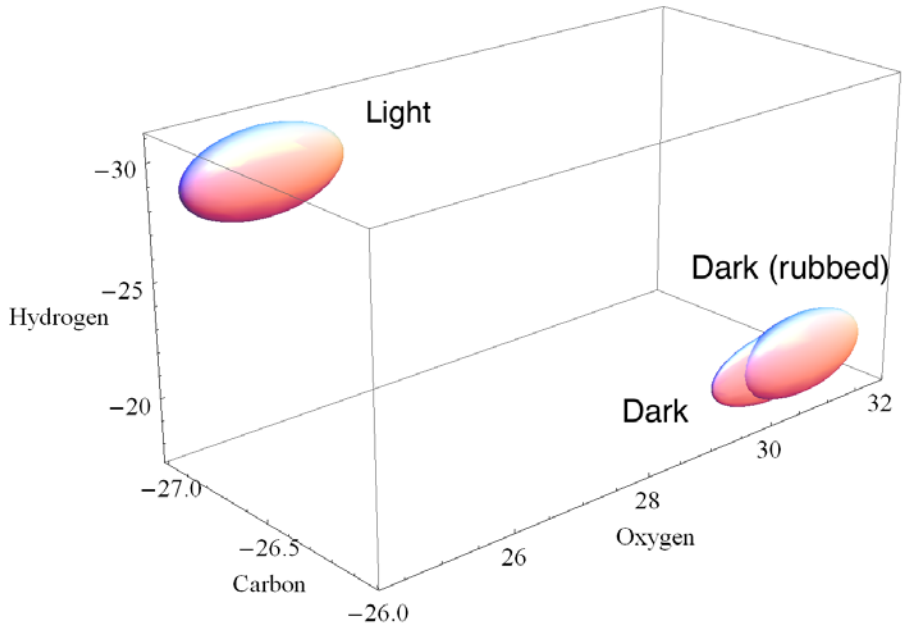

Figure 7. Carbon, hydrogen, and oxygen isotope ratios (‰) of threads from a pair of jeans, including cross-stitch (Light), blue thread (Dark), and blue thread with dye physically rubbed-off (Dark (rubbed)).

3.C. Other Natural Fibers

3.C.1. Wool

Five non-colored wool samples were purchased from TestFabrics from two different years. These samples included two different types of wool (worsted and jersey) that were each purchased in both years in order to determine if there were isotope ratio differences between temporally distinct samples made by the same manufacturer. The results of the isotope ratio measurements are shown in Table 18. Using nitrogen, oxygen and hydrogen isotope ratios, all five of these wool fibers were differentiable, as shown in Figure 8 and Table 19. Each ellipsoid is centered at the average isotope ratio for the measurements, with the standard deviation of each isotope represented by the ellipsoid width. As none of the ellipsoids overlap, all five samples are deemed to be differentiable.

Table 18: Average ± standard deviations of $\delta^{13}C$, $\delta^{15}N$, $\delta^{2}H$, and $\delta^{18}O$ values for wool

	$\delta^{13}C$	**$\delta^{15}N$**	**$\delta^{2}H$**	**$\delta^{18}O$**

Jersey 2011	-23.1 ± 0.7 (n=12)	7.1 ± 0.2 (n=11)	-59 ± 5 (n=12)	19.8 ± 0.8 (n=8)
Jersey 2013	-23.5 ± 0.3 (n=12)	7.0 ± 0.4 (n=12)	-92 ± 4 (n=11)	13.8 ± 0.5 (n=8)
Worsted 2011	-22.9 ± 0.3 (n=11)	7.4 ± 0.2 (n=11)	-61 ± 4 (n=12)	19.2 ± 0.8 (n=8)
Worsted 2013	-22.8 ± 0.4 (n=11)	9.2 ± 0.3 (n=11)	-54 ± 1 (n=12)	20.6 ± 0.7 (n=8)
Flannel 2013	-24.8 ± 0.5 (n=11)	8.8 ± 0.4 (n=12)	-56 ± 3 (n=12)	18.2 ± 0.8 (n=8)

