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Raman Spectroscopy of Aged Waterlogged Bones: Protocol Optimization

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Raman Spectroscopy of Aged Waterlogged Bones: Protocol Optimization

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Abstract

Current methods used for determining the age buried bones for forensic purposes are heavily affected by environmental conditions, can be susceptible to scientific bias and the interpretation can vary from investigator to investigator. Raman Spectroscopy can potentially be used as an automatic method to analyze the composition of bone eliminating the human factor. To test this hypothesis, we will use Raman spectroscopy to potentially identify the burial time of bones. The change in the chemical composition of the pig bones was probed using a Raman spectrometer with a 785nm excitation wavelength. Other parameters of the Raman instrument were optimized to create a protocol that would be universal for all bone samples, regardless of the age. The best parameters used were 100% laser power, one accumulation, and a 120-second accumulation time. This method was confirmed to be the ideal method for this sample by testing both the youngest and oldest samples in the group. Using this method, the youngest sample was tested in different spots on the particle, and it was seen that there were differences in the spectra, which is another factor that must be accounted for when making the model in the future.

Keywords: *Raman Spectroscopy, Aging, Osteology*

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Introduction

Determining the age of bones is an important aspect of forensic science. Knowing the time since deposition of the bones can be vital in investigations because it offers a period that can be used to narrow suspects or help identify the victim. This branch of forensic science is anthropology, which further branches into forensic osteology- to determine the age, sex, ancestry, and other unique information about bones. Bone age is usually confirmed by radiocarbon techniques but current methods for determining information other that age are less finalized and rely on postmortem index (PMI). This can be determined by scanning electron microscopy, radiographic techniques, and physical investigations[(1)]. PMI is usually determined by a trained anthropologist using the morphological changes of the bones and then supporting findings with other chemical or physical methods. However, if this is the only method then potential bias and person to person variations can be introduced. Other methods can support physical findings include chemical methods such as, radiocarbon, luminol chemiluminescence, and infrared spectroscopy[(2)]. Radiocarbon dating is an important method; however, it is more useful in studies of ancient bones because the long half-life of carbon and it is an expensive technique that may be out of the price range for crime labs to use. Luminol chemiluminescence methods can be used for screening but are prone to bias and false positives[(3)]. Lastly, IR is a useful method but has limitations with water and extensive prep for analysis, Raman would be a better choice when investigating bones that may have been in an aqueous environment. Raman has many benefits that would be ideal for investigations involving the burial of bones including that it is non-destructive, portable, relatively inexpensive, immune to interference from water, and it could be used as a potential confirmatory test that identifies chemical structures[(4)].

Unlike other methods, such as IR, Raman spectroscopy is mostly immune to interference from water. This is important for biological samples and can lower the amount of preparation. Raman analysis can also be done on fixed fresh or live tissues, which expands its use to different applications beyond forensics. Raman spectroscopy measures scattering of light and shows the vibrational modes of molecules, giving valuable information about the chemical structure of compounds. A common wavelength used in the case of organic materials is 785nm. Characteristics of the chemical structure can be determined by the stokes and anti-stokes scattering of light, two inelastic sources of scattered light that are measured with Raman spectroscopy. Stokes scattering occurs when the excitation radiation has a higher frequency than the scattered light, anti-stokes is when the excitation radiation has a lower frequency than the scattered light. When the scattered light is the same frequency as the incident radiation than this is called Rayleigh scattering, which is elastic because no energy is lost when the light is scattered. Elastic scattering is more frequent, so Raman signal is weaker when compared to this type of scattering. All of this information can be applied to determining the age of bones and characterizing the components[5] .

Selecting the correct range of wavelengths is important. Most of the peaks for biological samples fall withing 400-2000 Raman wavenumbers for typical bond vibrations. Ranges for compounds that are specifically studied in biological samples are bond vibrations of proteins, which show at a range of 1,500-1,700 cm^{-1}, carbohydrates at 470-1200cm^{-1}, and CH, NH, and OH stretching in lipid and proteins that have a range above the others at 2,700-3,500cm^{-1}. Phosphate groups on DNA show up at a variety of areas on the spectrum. This diverse range of compounds allows Raman to be useful in many biological samples and can apply to fields such as pharmacology, microbiology, toxicology, plant science, and human biology[6].

