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The Impact of Protein Biomarkers on Time Since Death Estimation Using Diverse Molecular Techniques

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Abstract:

The estimation of time since death (TSD) is a critical component of forensic science, aiding in criminal investigations and legal proceedings. Accurate estimation involves considering factors such as Algor Mortis, Rigor Mortis, Lividity (Livor Mortis), chemical changes, metabolic processes, RNA, DNA, protein degradation, and radiological imaging systems. This study explores the role of biomarkers, specifically proteins, in determining TSD through various analytical techniques applied to human and animal tissues. As decomposition progresses post-mortem, specific biochemical changes occur, allowing for the identification of reliable biomarkers. Certain biochemical alterations take place as post-mortem decomposition advances, making it possible to identify trustworthy biomarkers. We examine well-known techniques such as Immuno-histochemical (IHC), ATR-FTIR, Mass spectrometry, liquid chromatography, Western blotting, and enzyme-linked immunosorbent assay (ELISA), emphasizing how well they measure the composition and degradation of proteins. We show how the identification of protein biomarkers can improve the precision of PMI estimates by combining various methods. Biomarkers and protein estimation techniques are invaluable in forensic science for estimating the time since death. By concluding, we can understand the biochemical changes that occur post-mortem and by employing advanced analytical techniques, forensic scientists can provide more accurate TSD assessments, aiding investigations and legal proceedings. The different techniques were used widely in which SDS-PAGE, Gel Electrophoresis, and Western Blot were used mostly due to their precise estimation of protein level.

Keywords: *Protein Estimation, PMI, Biomarkers, Molecular Techniques, Time Since Death Estimation*

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Introduction

The estimation of time since death (TSD) is a fundamental aspect of forensic science, essential for criminal investigations and legal determinations (Madea). Precise TSD estimation requires the assessment of multiple postmortem physiological and biochemical parameters, including algor mortis, rigor mortis, and livor mortis, as well as molecular and metabolic alterations such as RNA, DNA, and protein degradation **(Donaldson and Lamont, 2013).**

A biomarker is a defined characteristic evaluated as an indicator of a pathogenic process, a normal physiological process, or a response to exposure or intervention, including therapeutic intervention. The use of biomarkers in epidemiological investigations enhances the validity by reducing measurement bias in neurological illnesses **(Megyesi *et al.*, 2005).** The use of biomarkers enhances the sensitivity and specificity of risk factor exposures. Molecular biomarkers provide the capability to identify individuals predisposed to disease **(Müller and Graeber, 1996).**

Fundamentals of PMI in Forensic Medicine

The discipline of forensic science includes forensic biology as a branch. It employs biological expertise to detect and study biological evidence acquired from the crime scene, the victim, or the suspect to show that the crime happened. The examination of evidence linked to living beings and the biological components they are frequently found with at crime scenes is the focus of the field of forensic biology **(Henssge and Madea, 2004).**

Traditional postmortem interval (PMI) estimation methods, including algor mortis, rigor mortis, and livor mortis, are often influenced by environmental conditions, individual physiological variations, and external factors such as temperature and microbial activity, leading to inconsistencies and reduced accuracy in forensic investigations **(Payne-James *et al.*, 2003; Secco *et al.*, 2025).** Biochemical and enzymatic approaches, while more advanced, still face limitations in standardization and reproducibility. Proteomics has emerged as a powerful tool in forensic science, providing molecular insights into PMI estimation through the degradation kinetics of specific proteins. Advanced analytical techniques such as mass spectrometry, two-dimensional gel electrophoresis, and enzyme-linked immunosorbent assays (ELISA) enable precise identification and quantification of postmortem protein biomarkers, offering a more objective and reproducible approach to time-of-death estimation (**Secco *et al.*, 2025; Donaldson and Lamont, 2014).** This review aims to examine the role of protein biomarkers in forensic investigations, assess various proteomic methodologies applied to PMI determination in human and animal models, and evaluate their advantages over traditional techniques.

