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PAPER

ANTHROPOLOGY

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An Investigation of Model Forensic Bone in Soil Environments Studied Using Infrared Spectroscopy

ABSTRACT: Infrared spectroscopy has been used to examine changes to bone chemistry as a result of soil burial. Pig carcasses were buried as part of a controlled field study, and pig bone was used in soil environments established in the laboratory. The variables of species type, bone pretreatment, soil type and pH, moisture content, temperature, and burial time were investigated. The crystallinity index (CI) and the organic and carbonate contents of the bones were monitored. The data revealed decreasing trends in the organic and carbonate contents and an increase in the CI of the bone with burial time. An acidic soil environment and soil type are the factors that have the most influence on bone chemistry as a result of burial. The study demonstrates the potential of infrared spectroscopy as a straightforward method of monitoring the changes associated with aging of bones in a variety of soil environments.

KEYWORDS: forensic science, bone, aging, soil, burial, infrared spectroscopy

Gaining an understanding of the aging of bone fragments or decomposed skeletal remains provides a challenge for forensic practitioners. Bones are complex in structure and are sensitive to environmental factors. Although forensic studies of the visual and physical properties of bones have been carried out, there have been fewer investigations into the changes to the chemical structure of bones in a forensic context. As bones contain both inorganic and organic components, there is potential to gain an insight into the postmortem decomposition processes occurring using techniques that are sensitive to changes to such components.

A thermal analysis of bones of the age range of interest in this study has demonstrated that changes to the structure can be detected (1). The mass loss because of the organic component of the bone has been shown to be sensitive to postmortem age. Likewise, an examination of the same bones using pyrolysis gas chromatography–mass spectrometry showed changes in the data could be correlated with bone age (2). Although both these techniques have demonstrated the potential for determining postmortem bone age in a forensic time frame, there is an interest in establishing an analytical method that requires minimal sample preparation and where the interpretation of the data produced is straightforward. Infrared spectroscopy is a technique that could meet these requirements and was utilized for this study.

Infrared spectroscopy lends itself to the examination of both the inorganic and organic components of bone. The characteristic bands associated with collagen and hydroxyapatite have been well characterized. Several means of monitoring changes in bone structure using infrared data have been developed. The organic content of bone can be estimated using the ratio of the amide I band of collagen to the large apatite phosphate band at 1030 cm^{-1} (3–6). The crystallinity index (CI) is a combination of the crystallite size and the degree of order within the lattice and is calculated using the asymmetric phosphate bending stretching band in the 500–600 cm^{-1} region (7–10). The CI is usually calculated by adding the heights of the bands at 605 and 565 cm^{-1} and dividing by the height of the trough between the two bands. In modern bone, the CI has been observed to show values in the range 2.50–3.25, and in general, higher CI values indicate a higher degree of order within the crystal lattice (6–8,11). Infrared spectroscopy can also measure the ratios of the carbonate content, both types A and B. Type A represents the substitution of hydroxide with carbonate in the hydroxyapatite structure, and type B occurs when phosphate is replaced by carbonate (12). The ratios of the two types of carbonate relative to a phosphate band at 1030 cm^{-1} can be calculated using an absorption band at 1545 cm^{-1} for type A carbonate and 1415 cm^{-1} for type B carbonate (6).

In addition to an understanding of the influence of burial time, there is an interest in the evaluation of how different bone types and pretreatments influence the interpretation of changes observed in specimens buried in a soil environment. Species type has been investigated in this study because of the potential for other species to be exhumed in forensic scenarios, with pig, sheep, and cattle bone utilized here. Pig bone was chosen as the primary model to represent human bone as it has been established that pig provides a suitable practical alternative because of anatomic similarities (13). Various bone pretreatments including defleshing, degreasing, and boiling have been examined in the current study. The reasoning for the examination of such

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treatments is twofold. First, it is possible that bones of forensic interest may have been exposed to extreme disposal methods by a perpetrator, and this may affect the interpretation. Second, there is an interest in establishing whether or not the means of preparing a bone for storage or examination by forensic practitioners influences the bone structure and, hence, the interpretation of bone history. Soil is a complex burial environment, with many variables that may potentially affect bone structure over a period of time. For this study, the factors that have been considered are soil composition, soil pH, moisture content, and temperature.

