ATR-FTIR spectroscopy and chemometric complexity: unfolding the intra-skeleton variability

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Abstract
Spectroscopy of skeletal tissues is increasingly used in various fields, including legal medicine, forensic science and archaeology. As it is fast and technically and financially affordable yet accurate and widely applicable, spectroscopy is often used when investigating skeletal tissues. Despite its usefulness, the heterogeneity of skeletal tissues highly affects their chemical composition and complicates the interpretation of the spectroscopy results. Though the research on the use of spectroscopy and skeletal tissues from various contexts is growing, little is known regarding the differences in the chemometric indices caused by intra-skeleton variability. The objectives were a comparison of the chemometric indices between teeth and bones, between different bone classes and between the skeletal elements within a bone class as well as an attempt to correlate the observed similarities or differences in chemometric indices to the functional or structural differences of skeletal elements. Different skeletal elements of three individuals were analysed with attenuated total reflectance (ATR)–Fourier transform infrared (FTIR) spectroscopy and compared. Significant differences between the chemometric indices of the bones and teeth were observed and meaningfully correlated with their structural differences. Though more subtle and harder to understand, significant differences were also observed between and within the bone classes and were tentatively correlated with their structural and functional differences. The observed variability agrees with other studies that stress the importance of intra-skeleton variability, which should be acknowledged when using spectroscopy to investigate skeletal tissues.

KEYWORDS
ATR-FTIR spectroscopy, bone type, chemometrics, intra-skeletal variability, skeletal element
1 INTRODUCTION

The Fourier transform infrared (FTIR) spectroscopic analysis of bones and teeth is an increasingly used technique in various fields, including archaeology, biological anthropology, forensics and medicine.\textsuperscript{1–14} Its popularity, particularly when used in conjunction with attenuated total reflectance (ATR), has to do with its wide applicability, low costs, ease of use and small sample requirements with minimal preparation and destruction. Additionally, its high sensitivity enables detection of small chemical and/or structural variation in the samples. A small amount of sample (~3–5 mg) is pressed on an optic window and analysed with a spectrometer. The technique uses the absorption of IR radiation and the detection of molecular vibrations of chemical bonds between atoms and/or ions to produce characteristic FTIR spectra. The obtained spectra consist of peaks, the wavenumber of which is determined by the intrinsic physiochemical properties of the corresponding molecule.\textsuperscript{15} Further peak analysis provides an opportunity to assess the chemical composition of the sample. In the case of skeletal tissues, this provides information on the organic and inorganic components, namely, the collagen and bioapatite. The assessment is based on various chemometric indices: the crystallinity index,\textsuperscript{1,16–18} carbonate-to-phosphate ratio,\textsuperscript{19} relative concentrations of A- and B-type carbonates,\textsuperscript{20–22} relative concentrations of collagen\textsuperscript{2,23} and collagen quality.\textsuperscript{24–26} Indices provide information on the nature of the molecular bonds, their environment and their relative content\textsuperscript{2} and, thus, on the differences and changes in the bone components caused by intrinsic and extrinsic factors. Consequently, they are often used as a pre-screening method to assess the preservation state of skeletal tissues and to decide on the most appropriate samples for further, more laborious and expensive analyses, such as DNA, protein and isotope analyses,\textsuperscript{4,9,27–29} to understand the taphonomic history\textsuperscript{17,30–32} and to discriminate between archaeological and forensic remains.\textsuperscript{5,33–35}

Bones and teeth have composite heterogeneous structures that consist of cells, an inorganic matrix and an organic matrix. Bones are formed by cortical and trabecular bone. The first is a strong and dense tissue, whereas the latter is a porous, more flexible and reinforcing tissue that frequently contains red bone marrow. Teeth components include dentine (the major component of tooth located below the enamel—an extremely hard material with almost no protein)—and cementum—a bone–dentine composite material. The organic part in the bone is formed by collagen and non-collagenous proteins, which are also major constituents of tooth dentin and cementum, but not enamel. The inorganic part is represented by the mineral analogue of hydroxyapatite, with a variety of substitutions and vacancies in the crystal matrix, numerous in the bone and dentin mineral and less numerous in the enamel apatite.\textsuperscript{36} On the nanoscale, crystals of bone and dentin are similar in size (20–50 $\times$ 12–20 nm), whereas enamel has 10 times larger crystals.\textsuperscript{37,38} Bones in the human skeleton form five main categories: long, short, flat, irregular and sesamoid bones, which differ based on their shape and function (Table 1).\textsuperscript{39} Consequently, their composition is also different, as some have thicker, more mineralised compact bone, whereas others have predominantly trabecular bone, covered by thin to medium-thick compact bone. Additionally, histomorphological variability between different bones and parts of the same bone exists,\textsuperscript{40} and there are important differences in the nanoscale characteristics of compact and trabecular bone.\textsuperscript{41}

