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Examination of the temporal variation of peptide content in decomposition fluid under controlled conditions using pigs as human substitutes

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ABSTRACT

We report the preliminary observations of the peptide content of decomposition fluid produced under controlled laboratory conditions and in the absence of a soil matrix. Four domestic pig (*Sus scrofa domesticus*) cadavers were used to model human decomposition over a four-week trial period; physical characteristics were recorded and the peptide components of decomposition fluid was analysed using high performance liquid chromatography-time of flight mass spectrometry. Preliminary data analysis indicated that a range of peptides were consistently detected across the course of the trial period and 27 of these were common to all four cadavers; 22 originating from haemoglobin. The peptides associated with haemoglobin subunit alpha and beta displayed a breakdown pattern that remained consistent for all cadavers for the duration of the trial. Though identification of peptides during decomposition has potential for estimating the time since death, quantification of selected peptides is likely to be essential to identify time-dependent trends.

1. Introduction

Estimating the post-mortem interval (PMI) is often essential in the investigation of homicides or unexplained deaths. An accurate PMI can assist an investigation in establishing the whereabouts and final movements of the deceased, exclude or include possible persons of interest and if homicide is suspected, corroborate witness testimonies. Knowing a PMI can also permit a more thorough taphonomic analysis, as the range of environmental factors can be retrospectively determined [1]. Traditionally, the observation of gross disintegration of soft tissue has been relied upon to estimate PMI by dividing the decomposition process into stages, the total number of which ranges from four to six [2–6]. However, defining the start and end points of each stage has proven difficult and the rate of progression through the stages is highly influenced by both intrinsic and extrinsic factors. This makes them unreliable and inaccurate for PMI estimation [7–9]. Although more quantitative botanical, entomological and biochemical methods to estimate PMI have been developed, these approaches still have their limitations [1].

A promising alternative to the established approaches is the investigation of degradation products in decomposition fluid. In 1992, Vass et al. reported the first study involving decomposition fluid and its potential to provide an alternative method for PMI estimation. The analysis of volatile fatty acids (VFAs) in decomposition fluid within a soil matrix was the primary focus. They concluded that VFAs were accurate and reliable biomarkers for certain stages of the decomposition process and that their determination in decomposition fluid may be of value in the estimation of PMI for human cadavers [10]. However, as this research was only conducted in one location (Tennessee, USA), the results may have been affected by soil type and other environmental factors. Subsequently, Swann et al. analysed the fatty acid and amino acid composition of decomposition fluid from pigs in the absence of a soil matrix [11–13] and found that the compounds were released in a cyclical fashion. They reported that, although the number of cycles observed was time-dependent and could be related to PMI, it also varied with temperature. Consequently, the approach was not considered definitive for PMI estimation [11,13].

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To date, most of the work directed at identifying biomarkers during decomposition has focused on fatty acids and carbohydrates in both humans and pigs [8,10–15]. However, more recently, several studies have demonstrated that protein biomarkers may be more useful predictors of PMI than these compounds. Most promising are the studies by Pittner et al., who demonstrated, using SDS-PAGE, Western blotting and casein zymography that the detection of protein degradation products in porcine muscle could be correlated with the post-mortem interval [16]. Similar patterns of degradation were reported, subsequently, in decomposing human muscle. Desmin and cardiac troponin T were identified as candidate proteins with high potential for estimating the PMI [17]. The approach, when applied to a case of murder suicide, provided information that suggested which of the deceased had died first [18].

In this study a "whole body" approach was adopted in which more sophisticated proteomic techniques were applied to evaluate the rate of protein degradation in decomposition fluid with the aim of assessing the extent to which the production of specific peptides could be correlated with time since death. The present study reports preliminary observations of the peptide content of fluid generated throughout the decomposition process under controlled laboratory conditions in the absence of soil, with the aim of determining if peptide content varies in relation to the decomposition process.

2. Materials and methods

2.1. Research facilities

Preliminary decomposition studies were carried out within an enclosed, temperature controlled (24 °C) environment located at Murdoch University, in Western Australia. A data logger (EL-USB-2+) was used to record and monitor temperature and humidity every 10 min for the duration of the study. The study was approved by the Murdoch University Animal Ethics Committee (Cadaver approval number: 376).

2.2. Cadavers

Domestic pigs (*Sus scrofa domesticus)* were utilised within this study to model the human decomposition process. The domestic pig is considered an appropriate substitute due to their similarity to humans with respect to weight, muscle to fat ratio, hair coverage, internal anatomy and gut fauna [19]. Four adult female pigs (20 kg) were euthanised by bolt gun to the temple two hours prior to commencing the study.

