ABSTRACT

Purpose: In fire scenarios, the application and accuracy of traditional odontological methods are often limited. Crystalline studies and elemental profiling have been evaluated for their applicability in determining biological profiles (age and sex) from human dentition, particularly fire- and heat-affected dental remains.

Methods: Thirty-seven teeth were paired according to tooth type and donor age/sex for the analysis of crown and root surfaces pre- and postincineration using X-ray diffraction (XRD) and scanning electron microscopy (SEM/EDX).

Results: In unburned crowns, carbon (C) content showed a positive correlation with age, whereas phosphorus (P) and calcium (Ca) contents showed a negative correlation with age. In unburned roots, C, P and Ca contents were inversely correlated with crown age. In relation to sex, females exhibited a higher C ratio than males, whereas males showed significantly higher levels of oxygen (O), P and Ca in unburned roots. Incineration resulted in an increase in the crystallite size that correlated with increasing temperature. No differences in hydroxyapatite (HA) crystallite size were found between age groups; however, unburned teeth from females exhibited a larger crystallite size than did those from males.

Conclusions: The challenges of using XRD with a 3D sample were overcome to allow analysis of whole teeth in a nondestructive manner. Further studies may be useful in predicting the temperature of a fire.

KEYWORDS:

Scanning electron microscopy, X-ray diffraction, heat, teeth, biological profile

INTRODUCTION

In mass disasters, high-speed crashes and other highly traumatic incidents, human dentition often serves as the only remaining and most reliable form of identification. In disaster victim identification (DVI) operations, establishing a biological profile by estimating age, sex, ancestral ties and individual characteristics is critical to determining identification. Traditional odontology (and anthropology) methods rely heavily on metric and morphological traits to determine a biological profile; however, these approaches are time consuming, require a high degree of expertise, and subject to low certainty given the propensity for international travel and intermingling of populations (fewer discrete population groups). Extreme heat and fire in such scenarios cause a range of physical and chemical alterations to teeth [1, 2], thus making identification more difficult and precluding the use of traditional odontological methods. Many of these methods are also destructive in nature and therefore unsuitable for DVI scenarios or not applicable to burned or fragile remains. To more readily and accurately predict biological profiles and reduce the burden on resources and response personnel, improvements and alternatives to the currently available methods for identification are being researched.

Nondestructive techniques such as scanning electron microscopy (SEM/EDX) allow micromorphology evaluations to differentiate dental from osseous and nonorganic tissue samples, even after incineration at high temperatures [3, 4]. An examination under SEM has revealed ultrastructural changes to enamel and cementum tooth surfaces at particular temperatures (100, 300 and 600ºC) [5]. In addition, individual elemental properties in burned restorative dental materials have been easily distinguished using SEM [6]. Likewise, X-ray diffraction (XRD) has led to the identification of human cremains [7] and has played a role in the dating of osseous remains [8]. More recent studies have underlined the importance of such techniques in the analysis of crystal structure and enamel crystal size [9,10].

Our study examined the feasibility of using XRD and SEM/EDX for elemental analysis of teeth. These techniques are rarely used to determine biological information for unknown victims. These approaches were used to estimate age and sex from dental remains, and their applicability to fire-and heat-affected dental remains was evaluated.

MATERIALS AND METHODS

Thirty-seven teeth (incisors: 9, canines: 6, premolars: 12, molars: 10) from 17 Spanish individuals of known age (min: 16 years, max: 72 years, average: 43.11 years) and sex (68% males and 32% females) were paired for analysis according to tooth type, donor age and sex. The teeth were stored in 0.05% chlorhexidine following extraction. Prior to analysis, the teeth were washed under running tap water with a soft bristled toothbrush, and a surgical scalpel was used to remove tartar when necessary. The teeth were then rinsed and stored in distilled water at room temperature.

X-ray diffraction (XRD)

Differences in the diffraction patterns of various dental structures (enamel, dentine and pulp cavity) were investigated using XRD and micro-XRD (μ-XRD) point analyses. The XRD instrument was a Bruker D8 Advanced with Cu Kα radiation and a LYNXEYE XE detector. The instrument was provided with a circular slit (0.3 mm diameter) and Goebbels mirror for μ-XRD experiments.

For compound identification, the patterns were recorded in the 2θ range from 25 to 66° with a 2θ step size of 0.025°, and a recording time of 300 s/step. Identification was performed using the software Diffract Eva from Bruker and the ICDD-PDF datafile. For routine XRD and μ-XRD at selected locations, the recording time was 4 s/step. Locations within the enamel (labial and lingual) and dentine (body and apex of the root, crown and within the pulp cavity) were analyzed using a longitudinally sectioned canine (tooth 2|3, 23 y/o, ♀).