| Class          | Features                              | Structure                                             | Functions                                           | Examples                          |
|----------------|----------------------------------------|-------------------------------------------------------|-----------------------------------------------------|-----------------------------------|
| Long           | Cylindrical in shape, longer than wide | Thick compact bone predominates, limited amount of trabecular bone, mainly towards the ends | Levers—move when muscles contract                   | Humerus, ulna, radius, clavicle, femur, tibia, fibula, metacarpals, metatarsals, phalanges |
| Short and sesamoid | In shape of a cube or round, approximately equal in length, width and thickness | Trabecular bone, covered by a thin layer of compact bone | Stability, support, limited motion, Protection of tendons | Carpals, tarsals, Patella          |
| Flat           | Thin, often curved                      | Two layers of thin compact bone enclosing a variable quantity of trabecular bone | Points of attachments for muscles, protection of internal organs | Flat cranial bones, scapula, sternum, ribs, pelvic bones |
| Irregular      | Various, irregular shape               | Trabecular bone enclosed within a thin layer of compact bone | Support, protection of internal organs               | Vertebrae, sacrum, mandible maxilla, temporal |

TABLE 1 Classification of bones and their main characteristics\textsuperscript{39}
These compositional differences also influence post-mortem changes, such as the susceptibility to taphonomy. For example, the highly mineralised thick compact bone of the diaphysis makes long bones less susceptible to taphonomic processes in comparison with ribs that have thin compact bone and relatively high amounts of spongy bone. Teeth are less susceptible to taphonomic processes than bones due to the protection from the highly mineralised enamel. Similarly, less mineralised bones of immature individuals with thinner cortices are more susceptible to post-mortem changes than the bones of adults. Certain pathological conditions can cause bone modifications that affect the structural and chemical composition of bones.

All this variation, be it from the natural composition of different skeletal elements, pathologically derived modifications or post-mortem alterations, affects the chemical composition of skeletal elements. Despite numerous studies combining FTIR spectroscopy and skeletal remains from various contexts, little is known regarding the differences in the chemometric indices caused by intra-skeleton variability. This is concerning, as it is well known that skeletal elements differ in their composition due to development and remodelling processes, sex, age, activity and health of the individual, as well as in their susceptibility to post-mortem taphonomy. The results of different spectroscopic studies acknowledging the intra-skeleton and intra-bone variability indicate that this variability can have a significant influence on the chemometric indices and their interpretation. This calls for more specific and detailed studies, possibly also performed on fresh bone to create a sort of a baseline, which will help us to understand the intrinsic and extrinsic factors affecting the results of FTIR spectroscopic analyses.

To contribute towards this understanding, we analysed different skeletal elements of three individuals using ATR-FTIR spectroscopy. The main objectives of the study were a comparison of the chemometric indices between teeth and bones, between different bone classes and between the skeletal elements within a bone class and an attempt to correlate the observed similarities or differences in chemometric indices to the functional or structural differences of skeletal elements. Additionally, we tested a clustering algorithm and prediction model to determine if the obtained data were sufficiently accurate and detailed for the separation of bones and teeth and for the classification of the samples to a correct bone class. The goal of this study was to assess and highlight the potential significance of the intra-skeleton functional and structural variation on the chemometric indices, as their interpretation can affect decisions in the following steps of medico-legal, forensic and heritage protection protocols.

We are fully aware that it would be ideal to perform this kind of analysis on more than three individuals and on the remains without any post-mortem changes. However, it would be extremely difficult to gain ethical approval and access to such remains. We believe that our sample set, originating from three individuals with similar biological profiles and almost the same, relatively short and stable taphonomic history, is a good compromise that yielded accurate and usable results.

