



The use of laser spectroscopy to investigate bone disease in King Henry VIII's sailors



Jemma G. Kerns ^{a,*}, Kevin Buckley ^{b,a,**}, Anthony W. Parker ^{b,a}, Helen L. Birch ^a, Pavel Matousek ^{b,a}, Alex Hildred ^c, Allen E. Goodship ^a

^a UCL Institute of Orthopaedics and Musculoskeletal Science, Royal National Orthopaedic Hospital, London, HA7 4LP, UK

^b Central Laser Facility, Research Complex at Harwell, STFC Rutherford Appleton Laboratory, Harwell Oxford, OX11 0FA, UK

^c Mary Rose Trust, College Road, HM Naval Base, Portsmouth, Hampshire, PO1 3LX, UK

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ABSTRACT

The *Mary Rose* was King Henry VIII's flagship before it sank in battle on the 19th July 1545. Over four hundred men went down with the ship and the environment of the Solent meant their remains were quickly covered in silt. Between 1979 and 1982 the remains of 179 individuals were recovered and examined as part of the excavation of the *Mary Rose*. The anaerobic environment created by the silt preserved the sailors' bones in remarkable condition and to date much has been learnt about life on the ship. In this study we used Raman spectroscopy (a non-destructive technique), to investigate the chemistry of the human bones, specifically for the identification of disease in archaeological specimens, for the first time. Raman data were collected from five anatomically normal tibiae and five tibiae that were bowed (individuals suspected to have suffered from bone disease in childhood). The data were processed using multivariate analysis (principal component analysis) and results showed the presence of chemical abnormalities in the bowed bones which resulted in the separation of the bones into two clearly defined groups, normal and bowed.

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1. Introduction

The *Mary Rose*, King Henry VIII's flagship, was built in Portsmouth in 1509–10 and was in service until she sank just outside Portsmouth Harbour during the Battle of the Solent on the 19th July 1545 (Rule, 1982; Stirland, 2000). The crew of over 400 men were trapped by the anti-boarding netting fitted above the upper deck and the vast majority drowned (only 35–40 survived). The geography of the Solent creates a unique tidal regime which deposited soft sediments on the wreck of the *Mary Rose*. The layers of sediments created anaerobic conditions, which preserved much of the organic material, including the human bones, exceptionally well (Stirland, 2000).

Abbreviations: PCA, principal component analysis.

* Corresponding author. Present Address: Lancaster Medical School, Lancaster University, UK. Tel.: +44 1524 592285.

** Corresponding author. Central Laser Facility, Research Complex at Harwell, STFC Rutherford Appleton Laboratory, Harwell Oxford, OX11 0FA, UK. Tel.: +44 1235 44 5377.

E-mail addresses: jemma.kerns@ucl.ac.uk, j.kerns@lancaster.ac.uk (J.G. Kerns), [kevin.buckley@stfc.ac.uk](mailto:kевин.buckley@stfc.ac.uk) (K. Buckley).

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A major excavation of the *Mary Rose* between 1979 and 1982 yielded more than 25,000 objects, including a large number of human bones. Although the soft tissue no longer remained, the bones were preserved, however no completely articulated skeletons were found. Although many areas contained dense accumulations of bones from a number of individuals, post excavation sorting revealed an assemblage of at least 179 individuals, 92 of which have been partially reconstructed based on bone morphology and archaeological association. All individuals on board were men with an estimated biological age range of 12–40 years, but the majority was aged 18–30 years. There is no crew list from the ship and only three individuals are known by name, unfortunately none of these can be identified from the assemblage recovered. As re-constructing individuals required fitting the bones together, specific bones such as shoulders, hands, feet, ribs and breastbones could not be included unless the archaeological association was overwhelming. Similarly, unless most of the spinal column was present, skulls were not assigned to individuals (Stirland, 2005).