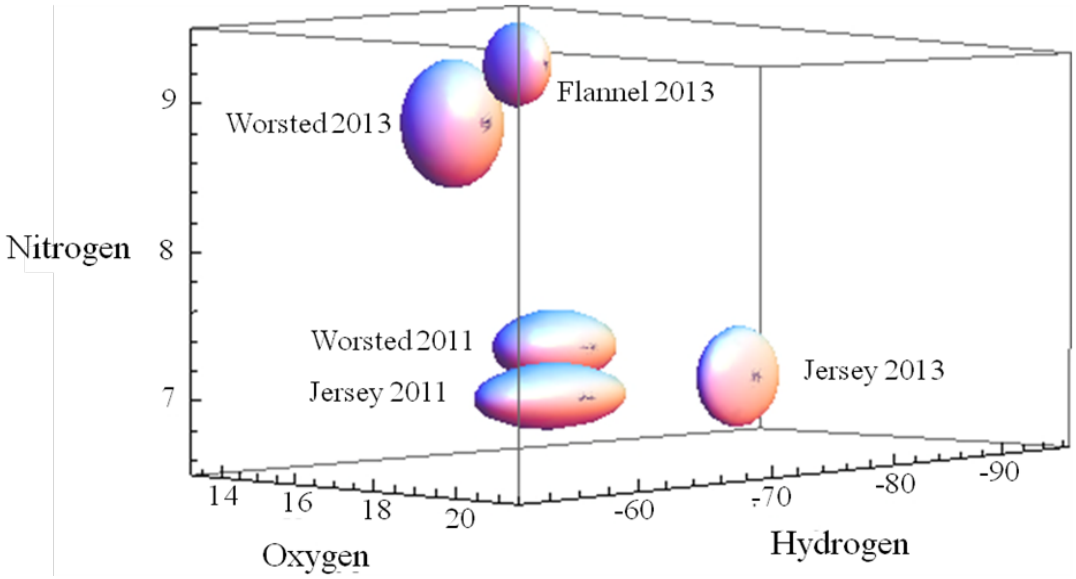

Figure 8. Nitrogen, oxygen, and hydrogen isotope ratios of samples taken from different wool bolts (Jersey, Worsted, and Flannel) purchased in 2011 and 2013.

Table 19. P-values showing differentiability of five wool samples. The order of values from top to bottom in each cell is $\delta^{13}C$, $\delta^{15}N$, $\delta^{2}H$, and $\delta^{18}O$ values. Highlighted values are higher than a p-value of .05 and are non-differentiable at the 95% confidence level.

	Jersey 2013	**Jersey 2011**	**Worsted 2013**	**Worsted 2011**	**Flannel 2013**
Jersey 2013	x	0.108 0.369 <0.001 <0.001	<0.001 <0.001 <0.001 <0.001	<0.001 0.006 <0.001 <0.001	<0.001 <0.001 <0.001 <0.001
Jersey 2011		x	0.235 <0.001 0.005 0.046	0.370 0.006 0.280 0.200	<0.001 <0.001 0.150 0.002
Worsted 2013			x	0.599 <0.001 <0.001 0.002	<0.001 0.034 0.013 <0.001

Worsted 2011		x	<0.001 <0.001 0.004 0.029
Flannel 2013			x

3.C.2 Silk

Eight different silk samples were purchased from TestFabrics, with the same four fabric types purchased in 2011 and again in 2013. Fibers from each fabric were analyzed with the nitrogen, oxygen, and hydrogen isotopes used to differentiate all eight fibers, with the results shown in Table 20. Hydrogen isotope ratios for chiffon and natural noil fibers from both years were analyzed in the same batch, with bleached noil and twill fibers from both years being run in a second batch. Using a combination of isotope ratio measurements, all eight silk samples were differentiable from one another as shown in Figure 9 and Table 21.

Table 20: Average ± standard deviations of $\delta^{13}C$, $\delta^{15}N$, $\delta^{2}H$, and $\delta^{18}O$ values for silk

	$\delta^{13}C$	**$\delta^{15}N$**	**$\delta^{2}H$**	**$\delta^{18}O$**
Chiffon 2013	-26 ± 2 (n=9)	1.6 ± 0.2 (n=8)	-93 ± 3 (n=12)	17 ± 1 (n=12)
Chiffon 2011	-27 ± 1 (n=9)	3.4 ± 0.1 (n=9)	-61 ± 2 (n=12)	23 ± 1 (n=12)
Natural Noil 2013	-27 ± 1 (n=9)	5.8 ± 0.2 (n=9)	-71 ± 2 (n=11)	18.7 ± 0.8 (n=10)
Natural Noil 2011	-27 ± 2 (n=9)	4.6 ± 0.3 (n=9)	-58 ± 2 (n=12)	23.8 ± 0.8 (n=12)
Bleached Noil 2013	-27 ± 1 (n=9)	5.3 ± 0.2 (n=9)	-72 ± 2 (n=12)	19 ± 1 (n=11)
Bleached Noil 2011	-26 ± 1 (n=9)	4.7 ± 0.2 (n=9)	-67 ± 3 (n=12)	19.2 ± 0.7 (n=12)
Twill 2013	-28 ± 1 (n=9)	5.3 ± 0.2 (n=9)	-75 ± 2 (n=12)	19.6 ± 0.9 (n=12)
Twill 2011	-26 ± 1 (n=9)	4.9 ± 0.2 (n=9)	-69 ± 1 (n=12)	20.8 ± 0.8 (n=12)