Limitations of the use of Raman analysis for biological samples could be interference with fluorescence, which is due to interference from an outside source of light. Another issue is that the burning of a sample is possible, especially in samples that have a darker pigmentation or due to the presence of amorphous carbon bands[6].

There are many applications of Raman spectroscopy beyond biological samples such as the identification of different varieties of white household paint[7], differentiation of similar cotton fibers[8], drug identification and analysis[9], and determination of trace evidence found on a crime scene. Previous studies that prove the use of Raman analysis for biological samples at a crime scene include the determination of time since deposition of blood[10] which species the blood came from[11], and discrimination of different body fluids such as semen, saliva, and blood[4]. Raman spectroscopy is nondestructive, only requiring a scan and not an invasive procedure. Unbiased, well published, and has known error rates. Because of this it meets the Daubert standard for admissibility in court. Furthermore, Raman is accepted as a confirmatory technique, which means that it can give information on the structure of the molecule and can help identify it based on databases and comparison of spectra[4].

The samples tested in this project are waterlogged bones. Bones differ from many other biological samples, as they are heterogenous composite material made up of a mineral phase, hydroxyapatite phase, organic phase, and water. Hydroxyapatite is a mineral common in bone matrix and has a formula $Ca_5(PO_4)_3OH$, and it add strength to hard tissues such as bones. Hydroxyapatite accounts for 30-98% of the mass of the bone, with the average falling at around 60-70%. Bone composition can change due to the type of bones, species, and stage of development. Changes in the mineral content of bone can also be affected by diseases such as osteoporosis or other diseases that increase the fragility of bones. Organic components of bones are mostly

proteins. Collagen I is the most abundant protein in bone. It crosslinks, which provides elasticity, stabilization, and support[12,13]. Both of these components contribute to the spectra taken of bone samples.

The end goal of this project is to create a model for determining the age of waterlogged bones using Raman measurements to assist forensic investigations. In order to create a protocol laser power, aperture, exposure time, accumulations, cosmic ray removal, and sample set up must be the same in all bones in the sample group. Determining these factors requires research into other articles and investigation of Raman spectra. Once a protocol is set then the number of scans required for an accurate representation of the data must be determined, and then final from this data a model can be made for age determination of bone buried in an aqueous environment.

Materials and Methods

A. Samples

Samples were collected from the biology department at the university at Albany and have been used in different studies, including testing the methods for extracting DNA with the aging of bones[14]. In the original study the samples were from fresh pigs(*Sus scrofa*), and the bones collected were twelve humeri and twelve ribs, these were split in half to give a total sample size of 24 humorous and 24 rib samples. These samples were then submerged in water with grating to ensure complete submergence. Two bodies of water were used in this study Henley Lake and James River. Initially the bones were cleansed of non-bone tissue by cutting the tissue away and then rinsing with ethanol, and if more cleaning was needed then sandpaper was used. These bones were then ground with a mortar and pestle with liquid nitrogen to get small fragments of bones. The group of bones used in this study were the scapula bones from Henley Lake, with different aging times. The way that time was recorded was with Accumulated degree days (ADD) which is when the temperature changed for each day, and each sample was collected every 250 ADD. The bones were then stored in a freezer set at -20ºC till analysis. Table 1 below shows the accumulated degree days for each of the samples.

Table 1

Bones Selected for Analysis

Date Collected	ADD Interval	Day Collected	Adjusted ADD interval	Season	Location	Sample type	Collection	Sample name
12/10/2016	250	249	250	Fall	Henley Lake	Scapula	1	HLS3C1
4/16/2017	1000	989	1000	Spring	Henley Lake	Scapula	4	HLS3C4
7/02/2017	1750	1738	1750	Summer	Henley Lake	Scapula	7	HLS3C7
9/04/2017	2500	2496	2500	Summer	Henley Lake	Scapula	10	HLS3C10
11/05/2017	3250	3434	3500	Fall	Henley Lake	Scapula	13	HLS3C13
3/09/2018	4000	4229	4250	Winter	Henley Lake	Scapula	16	HLS3C16

B. Instrumentation

Samples were tested with the dispersive Renishaw inVia Raman Spectrometer equipped with a Leica confocal Microscope and a 785-nm excitation wavelength and 50x objective.

