Bone natural autofluorescence and confocal laser scanning microscopy: preliminary results of a novel dating method to distinguish between forensic and ancient human skeletal remains

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ABSTRACT

The fast, high-throughput distinction between palaeoanthropological/archaeological remains and recent forensic/clinical bone samples is of vital importance in the field of medico-legal science. In this paper, a novel dating method was developed using the autofluorescence of human bones and the confocal laser scanning microscope as the means to distinguish between archaeological and forensic anthropological skeletal findings.

Human bones exhibit fluorescence, typically induced by natural antibiotics that are absorbed by collagen, and provide secondary, exogenous fluorophores. However, primary natural fluorescence (or autofluorescence) caused by enigmatic endogenous fluorophores is also present as a micro–phenomenon, whose nature is still obscure. Here, we show that the endogenous fluorophores are mucopolysaccharides of the Rouget–Neumann sheath and, more relevant, that the intensity of the natural fluorescence in human bone decreases in a relationship to the antiquity of the samples. These results suggest that the autofluorescence of bone is a promising technique for the assessment of skeletal remains that may be potentially of medico-legal interest. A larger study is proposed to confirm these findings and to create a predictive model between the autofluorescence intensity and the time since death.

Introduction

The analysis of skeletal remains is a highly specialized field in the medico-legal science. A forensic anthropologist is usually tasked with determining the biological profile of skeletal remains (e.g., ancestry, sex, age and stature) that have been identified as human. Morphometrical features of bones and teeth can help to answer these questions (Ubelaker, 1989; Dirkmaat, 2012; Işcan and Steyn, 2013). A more difficult parameter to estimate is the time since death (TSD). As quoted from Nagy et al. (2008), "dating human skeletal remains is one of the most important and yet unreliable aspects of forensic medicine. The identification of an unknown individual is a complex puzzle made up of many parts and one of the most significant of them is the dating of skeletal findings", even if the goal is only to distinguish between palaeoanthropological/archaeological remains and recent forensic/clinical bone samples.

In the scientific literature, one encounters several methodological approaches and procedures covering this topic without, however, finding a reliable solution to the problem of determining the TSD. Several authors have focused their studies on different techniques, such as X-ray diffraction, radiometrics, supersonic conductivity, Raman spectroscopy, luminol chemiluminescent reaction, Fourier transform infrared spectroscopy, or fluorescence of the bone, among others. (Taylor et al., 1989; Bartsiokas and Middleton, 1992; Bertoluzza et al., 1997; Facchini and Pettener, 1997; Swift, 1998, 2001; Introna et al., 1999; Howes et al., 2007; Creamer and Buck, 2009; Ramsthaler et al., 2009, 2011; McLaughlin and Lednev, 2011; Schrag et al., 2012; Hoke et al., 2013; Patonai et al., 2013; Cappella et al., 2015).

Fluorescence of the bone

Fluorescence is the property of certain chemical elements, called fluorophores, to emit visible light when excited by incident intense radiation. Some materials, when irradiated at a specific wavelength, are able to emit another light of a longer wavelength. This phenomenon is a short-lived luminescence, emitted simultaneously with the excitation light (Lakowicz, 1983; Müller, 2006).

Certain biological macromolecules and structures fluoresce themselves (*primary fluorescence* or *autofluorescence*), whereas other materials that do not exhibit primary fluorescence must be impregnated with fluorescent substances (fluorochromes; *secondary fluorescence*) before they can be examined (Lakowicz, 1983; Capasso et al., 2001).

Primary fluorescence emission in human bone was noted many years ago in clinical samples from patients treated therapeutically with antibiotics, primarily tetracycline (Prentice 1965, 1967). The pattern of fluorescence in tetracycline–labelled human bone was also observed in ancient human bones from archaeological contexts (Capasso et al., 2001; Capasso, 2007). In some cases, the fluorescence was attributed to the possible use of natural antibiotics in ancient societies: in a Sudanese Nubian population dated to 350-550 CE, the autofluorescence of human bones was related to the use of stored grains contaminated by tetracycline–producing *Streptomycetes* (Bassett et al., 1980). In the Roman population from Herculaneum, dated to 79 CE, the intense fluorescence of bone was related to the frequent (and possibly deliberate) use of tetracycline produced by *Streptomycetes* artificially cultivated on a pomegranate substrate, as described by ancient Roman physicians (D'Anastasio and Capasso, 2007). However, many studies reported the presence of an autofluorescence phenomenon in bone samples probably

due to the organic matrix, mainly collagen, of bone tissue (Prentice, 1967; Dekanic et al, 1977; Pilolli et al., 2008), although the nature of this autofluorescence is not well defined currently.

Assessing the UV-induced autofluorescence of bone fragments is often recommended as a first step to estimate the TSD of skeletal remains. However, opinions differ concerning the cause of the fluorescence, determining how to categorize the fluorescent properties, and the significance of the fluorescent characteristics in correlation with the TSD (Ramsthaler et al., 2011; Hoke et al., 2013).

For this reason, we examined samples of ancient bones from different archaeological periods in comparison with samples of recent human bones to (i) define the real nature of natural autofluorescence (NA) and (ii) delineate a possible relationship between NA intensity and bones dating.

Material and methods

Sample collection and preparation of histological thin sections

In our study, human bone samples were selected from the mid shaft of the femurs from 13 adult individuals from Italian archaeological sites and ossuaries ranging from the X–VIII century BCE to modern periods (D'Anastasio et al., 2004, 2009; D'Anastasio and Vitullo, 2008) (Table 1), for which no possible sources of natural antibiotics had been documented or identified.

With a circular blade diamond–edged (Leica 1600), three thin sections of thickness of 150 µm were obtained of each sample. Each section consists of non–decalcified, macerated compact bone including the *linea aspera*. Next, the samples were prepared

using two procedures according to the different goals of the study. Figure 1 shows a brief summary of the methodological procedures.

