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Research Article

The Truth in the Lye: Optimizing DNA, RNA, and Protein Extraction from Human Teeth Under Different Incubation Times and Environmental Conditions

Maria J Castagnola[1], Mayaas Hassan[1], Alixs M Pujols[1], Shelby Feirstein[2], Luis L Cabo[2], Joe Adserias-Garriga[2] and Sara C Zapico[1,3]*

[1]*Department of Chemistry and Environmental Sciences, New Jersey Institute of Technology, Newark, NJ, United States*

[2]*Applied Forensic Sciences, Mercyhurst University, Erie, Pennsylvania, United States*

[3]*Anthropology Department and Laboratories of Analytical Biology, NMNH, Smithsonian Institution, Washington, DC, United States*

***Corresponding Author:** Sara C Zapico, Department of Chemistry and Environmental Sciences, New Jersey Institute of Technology, Newark, NJ, United States and Anthropology Department and Laboratories of Analytical Biology, NMNH, Smithsonian Institution, Washington, DC, United States.

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Abstract

Human identification is of utmost importance in criminal investigations, mass disasters, and humanitarian contexts. Short Tandem Repeats profiling has been the gold standard for DNA-based identification, but integrating RNA and protein analysis for post-mortem interval estimation could significantly improve forensic investigations, particularly when traditional methods fall short. Advances in -omics technologies for skeletal remains highlight the importance of simultaneously extracting DNA, RNA, and proteins to maximize information from limited forensic samples. However, environmental conditions can significantly impact biomolecule recovery.

This study investigates the simultaneous extraction of DNA, RNA, and proteins from human teeth under different conditions. 23 third molars were analyzed in two phases. First, incubation times of 2, 5, 12, and 24 hours were tested to optimize biomolecular extraction using teeth from living donors. Second, the impact of chemical treatments (sodium hydroxide and hydrochloric acid) was evaluated on curated teeth, with a 24-hour incubation period. The Quick-DNA/RNA Miniprep Plus Kit (Zymo Research, Irvine, CA, USA) was used for extraction. Quantification was performed applying spectrophotometric and fluorometric techniques, along with a human DNA-specific quantitation method. STR analysis was performed, and statistical comparisons were carried out to compare the effect of time and treatments on the extractions.

In phase 1, DNA yield at 24 hours was significantly higher than at 2 hours and 5 hours. However, RNA and protein recovery showed no significant differences across incubation times. In phase 2, NaOH treatment significantly reduced DNA and RNA recovery compared to controls and HCl treatment. Protein recovery showed no significant differences. STR analysis produced complete DNA profiles for all samples, except two cases. Quality parameters (Degradation Index, Inhibition Control Index, Total Peak Height, Peak Height Ratio and possible dropout) indicated good-quality profiles, with the lowest values in NaOH-treated samples.

To the best of our knowledge, this is the first study to assess the simultaneous extraction of DNA, RNA, and proteins from human teeth under different environmental conditions. The results confirm that high-quality/quantity extraction is achievable, offering valuable insights for human identification and time-since-death estimation.

Keywords: *Forensic Odontology; Human Identification; Human Teeth; Simultaneous Extraction; STR Profiling; Time-Since-Death Estimation; Chemical Damage; Environment*

Introduction

Human identification plays an essential role in criminal investigations, mass disaster scenarios and humanitarian contexts [1]. While Short Tandem Repeats (STRs) profiling has long been the gold standard for DNA-based identification, the incorporation of RNA and protein analysis for postmortem interval (PMI) estimation could greatly enhance forensic investigations, especially in cases where traditional methods are limited. In this context, transcriptomics and proteomics are gaining recognition as valuable tools, particularly for analyzing skeletal remains [2].

The simultaneous recovery of high-quality and high-quantity DNA, RNA, and proteins from tissue presents a great challenge. For instance, the inherent instability of RNA, compared to DNA, underscores the need for optimized extraction methods [3]. The complexity of extracting these biomolecules in a single process is compounded by the limited available information on improved techniques [3-5].

