Title: RNA degradation of blood reveals a time diagnostic window in a controlled environment.

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Declaration of Competing Interest

The authors report no declarations of interest.

Highlights

- Total RNA quality and quantity metrics measured in degrading bloodstains.
- RNA quality is correlated to time.
- Diagnostic drop in RNA quality metrics after 24 hours.
- Biological replicates produced similar results for RNA quality metrics.
- Cold temperature better preserved larger RNA fragments than ambient temperature.

Abstract

Determining the age, or time since deposition (TSD), of bloodstains would provide forensic scientists with critical information regarding the timeline of the events involving bloodshed. The physicochemical changes that occur to major biomolecules as a bloodstain degrades can be used to approximate the TSD of bloodstains. Our study aims to quantify the timewise and temperature degradation trends found in total RNA from bloodstains, expanding the scope of the TSD research which has previously explored DNA and targeted mRNA molecules. Whole bovine blood was stored in plastic microcentrifuge tubes at 21°C or 4°C and tested over different timepoints spanning one week. A total of nine RNA metrics were visually assessed and quantified using linear and mixed models. The RNA Integrity Number equivalent (RINe) and the DV200 demonstrated strong relationships with time and independence from one another. Both exhibited negative trends over time, showing a decrease in both the quality and quantity of larger RNA fragments that varied with temperature. The RINe model fit was high ($R^2 = 0.60$), and while including the biological replicate as a random effect increased the fit for all RNA metrics, no significant differences were found between biological replicates stored at the

same temperature for the RINe and DV200 metrics; importantly, this suggests that both these standardized metrics can likely be directly compared between scenarios and individuals. The presence of a significant decrease in the retained RNA quality metrics after 24 hours suggests that this method could be used to differentiate day-old bloodstains from older bloodstains. Our models also demonstrated a faster rate of RNA degradation at room temperature than at 4°C, supporting the need to develop regional models. This study provides a novel approach for blood TSD estimates, producing metrics that are not affected by inter-individual variation and improving our understanding of the degradation occurring in bloodstains, bringing us one step closer to eventual crime scene application.

Keywords

RNA Integrity Number equivalent, Time since deposition, DV200, Bloodstain, Temperature, Aging

1. Introduction

Accurately determining the age of biological evidence, including soft tissue and bodily fluids, remains a complex task in forensic investigations [1,2]. Bloodstained evidence is commonly observed at the scenes of violent crimes, and determining the age, or time since deposition (TSD) of the blood, would provide context in criminal investigations, including the development of investigative leads and the assessment of alibis [1–4]. Currently, blood observed at crime scenes can be subjected to bloodstain pattern analysis (BPA), which provides

potential interpretations of the physical events that gave rise to the bloodshed [5]. DNA profiling, used for attribution purposes, can be carried out from collected bloodstains [6–9]. These tools do not provide TSD estimates of blood, requiring additional analytical approaches to be investigated. A bloodstain ages in a series of complex processes influenced by many factors, ranging from its formation on a surface [10–13] to the degradation it undergoes over time [14–17]. Previous studies have developed chemometric [2,14,15,18] and genetic methods [3,19–21] that estimate the TSD of bloodstains in controlled laboratory conditions. These methods generally exhibit low sensitivity and specificity in differentiating key timepoints, as well as high variability between samples and environmental conditions [3,14,15,18–20,22].

Ex-vivo, blood undergoes a series of time-dependent physicochemical and morphological changes, such as hemoglobin oxidation and conformational changes [14,23], drying [13,24], clotting [25,26], and DNA and RNA degradation [3,16,27–29]. Total RNA encompasses ribosomal RNAs (rRNA), messenger RNAs (mRNA), transfer RNAs (tRNA) and microRNAs, among others [30,31]. Eukaryotic rRNA makes up for 80% of the total RNA in a cell and consists of 5S, 5.8S, 18S and 28S rRNA [32]. mRNA expression patterns have been used to identify body fluids found at crime scenes [33,34] and might also correlate with time [35]. Fu *et al.* [21] found that, when analyzing blood-specific mRNA transcripts, TSD for bloodstains could be estimated to within a period of 2 to 6 weeks, depending on the age of the sample. Anderson *et al.* [3] distinguished 6-day old blood from fresh and aged (30 days) blood, while Lech *et al.* [19] classified deposition by three time-of-day periods with high prediction accuracy. High throughput sequencing by Weinbrecht *et al.* [20] demonstrated that mRNA transcripts

decreased over time, but blood-specific transcripts were detected up to 12 months after deposition.

