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Physicochemical changes to surface deposited decomposing bone over different timescales: Investigating the influence of bone fractures and the use of non-destructive analytical techniques

C. Mein *, J.R. Jones, C. Tennick, A. Williams

Research Centre for Field Archaeology and Forensic Taphonomy, School of Law & Policing, University of Lancashire, Preston PR1 2HE, UK

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ABSTRACT

Considerations on the drivers of bone diagenesis have received a lot of attention, yet there is still more to understand, particularly in relation to chemical changes that can occur post-mortem, and the rate at which these occur. The physicochemical composition of bone is altered during the post-depositional period, leading to a more thermodynamically stable crystal lattice, thus increasing the long-term survivability of the bone. Research has shown the potential for soft tissue trauma to affect the decomposition process, but the effect of bone trauma and fractures on diagenesis has not yet been considered. Most bone diagenesis research uses destructive analytical techniques, resulting in the loss of samples and the inability to perform repeat analyses. Presented here is a study investigating changes in the physicochemical composition of disarticulated *Sus scrofa* ribs, with and without fractures, using non-destructive analytical techniques. The aim was to explore the timescales in which physicochemical changes occur and to investigate the potential influence of bone fractures. Intact (control) or fractured (blunt-force or sharp-force) bone samples were deposited on a grassy surface for up to 240 days. Physicochemical changes to the bone sections were analysed using scanning electron microscopy – energy dispersive spectroscopy and Fourier transform infrared spectroscopy with attenuated total reflectance. It was hypothesised that physicochemical changes could be quantified in < 240 days using these techniques, and that the presence of fractures would affect the observed changes. Statistically significant ($p < 0.05$) losses in Na, K, and Mg and increases in crystallinity were seen over time, as well as significant changes in carbonate content and a significant loss of proteins. Differences physicochemical composition were observed between the undamaged and fractured samples, and the samples with BFT appeared to be the least affected for many elemental and IR parameters indicating BFT could potentially inhibit physicochemical change. The analysis of Na and K showed potential for PMI estimation, as these changed significantly over time, but as these were influenced by the presence of bone fractures, more research is needed fully understand how different variables can affect physicochemical change in bone, particularly the presence of bone fractures/damage.

1. Introduction

Literature shows that the decomposition process continues after skeletonisation has occurred, with bones changing, both in their physical appearance and chemical composition over time [1-7]. This is *bone diagenesis*. The presence of trauma to the soft tissues can affect the pattern of decomposition, if not the rate of the decomposition [8-10]. However, to date, research to determine whether fractures affect bone diagenesis has been limited [11], and particularly the rate at which physicochemical changes occur is little understood. As bone diagenesis occurs due to microbial interactions and chemical reactions between the bone and the surrounding environment [12], fractures, or bone damage, can influence the rate of diagenesis [11], as environmental agents, such as soil, water, and microbes can gain easier access to the bone via the trauma site(s). This could potentially trigger increased microbial damage and destruction of the microstructural integrity of the bone, and increased ion exchange resulting in changes to the physicochemical composition of the bone, via dissolution of the bone minerals and hydrolysis of collagen [13].

There is much literature that shows that taphonomic changes occur to skeletal tissue over time, particularly in archaeological contexts [14-16,1,2,4,17]. More recent studies have focused on remains from

* Correspondence to: School of Law & Policing, University of Lancashire, Fylde Road, Preston, Lancashire PR1 2HE, UK.
E-mail address: cmein@lancashire.ac.uk (C. Mein).

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shorter timescales, more pertinent to forensic scenarios, focusing on the causes and rates of skeletal degradation in the hope of establishing more accurate methods of post-mortem interval (PMI) estimation [12,18,5,19]. Research into soft tissue decomposition has allowed the development of reasonably accurate PMI methods (Wilson-Taylor & [20]), but these methods become less accurate as the PMI increases [21,22]. The ability to accurately estimate PMI is of great importance; it can help establish timelines, narrow suspect lists, lead to locations of significance, and help identify the victim [23]. While many factors have been included in diagenetic research, ranging from characteristics of the deposition environment, to the condition of the remains (whole vs partial; articulated vs disarticulated), the presence of fractures and their influence of PMI estimations has not been considered. Given that it has long been hypothesised that the study of diagenetic alterations, such as the histological integrity of bone [18,19]; or biochemical techniques, such as proteomics [5,24], or elemental analysis [25] could one day lead to more accurate methods of PMI estimation on skeletal remains, this is surprising. Diagenetic changes are attributed to microbial attack, and originate from both the remains and the deposition environment [3,7]. If, as hypothesised here, there is a correlation between bone fractures and microbially- and chemically-driven bone diagenesis, then the presence of fractures could affect histological and biochemical methods of PMI estimation. If physicochemical changes are triggered earlier than previously thought due to compromised tissues allowing easier access to the bone, then bone diagenesis could occur sooner, and to a greater extent than previous research has shown. There is potential for the presence of fractures to cause over-estimation of the PMI.

Bone is a composite material, made up of organic and inorganic factions [26]. Bone mineral is composed of hydroxyapatite, a crystalline matrix which is often shown to have the chemical composition, $Ca_{10}[PO_4]_6[OH]_2$ [27-29], while collagen accounts for 90 % of the protein content [26]. Hydroxyapatite has an unusual feature in that it can accommodate substitutions at every site of the crystal lattice [30,31].

As hydroxyapatite can accommodate substitutions along the crystal lattice [30,32,31], analysing specific elemental contents within the cortical bone could help in understanding the process and extent of diagenetic change [13], and it is believed by some it could have the potential to identify whether remains have been moved from a primary deposition site due to the specificity of ion exchange [25,33]. Yet the elemental analysis of post-mortem bone has received little attention [25]. It has been established that bone can, and will, exchange elements with the deposition environment, and such changes have been found in bones from archaeological contexts [34,13,35], and short timescale studies [36,33,37,31,25]. Elemental studies have focused on various elements, including calcium and phosphate due to their abundance in bone mineral, iron, sodium and potassium as they are related to bodily fluids, and other elements have been chosen due to their presence in soils (for example, magnesium, manganese, barium, zinc) [25,31]. The loss of sodium and potassium has been observed to occur within one year post-mortem [25,31] and has been linked to dehydration of the bone [31]. A loss of iron has also been noted to occur most likely as a result of the breakdown of blood cells [25], while changes to zinc [37], manganese and magnesium [31] have also been observed.

Previous studies have shown changes to the structural composition of bone occur over archaeological timescales [38-41,17], and these processes are believed to be key in the fossilisation process [17] due to the recrystallisation of the mineral lattice over time resulting in a more thermodynamically stable mineral matrix [36,40-42]. As well as increases in the size and order of the mineral crystals, substitutions occur along the crystal lattice, at the A-site (hydroxyl) position, and/or the B-site (phosphate) position, resulting in carbonate content changes [43,30,38,44,32,45]. Decreases in the carbonate-to-phosphate ratio (C/P) have been observed in several studies [31,36], and are attributed to carbonate loss, an increase in phosphate, or a loss in proteins. An inverse relationship has been noted in the literature between bone crystallinity and the C/P (C/P decreasing as crystallinity increases) [38,46,31]. The loss of CO_3^{2-} has also been observed as a result of dissolution [38]. Analysing the structural composition of bone has allowed the protein content of the bone to be measured. As collagen accounts for 90 % of the organic matrix of bone, this measurement of the protein content can give valuable information about collagen content [47]. Protein content, and therefore collagen, can decrease over time, in short timescales [48,36,31] or archaeological timescales [49,38,46] increased porosity and microbial attack, all of which are affected by various extrinsic and intrinsic factors relating to the remains and deposition environment [50,46,39].

The study presented here aimed to evaluate the potential effect of bone trauma on physicochemical changes occurring during the post-mortem period, in the hope of enhancing PMI research. This research focused on the use of the non-destructive analytical techniques scanning electron microscopy – energy dispersive spectroscopy (SEM-EDS) and Fourier transform infrared spectroscopy with attenuated total reflection (FTIR-ATR). It was hypothesised that changes would occur to the physicochemical composition of the bone over time, and this would be influenced by the presence of fractures, through the exchange of endogenous and exogenous ions because of bone degradation, the movement of soil water and microbial activity.

