forensic sciences

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Brief Report

An Assessment of Intra- and Interobserver Error in Luminol Chemiluminescence as a Presumptive Test for Postmortem Interval Estimation

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Abstract: Postmortem interval (PMI) estimation constitutes a challenge for forensic anthropologists. The application of the luminol technique as a preliminary test for PMI estimation is considered easy to use and inexpensive. The objective of our study is to validate luminol chemiluminescence testing through the assessment of intra- and interobserver error. Our sample included 266 human clavicles, with known PMIs ranging between 2655 days and 450 years. After sample preparation, luminol was applied, and the results were observed by two different observers. The intensity of the reaction was measured using a binary scale and a 5-level scale, according to the increasing degree of chemiluminescence. The Kappa statistic was used for the assessment of the intra- and interobserver agreement. The obtained results showed total interobserver error agreement regarding the binary scale and a K = 0.98 (95% CI: 0.97–0.99) regarding the 5-level scale. Additionally, Observer 1 and Observer 2 obtained a 100% agreement concerning the binary scale and a K = 1 (95%CI 0.99–1) and K = 0.99 (95% CI 0.99–1), respectively, concerning the 5-level scale. According to our research, it is possible to conclude that luminol chemiluminescence testing is suitable as a rapid diagnostic test, revealing this method as practically independent of observation error.

Keywords: postmortem interval; luminol; chemiluminescence; interobserver error; intraobserver error

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1. Introduction

The estimation of the postmortem interval (PMI) presents itself as a significant challenge within the realm of forensic sciences. This parameter holds a critical role in establishing the forensic relevance of human remains. Hence, the importance of conducting this evaluation becomes evident, as establishing the forensic origin of skeletal remains would demand a distinct investigative approach compared to archaeological remains. However, its precision is intrinsically linked to the time since death, as the accuracy of PMI estimation becomes increasingly intricate in longer postmortem intervals [1,2]. Despite the growing number of studies aiming to develop suitable methodologies for estimating the PMI, the effort to date skeletonized remains persists as a complex task for forensic anthropologists [3].

When dealing with forensic situations, a range of issues emerges involving expenses, time constraints, the quantity and dimensions of the fragments, their origin, the state of preservation and the decomposition conditions, and the feasibility of employing certain techniques over others, among other factors [4]. Considerable attention has been directed toward investigating the luminol technique for estimating the PMI in skeletonized remains

as a preliminary assessment. This is due to its perceived simplicity, affordability, and applicability to visual interpretation, even without sophisticated equipment [5–7].

On account of its high sensitivity, luminol is commonly employed in forensic contexts, primarily for detecting blood traces imperceptible to the naked eye, turning them visible due to its chemiluminescent properties [8]. More recently, its application in forensics has expanded to estimating the postmortem interval of skeletal remains, by correlating the time since death with the persistence of hemoglobin traces within the bone tissue [7,9–11]. Previous studies have showed that even minute concentrations of hemoglobin (ranging from 1:100,000 to 1:5,000,000) can induce a detectable chemiluminescent reaction [10]. Nonetheless, it is crucial to remember that this method is susceptible to potential interference such as household bleach (sodium hypochlorite), turnips, horseradish or parsnips, furniture polishes and enamel paints, or even motor vehicles' interior fabrics [12,13]. Thus, by applying luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) to traces of hemoglobin within bone tissue, a reaction of chemiluminescence occurs through the production of molecular nitrogen and an excited 3-aminophthalate dianion, which is the emission of chemiluminescent light attributed to the radiative deactivation of this dianion from its singlet excited state (see Figure 1) [14,15]. The occurrence of this reaction demands a redox catalyst, specifically the iron ions in the hemoglobin composition found within bones [13,16]. This phenomenon leads to the emission of light in the visible spectrum ranging from violet to blue, arising from the chemical reaction [5,9,15,17]. Over time, the quantity of hemoglobin present in skeletonized remains reduces [5,10,15], resulting in an anticipated reduction in the intensity of the chemiluminescence reaction as the postmortem interval increases [7,10,11].