Human remains are often subjected to a variety of external factors that impact degradation. Soil composition, pH levels, temperature, humidity, and microbial activity are crucial in the decomposition and preservation of human remains [6-8]. In addition, over the years, aggressive commercial household solutions have been employed at crime scenes to destroy human remains [9,10]. This complicates the task of human identification, as the extent of damage to the recovery of biomolecular material is still not fully understood. It is believed that these external factors can significantly accelerate the degradation of genetic material [11], leaving skeletal remains-particularly teeth and bones-as the only available sources for analysis.

Research on chemical damage to human remains is limited. While some studies focus on visual examinations of various tissues [9,10,12,13], others investigate microscopic and chemical changes in bones and teeth [14-17]. Similarly, research on DNA recovery following chemical treatments is also scarce. Some studies examine soft tissue and bone using animal models [18,19], which could complicate the extrapolation of findings to human remains, while only a few specifically address human teeth [20-22]. To our knowledge, no studies have investigated the simultaneous extraction of these biomolecules from chemically treated remains.

Over the years, multiple DNA extraction methods for skeletal remains have been explored, including silica column techniques [23-25], magnetic bead methods [26,27], and phenol–chloroform methodologies [28-30]. However, none has been universally recognized as the most effective. Teeth, being the hardest and most decomposition-resistant tissues in the human body, are ideal samples for human identification. Composed of four distinct tissues-cementum, enamel, dentin, and pulp-each with unique compositions, teeth offer valuable insights. The dental pulp, due to its central location and protection by hard tissues, and its advantage of containing nerves, blood vessels, and multiple types of cells, is particularly preferred for DNA analysis [31,32]. However, the pulp can sometimes decompose and rapidly be repleted, despite being protected by hard tissues [33]. Therefore, it is of utmost importance to evaluate not only the pulp but also the dentin as potential tissues for human identification.

This study aims to optimize the simultaneous extraction of DNA, RNA, and proteins from the dentin and pulp of teeth subjected to different chemical treatments. Initially, the focus will be on refining the extraction protocol by testing different incubation times. In the second phase, the effect of environmental factors on the recovery of DNA, RNA, and proteins will be studied through the use of commercial household chemicals, specifically acidic and basic solutions. The ultimate goal is to obtain high-quality, high-quantity DNA, RNA, and proteins for both STR profiling and time-since-death estimation, thereby advancing human identification efforts by maximizing the information that a single sample can provide.

Materials and Methods

Sample collection and treatment

Twenty-three healthy erupted third molars were obtained from two sources: teeth from living donors and modern skeletal collection specimens from Mercyhurst University (postmortem interval of 5 to 10 years). The New Jersey Institute of Technology Institutional Review Board (IRB) granted an exemption of these experiments under 45 CFR 46.104(d)(704), category 4 (protocol number: 2205021027).

The study was conducted in two phases: the first focused on optimizing incubation time using samples from living donors, while the second assessed the effects of chemical treatments under different environmental conditions using skeletal collection specimens, referred as "curated teeth".

In the first phase, six teeth from living donors were used to determine the optimal incubation time for maximizing the DNA, RNA and protein recovery. A total of 30 samples were obtained both from dental pulp (6) and dentin (24).

In the second phase, 17 curated teeth from the Mercyhurst collection were employed to assess the impact of alkali and acid conditions on biomolecule extraction efficiency. Of these, 8 teeth were treated with sodium hydroxide (NaOH) and 8 with hydrochloric acid (HCl) for periods between 30 minutes to 2 hours. The NaOH treatment was performed by placing the teeth in a 100 mL container containing a 1:1 mixture of distilled water and Instant Power® Crystal Lye Drain Opener (Scotch Corporation, Dallas, TX). The HCl treatment was conducted by immersing the sample in 100 mL of undiluted Transchem muriatic acid (Harcros Chemicals, Inc., Kansas City, MO). In total, 30 samples were processed in this phase, including 18 samples from dentin and 12 from pulp.