For in situ XRD analyses of heat-affected teeth, the samples were placed in a high-temperature oven chamber (HTK 1200N, from Anton Paar) coupled with a thermal control unit (TCU 1000N, Anton Paar). A stepwise heating profile was used to collect diffraction patterns at multiple stages of heating and cooling ranging from 40 to 600°C for the crown and up to 800°C for the root under an air atmosphere. The diffraction conditions were the same as those used for routine analysis.

To obtain further information about the size of the coherent diffraction domains (i.e., crystallite size), the (2,2,0) Bragg reflection was selected, and a single low-overlapping ubiquitous peak served as the representative peak for comparison across all samples. The recording of this reflection was carried out in the 2θ range from 25.2 to 27.0°. The crystallite size was estimated using the Scherrer equation.

The analytical point was defined on the vestibular surface along the midline of the tooth, approximately one-third of the total height of the tooth above and below the cementoenamel junctions (CEJs) of the crown and root, respectively. Individual teeth were tested at intervals of 0.5 mm from the analytical point in each *x* and *y* direction, and *z* was adjusted to achieve the best focal plane for each position. The sample was remounted, and the analyses were repeated to assess the influence of positional variation.

Scanning electron microscopy (SEM)

Teeth were dehydrated using increasing concentrations of acetone prior to undergoing the SEM/EDX analysis. The instrument used was a JSM 6300 scanning electron microscope (JEOL) coupled with an Inca Energy 250 microanalysis system (Oxford Instruments), which was operated at 20 kV with a 100 s live time for the microanalysis with 5 iterations.

The vestibular analytical point corresponding to that used for the XRD analysis was selected for the SEM micrographs, and the elemental ratio profiles were collected pre- and postincineration to compare heat-induced changes in the surface morphology and compositional changes.

Incineration conditions

Teeth were paired according to tooth type and donor age/sex and subjected to incineration at either low (650°C) or high (1000°C) temperatures. The teeth were placed in individual porcelain crucibles and then introduced to the furnace, and the temperature was increased at a rate of 10°C/min. The temperature was held at the assigned maximum value for one hour before the furnace was turned off, and then, the door was opened to allow the samples to cool overnight. Once cooled, the samples were photographed and assessed for their structural integrity, color and fracture patterns. The SEM/EDX and XRD protocols were repeated as per the preincineration assessments.

Statistical analysis

SigmaStat 3.5 and StatPlus software were used to identify differences and trends between groups (ANOVA, linear regression and significance testing). When the data followed a normal distribution, the differences between two groups were assessed using Student's t-test, and when multiple groups existed, one-way ANOVA was employed. When a nonnormal distribution was found, the Mann-Whitney Rank Sum test (two groups) or the Kruskal-Wallis one-way ANOVA (multiple groups) was used. Intergroup differences were assessed using Dunn's method (nonnormal distribution) or the Holm-Sidak test (normal distribution).

RESULTS

Incineration survivability studies carried out as part of this study (results not shown) demonstrated that the roots were the most survivable and readily identifiable dental structures following heat trauma. Crowns offered variable degrees of survivability but had a higher degree of fragmentation than the roots after exposure to heat trauma.

SEM- Elemental differences

The elemental composition in teeth is shown in Table 1. In all cases, C content showed an inverse relationship to Ca, P and O contents. Linear regression analyses showed that C and P contents were strongly correlated in the roots ($p < 0.001$, $R^2 = 0.937$) and crowns ($p = 0.001$, $R^2 = 0.901$). Both Ca/C and Ca/P displayed a correlation in crowns and a much stronger correlation in roots. Ca/C showed an inverse relationship, and C/P showed a direct proportional relationship (Fig. 1). The Ca to P ratio has been shown to change with temperature to approach the theoretical values of hydroxyapatite. The results obtained showed that incineration at 1000°C generally decreased the Ca/P ratio in crowns.

Elemental differences by age are shown in Table 2. In unburned crowns, significant changes in the relative weight percentages of C, P and Ca were found. C content showed a positive correlation with age, while P and Ca contents showed a negative correlation with age. Following incineration, the trends in C and P contents in the crowns were reversed with respect to that observed for the unburned crowns; C content

decreased with increasing age, and P content increased with increasing age. In preincineration roots, C, P and Ca contents also showed significant changes that were opposite of the changes observed in the crowns. C content decreased with increasing age, and significant differences were observed between all age groups. P and Ca contents showed overall increasing trends with increasing age; however, the middle age group in both cases demonstrated a higher average value. In postincineration roots, magnesium (Mg) was the only element with significance and clearly decreased with increasing age.