To develop a method to determine the age of bones a protocol must be developed. This method must the same method that is applied to all bones in the sample group. Aspects of the protocol that must be addressed include laser power, aperture, exposure time, accumulations, cosmic ray removal, and sample set up. Laser power is proportional to the power of the laser that is exciting the sample, so the larger the laser power the stronger the signal will be. However, an important aspect, especially with organic materials and materials that are dark in color, is to not burn the sample. To test the laser power first start with a low laser power and then increase till damage occurs. Exposure time and number of accumulations go together. Increasing the exposure time increases the spectrum quality and is useful in weak spectra samples. The same goes for accumulations. Also using longer exposure times or increasing the accumulations reduces noise as well. However, when making a model many scans are needed and time is a crucial factor.

Increasing the exposure time and accumulations increases the time as well. Another factor to consider is the presence of cosmic rays, which show up as spikes in the Raman spectra and are caused by random natural events but can be removed individually or by the scanning software. Using the scanning software also adds time to the scan and requires at least two accumulations to be run, further increasing the time per scan. Cosmic rays can also be removed manually from the spectra after the scan is done[14,15].

C. Methods Tested to Create a Protocol

Methods 1-3

The parameters for the first method were directly from the proof-of-concept paper[16]. This method tested the 250ADD sample. The sample was prepared by placing the sample on flat piece aluminum foil. The range of wavenumbers used was 3200-100 cm^{-1}, with 0.5% laser power, 35second exposure, and five accumulations. Method two increased the laser power,5%, with the other same parameters. Method three tested 10% laser power.

Methods 4-7

Method four was similar to methods 1-3, with the sample preparation on a flat aluminum foil surface a range of wavenumbers from 3200-100 cm^{-1}, 5% laser power, 35 second exposure, 5 accumulations, but cosmic ray removal was used to determine peaks that may have been obscured by cosmic rays. Method five changed the laser power used to 100% power but kept the other parameters the same. Method six changed the laser power to 50%, and method seven changed the laser power to 10% while keeping the other parameters the same.

Methods 8-9

The sample for method eight was prepared by placing a fragment on aluminum foil and was tested with a range of wavenumbers from 3200-100 cm^{-1}, 100% laser power, 35 second exposure, 3 accumulations, and cosmic ray removal was used. Method nine decreased the accumulations to one and kept the other parameters the same.

Method 10

The sample was prepared by placing a fragment in an aluminum foil cup and was tested with a range of wavenumbers from 3200-300 cm^{-1}, 100% laser power, 10 second exposure, and 5 accumulations. This differs from method nine because cosmic ray removal was turned off.

Methods 11-14

The sample was prepared by placing a fragment in an aluminum foil cup and was tested with a range of wavenumbers from 3100-300 cm^{-1}, 100% laser power, 30 second exposure, and 3 accumulations. Method twelve changed the exposure to 20 seconds. Method thirteen changed the exposure time to 25 seconds, and method fourteen changed the exposure time to 15 seconds.

Methods 15-21

The sample was prepared by placing a fragment in an aluminum foil cup and was tested with a range of wavenumbers from 3100-300cm^{-1}, 100% laser power, 10 second exposure, and 1 accumulation. The exposure time was tested in increments of 10 seconds: 20, 30, 40,50, 60 and 120 second exposures were tested as different methods.