The experimental setup is illustrated in figure 1.

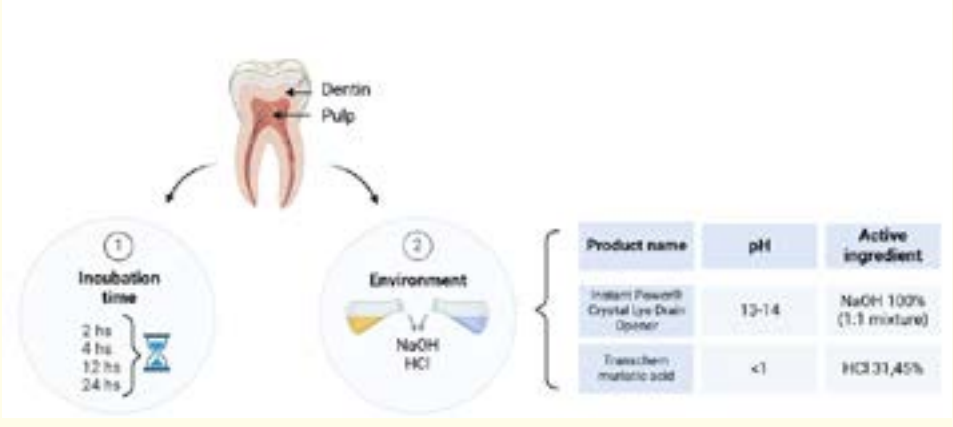


Figure 1: *Experimental setup for biomolecule extraction from human teeth. Phase 1: Effect of incubation of six teeth from living donors. Phase 2: Effect of chemical treatment on 17 curated teeth under alkaline and acidic conditions.*

Sample preprocessing

To eliminate exogenous DNA, each tooth was first exposed to UV light at 254 nm for 15 minutes each side of the tooth. The cementum and enamel were removed using a diamond cutting disk, which was also used to separate the crown from the root. The roots were then cut along the midline, and the pulp was carefully extracted with tweezers. For dentin processing, an agate mortar and pestle were employed to break the dentin into thin fragments. The resulting samples were weighed and transferred into 1.5 mL Eppendorf tubes. Dentin samples were aliquoted at 200 mg per tube, while pulp was processed as a single sample per tooth. The number of dentin samples obtained varied depending on the total dental mass of each tooth.

Simultaneous DNA, RNA and protein extraction

DNA, RNA, and proteins were extracted with the Quick-DNA/RNA Miniprep Plus Kit (Zymo Research). For each sample, 600 µL of DNA/RNA Shield, 30 µL of Proteinase K, and 60 µL of Proteinase K Digestion Buffer were added. Each sample was assigned a specific incubation

interval of 2, 5, 12, or 24 hours, and was incubated at room temperature on a thermal mixer (Thermo Scientific) with continuous agitation. The tubes were then vortexed, and the supernatant was carefully transferred to new 1.5 mL Eppendorf tubes. The extraction process was then continued in accordance with the manufacturer's instructions. DNA and RNA were eluted with 15 µL of DNase/RNase-Free Water, whereas proteins were eluted with 200 µL of PBS (Phosphate-Buffered Saline).

Biomolecule quantification

The biomolecule quantification was performed using different techniques. Initially, DNA, RNA and protein yields were measured using the Nanodrop™ One Microvolume UV-Vis Spectrophotometer (Thermo Fisher Scientific) for preliminary detection.

