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Peptide analysis of mammalian decomposition fluid in relation to the post-mortem interval

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ABSTRACT

We report the results of a semi-quantitative peptide analysis of decomposition fluid under field-based conditions in the absence of a soil matrix. Sixteen domestic pig (*Sus scrofa domesticus*) cadavers were used to model human decomposition in trials conducted in the summer and winter months in Western Australia. Physical characteristics were recorded and targeted peptide components of decomposition fluid were analysed using high performance liquid chromatography-triple quadrupole mass spectrometry. Principal component analysis identified 29 peptides, originating from haemoglobin subunits alpha and beta, creatine kinase, beta-enolase and lactate dehydrogenase, that contributed to differences in the mean peak areas of samples collected during the early period of decomposition (days 6–12 and day 2 in winter and summer, respectively) and during the later period (days 24–34 and days 8–10 in winter and summer, respectively). Fold changes for 8 peptides between these periods were significantly different. Three peptides derived from haemoglobin subunit beta, one from beta-enolase and two from lactate dehydrogenase displayed consistent trends, in that a notable increase in mean peak area was followed by a marked decrease in both the summer and winter samples. When temperature was accounted for, these trends occurred at different time points in summer and winter, indicating that factors other than temperature had impacted the rate of degradation of the proteins involved. The single peptides derived from haemoglobin subunit alpha and creatine kinase displayed consistent increases in mean peak area for the summer samples, suggesting that temperature played the most significant role in their degradation. Further analyses revealed that 7 peptides (one originating from haemoglobin subunit alpha, three from haemoglobin subunit beta and three from lactate dehydrogenase) displayed consistent trends that could be correlated with total body score and with the early stages of decomposition. The consistent trends (mean peak area versus time) for peptides derived from several proteins during decomposition trials conducted under different temperature regimes further emphasised the potential of peptide analysis in time since death estimation.

1. Introduction

Estimating time since death, or post-mortem interval (PMI), is fundamental in forensic investigations when reconstructing events surrounding suspicious deaths. An accurate PMI estimation can contribute significantly to such investigations by establishing a timeframe to which efforts and resources can be directed [1]. The decomposition process is highly variable and complex, making the determination of time since death difficult to estimate. Factors such as temperature [1–3], humidity and rainfall [4,5], trauma [6], insect activity [7,8], body size [9] and clothing [7] are all known to impact decomposition. Current methods to estimate PMI include visualisation of macroscopic changes [10–13], forensic entomology [14,15] and morphoscopic changes [7,16]; methods that are not without limitations. More recently, investigations into the biochemical products produced during decomposition have been undertaken. In contrast to more traditional methods, using biochemical biomarkers eliminates the potential for examiner bias. Additionally, biomarkers are known to remain biologically active and detectable for extended periods of time, making them suitable for PMI estimations [17–21]. Although the majority of techniques explored have displayed encouraging results, their current application to PMI estimations is limited [17–26].

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However, it is suggested that of the current biochemical approaches investigated, measurement of the rate and extent of protein degradation is a promising candidate [17–19,25,27,28]. Pittner et al. demonstrated that the degradation of several skeletal muscle proteins occurred at different time points during the decomposition process, allowing for more precise estimations of PMI during the intermediate and late postmortem stages [25]. Although promising, these studies were limited by the proteomic techniques applied and by the use of muscle tissue, restricting estimations of time since death to 10 days post-mortem [17,25]. In contrast, Procopio et al. used LC–MS/MS analysis to identify several protein biomarkers in bone samples collected over a six-month period. The proteins identified were found to correlate with PMI in that they decreased in concentration in bone as time progressed [27,28].

In previous studies, we have also highlighted the potential of peptide biomarkers for use in PMI estimation [18,19]. In both studies we utilised high performance liquid chromatography-time of flight mass spectrometry to analyse the peptide profile of decomposition fluid samples collected from trials conducted under different temperature regimes. Our preliminary study, conducted under laboratory-based conditions, identified 27 peptides that were consistently generated throughout the decomposition process. These peptides were derived from haemoglobin subunits alpha and beta, beta-enolase, creatine kinase and pyruvate kinase. In addition to this, the peptides associated with the haemoglobin subunits displayed breakdown patterns that remained consistent across multiple replicates [18]. Following these findings, a second study investigating the impact of environmental factors on the peptide profile of decomposition fluid was undertaken. In this study, 37 peptides were consistently detected in both the summer and winter trials; 22 originating from haemoglobin subunit beta, 1 from haemoglobin subunit alpha, 4 from beta-enolase, and 2 from creatine kinase. Most importantly, 13 of these peptides were consistently detected in all trials, regardless of trial conditions. In addition, the degradation patterns for haemoglobin subunits alpha and beta were not only consistent with those found in the preliminary study, but were similar when expressed in accumulated degree days (ADD) in the summer and winter trials [19]. The consistent identification of these protein-specific peptides during decomposition trials conducted under different temperature and rainfall regimes, suggests that peptide analysis could be a valuable indicator of time since death. This study applies a semi-quantitative approach to further evaluate the efficacy of the protein-specific peptides identified in our previous work.

2. Materials and methods

2.1. Experimental design

The full details of the experimental protocols were as described in Nolan et al. (2019) [19]. Decomposition studies were carried out at a bushland site located at Murdoch University, Western Australia. Studies were conducted within a pre-existing enclosed caged structure, to restrict access to the carcasses by vertebrate scavengers. The structure also provided shelter from the rain to minimise sample dilution and afforded protection from direct sunlight so as to minimise evaporation. It is important to note that cages were not fully enclosed so as to avoid the generation of a microclimate. A data logger (EL-USB-2+) was used to record and monitor temperature and humidity every 10 min for the duration of the study; this was suspended above the carcasses and measured humidity and ambient temperature. Trials concluded when carcasses reached complete mummification (inclusive of internal organs and skin).

Domestic pigs (*Sus scrofa domesticus)* were used to model the decomposition process. Eight sub-adult female pigs (mean weight 19.65 ± 2.16 kg) were euthanised by bolt gun to the temple 2 h prior to commencing each study. The study was approved by the Murdoch University Animal Ethics Committee (Cadaver approval number: 376).

Each carcass was placed on a raised, galvanised metal platform, exposed to insect activity. Openings in the platform enabled the fluid to drain away from the body into a plastic collection container. These containers were monitored daily and fluid samples were collected, when available, between 0800 and 0900 for the first two weeks and, thereafter, on every second day until the conclusion of each study (mummification stage). Five $\times$ 2 mL fluid samples were collected from each container and stored at $-80\,^{\circ}$C until analysed. Any remaining fluid not sampled was discarded and the containers were rinsed thoroughly with water before being replaced beneath each carcass.