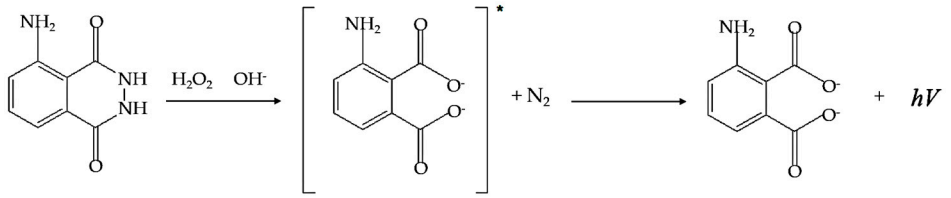

Figure 1. Chemiluminescence reaction formula of luminol (adapted from Barni et al., 2007 [15]).

The aim of this study is to evaluate the feasibility of luminol chemiluminescence testing as a method that is both simple and economical for estimating time since death, undertaking an assessment of the chemiluminescence repeatability within a significant and well-sized sample, including a broad range of PMI intervals, through intra- and interobserver error calculation.

2. Materials and Methods

Our study included human bones from 266 adult individuals, with ages between 20 and 100 years old, of both sexes, without visible or known bone pathology or antemortem/perimortem trauma, with different and known postmortem intervals, including subsamples from different origins: fresh bones from autopsies performed at the Portuguese National Institute of Legal Medicine and Forensic Sciences (INMLCF.IP.), South (n = 12) and Centre Branches (n = 8); dry skeletonized remains from 21st Century Identified Skeletal Collection [18] (n = 208); dry skeletonized remains from autopsies performed in the 20th century at the INMLCF.IP., South Branch (n = 29); remains from the Valle da Gafaria's osteoarcheological collection [19] (n = 9). Among the subsamples, the PMI ranged between 2655 days and 450 years. To ensure sample standardization, the study focuses solely on a single bone type, namely, the clavicle (commonly known as the collar bone).

The fresh clavicles from autopsy were formerly prepared and reduced to powder by our team in a previous study [5]. They were obtained from fresh cadavers during the autopsy (after consulting RENNDA—National Register of Non-Donors—and receiving approval from the Ethics Commission of the Portuguese National Institute of Legal Medicine and Forensic Sciences – CE-010/2018) and preserved by freezing at 4 °C (to meet

the mandatory conditions for follow-up work) in labelled zip-lock freezer bags. These bones were defrosted and macerated in water and cleaned manually with fresh sandpaper, brushes, and scalpels.

The sample treatment, conducted entirely by the same operator, involved manual cleaning with sandpaper and brushes, and for the reduction into powder, the bones were scraped with a scalper (Figure 2). For each clavicle, 50 mg of powder was collected in each Eppendorf tube (three replicas per individual) for the luminol technique. The Luminol 16 oz solution was prepared according to the directions included in the reagent (pre-prepared kit). Each sample was placed on a clean white paper sheet and luminol was applied using a spray bottle at room temperature, in a dark room. As a positive control, a drop of blood was used.

Figure 2. Reduction of the clavicle into powder.

The results were examined in situ at the moment of the chemiluminescence reaction with the naked eye by two different observers with different levels of expertise (Observer 1 had a higher level of expertise and Observer 2 had no previous experience), and photographed with a Canon EOS 500D (F/5, 30sec, ISO 3200, 27 mm) (Canon Inc., Tokyo, Japan) in a total absence of light. The intensity of the reaction was measured using a binary scale (positive or negative) and a 5-level scale according to the increasing degree of chemiluminescence (see Table 1, and Figure 3, for details). The observations by the two observers were carried out simultaneously and blindly when the reaction occurred, and the recording of the results occurred independently, with no influence from each other. For the intraobserver error, three replicas of each sample were blindly analyzed by each observer. At the time of the observations, the samples underwent anonymization, which meant that the postmortem interval remained undisclosed to both of the distinct observers.

Table 1. Interpretation of the 5-level scale for the chemiluminescence reaction (adapted from Ermida et al., 2017 [5]).

