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Estimation of the post-mortem interval of human skeletal remains using Raman spectroscopy and chemometrics

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ABSTRACT

An important demand exists in the field of forensic analysis to objectively determine the post-mortem interval (PMI) when human skeletal remains are discovered. It is widely known that bones undergo different chemical and physical processes after death, mainly due to their interaction with the environment in which they are found, although it is not known exactly what these processes consist of. Multiple techniques have been used so far to follow up these and other post-mortem changes and thus establish the time elapsed since the individual's death, but they present important drawbacks in terms of reliability and accuracy. The aim of this research was to propose an analytical methodology capable of determining the PMI by using non-destructive Raman spectroscopy measurements of human skeletal remains. The recorded Raman spectra provided valuable and potentially useful information from which a multivariate study was performed by means of orthogonal partial least squares regression (OPLSR) in order to correlate the PMI with the detected spectral modifications. A collection of 53 real human skeletal remains with known PMI (15 years ≤ PMI ≤ 87 years) was analysed and used for building and validating the OPLS model. The PMI of 10 out of 14 validation samples could be determined with an accuracy error of less than 30%, demonstrating the adequate predictive performance of the OPLS model even in spite of the large inter-individual variability it handled. This opens up the possibility of applying the OPLS model in combination with non-destructive techniques to the determination of the PMI of human skeletal remains that have been buried in conditions similar or equal to those of cemetery niches and in a geographic location with a Mediterranean climate, which is an important achievement for forensic medicine and anthropology.

1. Introduction

Multiple studies have shown the importance of achieving a methodology for establishing exactly how long the remains of a human body have been buried. This aim, which is an important demand for many researchers in the field of forensic science, is justified by anthropological and legal interests.

The time for a case to be relevant (not legally prescribed) varies according to each country's legal system. In the Spanish legal system,

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the knowledge of the PMI is fundamental in criminal law, since the Organic Law 10/1995 of 23 November 1995 establishes a maximum of 20 years for the prescription of a case, in which the judicial sentence is 15 years or more [1,2]. In Germany, the evidence is considered relevant when the PMI is less than 30–50 years [3], whereas for the jurisdiction of England and Wales the samples are legally valid if the human skeletal remains are not older than 75 years [4,5].

Most of the studies carried out so far agree on the justification for the lack of these methods, referring to the difficulty of establishing a reliable method to accurately determine the PMI, as Nagy et al. cited in their research study "Dating human skeletal remains is one of the most important and yet unreliable aspects of forensic medicine" [6]. The cause of this difficulty lies in the complex degradation processes undergone by the bones. These degradation processes known as diagenesis consists of a set of chemical, physical and biological interaction processes between bones and the environment in which they are found (water, soil, humidity, gases, suspended particles, pH and/or bacteria). The complexity and variety of these processes make this type of degradation not exactly known, in addition to being complicated to simplify these degradation processes to a few [7].

Based on the characteristics and chemical composition of the bone, it is important to note its different parts. Inside the bone, a medullary part called the organic phase can be observed, in which components such as collagen, lipids, proteins, etc. are found. The outer part, the cortical, is that which contains organic elements and minerals, such as magnesium and hydroxyapatite crystals, which provide hardness to the bone. The cortical area is therefore made up of an inorganic phase embedded in an organic one [1,8,9]. This research will focus on this area, since it presents an inorganic phase that will take longer to degrade and an organic phase that, being the first to degrade, can potentially be more informative in forensic contexts [7].

The factors mentioned above that influence degradation can be classified as internal and external [1,10]. The internal factors are those specific to the person, such as age, diseases suffered or the diet followed [1]. The externals are those factors related to the environment and the surroundings, i.e. temperature, humidity, insects, and above all, the characteristics of the soil where the body is buried [10]. These processes are called diagenetics and are not fully and accurately known, but affect both the organic and mineral phases. They can be related to the loss of collagen and, therefore, to a decrease in nitrogen and amino acids, among many other phenomena [11].

In recent years, the forensic field has expanded rapidly as a branch of analytical chemistry, both in research on PMI calculation and in the different areas that this science raises [7]. Besides empirical principles based on the examiner's experience and subjective comparisons [12], multiple techniques have been applied to determine the time elapsed since death of skeletal remains, such as luminol chemiluminescent reaction [2,3,12–14], radioisotope measurements [5,12,15], proteomics [16,17], metabarcoding [15,18], high performance liquid chromatography-tandem mass spectrometry (HPLC/MS/MS) [19], X-ray diffraction (XRD) [1], UV-Vis spectroscopy [8,20,21], UV-Vis-induced fluorescence [22], stereomicroscopy and digital imaging [23], Fourier transform infrared (FTIR) spectroscopy [6,21,24–26] and Raman spectroscopy [7,25,26]. In addition to the lack of objectivity of several of the methods mentioned, some of them are of a destructive nature. For this reason, among others, Raman spectroscopy has established itself as a very versatile technique for the analysis of bone tissue composition [27]. The Raman spectrum of skeletal remains not only provides us with extensive information about the structure and composition of their minerals and collagen, but also with a complete examination of their diagenesis [9,28–30]. Moreover, Raman spectroscopy has demonstrated its application in determining the PMI of bones and teeth by studying their dynamic chemical modifications and correlating them with the time of burial [7,25,26,28]. Further advantages of Raman spectroscopy are that it is microscopic, fast and non-destructive, which are of utmost importance in cases of forensic or anthropological analysis [10], in addition to the fact that it can be portable [7,9,27,31].