Table 2

Methods Tested

Method	Laser power	Exposure time	Accumulations	Cosmic Ray Removal
1	0.5	35	5	No
2	5	35	5	No
3	10	35	5	No
4	5	35	5	Yes
5	100	35	5	Yes
6	50	35	5	Yes
7	10	35	5	Yes
8	100	35	3	Yes
9	100	35	1	Yes
10	100	10	5	No
11	100	30	3	No
12	100	20	3	No
13	100	25	3	No
14	100	15	3	No
15	100	10	1	No
16	100	20	1	No
17	100	30	1	No
18	100	40	1	No
19	100	50	1	No
20	100	60	1	No
21	100	120	1	No

D. Testing the Heterogeneity of the Bones

After methods for the parameter were tested then the different particles and areas of the bone fragments were tested to see if the spectra yielded any different peaks or intensities. To do this, five scans on different spots located on different particles of the 250 ADD sample were taken and compared to one another. Two methods were chosen to test these parameters, method nineteen and twenty-one were best suited for analysis.

Results

When analyzing spectra of bone there are characteristic peaks to acknowledge. Mineral components have been known to have peaks at 960 cm^{-1}, and 485-400cm^{-1}, and these can be attributed to the phosphate groups in the bones. The collagen content in the bones have shown peaks at around 3040-2810 cm^{-1} for the CH_2 region, 1715-1610cm^{-1} for the amide I region, 1358-1217cm^{-1} for the amide III region, and 1500-1415cm^{-1} for the NH component of the organic content[16]. The table below shows the range of peaks common in bone spectra.

Table 3

Raman Ranges for Components of Bones

Compound	**Spectral Range (cm^{-1})**
Mineral component: Phosphate group v_1 PO_4^{3-}	960
Mineral component: Phosphate group v_2 PO_4^{3-}	485-400
Organic Component: C-H stretching	3040-2810
Organic Content: Amide 1	1715-1610
Organic Component: Amide III	1358-1217
Organic Component: NH	1500-1415

In previous studies that have aged bones using Raman spectroscopy, peak area ratios were used. These ratios usually compared an inorganic peak, which contains hydroxyapatite, to an organic peak that contains collagen components. Commonly used ratios include $v2PO_4^{3-}$ compared to CH_2 stretching, which can help quantify the degradation of collagen in bones[16].

Methods 1-4 aimed to test different laser powers, starting with low power. These methods did not yield spectra with peaks. These spectra were overrun with cosmic rays that would also

inhibit any peaks from being visualized. Because of this the laser power was increased for future scans, which increased the intensity of the peaks. Spectra with the greater peaks were still dominated by cosmic rays, which is why cosmic ray removal was added. At this stage in there were no discernible peaks to measure.

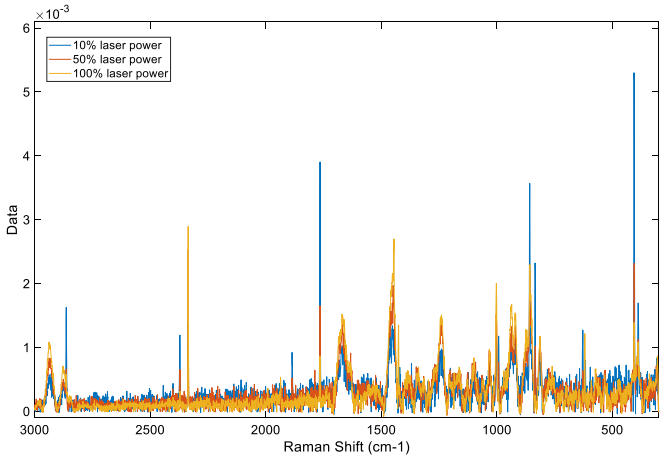

Figure 1: Spectra for Methods 5-7

Method 5-7 compared to one another. The intensity of peaks seen in method seven using 100% laser power was chosen for continued analysis

Figure one compares methods five, six, and seven, which have a higher laser power than the previous methods. This was done to increase the intensity of the peaks. These methods also included the cosmic ray removal. It is seen that the 10% laser power does not have many visual peaks and the 50% and 100%laser power methods have an increase in intensity of the peaks. It was also seen that there was no visual burning of the bones, which is the main concern of increasing the laser power with organic materials. Because of the lack of burning in the bones and the intensity

of the peaks the 100% laser power was chosen as the best to use. The preprocessing was done by Baseline correction Whittaker filter.