2 MATERIALS AND METHODS

2.1 Samples

The samples derived from three completely mummified bodies discovered in 2009 in the Huda Jama Second World War mass grave. All three individuals were young adult males, whose bodies were resting in a relatively stable cave environment for approximately 65 years. Prior to skeletal sampling, the remains were washed with water and a toothbrush, air-dried and stored at constant room temperature in cardboard boxes.

Altogether, 168 tooth and bone samples were collected, with 56 from each individual. The samples were taken from different body regions, in each case from the right side (Table S1). A 5-cm fragment of bone was cut from the diaphysis of the long bones, from the skull bones (in the case of temporal bone, petrous bone was used), from the bones of the torso and from the calcanei and taluses. Half of the metacarpals and metatarsals, patellae, cuneiforms naviculars and cuboids and whole teeth, capitates and phalanges were used. The whole (fragment) of bone was used, combining cortical and trabecular bone.

The collected skeletal elements were cleaned mechanically and chemically. The latter was used as contaminants can protrude deeper into the bone structure and mechanical cleaning does not suffice to remove them. After the removal of the outer surface by drilling, the elements were washed in detergent, bi-distilled water and ethanol. A Bead Beater MillMix 20 tissue homogeniser was used to obtain a fine powder. The bones were ground for 1–2 min at a frequency of 30 Hz. To avoid overheating during powdering, the metal vials and bones were cooled in liquid nitrogen.
The powder was placed in sterile tubes until the analysis. Though the preparation process could cause changes of the chemical components, all the samples were treated in the exactly same manner, and previous experiments confirmed that no significant changes were caused by the chemical cleaning and liquid nitrogen.\textsuperscript{4}

The samples were classified based on the bone classes determined in Gray’s anatomy.\textsuperscript{39} However, there were some obvious macroscopic differences that must be addressed. Petrous bones of the temporal bone, mandible and maxilla were not classified as irregular bones. They cannot be pooled together with the rest of the irregular bones as they have thicker compact bone and/or a lower amount of trabecular bone.

As such, the mandible and maxilla were classified as flat bones (together with the rest of the skull), whereas petrous bone was considered as a separate class (PETROUS BONE). Due to the clear differences in the size and thickness of the compact bones, the metacarpals, metatarsals and phalanges (HAND/FOOT) were separated from the arm and leg long bones.

### 2.2 Spectroscopic analyses

FTIR spectroscopic analyses were performed using a Bruker Vertex 70 equipped with a diamond ATR (attenuated total reflection) accessory and MCT (mercuric cadmium telluride) detector. The samples were scanned in the domain between 400 and 4000 cm\(^{-1}\) at a resolution of 1 cm\(^{-1}\). The spectrum of each sample was collected from an average of 64 scans. In the OPUS software, the baseline was subtracted using iterating averaging,\textsuperscript{61} and each spectrum was normalised to the highest peak (\(v_3\)PO\(_4\) at \(\sim1010\) cm\(^{-1}\)).

A Savitzky–Golay second derivative with five points of window was performed on the domain between 500 and 1800 cm\(^{-1}\) to overcome the overlapping of some of the peaks. Chemometric indices used to study human skeletal remains were extrapolated from the normalised spectra and second derivative (marked with *) spectra (Table 2). Though Querido et al\textsuperscript{1} suggested that other indices (e.g. R960/1115) are more accurate to assess bone crystallinity when using ATR-FTIR spectroscopy, splitting factor (SF) is one of the most used ratios when studying bone. Thus, both indices were included in the analysis.

### 2.3 Statistical analyses

Statistical analyses were performed in Orange\textsuperscript{62} and IBM SPSS. To investigate the potential differences between the teeth and bones, different bone classes and skeletal elements within a bone class, the chemometric indices were examined and compared. The data were first examined for normality using the Shapiro–Wilk test. Based on the results (Supporting Information), the non-parametric independent-samples Mann–Whitney \(U\) test was used for comparisons, setting the significance level at \(p \leq 0.05\).