In spite of the use of Raman spectroscopy and a wide variety of other analytical techniques in the determination of the PMI, only few research studies have applied them in combination with chemometrics. Chemometrics transforms the recorded data into more useful and interpretable information with which a predictive model can be built. This predictive model relates several variables or parameters allowing the examination of the hidden information in a set of data related to each other but influenced by numerous variables. With these chemometric treatments, useful information is extracted from the trends in the multivariate data set [27,32]. Wang et al. [24] used FTIR spectroscopy together with chemometrics to date human skeletal remains of up to 552 days. Predictions with a root mean square error of 50.93 days for buried bones and 71.03 days for unburied bones were obtained using the genetic algorithm combined with partial least squares (GA-PLS). In addition, the modifications that occur in the most significant FTIR regions and that are potentially applicable to the estimation of the PMI were identified and interpreted. However, this study did not take into account the influence of inter-individual differences on the FTIR spectrum variations and, even more important, the mathematical model was built from samples subjected to two controlled experimental burial conditions, which will differ from the natural ones, so the prediction set could have been giving by far over-optimistic results. Woess et al. [26] tested the suitability of various IR and Raman microscopic imaging techniques to draw conclusions about the PMI of human skeletal remains. The techniques were combined with multivariate imaging analysis (MIA) and principal component analysis (PCA) for a more sophisticated characterisation of the bone material as well as to differentiate between forensic and archaeological remains. Issues such as the impact of environmental factors still need to be explored and future studies will have to consider a larger sample size to further narrow down the PMI. Moreover, Longato et al. [33] used micro-computed tomography (micro-CT), mid-infrared (MIR) microscopic imaging and energy dispersive X-ray (EDS) mapping to investigate bone density, analyse organic and mineral components, determine elemental composition and, finally, apply and compare the results obtained by these analytical techniques together with PLSR to determine the PMI of human skeletal remains. The results obtained proved to be usable to predict the Ca/C ratio and bone volume (BV) over total volume (TV) for the estimation of the PMI. Future research will have to consider more individuals in order to develop a PMI screening tool based on the outcome of this multidimensional approach.

The aim of this research was to model the Raman spectral modifications experienced by human skeletal remains in relation to the PMI (15 years ≤ PMI ≤ 87 years) in order to provide a reliable method of quantifying the PMI. Likewise, the methodology was intended to be an invaluable tool for the identification and characterisation of the most influential spectral regions throughout the burial period, which could provide important additional information for the dating of the PMI. Furthermore, in order to fulfil these scopes and be applicable in real scenarios, the OPLS model was externally validated with bone samples coming from cemetery niches with a large inter-individual variability.

2. Materials and methods

2.1. Fieldwork

A collection of 53 human skeletal remains with known PMI (15 years ≤ PMI ≤ 87 years) was used in this study. The sampling for the collection of human skeletal remains with defined PMI was carried

out from niches of the San José (Granada, Andalucía, Spain) and Lucena (Córdoba, Andalucía, Spain) burial sites. The bone collection from the San José's burial site began to be exhumed in 1991 and was completed in 2010, while the collection from Lucena's burial site was fully gathered in 2018. The skeletal remains were kept piled up in bags in a warehouse at the cemetery. The exact date of their exhumation was unknown. It must be emphasised that since the human skeletal remains were buried in niches, they did not come into contact with the soil, nor were they exposed to adverse climatic conditions, thus minimising the diagenetic processes that occur after burial [1,19].

Both cities have a Mediterranean climate characterised by being warm and temperate with higher precipitation in winter than in summer. The mean annual temperature in Córdoba is 17.8 °C with a mean rainfall of 612 mm, whereas in Granada, the mean annual temperature is 15.5 °C with a mean rainfall of 450 mm [34].

2.2. Sample pre-treatment

The chemical treatment applied to the bones was a water bath with sodium hypochlorite and a brushing of each bone in order to eliminate the fungi and bacteria that had or could attack them in their storage causing an accelerated degradation of the bone. This treatment was carried out as a way to preserve both the organic and inorganic properties of the bone, avoiding bacterial growth and microbiological action. Once the pre-treatment of the samples was carried out, they were kept at a constant temperature (20–25 ºC) inside wooden boxes for their conservation, being only removed for analysis. The latency time until research was added to the known PMI. It is worth noting that all procedures in this study meet the requirements of local laws and institutional guidelines and were approved and supervised by the Ethics Committee of the University of Granada.

2.3. Bone subsampling

Long bones are mainly selected for sampling due to their relative size and strength as well as being more resistant to diagenesis [8,20,25]. The left tibias of 37 individuals (50% male and 50% female) from Lucena's burial site and 15 individuals (60% male and 40% female) from San José's burial site with age at death ranging between 32 and 100 years (mean 70 years; standard deviation 18) were collected. The skeletal remains had a PMI ranging from 15 to 87 years (mean 37 years, standard deviation 14) at the time of analysis. Anthropological data and burial sites of all measured human skeletal remains are shown in Table 1.