In this study, infrared spectroscopy has been used to investigate the changes to bone structure over a range of burial times, bone types and pretreatments, and environmental conditions. The determination of the organic and carbonate contents and the CI of the exhumed bones have been carried out using this spectroscopic approach and trends in the data used to determine the most influential factors on bone structure.

Materials and Methods

For the investigation of the effect of burial time on bone chemistry in a field study, pig carcasses were decomposed for a range of time periods. Bone specimens with postmortem ages ranging between 3 and 23 months were obtained. All samples were flat rib bones from female pigs obtained from the same farm, fed with an identical diet, and weighing 40–45 kg. The pigs were buried in slightly acidic soil 60 cm below the surface for a designated amount of time. The pig decomposition study was conducted in the Jandakot District of Western Australia, where the minimum and maximum temperatures for the study location were 11.3 and 24.3°C, respectively, and the mean annual rainfall was 833 mm. After exhumation, the bones were stored in sealed plastic bags at 4°C prior to analysis.

To investigate species type, bone pretreatment and soil environment of bone structure, model environments were established in the laboratory for a period of 8 months. Ribs of cattle, sheep, and pig were purchased from the Glenmore Meat Company, Sydney. Bones were buried in different soil environments contained within sealed polyethylene containers maintained at a constant temperature and in the absence of light. The reference soil environment was composed of defleshed bones buried in a loam soil (organic top soil purchased from Bunnings Warehouse, Sydney, NSW, Australia) at pH 7 and 20°C. Bones were defleshed using a mixture of 5 g "Surf-Sunshine Fresh" anionic detergent and 4 g Na_2CO_3 maintained at 50°C for 24 h using the method described by Fenton et al. (14). The other soil types investigated were silt ("brickies sand"), sand (washed Sydney River sand), and clay ("builder's clay"), all obtained from Bunnings Warehouse. For fleshed specimens, the flesh was left on the bones. For the degreased bones, defleshed bones were placed in an ammonium and water solution in a 50°C water bath overnight. For the boiled bones, defleshed bones were boiled in a water bath for 3 h. Defleshed bones exposed to an acidic environment were buried in loam soil with powdered sulfur added to maintain a pH of 5. For a basic soil environment, loam soil with lime and gypsum added to maintain a pH of 9–10 was used to bury defleshed bones. To create a wet soil environment, water was added to loam soil until saturated, and the wet soil was maintained for the duration of the burial. To establish a dry soil environment, loam soil was dried in a vacuum oven at 50°C for 2–3 h. A cold soil environment was established by maintaining buried defleshed bones in loam soil at 4°C. Duplicate bones were buried in each environment.

The exhumed bone specimens were cleaned by scraping the surface with a scalpel to remove fatty bone marrow from the interior and any residues from the exterior of the specimens (15). All samples were washed with water and sliced into thin sections using a diamond saw and freeze-dried for 24 h in a John Morris Scientific Alpha 2-4 LD plus freeze-drier (Sydney, NSW, Australia). The cancellous inner part of the bone sections was then removed with a scalpel before the cortical outer part of the bone sections was hand ground into a fine powder with a mortar and pestle.

For the Fourier transform infrared spectroscopy, the powdered bone was mixed with KBr in a 1:100 mass ratio and pressed with 10 ton in^{-2} pressure to produce disks. Mid-infrared spectra were recorded using Nicolet Magna-IR 760 Fourier transform infrared spectrometer (ThermoFisher Scientific, Waltham, MA). A resolution of 4 cm^{-1} was obtained, and 64 scans were collected for each sample. Baseline correction was carried out between approximately 4000 and 2000 cm^{-1}, 2000 and 1250 cm^{-1}, 1250 and 750 cm^{-1}, and 750 and 400 cm^{-1}. All samples were run in duplicate.