Elapsed time was calculated in accumulated degree days (ADD) to account for fluctuations in temperature [16]. Observations of physical appearance, including skin discolouration, bloating and deflation, skin ruptures, hair loss and bone exposure were recorded on each sampling day and were documented, photographically. The stage of decomposition was assessed and evaluated using the Total Body Score (TBS) method developed by Keough et al. (2017) [29].

2.2. Sample preparation and analysis

Prior to preparation, the protein concentration of each crude sample was determined using the Nanodrop One method (Thermo Fisher Scientific) by measuring the absorbance of each at 260 and 280 nm. Preparation of the samples for peptide analysis was as described in Nolan et al. (2019) [18]. Because sufficient decomposition fluid (>2 mL) was not always generated on a daily basis, samples were collected every second day for analysis.

Samples for Multiple Reaction Monitoring (MRM) were loaded onto an Eksigent ChromXP C18 column, 3 μm particle, 120 Å pore size $\times$ 50 mm (AB Sciex) and separated using a linear gradient of 10–40% acetonitrile in 0.1% (v/v) formic acid at 5 μL/min over a 20–min runtime on a Prominence HPLC (Shimadzu) coupled to a 5500 Q-TRAP mass spectrometer (AB Sciex). MRM transitions for peptides were created based on the initial discovery data described in Nolan et al. (2019) [23]. There were 36 peptides derived from 6 proteins with a total of 110 scheduled transitions (90 s window).

2.3. Data processing and statistical analysis

Analysis of MRM data was performed using Skyline (v19.1 MacCoss Lab Software) [30]. Manual inspection of peaks was carried out to reduce the likelihood of peaks being misallocated thus generating false positives. Identification of MRM peaks was achieved by comparing MS and retention times with reference compounds detected in our previous study [19]. Peak areas were normalised by correcting for the variable protein concentration of original samples using Microsoft Excel 2019 (v1907).

Multivariate analysis was performed, by importing the data into The Unscrambler X (v10.3, CAMO, Ausland Norway). Spectral data were log transformed and mean centred. Principal component analysis (PCA) was performed using the NIPALS algorithm in order to visualise and interpret spectral groupings present. The peptides that contributed most substantially to the variance observed between early and later post-mortem samples were identified from the X-loadings data. Two-sample t-tests assuming unequal variances were used to determine the significance of mean peak area in early and later decomposition fluid samples. Differences in mean peak area were regarded as statistically significant if $P < 0.05$.

Fold changes in mean peak area were calculated using normalised peak areas.

3. Results and discussion

3.1. Trial observations

3.1.1. Summer trial

The first trial was performed during summer (January-February 2018). The mean daily temperature recorded over the entire collection period was 23.67 ± 5.07 °C. Average daily temperatures ranged from 20.75–27.50 °C, with a maximum of 36.50 and a minimum of 15.50 °C recorded during the monitoring period. An overall relative humidity of 56.98 ± 16.24% was recorded, with average daily humidity ranging from 37.83 to 71.75% (Fig. 1). At the conclusion of the trial, cadavers exhibited mummification of the head, neck, trunk and limbs; skin was brown and leathery in appearance and was adhered to the bone. An average total body score of 24 was recorded. Decomposition fluid was collected, when available, from the eight domestic pig cadavers. Fluid was collected from cadavers 1, 3, 7 and 8 until day 7 (ADD 175), from cadavers 2 and 5 until day 9 (ADD 218), and cadavers 4 and 6 until day 10 (ADD 240).

3.1.2. Winter trial

The second trial was conducted in winter/spring (July-November 2018). The mean daily temperature over the entire collection period was 12.61 ± 4.83 °C. Average daily temperatures ranged from 8.40–19.58 °C, with a maximum of 29.00 and a minimum of 3.00 °C recorded during the monitoring period. The overall relative humidity was 83.92 ± 15.91%, with average daily humidity ranging from 57.88 to 94.12%. A total of 179 mm of rain was registered during the monitoring period. (Fig. 2). At the conclusion of the trial, cadavers exhibited mummification of the head, neck, trunk and limbs. Skin was black, brown in appearance and dry to the touch (hair was still present). A small amount of bone exposure was seen in the face and limbs. An average total body score of 24 was recorded. Decomposition fluid was collected, when available, from eight domestic pig cadavers. Fluid was collected from cadaver 7 until day 22 (ADD 274), cadaver 2 until day 26 (ADD 327), cadavers 1, 3 and 5 until day 30 (ADD 385), cadaver 6 until day 32 (ADD 406), and cadavers 4 and 8 until day 34 (ADD 429). Throughout the trial, decomposition fluid samples recovered from cadavers 4, 5 and 6 were heavily diluted by rain due to cadaver positioning within the caged structure. All samples collected from these cadavers, were therefore excluded from any analyses.

3.2. Targeted peptides

Our previous studies identified several protein-specific peptides that exhibited potential in estimating the post-mortem interval. Twenty-five peptides were selected for quantification based on their consistent identification in decomposition trials conducted under different temperature regimes [18,19]. This commonality, together with the identification of peptides that appeared at later periods of the decomposition process, resulted in an additional 9 peptides being selected for analysis (Table 1) [19].

3.3. Principal component analysis

Prior to principal component analysis, samples were assigned to one of three groups: early, intermediate and late as defined in Table 2. Samples were assigned to the groups so as to align with the stages of decomposition described by Keough et al. [29]. The early group, corresponded to the initial period of the "early decomposition "stage (mean TBS = < 10); the intermediate group aligned with the concluding period of the "early decomposition" stage (mean TBS = 10–16), while the late group equated to the "advanced decomposition" stage (mean TBS = 20+) as defined by these authors [29].

3.3.1. Winter trial

Principal component analysis of mean peak areas of peptides that were consistently identified in the winter trial showed a clear distinction between samples collected on days 6–12 and days 24–34, with 48% of the variance between the two groups explained by PC-1 (Fig. 3). Of the thirty-five peptides targeted, twenty-two peptides, identified by PCA loadings (X-loading > 0.1), were found to be the major contributors to the variance observed between days 6–12 and days 24–34. The fold change in mean peak area for each of these peptides between the samples collected in the early (days 6–12) and later (days 24–34) periods of the trial is shown in Table 3. Fold change for a further 2 peptides was unable to be calculated due to the absence of data on days 6–12.