Mechanism of Protein degradation and Proteolysis

After death, the quantity of protein that is present in tissues undergoes degradation due to enzymatic activity and microbial action, which leads to the breakdown of muscle structure and quality deterioration. The primary mechanisms involved in post-mortem protein degradation include are as follows huff **(Huff-Lonergan and Lonergan, 2005; Koohmaraie, 1992).**

- **Autolysis**

Endogenous enzymes (e.g., cathepsins, calpains) start to break down muscle proteins as cellular homeostasis is lost **(Huff-Lonergan and Lonergan, 2005).**

The Proteolytic enzymes from lysosomes are mostly responsible for protein degradation. The process of autolysis is the self-digestion of cells which is triggered by intracellular enzymes. Many times, the Lysosomes can contain cathepsins and other hydrolases that contribute to post-mortem tissue breakdown **(Duong *et al.*, 2021).**

- **Proteolysis by Calpains and Cathepsins**

Calpains is a calcium-dependent protease, that degrades cytoskeletal proteins like titin, nebulin, and desmin, influencing muscle tenderness **(Koohmaraie, 1992).** Cathepsin, a type of lysosomal enzyme further degrades myofibrillar proteins. The Calpain System, mainly contains Calcium-dependent proteases, such as calpains, which play a vital role in degrading cytoskeletal proteins, leading to the loss of structural integrity of proteins **(Huff-Lonergan and Lonergan, 2005).**

- **Microbial Proteolysis**

Post-mortem microbial growth familiarizes proteolytic enzymes that subsidize protein breakdown, and it is a key component in producing spoilage compounds like biogenic amines. After the death of the living being for PMI estimation, bacterial colonization accelerates protein degradation by secreting protease enzymes which leads to early PMI As the bacterial enzymes, the Clostridial, and enteric bacteria produce proteases which leads the level of degradation to the highest peak and accelerate the degradation of muscle proteins

(Gagaoua *et al.*, 2019; Huff-Lonergan and Lonergan, 2005).

The pH can also influence the degradation rate of PMI. A drop in pH due to glycolysis post-mortem affects enzyme activity, it can be the key factor affecting protein breakdown rates.

- **Degradation of Myofibrillar Proteins**

There are different types of Structural proteins like troponin, actin, and myosin that break down into smaller peptides. This process affects meat tenderness and quality in food industries, while also helping in the case of animal protein analysis **(Mathur and Agrawal, 2011).**

The post-mortem degradation of proteins is determined by various means, in combination with autolytic processes, microbial activity, and environmental conditions it affects the PMI rate. Further research into detecting the specific biomarkers and enzymatic pathways will enhance forensic and biomedical applications in Forensic medical disciplines **(Mathur and Agrawal, 2011).**

Protein Biomarker in Postmortem Interval Estimatio

Generally, the biomarkers are defined as protein portions that can used for identifying the specific gene segment with wide characteristics **Donaldson and Lamont, 2014).** There are different biomarkers present in the living body, the different organs in the human body like the heart, liver, lungs, kidney, and skeletal muscles have a variety of segments that define the different characteristics respectively. The brain contains GFAP, Neuron-specific enolase, the heart contains cTnT, cTnI, Creatinine Kinase MB, Myoglobin, Kidney Cystatin C, NGAL Renin enzymes, etc can be taken as biomarkers. The liver contains biomarkers like Albumin, Globulin, Cyt. P 450, Alanine, etc, Skeletal Muscles contain actin, Myosin, Titin, Desmin, and many other biomarkers found.

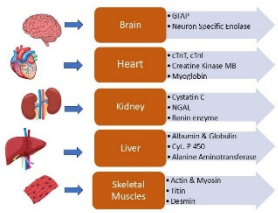

Figure No. 1: Types of Biomarkers present in different tissues

1. Brain Biomarkers

The Brain Biomarkers used for PMI detection include GFAP, or glial fibrillary acidic protein unique to astrocytes aids in the maintenance and repair of neurons. detectable for 24 to 72 hours after death **(Cecchi *et al.*, 2024).**

Gradual degradation is used to evaluate brain injury and estimate early PMI. An indication of the integrity of neurons is the glycolytic enzyme known as neuron-specific enolase (NSE). drastically drops between 12 to 48 hours. PMI estimation is aided by a time-dependent drop in concentration **(Cecchi *et al.*, 2024).**

Tau is a protein that is connected with microtubules in neurons and is essential for structural stability. starts to deteriorate after 24 hours, and by 72 hours, it has significantly broken down. Modifications to the postmortem aid in determining PMI and distinguishing between traumatic and natural death **(Aston *et al.*, 2004).**