Results and Discussion

Field Burials

The infrared spectra of the bones buried during the field study for varying time periods of time up to 23 months were recorded. Figure 1 provides an example of the spectra observed in the study and illustrates the infrared spectra obtained for bone buried for 3 months in loam soil. All the spectra obtained show the characteristic infrared bands illustrated in Fig. 1 associated with the collagen and hydroxyapatite components of the bone structure. To monitor the changes to the spectra resulting from increased burial time, three measures were made: organic content, CI, and carbonate content.

The organic content of the bones was measured using the ratio of the maximum absorbance of the amide I band at 1655 cm^{-1} to that of the phosphate band at 1030 cm^{-1}. Figure 2 shows the organic content measured as a function of bone burial time. There appears to be a decreasing amount of collagen observed with increasing time based on the decreasing ratio. A decrease in organic content with time has been previously reported for aging bones and indicates a loss of protein (16). The possibility of a logarithmic change with time was also investigated; the results are also shown in Fig. 2, and a decreasing trend is also indicated.

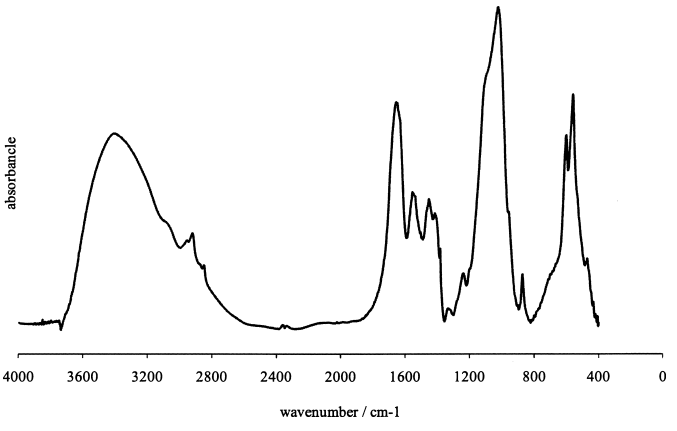

FIG. 1—*Infrared spectrum of bone buried for 3 months in loam soil.*

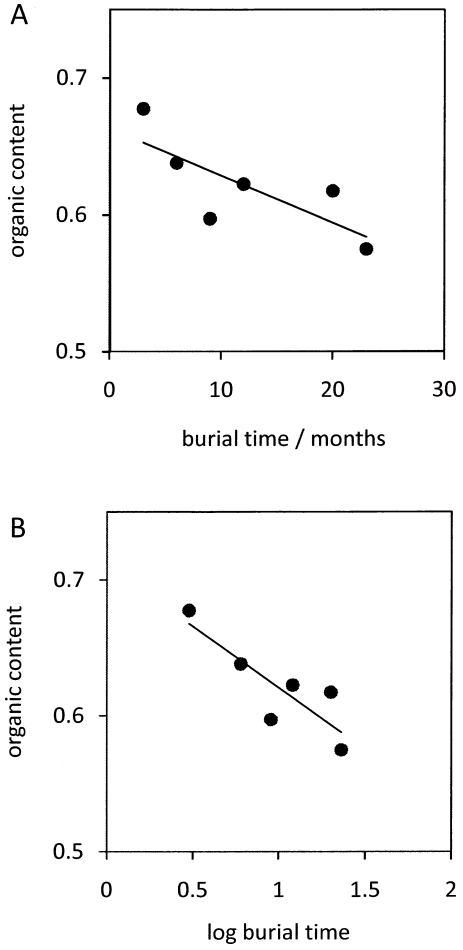

FIG. 2—*Organic content of bone as a function of burial time (A) and log burial time (B).*