Result	Interpretation
Negative (−)	No chemiluminescence. The reaction is not visible to the naked eye.
Barely positive (+)	The reaction is barely visible to the naked eye.
Weak positive (++)	The reaction is visible to the naked eye but with low intensity.
Positive (+++)	The reaction is easily visible to the naked eye.
Strong positive (++++)	The reaction is strongly visible to the naked eye.

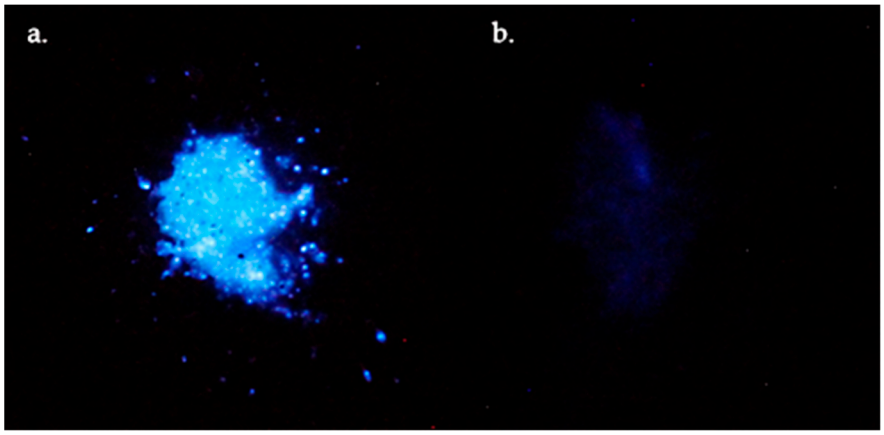

Figure 3. Chemiluminescence reaction. (**a**) Strong positive reaction; (**b**) weak positive reaction.

The Kappa statistic is frequently used when data requires statistical methods to assess the technique's repeatability. It calculates the proportion of agreement, taking chance agreement into account. It is calculated as: $\frac{(observed\ agreement - expected\ agreement)}{(1 - expected\ agreemnt)}$. Kappa is equal to 1 when two measurements perfectly agree and to 0 when two measurements only agree at the chance level [20]. In the present research, Cohen's Kappa statistic was used for the assessment of the intra- and interobserver agreement, with the usage of the unweighted values for the binary scale results and the weighted values for the 5-level scale (ordinal scale), evaluating the agreement between observations that takes chance into account, in order to improve the assessment of the luminol's chemiluminescence repeatability.

3. Results

The obtained results, recorded by both observers, are displayed in the plot illustrated in Figure 4. In this plot, it is possible to examine the overlap between what was observed by Observer 1 and Observer 2. Based on the obtained results, it is evident that the intensity of chemiluminescence reactions diminishes in individuals with an extended postmortem interval.

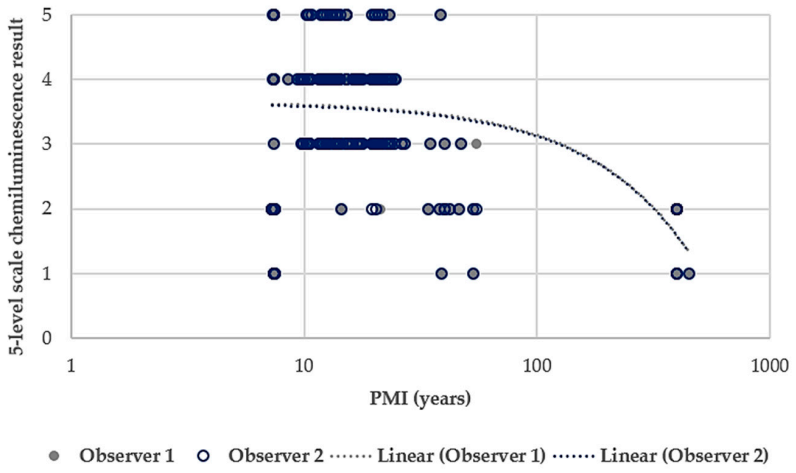

Figure 4. Perceived luminol's chemiluminescence using the 5-level scale (Observer 1 and Observer 2).