Of the 22 peptides analysed, 7 were found to be significantly different in mean peak area between samples collected on days 6–12 and days 24–34. Peptides derived from haemoglobin subunit beta showed significant increases (all $p < 0.05$) of 13.3-, 4.6- and 2.7-fold for ESFGDLSNADAVMGNPK, FGDLSNADAVMGNPK and

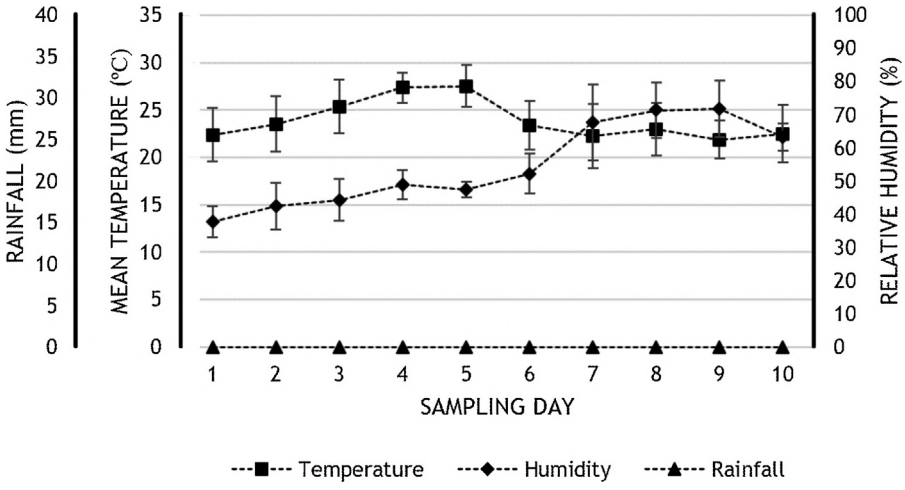

Fig. 1. Mean daily temperatures (squares), mean daily relative humidity (diamonds) recorded and daily rainfall (triangles) in summer trial.

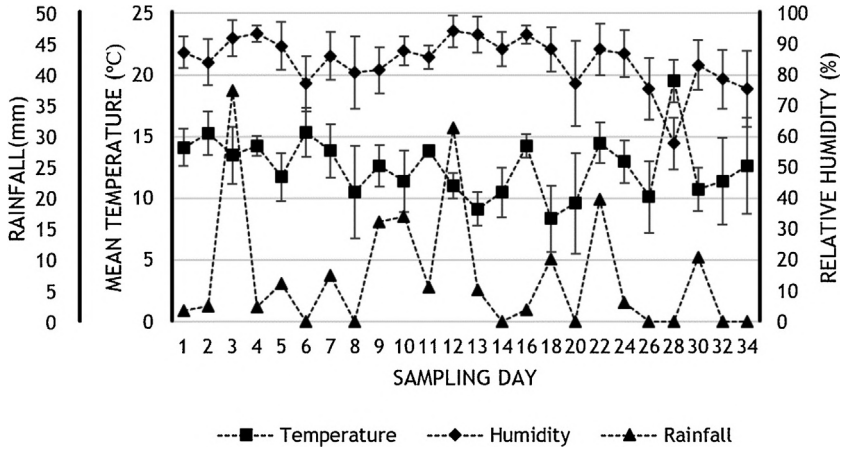

Fig. 2. Mean daily temperatures (squares), mean daily relative humidity (diamonds) recorded and daily rainfall (triangles) in winter trial.

Table 1
Peptides selected for data processing and statistical analysis based on detection in previous studies [18,19].

Protein	Peptide sequence
Haemoglobin subunit alpha	AAWGKVGGQAGAHGAEALER
	AVGHLDDLPGAL
	FLGFPTTKT
	GHLDDLPGAL
	LSHGSDQVKAHGQKVADALTK
	VGGQAGAHGAEAL
	VGGQAGAHGAEALERM
	VGHLDDLPGAL
	VLSAADKANVKAAWGK
Haemoglobin subunit beta	ESFGDLSNADAVMGNPK
	ESFGDLSNADAVMGNPKVK
	FGDLSNADAVMGNPK
	FGDLSNADAVMGNPKVK
	GDLSNADAVMGNPKVK
	GKVNVDEVGGEALGRL
	KVNVDEVGGEALGRL
	NVDEVGGEALGRL
	SFGDLSNADAVMGNPK
	VNVDEVGGEALGRL
Creatine kinase	DLFDPIIQDR
	KDLFDPIIQDR
Beta-enolase	IQIVGDDLTVTNPK
	IVGDDLTVTNPK
	IVGDDLTVTNPKR
	SGVNIQIVGDDLTVTNPKR
Lactate dehydrogenase	**A**TLKDQLIH
	ATLKDQLIHN
	ATLKDQLIHNL
	ATLKDQLIHNLLK
	DLQHGSLF
	ILGQNGISDVVKV
	KNLHPELGTDADKEHWK
	KVTLTPEEEAHLKK
	NLHPELGTDADKEHWK

Table 2
Number of samples assigned to principal component analysis groups early, intermediate and late.

Trial	Group assigned	Days collected	No. of samples
Winter	Early	6–12	16
	Intermediate	14–22	15
	Late	24–34	12
Summer	Early	2	8
	Intermediate	4–6	16
	Late	8–10	5

GDLSNADAVMGNPKVK, respectively. A 109.4-fold increase ($p < 0.05$) was also observed for KDLFDPIIQDR, a peptide derived from creatine kinase. Peptides derived from lactate dehydrogenase also displayed significant increases. These included DLQHGSLF (14.1-fold, $p < 0.05$), ILGQNGISDVVKV (142.7-fold, $p < 0.01$) and NLHPELGTDADKEHWK (1.7-fold, $p < 0.01$). PCA loadings suggest that these 7 peptides were largely responsible for the separation observed between the decomposition samples collected on days 6–12 and days 24–34.

Principal component analysis of mean peak areas of the peptides consistently identified in the winter trial did not show a clear trend over the duration of the trial (Fig. S1). This may have been due to the small sample size used and future studies, employing larger numbers, should be conducted to investigate this further. Because no overall trend was apparent, no further analyses of samples collected between days 12 and 24 were undertaken.

Due to the larger data set, samples collected during the winter trial could be used to determine if a clear distinction existed between the peptides produced in samples collected on days 6–12 and days 24–34 of the decomposition trial. As decomposition occurs at a much slower rate in the winter months, it was possible to conduct a more thorough interpretation of the data over a longer time-frame than in summer. Due to the promising results derived from the analysis of the winter data, the summer data was subsequently analysed, taking into account its compressed time-frame.

3.3.2. Summer trial

Principal component analysis of mean peak areas of peptides from the summer trial samples showed a difference between samples collected on day 2 and days 8–10, with 46% of the variance between the two groups explained by PC-1 (Fig. 4). Twenty peptides identified by PCA loadings (X-loading > 0.1) were found to be largely responsible for the variance observed between the samples collected on day 2 and days 8–10. Four of these peptides differed from those identified in the winter samples.