Elemental differences by sex are shown in Table 3. No significant changes in elemental content were identified in tooth crowns either pre- or postincineration. In unburned roots, a number of differences were found, and females exhibited a higher ratio of C than males. However, males had significantly higher levels of O, P and Ca.

Examining the elemental differences by temperature showed the crowns incinerated at 1000°C had higher levels of sodium (Na), Mg and Ca than those burned at 650°C. Conversely, chlorine (Cl) was found to decrease with increasing incineration temperature. In the roots, only Mg had a significant difference and was higher in the samples incinerated at 1000°C.

XRD results

The major compound identified was hydroxyapatite, $Ca_5(PO_4)_3OH$. Alternative compounds identified by matching the diffraction patterns were chlorofluorohydroxyapatite (chlorian), $Ca_5(PO_4)_3(OH, Cl, F)$, calcium sulfide phosphate and sodium calcium fluoride sulfate. In addition, a scan of the root of a tooth subjected to 1000°C revealed a diffraction pattern that also matched those of hydroxyapatite, fluorapatite, sodium calcium fluoride sulfate ($Na_6Ca_4F_2(SO_4)_6$, carbonatehydroxyapatite, and fluorian (Fig. 2). All of these compounds have a $P6_3/m$ space group symmetry with unit cell parameters of ca. a = 9.4 Å and c = 6.8 Å.

Crystallite size differences are shown in Table 4. In unburned teeth, both the crowns and roots of females displayed a larger crystallite size than the males. Incineration of the teeth increased the crystallite size in a temperature-dependent fashion. Comparisons of crowns and roots pre- and postincineration also showed an increase in the crystallite size postincineration.

DISCUSSION

The elements C, O, P, and Ca were found in the crowns and roots of all teeth pre- and postincineration. Given the composition of hydroxyapatite (HA) and the organic matrix, the frequency and abundance of these elements are expected, but the inability to detect hydrogen (H) was due to instrumental limitations. Additional elements Na, Mg, and Cl were also detected and are commonly involved in HA crystal substitutions; likewise, carbonate also contributes to the increased C and O levels. The relative frequencies and abundances of these elements were similar to those in previous studies on unburned tooth enamel and dentine [1, 11, 12].

It is worth noting the absence of fluorine, which is surprising because fluorine is strongly electronegative and one of the most common substitutes for hydroxyl groups. Interestingly, many other studies [11, 13, 14, 15] also failed to detect fluoride in teeth using various methods, and the reason for this observation is still under consideration.

Silicon (Si) and sulfur (S) were also frequently detected following incineration. As identified by Zenobio et al. [1], S has been notably absent in many studies but prominently features in roots both pre- and postincineration. The reason for this observation remains unclear but may be linked to changes during decomposition, which require further investigation.

Interestingly, the roots demonstrated a more varied elemental composition than the crowns, and this finding is supported by those of Soares et al. [12] and Arnold and Gaengler [16] who also observed differences between permanent and deciduous teeth. Low-level elements, such as aluminum (Al), potassium (K), copper (Cu), iron (Fe), zinc (Zn), tungsten (W), bromine (Br), iodine (I) and molybdenum (Mo), have been variably detected in this and other studies [1]. While their infrequent presence limits the use of these elements as discriminators, the detection of these elements may assist in individualization based on knowledge of an individual's diet, lifestyle and environmental exposure.

Because elements and their relative abundances have been shown to differ between surface and underlying enamel [16], a direct comparison of the surface analyses performed here with those of the powdered samples is not logical. However, as whole-tooth-surface studies are scarce, data obtained from powdered tooth samples may offer some insight. Comparisons of the current data with studies using powdered tooth segments [1,11,12] show great similarities in the ratios (% weight) with the exception of Na, which was determined in this study to be an average of three times lower than reported values.

Although our results are generally in agreement with those of many other enamel and dentine studies, as mentioned previously, studies by Lou et al. [17] are the most directly related to this study. A comparison of data obtained here from molars (by atomic %) with those of Lou et al. [17] showed largely different elemental ratios, most notably O (here found to be 9.77%, Lou et al. reported 31.37%), C (here 14.87%, Lou et al. 50.66%), and Cl (here 0.4%, Lou et al. 0.04%). The reasons for these differences are not yet clear, and additional surface studies may help to explain this discrepancy.