Procedure 1. Origin of the natural fluorescence

The aims of this procedure are (i) to determine the chemical of the endogenous fluorophores and (ii) to test if the natural autofluorescence (NA) was influenced by the mineral components or organic matrix of the tissue. For this purpose, two thin sections from the contemporary bone sample Sicily #2 (second half of XX century CE) received different treatments before dehydration and subsequent analysis.

Section #1: thin section of bone incubated in a solution of EDTA (0.05 M, pH 8) for four days to remove the mineral component of bone.

Section #2: thin section of bone incubated in a solution containing bacterial collagenase (1 mg/ml of the lyophilized enzyme in Hank's balanced salt solution) for 24 h at room temperature to digest the collagen.

Procedure 2. Dating of bone samples

To test empirically that (i) the observed NA trend of a decrease of the fluorescence intensity in relation to the bone samples dating and (ii) to quantify the fluorescence levels representative of each sample, we analysed a collection of selected human bone samples, coming from vary antiquity periods (Table 1).

Thin untreated sections were prepared from all of the bone samples from archaeological sites and modern ossuaries, to quantify the NA intensity and its relationship with the antiquity of the bone samples.

Section #3: thin section of untreated bone.

All of the histological thin sections (*sections #1*, *#2* and *#3*) were dehydrated using alcohol with an increasing gradation (from 70º to 100º, with alternating washes of 15 min) and incubated with Histolemon (at least 15 min). Next, the thin sections were mounted on a microscope glass slide with Canada balsam and a 0.17 mm cover slip. Near each thin section 10 µl of a suspension of microspheres was added for the fluorescence calibration. The microspheres suspension consisted of 100 µl of stirred suspension of fluorescent microspheres InSpeck Green (6-µm diameter; 505/515 nm of approximate excitation and emission maxima) (InSpeck Microscope Image Intensity Calibration Kit, component G; Molecular Probes, Eugene, OR USA), 1 ml of its proper medium of inclusion (Tween 20, water, 2 mM Sodium Azide), 1 ml of Histolemon and a drop of Canada balsam.

Confocal laser scanning microscopy and image analyses

The thin sections from human bone samples (Table 1) were observed using a confocal laser scanning microscope (Zeiss 510 META System connected to an AXIOVERT 200 microscope with the optical apparatus Plan–Neofluar 40X N.A. 1.4 Oil). The samples were excited with argon-ion laser light (15 mW, 488 nm; AOTF: 15%). The emitted light was first filtered using a HTF 488/543-nm to block the reflected excitation from the sample. Using a low pass filter (NTF 545-nm), the emitted light was channelled to the photomultiplier through a band pass filter set at 505/530-nm. Images were acquired and digitized at 12 bits, with a resolution of 512×512 pixel. The same parameters were used for all of the acquired images. The images analysis was performed off–line with the Zeiss LSM 3.0 software.

Three different fields of each thin section, including a Haversian canal, were observed and acquired. In each field, five regions of interest (ROIs) (505 μm^2) (Fig. 2) were randomly selected and, for each area, the relation between the fluorescence intensity (measured in arbitrary intensity units, AIU) and its frequency at intervals of 64 bit was measured. For each thin section, the fluorescence intensity was expressed as the modal value (corresponding to the more frequent value of NA intensity). Finally, for each population, a single relation curve between fluorescence intensity (AIU) and its absolute frequency was plotted, using the pooled data of individuals within each population. All of the descriptive statistical analyses and graphics were performed using the IBM SPSS Statistics 22.0 software (SPSS Inc., Chicago, IL, USA).

Chemical microanalysis

To evaluate a possible influence on the emission of fluorescence by the taphonomic transformations, we performed the microanalysis of burial grounds (all the skeletal individuals were earthen) and bones. The microanalyses of the bone and soil samples were conducted using a scanning electron microscope equipped with an electron probe microanalyser (Goldstein, 2003) at the Microscopy Centre of the University of L'Aquila (Italy).

Results

Origin of natural fluorescence

In complete, untreated bone sections, a near–uniform distribution of the NA was observed (Fig. 3A). The intensity curve shows an exponential shape-like, showing a

peak of fluorescence of 4064 AIU, with an average absolute frequency of 39.93. The NA intensity appeared increased in sections after EDTA–histochemical removal of hydroxyapatite (Fig. 3B). Reducing the scale of the graph, the intensity curve is similar to the previous one. The peak intensity also reaches 4064 AIU; however, the average absolute frequency rises to 1659.53. Conversely, in the thin sections in which collagen was removed by collagenase digestion, the intensity of NA was drastically reduced, and its distribution in the bone was highly localized to the walls of the cellular lacunae, as well as to the walls of the canalicular system (Fig. 3C). In this case, the intensity curve shows a Gaussian-shape-like curve, showing a peak of fluorescence of 2272 AIU, with an average absolute frequency of 19.33.

The increase of NA observed after removal of the hydroxyapatite revealed that the endogenous fluorescence does not depend on the mineralized portion of the human bone. In addition, this NA increment could be explained hypothesizing that the minerals present in the bone microstructure could be a light–filter of the NA produced by organic material. Consequently, the removal of the hydroxyapatite appears to produce a peculiar 'filter–free' effect.

In contrast, the NA is strictly dependent on the collagen, which represents the main organic material in both modern human bones and in ancient remains, even if the localized NA persistence, after collagen removal, demonstrates that NA is due also to other endogenous fluorophores. The localization of the residual NA suggests that the endogenous fluorophores are present in organic substances characteristically distributed in a thin layer around the walls of both the canalicular bone system and the osteocytic lacunae.

Dating of bone samples

The intensities of fluorescence signals were different in the analysed samples, even if the experimental protocols and technical parameters of image acquisition were kept the same. In other words, the modal values of the NA intensity (corresponding to the more frequent values of NA intensity) diverge from one to another (Table 2).

