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An optimization-based study of the impact of different parameters on DNA degradation

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Abstract: Human remains exposed to various conditions throughout time are frequently utilized for DNA analysis of tissue or bone for identification reasons. The deterioration and loss of DNA in particular environmental settings have previously created a dilemma for investigators. The post-mortem interval (PMI) or time since death is sometimes the most sought-after piece of information in a medical death investigation. Based on the discovery that DNA degradation has a disproportionate effect on the analysis of bigger genetic loci in particular studies, it was postulated that DNA degradation as a result of autolysis or putrefaction might be useful as a possible rate-of-change indicator of PMI. As a sample, goat liver tissue was used. It was incubated in three testing factors, namely pH, salt content, and sugar concentration, and was compared to a control sample that had not been incubated in any of these parameters. The samples were incubated for 24, 48, and 72 hours. An appropriate Lysis Buffer was used to isolate the DNA. Following the isolation of the DNA, quantitative analysis was performed in a UV-Visible Spectrophotometer (for both the control and incubated samples), followed by Agarose Gel Electrophoresis. It was discovered that the data provided by these studies, taken as a whole, show that we can give information relevant for calculating the post-mortem interval, mostly within the first 72 hours following death. Later, it was decided to use Response Surface Methodology (RSM) to optimize the parameters we had picked, with the BBD design model being the favoured choice. This was done with the help of software named, Design-Expert v11. A Fit Summary that showed a linear model fit for the data. ANOVA was used to generate an equation that may be used to quantify the amount of DNA present in a tissue sample that has been exposed to a specific value of pH, salt concentration, and sugar concentration. Finally, a 3-D response surface curve was produced, two factors at a time, to highlight the variance in DNA loss when those two parameters are taken into account.

Introduction

The death of an individual or any living species on Earth has a cause, a manner, and a time on that particular day. Death may be natural or unnatural. Mostly, it's the unnatural deaths that call for forensics. When forensics gets involved, the cause and manner of the death and the time of death play a very important role (Mona et al., 2019). The time of death is sometimes the most sought-after piece of information in a medical death investigation. As a result, forensic investigators require a method of determining when a person's body is discovered and the exact time of death, often referred to as the post-mortem interval. Establishing the time of death may prove a suspect innocent or guilty. If the time of death and the suspect's whereabouts in that are a match, it may prove their guilty. Again, if the suspect provides an alibi that turns out to be legit compared to the time of death, they may be proven innocent (Creighton, 2013).

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A decaying body experiences profound, inescapable, and irreversible biochemical, physical, and physicochemical changes after death due to a lack of circulating oxygen, the termination of anabolic metabolite formation, changed enzyme reactions, and increasing cellular breakdown. Post-mortem alterations mostly occur in a cyclical pattern of successive deterioration phases. However, significant variances are owing to a diverse set of influencing elements originating in both the human body (intrinsic factors) and the environment (extrinsic factors) (Kirk, 2012). Intrinsic variables include, among other things, body mass and surface area, but also age, sex, ante mortem medical problems, the presence of injuries/trauma, the amount of bacterial activity, and the cause of death. A thorough understanding of the susceptibility of post-mortem changes to such factors is critical because these are likely to affect both the appearance of the changes as well as the rate of decay, thus either speeding up or slowing down the progression of post-mortem events (Hau et al., 2014; Kagzi et al., 2022). This is undoubtedly one of the most important reasons why estimating the PMI has remained a difficult problem in forensic science for hundreds of years. Despite the fact that various 'classical' methods have been established to date, and new approaches are constantly being offered, all methods now accessible have limits that are frequently linked to influencing variables. If not taken into account, this renders a procedure prone to mistake and inaccuracy, limiting its use in ordinary forensic applications. A key to improving the approach is gaining a better knowledge of the impacts of intrinsic and extrinsic variables, which allows for the definition of appropriate correction factors and/or exclusion criteria, which improves the method's reliability and applicability (Lebreton et al., 1992).

DNA degradation starts right after death. The degradation of DNA depends upon multiple factors. Among various other factors, the impact of salt concentration, pH, and sugar concentration is notable. Accordingly, in this study, an inspection has been carried out to investigate the impact of the pH, salt concentration, and sugar concentration on the DNA degradation (which has been extracted from goat liver). Subsequently, optimization of the three different input parameters (pH, salt concentration, and sugar concentration was carried out to establish a model through which the DNA degradation (as the result of pH, salt concentration, and sugar concentration can be estimated within the defined factor space which is the novelty of the proposed study. Such optimization of the DNA degradation against the aforesaid input parameters has not been reported earlier as per the author's best belief.