As previously mentioned, Cohen's Kappa statistic test was used for the assessment of the relationship between the error variables (please refer to Tables S1–S14 in the Supplementary Material). The subjectivity inherent in this technique was considered for two different circumstances: between the two observers responsible for evaluating the chemiluminescence reaction—interobserver error (Tables 2 and 3), and between repetitions of

the same sample by the same observer—intraobserver error (Tables 4–6). Both inter- and intraobserver error were conducted for the binary scale and 5-level scale.

Table 2. Kappa statistics: interobserver error applying the binary scale. K = 1 (95% CI: 1–1); (− negative; + positive).

		Observer 1	
		−	+
Observer 2	−	10	0
	+	0	256

Table 3. Kappa statistics: interobserver error applying the 5-level scale. K = 0.98 (95% CI: 0.97–0.99); (− negative; + barely positive; ++ weak positive; +++ positive; ++++ strong positive).

		Observer 1				
		−	+	++	+++	++++
Observer 2	−	10	0	0	0	0
	+	0	21	4	0	0
	++	0	1	90	2	0
	+++	0	0	1	100	3
	++++	0	0	0	1	33

Table 4. Kappa statistics: intraobserver error (Observers 1 and 2) between replicas 1 and 2 applying the binary scale. K = 1 (95% CI: 1–1); (− negative; + positive).

		Replica 1	
		−	+
Replica 2	−	10	0
	+	0	256

Table 5. Kappa statistics: intraobserver error (Observer 1) between replicas 1 and 2 applying the 5-level scale. K = 1 (95% CI: 0.99–1); (− negative; + barely positive; ++ weak positive; +++ positive; ++++ strong positive).

		Replica 1				
		−	+	++	+++	++++
Replica 2	−	10	0	0	0	0
	+	0	21	0	0	0
	++	0	1	95	0	0
	+++	0	0	0	103	0
	++++	0	0	0	0	36

Table 6. Kappa statistics: intraobserver error (Observer 2) between replicas 1 and 2 applying the 5-level scale. K = 0.99 (95% CI: 0.99–1); (− negative; + barely positive; ++ weak positive; +++ positive; ++++ strong positive).

		Replica 1				
		−	+	++	+++	++++
Replica 2	−	10	0	0	0	0
	+	0	24	0	0	0
	++	0	1	92	1	0
	+++	0	0	1	103	0
	++++	0	0	0	0	34

Tables 2 and 3 display the Kappa statistic quantification of the two different observers' disagreement between chemiluminescence reactions after luminol. Concerning the inter-observer error, the binary scale obtained results that indicate a K = 1 (95% CI=1–1). The 5-level scale, on the other hand, has a K = 0.98 with a 95% confidence interval ranging from a minimum of 0.97 to a maximum of 0.99. Regarding the 5-level scale, as an ordinal scoring of luminol chemiluminescence, linear weights were considered, given it is important for the degree of disagreement to be reflected, introducing a greater emphasis on large differences between categories and less on small differences [20].

Additionally, Tables 4–6 show the quantification, through Kappa statistics, of the disagreement between two chemiluminescence analysis performed by the same observer, obtained from the same sample (two replicas). Although three replicas were conducted for each sample, no statistically significant differences were found among the three repetitions (see the Supplementary Material for more information), so the authors decided to only present the results for the intraobserver error between the first two replicas. Only in a single circumstance did the repetition of the luminol testing lead to a different result regarding Observer 1′s three replicas. The less experienced observed, Observer 2, had a different result in only three cases. Both observers obtained the exact same calculated intraobserver bias regarding the binary scale, with a 100% agreement (Table 4). Regarding the ordinal 5-level scale, Observer 1 attained a K = 1 with a 95% confidence interval between 0.99 and 1, and Observer 2 a K = 0.99 with a 95% confidence interval between 0.99 and 1.

4. Discussion and Conclusions

The assessment of luminol's chemiluminescence intra- and interobserver bias supports the potential of this method as a presumptive test for estimating time since death, and the obtained results are in line with the ones obtained in previous investigations [5,7]. It is important to highlight the robustness of the sample used, both in size and in its composition, including a wide range of PMI intervals, further emphasizing the importance of the results arising from this investigation. The Kappa statistical analysis illustrated an intraobserver and interobserver agreement superior to 0.9 in all the experiments, which, according to the literature, is suggestive of a nearly perfect agreement (>0.80) [21]. These results support the idea that even a less experienced observer, or one with no experience at all (as was the case with Observer 2), can use this technique with a reliable outcome. Thus, the current investigation can corroborate luminol chemiluminescence, not only as an inexpensive and simple technique, but also as easy to interpret, confirming its validity.