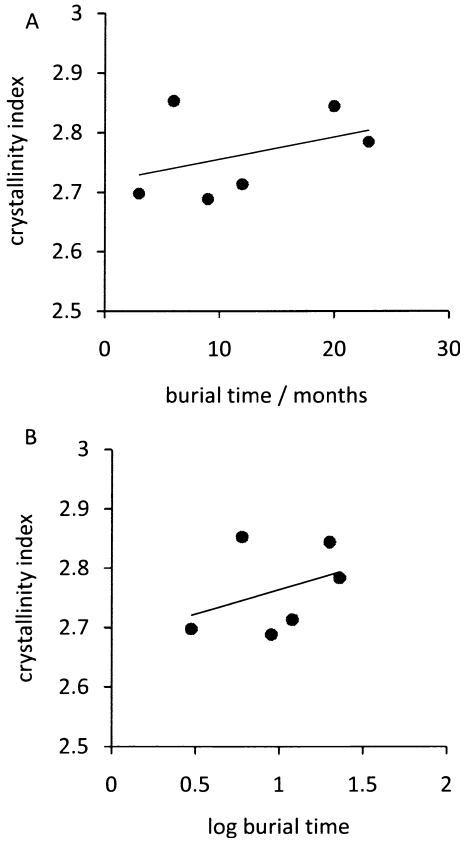

FIG. 3—*CI of bone as a function of burial time (A) and log burial time (B).*

The CI for the bones was determined using the maximum absorbance of the asymmetric phosphate split bands as follows:

$$CI = (A_{605} + A_{565})/A_{595}$$

Figure 3 illustrates the CI as a function of burial time and as a function of the logarithm of burial time, respectively. An increasing trend is observed, but there appears to be quite a degree of scatter in the data. The trend is in agreement with previous reports that have found that the CI of bone increases over time as the crystal structure becomes more ordered (6–9,17).

The type B carbonate of the bones was investigated by measuring the ratio of the maximum absorbance values of the 1415 and 1030 cm^{-1} bands. Although other studies have used the phosphate band at 605 cm^{-1} for this calculation, for this study, it was determined that the phosphate band at 1030 cm^{-1} was a better choice as its use reduces errors associated with a difference in the degree of splitting in the asymmetric phosphate band. The carbonate content was also monitored as a function of time and log time, and the results are shown in Fig. 4. A decreasing trend is observed for the carbonate content in both cases. A decrease in carbonate content with time has been previously reported for aged bones and indicates a loss of carbonate content in the inorganic phase and more ordering of the hydroxyapatite (5,6).

Both the time and logarithm of time models were tested to identify a relationship. The difference between the value predicted from the linear or logarithmic dependencies and the measured experimental data was determined, and a standard deviation as a percentage of the estimated mass loss for zero months was determined as a representation of the relative error of the model. The calculated y-intercept was taken as the value at the initial burial time, and the standard deviation was found as a percentage of that original amount (% SD). The calculated % SD values are shown in Fig. 5, and similar values are observed for each plot. Figure 6 illustrates the calculated slopes for each plot. A comparison with the variance can indicate whether a larger slope means the data are scattered or exhibiting a trend. In general, the larger the slope value, the sharper the trend.

Another means of examining the data is to assume two possible dependencies of the data; the bone specimens may be assumed to not decompose in the time frame of analyses, or the bone specimens are assumed to decompose, and the rate of decomposition follows a linear dependence. Given these two extremes, the % SD for each can be determined by averaging the data and measuring SD as a percent of the mean (i.e., the "raw data % SD") or by determining the SD from a linear fit as a percentage of the intercept (i.e., the "linear % SD"). The ratio of the "raw" % SD to the linear % SD was determined, and the values are shown in Table 1. The raw data % SD shows how much the data vary from the average of the data, while the linear % SD shows the variance in the data from a linear trend. Therefore, the higher the ratio of raw data % SD to linear % SD, the more the data follows a linear trend because the higher the % SD raw indicates a greater change and a small % SD linear indicates less variance from the linear model. Because the higher the values shown in Table 1, the higher the likelihood of a linear trend occurring, a qualitative assessment of the data is that a