As with the winter samples, fold changes between samples collected in the early (day 2) and later (days 8–10) periods of the trial were compared (Table 4). Of the 20 peptides analysed, 6 were found to have significant differences in mean peak area, 2 of which were also significant in the winter trial. Only one peptide derived from haemoglobin, GHLDDLPGAL, showed a significant increase in mean peak area (5.2-fold; $p < 0.01$). Peptide KDLFDPIIQDR, derived from creatine kinase, was found to increase 9.1-fold ($p < 0.05$) between days 2 and 8–10, but this increase was much less than that observed in winter (109.4-fold). Peptides derived from

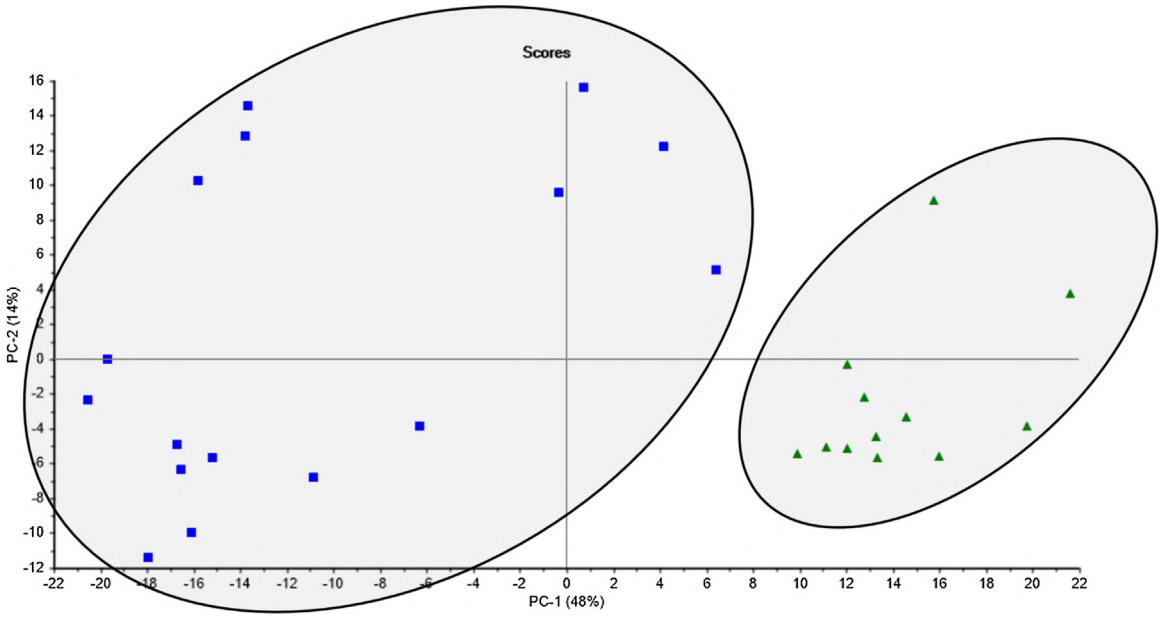

Fig. 3. Scores plot from principal component analysis of winter samples collected on days 6–12 (squares) (n = 16) and on days 24–34 (triangles) (n = 12).

beta-enolase displayed significant increases: IVGDDLTVTNPK (5.3-fold, $p < 0.01$); IVGDDLTVTNPKR (7.8-fold, $p < 0.05$) and SGVNIQIVGDDLTVTNPKR (7.7-fold, $p < 0.05$). In comparison to the winter trial, peptide DLQHGSLF, derived from lactate dehydrogenase, displayed a similar significant increase in mean peak area, (3.4-fold; $p < 0.05$). PCA loadings suggest that these 6 peptides were largely responsible for the separation observed between the samples collected on day 2 and days 8–10 in the summer trial. Of the 16 peptides found to be common to the winter and summer trials, 8 displayed the same trend in both winter and summer, but only 2 of these peptides changed significantly in concentration in both data sets. The incomplete correspondence between summer and winter may reflect the small sample size employed and needs to be clarified in future studies using a larger number of samples.

As was the case for the winter trial, principal component analysis of mean peak areas of peptides consistently identified in summer did not show a clear trend over the duration of the trial (Fig. S2). Therefore, no further analyses of samples collected between days 4 and 6 were undertaken.

Table 3
Fold change in mean peak area of peptides from samples collected in the early (days 6–12) and later (days 24–34) periods of the winter trial. + indicates a fold increase in mean peak area between samples collected on days 6–12 and days 24–34; – indicates a decrease. Significantly different mean peak areas between early (days 6–12) and later (days 24–34) sampling periods are shown by *P < 0.05 and **P < 0.01. Δ indicates the absence of a peptide in samples collected on days 6–12 or days 24–34, and as a consequence, fold change could not be calculated.

Protein	Peptide		Fold Change
Haemoglobin subunit alpha	GHLDDLPGAL	–	1.482241712
	VGGQAGAHGAEALERM	–	1.795032199
Haemoglobin subunit beta	ESFGDLSNADAVMGNPK	+	13.35789474*
	ESFGDLSNADAVMGNPKVK	+	2.079155673
	FGDLSNADAVMGNPK	+	4.561967879*
	FGDLSNADAVMGNPKVK	–	1.238440617
	GDLSNADAVMGNPKVK	+	2.714383328*
	GKVNVDEVGGEALGRL	+	2.431852523
	KVNVDEVGGEALGRL	–	2.472369743
	SFGDLSNADAVMGNPK	+	6.68976558
Creatine kinase	DLFDPIIQDR	+	2.202801927
	KDLFDPIIQDR	+	109.3984772*
Beta-enolase	IVGDDLTVTNPK	+	1.128819187
	IVGDDLTVTNPKR		Δ
	SGVNIQIVGDDLTVTNPKR		Δ
	IQIVGDDLTVTNPK	+	1.099010521
Lactate dehydrogenase	**A**TLKDQLIHNL	–	4.473205458
	ATLKDQLIH	–	1.661601101
	DLQHGSLF	+	14.11788618*
	ILGQNGISDVVKV	+	142.7222222**
	KVTLTPEEEAHLKK	–	1.385890895
	NLHPELGTDADKEHWK	+	1.651329243**

3.4. Analysis of protein-specific peptides and their changes in relation to time

To identify common trends in the summer and winter trials, mean peak areas were assessed for the peptides found to contribute, significantly, to the differences observed in decomposition samples derived from the early (days 6–12 and day 2 in winter and summer respectively) and later periods (days 24–34 and days 8–10 in winter and summer respectively). As a semi-quantitative approach was undertaken, comparisons were made in relation to (a) the general shape of the trends displayed, and (b) when they occurred during the decomposition process.

3.4.1. Haemoglobin subunits alpha and beta

In a healthy vertebrate, haemoglobin is contained within the red blood cells and is responsible for distributing and storing oxygen. Studies have shown that haemoglobins are known to change significantly after death as a result of cell lysis [31]. Due to the lack of ATP post-mortem, sodium accumulates inside the cells, which begin to lyse, resulting in widespread leakage of intracellular fluid into extracellular tissue spaces [32]. Jetter (1943) suggested that the changes in concentration of haemoglobins alpha and beta between ante- and post-mortem blood samples were of sufficient magnitude that they could be used as indicators of PMI within the first three days post-mortem. However, it was not stated whether the concentration of these proteins increased or decreased, or if they were useful indicators beyond that timeframe [33].