The measurements were performed in the diaphysis of the tibia in an area where the anthropometric measurements required by Anthropologists and Forensic Doctors were unaffected. An electric drill (Dremel® MultiPro®, United States (USA)) was used to scrape a region measuring approximately 5 mm wide, 10 mm long and 1–2 mm deep from the cortical surface of each bone. This way, any contaminants, such as the minerals deposited superficially in the outer layers of the bone and the residual haemoglobin in the bone tissue, which may be responsible for the emission of fluorescence and thus make the Raman spectra qualitatively and quantitatively unusable, could be removed quickly and in an almost non-destructive manner [31].

2.4. Raman spectroscopy measurements

The measuring equipment was a NRS-5100 Dispersive Raman Spectrometer (JASCO Analítica Spain S.L., Spain) equipped with a confocal microscope, using an x100 objective, and a 785 nm excitation wavelength laser, operating at 53.1 mW power. The scan range was 3200–200 cm^{-1} and the spectral resolution was 3.24 cm^{-1}. The measurement conditions were set to 3 scans of 20 s of acquisition per spectrum. Due to the high fluorescence radiated by the bones, a 3-minute photobleaching was performed prior to recording the spectrum, thus minimising the fluorescence background [9,35]. Three randomly selected points were measured in the scraped area of each sample to overcome bone heterogeneity. Cosmic rays were removed and the baseline was subtracted by applying a polynomial fit of order 3. The 3 recorded spectra were averaged to obtain a final Raman spectrum that was representative of a wide part of the cortical zone of each bone. The range of the Raman spectrum employed for the chemometric processing was 2000–350 cm^{-1}. The software used for system control, data acquisition and data analysis were the

Table 1
Description of the human skeletal remains sampled.

Sample code	Sex	Burial Site	Age at death (year)	PMI (year)
LU3	Male	Lucena	–	49
LU7	Male	Lucena	46	27
LU11	Male	Lucena	57	17
LU13	Male	Lucena	–	43
LU21	Female	Lucena	–	53
LU22	Male	Lucena	71	29
LU24	Male	Lucena	73	20
LU27	Male	Lucena	74	15
LU29	Female	Lucena	89	34
LU33	Female	Lucena	–	48
LU36	Male	Lucena	–	65
LU39	Male	Lucena	–	53
LU43	Male	Lucena	71	25
LU44	Female	Lucena	81	21
LU52	Female	Lucena	82	31
LU54	Male	Lucena	66	22
LU59	Male	Lucena	81	33
LU65	Male	Lucena	67	33
LU70	Male	Lucena	–	50
LU71	Female	Lucena	–	48
LU76	Female	Lucena	–	41
LU78	Male	Lucena	–	51
LU84	Male	Lucena	54	38
LU89	Male	Lucena	74	36
LU91	Male	Lucena	77	35
LU101	Female	Lucena	75	37
LU103	Female	Lucena	75	33
LU105	Female	Lucena	–	42
LU111	Female	Lucena	91	19
LU114	Female	Lucena	–	44
LU115	Female	Lucena	100	15
LU136	Female	Lucena	90	28
LU148	Female	Lucena	81	26
LU161	Male	Lucena	–	45
LU186	Female	Lucena	–	57
LU193	Female	Lucena	–	39
LU197	Female	Lucena	99	25
G14	Female	San José	73	51
G15	Male	San José	90	58
G56	Male	San José	75	51
G64	Female	San José	61	44
G67	Female	San José	41	44
G69	Male	San José	61	46
G170A	Female	San José	–	87
G261	Female	San José	81	22
G427	Male	San José	94	20
G490	Male	San José	32	18
G497	Female	San José	68	31
G498	Male	San José	33	31
G523	Male	San José	41	30
G528	Male	San José	65	24
G531	Male	San José	34	29

Spectra Manager II and WiRE 3 Renishaw® (Gloucestershire, United Kingdom (UK)).