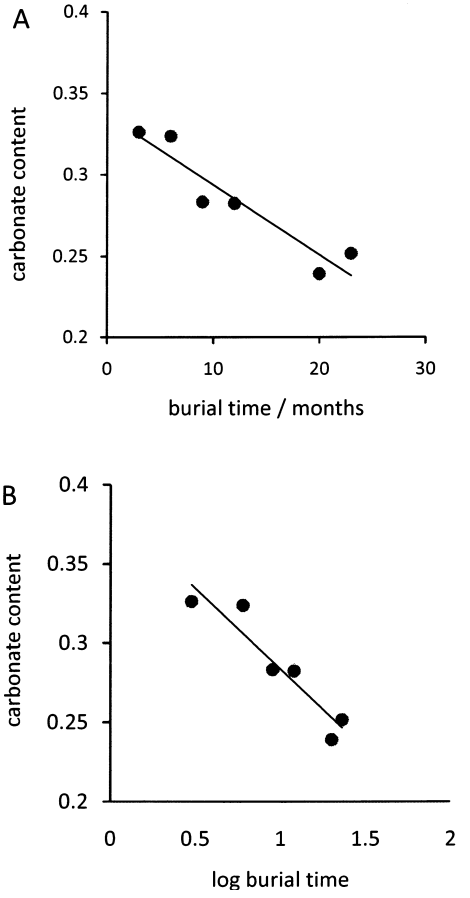

FIG. 4—*Carbonate content of bone as a function of burial time (A) and log burial time (B).*

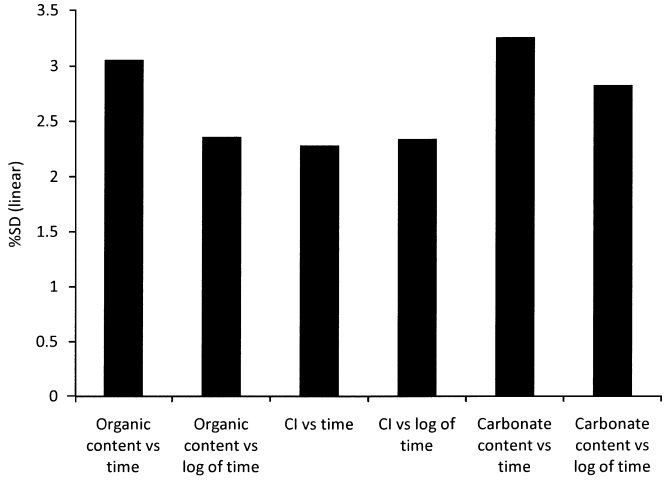

FIG. 5—*Standard deviation values for burial time data.*

ratio between 0 and 1.5 shows no trend, 1.5–2 may show a trend, and >2 suggests a tendency toward a linear trend. Trends are indicated for the organic content data, with ratios of 1.85 and 2.40 for time and log time plots, respectively. Table 1 also indicates that a trend is predicted for the carbonate content with a ratio value of 3.87. A logarithmic function is even better fit with a ratio value of 4.47 determined. The values observed for CI imply that trends are less likely for these variables.

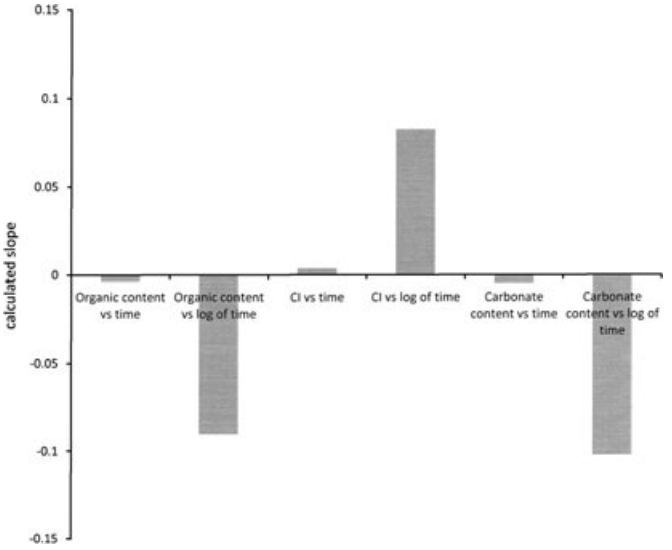

FIG. 6—*Calculated slopes for burial time data.*

TABLE 1—*Ratios of raw data % SD and linear % SD for field study bones.*

Plot	% SD Raw /% SD Linear
Organic content (time)	1.85
Organic content (log time)	2.40
CI (time)	1.17
CI (log time)	1.14
Carbonate content (time)	3.87
Carbonate content (log time)	4.47

Model Laboratory Burials

The variances and slopes for each model soil environment were also determined, and the results are illustrated in Figs 7 and 8, respectively. Table 2 lists the ratios of the raw % SD and linear % SD for each of the burial environments. Overall, the CI values have consistently less error than the organic and carbonate content values. The organic content appears to vary the most from a linear trend: The wet soil and clay results show high % SDs over 13%.