2. Methods

Samples were placed outside between September 2020 and May 2021. Temperature data was recorded using Blue Maestro Tempo Disc Bluetooth dataloggers and rainfall data was obtained through a local weather station accessed through World Weather Online (https://www.worldweatheronline.com/v2/weather-averages.aspx?q=hd1) (Fig. 1). Racks of porcine (*Sus scrofa domesticus*) ribs (n = 149) intended for human consumption were sourced from a local butcher. They were fresh and had not been previously frozen. The ribs were stored in a refrigerator overnight at 4 °C. Using a PM40 scalpel, the ribs were partially defleshed as close to the bone as possible without damaging the bone surface. Fresh, partially fleshed pig ribs (n = 4) were saved to be used as day 0 controls.

The pig ribs were separated into three groups; unfractured (n = 45), blunt-force fractured (n = 50), sharp-force fractured (n = 50). To create the blunt-force fractures a small hammer (Rolson; 342 g, 2.5 cm hammer head diameter) was attached to a purpose-built weapon holder along with a 5 kg weight (to give a total weight of 1696 g under the hammer head). The weapon holder consisted of a flat wooden board to place the sample onto with a pole attached at the opposite end. The weapon was attached to the end of the arm which allowed it to be dropped consistently from the chosen height. The ribs were placed one at a time onto the bottom of the weapon holder and the weapon and weight were raised to a height of 75 cm and dropped to produce extensive fractures (Fig. 2**A**). For the sharp-force fractures, a small camping axe (Rolson; 744 g, axe head 6.9 cm long) was attached to the weapon holder with no extra weight. The weapon was raised to a height of 45 cm and dropped onto the ribs to produce small fractures (Fig. 2**B**). All fractures were inflicted either when the bones were fresh (i.e. displaying wet bone characteristics), or 60 days after deposition.

The partially fleshed ribs (n = 145) were deposited onto a small patch of grass in a domestic garden in Huddersfield, West Yorkshire, UK and left to decompose. Stone bricks and wire nesh was used to prevent scavenger access. Samples were collected at 30, 90, 150, 180, and 240 days (n = 29 per PMI). The pH of the soil was measured monthly using a *Beslands 4 in 1 soi tester*, which showed the soil pH reduced from 7.5 at the start of the study to 6.5 by the end.

Upon collection, the samples were cleaned. The samples with shorter (<90 days) PMIs still had flesh attached and were macerated. For this, the samples were placed in padded bags and lowered into a maceration pot with a Buffalo mechanical stirrer (DN868) set at 40°C for up to 90 min. To ensure the integrity of the samples, high temperatures and

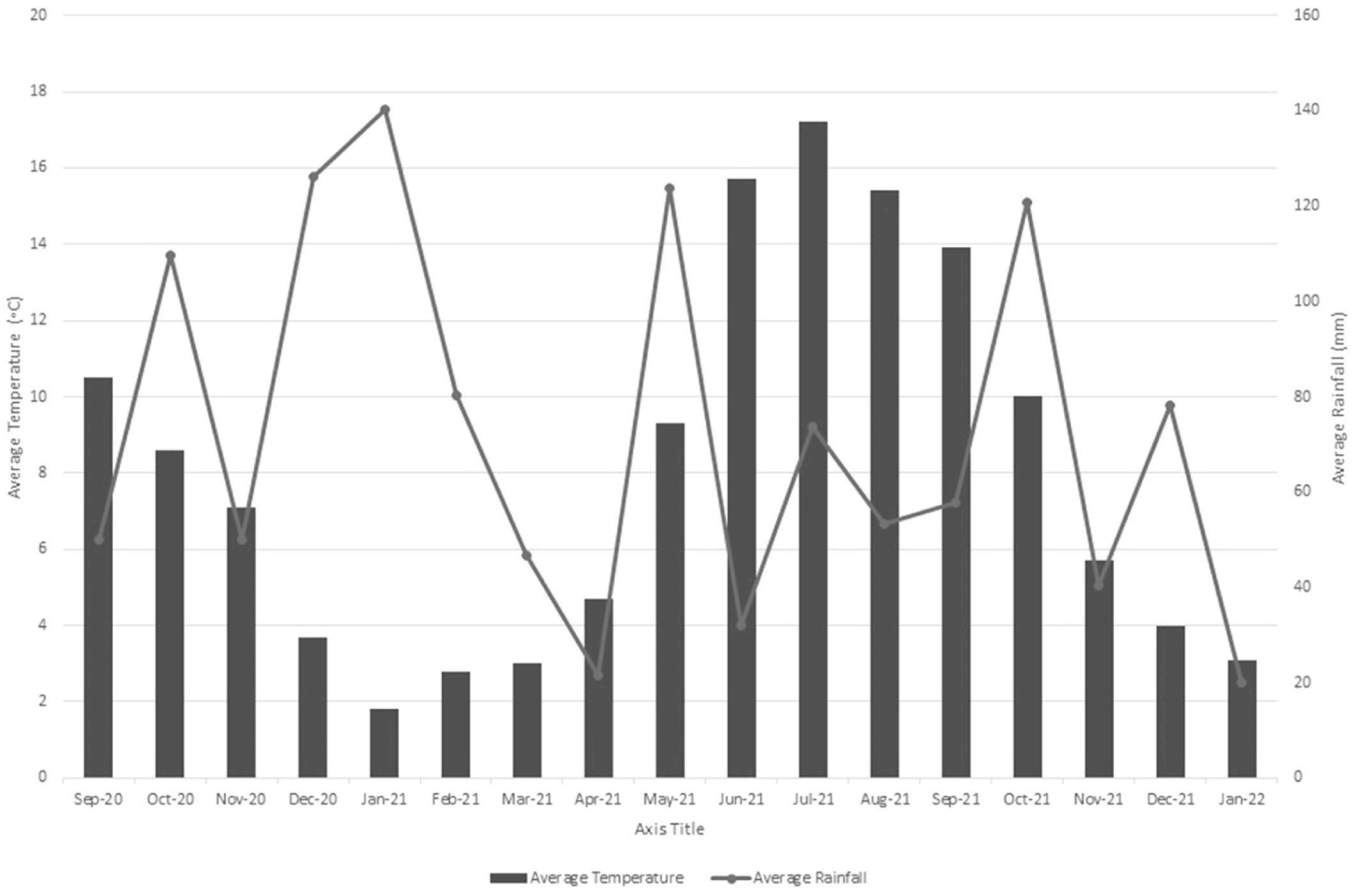

Fig. 1. Weather data for the duration of the study. Average monthly temperatures and rainfall are given.

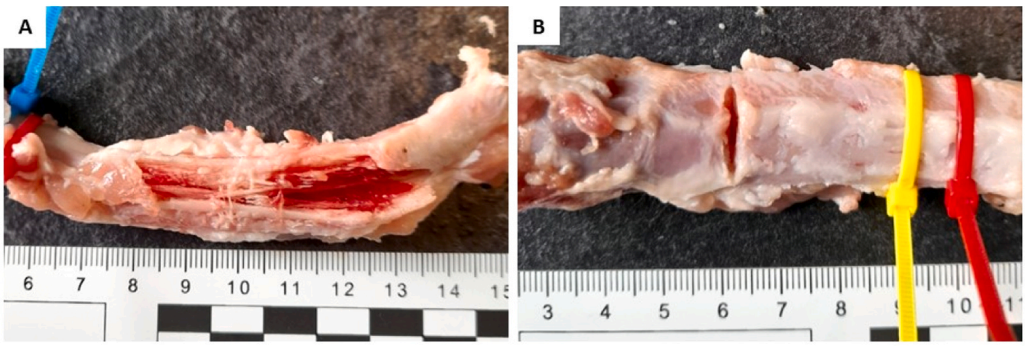

Fig. 2. Fractures on fresh porcine ribs, A) Blunt-force, B) Sharp-force.