On pooled data by population, the archaeological sample of Comino, the oldest among those examined, shows a fluorescence peak of 896 AIU (Fig. 4A). The samples of Opi shows a peak of fluorescence of 2320 AIU (Fig. 4B), while the samples of Monte d'Argento, from medieval times, present a fluorescence peak slightly lower than the Comino one (848 AIU) (Fig. 4C). The modern bone samples of Castel di Sangro and Sicily have fluorescence values much higher than the previous ones (Figs. 4D and 4E), of 4048 AIU and 4064 AIU, respectively.

Table 2 lists the peaks (corresponding to the modal values) of natural autofluorescence intensity described above: there is a general tendency of an enhancement of fluorescence intensity from the most ancient samples to the modern ones.

These results support the hypothesis that a relationship exists between NA fluorescence intensity and the antiquity of human bone. Also considering the NA variations produced by collagen digestion and by hydroxyapatite dissolution, both these chemical treatments did not affect the decrease of NA linked to the antiquity of samples (data not shown).

The peaks of the NA intensity of each bone sample were plotted to the historical dating of the archaeological sites and ossuary (Fig. 5). The bone samples from Castel di Sangro (first half of XX Century CE) and Sicily (second half of XX CE) show the

fluorescence intensity markedly higher than that observed in the other samples from classical archaeological contexts. The sample of Comino (IX Century BCE) shows the lowest fluorescence intensity. The sample of Monte d'Argento fluoresces with an intensity less than that found in older samples, thus constituting an exception to the general trend of an increase of fluorescence intensity in the skeletal remains of more recent periods.

In addition, the condition of inhumation should be taken into account. No differences were found in the chemical composition of soils, and microanalytical study of the bones showed no difference (Fig. 6); these findings, at present, exclude possible inferences of taphonomic phenomena on NA measurements in our samples.

Discussion

Origin of the natural fluorescence

According to Maggiano et al., 2006, 2009, the NA is strictly dependent on the collagen, which represents the main organic material in both modern human bones and in ancient remains. However, localized NA persistence observed in our study, after collagen removal, demonstrates that NA is due also to other endogenous fluorophores. The localization of the residual NA suggests that the endogenous fluorophores are present in organic substances characteristically distributed in a thin layer around the walls of both the canalicular bone system and the osteocytic lacunae. In particular, the NA distribution observed in the present study follows the topographic distribution of the so–called Rouget–Neumann sheath, which occupies (i) the space between the surface of the osteocytes and the walls of the cellular lacunae and (ii) the space between the cytoplasmic extensions of the osteocytes and the walls of the canalicular system. This

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sheath is an unstructured, amorphous and afibrillary substance consisting of a mixture of acid mucopolysaccharides (Ascenzi and Bonucci, 1977). It has been clearly demonstrated that the Rouget–Neumann sheath persists in ancient human bone, even after a very long period of inhumation (Capasso and Di Tota, 1993).

Dating of bone samples

The NA decreases over time: the ancient bones show lower value of the NA than the modern ones. Because the fluorescence is due to the organic component of the bone, it is reasonable to assume that the decrease of fluorescence intensity is due to the deterioration of the organic substances over the years.

A difference was noted in the modal values of the NA intensity: the NA intensity of the modern samples from Sicily and Castel di Sangro are higher than the ones registered in the archaeological samples. The distribution of the NA intensity of the archaeological samples resembles a Gaussian-shape-like curve, while the distribution of the NA intensity of the modern samples is similar to the graph of an exponential function.

Regarding the archaeological samples, the modal value of the NA intensity of the samples from Comino (X-VIII BCE) is similar to the one of the medieval samples from Monte d'Argento (1034–1470 CE); furthermore, the intensity values of the NA do not show significant intra-individual differences, but inter-individual differences. The inter-individual variability of the NA intensity and its similar values in ancient human populations of different historical periods could be the consequences of: (i) the different stratigraphic position of the skeletal individuals excavated from the same necropolis that could have been used for a long period of time; (ii) the taphonomic changes due to chemical, physical and/or biological factors that were not detected by the chemical

analyses of bone tissues and burial soils; and (iii) pathological conditions that do not involve the skeleton. For these reasons, at this stage of the research, the novel dating method is not useful for accurately dating human bones from archaeological or forensic contexts; in any case, the procedure is applicable to distinguish between forensic and ancient human skeletal remains.

Conclusions

Although forensic anthropologists are typically more concerned with modern, medico-legal cases, historical and archaeological remains are often examined as well. Thus, the anthropologist must determine if skeletal remains are of forensic interest and should be investigated by the judicial authorities.

Establishing the time since death of skeletal remains often involves a best guess, based on the experience of the anthropologist, familiarity with the recovery environment, knowledge of the recovery context and the results of the forensic and police investigation. Of immediate importance is determining whether remains are forensically significant or of non-significant origin (i.e., historical and archaeological). Non-significant remains are generally not a priority for law enforcement because the relevant parties (e.g., suspects and next of kin) are unlikely to be alive. Thus, the ability to identify the cut-off point is a major topic of interest, i.e., skeletal remains that fall to one side of the point are significant, whereas those on the other side are non-significant.

The defined "cut-off" between modern and historical is somewhat arbitrary and variable in different countries, ranging from 10 to approximately 100 years. For example, the Italian Penal Code (art .410) stipulates, in fact, the statute of limitations for the offense committed more than 50 years ago. Moreover, the Criminal Code in Spain

(Articles 131, 132 and 133; and Law 10/1995) stipulates a time limitation of 20 years for a homicide (Márquez-Grant et al., 2010). In France, after 10 years (20 years for minors) human remains are not subjected to criminal investigation (articles 716–47 of the Criminal Procedure Code). In Germany, usually after an exposure time of recent human remains of no more than 30–50 years, no forensic investigation will be started and the case will be closed (Márquez-Grant et al., 2010). In the United Kingdom, in legal terms, the temporal cut–off point between forensic and archaeological provenance is arbitrarily set at between 70 and 75 years before the present time (Cox and Mays, 2000).