Of the 1241 long limb bones that were excavated from the wreck, two femora out of 319 were significantly bowed

anteroposteriorly, and 12 tibiae out of 310 showed significant bowing mediolaterally, suggestive of residual rickets (Roberts, 2008; Brickley and Ives, 2008). Rickets is a childhood disease resulting from a lack of dietary phosphate and/or calcium and reduced sun exposure. The skeletal manifestation is bending and flaring of the bone: active rickets. Once the deficiency is treated as the bone turns over and remodels a full recovery is usually expected (healed rickets). However, when this does not occur individuals may have residual rickets. For definitive diagnosis of rickets (active and residual) in archaeological skeletal remains, evidence would also need to be confirmed in the skull and ribs; although radiological evidence of thickening of the concaved cortex with a shaped cortical outline would reduce the likelihood of a differential diagnosis (Brickley and Ives, 2008; Mays et al., 2006). In addition to bowing and swelling, increased porosity would also be evident in active rickets (Mays et al., 2006). To note, the majority of diagnosed rickets from bending deformities in archaeological adult specimens are cases of residual rickets (Mays et al., 2006). In the majority of cases, from the bones retrieved from the *Mary Rose*, the three types of specimen were not present in a single reconstructed individual. The other possible conditions that are associated with “long bone bending deformities and metaphyseal swelling” are osteogenesis imperfecta, metaphyseal chondrodysplasia and Blount's disease (Brickley and Ives, 2008). These disorders are either very rare and result in multiple fractures and/or short stature (osteogenesis imperfecta and metaphyseal chondrodysplasia) or they progress in such a way as to leave individuals severely disabled by military age (Blount's disease). There was no evidence of multiple fractures or that the bones were from individuals of short-stature: tibiae with fused epiphyses were 25–30 cm in length. Although the bones have bending deformities, there was no evidence that they were severe and therefore unlikely to result in disability.

Raman spectroscopy is a non-destructive technique, in which laser light of a single wavelength is scattered from the surface of an object, collected, and analysed. The scattered light contains wavelength-shifted components and this spectrally shifted light, when plotted as a spectrum (magnitude of shift versus intensity of scattered light), represents a unique chemical ‘fingerprint’ for a particular substance. For simple substances with a small number of chemical bonds the Raman spectra may be easy to elucidate but for complex molecules and mixtures (e.g. biological samples) and for cases where differences are subtle, more advanced analysis techniques are required. Commonly, principal component analysis (PCA) is used to reduce the number of variables by forming linear combinations of the wavenumbers and ranking them by degree of variance. The data may then be plotted in scatter plots along these new axes to look at how the data are grouped or spread. The inspection of these new axes may facilitate the identification of the spectral region, and therefore the biochemical(s), which are responsible for the grouping or spreading of the data. Further analyses can also be performed on the PCA-transformed data set.

Raman spectroscopy as a non-destructive technique represents an excellent method for characterising the chemical nature of valuable archaeological samples and has been applied to artworks and artefacts, (Smith and Clark, 2004) fossilised charcoal from archaeological sites, (Cohen-Ofri et al., 2006) and Roman age pottery, stone and glass (Ricciardi et al., 2009). Raman spectroscopy has also been applied to the study of ancient and fossilized teeth with two studies showing that the carbonate and collagen content of teeth decreases with time, and another to study pigmentation of human remains (Carden and Morris, 2000).

The dental studies illustrate an analytical strength of Raman spectroscopy: it can be used to probe both the mineral and organic phases of mineralised tissues (in contrast to X-ray techniques which probe the mineral phase). In recent years Raman

spectroscopic studies of bone diseases in the medical and biomedical fields have led to new insights in the etiology/diagnosis of osteoarthritis, osteoporosis and osteogenesis imperfecta (Morris, 2010).

The purpose of the present study is to use (non-destructive) Raman spectroscopy and multivariate analysis to obtain chemical information from the protein and mineral components of the human bones that have spent 437 years under the sea. Specifically, we aim to compare bowed archaeological specimens with anatomically normal control bones to determine if there are chemical modifications present which may enhance our understanding of the presence of metabolic bone disease in 16th century human populations.

2. Materials and methods

2.1. Specimens

Ten tibiae from adult men were obtained from the *Mary Rose* Trust in Portsmouth, UK (See Suppl. Table 1 for details). Five of the tibia had a normal anatomical appearance (Fig. 1, top) and five showed signs of significant proximal mediolateral bending suggestive of one of “various metabolic bone diseases in which bone shape can be altered by mechanical loading in response to an underlying pathological condition” (Fig. 1, middle) (Brickley and Ives, 2008). Bones with evidence of multiple pathologies, including osteoarthritis, were excluded from this study.