Focusing on total RNA has the advantage of circumventing potential PCR inhibition from the heme in blood, and the stochastic effects and amplification biases caused by reverse transcriptase and PCR [36–38]. The vast majority of mRNA molecules have a half-life of just minutes [39], and thus genomic DNA contamination is a concern in amplification experiments [40].

Total RNA degradation can be observed by examining the differently sized fragments with highly sensitive RNA electrophoresis, similar to how Cossette *et al.* quantified DNA concentration in base-pair size bins [16]. Processes that induce degradation in RNA can be endogenous or exogenous [41]; ribonucleases (RNases) are ubiquitously present enzymes that cleave the phosphodiester bonds of RNA [42], increasing the presence of small RNA fragments, and are found on most surfaces and airborne dust particles [43,44]. Further, and of particular relevance to forensic studies, RNases are more effective in cleaving their targets in hydrated environments [41]. Additionally, reactive oxygen species (ROS), alkylating agents and ultraviolet (UV) irradiation damage RNA nucleotides and disrupt the base-pairing occurring in tRNA and rRNA [45,46]. Other exogenous factors, such as humidity [17,47], pH [48] and temperature [17,49] also influence RNA degradation.

This study aims to explore the use of total RNA degradation in bloodstain TSD estimates and quantify the effect of temperature on RNA degradation and TSD models. We examined the time-dependent changes in the concentration and quality metrics of total RNA at two different

temperatures. We hypothesized that the concentration of total RNA in the blood samples will decrease with a longer TSD due to the activity of exogenous RNases [41,42] and the deformation of white blood cells as the blood dries [11,12]. We predicted that quality metrics, such as the RNA Integrity Number equivalent (RINe), the DV200 (percentage of RNA fragments greater than 200 nucleotides), and the 28S/18S rRNA peak area ratio, will decrease over time due to fragmentation of larger RNA fragments and a corresponding increase of smaller RNA fragments [42]. We expected that these changes would occur at a faster rate - represented by a steeper slope in the linear regression models - for the bloodstains stored at the warmer temperature compared to those stored at the cooler temperature. Additionally, for both concentration and quality metrics, we predicted that the greatest decrease would occur before the complete drying of the bloodstains due to RNA's structural instability and enzymatic activity [41]. Previous studies have shown that mRNA persists longer than a week [3,17]; however, without qPCR amplification, we do not expect to detect total RNA at this point.

2. Methods

2.1 Blood sample collection and deposition

Pathogen-free bovine blood was collected from Otonabee Meat Packers, an abattoir in Peterborough, Ontario, Canada. Bovine blood has previously been optimized for forensic research when acid dextrose anticoagulant solution A (ACD-A) is added at a concentration of 12.5% v/v, as it exhibits similar fluid properties to that of human blood [50]. Blood treated with ACD anticoagulants has demonstrated better nucleic acid extraction efficiencies than heparin-based anticoagulants and has not been shown to affect the cell counts of the different blood components [51]. The ACD-A used in this study was made by dissolving 6.6 grams of sodium

citric dextrose, 2.4 grams of citric acid and 6.68 grams of dextrose anhydrous, all purchased from ACP Chemicals Inc., in 500 mL of distilled water. We collected the bovine blood in an amber Nalgene bottle, which was subsequently placed on ice for transportation back to the laboratory (approximately 15 minutes).