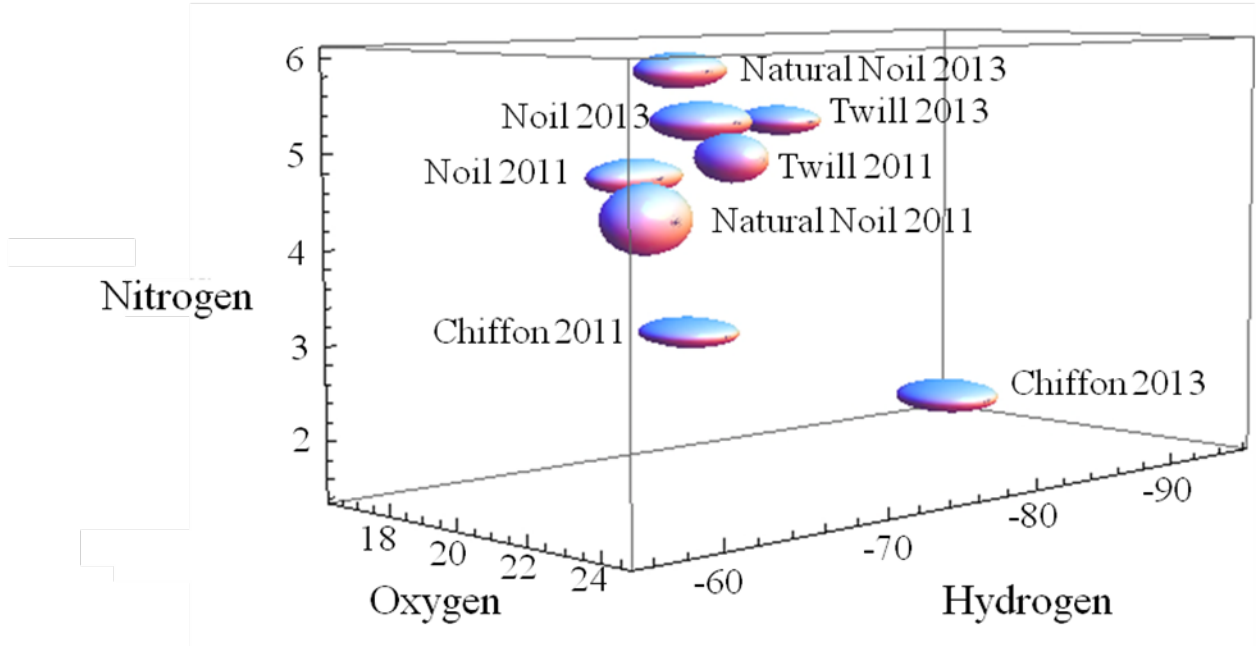

Figure 9. Nitrogen, oxygen, and hydrogen isotope ratios of samples taken from different silk bolts (Chiffon, Twill, Natural Noil, and Bleached Noil) purchased in 2011 and 2013.

Table 21. P-values showing differentiability of eight silk samples. The order of values from top to bottom in each cell is $\delta^{13}C$, $\delta^{15}N$, $\delta^{2}H$, and $\delta^{18}O$ values. Highlighted values are higher than a p-value of .05 and are non-differentiable at the 95% confidence level.

	Chiffon 2013	**Chiffon 2011**	**Natural Noil 2013**	**Natural Noil 2011**	**Bleached Noil 2013**	**Bleached Noil 2011**	**Twill 2013**	**Twill 2011**
Chiffon 2013	x	0.841 <0.001 <0.001 <0.001	0.263 <0.001 <0.001 0.012	0.728 <0.001 <0.001 <0.001	0.229 <0.001 <0.001 0.013	0.931 <0.001 <0.001 <0.001	0.041 <0.001 <0.001 <0.001	0.879 <0.001 <0.001 <0.001
Chiffon 2011		x	0.332 <0.001 <0.001 <0.001	0.872 <0.001 <0.001 0.097	0.278 <0.001 <0.001 <0.001	0.742 <0.001 <0.001 <0.001	0.045 <0.001 <0.001 <0.001	0.691 <0.001 <0.001 <0.001
Natural Noil 2013			x	0.419 <0.001 <0.001 <0.001	0.850 <0.001 0.226 0.938	0.419 <0.001 0.001 0.135	0.234 <0.001 <0.001 0.026	0.136 <0.001 0.083 <0.001
Natural Noil 2011				x	0.359 <0.001 <0.001 <0.001	0.619 0.266 <0.001 <0.001	0.068 <0.001 <0.001 <0.001	0.574 0.023 <0.001 <0.001
Bleached Noil 2013					x	0.138 <0.001 <0.001 0.208	0.369 0.982 0.002 0.046	0.124 0.014 0.003 <0.001
Bleached Noil						x	0.013 <0.001	0.937 0.068

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2011		<0.001 0.275	0.010 <0.001
Twill 2013		x	0.011 0.007 <0.001 0.002
Twill 2011			x

Since they are natural fibers, the same environmental factors that affect cotton from different locations and/or years would be expected to have similar effects on both silk and wool. Silk and wool from different years could be differentiated using isotope ratios as could different fabric types, which could either be the result of different manufacturing processes or of raw material from different initial sources. It therefore appears that naturally-derived fibers in general may be able to be distinguished from one another using isotope ratio analysis.