2.5. Chemometrics

SIMCA 15.0.2 Umetrics® (Umeå, Sweden) was used for multivariate analysis. A preliminary exploratory analysis was performed by PCA using the data of various ratios and mineral crystallinity calculated from the 53 samples of the set. PCA is a multivariate projection method designed to extract and display systematic variation in an X data matrix. The most important use of PCA is to represent a multivariate data table as a low-dimensional plane so that an overview of the data is obtained. This overview can uncover groups of observations, trends, relationships between observations and variables and outliers [32]. The detection of the latter was the objective of this exploratory analysis. Once the potential outliers were detected and eliminated, the modelling of the PMI was performed by means of OPLSR. OPLS is an extension of PLS that splits the systematic variation in the X block into two parts, one that models the correlation between X and Y (predictive) and another that shows the systematic variation in X not related to Y (orthogonal). Orthogonalisation can reduce the complexity of the model, as it eliminates the systematic variation in the X matrix that is not correlated with the property to predict [32]. Therefore, an OPLS model was built correlating the Raman spectra of the various bones (X matrix) with the known PMI of each of them (Y vector). This model will be used to predict the unknown PMI of new human skeletal remains from their spectroscopic data. To this end, the total sample set, reduced to 47 samples after the elimination of outliers, was split into three groups depending on their PMI: 15 years ≤ PMI ≤ 30 years (17 samples), 30 years < PMI ≤ 50 years (23 samples) and 50 years < PMI ≤ 87 years (7 samples). To construct the X matrix, the intensity values of the Raman shifts of the entire spectrum range of each bone sample were used. The X matrix was split into two sample sets using the Kennard-Stone algorithm [36]: a training set (calibration samples) consisting of 70% of the samples from each group and a test set (validation samples) consisting of 30% of them. The training set was used for the construction of the OPLS model. For its optimisation, the X matrix was scaled and transformed by applying different mathematical treatments, such as Savitzky-Golay, multiplicative scatter correction (MSC), standard normal variate (SNV), row center, exponentially weighted moving average (EWMA) and first and second derivatives. The Y variable (PMI) was transformed to a logarithmic function due to its non-normal distribution. In addition, the Raman spectrum was examined for non-informative and noisy X variables responsible for interfering with those that were determinant for the PMI modelling. The number of latent variables (LVs) constituting the model was set by internal validation with the cross-validation method. The statistical parameters used for evaluating this step were the root mean square error of cross-validation (RMSECV) and the coefficient of determination of cross-validation (R^2 CV). RMSECV was calculated using Eq. (1):

$$\mathrm{RMSECV} = \sqrt{\frac{\sum_{i=1}^{n} (y_i - \hat{y}_i)^2}{n}} \quad (1)$$

Where y_i is the known PMI value, $\hat{y}_i$ is the PMI value predicted by the model and n is the total number of samples used in the training set. The external validation of the OPLS model was subsequently performed using the test set. To this end, the RMSE of prediction (RMSEP) and the R^2 of prediction (R^2 P) were assessed. The RMSEP was calculated with Eq. (2):

$$\mathrm{RMSEP} = \sqrt{\frac{\sum_{i=1}^{n_t} (y_{t,i} - \hat{y}_{t,i})^2}{n_t}} \quad (2)$$

Where $y_{t,i}$ is the known PMI value, $\hat{y}_{t,i}$ is the PMI value predicted by the model and n_t is the number of samples in the test set. The RMSEP expresses the average error to be expected in future predictions when the OPLS model is applied to unknown human skeletal remains. In addition, the predictive accuracy error was calculated for the test set using Eq. (3):

$$\mathrm{Error}\ (\%) = \left(\frac{(y_r - y_p)}{y_r}\right) x\ 100 \quad (3)$$

Where, y_p is the PMI value predicted by the OPLS model and y_r the known PMI value of the bone. Taking into account the above statistical parameters, the optimal model would have to have high R^2 and Q^2 values, low RMSECV and RMSEP values as well as a small difference between these last two statistical parameters. A large difference between RMSECV and RMSEP would indicate the possibility that too many LVs were being used in the model (over-fitting) and that the noise was being modelled. The number of LVs was intended to be as low as possible, as larger number of LVs might include some irrelevant information. The R^2 parameter varies from 0 to 1, indicating the degree of adjustment to perfect fitting, and the Q^2 parameter shows the robustness and predictive capacity of the model, being good when $Q^2 > 0.5$ and excellent when $Q^2 > 0.9$ [32,37]. In this way, it was possible to check which of the treatments obtained the best and most reliable model with the lowest error rate to date the PMI.

3. Results and discussion

3.1. Characterisation of the Raman spectrum

Human skeletal remains are composite materials containing a 20–30 wt% organic fraction embedded in a 70–80 wt% inorganic phase of hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_x$) partially substituted by carbonate (approx. 7 wt%) at both the hydroxyl (A-type) and phosphate (B-type) sites [24,30]. Taking this into account, the identified characteristic bands of the Raman spectrum of a human bone are shown in Fig. 1.

The amide I band between 1605 and 1720 cm^{-1} was mainly due to the C═O stretching within the collagen polypeptide [7,24]. The δ N-H vibration of amide III within the protein α-helix was detected in the region between 1215 and 1360 cm^{-1}, while the CH_2 deformation of proteins was identified between 1410 and 1500 cm^{-1} [7,26,38]. The proline band assigned to the ν C-C vibrational mode was detected at 880 cm^{-1} and was surrounded by two collagen bands attributed to the δ CCH (855 cm^{-1}) and ν C-C (923 cm^{-1}) vibrational modes [9,38]. The phenylalaline band at 1003 cm^{-1} may be due to the breathing mode of the ring [9]. In addition to the organic Raman bands, those of the phosphate ions (PO_4^{3-}) corresponding to hydroxyapatite, which constitutes the inorganic phase of the bone, were detected [24,25,29]. The strongest and narrowest band at 925–992 cm^{-1} was assigned to the ν_1 vibrational mode of PO_4^{3-}, whereas the bands at 400–490 cm^{-1} and 550–615 cm^{-1} were attributed to the ν_2 and ν_4 vibrational modes of PO_4^{3-}, respectively [7,26]. The carbonate ion (CO_3^{2-}) band in the region between 1015 and 1095 cm^{-1} was due to the ν_3 vibrational mode and may result from the substitution of these ions into the phosphate positions in the hydroxyapatite mineral lattice [9,29]. Furthermore, the bones had a minimal fat composition with the δ CH vibrations of phospholipids being barely detectable at 1295 cm^{-1} [7].