This preliminary study demonstrates that human bone shows an intense natural fluorescence (NA) or autofluorescence that is clearly visible under the confocal laser scanning microscope in thin sections of untreated (undecalcified) bone, in agreement with previous results (Capasso et al., 2001; Pawley, 2006). The NA is linked to endogenous fluorophores of organic nature; this fact explains, at least in part, the phenomenon of the reduction in the intensity of NA in ancient samples, resulting in the gradual decay of its organic component. The data presented here, even if at this early stage, strongly support a relation between the decrease of NA of human bone and the antiquity of the samples. In particular, the contrast between the very high NA intensity typical of modern and recent bone samples (XX century CE) and the very low NA intensity of medieval and pre–medieval bone samples (earlier than the XIV century CE) is clearly evident.

According to Forbes and Nugent (2009), while there have been numerous methods investigating techniques for dating skeletal remains of forensic interest (e.g., Facchini and Pettener, 1997; Swift et al. 2011; Creamer and Buck, 2008; McLaughlin and Lednev, 2011; Schrag et al., 2012; Patonai et al., 2013), radiometric analysis, the most

widely and conventionally used dating technique to distinguish recent from ancient samples, has become the most reliable also from a judicial point of view; however, the procedure is expensive. The findings of our study lead the way to a new perspective on the topic of absolute dating of human bone in archaeology/palaeoanthropology and forensic context, at a time when standard radiometric dating appears to be increasingly uncertain because of the fluctuations in radioactivity due to variations in the intensity of cosmic rays and to the radioactive pollution introduced by human activities, both on the earth's surface and in the atmosphere (Kitagawa and van der Plicht, 1998). A new method to obtain absolute dating could be useful and complementary to the radiometric method, which was introduced more than half a century ago by Libby's discovery (Arnold and Libby, 1949; Libby et al., 1949).

Although the method developed by us is not currently usable for dating archaeological samples, it still allows for distinguishing the samples from the skeletal remains of recent humans. As mentioned above, this ability to rapidly distinguish the age is an important application in the medico-legal field, as modern human remains and archaeological samples follow different judicial processes. In addition, the potential applications of the method have been recognized, and the method itself is the subject of a European patent in 2011 (N° 12157634.2 – 1234 – 01.03.2011).

Undoubtedly, at this stage, our method presents many limitations, for example, destruction of the requested samples. In any case, this factor is also restrictive with respect to the standard radiocarbon analysis. Other aspects should provide possible advantages, in particular, the easier protocols of sample preparation in our method compared with the extremely complex procedures required for the standard radiometric methods, involving the transformation of carbon into gas. Another advantage is the use

of a confocal laser scanning microscope, which could be developed in the future as a transportable instrument to enable the absolute dating of samples without the structured and expensive laboratory equipment currently required for standard radiometric methods.

Continuing work and development of a predictive model

Our laboratory is devoted to continue this research to: (i) provide a more detailed curve of NA through time, increasing the sample series of human bones, possibly with remains as precisely as possible dated with other dating methods (i.e., using identified skeletal collections for recent human remains and radiocarbon analysis for ancient human remains); the current sampling is insufficient to define the mathematical function that correlates the two variables "dating" and "fluorescence intensity"; (ii) determine if there is a relationship between the intensity of NA and the sex and/or age at death of the sampled skeletal individuals; and (iii) assay the possible relationship between the intensity of NA and diagenetic factors, such as the type of inhumation, soil characteristics, etc.

With further research in this direction, information derived from the analysis of bone autofluorescence could be used to complement existing techniques and procedures for the dating of human skeletal remains. This application of bone autofluoresence would be a significant contribution to the field and a valuable tool for medico-legal research.

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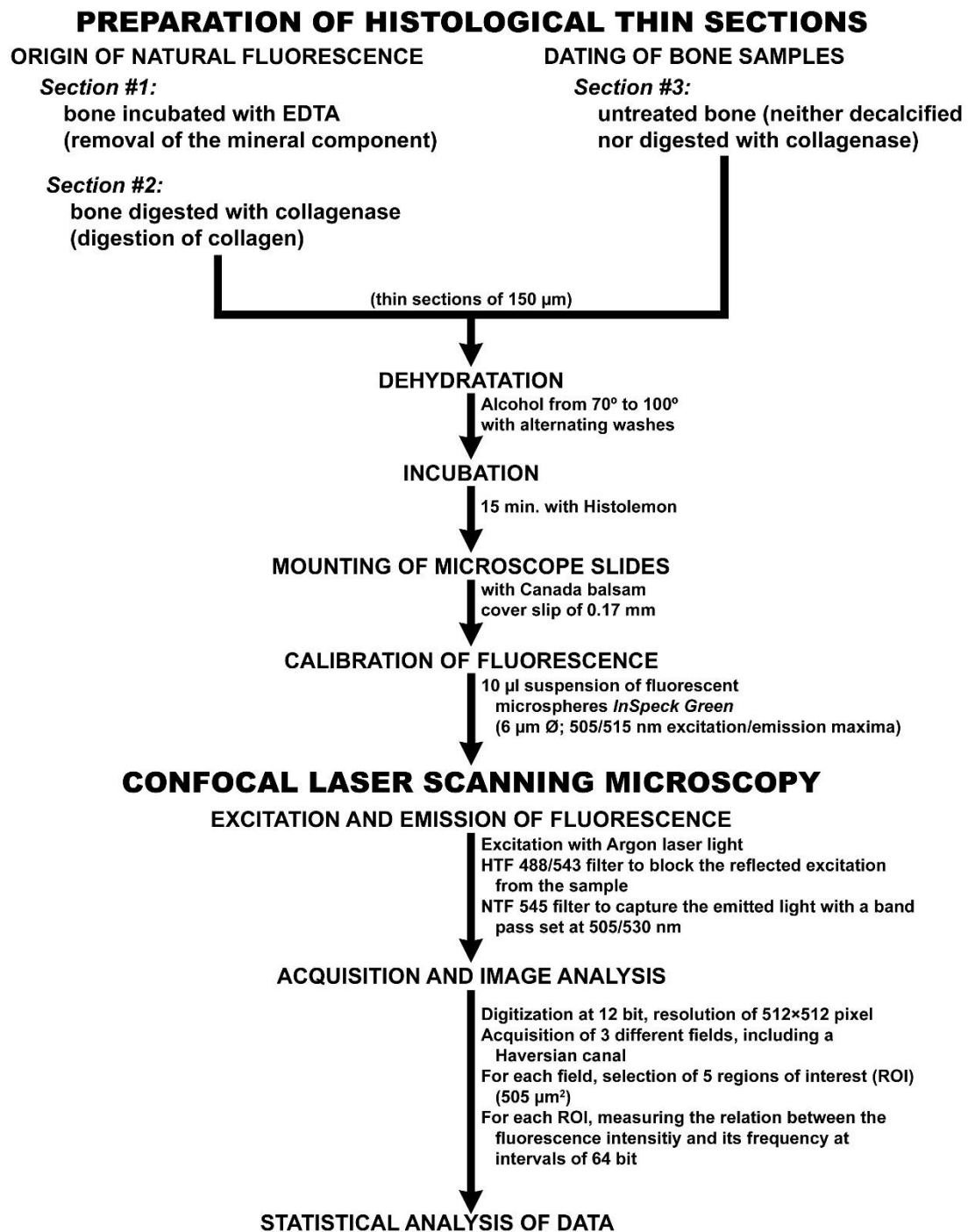
PREPARATION OF HISTOLOGICAL THIN SECTIONS