In the second step, the quantification of RNA and proteins was performed using the Qubit RNA Assay Kit and Protein Assay Kit (Life Technologies, Carlsbad, CA, USA), based on a fluorescent detection, along with the Qubit™ Fluorometer 1.0 (Thermo Fisher Scientific), according to the manufacturer's protocol. Human-specific DNA quantification was performed using qPCR (Quantitative Polymerase Chain Reaction) with the Promega PowerQuant® Kit (Promega Corporation) on the QuantStudio (Thermo Fisher Scientific), following the manufacturer's protocol. The PowerQuant® Kit also allowed for the determination of the Degradation Index (DI) and the detection of potential inhibitors through the Internal PCR Control (IPC), both of which functioned as quality parameters.

The subsequent analyses are based on the DNA concentration obtained using the PowerQuant® Kit and the RNA and protein concentrations measured with the Qubit™ Fluorometer 1.0.

Nuclear DNA profiling

Nuclear DNA profiling was performed using the Promega PowerPlex® Fusion 6C System (Promega Corporation, Madison, WI, USA) on the ProFlex™ PCR System (Thermo Fisher Scientific) to amplify and analyze 23 autosomal STRs and the Amelogenin gene, and three Y-STRs. Each sample was processed with 1 ng of DNA in a total volume of 25 µL and subjected to 29 amplification cycles. Fragment analysis was conducted on the SeqStudio Genetic Analyzer (Thermo Fisher Scientific, Waltham, MA, USA), with the following parameters: 7-second injection time, 1200 V injection voltage, 1440-second run time, and 9000 V run voltage. Final DNA profile analysis was carried out using Microsatellite Analysis Software in Thermo Fisher Cloud, with a peak detection threshold set at 150 RFU (Relative Fluorescent Units).

Data analysis

Statistical analyses were conducted using SPSS® Statistics version 26 (IBM), assessing data normality and homogeneity of variance. Based on the results, appropriate parametric and non-parametric tests were selected. Statistical significance was determined at $p \leq 0.05$.

To assess the quality of the DNA, three parameters were calculated based on previous studies [34-36]: total peak height (TPH), defined as the sum of the peak heights in a profile (measured in RFU); peak height ratio (PHR), the ratio of the smaller peak height to the larger peak height for each marker in a heterozygote; and possible dropout, calculated as the loss of signal in one of the alleles of a heterozygous marker when the RFU was below the threshold of 150. The average TPH and PHR were then calculated for control, alkali treatment, and acid treatment conditions.

The entire process, starting from sample processing, is outlined in figure 2.

Results

Comparison of DNA, RNA, and protein recovery between pulp and dentin samples

Differences between dentin and pulp samples from all teeth were evaluated. DNA concentrations were significantly higher in pulp (45.50 ± 5.31 ng/µL) compared to dentin (15.73 ± 2.20 ng/µL) at all incubation times (Mann-Whitney, $p < 0.001$). Similarly, DNA yields across all treatments were significantly greater in pulp (39.47 ± 6.76 ng/µL) than in dentin (12.85 ± 2.53 ng/µL) (Mann-Whitney, p =

Figure 2: *Schematic representation of the main stages in the experimental process: pre-processing, extraction, quantification, DNA profiling, and analysis. For further details, refer to materials and methods.*

0.003). Hence, DNA yield was consistently higher in pulp than in dentin, regardless of incubation time or treatment (Figure 3). No significant differences were observed between tissues for RNA or protein recovery (data not shown).