2.3. Decomposition trials

Each carcass was placed in a plastic container on a raised platform. Openings in the platform enabled the fluid to drain away from the body to ensure that the decomposition process was not influenced by fluid accumulation. The platforms on which the carcasses rested remained parallel to the ground to prevent decomposition fluid flowing to different areas within the carcass.

Insects were permitted access to two of the four carcasses. In order to simulate the colonisation of these carcasses by flies in an exposed environment, eggs of the calliphorid, *Lucilia sericata,* were introduced manually. The eggs were deposited on sites known to be colonised first (eyes, ears, mouth and nose) [3,7,20]. To exclude insects, the remaining two carcasses were covered with a breathable, white cotton fabric that was sealed to the containers. A mosquito net was also placed over each container as a primary barrier to insects. Cadavers from which insects were excluded were designated 1 and 2, while the insect-inclusive cadavers were designated 3 and 4.

2.4. Observations

Carcasses were monitored daily for the first two weeks and thereafter every second day until the conclusion of the study (four weeks post-deposition). Elapsed time was documented in accumulated degree days (ADD) to account for temperature. ADD represents the heat energy units available to sustain the chemical and biological processes required for decomposition to take place

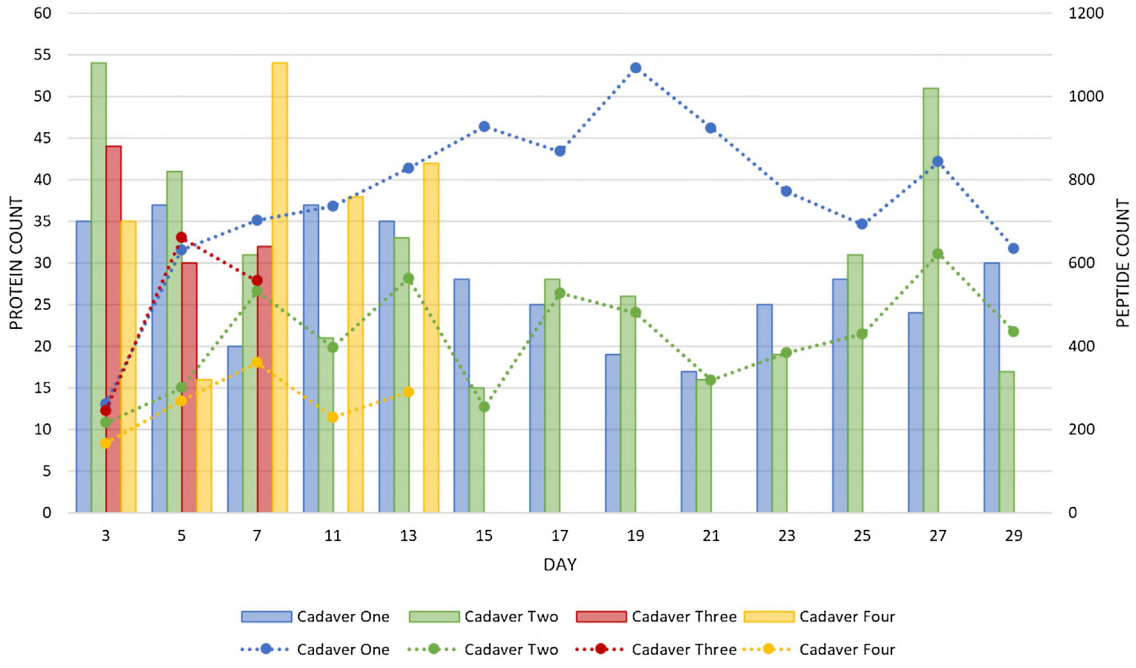

Fig. 1. Total number of proteins (histograms) and peptides (lines) (>95% CI) detected in decomposition fluid samples collected from Cadavers 1 and 2 (insects excluded) and Cadavers 3 and 4 (insect inclusive) on analysis days 3–29 (ADD 72–696).

Table 1
Identification of the 17 proteins found to be present (detected ≥50% of the time across trial period) in decomposition fluid collected from Cadavers 1, 2 and 4.