ORIGIN OF NATURAL FLUORESCENCE

Section #1:
   bone incubated with EDTA
   (removal of the mineral component)

Section #2:
   bone digested with collagenase
   (digestion of collagen)

DATING OF BONE SAMPLES

Section #3:
   untreated bone (neither decalcified
   nor digested with collagenase)

(thin sections of 150 μm)

DEHYDRATATION
Alcohol from 70º to 100º
with alternating washes

INCUBATION
15 min. with Histolemon

MOUNTING OF MICROSCOPE SLIDES
with Canada balsam
cover slip of 0.17 mm

CALIBRATION OF FLUORESCENCE
10 μl suspension of fluorescent
  microspheres *InSpeck Green*
  (6 μm Ø; 505/515 nm excitation/emission maxima)

CONFOCAL LASER SCANNING MICROSCOPY

EXCITATION AND EMISSION OF FLUORESCENCE
Excitation with Argon laser light
HTF 488/543 filter to block the reflected excitation
  from the sample
NTF 545 filter to capture the emitted light with a band
  pass set at 505/530 nm

ACQUISITION AND IMAGE ANALYSIS
Digitization at 12 bit, resolution of 512×512 pixel
Acquisition of 3 different fields, including a
  Haversian canal
For each field, selection of 5 regions of interest (ROI)
  (505 μm²)
For each ROI, measuring the relation between the
  fluorescence intensitiy and its frequency at
  intervals of 64 bit

STATISTICAL ANALYSIS OF DATA

Figure 1. Summary of the methodological procedures to evaluate the origin of natural fluorescence and the dating of bone samples.
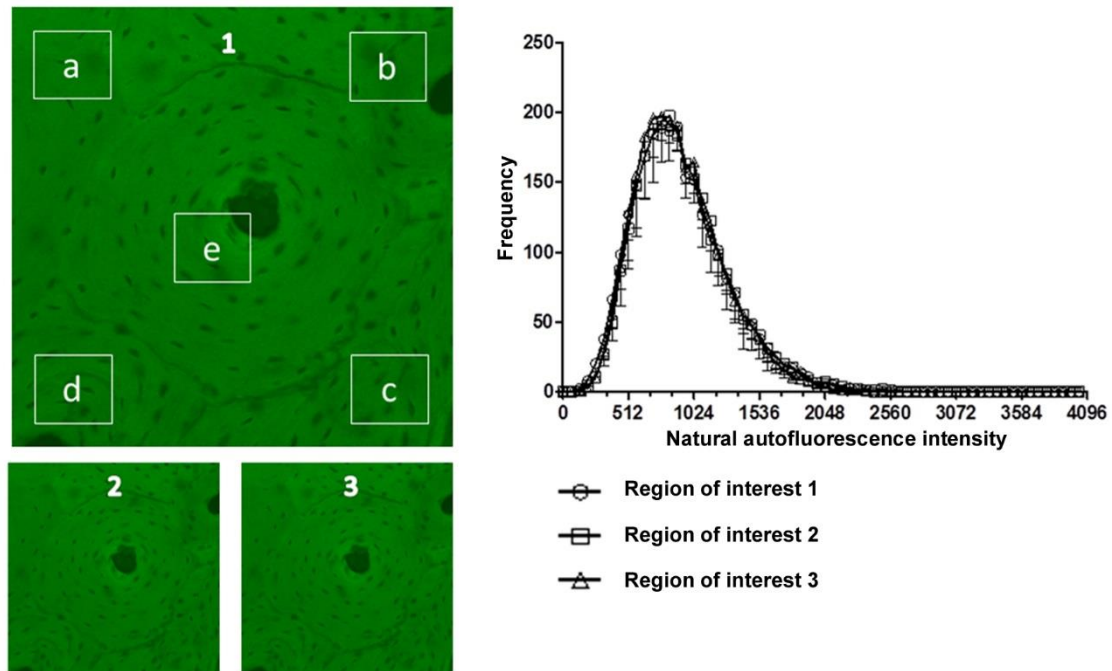
Figure 2. Images and graphic example of the analysis performed on bone thin sections. The acquired fields are numbered from 1 to 3. The five regions of interest (ROIs), selected to measure the natural autofluorescence intensity and frequency, are marked in lower case from "a" to "e". The graph represents the relation between fluorescence intensity and its absolute frequency in the three selected fields.