The process of reducing the fresh clavicles to powder from autopsy displayed some variances. Nevertheless, these small disparities in sample preparation have no discernible impact on the results obtained in the current study, as it is exclusively based on the assessment of chemiluminescent intra- and interobserver error.

It is also really important to emphasize our study's outcome, as it contributes to bringing the use of this method closer to its practical application. In a forensic case, standards of quality and reliability must be met in the methods applied [22]. According to the Daubert Standards (US federal standards specified in 1993 in Daubert vs. Merrell Dow Pharmaceuticals), in order for a methodology to be considered valid in a court of law, it must fulfil the following conditions: (1) it is capable of being tested and has undergone testing; (2) it has undergone peer review and publication; (3) its existing or potential error rate is known; (4) there are established standards for the control of the technique's operation; and (5) it has gained significant acceptance within the relevant scientific community [23–26]. These criteria intend to ensure the reliability, replicability, and relevance of scientific testimony in court. It also led the forensic disciplines to recognize the need to reconsider some of the techniques used in their analysis and follow these standards as good practice, validating their statements with scientifically tested methods substantiated by probability assessments [23,27]. Regarding these criteria, our findings bring the application of the luminol chemiluminescence method closer to potential acceptance in the legal set-

ting, as the intra- and interobserver assessment repeatability has an extremely high level of consistency.

However, for this technique to meet all the mandatory Daubert Standards, there is still a lot of work to be done. Although luminol chemiluminescence testing is considered a promising technique for PMI estimation, since it is simple, inexpensive, and easy to interpret, it is still not possible to properly relate the reaction intensity with PMI-specific intervals. Therefore, this method is mainly used as a presumptive test to identify human remains of forensic interest, since the intensity of the chemiluminescence reactions clearly decreases in archaeological remains, which can be supported by the results of this study. So, it cannot be used as a single method to date time since death (the obtained results need further confirmation from methods such as radiocarbon analyses). In order to achieve more accurate results, further research is required. Subsequent studies should delve into the quantification of the light derived from the chemiluminescence reaction, as well as on the influence of the taphonomic factors in the decomposition process that affect the results obtained through this method. However, the call for future research is substantiated by promising outcomes achieved by studies, as the one presented in this work, which highlight the value of this technique in terms of its repeatability.

In conclusion, according to our study, the luminol technique is suitable as a rapid diagnostic test, given the intra- and interobserver error obtained, revealing it as practically independent of observation bias. In future research, it would be advantageous to reproduce this experiment with a larger number of observers.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/forensicsci3040044/s1, Table S1: Cohen's Kappa analysis, Observer 1 vs. Observer 2, binary scale; Table S2: Cohen's Kappa analysis, Observer 1 vs. Observer 2, 5-level scale; Table S3: Cohen's Kappa analysis, Observer 1, Replica 1 vs. Replica 2, binary scale; Table S4: Cohen's Kappa analysis, Observer 1, Replica 1 vs. Replica 2, 5-level scale; Table S5: Cohen's Kappa analysis, Observer 1, Replica 1 vs. Replica 3, binary scale; Table S6: Cohen's Kappa analysis, Observer 1, Replica 1 vs. Replica 3, 5-level scale; Table S7: Cohen's Kappa analysis, Observer 1, Replica 2 vs. Replica 3, binary scale; Table S8: Cohen's Kappa analysis, Observer 1, Replica 2 vs. Replica 3, 5-level scale; Table S9: Cohen's Kappa analysis, Observer 2, Replica 1 vs. Replica 2, binary scale; Table S10: Cohen's Kappa analysis, Observer 2, Replica 1 vs. Replica 2, 5-level scale; Table S11: Cohen's Kappa analysis, Observer 2, Replica 1 vs. Replica 3, binary scale; Table S12: Cohen's Kappa analysis, Observer 2, Replica 1 vs. Replica 3, 5-level scale; Table S13: Cohen's Kappa analysis, Observer 2, Replica 2 vs. Replica 3, binary scale; Table S14: Cohen's Kappa analysis, Observer 2, Replica 2 vs. Replica 3, 5-level scale.