Replicate	Proteins present
Cadaver 1	Beta-enolase
	Calsequestrin
	Carbonic anhydrase 3
	Creatine kinase M-type
	Dihydropyrimidinase
	DJ-1 protein
	Fructose-bisphosphate aldolase
	Glucose-6-phosphate isomerase
	Haemoglobin subunit alpha
	Haemoglobin subunit beta
	Phosphatidylethanolamine binding protein 1
	Phosphoglycerate kinase 1
	Phosphoglycerate mutase
	Proteasome subunit alpha type
	Pyruvate kinase
	Serotransferrin
	Serum albumin
Cadaver 2	Beta-enolase
	Carbonic anhydrase 3
	Collagen type VI alpha 3 chain
	Creatine kinase M-type
	Glucose-6-phosphate isomerase
	Haemoglobin subunit alpha
	Haemoglobin subunit beta
	L-lactate dehydrogenase
	Myoglobin
	Phosphatidylethanolamine binding protein 1
	Phosphoglycerate kinase
	Pyruvate kinase
Cadaver 4	4-trimethylaminobutyraldehyde dehydrogenase
	Beta-enolase
	Calsequestrin
	Creatine kinase M-type
	Fibrinogen beta chain
	Fructose-bisphosphate aldolase
	Haemoglobin subunit alpha
	Haemoglobin subunit beta
	L-lactate dehydrogenase
	Peroxiredoxin-6
	Pyruvate kinase
	Tropomyosin alpha-1 chain

[1]. Observations were recorded on each experimental day focusing on physical appearance, including skin discolouration, bloating and deflation, skin ruptures, hair loss and bone exposure. Photographs were also taken to accompany observations. The stage of decomposition was assessed and evaluated, using the Total Body Score (TBS) method developed by Keough et al. [21]. As the manner and rate of decomposition is known to differ between anatomical regions, three regions of the carcass were scored independently: the head and neck, trunk and limbs. These scores were then summed to calculate the TBS to represent the overall stage of decomposition for each carcass.

2.5. Fluid collection

Collection containers were monitored daily and fluid samples collected between 0800 and 0900 for the first two weeks and, thereafter, every second day for the remainder of the study. The fluid was mixed thoroughly to ensure a homogenous mixture of the accumulated fluid was sampled on each monitored day. 5×2 mL fluid samples were collected from each carcass container using a 1 mL plastic pipette and placed in 2 mL graduated microtubes. The samples were stored at −80 °C until analysed.

2.6. Sample preparation and analysis

Fluid samples were allowed to equilibrate to room temperature. A 1:4 dilution of the sample was prepared using LC/MS grade water (Thermo Fisher Scientific, Australia). The mixture was filtered using a 0.2 μm filter and the filtrate loaded onto a Vivaspin 2 10,000 MWCO filter (Sartorius, Australia). Samples were centrifuged at 4000 × *g* for a minimum of 60 min; centrifugation was continued in 30-min increments until the volume of retentate was ~100 μL. A Strata-X 33 μm Polymeric Reverse Phase, 60 mg/3 mL column (Phenomenex, Australia) was pre-conditioned with methanol, acetonitrile, 0.1% formic acid and 80% acetonitrile/0.1% formic acid solution. The filtrate was then loaded onto the column, washed with 0.1% formic acid (Thermo Fisher Scientific, Australia) and eluted with 0.5 mL 80% acetonitrile/0.1% formic acid solution. The sample was then

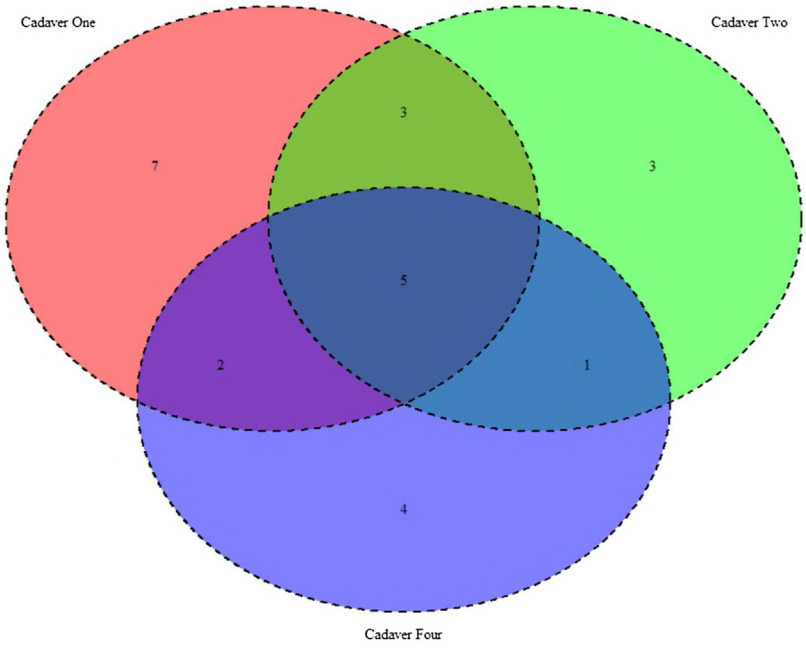

Fig. 2. Venn diagram reporting proteins present (detected ≥50% of the time across trial period) in decomposition fluid collected from Cadavers 1, 2 and 4.