3.2. Outliers detection through PCA

The carbonate-to-phosphate ratio (ν_3 CO_3^{2-} band (1070 cm^{-1})/ν_1 PO_4^{3-} band (960 cm^{-1})), the mineral-to-matrix ratio (ν_1 PO_4^{3-} band/amide I band (1605–1720 cm^{-1})), the collagen crosslinking ratio

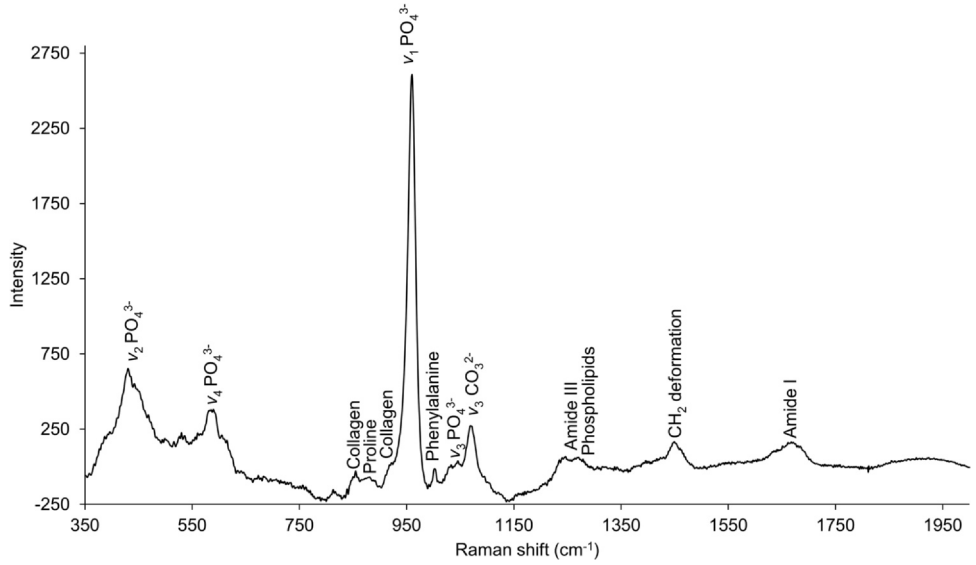

Fig. 1. Raman spectrum of the LU27 bone sample with the baseline subtracted by applying a polynomial fit of order 3. The characteristic Raman bands are identified.

(amide I band (1675 cm^{-1})/amide I band (1645 cm^{-1})) and the mineral crystallinity ($1/\nu_1$ PO_4^{3-} band width) were calculated. Ratios and mineral crystallinity have previously been used in other research studies to define bone quality. Such studies have reported anomalies in bone composition and, consequently, in the values of ratios and mineral crystallinity calculated from individuals with genetic disorders, osteoporosis, fractures, etc. [RW.ERROR - Unable to find reference:414], so that exploratory analysis of ratios and mineral crystallinity would allow us to detect outliers in the data set available. The intensities of the corresponding bands of the Raman spectrum of each bone sample were used to calculate the ratios due to the fact that they were more reproducible and less influenced by instrumental variations than those calculated from the integrated areas. The calculated ratios and mineral crystallinity were used as X variables in the PCA. Subsequent to being scaled to unit variance (UV), the Hotelling's T2 and distance-to-model plots at 95% confidence were used for outlier detection.

The sample set consisted of 53 samples, 6 of which (LU36, LU186, LU21, LU115, G69, G261) were detected as outliers, exceeding the critical limits (level 0.05) of the Hotelling's T2 and distance-to-model plots of the PCA (Fig. 2) and, therefore, eliminated from the set. Due to the large inter-individual variability (sex, age, diet, body size, etc.) of the analysed human skeletal remains, it was not possible to establish the cause of the anomalous and different behaviour of these