Fig. 3. Porcine ribs at 180 days PMI. A) Unfractured sample, B) Damaged sample (blunt-force), C) Damaged sample (sharp-force).

detergents were avoided as advised in the literature [51-57]. Once the flesh had been removed, the samples were rinsed with fresh water and air dried thoroughly. The samples with longer (>90 days) PMIs had no soft tissue present and were cleaned with cold water then air dried thoroughly. All samples were photographed after cleaning (Fig. 3). All samples were stored in a freezer at −20°C until analysis could be conducted. Freezing samples can potentially affect the physicochemical integrity of bone [58-60], however as all samples were subject to the same storage conditions the samples are all comparable. Note was taken of the conclusions by McElderry et al. [60] and only one cycle of freezing and defrosting was undertaken.

2.1. Physicochemical analysis

Sections of bone 3–5 mm in thickness were cut from each sample (from the middle of the control samples and at the fracture site of the experimental samples). The sections were dried overnight at 23°C then stored in plastic containers at an ambient temperature of 23°C until ready for analysis.

Trace elemental analysis was conducted using a *JEOL JCM 6000* + desktop SEM with EDS analyser (settings: filament current: high; probe current: high; accelerating voltage:15 kV; working distance:19 mm; magnification:500x). Three elemental measurements were taken from the outer edge of the cortical bone per sample, these were averaged to give one overall reading. Eight elements were selected using available literature [25,37] as guidance; calcium, phosphorus (bone mineral), potassium, sodium, iron (bodily fluids), magnesium, zinc, barium (trace elements). For analysis the mass (%) was calculated using analytical software in standardless quantitative mode.

To prepare for FTIR-ATR analysis, the dried bone sections were broken into small fragments (approx. 1 mm in size) using a hammer. The outer cortical bone fragments were collected for analysis. Measurements were conducted using a *Thermo Scientific NICOLET iS5* FT-IR spectrometer fitted with an *iD7* ATR accessory (parameters: range: 4000–400/ 450 cm^{-1}; no. of scans: 144; resolution: 4 cm^{-1}; mode: absorbance). Bone fragments were placed onto the optic window and pressed onto the diamond crystal using the pressure applicator. After each sample scan, the plate and pressure anvil were cleaned with ethanol. Six peaks were identified for analysis using protocols set out by Hollund et al. [61]; Kontopoulos et al. [44]; Sponheimer and Lee-Thorp [62]; Trueman et al. [47]; Weiner and Bar-Yosef [63]; Wright and Schwarcz [64] (Fig. 4) to give information on the changes occurring to the size and order of the crystals (IRSF) within the lattice as well as the carbonate (API, BPI, BAI) and protein (Am/P) content.

The background was measured before the start of each day, whenever a new set of samples were analysed (per PMI), and/or every 100 min, whichever was sooner.

The analytical chemistry software, *MestreNova* (MNova), was used to analyse the date. No ATR correction was performed. Baseline correction was applied between 450 and 2000 cm^{-1} at c. 470 > 650 > 800 > 1250 > 1750 using [31] as a guide. The peak-by-peak function was used to obtain the heights of the required peaks while manual threshold was used to determine the trough between peaks 560 and 600 for the IRSF measurement. IRSF was attempted using the protocol set out by [65], but this gave inconsistent results. The calculations used to establish changes to the mineral and amide content of the samples are shown in Table 1. The calculations for measuring the amount of Type A and Type B carbonate relative to phosphate were done using the phosphate band at 1010 cm^{-1} using the protocol set out in Howes et al. [48]. The phosphate band at 600 cm^{-1} has been used in other research [44,62], however the justification given by Howes et al. [48] that the 1010 cm^{-1} peak could give more accurate results made it the better option.

2.2. Statistical analysis

The statistical software package, SPSS, was used for all statistical testing. The Shapiro-Wilkes test for normality was conducted in the first

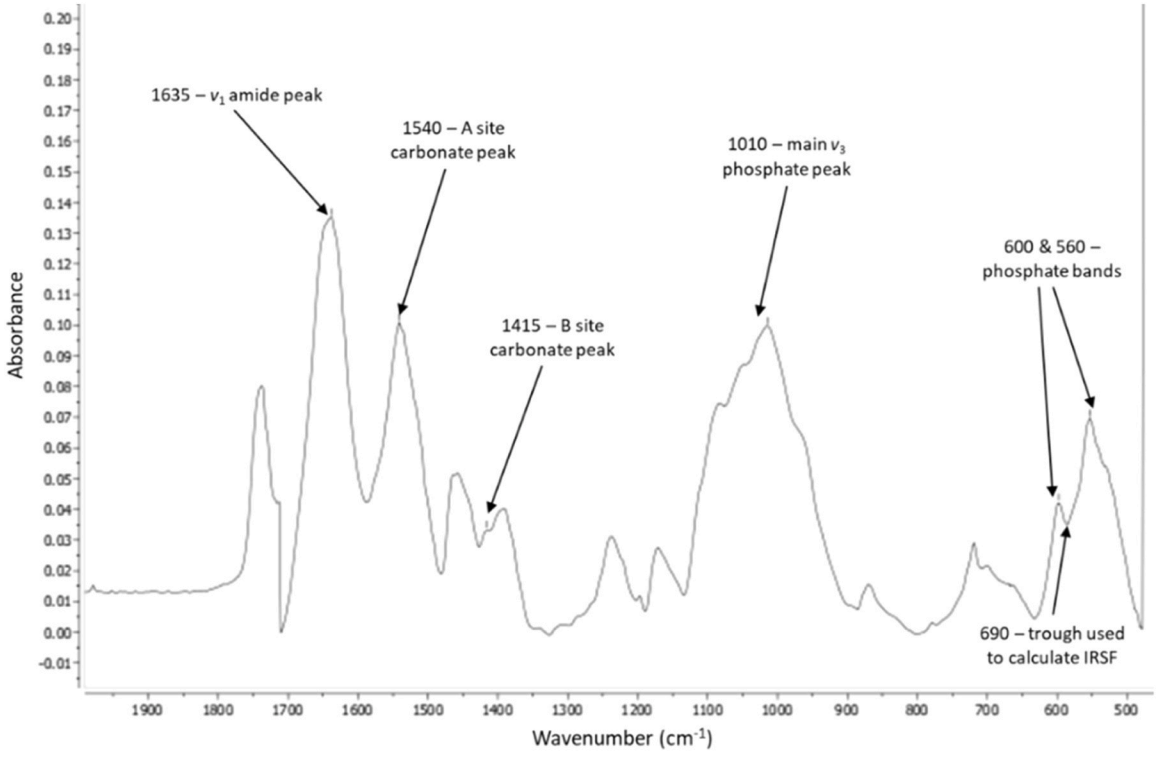

Fig. 4. FTIR spectra taken from MestreNova software. Arrows indicate the six peaks used for all calculations and what bonds/functional groups they correspond to; the trough used for IRSF. Baseline corrected at c. 470 > 750 > 1250 > 1720.

Table 1
Structural changes assessed using FTIR-ATR spectra.

Assessed Changes	Description	Peaks (cm^{-1}). Calculation used	References
Infra-red splitting factor (IRSF)	Observe changes to size and order of hydroxyapatite crystals within the mineral lattice.	$\frac{600+560}{590}$	Hollund et al. [61]; Kontopoulos et al. [44]; Weiner and Bar-Yosef [63]; Wright and Schwarcz [64]
Type-A carbonate-phosphate index (API)	Amount of type-A carbonate within the sample relative to phosphate.	$\frac{1540}{1010}$	Howes et al. [48]
Type-B carbonate-phosphate index (BPI)	Amount of type-B carbonate within the sample relative to phosphate.	$\frac{1410}{1010}$	Howes et al. [48]
Type-B-to-Type-A Index (BAI)	Amount of type-B carbonate to amount of type-A carbonate within the sample.	$\frac{1410}{1540}$	Sponheimer and Lee-Thorp [62]
Amide-phosphate ratio (Am/P)	Amount of protein within the sample relative to phosphate.	$\frac{1640}{1010}$	Hollund et al. [61]; Kontopoulos et al. [44]; Trueman et al. [47]

instance to determine which statistical tests to continue with. This showed the data was not normally distributed ($p < 0.05$) therefore non-parametric tests were performed. The Kruskal-Wallis test for Independent Samples was used. Pairwise comparisons were included to show where any significant differences were occurring. Comparisons were:

- Diagenetic changes occurring over time in the undamaged samples,
- Diagenetic changes occurring over time in the damaged samples.