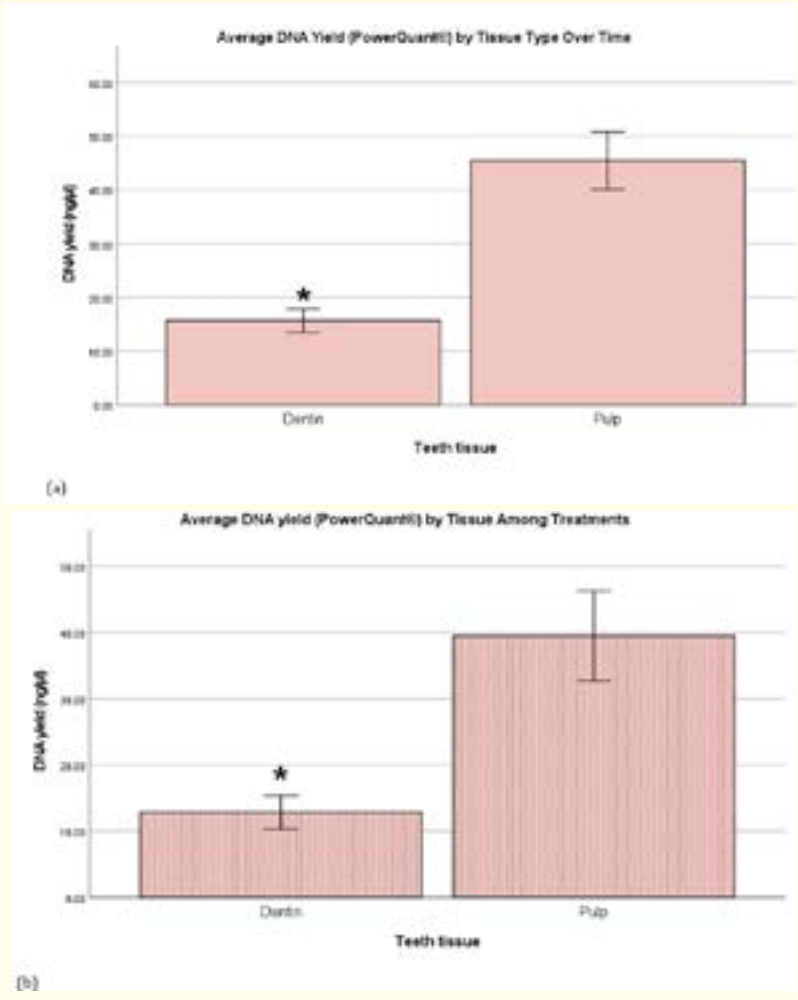


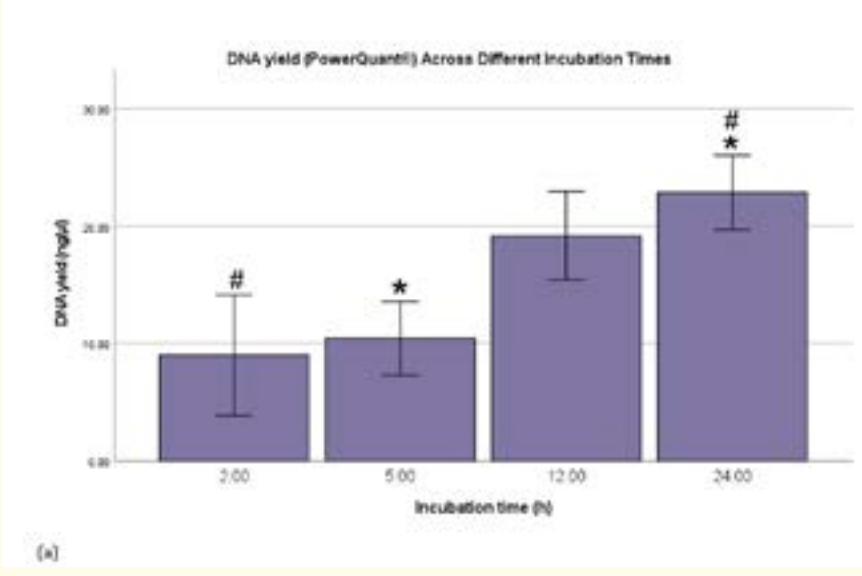
Figure 3: *DNA yield was significantly higher in pulp than in dentin. Significant differences are indicated by *p < 0.05 across all incubation times (a) and among all treatments (b). Error bars represent the standard error (SE).*