We immediately pipetted 50 μ L of blood into 1.5 mL plastic microcentrifuge tubes. All samples were left to air dry by keeping the microcentrifuge tubes open until RNA extraction. Samples were stored at one of two temperatures: room temperature ($\sim 21^{\circ}\text{C}$) or refrigerated at 4°C for a designated amount of time. The room temperature storage condition has an approximate 40% relative humidity (RH), while the RH is likely slightly greater for samples stored at 4°C . We performed five time-series experiments, three at 21°C and two at cool temperature (4°C); blood from a different cow (biological replicate) was used for each experiment. RNA extractions were completed at 0 hours (time of initial pipetting; ~ 30 minutes post-blood collection) and across various timepoints (Table 1); a subset of timepoints were altered after visual inspection of the data to provide better resolution at specific times. In all experiments, we used five technical replicates for each of the timepoints, resulting in a total of 200 samples.

Table 1: Overview of the time series conducted as well as each experiment's storage temperature for the bloodstains. Each biological replicate represents a different cow.

Biological Replicate	Temperature	Timepoints (hours after deposition)
A	21°C	0,1.5,3,6,9,24,48,72,96,168
B	21°C	0,1.5,3,6,9,24,36,48,72,96
C	21°C	0,6,20,24,28,36
D	4°C	0,3,6,9,24,48,72
E	4°C	0,3,9,24,48,72,96

2.2 RNA extraction

We extracted total RNA from the deposited blood using the PureLink Total RNA Blood Purification kit by Invitrogen [52]. We doubled the volume of lysis buffer (L5) recommended in the manufacturer's protocol to minimize blood clotting. We also completed an optional on-column DNase incubation following the manufacturer's protocol. Total RNA was eluted with 30 μ L of RNase-free water and prepared for analysis on the Agilent Technologies 4200 TapeStation [53]. We mixed 1 μ L of the high sensitivity RNA screen tape sample buffer with 2 μ L of the total RNA extracts following the manufacturer's protocol for the *High Sensitivity RNA ScreenTape Assay* [53]. We collected and examined a series of RNA metrics using the TapeStation Analysis Software A.02.02 (Table S1). Of note, the RINe metric is generated by an algorithm that calculates the ratio of the height of the 18S rRNA peak to the background RNA signal, which represents the signal for fragments found in the fast zone ranging from 200 bp to 1.8 kb [54,55]. The RINe metric is a dimensionless number that ranges from 1 to 10, with 10 representing intact and high-quality RNA, while a value of 1 represents low-quality and highly fragmented RNA [54,56]. The DV200 represents the percentage of RNA fragments greater than 200 nucleotides [57]. We also created a custom RNA bin to estimate concentration from 200 bp to 500 bp fragments, presumably representing the shorter mRNAs extracted from our samples [58].

2.3 Statistical analyses

Correlation matrices were created prior to building models to ensure that the response variables (RNA metrics) included in the analyses were not highly correlated to each other (Pearson's $r < |0.70|$). Variables showing independence and a correlation to time were used to

build a series of linear mixed-effects models, with log-transformed time and temperature as fixed effects and biological replicate (individual cow) as a random effect. An interaction term between log-transformed time and temperature was also included in our models. We assigned timepoint zero a value of 0.1 (6 minutes) prior to the log transformation. Effect size, R^2 and p-values were recorded for each model.

After preliminary analysis of our data for each temperature separately, we were interested in a shift in RNA metrics that was apparent after 24 hours. Following the assessment of our data's normality using the Shapiro-Wilk test, we applied the unpaired two-samples Wilcoxon test, a non-parametric test, to determine whether there was a significant difference in our RNA variables before and after the shift occurring at 24 hours. We also explored the non-linearity of RNA metrics over time by fitting non-linear curves and assessing the impact on the residual standard error. All statistical analyses and data visualizations were created using R Version 4.0.3. Raw data and scripts are publicly available and deposited at https://gitlab.com/WiDGeT_TrentU.