Materials and Methods

The goat liver sample was selected as the representative specimen in this study which was procured locally from Humaipur, Madhyamgram, West Bengal, India. The specimen was initially dipped into 70% ethanol for 15-20 minutes and thereby was hermetically refrigerated at -20°C as per previous standard literature reports (Beena, 2019). All the chemicals used in this were procured from S.D. Fine®. Double distilled water was used in the entire experimentation, which was produced in-house using Borosil® double distillation apparatus.

(A) The method of preparation of the different pH solutions used in this study is indicated below:

(i) 5 conical flasks of 250 ml were taken.

(ii) 100 ml of autoclaved distilled water was added to each conical flask.

(iii) The pH for each conical flask was checked by using a pH meter to ensure the pH of the distilled water is 7, considered the standard pH for distilled water.

(iv) In the conical flask, Glacial Acetic Acid was initially added dropwise to bring down the pH to 2.

(v) The same process was carried out to obtain pH 4.

(vi) The third conical flask was marked as pH 7.

(vii) For the fourth and fifth conical flask, pellets of Sodium Hydroxide were added, one by one, until the pH of 9 and 10.5 were obtained, respectively.

(viii) After the different pHs were obtained, the remaining 100 ml of autoclaved distilled water was added to each conical flask.

(B) Methods for the preparation of saline water used in this study are indicated below

(i) 5 conical flasks of 100 ml each were taken.

(ii) 25 ml of autoclaved distilled water was added to each conical flask.

(iii) Salt concentrations of 0.5%, 0.9%, 2%, 5% and 10% were prepared.

(iv) For preparing 0.5%, 0.125 grams of Sodium Chloride was added to 25 ml of autoclaved distilled water.

(v) For preparing 0.9%, 0.225 grams of Sodium Chloride was added to 25 ml of autoclaved distilled water.

(vi) For preparing 2%, 0.5 grams of Sodium Chloride was added to 25 ml of autoclaved distilled water.

(vii) For preparing 5%, 1.25 grams of Sodium Chloride was added to 25 ml of autoclaved distilled water.

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(viii) For preparing 10%, 2.5 grams of Sodium Chloride was added to 25 ml of autoclaved distilled water.

(ix) The 5 conical flasks were then autoclaved and stored for future use.

(C) Methods for the preparation of sugar solution.

(i) 6 conical flasks of 100 ml each were taken.

(ii) 30 ml of autoclaved distilled water was added to each conical flask.

(iii) Sugar concentrations of 50 mg/dl, 80 mg/dl, 120 mg/dl, 145 mg/dl, 180 mg/dl and 220 mg/dl were prepared.

(iv) For 50 mg/dl, 0.015 grams of glucose was added to 30 ml of autoclaved distilled water.

(v) For 80 mg/dl, 0.024 grams of glucose was added to 30 ml of autoclaved distilled water.

(vi) For 120 mg/dl, 0.036 grams of glucose was added to 30 ml of autoclaved distilled water.

(vii) For 145 mg/dl, 0.0435 grams of glucose was added to 30 ml of autoclaved distilled water.

(viii) For 180 mg/dl, 0.054 grams of glucose was added to 30 ml of autoclaved distilled water.

(ix) For 220 mg/dl, 0.066 grams of glucose was added to 30 ml of autoclaved distilled water.

For conducting the pH-controlled tissue degradation, the tissue samples weighing approximately 3 grams (each slice weighing 1 gram) were placed in five individual 100 ml conical flasks, each containing 25 ml solutions of pH 2, pH 4, pH 7, pH 9, and pH 10.5, respectively. The flasks were thereby incubated for 24 hours, 48 hours, and 72 hours. A control sample of 1 gram was immediately placed at -20°C.

In order to carry out salt concentration-driven tissue degradation, tissue samples weighing approximately 3 grams (each slice weighing 1 gram) were placed in five individual 100 ml conical flasks, each containing 25 ml solutions of different salt concentrations, 0.5%, 0.9%, 2%, 5%, and 10%, respectively. The flasks were incubated for 24 hours, 48 hours, and 72 hours with respect to each 1-gram sample. A control sample of 1 gram was immediately placed at -20°C.

Tissue samples weighing approximately 3 grams (each slice weighing 1 gram) were placed in six individual 100 ml conical flasks, each containing 25 ml solutions of different sugar concentrations, 50 mg/dl, 80 mg/dl, 120 mg/dl, 145 mg/dl, 180 mg/dl, 220 mg/dl, respectively. The flasks were incubated for 24 hours, 48 hours, and 72 hours with respect to each 1-gram sample. A control sample of 1 gram was immediately placed at -20°C. All the experiments were carried out aseptically.