2. Heart Biomarkers

The Heart Biomarkers used for PMI detection include a protein that regulates the contraction of the heart muscle called cardiac troponin I (cTnI). deteriorates postmortem in a time-dependent manner. Research has shown that cTnI levels gradually decline after death, with discernible alterations associated with certain PMIs. For a maximum of 72 hours **(Mathur and Agrawal, 2011).**

The oxygen-binding protein myoglobin is found in skeletal and cardiac muscles. shows rapid postmortem deterioration as a result of oxidative damage and proteolysis. Myoglobin degradation rate is a possible indication for early PMI assessment since it corresponds with the time since death. The phase of early postmortem up to 48 hours **(Zhang *et al.*, 2020).**

The enzyme creatine kinase-MB (CK-MB) aids in the regeneration of ATP in cardiac muscle cells. CK-MB activity gradually declines after death. Its usefulness in PMI estimates, especially in cardiac-related mortality, has been shown by quantitative evaluations that show a link between decreasing CK-MB levels and rising PMI. For a maximum of 72 hours (**Zhang *et al.*, 2020; K Zhang *et al.*, 2020**).

3. Kidney Biomarkers

A protein called neutrophil gelatinase-associated lipocalin (NGAL) is linked to stress response and renal tubular injury. It has been shown that NGAL levels

increase after death, and this increase is time-dependent and connected with PMI **(Keltanen *et al.*, 2016).**

PMI in the early to mid-range (up to 72 hours) Cystatin C An inhibitor of cysteine protease that controls tissue proteolytic activity. Research has shown that cystatin C levels gradually decrease after death, and the pace at which it degrades may be used to estimate PMI **(Keltanen *et al.*, 2016).**

PMI between 48 and 120 hrs Tissue damage triggers the production of the pro-inflammatory cytokine interleukin-18 (IL-18). It has been discovered that IL-18 levels rise soon after death and then gradually fall, providing a possible indicator for early PMI assessment **(Dinis-Oliveira, 2024).**

4. Liver Biomarkers

Albumin The liver produces this important plasma protein, which transports different chemicals and keeps oncotic pressure stable. According to postmortem investigations, albumin levels gradually drop as a result of proteolytic breakdown. Environmental variables like humidity and temperature might affect the pace of decrease. PMI between 2 to 72 hrs **(Parmar and Menon, 2015; Costa *et al.*, 2015).**

Globulin group of plasma proteins, such as antibodies, that are involved in immunological responses. little particular information on globulin degradation rates after death. Nonetheless, globulin levels seem to have gradually decreased after death, according to typical protein breakdown trends. Not well defined **(Hoole *et al.*, 2018).**

Enzymes of Cytochrome P450 enzymes that have a role in the metabolism of both endogenous and external substances. Cytochrome P450 enzymes degrade postmortem as a result of microbial activity and autolysis. Although precise timeframes are not well known, various isoforms may deteriorate at varying rates. Not well defined; further investigation is required **(Dinis-Oliveira, 2024).**

5. Skeletal Biomarkers

The work by **(Pittner *et al.*, 2020)** addresses the postmortem breakdown of skeletal muscle proteins as a unique technique to calculate the time after death, an important feature in forensic investigations. Actin structural protein that keeps cells mobile and in their proper form. Degradation becomes considerable after 8 days postmortem, making it less accurate for PMI estimates beyond 10 days **(Foditsch *et al.*, 2016).**

Muscle contraction requires the myosin motor protein. Although there are no clear PM intervals for liver tissue, degradation products do rise with time after death **(Sun *et al.*, 2010).**

The flexibility and structural integrity of muscles are influenced by the titin protein. Progressive degradation into T2 form takes place; noticeable alterations in muscle tissue may be seen up to 28 days after death. There aren't many specific liver tissue statistics available for Up to 28 days **(Wang et al., 2011).**

Muscle cells' structural integrity is preserved by the desmin intermediate filament protein. Postmortem degradation was seen; it was more noticeable in certain muscle groups. There is no clear evidence of specific degradation timescales in liver tissue.