3. Results

We collected extracted RNA metrics on 200 deposited bloodstains; four RNA metrics were retained for statistical analyses as they were not correlated to each other (Pearson's $r < |0.70|$; Fig. 1). The retained metrics included the RINe, the DV200, 28S/18S rRNA peak area ratio and total RNA concentration (Table 2).

Fig. 1. Correlations between the nine RNA metrics obtained from the 4200 TapeStation RNA analysis. Size of the dot increases with a greater correlation between variables. Blue-coloured dots indicate a positive correlation between RNA metrics, while red-coloured dots indicate a negative correlation between them. Shading of the dot increases with a greater correlation, with light colours indicating a weak correlation and darker colours indicating a strong correlation (see legend).

Table 2. Statistics for the mixed models of the four quantified RNA metrics. Note that there was an interaction between log-transformed time and temperature. Biological replicate was used as a random effect. Each regression was computed individually for each RNA metric and each row represents a separate model. Because of the interaction term, log(Time) in the table represents the relationship between the response variable and time at 21°C; Temperature (4°C) represents the difference between the intercept at both temperatures; and log(Time)*Temperature reflects the difference in correlation between retained RNA metrics and time between 21°C and 4°C. Significant p-values are bolded.

RNA metrics	Marginal R ²	Conditional R ²	log(Time)			Temperature (4°C)			log(Time)*Temperature		
			Estimate	Confidence Interval	p-value	Estimate	Confidence Interval	p-value	Estimate	Confidence Interval	p-value
RINe	0.51	0.60	-0.51	-0.58 – -0.43	<0.001	0.12	-0.74 – 0.98	0.78	0.17	0.04-0.29	0.01
DV200	0.31	NA	-2.4	-2.99 – -1.80	<0.001	-0.47	-3.59 – 2.64	0.77	1.99	1.03 – 2.96	<0.001
28S/18S Peak Area Ratio	0.061	0.24	0.34	-0.21 – 0.89	0.22	0.35	-3.50 – 4.20	0.86	0.19	-0.54 – 0.91	0.62
Concentration	0.069	0.30	-885.42	-1305.24 – -465.60	<0.001	-2142.06	-7554.02 – 3269.90	0.44	789.89	116.22 – 1481.56	0.022

3.1 RNA Integrity Number equivalent

The RINe metric demonstrated the best fit model ($R^2 = 0.60$; Table 2) and a relationship with log-transformed time (Table 2). RINe values were influenced by storage temperature, however, the effect of temperature on RINe varied according to time, as seen by the significant interaction effect in Table 2. The addition of biological replicate (donor) as a random effect slightly increased the model's goodness-of-fit (marginal vs conditional R^2 in Table 2); this was driven by a temperature-donor effect as RINe values obtained from the different biological replicates at the same temperature were not different from each other (Table 3). The timewise changes in RINe at different temperatures (Fig. 2) showed 21°C caused more degradation (exhibited a steeper slope) than the samples stored at 4°C samples (Fig. 2; Fig S1). Of note, samples deposited at both temperatures exhibited a steep decrease in the RINe values after 24

hours (Fig. 3). A Wilcoxon test supported clear differences in the RINe values before and after 24 hours for both temperatures (21°C: W = 3361, p-value < 2.2e-16; 4°C: W = 687, p-value = 5.3e-07).

Table 3. Unpaired two-sample Wilcoxon tests between the different biological replicates for the RINe, DV200 and total RNA concentration. Significant p-values are bolded.

RNA metric	Biological replicate pairs	Temperature (°C)	Test statistic (W)	p-value
RINe				
	A-B	21	1030	0.17
	A-C	21	574	0.67
	B-C	21	625	0.37
	D-E	4	335	0.36
DV200				
	A-B	21	1352	0.38
	A-C	21	658	0.14
	B-C	21	619	0.40
	D-E	4	367	0.69
Concentration				
	A-B	21	1592	0.010
	A-C	21	742	0.012
	B-C	21	626	0.36
	D-E	4	94	<0.001

Fig. 2. Relationship between RINe and log-transformed time. RINe values are grouped by storage temperature for comparison purposes; points and regression lines are coloured according to storage temperature. Shaded areas around the regression lines represent the standard error. The interaction effect between log-transformed time and temperature is not shown as data is already separated by storage temperature.