More changes appear to occur in the carbonate content, with five burial conditions showing linear trends. The reference, fleshed bone, and acidic conditions all show decreasing linear trends in carbonate content, while the cold environment shows an increasing linear trend. Initial inspection of the carbonate content of bone buried in sand shows no trend; however, the calculations would imply otherwise. There is a small amount of variance from a linear trend and the % SD raw:% SD linear ratio is the highest calculated. This may be a result of the fact that the calculated variance within the data (% SD raw) and the deviation from a linear model are both very high.

The organic content also shows certain trends within different environments. The results for the acidic soil environment show a very strong negative linear trend, while silty soil shows a strong positive linear trend. A positive trend implies an increase in collagen content, which is not probable. However, it has been shown that the relative intensity of the amide I band of collagen can increase on denaturation (18). The calculated slopes for both sample sets are high indicating a significant trend, while the variances from the calculated linear model appear to be in the middle of the spread of results. Degreased bones show a good

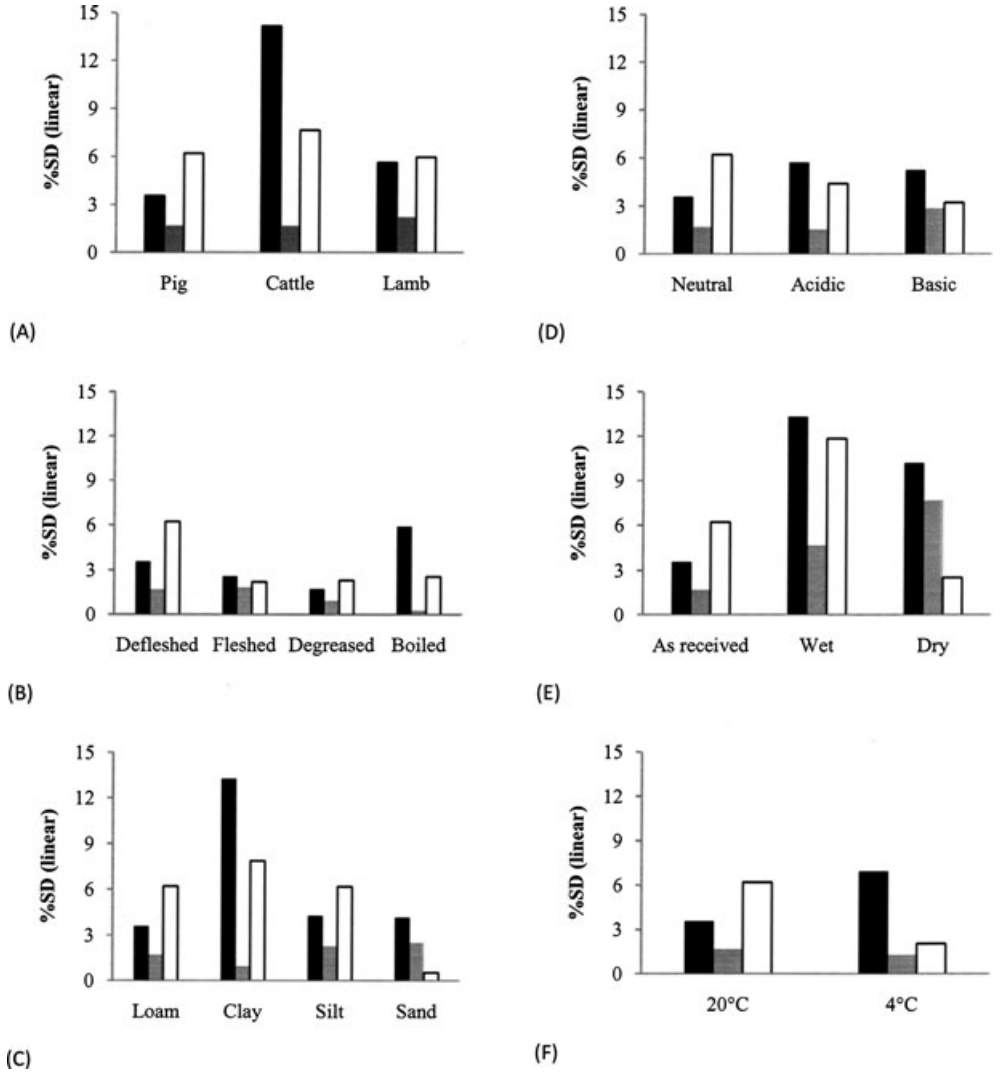

FIG. 7—*Standard deviation values for model burial environments: (A) species, (B) bone pretreatment, (C) soil type, (D) soil pH, (E) moisture content, and (F) soil temperature.*

correlation with a positive linear trend, but the variance and slope are relatively small, and so the increase is also not as significant as that determined for bone buried in the silty soil. The cold environment samples show a high calculated slope, but the variance is high indicating some scatter and the % SD raw:% SD linear ratio is low, so a trend is questionable.