| Index | Peaks (cm\(^{-1}\)) | Meaning | Reference |
|-------|---------------------|---------|-----------|
| \(SF\) | Ratio between the sum of 560 and 600, divided by the lowest point between them | Crystallinity index II (crystal order, strain organisation) | \cite{16,18} |
| \(R960/1115^*\) | Ratio between 960 and 1115 | Crystallinity index I (stoichiometric vs. non-stoichiometric apatite) | \cite{1} |
| \(H872\) | Height of 872 | Relative concentration of A + B type carbonates | \cite{2} |
| \(H1410\) | Height of 1410 | Relative concentration of B-type carbonate + collagen side band | \cite{2} |
| \(H1640\) | Height of 1640 | Relative concentration of amide I | \cite{2} |
| \(H1545\) | Height of 1545 | Relative concentration of amide II | \cite{2} |
| \(R1640/1410\) | Ratio between 1640 and 1410 | Amide I relative to B-type carbonate + collagen side band | \cite{22} |
| \(R1660/1630^*\) | Ratio between 1660 and 1630 | Degree of order in proteins | \cite{24} |
In the second step, all the samples were subjected to the k-means clustering algorithm using \textit{k-Means++} initialization, 10 Re-runs and 300 Maximal iterations, leaving the algorithm to find the optimal number of clusters on its own based on the Silhouette (contrast between average distance to elements in the same cluster with the average distance to elements in other clusters).

In the third step, random forest algorithm (RF) was run over the normalised spectra and chemometric indices of the bones. This machine learning supervised algorithm provides classification of the samples based on a large number of individual decision trees that yield a vote for the final decision.\textsuperscript{63,64} Following Oshiro et al.\textsuperscript{64} the \textit{number of trees} was set to 100. To allow reproducibility of the results, the \textit{seeds for the random generator} were fixed. The obtained classification results were then projected using linear projection (LP) combined with principal component analysis (PCA).

\section{RESULTS}

\subsection{Teeth and bone classes}

When compared to teeth, bones had higher PO$_4$ peaks in the range between 450 and 650 cm$^{-1}$, with a lower 960 cm$^{-1}$ PO$_4$ peak and 872 cm$^{-1}$ carbonate peak and lower amide–carbonate complex in the range between 1180 and 1720 cm$^{-1}$ (Figure 1).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{spectrum.png}
\caption{Comparison of the averaged normalised spectra of teeth and bones}
\end{figure}

\begin{table}[h]
\centering
\caption{Summary of relative teeth and bone class characteristics based on chemometric indices}
\begin{tabular}{|c|c|c|c|c|c|c|c|}
\hline
\textbf{Class} & \textbf{SF} & \textbf{Crystall. R960/1115} & \textbf{Carbonates H872; H1410} & \textbf{Collagen H1640; H1545} & \textbf{Coll. quality R1660/1630} \\
\hline
\textit{TEETH} & High & High & Low & Low & High & >1.5 \\
& >3.6 & >4.9 & <0.51 & <0.75; <0.54 & \\
\hline
\textit{PETROUS} & Low & Low & >0.54; >0.67 & High & <0.54; >0.78; >0.56 & <1.1 \\
& <3.35 & <3 & \\
\hline
\textit{LONG} & Medium & Medium & High & Medium & Poor & <1.1 \\
& 3.35–3.6 & 3–4.9 & >0.54; >0.67 & 0.75–0.78; 0.54–0.55 & \\
\hline
\textit{HAND/FOOT} & Medium & Medium & Medium & Medium & High & >1.5 \\
& 3.35–3.6 & 3–4.9 & 0.51–0.54; 0.64–0.67 & 0.75–0.78; 0.54–0.55 & \\
\hline
\textit{FLAT} & Medium & Medium & Low & Low & Medium & 1.2–1.5 \\
& 3.35–3.6 & 3–4.9 & <0.51; <0.64 & <0.75; <0.54 & \\
\hline
\textit{SHORT} & Medium & Medium & Low & Medium & Medium & 1.2–1.5 \\
& 3.35–3.6 & 3–4.9 & <0.51; <0.64 & 0.75–0.78; 0.54–0.55 & \\
\hline
\textit{IRREGULAR} & High & Medium & Low to medium & High & Medium & 1.2–1.5 \\
& >3.6 & 3–4.9 & <0.51; 0.64–0.67 & >0.78; >0.56 & \\
\hline
\end{tabular}
\end{table}
Similar observations were made in the chemometric indices (Tables S1 and S2; Supporting Information). Significant differences in the chemometric indices between bones and teeth were seen in H1640, H1545, H1410 and H872, which were all lower in teeth, and in R960/1110, the splitting factor (SF) and R1660/1630, which were all higher in teeth.