Fig. 3. Relationship between the RNA Integrity Number equivalent (RINe) and time. Time was divided into two groups: less than or equal to 24 hours of deposition, and above 24 hours of deposition. Boxplots have been coloured according to the temperature at which the bloodstains were stored.

3.2 DV200

The DV200 demonstrated the second-best model fit ($R^2 = 0.31$); the random effect of biological replicate did not improve the model (Table 2). DV200 showed a statistically significant relationship with time and was influenced by storage temperature, the effect of which varied according to time (Table 2). The positive estimate for the interaction effect indicated that over time, bloodstains kept at 4°C provided greater DV200 values relative to those stored at 21°C (Table 2, Fig. 4). DV200 values obtained from the different biological replicates at the same

temperature were not different from each other (Table 3), but there was a difference between DV200 values recovered from bloodstains deposited at 21°C and 4°C ($W = 2572$, $p\text{-value} = 0.015$; Fig. 4A-C). Bloodstains stored at room temperature also exhibited greater levels of fragmentation over time (Fig. S2, Fig. 4A-C). DV200 values from bloodstains deposited at room temperature also exhibited a non-linear trend over time; we determined that the log-logistic curve – akin to a dose-response curve – provided the best fit at 21°C. The residual standard error (RSE) was smaller (5.9) in the log-logistic curve than the linear model (7.7). Samples deposited for up to 24 hours could be distinguished from those deposited for longer periods of time, but only at room temperature (Fig. 4C). DV200 values obtained from bloodstains stored at 4°C were not significantly different from each other prior to and after 24 hours ($W = 478$, $p\text{-value} = 0.12$; Fig. 4C).

Fig. 4. Relationship between the DV200 and time and temperature. **A)** Linear regression curve for the DV200 against log-transformed time at 4°C. A negative slope is observed. **B)** Log-logistic curve for the DV200 against time at 21°C. The curve decreases at 24 hours, highlighting the diagnostic drop occurring at this timepoint. Shaded areas in **A)** and **B)** represent the standard error. Note that the y-axis is scaled differently for **A)** and **B)**. The interaction effect between log-transformed time and temperature is not shown. **C)** DV200 values for bloodstains deposited for 24 hours or less and those deposited longer than 24 hours. Boxplots are coloured according to storage temperature.

3.4 Total RNA concentration

The concentration of total RNA was shown to have a lower model fit than the RINe or DV200 (Table 2); the addition of biological replicate as a random effect greatly improved the model's fit, suggesting high inter-individual variation (Table 2). We detected a significant relationship between total RNA concentration and time and temperature (Table 2). Total RNA concentrations obtained from bloodstains deposited for 24 hours or less, and those deposited longer than 24 hours were different from each other at room temperature, but not at 4°C (21°C: $W = 3100$, $p\text{-value} = 9.4\text{e-}14$; 4°C: $W = 372$, $p\text{-value} = 0.85$). We detected differences between concentrations from biological replicates originating from the same temperature (Table 3, Fig. S3). Density plots demonstrated bimodal distributions for temperature data, concordant with the result that biological replicates from the same temperature were different (Table 3, Fig. 5). Most concentrations recorded for bloodstains deposited for less than 24 hours were above the limit of quantification (500 pg/μL), while many concentrations at room temperature fell below this threshold after 24 hours (Fig. 5).

Fig. 5. Boxplots and density plots illustrating the distribution of total RNA concentration (log-transformed) for bloodstains deposited for 24 hours or less and those deposited for longer periods of time. Raw concentration data are overlaid on the boxplots. The red dashed line represents the 4200 TapeStation's limit of quantification (500 pg/μL) and the navy dashed line represents the instrument's limit of detection (100 pg/μL).