Correlation analysis was undertaken to assess the relationship between physicochemical changes and time using the Spearman's rho test.

3. Results

3.1. SEM-EDS results

Over 240 days, the elemental composition of the samples changed significantly, regardless of the condition of the bones (Fig. 5; Table 2). Decreases in Na and K were noted in all three bone condition groups (unfractured, blunt-force, and sharp-force fractures) over time. When analysing the statistical significance of any changes occurring over time, the samples fractured with the axe appeared to affect the chemical composition of the samples, with four significant elemental changes observed.

The unfractured bones showed statistically significant decreases in Na, K, and Mg levels by 240 days PMI (Fig. 5**A-C;** Table 2), although

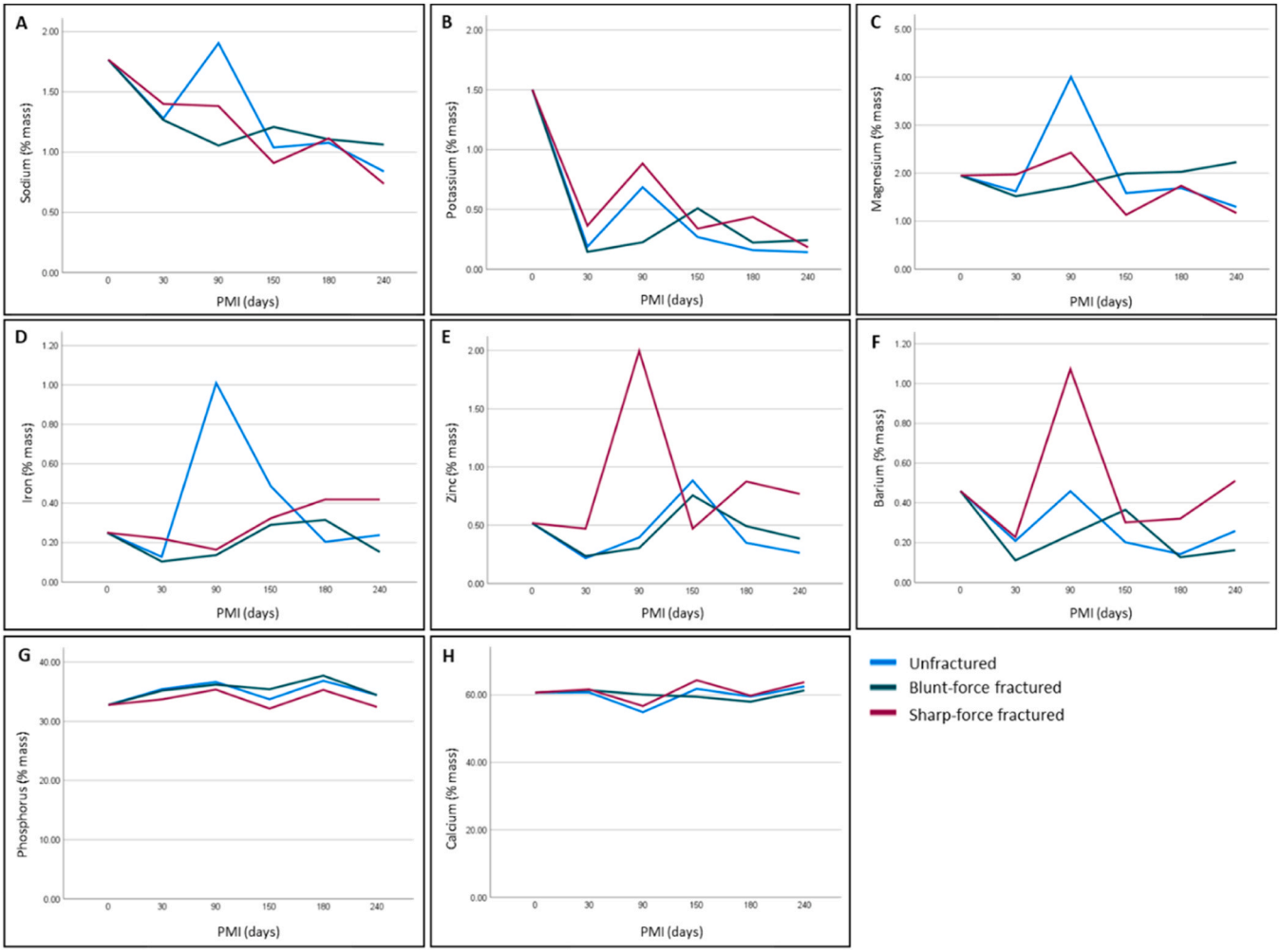

Fig. 5. Line graphs representing elemental changes occurring to the bone samples over time for each element tested, **A)** sodium, **B)** potassium, **C)** magnesium, **D)** iron, **E)** zinc, **F)** barium, **G)** phosphorus, **H)** calcium. Note that the scale for each element is different.

Table 2
Statistical data for changes occurring to the elemental composition of the bones from fresh up to 240 days PMI.

Element	Unfractured (n = 45)		Blunt-force (n = 50)		Sharp-force (n = 50)	
	Significance (*p*)	Correlation	Significance (*p*)	Correlation	Significance (*p*)	Correlation
Sodium (Na)	**< 0.001**	**-.599*****	0.133	-.251	**< 0.001**	**-.590*****
Potassium (K)	**0.002**	**-.462*****	**0.005**	-.187	**0.022**	**-.420****
Magnesium (Mg)	**0.005**	**-.369****	0.540	-.076	**< 0.001**	**-.466*****
Iron (Fe)	0.141	.109	0.079	.071	0.309	**.272***
Zinc (Zn)	0.301	-.149	0.618	.158	0.629	.117
Barium (Ba)	0.503	-.082	**0.032**	-.058	0.168	.211
Calcium (Ca)	0.112	.229	0.793	-.046	**0.017**	.211
Phosphorus (P)	0.165	.070	0.272	-.125	0.085	-.054

* Correlation is significant at < 0.05;
** correlation is significant at < 0.01;
*** correlation is significant at < 0.001

increases in these elements were observed at 90 days PMI. Post-hoc tests showed Na did not significantly decrease until 150 days PMI, but K had decreased significantly by 30 days PMI. Fluctuations occurred in Fe, Zn, Ba, P and Ca levels (Fig. 5**D-H**) throughout the study but none of these resulted in statistically significant differences. There was a slight non-significant increase in Ca and P content by the end of the study, alongside a slight, but non-significant decrease in Zn and Ba levels. Fe content did not show any changes in content overall, despite fluctuations at various PMIs. Correlation analysis showed moderate, but significant relationships between three elements, Na, K, and Mg, and time (Table 2).

The samples with blunt-force fractures showed significant changes in K and Ba levels over time (Table 2), although there were other elements that appeared to fluctuate over time (Fig. 5). Na decreased throughout the study. Mg increased very slightly overall, but fluctuations were seen at various PMIs, and one sample showed an unusually high Mg level at 240 days PMI. P also fluctuated throughout, and an increase was seen by 240 days PMI. While Ca, Fe, and Zn contents fluctuated throughout the study, no change was observed by the end of the study. K and Ba levels showed statistically significant decreases by 240 days PMI; both elements fluctuated at various PMIs and were significantly different by 30 days PMI compared to fresh bone. Correlation analysis showed weak relationships between time and all eight elements, which were not significant (Table 2).

The sharp-force fractured samples showed the most change in elemental composition. Na, K and Mg levels (Fig. 5**A-C**) were all significantly decreased over time (Table 2). It took 150 days for Na and Mg levels to be significantly changed compared to fresh bone, but only 30 days for K. Ca levels increased significantly over time (Fig. 5**H;** Table 2), although fluctuations were observed at various PMIs and post-hoc tests did not find any significant differences between fresh bones and any timescale analysed. Fe content showed a non-significant increase over time, while P content fluctuated but no obvious change was observed by 240 days PMI. Zn and Ba both showed fluctuations occurring at various PMIs, but no significant change was observed overall. Correlation analysis showed negative, moderate and significant relationships between time and three elements (Na, K, Mg), while a weak but significant relationship was found to occur between time and Fe (Table 2).