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Institutional Review Board Statement: The authors declare that this study was approved by the Ethics Commission of the Portuguese National Institute of Legal Medicine and Forensic Sciences (CE-010/2018) and certify that the study was performed in accordance with the ethical standards as laid down in the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards.

Informed Consent Statement: Not applicable.

Data Availability Statement: Further data that support the findings of this study are available from the corresponding author upon reasonable request.

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References

1. Cunha, E.; Cattaneo, C. Forensic Anthropology and Forensic Pathology. In *Forensic Anthropology and Medicine: Complementary Sciences from Recovery to Cause of Death*; Schmitt, A., Cunha, E., Pinheiro, J., Eds.; Humana Press: Totowa, NJ, USA, 2006; pp. 39–53; ISBN 978-1-59745-099-7.
2. Forbes, S.; Nugent, K. Dating of Anthropological Skeletal Remains of Forensic Interest. In *Handbook of Forensic Anthropology and Archaeology*; Blau, S., Ubelaker, D.H., Eds.; Routledge: New York, NY, USA, 2016; pp. 213–225; ISBN 978-1-315-52893-9.
3. Ermida, C.; Cunha, E.; Ferreira, M.T. Dating Death: Post Mortem Interval Estimation of Human Skeletal Remains. *Antropol. Port.* **2022**, *39*, 45–72. [CrossRef]
4. Cappella, A.; Gibelli, D.; Muccino, E.; Scarpulla, V.; Cerutti, E.; Caruso, V.; Sguazza, E.; Mazzarelli, D.; Cattaneo, C. The Comparative Performance of PMI Estimation in Skeletal Remains by Three Methods (C-14, Luminol Test and OHI): Analysis of 20 Cases. *Int. J. Leg. Med.* **2018**, *132*, 1215–1224. [CrossRef]
5. Ermida, C.; Navega, D.; Cunha, E. Luminol Chemiluminescence: Contribution to Postmortem Interval Determination of Skeletonized Remains in Portuguese Forensic Context. *Int. J. Leg. Med.* **2017**, *131*, 1149–1153. [CrossRef]
6. Quickenden, T.I.; Cooper, P.D. Increasing the Specificity of the Forensic Luminol Test for Blood. *Luminescence* **2001**, *16*, 251–253. [CrossRef]
7. Ramsthaler, F.; Kreutz, K.; Zipp, K.; Verhoff, M.A. Dating Skeletal Remains with Luminol-Chemiluminescence. Validity, Intra- and Interobserver Error. *Forensic Sci. Int.* **2009**, *187*, 47–50. [CrossRef]
8. Caudullo, G.; Caruso, V.; Cappella, A.; Sguazza, E.; Mazzarelli, D.; Amadasi, A.; Cattaneo, C. Luminol Testing in Detecting Modern Human Skeletal Remains: A Test on Different Types of Bone Tissue and a Caveat for PMI Interpretation. *Int. J. Leg. Med.* **2017**, *131*, 287–292. [CrossRef]
9. Creamer, J.I.; Buck, A.M. The Assaying of Haemoglobin Using Luminol Chemiluminescence and Its Application to the Dating of Human Skeletal Remains. *Luminescence* **2009**, *24*, 311–316. [CrossRef]
10. Introna, F.; Di Vella, G.; Campobasso, C.P. Determination of Postmortem Interval from Old Skeletal Remains by Image Analysis of Luminol Test Results. *J. Forensic Sci.* **1999**, *44*, 535–538. [CrossRef]
11. Ramsthaler, F.; Ebach, S.C.; Birngruber, C.G.; Verhoff, M.A. Postmortem Interval of Skeletal Remains through the Detection of Intraosseal Hemin Traces. A Comparison of UV-Fluorescence, Luminol, Hexagon-OBTI®, and Combur® Tests. *Forensic Sci. Int.* **2011**, *209*, 59–63. [CrossRef]