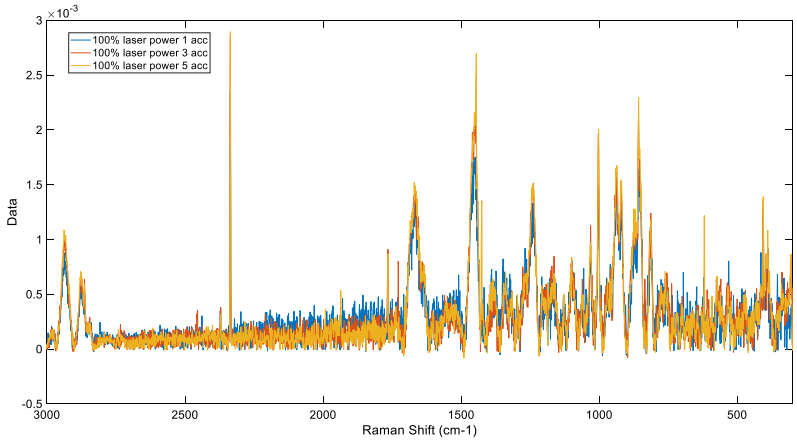

Figure 2: Spectra for Methods 8-9

Methods 8-9 compared to one another, showing different accumulations and their corresponding intensities of peaks

Figure two shows the overlay of the spectra from method seven, eight, and nine. This is comparing the accumulations. Increased accumulations would increase the intensity of the peaks, but it also increases the time. It is seen that five accumulations have the most intense and visual peaks, but the three accumulations have an acceptable amount of visible peaks, and the scan was significantly shorter. The five-accumulation scan took around 20 minutes, and if multiple spectra need to be taken per sample because of the heterogeneity then a shorter method must be developed. One accumulation with a higher exposure time was chosen after testing method 10-14 because all of these methods showed similar results to 1 accumulation with higher exposure time. The preprocessing was done by Baseline correction Whittaker filter.

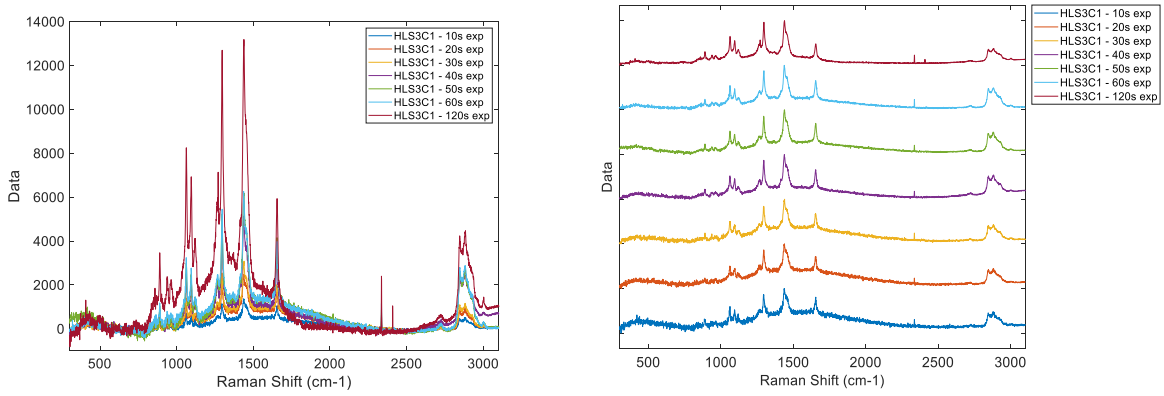

Figure 3: Spectra for Methods 15-21

Raman spectra show methods 15-21 compared to one another. The intensity of the peaks in 50-seconds exposure and 120-seconds exposure were chosen for continued analysis

Figure three shows the spectra that compares methods 15-21, which changes the exposure time. 1 accumulation was chosen because increasing the exposure time and increasing the accumulations results in similar intensities, but both increase the time per scan, so only one parameter from the two needed to be increased. The only difference between the different exposure times shown above was the intensity of the peaks, with the intensity increasing proportionally with the increasing exposure time. By comparing the quality of the spectra to one another it was determined that the 120 second exposure time and the 50 second exposure time were the best suited for continued analysis because of the intense peaks present. The 50 second exposure time had similar intensities of peaks to the 60 second exposure time method, so the shorter method was chosen to test different spots on the same sample because many spectra would need to be taken to help determine the heterogeneity of the samples. The preprocessing method using for these spectra is automated weighted least squares analysis.