Determination of the rate and extent of haemoglobin proteolysis in decomposition fluid, to which blood is a major contributor [23], may provide useful information relevant to the estimation of PMI. In this study, three peptides associated with haemoglobin subunit beta (ESFGDLSNADAVMGNPKVK, FGDLSNADAVMGNPK

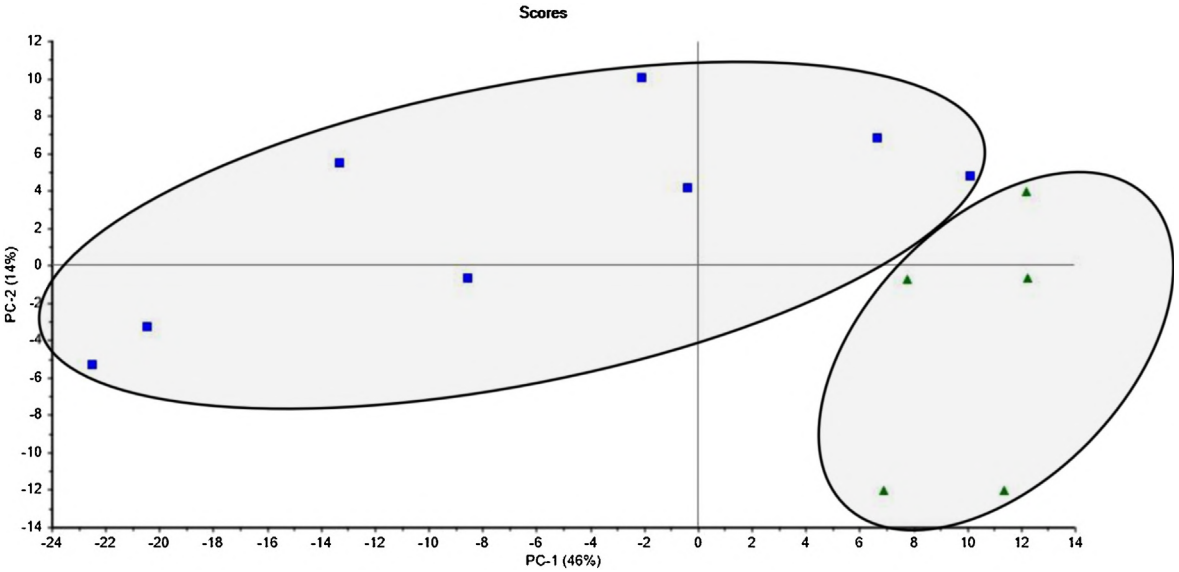

Fig. 4. Scores plot from principal component analysis of summer samples collected on day 2 (squares) (n = 8) and on days 8–10 (triangles) (n = 5) periods of decomposition.

and GDLSNADAVMGNPKVK) displayed consistent trends in that a notable increase in mean peak area was followed by a marked decrease (Fig. 5b–d) in both the summer (at approximately 100–150 ADD) and winter samples (at approximately 230 ADD). Though the trend was similar, the increase in average peak area occurred at different time points in summer and winter, even when temperature differences, via ADD, were taken into account. In contrast, the haemoglobin alpha-derived peptide GHLDDPGAL displayed a consistent increase in mean peak area for the summer samples, while the winter samples were more variable (Fig. 5a). This suggests that temperature played the most significant role in the degradation of haemoglobin subunit alpha in the summer trial, while an additional factor appears to have impacted the rate of degradation of haemoglobin subunit beta in both trials. If these peptides are quantified at regular intervals during the decomposition process, their concentrations or concentration ratios could reveal time-specific trends which may provide useful information relevant to time since death beyond the 3 days specified by Jetter [33].

Table 4
Fold change in mean peak area of peptides from samples collected in the early (day 2) and later (days 8–10) periods of the summer trial. + indicates a fold increase in mean peak area between samples collected on day 2 and days 8–10; – indicates a decrease. Significantly different mean peak areas between early (day 2) and later (days 8–10) sampling periods are shown by $^{*}P < 0.05$ and $^{**}P < 0.01$.

Protein	Peptide		Fold Change
Haemoglobin subunit alpha	AVGHLDDLPGAL	+	1.795690385
	GHLDDLPGAL	+	5.174942741**
	VGHLDDLPGAL	+	1.456415593
Haemoglobin subunit beta	ESFGDLSNADAVMGNPKVK	–	1.381271993
	FGDLSNADAVMGNPKVK	–	2.48859958
	GDLSNADAVMGNPKVK	–	1.089978418
	KVNVDEVGGEALGRL	+	2.418934269
	NVDEVGGEALGRL	+	1.40636541
	VNVDEVGGEALGRL	–	1.234509192
Creatine kinase	DLFDPIIQDR	+	1.484445569
	KDLFDPIIQDR	+	9.137874897*
Beta-enolase	IVGDDLTVTNPK	+	5.325244606**
	IVGDDLTVTNPKR	+	7.881081111*
	SGVNIQIVGDDLTVTNPKR	+	7.698466085*
	IQIVGDDLTVTNPK	+	4.297619391
Lactate dehydrogenase	**A**TLKDQLIH	+	1.084943473
	DLQHGSLF	+	3.37286389*
	ILGQNGISDVVKV	+	2.975556742
	KVTLTPEEEAHLKK	+	1.02166067
	NLHPELGTDADKEHWK	+	11.37222941

3.4.2. Creatine kinase

Creatine kinase catalyses a reversible reaction between creatine and ATP to generate phosphocreatine. There are two cytosolic forms of creatine kinase, named for the tissue from which they are typically isolated, muscle (MM-CK) and brain (BB-CK) [34]. Although creatine kinase is found in muscle, the majority of studies involving post-mortem changes have focused on its concentration in cerebrospinal fluid (CSF). Paulson et al. (1971) reported creatine kinase to increase in the CSF after death, but stated that the increase bore no relationship to the PMI [35]. In contrast, several other studies found that there was a significant correlation between the post-mortem concentration of creatine kinase in CSF and time since death [36–38].

The creatine kinase-derived peptide KDLFDPIIQDR exhibited a trend similar to that observed for the peptide derived from haemoglobin subunit alpha. An increase in mean peak area was observed in the summer samples, while the winter samples were, again, more variable (Fig. 6). Although the correlation between time and mean peak area did not display the linearity reported by Karkela et al. (1993). [37], the trends suggest that creatine kinase could prove useful in PMI estimation. Again, this suggests that temperature was the most dominant factor in the degradation of creatine kinase in the summer trial, while an additional factor impacted its degradation in the winter trial. The consistency in the trends observed (up to approximately 250 ADD), coupled with the statistically significant differences observed in summer and winter in the post-mortem samples derived from the early and later periods, indicate that, if quantified, KDLFDPIIQDR could prove to be a potential marker for time since death estimation.