When considering bones, one should bear in mind that skeletal elements of only three individuals were included. Yet, significant differences were seen in R1640/1410, H1410 and SF. R1640/1410 was the lowest in the long bones, due to the highest H1410 and one of the lowest H1640. On the other end were irregular bones with the highest R1640/1410, with a medium H1410 and one of the highest H1640. H1410 was lowest in the flat and short bones and highest in the long and petrous bones. The SF was lowest in the petrous bone and highest in the irregular bones.

 Barely non-significant were R1660/1630 ($p = 0.051$), which were highest in the hand/foot bones and lowest in the petrous bones, and H872 ($p = 0.061$), which was highest in the petrous bones and lowest in the short bones.

Observing the SF and crystallinity index R960/1115, the relative concentrations of carbonates (H1410 and H872), relative concentrations of collagen (H1640 and H1545) and collagen quality (R1660/1630), it was possible to deduce that (Table 3):

- **TEETH** are characterised by high SF and crystallinity and low relative concentrations of carbonates and collagen, with the latter of high quality.
- **PETROUS BONE** has low SF and crystallinity and high relative concentrations of carbonates and collagen, with the latter of poor quality.
- **LONG BONES** are characterised by medium SF, medium crystallinity, high relative concentrations of carbonates and medium concentrations of collagen, with the latter of poor quality.
- **HAND/FOOT** bones are characterised by medium SF, crystallinity, relative concentrations of carbonates and collagen, with the latter of high quality.
- **FLAT BONES** are characterised by medium SF, medium crystallinity and low relative concentrations of carbonates and collagen, with the latter of medium quality.
- **IRREGULAR BONES** are characterised by high SF, medium crystallinity, low to medium relative concentrations of carbonates, and high relative concentrations of collagen, with the latter of medium quality.
- **SHORT BONES** are characterised by medium SF, medium crystallinity, low relative concentrations of carbonates and low to medium relative concentrations of collagen, with the latter of medium quality.

### 3.2 Variations within the bone classes

#### 3.2.1 Long bones

Comparing the long bones of the arm and the leg, the latter had significantly lower SF and R1640/1410. Though non-significantly, the leg also had lower H1640 and H1545 and higher H872, H1410 and R1660/1630 when compared to the arm (Table S3).

#### 3.2.2 Hand/foot bones

There were no significant differences between the metacarpals and metatarsals. When comparing the metacarpals and metatarsals with phalanges, significant differences were seen in H872 and H1410, which were lower in the phalanges, and in R960/1115, which were higher in the phalanges. Though lacking significance, the phalanges also had the lowest H1640, H1545 and R1660/1630 (Table S3).

#### 3.2.3 Flat bones

Comparing the flat bones of the skull, torso and pelvis, significant differences were observed in R1640/1410 and H1640, which were highest in the pelvis, followed by the torso and skull. Barely non-significant was H1545, $p = 0.58$, which was also highest in the pelvis and lowest in the skull. Though lacking significance, R1660/1630 was highest in the skull and lowest in the pelvis (Table S3).
3.2.4 | Short bones

Comparing the carpals and tarsals, the only significant differences were seen in R1660/1630, which were highest in the foot and lowest in the hand. Though non-significantly, H1640, H1545, H872, H1410, SF and R960/1115 all presented the same order (Table S3).

3.2.5 | Irregular bones

There were no significant differences when comparing irregular bones. However, when only comparing cervical and lumbar vertebrae, there were significant differences in the SF, higher in the former. Though non-significant, differences were also seen in H1640, which was higher in the lumbar vertebrae, and in R1660/1630, which were higher in the cervical vertebrae. Lacking significance but still present were higher H1410 and H872 in the lumbar vertebra and higher R960/1115 in the cervical vertebrae. Slightly outstanding was also the sacrum, with H1640 and H1410 close to the lumbar vertebrae, with R960/1115 close to the cervical vertebrae, with SF in the middle, and with the highest R1660/1630 (Table S3).

3.2.6 | Classifications

Separation between the bones and teeth based on normalised spectra and chemometric indices Figure 2A using the k-means algorithm presented two clusters Silhouette scores 0.539 in case of spectra and 0.617 in the case of indices with all the bones in Cluster 1 and all the teeth in Cluster 2. The only exceptions were the rib XII of Skeleton B and ischium of Skeleton A, which were clustered in Cluster 2. When the exceptions were compared with the other two samples from the rib XII and ischium, the exceptions presented significantly higher relative concentrations of phosphates (R960/1115, SF), but significantly lower carbonates (H1410, H872) and collagen (H1640, H1540). The values from the exceptions were closer to the values of the samples from the teeth than to samples from the bones (Table S4).