3.D. Synthetics

Six different synthetic fabrics were purchased in 2013 and again in 2014. As fibers from these fabrics are chemically different from one another, other simpler methods can be used to differentiate the fibers from the same year from one another. In order to determine if synthetic fibers of the same chemical composition can be distinguished using isotope ratios, the $\delta^{13}C$ and $\delta^{15}N$ ratios were measured for each fiber and statistically compared to the same fiber type from the other year. The results are given in Table 22.

Table 22. P-values showing differentiability of six synthetic samples (n=9). Values lower than a p-value of .05 are differentiable at the 95% confidence level. N/A indicates fiber doesn't contain nitrogen

Synthetic Fiber	**Carbon p-value**	**Nitrogen p-value**
Dacron Type 54	<0.0001	N/A
Creslan Acrylic Type 61	<0.0001	0.4541
Spun Orlon Type 75	0.0986	<0.0001
Spun Polypropylene	<0.0001	N/A
Spun Nylon 6.6 Dupont Type 2	0.1046	<0.0001
Spun Rayon	<0.0001	N/A

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The combination of carbon and nitrogen isotope ratios allows for all fiber combinations to be distinguished. Therefore, hydrogen and oxygen isotope analysis was not done, but adding these elements could add further discrimination power. As was found with cotton fibers, chemically consistent synthetic fibers can likely be differentiated using isotope ratio analysis.

3.E. Carpet

Since synthetic clothing fibers were found to be differentiable, six different nylon carpet samples were obtained and eight replicate samples were analyzed for each. These six carpet samples included two each of three different colors. Using carbon, oxygen and hydrogen isotope

ratios, all six carpet samples could be differentiated, even when the color was visibly identical, as shown in Figure 10.

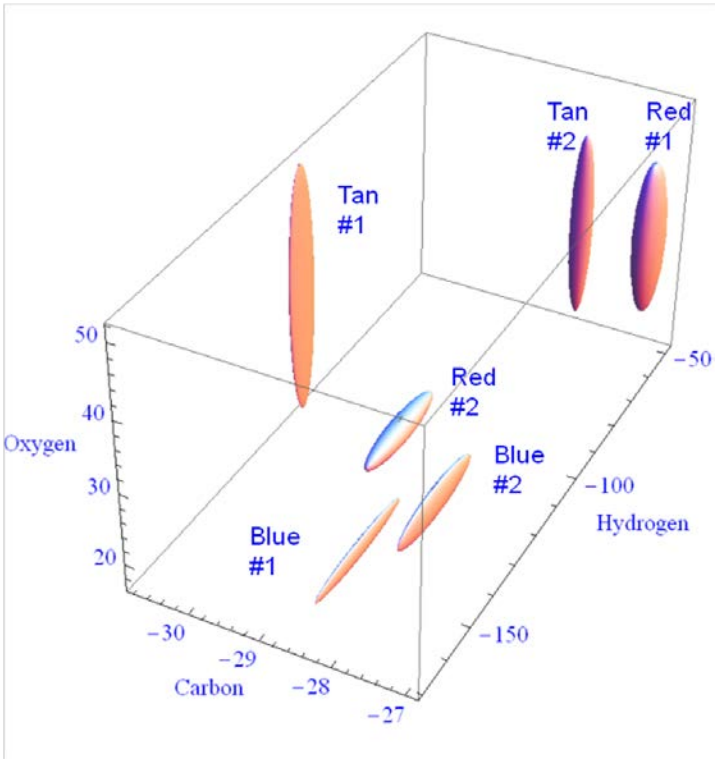

Figure 10. Carbon, oxygen, and hydrogen isotope ratios from a variety of carpet samples.

Thus, as would be expected based on the synthetic fiber analysis discussed above, different carpet fibers could be differentiated, even when they had similar coloring patterns. Thus, isotope ratio analysis can be applied to non-clothing fibers, such as carpet from a crime scene or from a car trunk, in order to potentially show similarity between two fibers or to exclude two fibers as having originated from the same source.

3.F. Effect of Surface Stains

Different samples from a t-shirt were stained with grass and dirt. Since all samples came from the same t-shirt, the only changes to the isotope ratios would occur through its stain treatment and preparation. When the five groups of t-shirt samples: original (control), grass stained, dirt stained, grass stained-washed, and dirt stained-washed, were analyzed, $\delta^{13}C$, $\delta^{2}H$, and $\delta^{18}O$ values were found to range from -27.105 to -26.263, -16.573 to -2.622, and from 28.707 to 30.258, respectively.

The results from the runs are shown in Table 23. The p-values for the comparison of each fabric type are shown in Table 24. All five analytes were found to be indistinguishable from each other. A three-dimensional trivariate plot, shown in Figure 11, allows for easy visualization of this. The overlap between the 'bubbles' in the plot is consistent with the lack of differentiability between analytes. Thus, the introduction of stains, at least surface stains such as grass and dirt, does not appear to significantly change the isotope ratio values for the fibers. This is most likely due to the relatively small mass of the stain material compared to the mass of the cotton fibers. Since the stain material makes up such a small percentage of the overall total mass, it does not affect the overall measurement, even though the stain material most likely has a different isotope ratio value than does the cotton fiber itself.