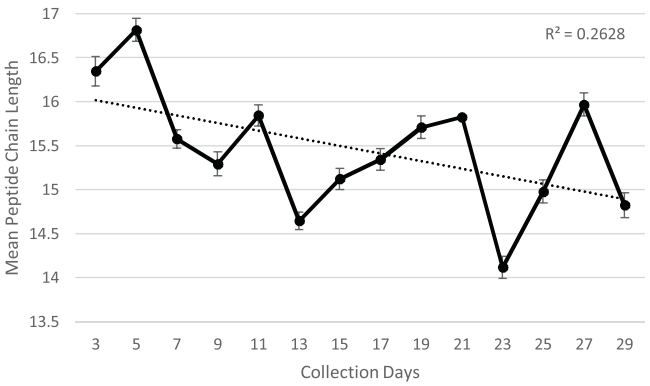

Table 2
Total number of peptides associated with the five proteins identified as being present (detected ≥50% of the time across trial period) in Cadavers 1, 2 and 4.

Protein	Total no. peptides
Creatine kinase	437
Beta-enolase	398
Pyruvate kinase	476
Haemoglobin subunit alpha	875
Haemoglobin subunit beta	752
Total	2938

evaporated to dryness using an Eppendorf Concentrator Plus vacuum concentrator and stored at −80 °C until analysed.

The sample was reconstituted in 100 μL of 2% acetonitrile and 0.1% formic acid and analysed using a Shimadzu Prominence nano HPLC system (Shimadzu) coupled to a 5600 TripleTOF mass spectrometer (Sciex). Peptides were loaded onto an Agilent Zorbax 300SB-C18, 3.5 μm, 0.075 × 150 mm column (Agilent Technologies) and separated using a 40 min linear gradient of water/acetonitrile/0.1% formic acid at a flow rate of 300 nL/min. Peptides were eluted through a nanospray interface into a 5600 TripleTOF mass spectrometer (Sciex). The data were acquired in an information-dependent acquisition (IDA) mode with Analyst TF 1.6 software (Sciex). The MS settings were as follows: Ionspray Voltage Floating (ISVF) = 2300 V, curtain gas (CUR) = 20, ion source gas 1 (GS1) = 20, interface heater temperature (IHT) = 150, and declustering potential (DP) = 70 V. The first TOF MS scan (experiment 1) was performed in the mass range of 300–1250 Da with a 0.25 s TOF MS accumulation time, whereas the MS/MS product ion scan was performed in the mass range of 100–1800 Da with a 0.05 s accumulation time. The criteria for product ion fragmentation was set as follows: ions (>300 and <1250 m/z) with charge states of 2–5 and an abundance threshold of >250 cps. Former target ions were excluded for 10 s after one occurrence. The maximum number of candidate ions per cycle was 20 spectra. Spectral data was analysed using ProteinPilot™ 5.0 Software (Sciex) against the UniProt *Sus scrofa* database.

2.7. Data analysis

Peptide data was analysed using the VennDiagram package [22] in RStudio version 3.5.1 [23]. Peptide sequences were aligned using the following alignment settings in Geneious (v11.1.5): Alignment type = Global alignment with free end gaps, Cost Matrix = Blosum90, Gap open penalty = 22, Gap extension penalty = 3, Refinement iterations = 2 [24].

3. Results and discussion

3.1. Decomposition fluid analysis

Decomposition fluid was collected, when available, from the four domestic pig cadavers during a four-week monitored period. Due to a combination of insect activity and experimental design, fluid was collected from Cadavers 3 and 4 only until days 7 (ADD 168) and 13 (ADD 312), respectively, whereas fluids continued to be collected from Cadavers 1 and 2 until the end of the four-week period (ADD 696). Initial analysis of the samples revealed numerous peptides for each cadaver across the course of the trial period. There was no clear correlation between total peptide number and days progressed (r = 0.46, 0.24 and 0.32 for Cadavers 1, 2 and 4, respectively) nor the degradation of the specific proteins from which the peptides were derived (r = −0.58, 0.13 and −0.06 for Cadavers 1, 2 and 4, respectively). Stronger correlations were apparent for Cadaver 3 between total peptide number and days elapsed (r = 0.72) and between total peptide number and total parent protein (r = −0.99), however due to the small sample size, these were not included in further analysis (Fig. 1).