3.2. FTIR-ATR results

The spectra of all samples were analysed to assess changes occurring to the crystal lattice structure, and the mineral and protein content of the bone (Fig. 6). Over 240 days, the structural composition of the bones changed significantly, regardless of the presence of fractures (Fig. 7**;** Table 3). In particular, the protein and carbonate contents were observed to change in all three groups (unfractured, blunt-force, and sharp-force fractured). A broad peak at 1010 cm^{-1} was observed in all FTIR spectra due to the vibrational modes of phosphate ions [66]. This absorption band was easily distinguished as it is the most intense peak observed [66]. Peaks at 1415 cm^{-1} and 1540 cm^{-1} were seen due to the stretching of the carbonate ions, these are for type A and type B substitutions, respectively [49,67,66]. The API, BPI, and BAI were calculated using the phosphate band at 1010 cm^{-1} and carbonate bands at 1415 cm^{-1} and 1540 cm^{-1}. While other studies have used the phosphate band at circa 600 cm^{-1}, this study used the protocol as set out in Howes et al. [48].

All five IR parameters changed significantly in the unfractured samples (Table 3). The crystallinity increased over time (Fig. 7**A**) and this increase was significant by 90 days PMI. The API fluctuated but a slight decrease was seen overall (Fig. 7**B**). The BPI and BAI had both increased by the end of the study (Fig. 7**C-D**). A significant increase in the BPI was observed at 30 days PMI, but decreases were seen thereafter. The BAI increased over time until 90 days PMI, before appearing to stabilise. The Am/P was the only IR parameter to decrease over time. (Fig. 7**E**) This loss appeared to stabilise > 90 days PMI. Correlation analysis showed moderate, but significant relationships between four IR parameters (crystallinity, API, BAI, Am/P) and time (Table 3). A correlation between IRSF and Am/P was moderate and significant (r = -0.412, n = 48, $p = 0.004$) for the unfractured samples as the organic content of the bone decreased as the crystallinity increased.

Only three IR parameters changed significantly over time for the blunt-force fractured samples (Table 3). Crystallinity increased (Fig. 7**A**), but this was not significant. While fluctuations in API were seen at various PMIs (Fig. 7**B**), only a small non-significant increase was observed by 240 days PMI. The BPI significantly increased over time until 90 days PMI, after which it started to stabilise (Fig. 7**C**). The changes in BPI were significantly different to fresh bone by 30 days PMI. The BAI increased steadily until 240 days PMI when it suddenly decreased (Fig. 7**D**). Post-hoc tests showed BAI was significantly different from fresh bone by 90 days PMI. The Am/P significantly decreased over time. A sudden loss in protein was observed at 30 days PMI and smaller losses occurred at each subsequent PMI (Fig. 7**E**). However, protein loss was not statistically significant until 150 days PMI. Correlation analysis showed positive, moderate, and significant relationships between time and both the crystallinity and the BAI, while a negative, moderate and significant relationship was observed between time and the Am/P (Table 3). The relationship between IRSF and Am/P was weak and non-significant (r = -0.109, n = 52, $p = 0.440$) in these samples.

The sharp-force fractured samples showed significant changes over time for all five IR parameters (Table 3). The crystallinity increased at each PMI (Fig. 7**A**), and this measurement was significantly different from fresh bone by 90 days PMI. The API decreased over time after an initial increase at 30 days PMI (Fig. 7**B**). The BPI showed a large increase at 30 days PMI followed by fluctuations at each subsequent PMI, resulting in a higher content by the end of the study compared to fresh bone (Fig. 7**C**). The BAI steadily increased over time (Fig. 7**D**) and was significantly different 150 days PMI. The Am/P initially increased at 30 days PMI before steadily decreasing at each subsequent PMI, and then

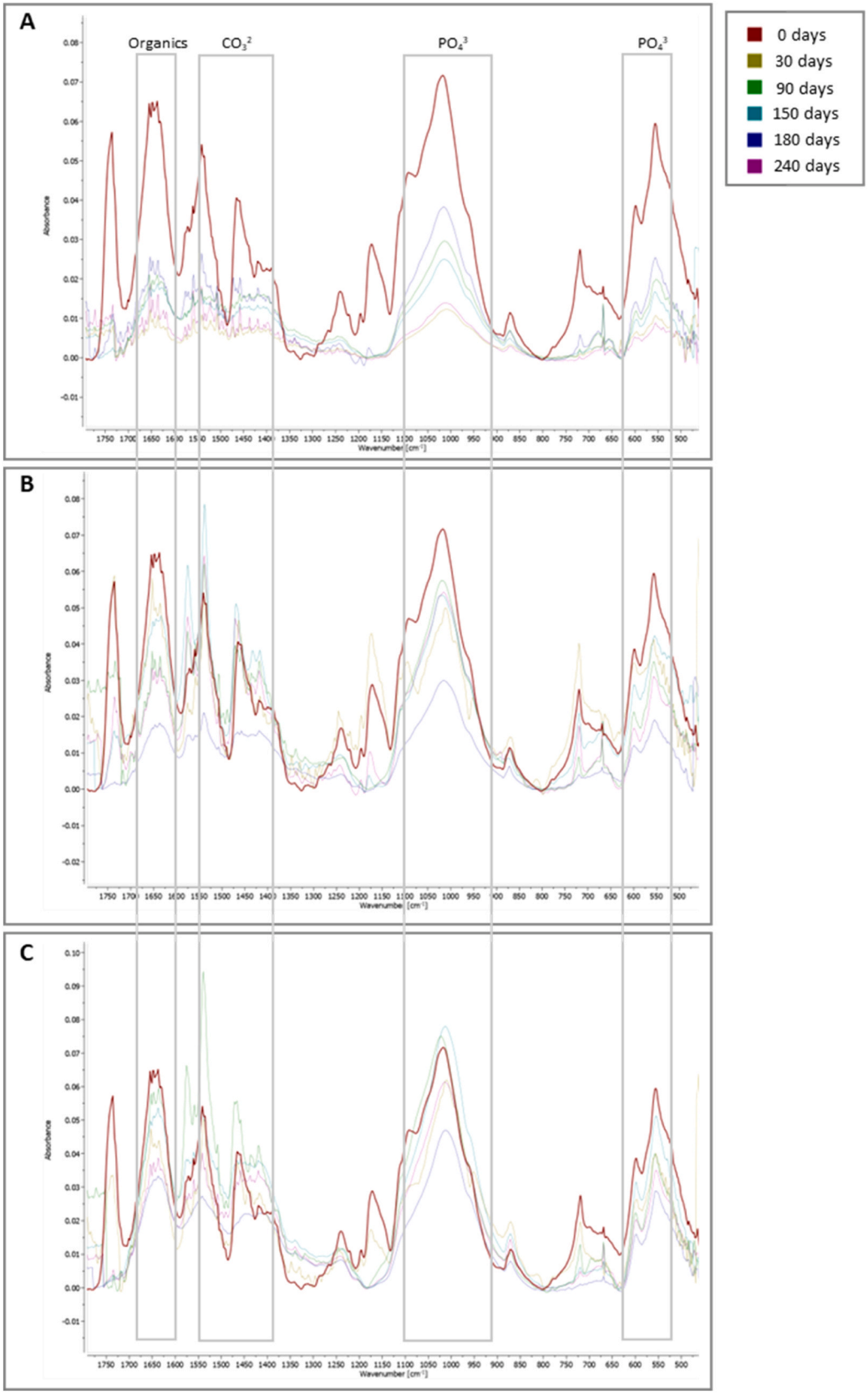

Fig. 6. FTIR-ATR spectra of samples from each condition and for each PMI investigated. Peaks of interest are highlighted **A)** unfractured, **B)** Blunt-force fractured, **C)** Sharp-force fractured.