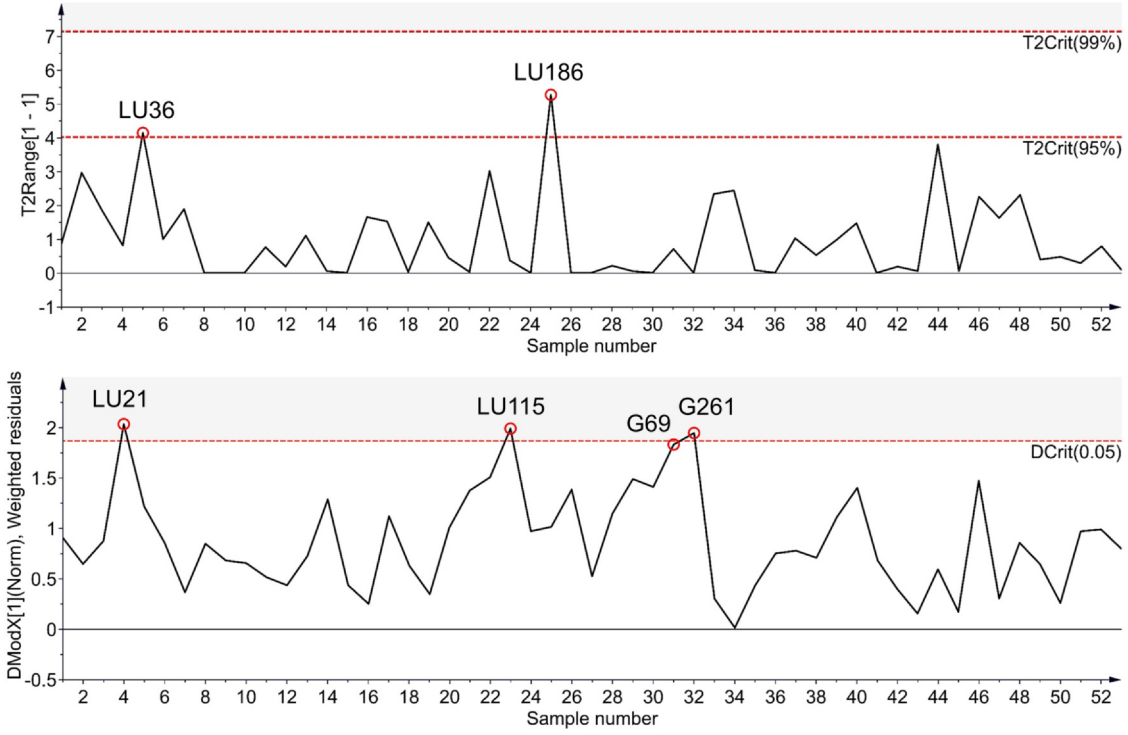

Fig. 2. Outliers detected in the Hotelling's T2 and distance-to-model plots at 95% confidence.

outliers. This way, the training set eventually included 33 samples, while the test set included 14.

3.3. OPLS model construction and validation

The UV scaling and the second derivative transformation of the X matrix were the mathematical treatments selected for the optimisation of the OPLS model. The aim of the second derivative transformation was to resolve the overlapping bands, which allowed for improved qualitative and quantitative data [24]. X variables with values between −0.04 and 0.04 in the loadings plot were eliminated, which resulted in an X matrix consisting of 295 variables, thus improving the robustness and performance of the OPLS model. The model was established with 3 LVs, explaining 43% for R^2X and 89% for R^2Y and predicting 67% for Q^2 of the variance of the population data within the training set, which indicates that the OPLS model had a valid fit and predictive ability. In addition, values of 0.10 (1.25 years) for RMSECV and 0.89 for R^2 CV were obtained. The low RMSECV value as well as the R^2 CV value close to 1 highlight the high predictive accuracy and the adequate fit of the OPLS model. It also had a great predictability, obtaining a value of 0.17 (1.48 years) for the RMSEP within the validation set. The RMSECV and RMSEP values were of the same magnitude, which demonstrates the robustness of the model.

3.4. OPLS model application to PMI estimation

The OPLS model was used to determine the PMI of the 14 samples of the test set. Predictions with an accuracy deviation of less than 30% were obtained for 10 of the samples studied, as shown in Table 2. The fact that it was possible to accurately predict 71% of the samples in the total set demonstrated the adequate predictive performance of the OPLS model. The large deviation of the remaining 29% of the bone samples (results not shown) could be justified due to the high inter-individual variability introduced, which could affect the chemical content of the bones and, therefore, lead to variations in the Raman spectra characteristic of each individual, thus not fitting well into the OPLS model. Nevertheless, the use of human skeletal remains with large inter-individual variability and exposed to uncontrolled burial conditions as calibration and validation samples enabled the OPLS model to be applied to the identification and determination of the PMI of skeletons encountered in real forensic scenarios and to the potential distinction between these and the archaeological ones, while ensuring the reliability and acceptable error rate of the predictions provided. In addition, the optimistic predictions that would have been obtained if the OPLS model had been built and validated with bone samples of certain inter-individual characteristics, non-human or buried under controlled experimental conditions are avoided. However, future studies will have to check whether the OPLS model could be applied to human skeletal remains from another geographical location and exposed to different burial conditions than those used in this study.

Table 2
PMI estimates and accuracy errors given in absolute value for the test set samples using the OPLS model.

Sample code	PMI (year)	Predicted PMI (year)	Accuracy error (%)
LU3	49	39	20
LU22	29	31	7
LU33	48	44	7
LU65	33	38	15
LU70	50	41	18
LU71	48	35	28
LU78	51	36	29
LU84	38	29	23
G497	31	30	2
G523	30	38	27

Moreover, the regions of the Raman spectra of human skeletal remains that were most significantly modified as a function of their PMI and, therefore, had the greatest contribution to the PMI modelling, were examined using the loadings plot. To understand these modifications, in addition to Raman studies, we have relied on those of FTIR performed on bone, since Raman spectroscopy researchers are known to have used and modified infrared correlations in order to be able to interpret Raman spectra of bone [38].