Finally, the DNA degradation of the specimen under evaluation (goat liver) was quantified spectrophotometrically as per established literature reports (Wang et al., 2012).

Finally, an optimization study was conducted by adopting the response surface methodology to determine the optimized process parameters corresponding to the DNA degradation. Among various literature-reported methods, the Box Behnken Design (or BBD) is one of the most promising tools of optimization. BBD is a rotatable second-order factorial design that places points on the midpoints of the cubical design region's borders as well as points in the center. The three-dimensional response surface curves were thereby obtained to determine the impact of the different factors (Gaur et al., 2014). The entire optimization was performed by using Design Expert® software version 11. All the experiments were carried out in triplicates to define the associated errors.

Thus, the present study involves a series of experiments to screen and optimize the various process parameters linked with DNA degradation, which in turn indicates the novelty of the present study.

Results and Discussion

The literature reports that a wide spectrum of factors is responsible for DNA degradation. In this study, the impact of various factors on DNA degradation was assessed, followed by their optimization.

The literature reported that pH plays an important role in DNA degradation (Shrode et al. 1997). Accordingly, the impact of pH on the DNA degradation (of goat liver sample) was evaluated in this study. The pH range was

Table 1. Impact of pH on DNA degradation isolated from goat liver.

Incubation Period (Hours)	pH	DNA Conc. At 260 nm (ng//µl)
24	2	2.101
	4	2.558
	7	3.515
	9	2.474
	10.5	2.122
48	2	1.149
	4	1.854
	7	2.001
	9	1.110
	10.5	1.009
72	2	0.005
	4	0.872
	7	1.551
	9	0.451
	10.5	0.226

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Table 2. Impact of salt concentration on DNA degradation isolated from goat liver.

Incubation Period (Hours)	Salt concentration (%)	DNA Conc. At 260 nm (ng//µl)
24	0.5	2.121
	0.9	2.212
	2	2.350
	5	2.385
	10	2.889
48	0.5	1.121
	0.9	1.333
	2	1.545
	5	1.667
	10	1.885
72	0.5	0.031
	0.9	0.072
	2	1.024
	5	1.412
	10	1.667

varied between 2-10.5 to cover a wider spectrum. The experiments were carried out at three-time intervals of 24, 48, and 72 hours. The respective DNA degradation was noted through a UV-vis spectrophotometer at 260 nm. The obtained results have been detailed in Table 1.

The data indicated in Table 1 indicated that the pH has a notable impact on DNA degradation. An inverse-urn-shaped pattern of the impact of pH on DNA degradation was noted for 24, 48, and 72 hours respectively. The obtained results were found to be in line with the other previously reported literature studies (Kagzi et al., 2022).

The salt concentration was also reported to have notable cognizance of DNA. Accordingly, the efficacy of the salt concentration towards DNA degradation was assessed in this study. The concentration of the salt varied within the range of 0.5- 10 % as per previous literature reports on degradation (Nayek et al., 2020). The entire set of experimentations was carried out at three different time intervals of 24, 48, and 72 hours. The obtained results have been presented in Table 2.

The obtained data indicated that a similar trend of DNA degradation as the impact of pH was also noted for salt concentration. The results were found to be in line with the other literature-reported studies (Nieves-Colón et al., 2018).

Finally, the impact of sugar concentration on the DNA degradation of the goat liver cells was analyzed. The range of sugar concentration varied within the range of 50-200 mg/dl as per previous literature reports (Jatlow et al., 2014). The results obtained are presented in Table 3.

Table 3. Impact of sugar concentration on DNA degradation isolated from goat liver.

Incubation Period (Hours)	Sugar concentration (mg/dl)	DNA Conc. At 260 nm (ng//µl)
24	50	2.751
	80	2.707
	120	3.080
	145	3.222
	180	3.350
	220	3.469
48	50	1.991
	80	1.952
	120	2.111
	145	2.354
	180	2.381
	220	2.417
72	50	1.528
	80	1.533
	120	1.749
	145	1.963
	180	2.333
	220	2.426

Table 4. The experimental design array was used for the optimization of DNA degradation.