A fresh cadaveric human tibia from an adult male was also obtained (from Vesalius Clinical Training Centre, University of Bristol; ethics approval no. 08/H0724/34), so the spectra could be compared. That tibia was from a 58 year old individual who had died due to a soft-tissue cancer (the bone was examined by an orthopaedic surgeon and showed no signs of bone disease). The cadaver was frozen on being donated (193 K) and after being excised the tibia was stored at 193 K. All the soft tissue associated with the tibia was removed with a scalpel before it was scanned.

2.2. Raman spectroscopy

The Raman spectra were collected with an instrument that was custom built (Cobalt Light Systems Ltd., Oxfordshire, UK). The instrument utilised an 830 nm laser (300 mW at sample), a low loss Optran WF fibre-optic bundle (CeramOptec, MA, USA) and a Raman

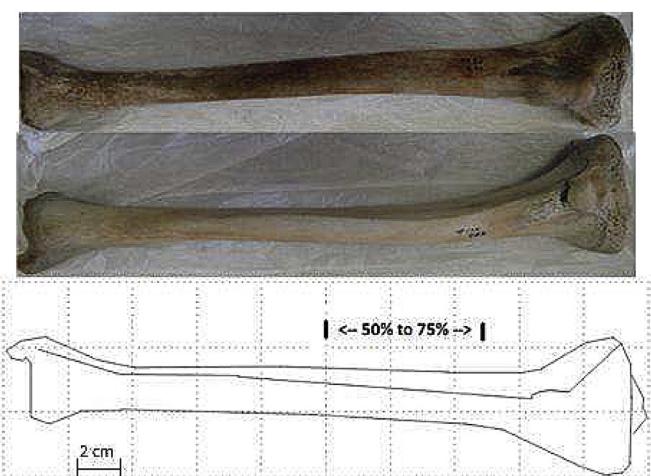


Fig. 1. Top – normal tibia bone from the *Mary Rose*; middle – bowed tibia bone from the *Mary Rose*; bottom – outline showing where Raman data was collected.

Explorer spectrograph (Headwall, MA, USA). At the output of the spectrograph was a charge-coupled device detector (Andor iDus 420 BR-DD, Andor, Belfast, Northern Ireland). The detector had a spectral resolution of $\sim 8\text{ cm}^{-1}$ and removed numerically spurious signals that were due to cosmic-ray events. The instrument is capable of recording spatially offset Raman spectra (SORS) (Buckley and Matousek, 2011) but in this study it was used with zero spatial offset, i.e. as a conventional Raman spectrometer.

For each bone, a number of 10-s Raman spectra ($0.5\text{ s} \times 20$) were collected along a line on the surface of the bone (at 1 mm intervals), from halfway to 75% along the total length (Fig. 1). The sections length and the accumulation time of each spectrum (10 s) meant total time to scan each bone was 15–20 min. The large collection zone, of the order $\sim 10\text{ cm}$, was used in order to reduce errors associated with bone's heterogeneous composition (Buckley et al., 2014) and this particular section was chosen because it coincided with the most pronounced areas of bowing. A minimum of 100 spectra were collected from each bone.

2.3. Spectral analysis

The broad fluorescence background was subtracted from each of the (approximately 1000) spectra by fitting a polynomial curve (Lieber and Mahadevan-Jansen, 2003). This was done using a MATLAB executable routine written in-house. The spectra were then normalised to the intensity of the v_1 phosphate band at 960 cm^{-1} , i.e. the intensity of the band was set to one (see Fig. 2).

The Raman data from each bone were then decomposed using principal component analysis (PCA), an unbiased technique (i.e. no *a priori* information) that separates and ranks the data points based on variance. It does this by forming linear combinations of the variables. PCA analysis allows access to the ranked variation 'spectra', called loadings or eigenvectors, and the weighting (the score) that is needed to recreate each spectrum from the eigenvectors.

3. Results

The averaged spectrum from each of the five anatomically normal *Mary Rose* tibiae is plotted alongside the averaged spectrum from the fresh tibia in Fig. 2 (the spectra are offset to aid

comparison). The spectra are remarkably similar and even small spectral features (for example the proline/hydroxyproline bands at $\sim 850\text{ cm}^{-1}$ and the phenylalanine band $\sim 1000\text{ cm}^{-1}$) can be retrieved. This is noteworthy considering the bones were buried within the seabed for 437 years and previous experiments using Raman spectroscopy to detect bone conditions have found subtle changes across these features (Kerns et al., 2014; Kazanci et al., 2006; Morris and Mandair, 2010). Importantly, the spectral comparison reveals no spectral contribution from surface contamination of the *Mary Rose* bones; the majority of the surface contamination that is present is iron staining (from corroding iron shot).