(a). (b).

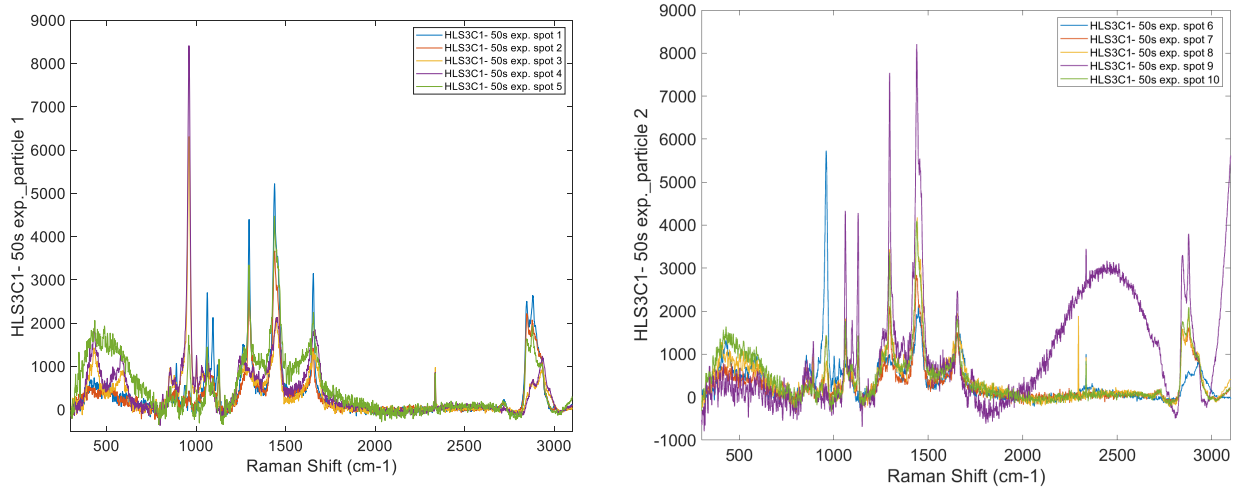

Figure 4: Spectra for Different Spots on 250 ADD Sample

Spectra from the same sample tested with method 19. (a) is particle 1 and (b) is particle 2 from the same sample.

Figure four shows the comparison of scans from the same sample taken at different locations. There are five scans on two separate particles. It is seen that there is both a change in intensity and Raman shift. The most noticeable change in peak intensity is the peak at 960 cm^{-1}, which is part of the mineral component of bone. This peak dominates spot four on particle one and spot six on particle two. In contrast peaks that are attributed to organic components of bones dominate the spectra of spot one on particle one and spot nine on particle two. There is also a shift of the 2900cm^{-1} peak, there are two distinct patterns of peaks in that area, which can be clearly seen by comparing spot four and spot one on particle one. This peak is usually attributed to CH stretching. The preprocessing on this spectrum is done by automated weighted least squares.

(a). (b).