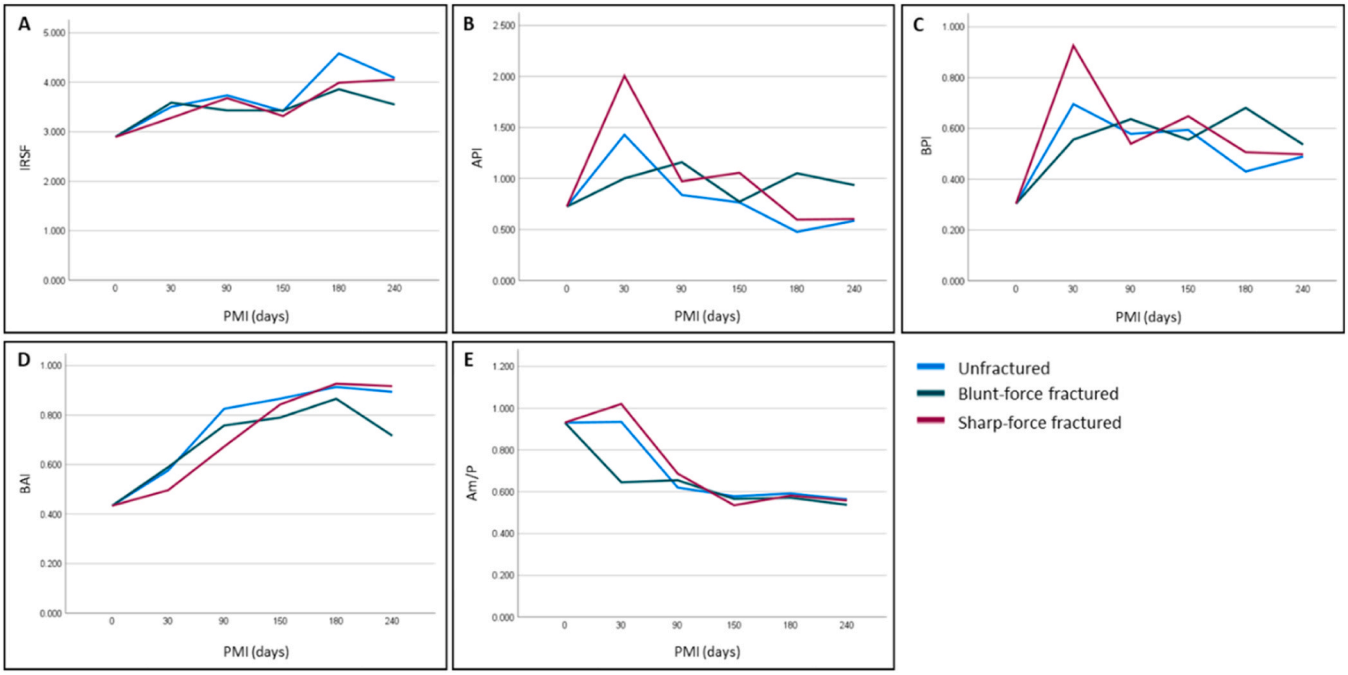

Fig. 7. Line graphs representing structural changes occurring to the bone samples over time, **A)** IRSF (crystallinity), **B)** API (type-A carbonate content), **C)** BPI (type-B carbonate content), **D)** BAI (carbonate content), **E)** Am/P (protein content). Note that the scale for each element is different.

Table 3
Statistical data for changes occurring to the structural composition of the bones up to 240 days PMI.

IR Parameter	Unfractured (n = 45)		Blunt-force (n = 50)		Sharp-force (n = 50)	
	Significance (*p*)	Correlation	Significance (*p*)	Correlation	Significance (*p*)	Correlation
IRSF	**0.006**	**.430****	0.130	**.308***	**0.001**	**.510*****
API	**0.004**	**-.464*****	0.681	-.096	**0.002**	**-.514*****
BPI	**0.001**	-.082	**0.045**	.192	**< 0.001**	-.180
BAI	**0.002**	**.529*****	**0.044**	**.302***	**0.002**	**.596*****
Am/P	**0.004**	**-.557*****	**0.028**	**-.460*****	**0.005**	**-.504*****

* correlation is significant at 0.05;
** correlation is significant at 0.01;
*** correlation is significant at 0.001

stabilised at > 150 days PMI (Fig. 7**E**). The Am/P took 150 days to show a significant difference from fresh bone. Correlation analysis showed positive, moderate, and significant relationships between time and three IR parameters (IRSF, API, BAI), while a negative, moderate and significant relationship occurred between time and Am/P (Table 3). A weak and non-significant relationship ($r = -0.202$, $n = 53$, $p = 0.146$) occurred between the IRSF and Am/P in these samples.

Inaccuracies in Type A carbonate calculations when using the 1540 cm^{-1} peak due to the potential overlap with amide II have been suggested [68], therefore the linear relationships between the 1540 cm^{-1} peak and the amide I peak at 1640 cm^{-1} was assessed. This showed a moderate relationship for the unfractured bones, and weak relationships for both fracture conditions (Fig. 8).

4. Discussion

Changes occur to the physicochemical composition of bone in the post-mortem period [36,11,37,47,31,25]. The study presented here focused on the physicochemical changes occurring to surface-deposited bone over time using non-destructive analytical techniques and considered bones that had been fractured. Over the course of this 240-day study, the physicochemical composition of the bone samples changed significantly showing that physicochemical changes to bone can be quantified within this timescale.

The three groups (unfractured, blunt-force-, sharp-force fractured) were differently affected in their elemental composition over time. Statistical analysis suggests that samples with blunt-force fractures are not as susceptible to elemental change over time as their sharp-force fractured and unfractured counterparts. The lack of statistically significant changes from the blunt-force fractured samples may indicate that the presence of extensive fractures (Fig. 2**B**; Fig. 3**B**) can limit dehydration of the bone, perhaps due to water having easier access to the inner cortex of the bone through fracturing sites.

4.1. Changes observed in Na, K, and Mg

Decreases in Na and K levels were seen throughout the timeframes studied, and this occurred whether the samples were fractured or not. However, the unfractured samples exhibited unusual increases in these elements at 90 days PMI after initially displaying losses at 30 days PMI. It is unclear why this increase occurred at 90 days PMI, but it is interesting that the blunt-force fractured samples did not show the same pattern. Similar changes were observed by Végh et al. [31], who noted an initial decrease in K in the first 14 days, followed by fluctuations thereafter. Na and K have been associated with the moisture content of the bone, and it is possible these samples did not lose as much moisture due to their placement within the deposition site as some areas were more prone to water build up than others. The blunt-force- and

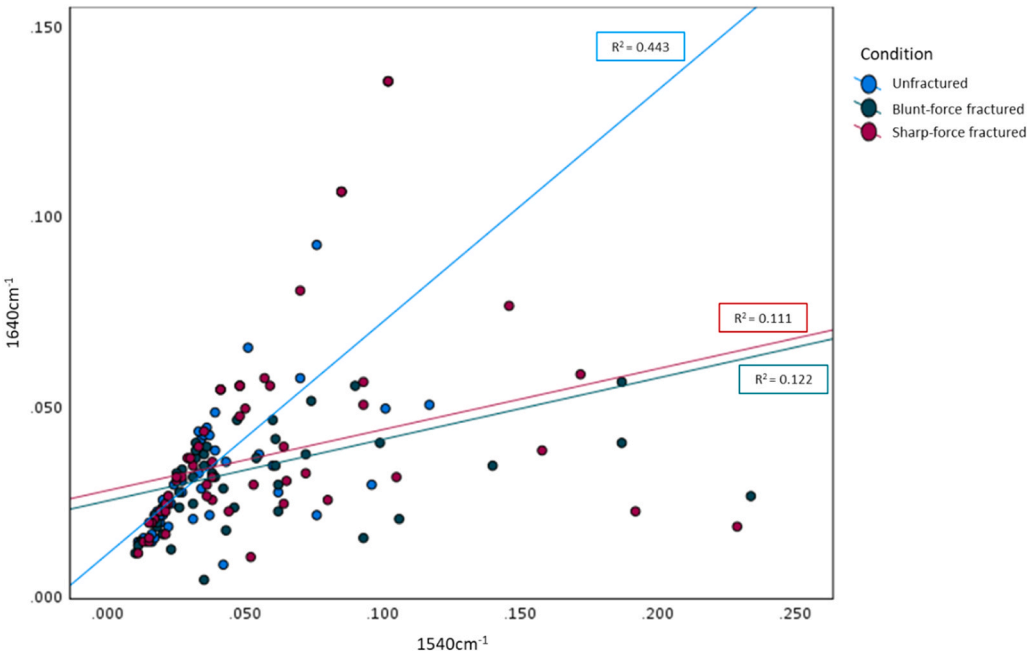

Fig. 8. Scatter plots to show the relationship between the absorbance heights at 1540cm-1 and 1640cm-1 for all samples.

sharp-force fractured samples showed decreases in these two elements, however while changes in Na content were significant for the unfractured and sharp-force fractured samples, they were not for blunt-force fractured samples. The overall loss of Na and K corroborate the results of Végh et al. [31] and Walden et al. [25]. As these elements are associated with extracellular fluids, a decrease would indicate dehydration of the bone occurring over time [25].