3.4 28S/18S rRNA peak area ratios

The 28S/18S rRNA peak area ratio was the retained RNA metric that displayed the weakest model fit and little relationship to time or temperature (Table 2). Peak area ratios were only obtained for timepoints up to 24 hours for bloodstains deposited at room temperature and 72 hours for those stored at 4°C (Fig. S4); the 28S/18S peak area ratios could not be

obtained for the later timepoints, as the 18S rRNA peak dropped out of the electropherograms (Fig. S5).

4. Discussion

Estimating the age of biological fluids found at crime scenes, such as bloodstains, remains a challenge within the forensic community [1,2]. Intra and inter-individual variation in blood composition, substrate type and storage conditions all influence bloodstain degradation and must be accounted for to accurately estimate TSD [2]. Our study examined the degradation of total RNA present in bloodstains up to 168 hours after deposition at two different temperatures using automated high-sensitivity RNA gel electrophoresis. We found that the RINe and DV200 both provided diagnostic relationships with time that varied with temperature, with a minimal effect of biological replicate on the model. A key observation here is that the limited effect of biological replicate implies these could be universal diagnostic metrics.

The RINe is an RNA quality metric that is equal to the ratio of the 18S rRNA peak height to the signal present in the fast zone (approximately 200 bp - 1.8 kb), which represents fragmented RNA [54]. This ratio normalizes the discrepancies observed in total RNA concentration and has a clear relationship to time (Fig. 2, Table 2). The addition of biological replicate as a random effect in the model only marginally increased the model fit (Table 2), which was driven by a temperature-donor effect (Table 3). This is important because previous DNA degradation models found a large effect of biological replicate [29], but the standardized nature of RINe makes it a universally comparable metric. Blood composition varies by individual, for example, research on human blood showed that the amount of RNA in healthy subjects varied from 6.7 to 22.7 µg/ml [59], this variability could lead to significant differences

in concentrations of total RNA obtained from biological replicates, as we observed during our study (Fig. 5). The RINe metric is not affected by this variation (Table 2, Table 3); additionally, the RINe is not highly correlated to total RNA concentration and is independent of sample volume, highlighting the potential for wide-scale applicability. The independent and negative correlation with time, with a clear decrease occurring after 24 hours, suggests the utility of RINe as a TSD diagnostic marker. We also have no reason to suspect that the patterns we observed would not be present in human blood; bovine blood exhibits similar fluid properties to that of human blood [50], and similar genome size and composition [60]. Bovine blood also has similar RNA concentration [59,61], white blood cell (WBC) count (human: $3.4\text{--}11.6 \times 10^3/\mu\text{L}$, bovine: $4.9\text{--}13.3 \times 10^3/\mu\text{L}$) and subtypes of WBCs as human blood (neutrophils and lymphocytes are the most abundant in each organism) [59,62]. Confirmatory analyses validating our results using human blood could make this technique applicable to crime scene samples in the future.

A common theme observed throughout our study is the preservation effect of cold temperature. The bloodstains at 4°C consistently improved quality metrics when compared to the bloodstains stored at 21°C. Colder temperatures have been shown to preserve nucleic acids and slow their degradation [16,17,63], a trend that was reproduced herein by comparing the slopes observed in the linear models for the RINe and the DV200 (Fig. 2, Fig. 4A, Fig. S2). In both instances, room temperature produced steeper slopes, indicative of faster degradation. Previous studies have shown that the DV200 provides an alternative to the RINe when analyzing highly degraded samples, where electropherograms lack the 18S and 28S rRNA peaks due to fragmentation [57,64]. The DV200 is often used as a quality indicator before library preparation for high-throughput sequencing, as it is not contingent on rRNA being present to