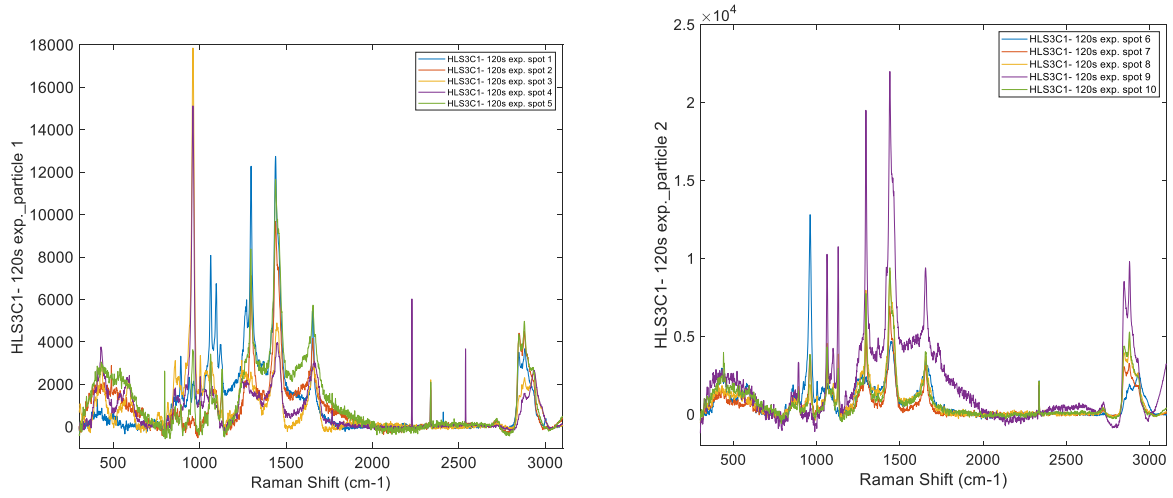

Figure 5: Spectra from Different Spots on 250 ADD Sample with Method 21

Spectra from different spots on the 250ADD sample tested with method 21 (a) is particle 1 and (b) is particle 2.

Figure five shows the same spots as figure six, but the method used was method 21. This method increase the exposure time with should increase the insensity of the peaks. Compared to the 50 second exposure the peaks are more intense, but the same trends seen in the 50 second exposure scans were seen, with intensity changes and peaks shifts indicating that the sample is not heterogenous. The preprocessing done for this sample was automated weighted least squares.

Discussion

To analyze bones using Raman Spectroscopy peak seen must be quantifiable and intense. Aspects of the protocol must be altered to achieve intense peaks, but also minimizing the amount of time needed per scan. The first method was used from the proof-of-concept paper[16], the only parameter that was not listed in this paper was the laser power used, so this was the first choice to test. To not burn the bone a low laser power was used to start and observe the results. No visible peaks were seen with the 0.5% laser power, this is because laser power is proportional to the signal of the Raman instrument and a low laser power such as 0.5% would not be enough to create peaks that were intense enough for data analysis. This lead to methods two and three, which changed the laser power to 5% and 10%, and kept the same parameters from the proof-of-concept paper. After using these methods, it was seen that there was a lot of interference from fluorescence, which gave sharp spikes in the spectra that are due to cosmic rays, which offer no information about the bones and interfere with the analysis of peaks that may be present in the spectra but are combined with a cosmic ray. One way to remove cosmic rays is to turn on cosmic ray removal, which is software present in the Raman instrument. This allowed the peaks that were relevant to be visualized easier, this helped determine what peaks would be the most prominent in the spectra and at what Raman shift they would be present at. It was seen in methods 1-4 with low levels of laser power 0.5%, 5%, and 10%, that the peaks are not intense enough for analysis, which lead to an increase of laser power which would increase the intensity of the peaks. It was seen they became more prominent; however, it is important to note that increased laser power could have the potential to burn the samples, but this was not seen in the samples tested repeatedly with higher laser power. For this reason, the 100% laser power was established as the ideal laser power for this sample.

The next parameter that was tested was the number of accumulations. This was tested because five accumulations with cosmic ray removal took around 20 minutes for one scan, seen with methods 4-7. This is not ideal because many scans are required for a complete model, and since bone is heterogenous many scans are needed to create a good estimation of the bone sample. Shortening the amount of time to create a relevant spectrum is one aspect of the parameter that is necessary, so accumulations, exposure time, and cosmic ray removal are all components that add a significant amount of time so balancing them to achieve the best spectra is necessary.

One spectrum that was not included was the rerun of the 100% laser power with cosmic ray removal and three accumulations. The rerun was done because there may have been movement in the sample while the spectra was being taken, which affected the quality of the spectra. To fix this instead of laying the bone fragment on a flat aluminum foil covered slide, a cup handmade from aluminum foil was used to prevent movement. Another way to fix the moving problem that was proposed was to adhere the bone to the foil with a glue. This was not ideal because the bone would not be able to be removed and only one side of the bone could be analyzed. Additionally, the fragments are fragile and could break apart. The cup was used because the fragments are large enough to move with tweezers and can be held securely with a cup.