A
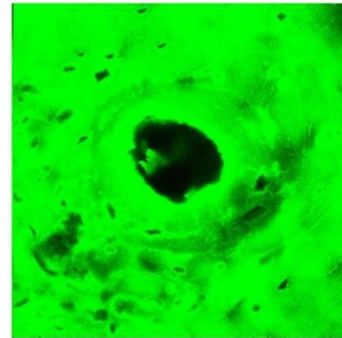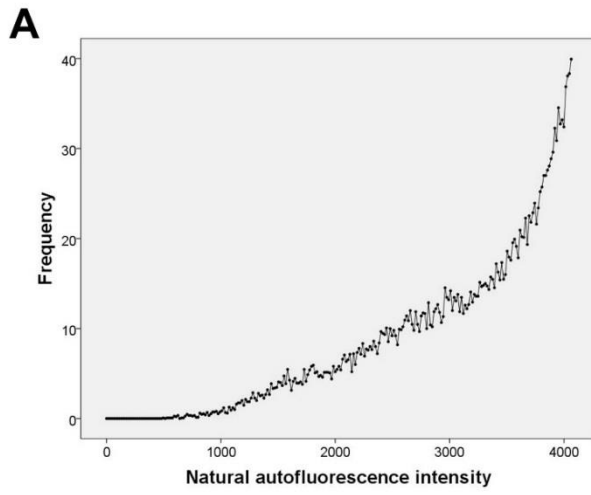
Autofluorescence intensity: 4064 AIU
Absolute frequenc:y 39.93

B
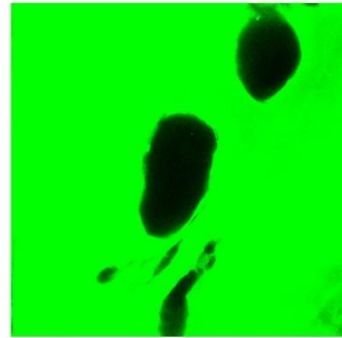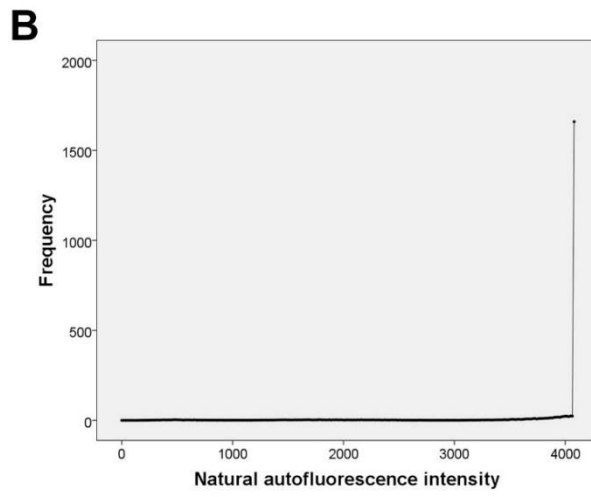
Autofluorescence intensity: 4064 AIU
Absolute frequency: 1659.53

C
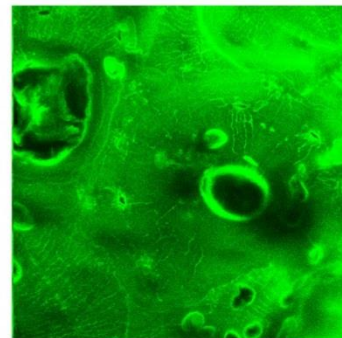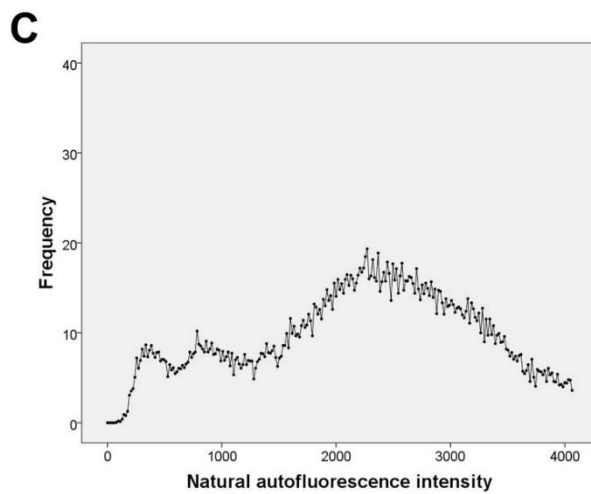
Autofluorescence intensity: 2272 AIU
Absolute frequency: 19.33