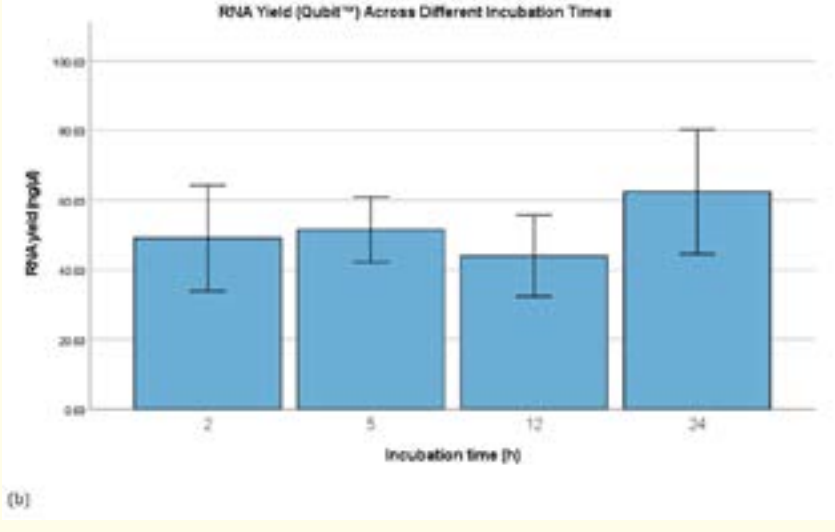
Phase 1: Evaluation of extraction efficiency at different incubation times using Zymo quick-DNA/RNA™ microprep plus kit

In this initial stage, teeth from living humans were employed to compare incubation times. Although both pulp and dentin were extracted from each tooth, only the 24 dentin samples were included in the analysis to eliminate variability introduced by using pulp tissue.

The average concentrations across all incubation times were 15.73 ng/µL (± 2.20) for human autosomal DNA, 51.42 ng/µL (± 6.74) for RNA, and 1.02 µg/µL (± 0.04764) for proteins. Specifically at 24 hours, human autosomal DNA yield was 22.85 ng/µL (± 3.16), RNA was 62.38 ng/µL (± 17.82), and protein was 1.04 µg/µL (± 0.132).

After performing the hypothesis tests for normality and homogeneity of variance, the Kruskal-Wallis non-parametric test was applied, showing significant differences between at least two groups ($p < 0.036$). Additionally, pairwise comparisons were conducted using the Mann-Whitney test. The results indicated that DNA yield at 24 hours was significantly higher compared to 2 hours ($p = 0.037$) and 5 hours ($p = 0.006$). No significant differences were observed in RNA or protein concentrations across the incubation times. Differences in incubation times are shown in figure 4.