Table 23: Average ± standard deviations of $\delta^{13}C$, $\delta^{2}H$, and $\delta^{18}O$ values for the original shirt, natural stains, and washed stains.

Original (Control)	-26.7 ± 0.2 (n=12)	-11 ± 4 (n=12)	29.4 ± 0.4 (n=12)
Grass Stained	-26.6 ± 0.2 (n=12)	-11 ± 4 (n=11)	29.2 ± 0.3 (n=11)
Dirt Stained	-26.7 ± 0.2 (n=11)	-10 ± 3 (n=11)	29.2 ± 0.4 (n=11)
Grass Stained-Washed	-26.8 ± 0.2 (n=12)	-9 ± 3 (n=12)	29.3 ± 0.4 (n=12)
Dirt Stained-Washed	-26.6 ± 0.2 (n=12)	-9 ± 4 (n=12)	29.5 ± 0.5 (n=12)

Table 24. P-values showing the non-differentiability of five cotton samples. The values at the top, middle, and bottom of each cell are $\delta^{13}C$, $\delta^{2}H$, and $\delta^{18}O$ values, respectively. Highlighted values are higher than a p-value of .05 and are non-differentiable at the 95% confidence level.

	Original	**Grass Stained**	**Dirt Stained**	**Grass Stained Washed**	**Dirt Stained Washed**
Original	x	0.286 0.853 0.361	0.746 0.668 0.399	0.466 0.315 0.858	0.520 0.311 0.463
Grass Stained		x	0.396 0.528 0.975	0.064 0.219 0.459	0.636 0.230 0.114
Dirt Stained			x	0.242 0.555 0.499	0.709 0.527 0.135
Grass Stained Washed				x	0.147 0.905 0.365
Dirt Stained Washed					x

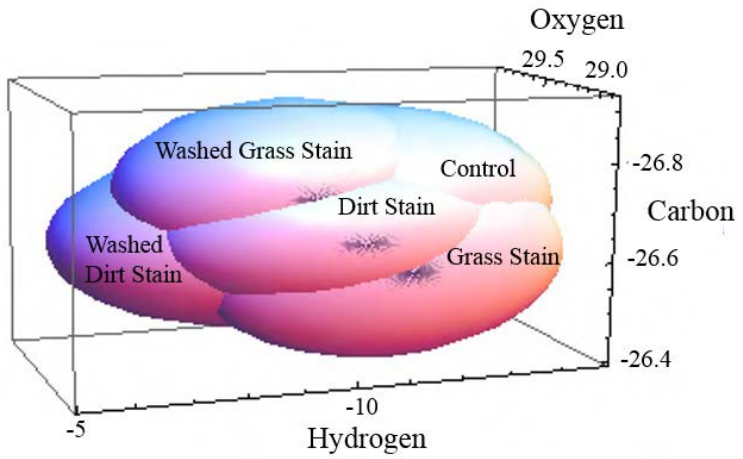

Figure 11: Trivariate plot of grass- and dirt-stained fibers compared to non-stained control sample

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These results were consistent with the addition of clothing dyes and the removal of dye material from blue jeans presented in section 3.B.

3.G. Effect of Blood stains

Another type of stain that could be expected at a crime scene is blood. Cow blood was introduced to sampled white cotton, allowed to soak into the fabric, dry and was then washed off with cold running water. The blood stained fabric was resampled and the isotope ratios analyzed along with the pre-stained fibers. The results of this analysis are shown in Table 25.

Table 25. Results of two-tailed t-tests on blood stained and pre-stained cotton fibers. A p-score <0.05 indicates that the samples are distinguishable.

	Pre-stained Cotton (Carbon)	Pre-stained Cotton (Hydrogen)	Pre-stained Cotton (Oxygen)
Blood Stained Cotton	<0.00001	0.03701	0.03702

In this case, all three isotope values can be used to distinguish the blood-stained fibers from the non-blood stained fibers. Thus, blood stained fibers show a difference in their isotope ratio values. In contrast to grass and dirt stains, the blood stains can penetrate the entire fiber, and may also deposit cells which can be retained in the fibers, even after washing with water. Therefore, blood stained fibers found at a crime scene should not be used to compare to non-blood stained fibers, unless care is taken to ensure all traces of blood have been removed.

3.H. Effect of DNA Extraction

Fibers are routinely analyzed for DNA content. This involves adding chemicals to extract the DNA. If these extraction chemicals do not affect the isotope ratio values, fibers could first be treated to remove any DNA present and then analyzed for isotope ratios. Ten fiber samples were obtained from six fabric types, including natural, synthetic, and blended fabrics. The DNA extraction protocol was then performed on each fabric and ten fiber samples were again collected. The statistical analysis of the isotope ratio results is shown in Table 26 and shows that carbon and nitrogen isotopes are not affected by the DNA extraction process. Two fabrics showed statistically significant differences in hydrogen isotope results and one fabric showed

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statistically significant differences in oxygen isotope ratio values between the pre- and post-treated fibers. These results should be further studied but indicate that isotope ratio analysis should not be undertaken, at least for oxygen and hydrogen, for fibers that have underwent DNA extraction as the processing step may change the isotope ratio results.