Trends do not seem to be present in the CI data, except for the acidic environment, which shows a significant positive linear trend. The results for bone buried in this environment show high calculated slopes with an average amount of variance from the linear model relative to the rest of the sample sets.

The most significant changes to buried bone structure were observed for specimens exposed to an acidic soil environment. The observed trends indicate that protein content is degraded, and there is ordering of the crystalline phase when the bone is exposed to an acidic environment over a relatively short period of time. It has been previously reported that a highly acidic soil pH can decompose bone more rapidly because of the dissolution of the inorganic matrix of hydroxyapatite (19). The lack of notable changes to the spectra obtained for the bones exposed to a basic or neutral pH soil is in line with previous observations about such environments (20).

Conclusions

Infrared spectroscopy has been applied to monitoring the microstructural changes that occur to bones after burial in a soil environment. The effects of time, bone types and preparation, and soil conditions were studied. Linear and logarithmic fits to the data were investigated for the bones exhumed from burials of up to 23 months as part of a field study. A decrease in organic and carbonate contents and an increase in the CI occurred in the time frame of the burials utilized for this study. An examination of the data variance indicates that the best correlations occur for the carbonate and organic content data.

The same analytical approach was applied to bones exposed to model soil environments. The environment that was shown to have a significant effect on the determination of burial time was an acidic soil. An examination of the organic and carbonate contents and the CI of the bones exposed to acidic soil revealed that bone decomposition is accelerated. The other