The RF method based on the normalised spectra and chemometric indices of the bones produced results with relatively poor accuracy and precision.

FIGURE 2  (A) Linear projection with principal component analysis (PCA) using the k-means algorithm to separate the teeth (Cluster 1—red) and bones (Cluster 2—blue) based on the chemometric indices. The XII rib and ischium are wrongly positioned among the teeth. The most relevant for clustering was the crystallinity R960/1115 on the one side and the relative concentration of collagen H1545 and H1640 and carbonates H872 and H1410 on the other side. (B) A linear projection with PCA based on the random forest (RF) algorithm using only bone samples (right). The hand/foot bones (red), long bones (orange) and flat bones (blue) are clearly separated. Petrous bones (yellow) are positioned between the hand/foot and long bones. Irregular (green) and short (purple) bones are clustered together, though separated from the rest of the bone classes.
The accuracy and precision of the algorithm when using spectra were 0.42 and 0.39, respectively. The accuracy and precision of the algorithm when using indices were 0.35 and 0.31, respectively. As the training and tested data originated from the same set, linear projection based on the RF results still managed to separate different bone classes, and only irregular and short bones were clustered together (Figure 2B; Supporting Information).

4 | DISCUSSION

4.1 | Chemometric profile

Many differences in the chemometric profiles were observed between the teeth and bones. The first had higher crystallinity, whereas the second had higher relative concentrations of carbonates and amides. This is not surprising as those are the basic differences in the structure of bones and teeth, particularly when considering the whole tooth, including the highly mineralised enamel with almost no collagen, minimal carbonate substitutions in the crystal matrix, and larger crystals. The exceptions, rib XII and ischium, presented very unusual values of the chemometric indices, which were closer to teeth than bones. There are two possible explanations—significant recrystallisation with loss of carbonates and collagen occurred, or a mistake was made during sample processing. As the values stand out significantly and all the skeletons originate from the same site with similar gross environmental conditions, the latter explanation seems more likely. However, variation in the environmental conditions cannot be excluded as it was previously shown that the remains from the same site can have different preservation state.

Though limited, some significant differences were observed in the chemometric profiles of different bone classes. Petrous bone had the lowest SF and crystallinity among bones, likely due to good preservation with high relative concentrations of collagen, carbonates and non-stoichiometric apatite. Surprising was the lowest collagen quality, because petrous bone is known to be resistant to diagenesis.

Petrous bone has its own characteristics, which are not easily comparable with the rest of the bones. The results might be explained with the unique macrostructure of the petrous bone—thick compact bone with little trabecular bone and a unique microstructure—highly mineralised apatite, a large network of vascular canals, high cellularity and a lack of remodelling.

In addition to the teeth, high collagen quality was only observed in the hand/foot bones with a medium SF, crystallinity and relative concentrations of collagen and carbonates. The long bones of the hand and foot presented a good balance between all the major components in bone, leading to a mutual protection between bioapatite and collagen, also seen in the highest DNA yield.

This could be explained in two ways. First, hand and feet have less biomass when compared with other parts of the body and are also the most distant from the gut, which could result in different post-mortem microbial activities. Second, metacarpals and metatarsals have a balanced ratio between the thickness of compact bone and the amount of trabecular bone, especially in relatively young remains exposed to only limited taphonomic processes, where soft tissue stored in the intertrabecular spaces might still be preserved.

When compared with metacarpals and metatarsals, the phalanges presented higher crystallinity and lower relative concentrations of carbonates and collagen, with the latter of lower quality. This is possibly due to the thinner cortex and less trabecular bone present in the phalanges, particularly because the sampled phalanges of the thumb were significantly smaller and shorter in comparison with the metacarpals and metatarsals (Figure 3).

The observed differences between carpals and tarsals all the indices were higher in the latter could be correlated to the structural and functional differences between hand and foot bones. In comparison with the carpals, the tarsals, especially the calcaneus and talus, were bigger, with more trabecular bone. Feet are also weight bearing and undergo more bone remodelling.