In relation to elemental differences by age, the C content showed a positive correlation with age, whereas the P and Ca contents decreased with age in unburned crowns. If the abundance of C is due to carbonate, this finding is in contrast to that of Brudevold and Soremark [18] who found the carbonate content in the tooth surface decreased with age. Speirs [19] noted that the carbonate content increases with abrasion of the surface of teeth and, thus, should increase with age. In preincineration roots, the C, P and Ca contents also showed significant changes with trends inverse to those observed in the crowns. The C content decreased with age and was significantly different among all age groups. The P and Ca contents showed an overall increasing trend with age. Because the cementum is a highly responsive mineralized tissue [20, 21], age-associated changes are likely to become more evident with age and increased use. As stress on the periodontal ligament (PDL) increases and periodontitis advances with age, it is possible that

calcification of the cementum, as seen in dentine, would also increase accordingly. Without further research regarding cement genesis and age/pathological responses, it is difficult to speculate.

Following the incineration of crowns, the C content decreased with increasing age, and the P content increased with increasing age. The incineration and preferential loss of carbon-based organic material likely results in a lower relative abundance of C compared with that of other elements. In postincineration roots, Mg was the only element that changed significantly and showed a clear decrease with increasing age. As mentioned above, a better understanding of age-related changes in cementum is required, although the decrease in Mg may be linked to increased mineralization with increasing age and the consequent reduction in organic components, leading to a loss of Mg.

In relation to differences by sex, no significant changes in elemental content were identified in tooth crowns either pre- or postincineration. In unburned roots, females exhibited a higher ratio of C than did males, whereas males showed significantly higher levels of O, P and Ca (Table 3). Few studies have addressed the differences in the elemental composition between male and female teeth, and none have compared differences in the cementum. If the ratio of C to Ca, O and P reflects the proportion of inorganic to organic material, this difference would indicate a higher level of mineralization in male tooth roots. No differences were found in the postincineration roots based on sex.

Elemental differences by temperature showed higher levels of Na, Mg and Ca in crowns incinerated at 1000°C relative to those burned at 650°C. Conversely, the Cl content was found to decrease with increasing incineration temperature. In the roots, only the Mg content was significantly different and was higher in the samples incinerated at 1000°C. This difference may be due to the large loss of C with increasing temperature. In all samples, C represented the greatest % loss during incineration, and all other elements appeared to increase in abundance to compensate for this loss. Thus, the concentrations of Na, Mg and Ca (all potentially crystal bound) may actually remain constant but appear to increase by virtue of the C loss.

With regard to the XRD analyses, this research focused on nondestructive methods that given the development of appropriate standards and controls may be applicable to all types of dental remains, e.g., burned, unburned, fleshed, or skeletonized, both in situ and isolated. To avoid sample destruction, whole teeth were selected for analysis instead of powdered samples. Using whole teeth brought about a number of challenges for analysis but offered significant advantages over powdered methods that are particularly useful in a forensic scenario. First, the sample was not destroyed or modified in any way, and this technique enabled analysis of the same surface of a whole tooth both pre- and postincineration. Additionally, the outer tooth surface (outer 100 μm), which the current literature shows is not fully understood, has been widely shown to possess structural, density and compositional properties that differ from those of the subsurface enamel [19]. Therefore, an investigation of only the tooth surface offers a potentially different perspective than that of a powdered or near-surface sectioned sample.

Dental enamel hardness has been suggested to be related to crystallite size, which is generally considered to be influenced by trace elements [22]. A number of elements are known to influence crystallite

size (fluoride, lead, titanium, and manganese), lattice parameters (selenium, chromium and nickel) and microstrain [19, 23].

The peak width and intensity of Bragg reflections are affected by the crystal properties in a substance. Therefore, peaks become narrower and more intense in a manner proportional to the crystallite size and crystallinity, respectively [24]. Accordingly, the higher intensity and narrower peaks observed in regions of tooth enamel (Fig. 3) indicate a higher crystallinity and larger crystallite size, respectively, relative to those of dentine. Using the single-peak method to explore various points on the vestibular surface of a tooth revealed the optimal point to be one-third above and below the CEJ (see the experimental section). This point offered the best baseline differentiation and lowest variability.