12. Creamer, J.I.; Quickenden, T.I.; Apanah, M.V.; Kerr, K.A.; Robertson, P. A Comprehensive Experimental Study of Industrial, Domestic and Environmental Interferences with the Forensic Luminol Test for Blood. *Luminescence* **2003**, *18*, 193–198. [CrossRef]
13. Quickenden, T.I.; Creamer, J.I. A Study of Common Interferences with the Forensic Luminol Test for Blood. *Luminescence* **2001**, *16*, 295–298. [CrossRef]
14. Giussani, A.; Farahani, P.; Martínez-Muñoz, D.; Lundberg, M.; Lindh, R.; Roca-Sanjuán, D. Molecular Basis of the Chemiluminescence Mechanism of Luminol. *Chem. A Eur. J.* **2019**, *25*, 5202–5213. [CrossRef]
15. Barni, F.; Lewis, S.W.; Berti, A.; Miskelly, G.M.; Lago, G. Forensic Application of the Luminol Reaction as a Presumptive Test for Latent Blood Detection. *Talanta* **2007**, *72*, 896–913. [CrossRef]
16. Creamer, J.I.; Quickenden, T.I.; Crichton, L.B.; Robertson, P.; Ruhayel, R.A. Attempted Cleaning of Bloodstains and Its Effect on the Forensic Luminol Test. *Luminescence* **2005**, *20*, 411–413. [CrossRef]
17. Sarabia, J.; Pérez-Martínez, C.; Hernández del Rincón, J.P.; Luna, A. Study of Chemiluminescence Measured by Luminometry and Its Application in the Estimation of Postmortem Interval of Bone Remains. *Leg. Med.* **2018**, *33*, 32–35. [CrossRef]
18. Ferreira, M.T.; Coelho, C.; Makhoul, C.; Navega, D.; Gonçalves, D.; Cunha, E.; Curate, F. New Data about the 21st Century Identified Skeletal Collection (University of Coimbra, Portugal). *Int. J. Leg. Med.* **2021**, *135*, 1087–1094. [CrossRef]
19. Ferreira, M.T.; Coelho, C.; Wasterlain, S.N. Discarded in the Trash: Burials of African Enslaved Individuals in Valle Da Gafaria, Lagos, Portugal (15th–17th Centuries). *Int. J. Osteoarchaeol.* **2019**, *29*, 670–680. [CrossRef]
20. Sim, J.; Wright, C.C. The Kappa Statistic in Reliability Studies: Use, Interpretation, and Sample Size Requirements. *J. Am. Phys. Ther. Assoc.* **2005**, *85*, 257–268. [CrossRef]
21. Landis, J.R.; Koch, G.G. The Measurement of Observer Agreement for Categorical Data. *Biometrics* **1977**, *33*, 159–174. [CrossRef]
22. Passalacqua, N.; Pilloud, M. The Need to Professionalize Forensic Anthropology. *Eur. J. Anat.* **2021**, *25*, 35–47.
23. Christensen, A.M.; Crowder, C.M. Evidentiary Standards for Forensic Anthropology. *J. Forensic Sci.* **2009**, *54*, 1211–1216. [CrossRef]
24. Daubert v. Merrell Dow Pharmaceuticals Inc., 509 US 579. 1993. Available online: https://supreme.justia.com/cases/federal/us/509/579/ (accessed on 29 September 2023).

25. Lesciotto, K.M. The Impact of Daubert on the Admissibility of Forensic Anthropology Expert Testimony. *J. Forensic Sci.* **2015**, *60*, 549–555. [CrossRef]
26. Rosa, J.; Marques, M.P.M.; Gonçalves, D.; Ferreira, M.T. Half a Century of Systematic Research on Heat-Induced Colour Changes in Bone—A Review. *Sci. Justice* **2023**, *63*, 573–580. [CrossRef]
27. Dirkmaat, D.C.; Cabo, L.L.; Ousley, S.D.; Symes, S.A. New Perspectives in Forensic Anthropology. *Am. J. Phys. Anthropol.* **2008**, *137*, 33–52. [CrossRef]