Design Point	Factor Level Settings			Output: DNA Conc. At 260 nm (ng/µl)
	pH	Sugar Conc (mg/ml)	Salt Conc. (%)	
1	3.5	50	5	1.201
2	10.5	50	5	1.227
3	3.5	220	5	1.311
4	10.5	220	5	1.345
5	7	145	1	1.395
6	10.5	145	1	1.412
7	3.5	145	10	1.667
8	10.5	145	10	1.795
9	7	50	1	2.015
10	7	220	1	1.881
11	7	50	10	1.871
12	7	220	10	1.855
13	7	145	5	1.825
14	7	145	5	1.825
15	7	145	5	1.825

Footnote: *Design point 13 was triplicated to determine the associated error.*

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From the obtained results, an ardent need for the optimization of the process parameters was identified to establish the reproducibility of the study within the defined factor space. Accordingly, the optimization of process parameters corresponding to the DNA degradation was conducted. The literature reported that various methodologies had been adopted for optimization, among which the response surface methodology (RSM) approach has been widely reported (Kumari and Gupta, 2019). Box Behnken Designing (BBD) is an established RSM approach that was used in this study to optimize the process parameters (Wu et al., 2012).

Initially, the standard BBD experimental matrix was adopted for the optimization procedure which is detailed in Table 4. Each factor (pH, Sugar conc., and Salt conc.) was evaluated at three different intervals (also known as levels). The higher and the lower level were considered the highest and the lowest values for their corresponding factors. The middle level for each factor was obtained by taking the average of the highest and the lowest values.

Table 5. Analysis of Variance (ANOVA) of the optimization study.

Source	Sum of squares	Degrees of freedom	Mean square	F-value	p-value
Model	0.5167	3	0.1722	3.28	0.0625
pH	0.1864	1	0.1864	3.55	0.0864
Sugar Conc.	0.2980	1	0.2980	5.67	0.0364
Salt Conc.	0.0324	1	0.0324	0.6163	0.4490
Residual	0.5781	11	0.0526		
Lack of Fit	0.4366	9	0.0485	0.6862	0.7170
Pure Error	0.1414	2	0.0707	-	-
Correlation- Total	1.09	14	-	-	-

The optimization result indicated the linear fit with a sum of squares 0.4366, along with 9 degrees of freedom, mean square of 0.0485, F-value of 0.6862, and p-value of 0.7170.

The analysis of variance (ANOVA) was thereby conducted to select the statistically significant model terms. The ANOVA results have been presented in Table 5.

The final optimized model equation after ANOVA has been presented below in equation 1;

DNA Conc. = 1.630 + 0.153*pH +0.193*Sugar Conc. + 0.064*Salt Conc. ……(1)

To the accuracy and precision of the proposed model equation, a normality plot was obtained by plotting the experimentally determined and the model predicted values under the given factor space. It was observed that the model-predicted response was found to be very close to the experimentally determined response. Thus, it may be conferred that the developed model has good accuracy and precision.

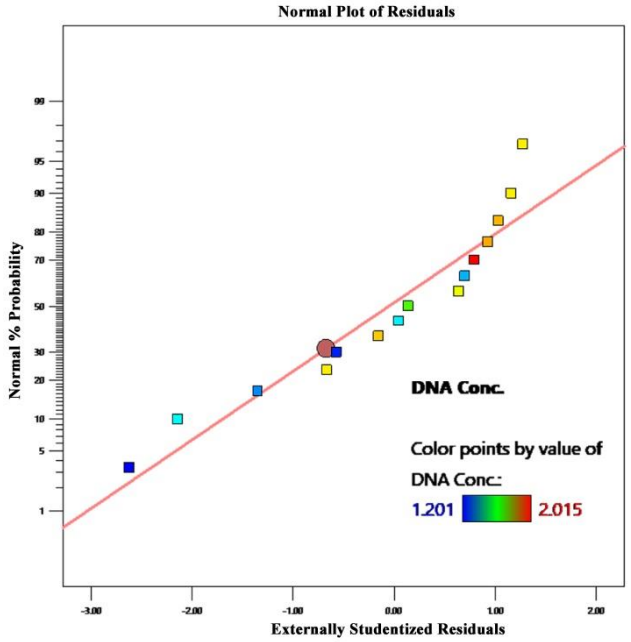

Figure 1. The plot of the experimentally determined and the model predicted response

Finally, the three-dimensional response surface curves were presented to check the impact of the individual components (using two factors at a time) on the process output. The obtained results have been presented in Figure 2. It is evident from the three-dimensional curves that the combined impact of pH and sugar concentration on DNA degradation at the lower level was high. As both the levels were increased, a reduction in DNA degradation was observed in the middle levels. Again, while moving to the respective higher levels, the combined impact of both the factors on the DNA