Long bones, with high relative concentrations of carbonates and poor collagen quality and a medium SF, crystallinity and relative concentrations of collagen, resemble petrous bones in one respect and metacarpals and metatarsals in another. As diaphysis of the long bones was sampled, the observed chemometric characteristics can be correlated to the constantly and slowly remodelling, thick, well-mineralised compact bone with a lack of trabecular bone and high osteon density. When compared with the arm bones, leg bones had lower relative concentrations of collagen but were of higher quality.

However, the only significant difference was the lower SF in the leg, likely due to slightly more carbonates being preserved in the lattice. The long bones of the leg and arm, thus, presented similar compositions, which is
understandable, as their shape, function and structure are also similar. Indices with higher values in the leg bones could be explained as the legs bear more weight than arms, leading to a thicker, more mineralised cortex with less collagen of better quality. Interesting differences were also seen when comparing the femur and fibula.

The chemometric indices indicated significantly higher relative concentrations of collagen in the femur when compared with the fibula (0.80 ± 0.03 vs. 0.69 ± 0.04), whereas the fibula in comparison with the femur presented a higher collagen quality (1.3 ± 0.51 vs. 0.88 ± 0.07), SF (3.38 ± 0.16 vs. 3.22 ± 0.01) and crystallinity (3.51 ± 0.73 vs. 2.55 ± 0.16). This might be correlated to the thicker cortex, more remodelling and higher osteon density in the femur. Though these are both long bones of the leg, they are subjected to different loads, resulting in different tensile strength, elasticity and histomorphology, with the femur having a higher osteon density, whereas the fibula has a more scattered Haversian system and more interstitial lamellae.

In contrast to the long bones, the flat bones presented significantly lower relative concentrations of carbonates and a significantly higher SF. This could be explained with the thinner cortex and higher amount of the trabecular bone in the flat bones, especially as irregular, short and flat bones, all with high amounts of trabecular bone and a thin cortex, had a higher SF in comparison with other bone classes. Gonçalves et al. noted that the SF was significantly higher in the epiphysis and metaphysis with more trabecular bone and thinner cortical bone when compared with diaphysis with thick cortical bone and a limited amount of trabecular bone.

There were only minor differences between the short, flat and irregular bones. This was to be expected, as the structure of these three bone types is quite similar. The only significant difference was the higher relative concentration of collagen in irregular bones when compared with flat bones. This might be correlated to the prevalence of trabecular bone in the irregular bones, as trabecular bone has lower relative amounts of inorganic phase and is less crystalline due to greater bone remodelling when compared with compact bone.

Flat bones were investigated further as they do differ in their structure: Pelvic bones had a thinner cortex and higher amounts of trabecular bone when compared with the bones of the skull (Figure 4). Pelvic bones had the highest relative concentrations of collagen, which was of the lowest quality, whereas the opposite was true for the skull. There appeared to be a correlation between thicker compact bone with some amounts of trabecular bone and better collagen quality, despite the lower collagen concentrations. This could be explained with higher amounts of trabecular bone contributing to more collagen, possibly due to the already mentioned remnants of soft tissue, with a thicker cortex limiting the collagen denaturation.
Similarly, there were some macroscopic differences in the irregular bones, which were the most obvious between the cervical and lumbar vertebrae. The former were smaller with predominantly compact bone, whereas the latter were larger, with predominantly trabecular bone (Figure 5). The sacrum also has a different shape and structure in comparison to the vertebrae. It is larger, more robust, with less porous compact bone in comparison with the lumbar vertebrae and with higher amounts of trabecular bone compared with the cervical vertebrae.

The observed differences in collagen agree with the observations made with the flat bones. Higher amounts of trabecular bone in the lumbar vertebrae and sacrum contributed to higher concentrations of collagen, whereas the thicker compact bone in cervical vertebrae and sacrum contributed to its better quality. The lower SF in the lumbar

**FIGURE 4** X-ray of (A) the skull, F = frontal bone, and (B) of the pelvic area of an adult. Case courtesy of Dr Andrew Dixon, Radiopaedia.org, rID: 62508 and 48083.

**FIGURE 5** X-ray of (A) cervical and (B) lumbar vertebrae of an adult. Case courtesy of Dr Andrew Dixon, Radiopaedia.org, rID: 32505 and 47355.
vertebrae could be correlated to the higher carbonates in the mineral matrix and more collagen in the bone, whereas the higher crystallinity could be correlated to the predominantly compact bone of the cervical vertebrae.