3.4.3. Beta-enolase

Enolase enzymes are abundantly expressed carbon-oxygen lyases known for their role in glycogenesis and glycolysis [39]. There are three subunits of enolase, each encoded by different genes. Non-neuronal enolase (alpha-enolase) is found in a variety of tissues including liver, brain and kidney [39]. Muscle-specific

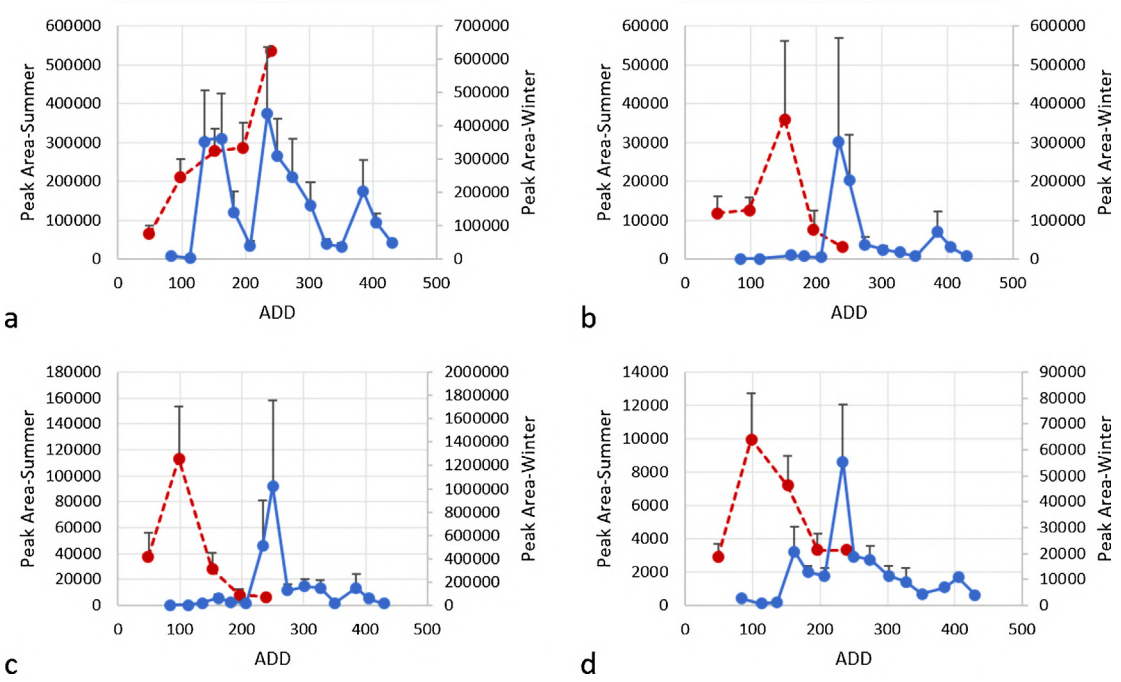

Fig. 5. Mean peak area for (a) GHLDDPGAL, (b) ESFGDLSNADAVMGNPKVK, (c) FGDLSNADAVMGNPK and (d) GDLSNADAVMGNPKVK on days 2–10 (ADD 49–240) in summer (red dotted line) (n = 8) and days 6–34 (ADD 84–429) in winter (blue solid line) (n = 5). Peak areas in each sample were normalised to the crude protein concentration.

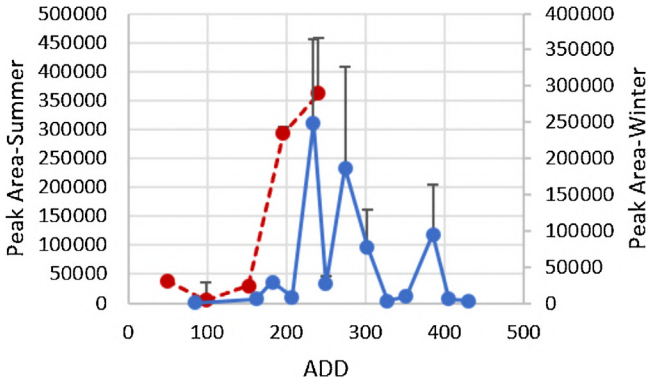

Fig. 6. Mean peak area for KDLFDPIIQDR on days 2–10 (ADD 49–240) in summer (red dotted line) (n = 8) and days 6–34 (ADD 84–429) in winter (blue solid line) (n = 5). Peak areas in each sample were normalised to the crude protein concentration.

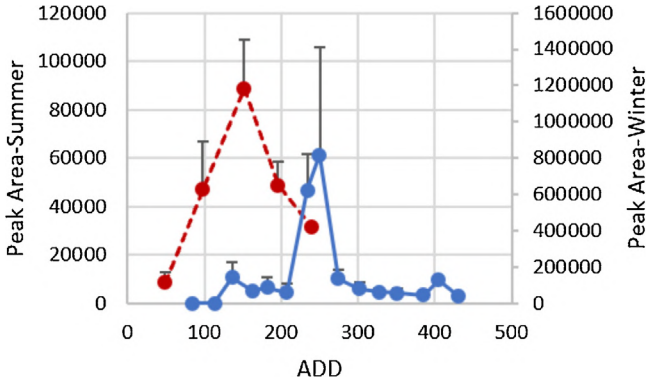

Fig. 7. Mean peak area for IVGDDLTVTNPK on days 2–10 (ADD 49–240) in summer (red dotted line) (n = 8) and days 6–34 (ADD 84–429) in winter (blue solid line) (n = 5). Peak areas in each sample were normalised to the crude protein concentration.

enolase (beta-enolase) and neuron-specific enolase (gamma-enolase) are restricted to muscle and brain, respectively [39,40]. There is little information in the literature concerning the presence of beta-enolase in post-mortem samples, though, a link between beta-enolase and the ageing of living cardiac and skeletal muscle has been reported [41,42].

A single peptide derived from beta-enolase was found to have contributed significantly to the differences observed between decomposition samples derived from the early and later periods. As described for the other peptides of interest, IVGDDLTVTNPK, displayed a consistent trend; an increase in mean peak area was followed by a sharp decrease (Fig. 7) in both the summer and winter samples. The trend mirrored that observed for the peptides generated from haemoglobin subunit beta (Fig. 5b–d). This is consistent with the notion that a factor (or factors) additional to temperature has contributed to the rate of degradation of beta-enolase.