The degradation of human skeletons is a complicated and multivariate process that begins immediately after the death of the individual and is highly dependent on the external conditions to which the body is exposed [29]. It has been reported that the PO_4^{3-} bands are not meaningfully modified within the first few years of post-mortem [7,24,25]. In the present research study, the human skeletal remains with a PMI greater than 15 years underwent changes in the Raman bands assigned to the ν_2 and ν_4 vibrational modes of PO_4^{3-} (Fig. 3), which may be due to the environmental factors to which the skeletons have been exposed and which have caused their inorganic phase to dissolve, leach, exchange ions, precipitate minerals and/or recrystallise [24]. In relation to the latter, modifications in the phosphate bands can be attributed to changes in the crystallinity index (C.I.), as reported by Patonai et al. [39]. Such authors found that forensic human skeletal remains had a poorly crystallised apatite content, while at higher PMI the C.I. index increased, undergoing modifications in the apatite and resulting in a more ordered and larger crystal structure. Likewise, the loss of organic matter in the post-mortem degradation process of the skeletons, as will be discussed below, can also cause an increase in the degree of crystallinity of the hydroxyapatite [1]. It is worth recalling that the human skeletal remains from the present research study had a PMI between 15 and 87 years and, therefore, modifications in the ν_2 PO_4^{3-} and ν_4 PO_4^{3-} bands were detected in skeletons with up to the aforementioned PMI. Nevertheless, Woess et al. [26] reported that such modifications (reduction in the intensity of Raman bands) continue to be experienced in archaeological remains with a PMI of up to 1260 years. Moreover, in the present research study the skeletal remains with a PMI between 15 and 50 years underwent changes in the PO_4^{3-} Raman band attributed to the ν_1 vibrational mode, however, those with a PMI greater than 50 years remained unchanged, as shown in Fig. 3. It is worth mentioning that Woess et al. [26] reported that this phosphate band was minimally modified in forensic samples with a PMI between 1 day and 85 years and in archaeological samples with a PMI between 650 and 1260 years. Nonetheless, King et al. [29] observed changes in the position and full width at half the maximum height (FWHM) of the primary phosphate band (ν_1 PO_4^{3-}) of the Raman spectrum of prehistoric skeletal remains due to the substitution of foreign ions from the soil in which they were buried and the adverse climatic conditions to which they were exposed. The human skeletons from the present research study were buried in niches and, therefore, were less susceptible to foreign ion substitutions into the mineral lattice, not undergoing such significant modifications and shifts in the ν_1 PO_4^{3-} band. Furthermore, Woess et al. [26] found that archaeological human skeletal remains had a more prominent and sharp IR band at 1042 cm^{-1} compared to the forensic ones due to the mineralisation of the bones. Although this band may be less obvious in forensic remains, in the present research study, the OPLS model was able to detect modifications in the Raman range from 1040 to 1048 cm^{-1}, except in those skeletons with a PMI between 30 and 50 years, as shown in Fig. 3.

Moreover, according to several scientific papers that report the alteration of the organic matrix (decrease of IR and Raman bands) in the skeletal remains with a PMI of up to a few years [7,24,26], the modifications in the Raman bands of the CH_2 deformation of proteins and of the amide I and III continued to be observed in the present research study up to the 87 years of PMI (Fig. 3). Due to the