3.2. Proteins

Proteins were classed as being present if they were identified more than 50% of the time over the course of the trial period (29 days) [25]. Seventeen proteins were classed as being present for Cadaver 1, while 12 were deemed to be present for Cadavers 2 and 4 (Table 1). Cadaver 3 was excluded from this analysis due to the small number of samples collected.

Of the 41 proteins identified (based on the presence of peptides) in the decomposition fluid from the three cadavers, 5 were found to be common to all cadavers (Fig. 2). Further investigation of the data set, focusing on proteins present in 3 out of 5 sample days did not add any further data.

3.3. Protein identification

The five common proteins were identified as creatine kinase, beta-enolase, pyruvate kinase, haemoglobin subunit alpha and haemoglobin subunit beta.

The presence of the above proteins in decomposition fluid can be explained by the process of autolysis in which digestive enzymes break down the tissues of the body following death [26–28]. Autolysis first begins in cells that are more metabolically active and thus possess more hydrolytic enzymes [26,27]. Though autolysis has been found to commence as soon as 4 min after death [29], the time at which the process begins varies between tissue and organ types [27]. The first organs to undergo decomposition are the intestine, stomach, pancreas, liver, heart and blood [26]. These are followed by the lung, kidney, bladder and brain. Skeletal muscle is the next tissue to decompose, followed by more durable connective tissue [26]. During autolysis, cells exhibit two major types of micro- and macroscopic changes in appearance. The first is coagulative necrosis, which is characterised by the preservation of the original structure of the tissue for several days following cell death. The second is liquefactive or colliquative necrosis, defined by the partial or complete disintegration of the dead tissue into a liquid, viscous mass [26]. Although the brain is the most recognised organ in which liquefactive necrosis takes place, any cell containing hydrolytic enzymes will undergo some level of liquefaction [26,28]. The liquefaction of multiple tissues is believed to be the source of decomposition fluid and, if the level of these proteins can be monitored over time, it may indicate their potential as biomarkers of time since death.