Methods 11, 12, 13, and 14 tested the number of accumulations. This was to shorten the scan time because the methods still took around 15 minutes each. A map was proposed but the method would not be ideal for bones because they have different surfaces that can become unfocused as the microscope is moving to take another scan, and as seen in the study with different spots on the same particle the different spots can give different spectra. Cosmic ray removal was also taken out of the parameter at this point because it added additional time to the scans which is unnecessary because the cosmic rays can be removed easily after the scan is done. The addition of

cosmic ray removal at the beginning of the experiment was ideal because it made it easier to visualize the peaks, but this was with lower laser power, so the peaks were already not intense and were harder to distinguish from the cosmic rays, but when higher laser power is used these peaks are easier to distinguish from the cosmic rays.

Methods 15-21 aimed to saturate the detector, and then backtrack to find the highest exposure time that would yield the best results with just one accumulation. It was seen that the detector did not become saturated, even with 120 seconds, instead the spectra looked high quality. Both the 120, 60, and 50 second exposure times yielded intense peaks so these were chosen as part of the method that would be used to test different particles of the bone. 120 and 50 seconds were chosen specifically to give intense peaks, and 50 was chosen over 60 seconds because they gave similar intensities, and the 50 second method was shorter. A short method at this point of the project was important because it was determined that many scans might be necessary because of the differences in the spectra from the same sample.

One aspect of bone that plays a principal factor in this project is the heterogeneity of the sample. This means that various parts of the bone samples may give different intensities of peaks and have different spectra. In all of the scans that were taken to test the methods, one spot of bone was measured, so the next step to test would be to look at different parts of the sample with the same method and determine if there was any difference. This was done with established methods 19 and 21. Spectra was compared between different particles and different spots on the bones. One source of this difference is the two components of bone, organic and inorganic. Bone is comprised of a collagen matrix with hydroxyapatite crystals throughout, which has peaks at loser Raman shift values than the collagen peaks. Some of the spectra with more intense peaks at 960 cm^{-1} could be due to the scan taking place at a point with one of the crystals, or in an area with a higher

concentration of hydroxyapatite. This also could be a reason for the inversely intense peaks for the organic and inorganic components. There is also a shift peak present at the 2900cm^{-1} peak, which could also be a result of this heterogeneity. It is also important that these samples are not whole samples, they are ground up and could have pieces of the outer layer of bone and the inner layer of bone mixed with one another, which could also lead to the difference in the spectra. The peak shift and intensity differences present also affect the commonly used ratios that can help quantify components of bones, since the ratios are no longer consistent between scans on the same sample. This is an important factor for creating a model and is used in many papers for analysis.

Conclusion

With many different parameters tested to create an ideal method for Raman Spectroscopy with bones, the best method that was determined was a 1 accumulation, 100% laser power, and 50 or 120 second exposure time. This method yielded intense peaks and remained a relatively short amount of time, which is necessary for samples that require many scans. The heterogeneity of the bones was seen when comparing scans on the same sample in different locations, and this was seen in intensity of the phosphate peaks and peaks shifts in the CH_2 stretching peak. Further investigation on how to create a sample size that will have the best accumulation of scans is needed, or an alteration of the bone samples to create a more heterogenous sample.

It is unknown if a model can be created for aging these specific bones, but perhaps with intact bones it may be a possibility or more scans per sample.

In the Future

This project is in its preliminary stage at this point. Future directions for this project include overcoming the sample heterogeneity issue. Ways that this could be done is through making the sample more homogenous by grinding the bone to a fine powder and then taking a map of the sample, which will utilize the short time for the scan in the method developed. Another way to overcome this issue is to determine what parts of the bone give different spectra and treat them as separate samples. These samples could also be compared to standards of the components of bone, and a sample of bone with the different components of bone already identified. If one of these methods works, then the number of scans needed per sample to have a good representation of the sample would need to be calculated. Then if this works a model could be developed from this data.

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