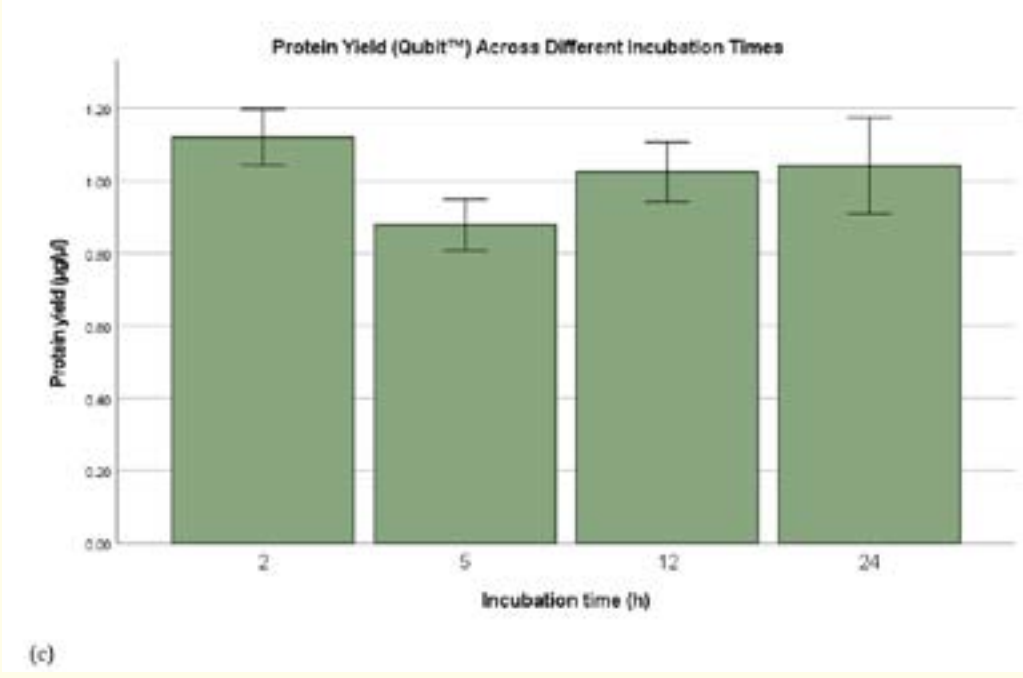


Figure 4: *DNA, RNA, and protein yield at different incubation times (2, 5, 12, and 24 hours). (a) DNA yield was significantly higher at 24 hours (22.85 ng/µL ± 3.16) compared to 2 hours (9.01 ng/µL ± 5.13) and 5 hours (10.43 ng/µL ± 3.12) (Phase 1: Evaluation of extraction efficiency at different incubation times using zymo Quick-DNA/RNA™ microprep plus kit). A significance level of #p < 0.05 indicates a difference between the 2 and 24h, while * p < 0.05 denotes a significant difference between 5 and 24h. (b) No significant differences were observed in RNA yield across incubation times. (c) No significant differences were found in protein yield across incubation times. Error bars represent the standard error (SE).*

Regarding quality parameters, all samples were below the threshold of the IPC shift. For degradation, the DI was above the threshold only in eight dentin samples from tooth T2 at different time points, as well as in one sample from tooth T6.

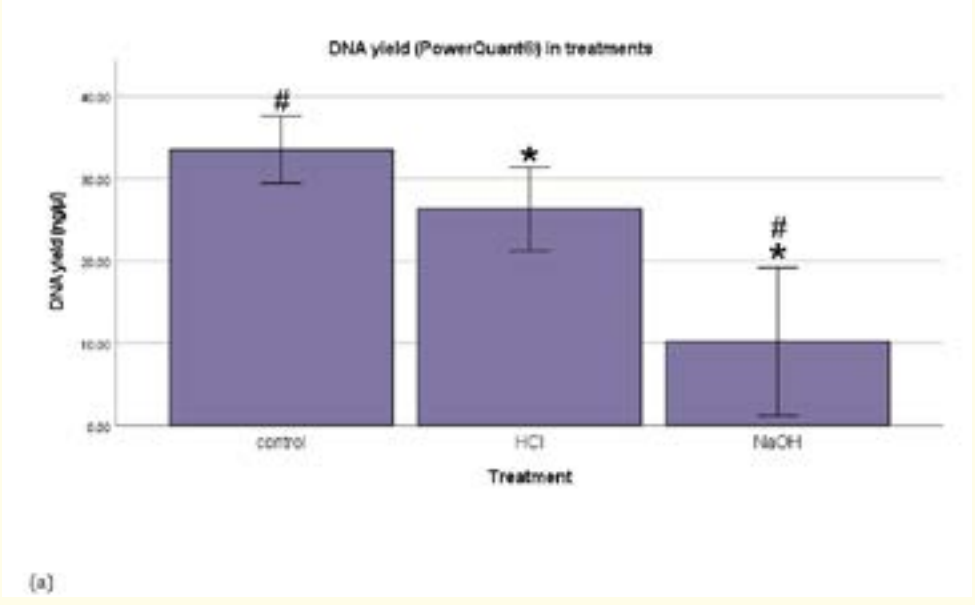
Phase 2: Effect of chemical treatments on DNA, RNA, and protein recovery