The sacrum, thus, combines both a thicker cortex and higher amounts of trabecular bone, which is seen in high concentrations of collagen of the highest quality. This fits with the proposed theory that hand/foot bones present a good balance between the thickness of the compact bone and the amount of trabecular bone for collagen quantity and quality preservation.

4.2 | Clustering and classification algorithms

The results show that the teeth and bones can be separated based on the spectra when using the clustering algorithm k-means. A learning algorithm RF must be introduced for the bone classes, and, even then, the results lack accuracy and precision. The reasons for the separation between teeth and bone can clearly be correlated to their obvious compositional differences. The complexity of bone classification is also understandable, as different skeletal elements have similar compositions despite their different shapes, functions and structures.

When training on the same dataset, RF was, nevertheless, able to separate metacarpals and metatarsals from the rest of the bone classes. Petrous bone was positioned close to the metacarpals and metatarsals, and to the long bones, which agrees with their composition. Relatively close together were the long and flat bones, likely due to the flat bones of the skull, as they were the ones closest to the long bones. Additionally, the SF and crystallinity of the skull bones were closer to the arm and leg bones than to the pelvic bones or torso.

This might be due to their thicker cortex and less trabecular bone, which makes them more comparable to long bones. Most problematic were the short and irregular bones, which were not separated by the learning algorithm. Even though irregular bones presented lower crystallinity and had a higher relative concentration of collagen when compared with the short bones, the differences were apparently not significant enough for separation.

5 | CONCLUSIONS

FTIR spectroscopic analysis is an increasingly used technique for the study of skeletal tissues. As skeletal elements vary significantly in their structure, intra-skeleton differences in the chemometric indices are expected and were previously confirmed. Yet, little is known regarding how to understand and acknowledge this variation when interpreting the spectra, and even less is known on how to correlate them with the intrinsic and extrinsic factors affecting the results of the analyses.

The main objectives of this study were to compare ATR-FTIR obtained spectra between teeth and different bone classes to evaluate if and/or how structural components, namely, the crystallinity, relative concentrations of carbonates, relative concentrations of collagen and collagen quality, differ among them, and if the observed differences could be correlated to the structure of the analysed skeletal elements. Additionally, a clustering algorithm and prediction model were tested to investigate if the obtained data were sufficiently accurate and precise for the correct classification of the samples.

Although the algorithms managed to determine the bone classes in most cases, the accuracy of the model was low, and the correct classification was only possible as the training and predictions were performed on the same dataset. For now, their use mainly serves the purpose of highlighting the significance of the differences between the skeletal elements. For the general application of the algorithms to classify elements, for example, unknown small fragments from other contexts, additional research is needed.

As expected, the results presented significant differences between bones and teeth, easily relatable to their structural differences, which also enabled the k-means clustering algorithm to separate them. Significant differences between and within bone classes were also observed, though these were more subtle and much harder to understand. Especially similar were the indices of short and irregular bones, likely due to their similar structures. On the other hand, the rest of the bone classes were relatively well separated based on their chemometric profile.

Though provided explanations for the compositional differences require further investigation with more objective measurements, it appears that the thickness of the compact bone and the amount of trabecular bone highly influences the chemometric profile. The implicated amount of trabecular bone is likely significant because the samples originated from relatively well-preserved remains where soft tissue might still be present in the intertrabecular spaces. In poorly
preserved remains, this might not be the case, and the thickness of compact bone could play a more important role. The observed variability agrees with other studies stressing the importance of intra-skeleton variability. The chemometric indices representing different bone components or characteristics crystallinity, carbonates and collagen appeared to be highly correlated and covaried with the bone class, bone sub-class and skeletal elements. The latter, and the different parts of the same element, as seen, for example, in the study of Gonçalves\textsuperscript{6} and Cummaudo,\textsuperscript{40} thus, require further investigation to circumvent misinterpretations of the chemometric indices. For now, to avoid bias when employing ATR-FTIR spectroscopy for the evaluation of the preservation state of remains and to decide on further steps, the contextual origin of the samples should be acknowledged, and sampling or comparing the same region of the same specific element is advised.

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**CONFLICT OF INTEREST**
The authors declare no competing interests.

**ETHICS STATEMENT**
The research project was approved by the Slovenian Medical Ethics Committee 0120-481/2018-11 and 0120-350/2018/6.

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Data available on request from the authors.

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