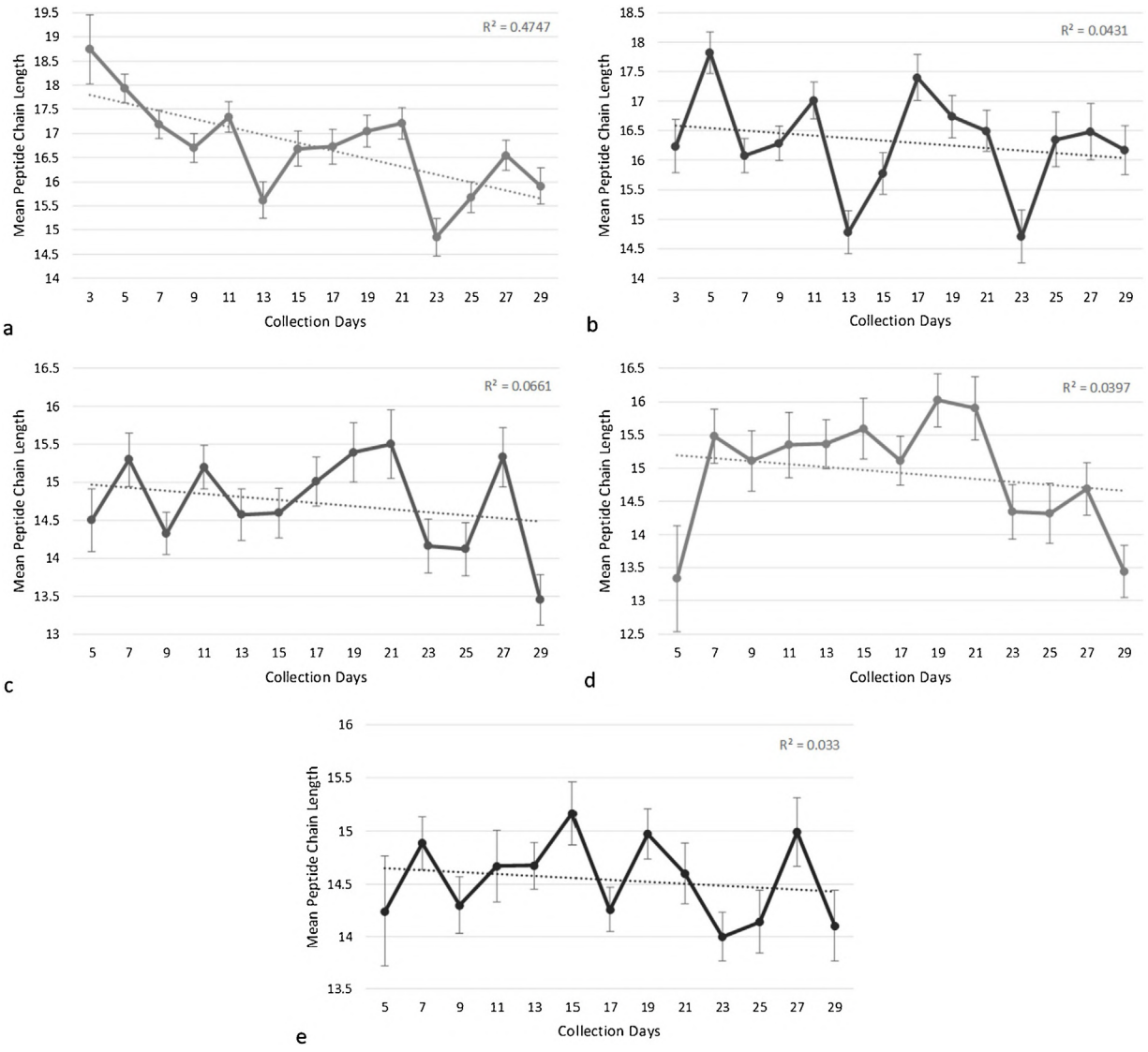

Fig. 4. Average chain length of peptides associated with (a) haemoglobin subunit alpha, (b) haemoglobin subunit beta, (c) beta-enolase, (d) creatine-kinase, (e) pyruvate-kinase detected in decomposition fluid samples. Samples collected from Cadavers 1 and 2 (insects excluded) and Cadaver 4 (insect inclusive) on analysis days 3–29 (ADD 72–696).

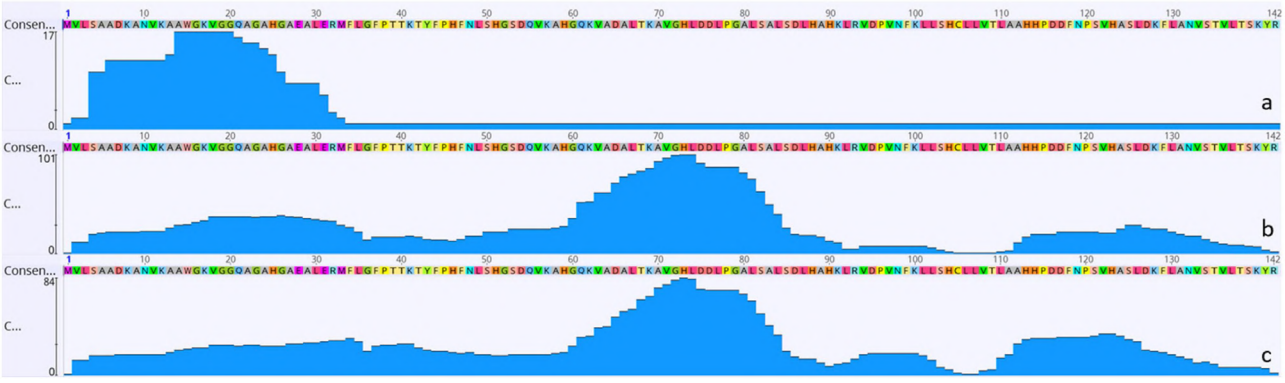

Fig. 5. Typical haemoglobin subunit alpha coverage. Peptides were detected in decomposition fluid samples collected from Cadaver 1 (insects excluded) on analysis days (a) 3 (ADD 72), (b) 15 (ADD 360) and (c) 27 (ADD 648).

