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Quantifying collagen quality in archaeological bone: Improving data accuracy with benchtop and handheld Raman spectrometers

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ABSTRACT

Chemical analysis of collagen and other endogenous protein in excavated bones is ever more common in paleontology and archaeology to determine dietary ecology, migration patterns, age, and diagenetic pathways. Decisions to sacrifice valuable samples to destructive stable isotope, radiogenic isotope, and proteomic analyses are easier to make if one knows ahead of time whether sufficient undegraded organic material is preserved in the bone for chemical analysis. Recent advances with near–infrared Raman spectroscopy to non-destructively pre-screen bones for the presence of undegraded endogenous protein can indicate whether original isotopic or proteomic signatures have remained intact in long-buried bones. However, it is crucial to examine the different emerging approaches with benchtop and handheld Raman spectrometers and to establish uniform data reporting standards that will allow laboratories to accurately compare results. Three recently published studies are compared to understand the factors that affect protein screening success and to establish best practices for the technique in the laboratory and in the field. A set of 37 archaeological human bone samples analyzed previously by the authors using FT-Raman spectroscopy are re-analyzed with a handheld 1064 nm Raman spectrometer such that the laser power was similar but the spectral resolution and signal-to-noise were lower. Two methods to identify peak heights were evaluated: 1) peak height fixed at a specific wavenumber and baseline anchored at fixed points bracketing the band and 2) averaging peak intensities over a short wavenumber range while also setting the baseline of the band by an average of several anchor points. This second method appeared to better classify whether the bones contained well-preserved protein signatures. In particular, peak height ratios of 960 cm^{-1} to 1636 cm^{-1} or 1450 cm^{-1} can indicate quality and abundance of bone protein, but have benefits and trade-offs that depend on the instrument used. The 960 cm^{-1}:1636 cm^{-1} ratio is more descriptive of collagen quality as defined by an extracted carbon:nitrogen ratio of 2.8–3.6, but the 1636 cm^{-1} peak can be difficult to resolve well with an inherently less sensitive handheld Raman spectrometer. The 1450 cm^{-1} peak is more prominent in the spectrum and therefore more easily resolved with a handheld spectrometer, but it describes a common C—H stretch found in collagen and other organic molecules.

1. Introduction

Chemical analysis of excavated bones is increasingly popular in paleontology and archaeology. Bones frequently are analyzed using stable isotopes, radiogenic isotopes and proteomic techniques to determine dietary ecology, migration patterns, age, and diagenetic pathways. Decisions to sacrifice valuable samples to these destructive techniques are easier to make if one knows ahead of time whether sufficient undegraded, endogenous protein is preserved in the bone for chemical analysis. Recent advances have been made with near–infrared Raman spectroscopy to non-destructively pre-screen bones for good collagen preservation (France et al., 2014a; Halcrow et al., 2014; Pestle et al., 2014, 2015), which is a strong indicator that original isotopic or proteomic signatures have remained intact in long-buried bones. However, these researchers have focused on different criteria for collagen quality and analytical parameters. As this technique continues to develop it is important to recognize these different approaches, examine their attributes, and establish uniform data reporting standards that will allow laboratories to compare results. This is a crucial step toward becoming a mature science with generally agreed upon standards that yield accurate and precise protein data (Killick, 2015). Furthermore, the advent of relatively inexpensive, easy to operate, handheld Raman spectrometers sets up new challenges for assessing data quality. The spectral sensitivity and resolution of these handheld instruments is impressive but nonetheless lower than research-grade benchtop spectrometers, and these instruments are likely to be

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deployed in the field by archaeologists with varying expertise in spectroscopy. Here, three recently published studies are examined, and the set of bone samples evaluated by France et al. in 2014 (2014a) with a high-resolution, benchtop FT-Raman spectrometer is re-analyzed with a handheld 1064 nm Raman spectrometer for comparison.

1.1. Raman spectroscopy of bone

Raman spectroscopy describes the energy distribution of light scattered inelastically by a bone. The organic and inorganic components of bone are made up of atomic bonds that vibrate in characteristic movements. The bonds might stretch or bend, for example, and each movement requires a small, very specific amount of energy. In Raman spectroscopy a laser is directed at the bone. Most of the light reflects off the bone with the same energy (i.e., color) as the laser light, and no energy is exchanged. However, a small amount of the laser light does interact with the bonds in hydroxyapatite, other inorganic salts, type I collagen, and other proteins in the bone. These bonds siphon off small packets of energy to make their characteristic movements, and the photons scattered back to the instrument's detector have slightly shifted wavelengths. A Raman spectrum is a plot of those shifted energies. Each peak describes a type of movement, called a *vibrational mode*, of a specific atomic bond or group of bonds. Together the peaks in a Raman spectrum are like a fingerprint of the material. The presence, position, relative size, or absence of certain peaks can indicate whether a molecule such as collagen is present, intact, or altered such that it has become unsuitable for further chemical analysis. A typical bone spectrum includes many peaks, the most prominent of which are indicated with their associated bonds in France et al. (2014a) and are reproduced here (Fig. 1).