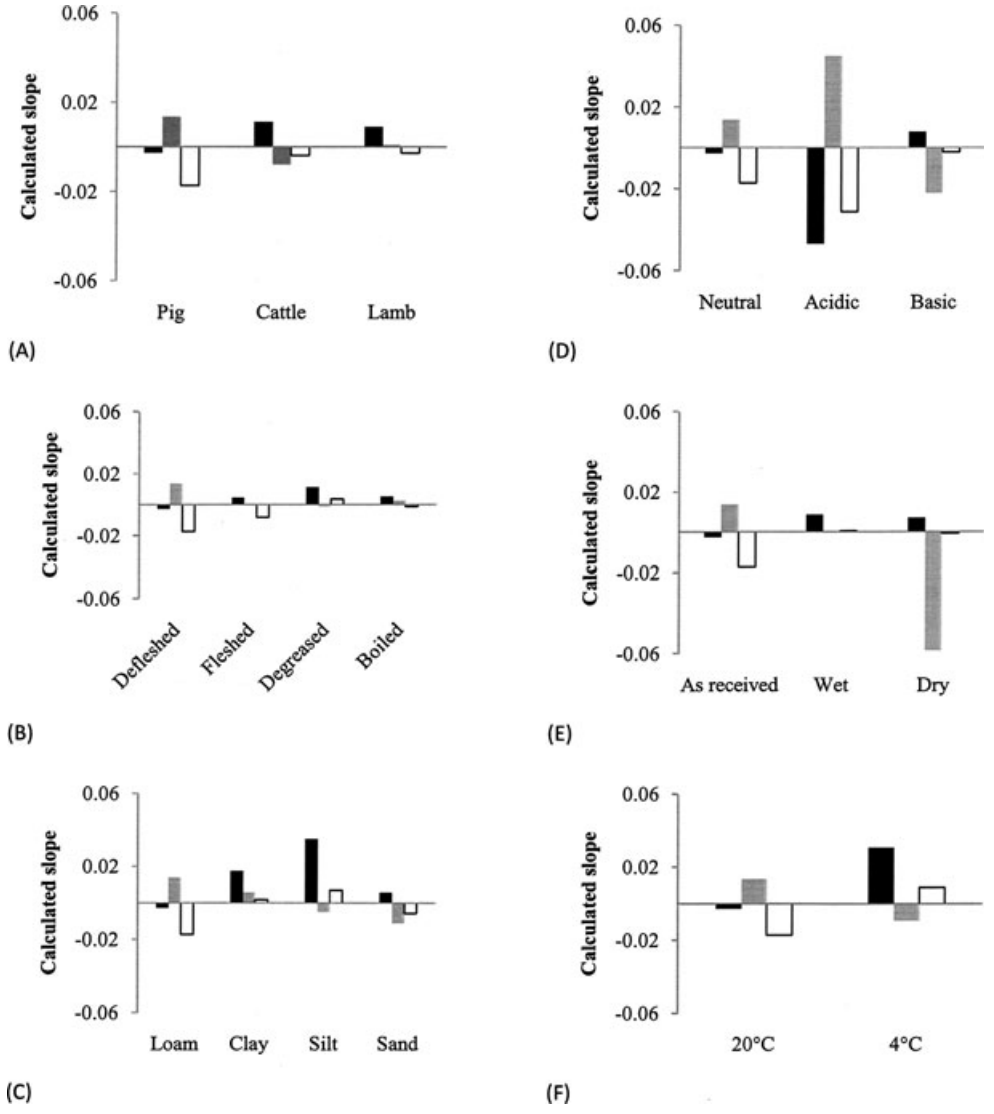

FIG. 8—*Calculated slopes for model burial environments: (A) species, (B) bone pretreatment, (C) soil type, (D) soil pH, (E) moisture content, and (F) soil temperature.*

TABLE 2—*Ratios of raw % SD and linear % SD for bones from model burial environments.*

Factor	Sample	Organic Content	CI	Carbonate Content
Species	Pig	1.18	1.43	2.39
	Cattle	0.86	1.26	1.21
	Lamb	1.21	1.12	1.20
Bone pretreatment	Defleshed	1.18	1.43	2.39
	Fleshed	1.27	1.12	3.19
	Degreased	2.69	1.13	1.53
	Boiled	1.14	1.51	1.19
Soil type	Loam	1.18	1.43	2.39
	Clay	1.11	1.29	1.11
	Silt	3.22	1.15	1.34
	Sand	1.21	1.24	1.50
Soil pH	Neutral	1.18	1.43	2.39
	Acidic	4.62	3.33	7.33
	Basic	1.24	1.44	1.27
Moisture content	As received	1.18	1.43	2.39
	Wet	1.09	1.12	1.11
	Dry	1.11	1.47	1.15
Soil temperature	20°C	1.18	1.43	2.39
	4°C	1.97	1.39	3.50

factors investigated, including species type, pretreatment methods, basic soil pH, soil moisture content, and temperature, were found not to have a significant effect on bone structure during burial.

The findings of this investigation provide a valuable approach to the examination of exhumed bones. Trends in infrared data are observed with time and can potentially be used to estimate the length of burial. Future work will involve further testing of the variability of data. Also, expanding this approach to different time frames will be investigated to test the validity of this approach to extended time periods. Importantly, most bone treatments and soil environments do not appear to affect the interpretation of the time data to any significant degree, apart from the exposure to an extreme acidic soil.

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