Table 26. Results of two-tailed t-tests on pre- and post-DNA extracted fibers (n=10). A p-score <0.05 indicates that the samples are distinguishable. N/A indicates fiber doesn't contain nitrogen.

Fiber Type	**Nitrogen p-value**	**Carbon p-value**	**Hydrogen p-value**	**Oxygen p-value**
Spun Rayon	N/A	0.8598	0.0015	0.0007
Silk Twill	0.6866	0.5789	<0.0001	0.1437
87/13 Nylon Lycra Knit	0.5851	0.2738	0.2838	0.9605
Bleached Cotton T-shirt Fabric	N/A	0.9863	0.6673	0.2842
50/50 Polyester Cotton Jersey Fabric	N/A	0.7663	0.6036	0.1346
65/35 Polyester Cotton Twill	N/A	0.7184	0.9449	0.5409

3.I. Effect of Environmental Exposure

For any fibers found outside, environmental exposure is a potential source of contamination and could lead to changes in isotope ratio values. In order to investigate the possible effect of environmental conditions, three fabrics were each exposed to different environmental conditions. One set was buried in the snow, a second set was exposed to sunlight and rain, and a third set was submerged in a pond. After a one month exposure, the fabrics were washed to remove any large visible contaminants and sampled. The observed $\delta^{13}C$ isotope ratio values were compared to those obtained from control fibers that had not been exposed to the elements. The results are presented in Table 27. Three of the nine comparisons with the control samples showed a difference in the carbon isotope ratio after environmental exposure. Upon closer inspection of the fibers, the exposed fibers were found to be stained, even after washing to remove larger particulate matter. These stains likely represent the inclusion of non-fabric matter that was not washed away and not just surface stains. Therefore, if isotope ratio analysis is to be used for fibers exposed to the elements for relatively long periods of time, a better washing protocol must be developed. Otherwise, fibers exposed to the elements may not be able to be compared with fibers that were not exposed to the same conditions.

Table 27. Results of two-tailed t-tests for carbon analysis of fabrics exposed to environmental conditions (n=9). A p-score <0.05 indicates that the samples are distinguishable.

	Snow Exposure	Sun/Rain Exposure	Pond Exposure
Cotton T-shirt	0.2058	0.3094	0.0040
Blue Cotton Denim	<0.0001	0.1634	<0.0001
50/50 Polyester/Cotton Jersey	<0.0001	<0.0001	<0.0001

3.J. Sample Size Analysis

The default fiber sample size of 200 µg was used for all studies to better allow for comparisons between samples and because this amount was found to be reasonably small but

still easy to handle when preparing samples. For an average thread, 200 µg represents approximately 1 cm of thread. Since many crime scenes may have considerably smaller fiber samples, a study was done to determine how small a sample amount could be analyzed without statistically changing the measured isotope ratio values. Samples were made from white cotton t-shirt material, blue denim material, and silk fabric containing 200 µg, 100 µg, 50 µg, 20 µg, 10 µg, and 6 µg. A total of eight replicate measurements were obtained for each sample mass of each fabric type. For each fabric type the results from each sample mass were statistically compared to each other using a two-tailed t-test at the 95% confidence interval. For the cotton t-shirt and denim fabrics, the isotope ratios for carbon, oxygen and hydrogen were consistent (at the 95% confidence level) between the 200 µg, 100 µg and 50 µg samples, with slight deviations observed when the 20 µg samples were compared to samples of larger mass. Therefore, a minimum sample size of 50 µg was assigned for the cotton and denim fibers in order to obtain statistically similar results as when larger samples were analyzed.

For the silk samples, the isotope ratio values obtained for the 200 µg, 100 µg, 50 µg, 20 µg, and 10 µg samples were statistically similar at the 95% confidence level, with deviations observed when the 6 µg samples were compared to larger samples. Silk was therefore determined to have a lower limit of 10 µg of sample in order to not change the measured isotope ratios due to the size of the sample. While the cause of the different sample lower limit size between the different types of fibers is unknown, all of these results indicate that significantly smaller sample amounts than the 200 µg default amount could be used to obtain isotope ratios from small fibers originating from a potential crime scene.

IV. Conclusions

The research presented here indicates that isotope ratio mass spectrometry might be a valuable forensic analytical tool for the analysis of fibers. Both natural and synthetic fibers were shown to be differentiable from chemically similar fibers using a combination of isotope ratio measurements. This was true for fibers made by different companies as well as for those made by the same company but in two different years. Fibers from the same regions of the same garment, or from garments packaged together, were indistinguishable from one another by IRMS. Thus, fibers from the same source would be expected to have the same isotope ratio profile, while fibers from different sources can likely be differentiated even if they are made from the same chemical composition. While isotope ratios are not as unique as fingerprints or DNA, they are more distinctive than fiber color or chemical composition and thus, isotope ratio analysis would add an additional method for the comparison of fibers to determine if two fibers potentially have common origins. Coloring, surface stains or the wearing away of color from fibers does not appear to alter the isotope ratios to a significant extent. Exposure to other conditions, such as blood, bleach, DNA extraction chemicals and the elements can affect the isotope ratios, so these need to be considered if IRMS is to be used to analyze fibers.