A key factor in Raman spectroscopy of bone is the choice of laser. A laser is monochromatic, meaning it emits a single color of light, typically described by its wavelength in nanometers (nm). Visible light lasers (i.e., 532 nm green and 785 nm red) can trigger fluorescence signals that distort spectral baselines and obscure the relatively weak Raman peaks. Instead near infrared lasers are chosen for bone analysis to avoid interference from fluorescence. For this reason, each of the recently published studies uses a 1064 nm laser. Other important instrument variables are detector sensitivity, spectral resolution, and instrument portability, and these are interrelated. The most sensitive spectrometers with the best ability to discern different vibrations (i.e., adjustable resolution up to 1 cm^{-1}) currently are designed for laboratory research and are not easily portable. This type of instrument is described in this article as high-resolution, research-grade, or benchtop. In addition, the specific instruments used in France et al. (2014a) and Halcrow et al. (2014) are Fourier-transform Raman (FT-Raman) spectrometers; the name describes the mechanism by which the instruments create a spectrum. Increasingly popular portable and handheld instruments are lightweight and less expensive, but they have less sensitive detection, and the resolution is lower (i.e., 8–15 cm^{-1}) and usually not adjustable. This affects the ability to collect Raman spectra without damaging a bone and the quality of those spectra. While these smaller instruments can be used for some research purposes, their ability to discern subtle differences between materials is more limited.

1.2. Bone collagen

Bone is a composite material composed of inorganic mineral grains in *collagen*, which is the main structural protein in bone and also the traditional catch all term for other organic compounds that are also present in lesser proportion. As the science of proteins develops ever more quickly, the ability to distinguish the minor organic components is improving and outpacing the language traditionally used to describe the bulk organic matrix. Thus the term *collagen* is still the convention, but it is not entirely accurate. In this article the authors have chosen to use the term collagen in the conventional way, but type I collagen is specified where appropriate and other endogenous proteins and organic compounds are as well.

In chemical studies of excavated bone, the presence of undegraded *collagen* has long been taken to indicate the overall preservation of original isotopic, radiogenic, and other chemical signatures. If the proteinaceous component of the bone is present, intact, and uncontaminated, it can be used to establish dates for skeletal remains or as evidence of an individual's environment or diet during life.

Nearly all the protein in a bone is type I collagen, which makes up approximately 20% of bone weight and is composed of strong and complex triple helical molecules organized into arrays of fibrils that protect the hydroxyapatite mineral grains from exposure to ground water and other diagenetic influences under most burial conditions (Fratzl et al., 2004; Nelson et al., 1986; Person et al., 1996; Shoulders and Raines, 2009; Tütken et al., 2008; Veis, 2003; Weiner and Wagner, 1998). The remainder of the bone is inorganic minerals including hydroxyapatite (approx. 65%), non-collagenous proteins (< 5%), and water (approx. 10%) (Olszta et al., 2007; Veis, 2003; Dorozhkin, 2011). The quality of type I collagen is typically assessed by measuring its elemental composition and the total amount of material extracted from a bone through a chemical process. Its unique amino acid profile contains ~11–16% nitrogen, ~30–45% carbon, and a carbon to nitrogen (C:N) ratio of 2.8–3.6 (Ambrose, 1990; DeNiro, 1985; DeNiro and Weiner, 1988; McNulty et al., 2002; van Klinken, 1999). Partially degraded type I collagen, other bone proteins, and post-burial humic, fulvic, and bacterial products have different compositions with C:N ratios outside this range (Balzer et al., 1997; DeNiro and Weiner, 1988; Harbeck and Grupe, 2009; Hare, 1980; van Klinken and Hedges, 1995). The C:N ratio is the typical criterion of choice for determining the presence of type I collagen with a well-preserved structure and whether

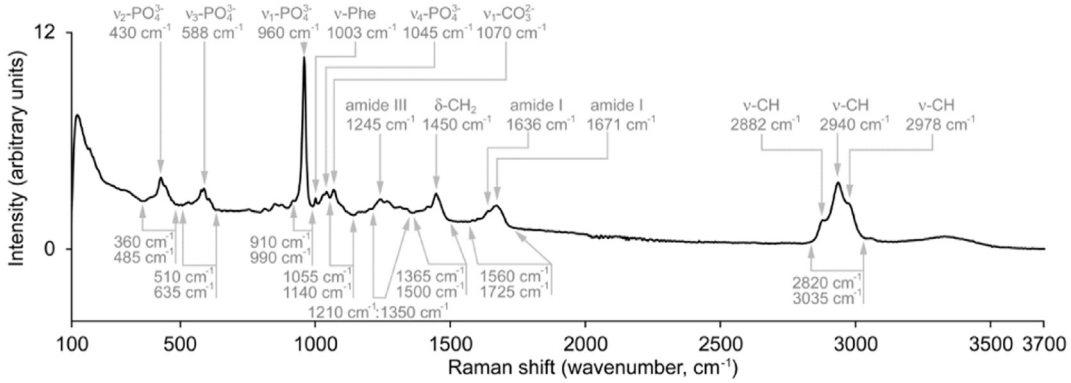

Fig. 1. Characteristic Raman spectrum collected from a well-preserved historic bone sample using a Thermo NXR FT-Raman spectrometer and 1064 nm laser excitation. Corresponding vibrational and stretching modes are indicated with wavenumber. (Reproduced from France et al., 2014a with permission from Elsevier.)