The average spectrum from each control (anatomically normal) *Mary Rose* tibiae and the average for each bowed *Mary Rose* tibia are shown in Fig. 3. The spectra are similar but show some differences in the mineral region below 700 cm^{-1} and in the protein region between 1200 cm^{-1} and 1500 cm^{-1} (Suppl. Table 1).

PCA analysis was performed on the 10 averaged spectra, 5 bowed and 5 controls, to investigate whether there was any systematic difference between the two sets of spectra. PCA is an unsupervised analysis technique, therefore the cohort labels were not used during the analysis, but only for visualisation afterwards in the resulting plots. The first three loadings (i.e. the three main sources of variation between the 10 different spectra) are shown in the inset of Fig. 4. They can be seen to have features in both the mineral and collagen regions of the spectrum. Each bone is then shown as a data point in the main window; its position being determined by its score for the three main loadings. It can be seen that the bowed bones (blue points) (in web version) are spatially separated from the control bones (black points) and thus have different chemical compositions (the ellipsoid envelopes around them are 90% confidence ellipsoids). This spread of data is based on the variance throughout all the spectra regardless of any characteristics differences e.g., shape, or user input. This supports the hypothesis that the individuals had suffered a bone disease rather than the bones just being different shapes. The ellipsoids do take into account the cohort labels, and do not overlap.

The 90% confidence ellipsoids show that the variance in the bowed group is much larger than in the control group. This intra-cohort variance suggests that there may be two groups present, but due to the size of the 'two groups' (i.e. three bones and two bones) the present study will not extrapolate anything about them.

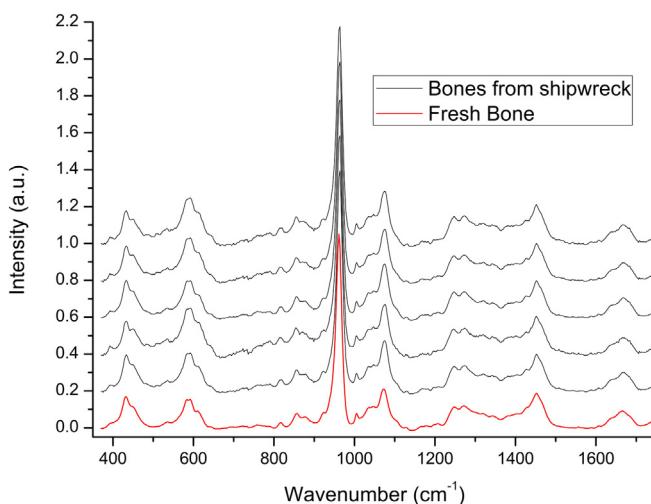


Fig. 2. Raman spectra from bones, which were buried within the seabed for 437 years, are remarkably rich in chemical information and are comparable to the spectrum of fresh bone. The lower trace (red) is the fresh bone. Spectra are offset in the y-axis for visualisation.

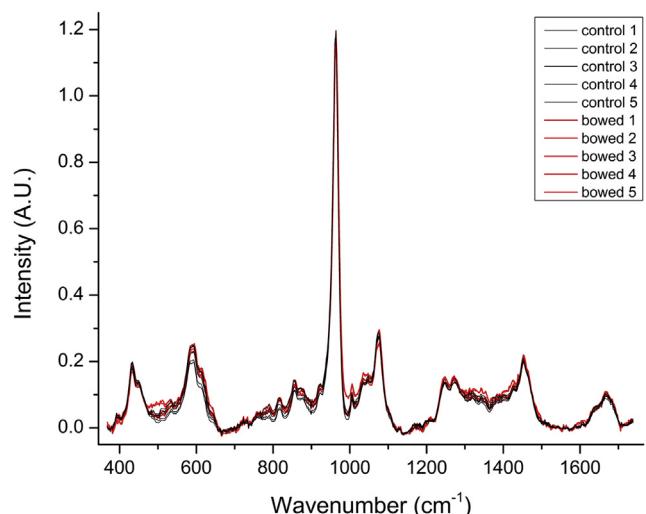


Fig. 3. Average spectrum of each of the five bowed tibiae and five control tibiae.