While fiber analysis by IRMS cannot conclusively indicate that two fibers came from the same source, it can exclude a common source for two fibers. For fibers that are found to have the same isotope ratios, this result provides additional circumstantial evidence that they might be related. This analysis would enhance the ability to compare fibers over what is currently done and thus could find use as a forensic analysis technique in certain circumstances. One of the limitations of this technique is the specialized instrumentation required. Most forensic labs do not have ready access to an isotope ratio mass spectrometer, although larger forensic facilities are equipped with this instrumentation. In addition, IRMS is a destructive technique, so care needs to be taken that all other analyses are complete before isotope ratio analysis is undertaken.

Additional studies should be performed on blood stained fibers and fibers exposed to the environment. Both of these types of samples showed some change in isotope ratios after exposure. It may be possible to develop a better washing protocol that will allow consistent isotope ratio values to be measured between pre- and post-stained fibers. This would be important if blood stained fibers or fibers that have been found outside are collected and need to be compared to other fibers using IRMS. The effect of DNA processing on isotope ratios should also be investigated further. Inconsistent results were obtained, with the isotope ratios of most fibers not affected by the DNA extraction protocol while two samples did exhibit a statistical difference between pre- and post-processed fibers. The study should be replicated and expanded to include more than six fiber types so that the effect of DNA-extraction can be studied more completely.

With these potential limitations noted, IRMS appears to be useful forensic analysis method for fiber analysis. While many instances may not require this technique, it could be useful in excluding or possibly including fibers from a common source.

V. References

[1] C. Gilbert, S. Kokot, U. Meyer. Application of DRIFT spectroscopy and chemometrics for the comparison of cotton fabrics. *Appl. Spectrosc.* 47 (1993) 741-748.

[2] C. Gilbert, S. Kokot. Discrimination of cellulosic fabrics by diffuse reflectance infrared Fourier transform spectroscopy and chemometrics. *Vib. Spectrosc.* 9 (1995) 161-167.

[3] S. Kokot, N. Anh, T. L. Rintoul. Discrimination of reactive dyes on cotton fabric by raman spectroscopy and chemometrics. *Appl. Spectrosc.* 51 (1997) 387-395.

[4] M. C. Grieve, J. Dunlop, P. Haddock. An Assessment of the Value of Blue, Red, and Black Cotton Fibers as Target Fibers in Forensic Science Investigations. *J. Foren. Sci.* 33 (1988) 1332-1344.

[5] J. Thomas, P. Buzzini, G. Massonnet, B. Reedy, C. Roux. Raman spectroscopy and the forensic analysis of black/grey and blue cotton fibres Part 1. Investigation of the effects of varying laser wavelength. *Forensic Sci. Int.* 152 (2005) 189-197.

[6] West, J.W., H. Kreuzer, and J.R. Ehleringer, Approaches to plant hydrogen and oxygen isoscapes generation, p. 161-178. In J. West, G.J. Bowen, T.E. Dawson, and K. Tu (eds.), Isoscapes: understanding movement, pattern, and process on Earth through isotope mapping. Springer Verlag, New York 2010.

[7] N. Nic Daéid, W. Meier-Augenstein, H. Kemp. Investigating the provenance of un-dyed spun cotton fibre using multi-isotope profiles and chemometric analysis. *Rapid Commun. Mass Spectrom.* 25 (2011) 1812-1816.

[8] W. Meier-Augenstein. Stable Isotope Forensics: An Introduction to the Forensic Application of Stable Isotope Analysis, John Wiley, Chichester, 2010.

[9] J. F. Casale, J. R. Ehleringer, D. R. Morello, M. J. Lott. Isotopic fractionation of carbon and nitrogen during the illicit processing of cocaine and heroin in South America. *J. Forensic Sci.* 50 (2005) 1-7.

[10] F. Angerosa, O. Breas, S. Contento, C. Guillou, F. Reniero, E. Sada. Application of stable isotope ratio analysis to the characterization of the geographic origin of olive oils. *J. Agric. Food Chem.* 47 (1999) 1013-1017.

[11] L. Bontempo, F. Camin, R. Larcher, G. Nicolini, M. Perini, A. Rossmann. Coast and year effect on H, O and C stable isotope ratios of Tyrrhenian and Adriatic Italian olive oils. *Rapid Commun. Mass Spectrom.* 23 (2009) 1043-1048.

[12] J. B. West, J. R. Ehleringer, T. E. Cerling. Geography and vintage predicted by a novel GIS model of wine delta ^{18}O. *J. Agric. Food Chem.* 55 (2007) 7075-7083.

[13] G. J. Bowen, L. Chesson, K. Nielson, T. E. Cerling, J. R. Ehleringer. Treatment methods for the determination of δ^2H and $\delta^{18}O$ of hair keratin by continuous-flow isotope ratio mass spectrometry. *Rapid Commun. Mass Spectrom.* 19 (2005) 2371-2378.