3.4.4. Lactate dehydrogenase

Lactate dehydrogenase (LDH) catalyses the interconversion of lactate and pyruvate. LDH consists of two distinct subunits, designated either 'H' or 'M' for the enzymes derived from heart or skeletal muscle, respectively [32]. In the living, elevated levels of LDH in the blood typically reflect myocardial infarction, liver disease, skeletal muscle damage or some cancers [43]. However, increased levels of LDH have also been reported in post-mortem blood and CSF samples [35–37,44]. Osuna et al. (1992) and Karkela et al. (1993) found that the increase in LDH activity in CSF samples collected up to 24 h post-mortem was significantly correlated with time since death [36,37].

In this study, two LDH-derived peptides (DLQHGSLF and ILGQNGISDVVKV) exhibited an increase in mean peak area followed by a decrease in both the summer and winter samples with ADD (Fig. 8a–b). In contrast to the results described earlier, these trends were apparent over a longer timeframe, which suggests that these peptides may have potential for predicting PMI over a more extended period.

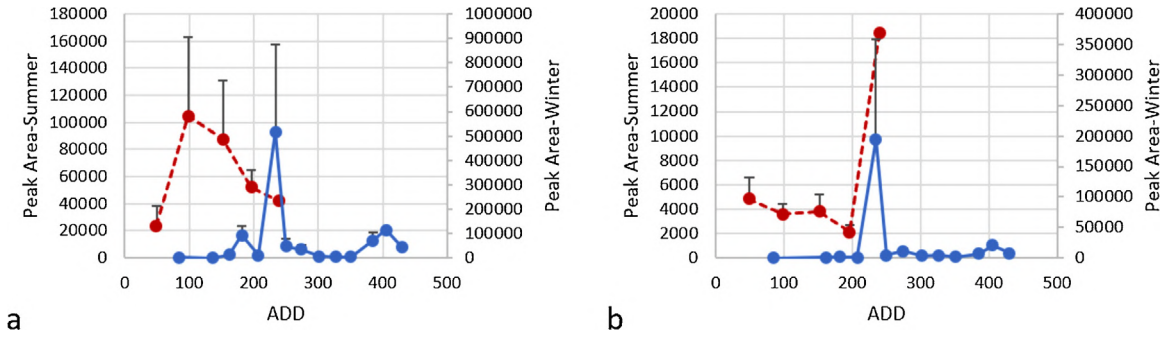

Fig. 8. Mean peak area for (a) DLQHGSLF and (b) ILGQNGISDVVKV on days 2–10 (ADD 49–240) in summer (red dotted line) (n = 8) and days 6–34 (ADD 84–429) in winter (blue solid line) (n = 5). Peak areas in each sample were normalised to the crude protein concentration.

3.5. Total body score vs. peptide production

To determine if peaks in peptide production occurred during specific stages of the decomposition process, mean peak areas were compared and correlated with total body score (TBS). Peptides found to contribute significantly to the differences observed in decomposition samples derived from the early period (days 6–12 and day 2 in winter and summer, respectively) were compared with those derived from the later period (days 24–34 and days 8–10 in winter and summer, respectively). As a semi-quantitative approach was undertaken, comparisons were made in relation to (a) the general shape of the trends displayed, and (b) when they occurred during the decomposition process.

A single peptide derived from haemoglobin subunit alpha (VLSAADKANVKAAWGK) and three peptides derived from haemoglobin subunit beta (FGDLSNADAVMGNPK, FGDLSNADAVMGNPKVK and GDLSNADAVMGNPKVK) displayed similar trends in both the summer and winter trials and these trends correlated with similar total body scores (Fig. 9a–d). Similar trends were observed for three peptides derived from lactate dehydrogenase (ATLKDQLIH, ATLKDQLIHN and DLQHGSLF) (Fig. 10a–c). In each case, a large increase was followed by a sharp decrease in both the summer and winter trials. The peaks were associated with mean total body scores ranging from 12.0–12.6 which corresponded to the stage of decomposition defined as “early” [29]. Physical features included brown discolouration of the head and neck, drying of the ears, purging of decomposition fluids, grey-purple discolouration of the abdomen with maximum bloat and brown/black discolouration of the limbs. Though these trends provide only minimal insight into PMI estimation, the occurrence of specific peptides at particular stages of the decomposition process may help to illuminate the biological processes that generate the physical features observed during each decomposition stage. Consequently, thorough mapping of these peptides throughout the decomposition process should be undertaken.

4. Conclusion

The analysis of peptides resulting from the degradation of proteins in decomposition fluid demonstrates potential for use in

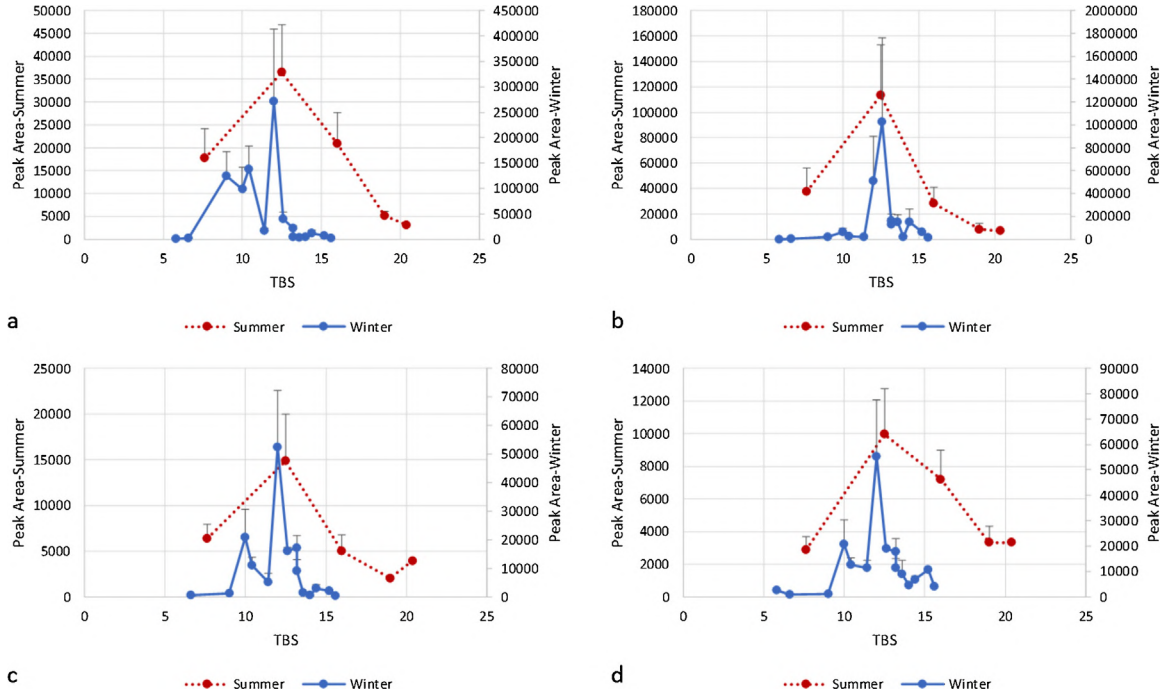

Fig. 9. Mean peak area for (a) VLSAADKANVKAAWGK, (b) FGDLSNADAVMGNPK, (c) FGDLSNADAVMGNPKVK and (d) GDLSNADAVMGNPKVK vs mean total body score (TBS) on analysis days 2–10 (ADD 49–240) in summer (red dotted line) (n = 8) and days 6–34 (ADD 84–429) in winter (blue solid line) (n = 5). Peak areas in each sample were normalised to the crude protein concentration.