Figure 3. Autofluorescence localisation on thin sections from bone samples (A: untreated bone; B: decalcified bone; C: bone treated with collagenase; Bar = 10 μm.), showing the relation curve between fluorescence intensity and its absolute frequency.
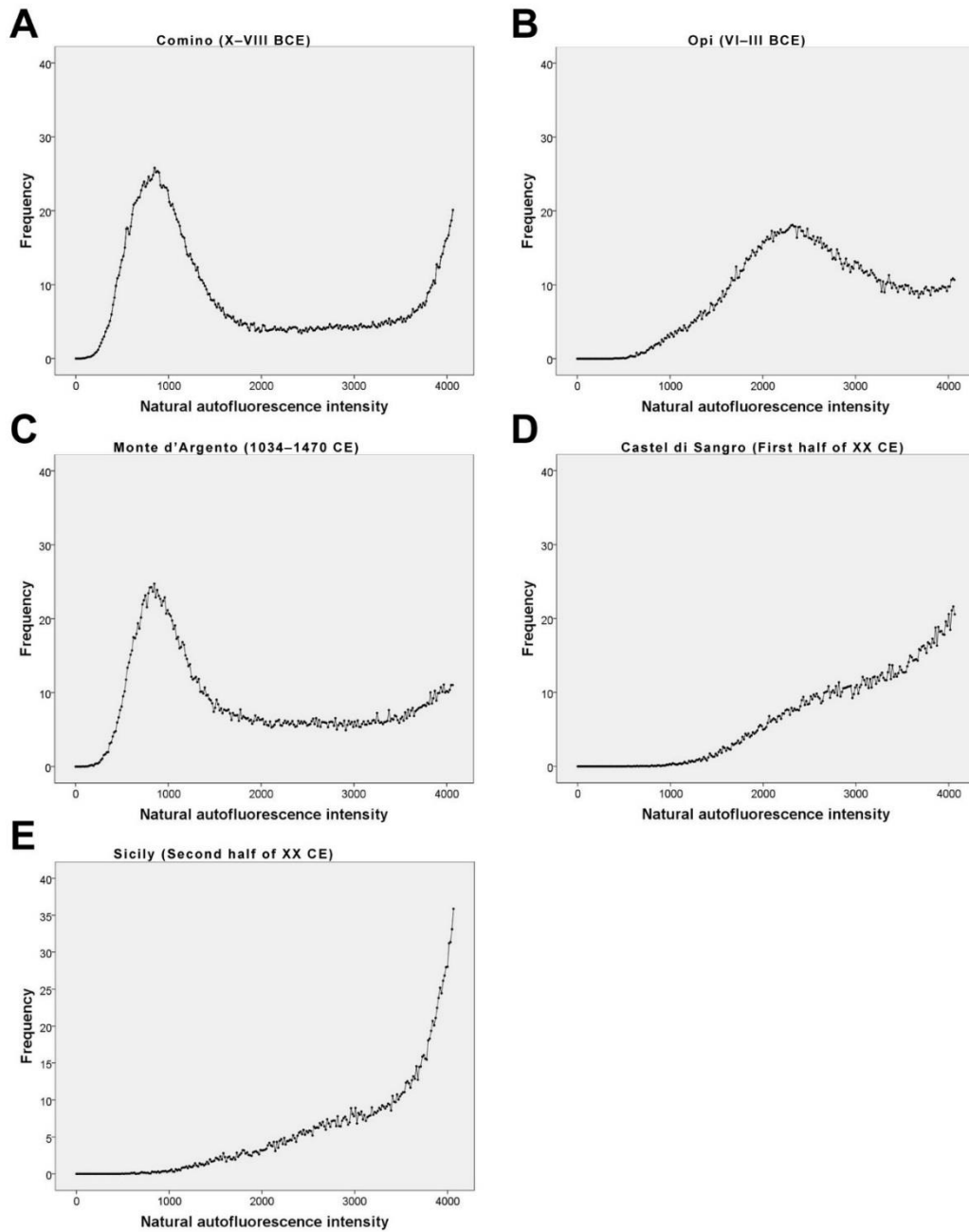
Figure 4. Natural autofluorescence (NA) intensity related to its frequency in: Comino (X–VIII BCE) (A); Opi (VI–III BCE) (B); Monte D'Argento (1034–1470 CE) (C); Castel Di Sangro (first half of XX CE) (D); Sicily (second half of XX CE) (E).
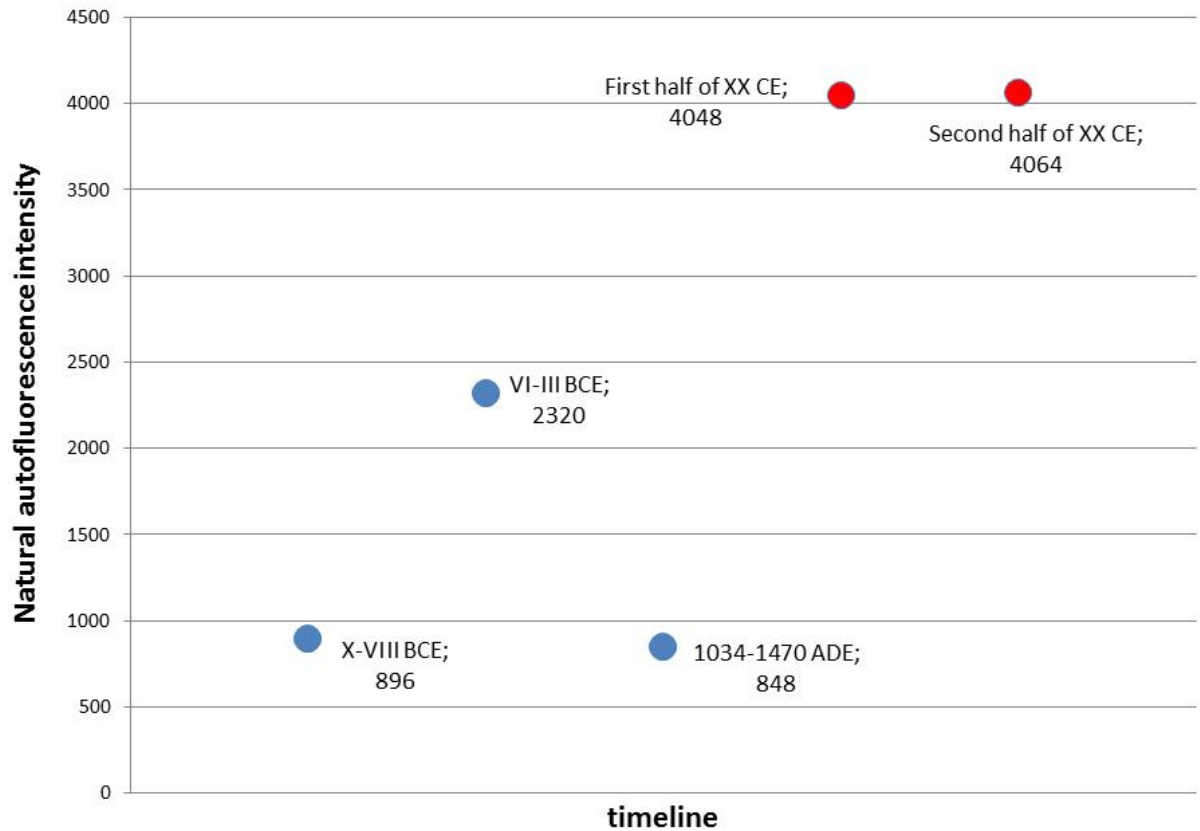