[14] H. A. S. Buchanan, N. Nic Daéid, W. Meier-Augenstein, H. F. Kemp, W. J. Kerr, M. Middleditch. Emerging use of isotope ratio mass spectrometry as a tool for discrimination of 3,4-methylenedioxymethamphetamine by synthetic route. *Anal. Chem.* 80 (2008) 3350-3356.

[15] Y. Saranga, I. Flash, A. Paterson, D. Yakir. Carbon isotope ratio in cotton varies with growth stage and plant organ. *Plant Science*. 142 (1999) 47-56.

[16] D. Yakir. Variations in the natural abundance of oxygen-18 and deuterium in plant carbohydrates. *Plant Cell Environ.* 15 (1992) 1005-1020.

[17] F. Ripullone, N. Matsuo, H. Stuart-Williams, S. Chin Wong, M. Borghetti, M. Tani, G. Farquhar. Environmental effects on Oxygen Isotope Enrichment of Leaf Water in Cotton Leaves. *Plant Physiol.* 146 (2008) 729-736.

[18] Commission on Isotopic Abundances and Atomic Weights.

[19] Indiana University Biogeochemical Laboratories.

[20] H. Qi, T.B. Coplen,H. Geilmann, W.A. Brand, J.K. Böhlke. (2003) Two new organic reference materials for $\delta^{13}C$ and $\delta^{15}N$ measurements and a new value for the $\delta^{13}C$ of NBS 22 oil. *Rapid Commun. Mass Spectrom.* 17 (2003) 2483-2487.

[21] J.K. Böhlke, S.J. Mroczkowski, T.B. Coplen. Oxygen isotopes in nitrate: new reference materials for 18O:17O:16O measurements and observations on nitrate-water equilibration. *Rapid Commun. Mass Spectrom.* 17 (2003) 1835-1846.

[22] L. I. Wassenaar, K. A. Hobson. Two new keratin standards (δ^2H, $\delta^{18}O$) for daily laboratory use in wildlife and forensic isotopic studies. Poster. Environment Canada.

[23] W.A. Brand, T.B. Coplen, A.T. Aerts-Bijma, J.K. Böhlke, M. Gehre, H. Geilmann, M. Gröning, H.G. Jansen, H.A. Meijer, S.J. Mroczkowski, H. Qi, K. Soergel, H. Stuart-Williams, S.M. Weise, R.A. Werner. Comprehensive inter-laboratory calibration of reference materials for $\delta^{18}O$ versus VSMOW using various on-line high-temperature conversion techniques. *Rapid Commun. Mass Spectrom.* 23 (2009) 999-1019.

VI. Dissemination of Research Findings

A total of eight presentations have been made based on this award, as listed below. In addition, an abstract has been accepted to present the final results from this award at the 2017 PittCon Conference in Chicago, IL in March. One manuscript has been submitted to the Journal of Forensic Science and is under review, and two more are in preparation.

1. Brown, H., Le, D., Beussman, D.J. "Isotope Ratio Mass Spectrometry Analysis of Natural and Synthetic Fibers and Effects of Chemical and Environmental Factors for Forensic Applications" SCIX 2016, Minneapolis, MN, September 2016.
2. Brown, H., Macon, E., Beussman, D. J. "Analysis of Fibers via Isotope Ratio Mass Spectrometry" 2016 Pittsburgh Conference and Exposition, Atlanta, GA, March 2016.
3. Brademan, D., Rolfs, Z., Beussman, D.J. "Fiber and Thread Analysis Via Isotope Ratio Mass Spectrometry" 2015 Pittsburgh Conference and Exposition, New Orleans, LA, March 2015.
4. Brademan, D., Rolfs, Z., Beussman, D.J. "Fiber and Thread Analysis Via Isotope Ratio Mass Spectrometry" 43rd Annual Fall Meeting of the Midwestern Association of Forensic Scientists, St. Paul, MN, Oct. 2014.
5. Saksa, B., Wang, J., Beussman, D.J. "Exploration of Isotope Ratio Mass Spectrometry as a Method for Thread Analysis" 66th American Academy of Forensic Sciences Annual Meeting, Seattle, WA, Feb. 2014.
6. Gangelhoff, K., Beussman, D. J. "Differentiation of Cotton Fibers Using Isotope Ratio Mass Spectrometry", 61st ASMS Conference on Mass Spectrometry and Allied Topics, Minneapolis, MN, June, 2013.
7. Beussman, D.J. "CSI Comes to Minnesota: Date-Rape Drug and Fiber Analysis using Mass Spectrometry" University of St. Thomas invited speaker, St. Paul, Minnesota, October 19, 2012.
8. Eckmann, J., Beussman, D. J. "Differentiation of Cotton Fibers from Clothing and Other Common Items Using Isotope Ratio Mass Spectrometry" 63rd Pittsburgh Conference and Exposition, Orlando, FL, March 2012.