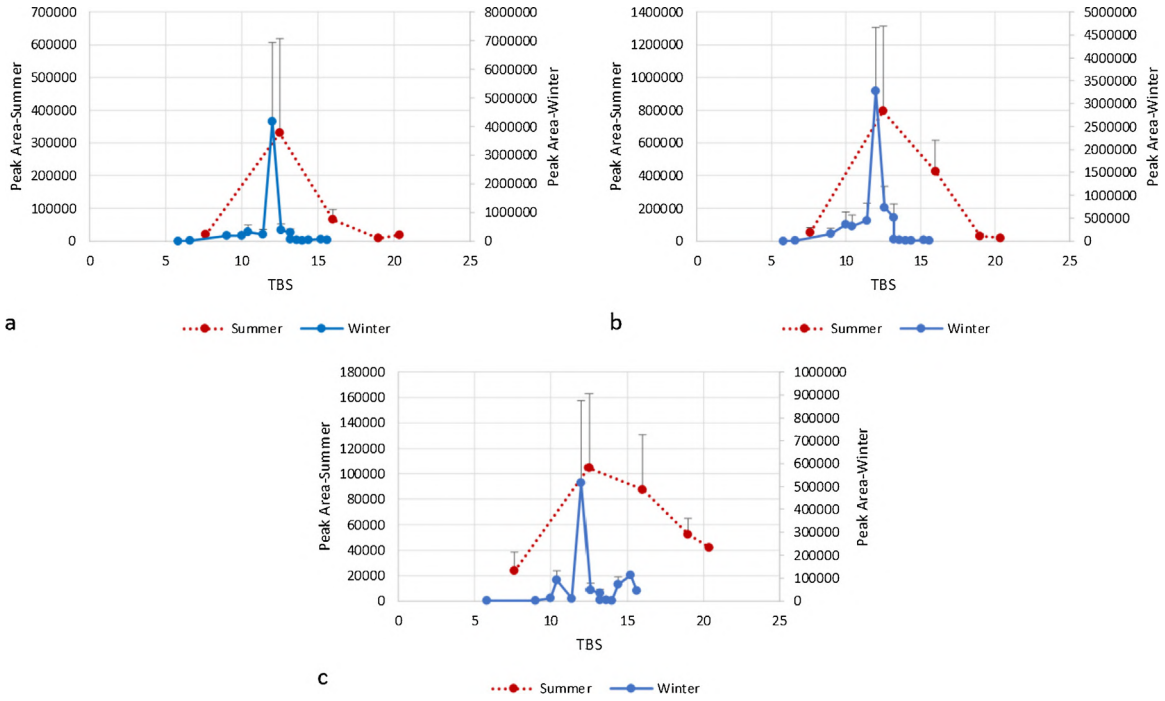

Fig. 10. Mean peak area for (a) ATLKDQLIH, (b) ATLKDQLIHN and (c) DLQHGSLF vs mean total body score (TBS) on analysis days 2–10 (ADD 49–240) in summer (red dotted line) (n = 8) and days 6–34 (ADD 84–429) in winter (blue solid line) (n = 5). Peak areas in each sample were normalised to the crude protein concentration.

PMI estimation. A semi-quantitative approach revealed that 29 peptides derived from 5 different proteins contributed to differences in the peptide content between decomposition fluid samples collected during the early (days 6–12 and day 2 in winter and summer respectively) and later periods (days 24–34 and days 8–10 in winter and summer respectively) of the decomposition process. Moreover, fold changes observed between samples collected in the early and later post-mortem periods for peptides derived from haemoglobin subunit alpha (GHLDDPGAL), haemoglobin subunit beta (ESFGDLSNADAVMGNPKVK; FGDLSNADAVMGNPK; GDLSNADAVMGNPKVK), creatine kinase (KDLFDPIIQDR), beta-enolase (IVGDDLTVTNPK) and lactate dehydrogenase (DLQHGSLF; ILGQNGISDVVKV) were significantly different and were responsible for most of the dissimilarities observed. Furthermore, these peptides displayed distinct trends in mean peak area with time, further emphasising their potential for use in time since death estimation. Seven peptides displayed consistent trends that could be correlated with TBS during the early stages of decomposition. Further mapping of these peptides may provide insight into the biological processes responsible for generating the physical features observed during decomposition.

We have established that there are clear differences in the peptide profile associated with the early and advanced stages of the decomposition process, but the inherent variability associated with the rate and extent of protein degradation requires additional research before it can be considered as an approach to PMI determination for forensic casework. Once the potential of the peptide profile of decomposition fluid as a PMI-indicator has been fully evaluated, studies on the recovery and analysis of fluid from soil matrices need to be undertaken to evaluate the practical applicability of this approach. Studies will need to be conducted over several years, during different seasonal periods and in different geographical locations. This will allow variability between different seasons and different years to be evaluated and will provide information on the protein degradation patterns associated with cold, temperate and tropical environments. In addition, other factors known to influence decomposition such as soil pH, clothing, wounds, and the impact of size, age and weight of cadavers should be assessed [4,13,15,20].

Future studies will also require a greater sample size, more analyses and a fully quantitative approach. An untargeted method should also be employed to allow the full extent of protein degradation in post-mortem samples to be explored and mapped. This would enable the calculation of peptide ratios, which may also reveal time-dependent trends. There is a clear need to develop viable alternatives to the methods currently used for the estimation of the post-mortem interval. Whilst studies focusing on other biochemical markers have defined limitations, this study, which has focused on the use of peptides, has generated encouraging results. Although further investigation is required, the potential exists for the application of this method to time since death estimations.

CRediT authorship contribution statement

Ashley-N'Dene Nolan: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Writing - original draft, Writing - review & editing, Visualization, Project administration. **Garth Maker:** Conceptualization, Methodology, Formal analysis, Writing - review & editing, Supervision, Project administration. **Robert J. Mead:** Conceptualization, Methodology, Writing - review & editing, Supervision, Project administration. **Scott Bringans:** Writing - review & editing, Supervision, Project administration, Funding acquisition. **Samuel J. Speers:** Conceptualization, Methodology, Writing - review & editing, Supervision, Project administration.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at https://doi.org/10.1016/j.forsciint.2020.110269.

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