Decreases in Na in buried samples was also observed by Keenan and Engel [36], and other studies have found much lower levels of Na in archaeological bone compared to fresh, modern bone [69]. Na and K ions have also been extracted from soil water beneath skeletonised remains indicating they are lost from decomposing remains over time [70]. Post-hoc tests indicate it took 150 days for the Na content to be significantly lost in the unfractured and sharp-force fractured samples. All three groups had significantly changed K levels over time, and post-hoc tests showed that this element was significantly decreased within the first 30 days of the study in all sample groups. The unfractured samples showed a similar pattern of change as the sharp-force fractured samples (Fig. 5), although the sharp-force fractured samples maintained higher levels of Mg compared to the unfractured samples throughout the study. The correlation analysis showed negative, moderate, and significant relationships between time and the loss of Na and K for both the unfractured and sharp-force fractured samples, but no significant relationship was noted for the blunt-force fractured samples. From observing the unfractured and sharp-force fractured samples, this data could indicate there is potential to use the analysis of Na and K as a parameter for PMI estimation as these elements decreased within 30 days PMI, which supports the conclusions made by Walden et al. [25]. As suggested above, it is theorised that extensive fractures or damage to bone early in the post-mortem period could inhibit dehydration and influence elemental change. More research is needed to fully understand this.

The Mg content decreased throughout the study for the unfractured and sharp-force fractured samples, although increases were observed at 90 days PMI in these samples. The unfractured samples showed unusually high Mg levels at this timeframe, which was not consistent with other timeframes measured. The cause of these increases is unclear, but it has been suggested that Mg could leach into the mineral matrix from soil [25]. Piga et al. [71] has suggested that Mg can replace Ca, although this is usually noted by the presence of a peak at 1123cm-1 and a shoulder at 547cm-1. These features were not observed in the FTIR-ATR spectra (Fig. 6), but for the SEM-EDS data, the increase in Mg does appear to coincide with slight decreases in Ca in the unfractured and sharp-force fractured samples (Fig. 5**C; H**). As with the Na content, the loss in Mg was significant in the unfractured and sharp-force fractured samples, but not in the blunt-force fractured samples. Mg content fluctuated throughout the study in the blunt-force fractured samples, which may account for the lack of significant changes observed. Fluctuations in Mg content were also seen by Walden et al. [25], although they do not mention any change in Mg content by the end of their study. Contrasting literature can be found for Mg content changes, with research in support of overall Mg increases occurring over time [31], and in support of decreases occurring over time [33], although it has to be noted that these decreases occurred in bone fragments from moist sediments over a period of 2.5 years. Post-hoc tests did not show the Mg changes to be significant in the unfractured samples between fresh bone and any PMI investigated, although this element significantly decreased by 150 days in the sharp-force fractured samples. Correlation analysis showed a negative, moderate and significant relationship between Mg content changes and time for both the unfractured and sharp-force fractured samples, which contrasts with the literature which reports a moderate, nonlinear but significant increase in Mg content over time [31]. As a trace element found in soil, Mg can be incorporated into the bone during diagenesis [31]; contradicting results could be due to the soil composition, however as no soil analysis was conducted, this could not be corroborated. The presence of Mg ions have been found in soil water collected from beneath skeletonised remains, indicating Mg can be lost from the body as a by-product of decomposition and bone breakdown [70]. These results indicate that assessing Mg in bone would be of limited use forensically, which supports conclusions drawn by Walden et al. [25], based on their experiments using intact bone.

4.2. *Changes observed in Ca, P, Fe, Ba, and Zn*

All three bone condition groups showed fluctuations in Ca, P, Fe, Ba and Zn throughout the timeframes studied. However, none of these elements were significantly changed in the unfractured samples, although Ca and P levels did appear to increase slightly, and a small decrease in Zn

and Ba levels was observed by the end of the study. Despite fluctuations throughout in the unfractured samples, Fe did not show any differences in content compared to fresh bone by 240 days PMI. The Ba content was significantly changed over time in the blunt-force fractured samples, although the other remaining elements did not show any significant differences over time. While the Ba content did fluctuate in this group, it was significantly lower by 240 days PMI compared to fresh bone, unlike the sharp-force fractured samples that did not show any difference in Ba content by the end of the study, despite observed fluctuations. Fluctuations in Ba content were observed by Nagai et al. [37], although at different timescales than was seen here, with slight non-significant increases in Ba occurring at 6 months PMI followed by significant decreases by 12 months PMI. Krajcarz [33] saw decreases in Ba in bone fragments left in dry sediments. Walden et al. [25] also observed changes to Ba content over time but found the levels to be negligible. Post-hoc tests showed the Ba content was significantly changed in the blunt-force fractured samples by 30 days PMI, but correlation analysis only showed a weak and non-significant relationship between Ba and time due to the fluctuations observed at the other timeframes measured.

Although Fe was not changed by the end of the study in the unfractured samples, or in the blunt-force fractured samples, fluctuations did occur at various PMIs. Fe levels decreased until 90 days PMI before increasing in the sharp-force fractured samples, resulting in a non-significant increase over time, and correlation analysis showed a weak but significant relationship between Fe and time. The two fracture groups appeared to follow a similar pattern of change for Fe content until 150 days when the sharp-force fractured samples started to show larger increases (Fig. 5). The contrasting results of Fe changes was interesting as contrasting data is available for changes to Fe content in post-mortem bone; Walden et al. [25] and Végh et al. [31] observed decreases in Fe content after an initial increase in the first instance, while Keenan and Engel [36] discuss non-linear increases in Fe content. Végh et al. [31] does mention not observing any significant changes in Fe associated with increasing PMIs, despite observing a decrease over time. The results presented here appear to disagree with the literature [36,31,25], although it may be the first PMI measured was too long to observe any change (30 days PMI). Walden et al. [25] saw an initial increase in Fe during the early stages of decomposition (between 0 and 28 days PMI), and Végh et al. [31] observed their increase in Fe at 14 days PMI.

Zn fluctuated in all three groups, and showed a small, non-significant decrease by 240 days PMI in the unfractured samples, while the two fractured groups did not show any change in Zn content by the end of the study. The unfractured and blunt-force fractured samples showed similar patterns of change in Zn content over time, although the blunt-force fractured samples had slightly higher levels of Ba by the end of the study (Fig. 5). Literature shows Zn content in post-mortem bone can fluctuate slightly [25], as seen here, possibly due to it's natural presence as a trace element in both bone and soil [72].

Ca and P levels underwent small, non-significant increases by 240 days PMI in the unfractured samples. Despite fluctuations in Ca and P at various PMIs, no change was observed in Ca content by the end of the study for the blunt-force fractured samples, but an increase in P content was observed by 240 days PMI. The sharp-force fractured samples, while also showing fluctuating levels for both elements, did not show any change in P content by the end of the study, but did show a significant increase in Ca by 240 days PMI. The literature shows varying results for Ca content changes with insignificant changes in Ca content occurring over time in the some studies [25,31], and non-linear increases in Ca content being seen in others [33,36]. Fluctuations in P, as noted here, have been observed previously [25]. Post-hoc testing did not show the Ca content to be significantly changed at any PMI investigated when compared with fresh bone levels, however, and correlation analysis only found a weak, non-significant relationships between Ca and time. The fluctuations in P content appear to have been very similar across all three groups (Fig. 5), while the Ca fluctuations were very similar in the unfractured and sharp-force fractured groups.