At the occlusal surface of a tooth, gnarled enamel is known to show a lower crystallinity in addition to surface irregularities due to the cusp and fissures that result in peak shifts, aberrant intensities and occasionally double peaks because of the altered refraction angles on either side of the cusp. The lack of signal obtained from the root apex and the consistently poor diffraction patterns across all tooth trials indicated the poor crystallinity and smaller crystallite size in this region. Vestibular enamel was selected as the analysis point within the crown and a second point in the vestibular root because the root is the most protected and often the most recoverable portion in severely burned remains [25, 26]. Because the samples for each SEM/EDX and XRD analysis are manually loaded and focused, it is not feasible to analyze the exact same point on a tooth each time using different techniques.

The major compound identified was hydroxyapatite. Alternative compounds identified by matching the diffraction pattern included chlorofluorohydroxyapatite (chlorian), calcium sulfide phosphate and sodium calcium fluoride sulfate. The overlapping reflections with those of pure hydroxyapatite were significant, and it was not possible to differentiate the dominance of one over the other [27, 28]. These findings were supported by previous studies that found hydroxyapatite to be the dominant phase in enamel (>60%) [10] across a range of temperatures (20-1010°C) [1, 7, 29].

Very few significant differences were found regarding the crystallite size. In unburned teeth, both the crowns and roots of females displayed a larger size than males. The reason for this result is unclear and may be a result of differing mineralization processes, which are not yet understood by the wider scientific community. This result is contrary to the preliminary findings of Leventouri et al. [27] who observed a decrease in the crystallinity of human dental enamel as a function of age; in our study, there was no correlation between age and crystallite size in human enamel. The crystallite size in crowns and roots was similar in all tested incineration scenarios, suggesting a direct relationship with temperature. Incineration of teeth increased the crystallite size in a temperature-dependent fashion. This trend continued, and the crystallite size in the teeth subjected to 1000°C was larger than that in teeth incinerated at lower temperatures (650°C). The variation in size was found to be much greater in the postincinerated samples and more so in the roots than the crowns, which reflects the higher mineralization and organization of enamel relative to those of the root cementum/dentine complex. Although enamel is typically shattered upon incineration, the brittle nature of enamel appears to result in its failure at lower temperatures, but its microstructure is partially retained. In the roots, the lower ratio of organic to inorganic components results

in a more flexible nature, allowing macrostructural survivability while accommodating heat-induced stressors at the microscopic level.

Additional research into the mineralization mechanisms of dental hard tissues and of cementum in general will perhaps shed some light on the reasons for the observed differences and provide grounds for greater differentiation in the future.

CONCLUSIONS

This study offers an original, rapid and nondestructive technique to determine trends in the biological profiles and individual features of dental structures. The study begins to elucidate key points of differentiation in dental structures, which have been shown to be the most relevant, informative and survivable remains following high-impact and heat trauma.

Dental roots showed excellent survivability at all tested temperatures and are the best candidate for postincineration analyses. In addition, the results presented here demonstrated that roots offered the strongest correlation to age and sex in both SEM and XRD analyses. Furthermore, dental roots offered the greatest variability in their elemental profiles, making them the most viable for differentiating individuals.

The frequency of elements detected by EDX and their relative abundances were reflective of the stoichiometric range of biological hydroxyapatite and known crystal substitutions (CO_3^{2-}, Na, Mg, and Cl); however, there was an unexpected lack of fluorine. This study has shown a number of significant differences exist according to age and trends evident of sexual dimorphisms.

The challenges of performing XRD with a 3D sample were overcome to allow whole teeth analysis in a nondestructive manner using a method that has not been previously investigated. Incineration and an increase in temperature were shown to result in an increase in the crystallite size. Further studies may be useful for predicting the temperature of a fire. No differences in HA crystal size were found between age groups; however, in unburned teeth, females exhibited a larger crystallite size than males.

The main limitation of our study is the small sample size. Given the variability of elements detected by EDX within the population, a large degree of overlap still exists between groups. A larger sample size and further validation are required.

KEY POINTS

- Elemental analysis of teeth using EDX showed differences correlating with age and sex.
- XRD methodology allowed analysis of whole teeth in a nondestructive manner.
- Increasing temperature correlated with an increase in HA crystallite size.
- Unburned teeth revealed that females had a larger HA crystallite size than males.

Compliance with Ethical Standards

Funding: none.

Conflict of Interest: none.

Ethical approval: This research was approved by the Ethics Committee of the University of Cordoba (Spain).

Informed consent: Informed consent was obtained from all participants.

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FIGURE CAPTIONS

Fig. 1 Linear regressions of key elements detected by EDX

Fig. 2 Diffraction pattern obtained from the vestibular analytical point of an unburned premolar (black) produced a well-defined alignment with the HA standard profile (red)

Fig. 3 Point analysis using XRD on various tooth structures