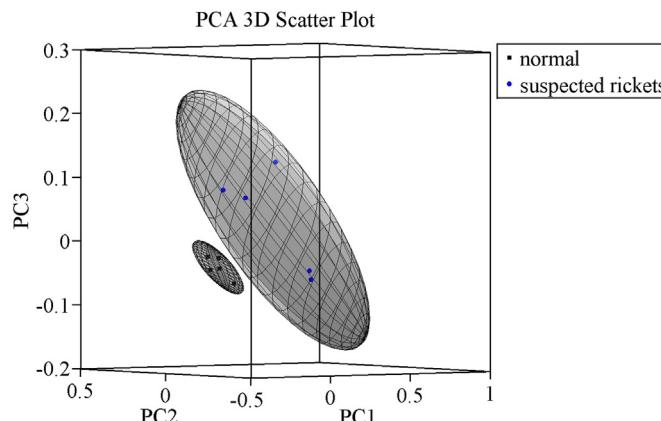


Fig. 4. The separation shows that there is a chemical difference between the five bowed tibiae and the five controls.

4. Discussion

Raman spectroscopy has been used to study bone disease in archaeological specimens for the first time. The chemical information retrieved from these samples was surprisingly rich and was almost identical to that retrieved from a fresh sample, which had been frozen directly after being excised. Importantly, the retrieval of chemical information from both the organic and inorganic components was assessed non-destructively; this would not be possible with physical examination or current techniques based on radiography.

The Raman analysis showed that the bowed bones are chemically different to the straight bones and allowed them to be separated into two groups based on their chemical composition, one group corresponding to normal macroscopic shape and one group corresponding to 'bowed' macroscopic shape. The difference between the two groups of bones can be identified from the loadings plot (Fig. 4 inset) and shows that the mineral phase accounts for the most variance between the groups.

Rickets is a disease of the growing skeleton affecting both the mineral and organic (extracellular collagen matrix) phases and can lead to large areas being affected at the growth plate of hypomineralised bone. In this region the increased phyeal cartilage and poorly mineralised bone are soft and unable to withstand normal mechanical loading, leading to development of skeletal structural deformities, specifically bending and swelling of the long bones. These pathognomonic signs are caused by disturbance of calcium and phosphate metabolism brought on by inadequate diet, and if left untreated, leading to long-term damage of the skeleton, which may be classified as residual rickets (Cianferotti and Marcocci, 2012; Sharp et al., 1997).

The chemical differences observed in our Raman study, when coupled with gross anatomy (i.e. the "long bone bending deformities and metaphyseal swelling [that] are characteristic and well-recognised skeletal changes of rickets") strongly suggest that these sailors suffered from residual childhood rickets (Brickley and Ives, 2008). The chemical changes identified indicate the presence of the adaptive adult response to severe rickets (now evident as residual rickets) sustained in childhood. These chemical changes are due to the response of the skeleton to the bent, less mechanically competent, bone.

The chemical signature measured in this study is most likely the long-term effect of rickets on the skeleton; the bowing would have resulted in a loading response that was different to non-bowed bone, and therefore the bone produced at the metaphysis would have altered composition to withstand the new loading conditions.

Nowadays, the presentation of rickets is rarely observed at the skeletal level, as it is diagnosed early and leads to rapid treatment (with vitamin D) resulting in a cure, assuming that the vitamin levels are maintained subsequently. The existence of skeletal deformity in military-age sailors illustrates the importance of early diagnosis and treatment in children.

5. Conclusion

To the best of our knowledge, this is the first Raman study of bone disease in archaeological human bone. Chemical information was obtained from both the protein and mineral components of human bone after 437 years under the sea using (non-destructive) Raman spectroscopy and multivariate analysis. The archaeological bones from the *Mary Rose* were chemically comparable to fresh bones. Importantly, the grossly abnormal *Mary Rose* bones identified as coming from individuals who suffered from a metabolic bone disease (likely rickets, because of their macroscopic shape) showed chemical differences from both normal *Mary Rose* bones and from recent cadaveric bone.

The work fully demonstrates the benefits Raman spectroscopy brings for the analysis of archaeological human bone due to its non-destructive nature. The present study validates that it can determine chemical information at a sufficient level to identify diseases and/or lifestyle adaptations and suggests that studies of other conditions such as osteoarthritis, osteoporosis and osteogenesis imperfecta in archaeological human bone could be possible, without resorting to destructive sampling for histological study (Mays et al., 2013).

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.jas.2014.11.013>.

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