provide measurements [57,65]. Our results indicated that the DV200 was influenced by the dropout of 18S rRNA with time (Fig. 4); specifically, the values obtained at room temperature exhibited non-linear behaviour over time that appeared to be directly driven by the degradation of the 18S rRNA peak occurring after 24 hours (Fig. 4B, Fig. S6). Non-linear relationships between response variables and time have been observed in previous blood TSD studies [14–16,21], indicating that non-linearity, potentially resulting from preferential degradation of certain RNA fragments (or parts therein) [21], oxidation or dehydration [14], is not uncommon. The non-linearity of the DV200 values at room temperature can possibly be exploited as a diagnostic tool to differentiate day-old bloodstains from older ones as there is a precipitous loss of RNA that occurs after 24 hours (Fig. 4B), with most measurements approaching the instrument's limit of quantification (Fig. 5), implying that little RNA is present in the sample. It is conceivable that the same curve and diagnostic signature would be present at 4°C over longer periods. Future work should extend the 4°C sampling period as this could prove useful for crime scene investigation in cold climates [66].

Our approach analyzes total RNA rather than mRNA, and thus direct comparisons between our results and those obtained in previous studies can be difficult. We observed low RNA concentrations and quality, often below the limit of quantification, as time progressed. Our size bin ranging from 200 bp – 500 bp, which should represent smaller mRNAs in our sample, followed the same trend as total RNA and produced concentrations near the limit of detection. This is consistent with known half-lives of mRNA [67]. Although trends in total RNA degradation tend to follow those found using mRNA transcripts, the latter is more sensitive as it targets a specific gene and can determine actual copy numbers [3,17,21]. It is also susceptible

to genomic DNA contamination [40]. Fu *et al.* [21], and Weinbrecht *et al.* [20], both detected blood-specific transcripts from bloodstains deposited for up to one year, while the total RNA concentration in our study fell below the quantitative range after 24 hours of deposition. Here, we suggest the faster degradation of total RNA observed in our study allows for better discrimination at early timepoints than mRNA studies.

It is also important to note that while qPCR can quantify copy number, such a long-term *ex-vivo* persistence of mRNA is still not well understood and is not concordant with our current *in-vivo* understanding of the molecule, as 80% of mRNAs have a half-life of 2 minutes or less [67]. Theories for such long-term persistence of RNA, outside of gDNA contamination [40], are that RNA undergoes less depurination than DNA (a rapid reaction) and can also form secondary and tertiary structures, which are more stable and resistant to degradation than their single-stranded conformations [41]. Dehydrated samples also have reduced RNase activity, limiting the enzymatic degradation of RNA present in these samples [41,68]. Coupled with the absence of *in-vivo* degradation pathways, long-term persistence of RNA in blood may be possible.

5. Conclusion

Our study demonstrated significant changes in the RINe and DV200 of RNA extracted from bloodstains deposited for up to 168 hours. A significant decrease in the RINe was observed after 24 hours at both room temperature and 4°C, while the significant decrease only occurred at room temperature for the DV200. This suggested that these RNA quality metrics could be used to differentiate day-old bloodstains from older bloodstains. The biological replicates that we included in this study produced similar results, indicating that this method was reproducible, transferrable, and diagnostic. Moving forward, in addition to longer time

series at colder temperatures and experiments using human blood, the effects of humidity and substrate should be evaluated as these factors have been shown to affect the RNA degradation and drying times of bloodstains, which could cause the clear drop in RNA metrics to occur at earlier or later timepoints [17,26,47]. Timewise changes in the rheological properties of blood, DNA and RNA could also be explored and combined with previously developed TSD models [69,70]. This type of combinatorial research could bring the forensic community one step closer to developing a sensitive and robust TSD model for bloodstains that could one day be applied to crime scene samples.

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Author Contributions

Colin I. Elliott: Visualization, Writing - original draft, Formal analysis, Investigation, Funding acquisition, Software, Data curation, Methodology. **Theresa E. Stotesbury:** Conceptualization, Supervision, Funding acquisition, Resources, Writing - review & editing, Project administration, Methodology, Validation. **Aaron B.A. Shafer:** Conceptualization, Supervision, Funding acquisition, Resources, Writing - review & editing, Project administration, Methodology, Validation.

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