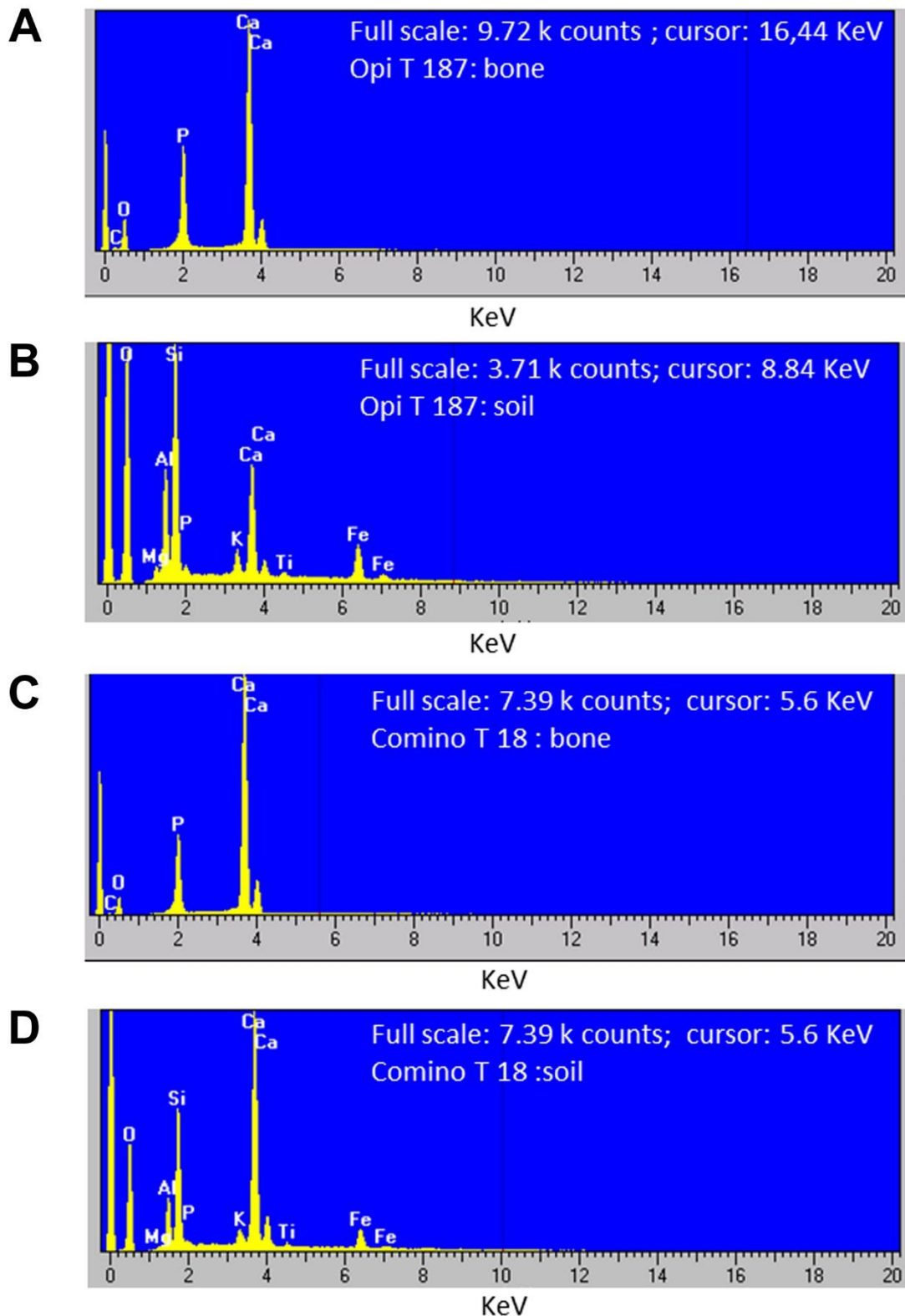Figure 5. The natural autofluorescence (NA) intensity and bone antiquity of the samples considered.

Figure 6. Chemical composition of bone samples (femurs) and burial soil from individual Opi #187 (A: bone; B: burial soil) and Comino #18 (C: bone; D: burial soil).

Table 1. Collection of human bone samples.

Population	Sample	Age at death	Sex	Antiquity
Comino	#25	20–25	♂	X–VIII BCE
Comino	#18	45–50	♂	X–VIII BCE
Comino	#45	35–40	♀	X–VIII BCE
Opi	#187	35–40	♂	VI–III BCE
Opi	#198	45–50	♂	VI–III BCE
Opi	#173	30–35	♀	VI–III BCE
M. d'Argento*	#32	30–35	♀	1034–1470 CE
M. d'Argento*	#70	25–30	♀	1034–1470 CE
M. d'Argento*	#35F	Adult	♀?	1034–1470 CE
Castel di Sangro	#Ind.1	Adult	?	First half of XX CE
Castel di Sangro	#Ind.2	Adult	?	First half of XX CE
Sicily	#1	87	♂	Second half of XX CE
Sicily	#2	44	♀	Second half of XX CE

*Radiocarbon dating (^{14}C) (D'Anastasio et al., 2004).

Table 2. Peaks of natural autofluorescence intensity in ancient and modern human populations.

Individuals	Modal values	
	Intensity	Frequency
Comino (X–VIII BCE)		
#25	2032	13.87
#18	896	51.60
#45	4064	60.60
Pooled data	**848**	**25.81**
Opi (VI–III BCE)		
#173	1824	22.27
#187	2416	30.27
#198	2736	14.80
Pooled data	**2320**	**18.09**
M. d'Argento (1034–1470 CE)		
#32	4048	30.53
#35F	1392	21.87
#70	800	63.00
Pooled data	**848**	**24.73**
Castel di Sangro (First half of XX CE)		
Ind.1	3360	21.47
Ind.2	4048	29.53
Pooled data	**4048**	**21.63**
Sicily (Second half of XX CE)		
#1	4064	39.93
#2	4064	31.80
Pooled data	**4064**	**35.86**

The modal values of pooled data of individuals within each population are boldfaced.