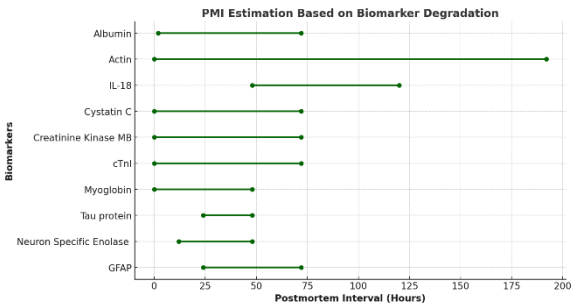

Figure No. 2: Protein Biomarkers with different PMI ranges

Additionally, their research highlights the importance of toxicological analysis in understanding the causes of death, especially in cases involving overdoses or poisonings, which accounted for a substantial number of accidental deaths in the U.S. in 2009 **(Mathur and Agrawal, 2011).** Several proteins, including actin, tropomyosin, myosin, and vimentin, have been discovered as effective biomarkers owing to their predictable breakdown rates. Advanced methods such as mass spectrometry, Western blotting, and proteomic analysis help quantify these proteins across time, giving a biochemical foundation for PMI estimate **(Merkley *et al.*, 2019).**

The study by **(Duong *et al.*, 2021)** highlights how forensic proteomics aids in identifying body fluids, estimating post-mortem intervals, and analyzing samples such as hair, bone, and fingernails. Similarly, it emphasizes that proteomics bridges genotype and phenotype information, making it valuable for

biological forensics, including criminal justice, archaeology, and national security **(Merkley *et al.*, 2019).**

Molecular techniques for protein biomarker analysis

Modern analytical techniques have significantly contributed to biomolecular research, which allows us to study different proteins in depth, different biomolecular interactions, and cellular structures are also studied. Proteomics, immunoassays, spectroscopic imaging, molecular techniques, etc have paved the way for improved diagnostics, disease monitoring, and therapeutic interventions. This paper discusses the latest developments in forensic medicine and highlights their advantages, challenges, and future perspectives by studying the different biomarkers. Key techniques include two-dimensional gel electrophoresis, Mass Spectroscopy, Liquid Chromatography-Tandem Mass Spectroscopy, Protein microarrays, and many more advanced technologies are in a row.

a) Proteomic Approaches

Proteomics is the large-scale study of proteins, their structures, and functions. Two-Dimensional Gel Electrophoresis (2D-GEL) Separates proteins based on isoelectric point and molecular weight. Proteome analysis is most commonly accomplished by a combination of two-dimensional gel electrophoresis (2DE) to separate and visualize proteins and mass spectrometry (MS) for protein identification **(Görg *et al.*, 2004).** Proteomics is the study of the subsets of proteins present in different parts of an organism and how they change with time and varying conditions **(Webb-Robertson *et al.*, 2008).**

Protein Microarrays have a high-throughput analysis of protein interactions and modifications **(Wang *et al.*, 2011).** Over the last 10 years, DNA microarrays have achieved a robust analytical performance, enabling their use for analyzing the whole transcriptome or screening thousands of single-nucleotide polymorphisms in a single experiment. High-throughput microarray technology platforms allow for simultaneous, multi-parametric analysis of complex protein mixtures **(Wang *et al.*, 2011).**

Immunoassay based techniques

Immunoassays detect and quantify biomolecules using antigen-antibody interactions. Common techniques include Enzyme-Linked Immunosorbent Assay (ELISA), which is highly sensitive and widely used in diagnostics and treating various biological diseases. A rapid and simple detection method for iprodione was reached by the combination of the sample dilution and the devised extraction method, which required no instruments for extraction **(Lee *et al.*, 2008).**

Table No. 1: Diverse Molecular techniques used for PMI Estimation

Molecular Techniques for Protein Biomarker Analysis	**Proteomic Approach**	**2D Gel Electrophoresis**
		Protein Microarray
		Quantitative Proteomics
	Immunoassay Techniques	**ELISA**
		Western Blot
		Lateral Flow Assay
	Spectroscopic Techniques	**FTIR**
		LC-MS/MS (Tandem)
		Fluorescence Spectroscopy