4.3. Changes observed in the structural composition

Observing the structural changes of the three groups over time suggested that samples exhibiting blunt-force fractures may not be as susceptible to structural changes as unfractured bones, or even those with sharp-force fractures, despite the extensive damage caused by blunt-force trauma. All five IR parameters were changed significantly for the unfractured and sharp-force fractured samples, but only three IR parameters were significantly changed in the blunt-force fractured samples.

Crystallinity within the bones increased throughout this study, regardless of the presence of fractures. Crystallinity increases indicate changes to the size and order of the crystals within the lattice structure [50,40,41,31], suggesting reorganisation of the mineral lattice is occurring. This has been found to occur within 12 months PMI [31]. It appeared that samples exhibiting blunt-force fractures were not as susceptible to crystallinity changes when compared with unfractured samples, or those with sharp-force fractures. While the unfractured and sharp-force fractures showed significant increases in crystallinity, this was not the case for the blunt-force fractured samples, which saw non-significant increases. Significant increases in crystallinity were seen by 90 days PMI. However, correlation analysis showed a positive, moderate and significant relationship between crystallinity and time for all three groups.

The carbonate content of the bones was observed to change over time. Changes to the type A and type B carbonate bands can indicate substitutions of CO_3^{2-} have occurred to the hydroxyapatite structure, with type A substitutions occurring at the OH^- site and type B substitutions occurring at the PO_4^{3-} site [43,48,38,44,32]. Fluctuations were seen in the API for all three groups and alongside these the BPI was also significantly increased. Post-hoc tests showed the BPI was significantly altered by 30 days PMI compared to fresh bone in all three groups. Correlation analysis showed a negative, moderate and significant relationship between API and time for the unfractured and sharp-force fractured samples, but not for the blunt-force fractured samples. The BPI did not show any significant relationship with time for any group. Changes to the carbonate-phosphate ratios can occur due to a number of reasons; increases/decreases in carbonate content due to substitutions, increases/decreased in phosphate, loss in the organic material [31,36]. It has been suggested that decreases in API can occur due the A site carbonates being displaced to the B sites during diagenetically induced dissolution [62], however this does not appear to have happened here. If the A site carbonates were being transferred to the B carbonate site, then a decrease in the BAI should also occur. This was not the case in this study. Instead, the BAI increased over time for all three groups. Increases in BAI were observed for all three groups until 240 days PMI when decreases were seen; this decrease was larger in the blunt-force fractured samples. These changes suggest the API is also affected by phosphate changes. Post-hoc analysis showed significant changes in BAI by 90 days PMI for the blunt-force fractured samples, and 150 days PMI for the sharp-force fractured samples. Correlation tests found a positive, moderate and significant relationship between the BAI and time for all three groups.

Analysis of the linear relationships between the amide I peak at 1640 cm^{-1} and the peak at 1540 cm^{-1} for the three conditions (unfractured, blunt-force, sharp-force fractured) showed a moderate relationship for the unfractured bones and weak relationships for both fracture conditions (Fig. 8). A strong correlation between these two peaks could suggest that the changes seen in API and BAI were potentially a result of changes in amide rather than changes in type A carbonate.

Using the large amide peak at 1635^{-1} and dividing it by the phosphate band at 1010 cm^{-1} [47], the organic content of the bone was assessed. As decreases in the organic content are thought to correspond

with the loss of proteins, this measurement can give valuable data about the condition of the bone [48], and thus give information about the survival of collagen within the bone [47]. The Am/P decreased over time in all three groups. This loss appeared to stabilise after 90 days PMI in the unfractured samples, but it didn't stabilise in either fracture group until 150 days PMI. Decreases in Am/P have been shown to occur within short timescales (<3 years) [48,36,31], longer timescales (>19 years) [47] and within archaeological samples [46,49]. Contrasting results were seen for the Am/P changes at 30 days PMI, there was only a negligible change in the unfractured samples, while the blunt-force fractured samples suffered their biggest protein loss at this timescale, and the sharp-force fractured samples showed an increase. Large Am/P decreases in the unfractured and sharp-force fractured samples occurred at 90 days PMI to bring their protein levels inline with the blunt-force fractured samples at this timescale. Post-hoc tests showed the Am/P was significantly decreased by 150 days PMI in all three groups, and correlation analysis showed a negative, moderate and significant relationship between Am/P and time. The same significant correlations between protein loss and time have been found in previous studies, also using pig bones [48], but a study utilising human samples did not show these correlations [66]. It has been found that taphonomic research using human subjects can show different results to animal studies due to the increased inter-variability within humans as a result of differing diets, geographical locations, general health [21,73].

Studies have discussed significant relationships between crystallinity and Am/P with research showing losses in organic content as crystallinity increases [34,46,47]. Correlation analysis showed a negative moderate and significant relationship between IRSF and Am/P for the unfractured samples, indicating there is a potential relationship between the two IR parameters. However, only a weak and non-significant relationship could be found between the IRSF and Am/P for the fracture groups.

5. Conclusion

The research presented here has focused on the potential for bone fractures to affect physicochemical changes that can occur to bone over forensic timescales. Due to the ethical and moral considerations of undertaking this type of research, particularly for the use of human remains or archaeological samples, the use of non-destructive techniques was a focus of this study, which preserved the integrity of the original samples. The development and promotion of non-destructive techniques is important within forensic science; the techniques used here allow for tests to be replicated, even on the exact same section of bone. The method applied SEM-EDS and FTIR-ATR as both techniques are easy to use, provide fast results, and are typically available in most forensic science or analytical laboratories enabling wider applicability of this approach. FTIR-ATR can provide an array of information about the structural composition of the mineral lattice, covering both the organic and inorganic components, while SEM-EDS provides quantitative elemental analysis. Both techniques were shown to be able to quantify physicochemical changes occurring to bone in < 240 days PMI. These techniques show promise as preliminary assessment tools to help inform practice when deciding which destructive analyses to apply to skeletal samples. More research needs to be conducted utilising these techniques with samples from different depositional contexts, and particularly with human skeletal remains.

Trauma is increasingly seen in medicolegal contexts. While research has been undertaken on the potential influence of soft tissue trauma on early decomposition, no previous research has considered how bone fractures could influence diagenetic change. This study showed that physicochemical changes occur over increasing PMIs with losses in various elements and protein content as well as changes to the carbonate content and crystallinity of the bone in all three groups. This agreed with the literature assessing diagenetic changes of animal bones without evidence of trauma [50,31,25]. In particular, the loss of Na, K, and Mg was observed, as well as an increase in crystallinity. The presence of bone fractures was found to affect the rate at which these physicochemical changes occur. Interestingly, there were many similarities in the pattern of elemental change between the unfractured and sharp-force fractured samples. This could indicate that the presence of fractures caused by blunt-force trauma may inhibit physicochemical changes, although why this occurs is unclear and needs investigating further.

Na and K showed the potential to be used as markers for PMI estimation, however these elements were also affected by the presence of bone fractures. This indicates that the presence of bone fractures could influence the rate at which bone diagenesis occurs and impact the accuracy of any PMI estimation. This is an under researched area of forensic research that requires more study if the full influence of bone fractures on diagenetic change are to be fully understood.

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CRediT authorship contribution statement

C. Mein: Writing – review & editing, Writing – original draft, Investigation, Visualization, Formal analysis, Methodology, Conceptualization. **J.R. Jones:** Writing – review & editing, Methodology, Resources, Supervision, Conceptualization. **C. Tennick:** Writing – review & editing, Resources, Conceptualization, Supervision, Methodology. **A. Williams:** Methodology, Supervision, Project administration, Conceptualization, Writing – review & editing, Resources, Funding acquisition.

Declaration of Competing Interest

The authors declare no conflicts of interest

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Data availability statement

The data related to this article is available upon request to the corresponding author.

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