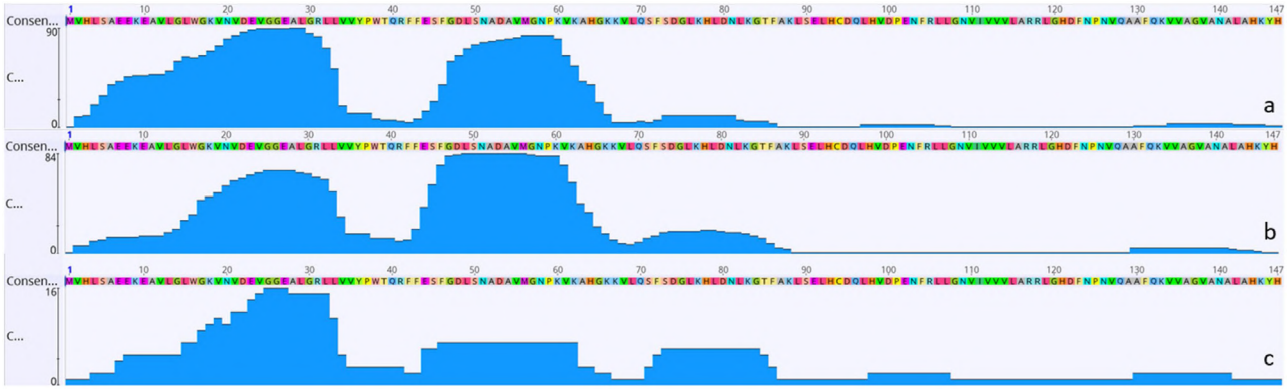

Fig. 6. Typical haemoglobin subunit beta coverage. Peptides were detected in decomposition fluid samples collected from Cadaver 1 (insects excluded) on analysis days (a) 3 (ADD 72), (b) 17 (ADD 408) and (c) 29 (ADD 672).

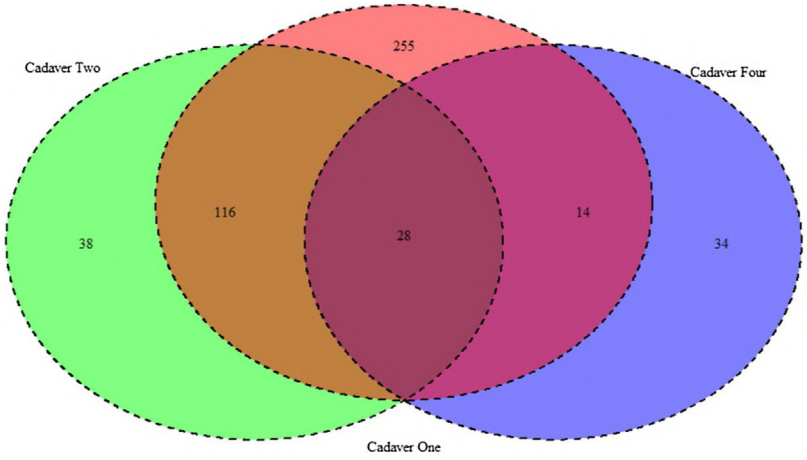

Fig. 7. Venn diagram reporting the common peptides present (detected ≥50% of the time across trial period) in Cadavers 1, 2 and 4.

3.4. Peptides

3.4.1. Total peptides

Peptide chain length for each sample day ranged from 14.1 to 16.8 amino acids, with relative standard deviations ranging from 27.5 to 32.9%. Average chain length was found to vary throughout the trial period with a trend towards longer chain length between collection days 13–19 (ADD 312–456) (Fig. 3). The average chain length did not correlate with days elapsed ($r = -0.51$), and was therefore excluded as a determinant of PMI.

3.4.2. Specific peptides

As shown in Table 2, the five common proteins yielded a total of 2938 unique peptides during the decomposition process. The chain lengths of these peptides also fluctuated throughout the trial (Fig. 4a–e).

Correlation between chain length and days elapsed ($r = 0.68$) was most apparent for the peptides generated from haemoglobin subunit alpha (Fig. 4a) with a trend towards shorter peptides as time progressed. However, correlation between chain length and time for the peptides generated from haemoglobin subunit beta ($r = 0.21$), beta-enolase ($r = 0.26$), creatine-kinase ($r = 0.20$) and pyruvate-kinase ($r = 0.18$) was less apparent (Fig. 4b–e) and relative standard deviations for the calculated means were larger (12.7–37.5%).

Peptide sequences were mapped against reference sequences of haemoglobin subunit alpha (XP_020942625) [30], haemoglobin subunit beta (NP_001138313) [31], beta-enolase (NP_001037992) [32], creatine-kinase (NP_00112342) [33] and pyruvate kinase (XP_001929104) [34] to identify breakdown trends on each sample day. Consistent breakdown patterns were identified for Cadavers 1, 2 and 4 for both haemoglobin subunit alpha and beta, however, breakdown patterns for beta-enolase, creatine kinase and pyruvate kinase showed inconsistencies between the three cadavers. The peptides associated with haemoglobin subunit alpha displayed a breakdown pattern that shifted from the amino terminal end to the carboxy terminal end of the reference protein as the trial progressed (Fig. 5a–c). The most abundant peptides generated on Day 3 (ADD 72) were derived from residues 4–27, while the most abundant peptides generated between Days 5–29 (ADD 120–696) were derived from residues 68–82. Further inspection of the structure of haemoglobin subunit alpha revealed greater potential exposure to proteolytic enzymes at these particular regions, likely increasing their susceptibility to proteolysis [30].