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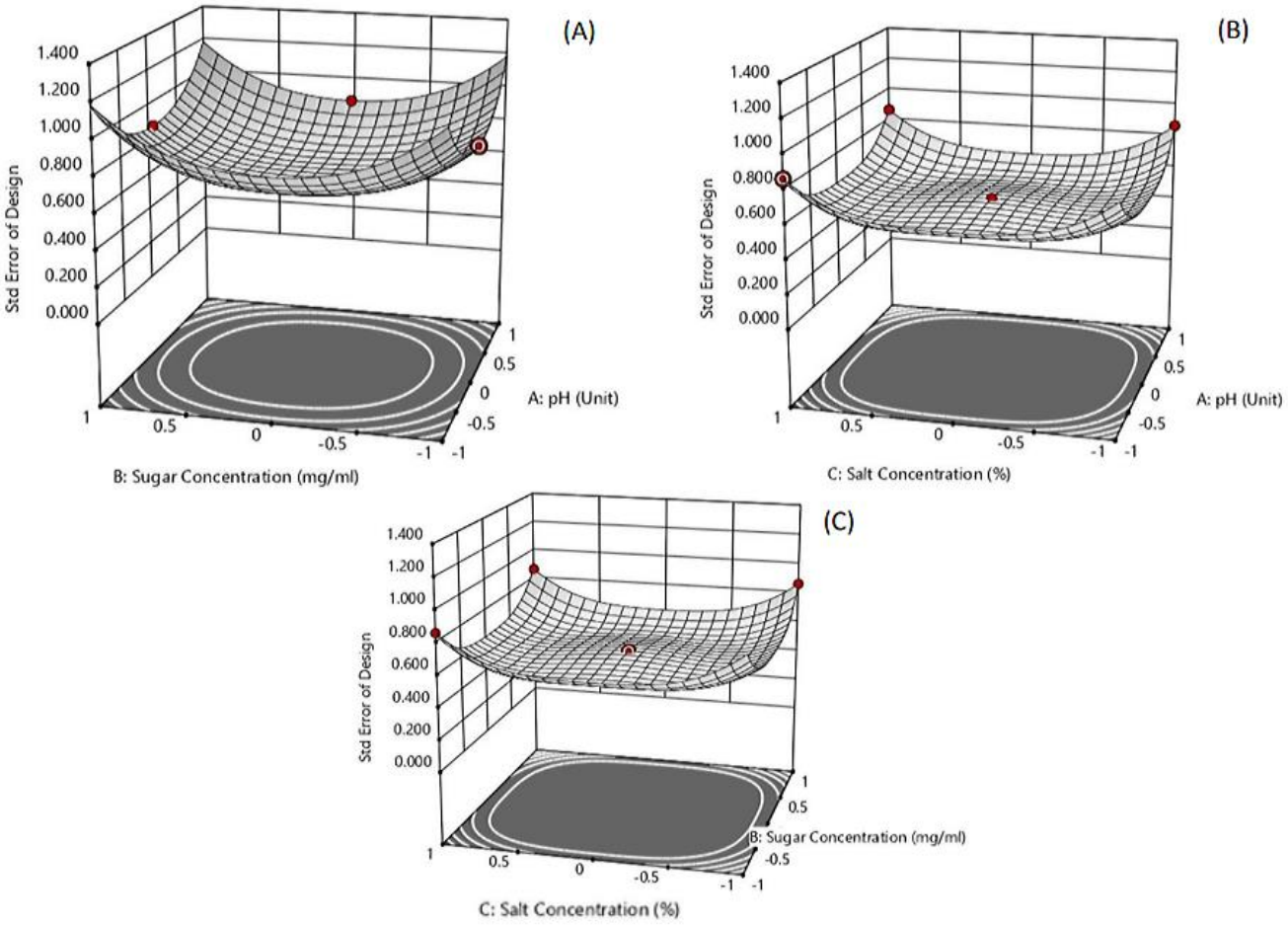

Figure 2. Three-dimensional plots indicate the impact of different factors (two factors at a time) on the response variable (DNA degradation).

degradation increases and reaches the apex. A similar impact was also noted for the synergistic impact of salt conc. & pH, and salt conc. & sugar conc.

Conclusion

The present study investigates the impact of various factors on DNA degradation. Three different parameters, namely pH, salt concentration and sugar concentration, were chosen through an exhaustive literature search. A wide range of each factor was chosen (by using one factor at a time) to determine the effective range of the parameters for DNA degradation. Accordingly, the range of the individual factors was normalized. Thereafter, an RSM-based optimization was carried out to optimize the DNA degradation. The final optimized model was statistically validated using variance analysis (ANOVA). The impact of the different parameters on the response variable (DNA degradation) was also presented through three-dimensional response curves. The unique deliverable of the study is the model equation through which the DNA degradation can be estimated within the defined factor space with minimum error. The proposed scheme of study thus provides an excellent approach to quantifying DNA degradation.

Conflict of interest

The authors declare no potential conflict of interest for the reported study.

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