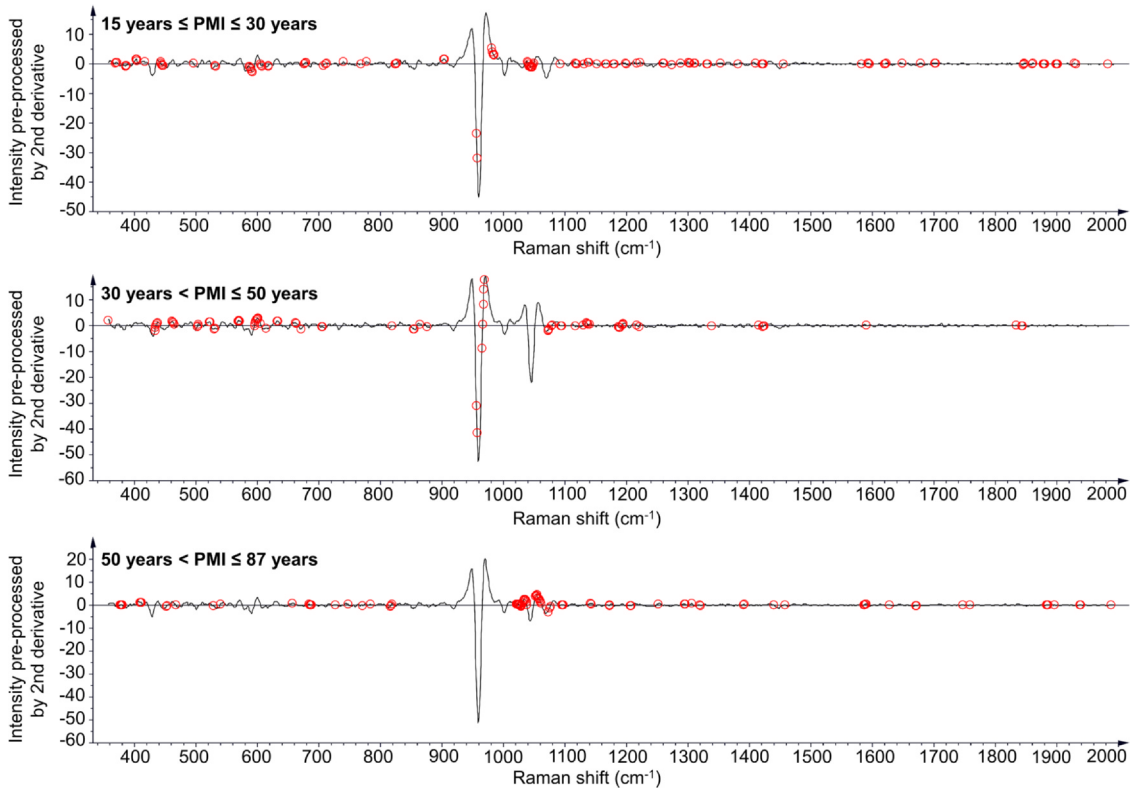

Fig. 3. Second derivative Raman spectra representative of the three groups of samples studied according to their PMI. The regions which undergo modifications throughout the burial period and which have the greatest influence on the PMI modelling are rounded in red.

decay of human skeletal remains, organic matter undergoes a complex process of disintegration, resulting in the formation of simple chemical substances [1]. Therefore, forensic bone samples would be expected to have more organic bands in their spectra compared to archaeological ones [26,33,39]. Amide I and III regions are highly prone to changes in the secondary structure of proteins. More specifically, alterations in the amide I band may be due to a structural change in collagen [7]. In this regard, it has been reported that skeletal remains buried in the soil have suffered collagen loss with increasing PMI, either by chemical breakdown or enzymatic digestion by bacteria followed by leaching [7,23,24]. Wang et al. [24] observed large differences between the FTIR spectra of bones buried in soil and those not buried and exposed to air, with the former undergoing a higher rate of degradation. As mentioned above, the human skeletal remains from the present research study were buried in niches and, therefore, less prone to attack by bacteria and fungi that could accelerate the removal of organic matter and weaken the structural integrity of the bone. In spite of this, human skeletons experienced modifications in the organic matrix consistent with those reported by Pérez-Martínez et al. [19], who observed a loss of collagen in the remains buried in niches, with a significant decrease in the number of peptides of Collagen type I in those with a PMI equal to or greater than 20 years. All the modifications observed during the period of burial in the present research study (15 years ≤ PMI ≤ 87 years) could be extrapolated and applied as indicative information to real cases with circumstances similar to those of human skeletons buried in niches, such as open-air burials, e.g. coffins and bodies found in caves.

4. Conclusions

The combination of Raman spectroscopy with OPLSR has proved to overcome the inefficiency of single analytical techniques in the study of the diagenetic processes to which human skeletal remains are subjected during burial by enabling the visualisation and interpretation of the modifications experienced in the spectra of human skeletons according to their PMI. Moreover, even though the coupling of such techniques had previously demonstrated its effectiveness in the determination of the PMI from the modelling of the modifications experienced by synthetic laboratory samples, it has also turned out to be successful when applied to real human skeletal remains, presenting itself as an additional and/or complementary tool to those already existing for the determination of the PMI. In spite of a previous exploratory analysis for the detection of outliers and a pre-processing of the analytical signals, the great inter-individual variability of the skeletons and the large number of external and internal factors that influence their diagenesis have been decisive to fail in the construction of an OPLS model capable of estimating with acceptable accuracy the PMI of all the samples in the validation set. Nevertheless, the estimates were highly promising for those samples to which the OPLS model was able to respond, opening up the possibility of determining the PMI of human skeletal remains (15 years ≤ PMI ≤ 87 years) that have been buried in similar or equal conditions to those of the cemetery niches and in a geographic location with a Mediterranean climate. The transferability of the proposed methodology depends on the availability of a large number of reference materials with burial conditions similar to the one(s) for which the PMI is to be determined. In the future, therefore, the results obtained in this research could be improved by increasing the number of real skeleton samples with which the OPLS model is constructed and validated. In conclusion, the non-destructive and confirmatory nature, versatility, portability, selectivity, accuracy and the ability to provide an estimated error associated with the result provided via chemometrics, make Raman spectroscopy in combination with

the OPLSR method a promising technique for determining the PMI in forensic settings where only human skeletal remains are available for analysis.

CRediT authorship contribution statement

L. Ortiz-Herrero: Methodology, Validation, Formal analysis, Investigation, Resources, Data curation, Writing – original draft, Visualisation. **B. Uribe:** Investigation, Writing – review & editing. **L. Hidalgo Armas:** Investigation, Writing – review & editing. **M.L. Alonso:** Writing – review & editing. **A. Sarmiento:** Resources, Writing – review & editing. **J. Irurita:** Conceptualisation, Resources, Writing – review & editing. **R.M. Alonso:** Funding acquisition, Writing – review & editing. **M.I. Maguregui:** Conceptualisation, Writing – review & editing, Supervision, Project administration. **F. Etxeberria:** Resources. **L. Bartolomé:** Conceptualisation, Writing – review & editing, Supervision, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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