The peptides associated with haemoglobin subunit beta displayed a breakdown pattern that remained consistent for the duration of the trial (Fig. 6a–c). Peptides found to be most abundant were derived from residues 15–34 and 45–63 for each cadaver. As for haemoglobin subunit alpha, the nominated ranges of residues are potentially more exposed and therefore may be more susceptible to hydrolysis by proteolytic enzymes [30].

Regardless of the presence or absence of insect activity, the breakdown of the haemoglobin subunits alpha and beta remained consistent for each cadaver group; in future, larger sample sets will be needed to verify these findings. Despite the promising findings that consistencies were present across multiple replicates, it is

Table 3
Identification of the 27 peptides found to be common in decomposition fluid collected across the trial period in Cadavers 1, 2 and 4.

Protein	Peptides associated
Creatine kinase	• DLFDPIIQDR • KDLFDPIIQDR
Beta-enolase	• QEFMILPVGASSF • IVGDDLTVTNPK
Pyruvate kinase	• GDLGIEIPAEKVF • LVTEVENGGFLGSK
Haemoglobin subunit alpha	• AVGHLDDLPGALSAL • FLGFPTTKT • VGGQAGAHGAEALERMF • VLSAADKANVKAAWGKVGGQAGAH
Haemoglobin subunit beta	• DEVGGEALGRLL • ESFGDLSNADAVMGNPK • ESFGDLSNADAVMGNPKVK • FFESFGDLSNADAVMGNPK • FFESFGDLSNADAVMGNPKVK • FGDLSNADAVMGNPK • FGDLSNADAVMGNPKV • FGDLSNADAVMGNPKVK • GDLSNADAVMGNPKV • GDLSNADAVMGNPKVK • GKVNVDEVGGEALGRL • GKVNVDEVGGEALGRLL • KVNVDEVGGEALGRL • NVDEVGGEALGRL • SFGDLSNADAVMGNPK • VDEVGGEALGRLL • VNVDEVGGEALGRL • VNVDEVGGEALGRLL

important to recognise that fluids were allowed to accumulate over the course of the trial period. It is therefore unknown whether the hydrolysis of these proteins was a direct consequence of the decomposition process or if breakdown also occurred within the collection containers. Future research will focus on the protein degradation that occurs as a direct outcome of the decomposition process. This can be achieved by sampling fluid produced within a set timeframe and then discarding any remaining fluid after sampling is complete. If the degradation of proteins is similar to that found in this pilot study, this approach could assist in the estimation of time since death, particularly in the earlier stages of decomposition.

Also promising is the consistent identification of peptides derived from residues 4–27 and 68–82 for haemoglobin subunit alpha and 15–34 and 45–63 for haemoglobin subunit beta over the course of the trial period. As for the proteins, peptides were classed as present if they were identified more than 50% of the time over the course of the trial period [25]. A combined total of 413 peptides were classed as being present for Cadaver 1, 182 for Cadaver 2 and 76 for Cadaver 4. Of the 671 peptides identified across the cadavers, 28 were found to be common (Fig. 7).

Of the 28 common peptides, 18 originated from haemoglobin subunit beta, 4 from haemoglobin subunit alpha and 2 each from creatine kinase, beta enolase and pyruvate kinase (Table 3). The fact that these were found consistently over the course of the trial period and that they fell within the range of residues most susceptible to hydrolysis suggests that, if quantified, they may be useful determinants of time since death.

4. Conclusion

Decomposition fluid has proven itself to be a complex and highly variable matrix to study, although initial trends show promise in that several peptides were consistently detected across the trial period. Further development of the methodology is required for the quantification of peptides present throughout the decomposition process in order to investigate their potential association with time since death. While this preliminary study has only focused on controlled conditions, there is a need to effectively evaluate the relevance of these peptide trends within the field, in both summer and winter. Future work will require the use of larger sample sets to provide a more robust approach for the identification of peptide trends in decomposition fluid; this will also ensure relevant trends are recognised and quantified.

CRediT authorship contribution statement

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

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