Western Blotting is one of the best techniques for detecting PMI from protein samples and it is a much more reliable technique when we have to detect some specific proteins from complex mixtures. Lateral Flow Assays (LFAs) is also a Point-of-care diagnostics method for rapid detection of proteins from tissues **(Posthuma-Trumpie *et al.*, 2009).** Electrochemiluminescence Immunoassay (ECLIA) has an enhanced sensitivity and dynamic range for clinical applications in laboratories **(Hajduk *et al.*, 2016).** Immunoassays have revolutionized clinical diagnostics by providing rapid and reliable biomarker detection for time since estimation.

b) Spectroscopic and imaging techniques

These techniques provide structural and functional insights into biomolecules and cells. Fourier-Transform Infrared (FTIR) Spectroscopy is used to identify functional groups present in biomolecules

Fourier transform infrared (FTIR) spectroscopy is an attractive tool for proteomics research as it can be used to rapidly characterize protein secondary structure in aqueous solution **(Aboul-enein *et al.*, 2014).** Mass Spectrometry (MS) Identifies proteins and post-translational modifications. In addition, simultaneous improvements to and development of mass analyzers and detectors have greatly increased the use of mass spectrometry for biological studies. Mass spectrometers measure masses of charged species, so the source must be able to produce ions **(Domon and Aebersold, 2006).**

Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) enhances protein quantification and identification, Liquid chromatography coupled with mass spectrometry (LC-MS) has been widely used for profiling protein expression levels **(Tsai et al., 2016).** In proteomic studies, liquid chromatography coupled with mass spectrometry (LC-MS) is a common platform to compare the abundance of various peptides that characterize particular proteins in biological samples **(Varghese and Ressom, 2011).**

One important parameter distinguishing Raman spectroscopy from many other spectroscopic methods is its applicability to systems containing high concentrations of proteins, which is critical for the investigation of structural changes during processes such as coagulum or gel formation. Raman Spectroscopy provides label-free molecular fingerprinting **(Zhang *et al.*, 2016; Li-Chan *et al.*, 1994).** Fluorescence spectroscopy enables the visualization of biomolecules using fluorophores. It is used in PMI estimation to study the conformational changes and dynamics of proteins. This method is particularly useful for analyzing the binding of ligands to proteins **(Lichtman and Conchello, 2005).**

c) Emerging Molecular Techniques:

Recent advancements in molecular biology have led to novel diagnostic and analytical methods. Emerging molecular techniques for estimating post-mortem interval (PMI) increasingly leverage proteomic approaches, highlighting the degradation patterns of specific proteins as potential biomarkers. Studies indicate that proteins such as alpha-actinin, GAPDH, and eEF1A2 exhibit distinct degradation kinetics post-mortem, with some proteins like tropomyosin remaining stable for extended periods (**Chhikara *et al.*, 2024 ; Choi *et al.*, 2019).** Techniques such as mass spectrometry and protein chip technology facilitate the identification and quantification of these proteins, allowing for the establishment of statistical models to correlate protein changes with PMI **(K. Zhang *et al.*, 2020).**

Crispr-based technology is very precise and also termed a gene editing tool, and rapid pathogen detection **(Doudna and Charpentier, 2014).** Next-Generation Sequencing (NGS) is a High-throughput sequencing technology used for genomics and transcriptomics. While CRISPR is primarily used for genome editing, it has potential applications in forensic science for identifying post-mortem molecular changes **(Stewart et al., 2009).**

Digital PCR (dPCR) is also one of the key techniques used in the time since estimation, it can easily quantify the absolute number of nucleic acids. These cutting-edge techniques are transfiguring disease research and personalized medicine **(Stewart et al., 2009).** Proteomic approaches, immunoassays, spectroscopic and imaging methods, and molecular techniques are critical tools in biomedical research and clinical diagnostics. Their continued development promises enhanced accuracy, sensitivity, and efficiency in disease detection and treatment.

Factor affecting Protein Biomarker Degradation

Protein biomarkers are measurable indicators of biological states or conditions and are extensively used in medical diagnostics. However, protein degradation is a major challenge that can compromise their detection and quantification. Protein degradation is influenced by a combination of intrinsic and extrinsic factors, necessitating stringent measures for sample collection, processing, and storage to ensure biomarker integrity.

- **Key Factor that affects PMI rate**

Human and animal (mouse, pig) skeletal muscles, brain, cartilage, heart tissue, lung, and pancreas were among the tissues examined; post-mortem intervals (PMIs) ranged from 0 to 38 days. Western blotting, SDS-PAGE, immunohistochemistry, casein zymography, and histopathology were among the methodological approaches used. Four studies showed that proteins broke down more readily at higher temperatures than at lower ones **(Kumar *et al.*, 2016; Poloz and O'Day, 2009; Foditsch *et al.*, 2016).**

Two Western blot studies using human skeletal muscle and cardiac tissue investigated the impact of the cause of death on protein breakdown. Only one study used ADD and targeted cluster analysis to investigate how age affects postmortem protein breakdown in human skeletal muscle. When examined in an age-adjusted

cohort, the relationship between protein degradation and ADD was shown to increase, indicating that degradation rates are greater in the middle-age range **(Kumar *et al.*, 2016; Pittner *et al.*, 2020).**

Once again, just one study examined how exposure conditions (such as shade, direct sunlight, or under a canopy of branches) affected the breakdown of skeletal muscles in pig cadavers, which were shown to be mostly resistant to exposure's effects **(Pittner *et al.*, 2020).**

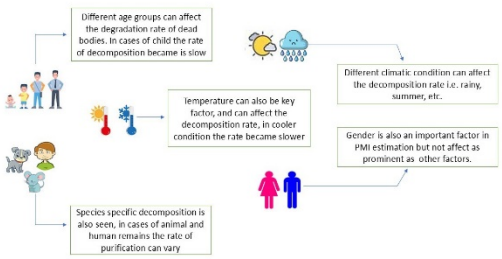

Figure No. 3: Factors that can affect the rate of PMI

The effect of microbes on the decomposition of collagen in bones was investigated. Therefore, hay infusions were poured into boxes containing buried bones, and the results were compared to a control group. The researchers found that over the three months under investigation, Co/NCo ratios decreased, but that the presence of microbes had no discernible impact.

Protein biomarker degradation poses a significant challenge in clinical and research applications. Various intrinsic and extrinsic factors contribute to this instability. By understanding and mitigating these factors, researchers and clinicians can improve biomarker reliability, leading to more accurate diagnostics and research outcomes **(Pittner *et al.*, 2020; K. Zhang *et al.*, 2020).**

Conclusion

The use of protein biomarkers for estimating the postmortem interval (PMI) is transforming forensic science by providing a more reliable and objective alternative to traditional methods like rigor mortis, livor mortis, and algor mortis. Unlike these conventional approaches, which can be influenced by external conditions, proteins degrade in a systematic and measurable way, making them powerful forensic tools. Through advanced techniques such as mass spectrometry, ELISA, Western blotting, and spectroscopy, researchers have identified key proteins—like actin, myosin, tropomyosin, desmin, cardiac troponins, and neuron-specific enolase—that break down in a predictable manner, helping forensic scientists estimate time since death with greater precision.

Despite these advancements, there are still challenges to overcome. Environmental factors, such as temperature and microbial activity, can affect protein degradation, and standardizing forensic proteomics for widespread use remains a hurdle. Future research should focus on identifying new, highly reliable biomarkers across different tissues, refining existing techniques, and integrating artificial intelligence (AI) and machine learning to analyze vast forensic datasets. AI-driven models could help detect subtle patterns in protein degradation, improving accuracy and minimizing human error.

The future of forensic science also lies in portable, real-time diagnostic tools that could allow investigators to analyze protein biomarkers at crime scenes, reducing dependency on complex laboratory setups. Additionally, integrating multiomic approaches—combining proteomics with genomics and metabolomics—could provide a more holistic view of the biochemical changes that occur after death. Understanding how proteins behave under different environmental conditions will further refine PMI estimation and improve forensic investigations.

However, as we push the boundaries of forensic proteomics, it is also essential to address ethical and legal considerations. Establishing global forensic databases and creating standardized protocols will ensure consistency and reliability across different forensic labs. Legal systems must also adapt to these advancements, ensuring that molecular-based forensic evidence is admissible in court.

In conclusion, protein biomarkers represent a promising frontier in forensic science, offering a scientific, data-driven approach to PMI estimation. With continued research, technological advancements, and cross-disciplinary collaboration, forensic proteomics has the potential to redefine how we determine time since death, bringing greater accuracy and reliability to forensic investigations and justice systems worldwide.

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