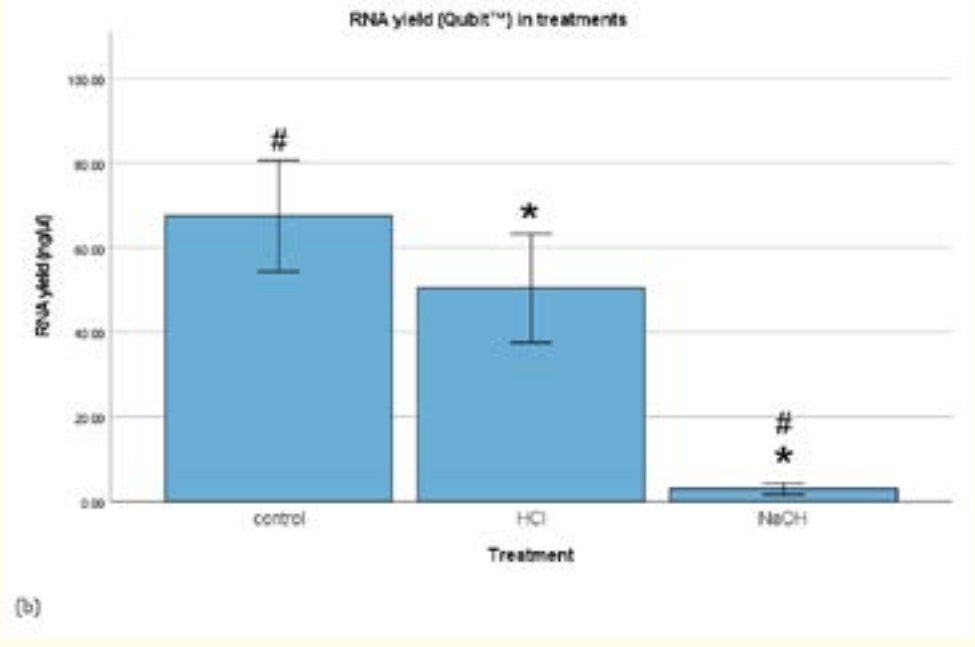
In a second phase, the impact of chemical treatments on the simultaneous extraction of DNA, RNA, and proteins was analyzed. Control and treated samples were incubated for 24 hours, based on the previously determined optimal incubation time (Phase 1: Evaluation of extraction efficiency at different incubation times using Zymo Quick-DNA/RNA™ Microprep Plus Kit). Recovery of DNA, RNA, and proteins was successful in all cases.

The average DNA yields were 10.18 ng/µL (± 8.99) for the NaOH group, 26.33 ng/µL (± 5.09) for the HCl group, and 33.53 ng/µL (± 4.06) for the control group. For RNA, the NaOH group yielded an average of 3.036 ng/µL (± 1.33), the HCl group 50.46 ng/µL (± 12.84), and the control group 67.49 ng/µL (± 13.12). However, protein recovery showed no significant differences among the treatment groups, with average yields of 1.39 ± 0.21 µg/µL in the control group, 1.66 ± 0.16 µg/µL in the HCl group, and 1.32 ± 0.18 µg/µL in the NaOH group.

The results indicate that NaOH treatment has a greater impact on the recovery of DNA and RNA. DNA yield was significantly lower in the NaOH-treated group compared to the control (Mann-Whitney, $p < 0.001$) and the HCl-treated group (Mann-Whitney, $p = 0.003$). Simi-

larly, RNA yield was significantly lower in the NaOH-treated group compared to the control (Mann-Whitney, $p < 0.001$) and the HCl-treated group (Mann-Whitney, $p < 0.001$). No significant differences were observed in protein yield among groups (Figure 5).





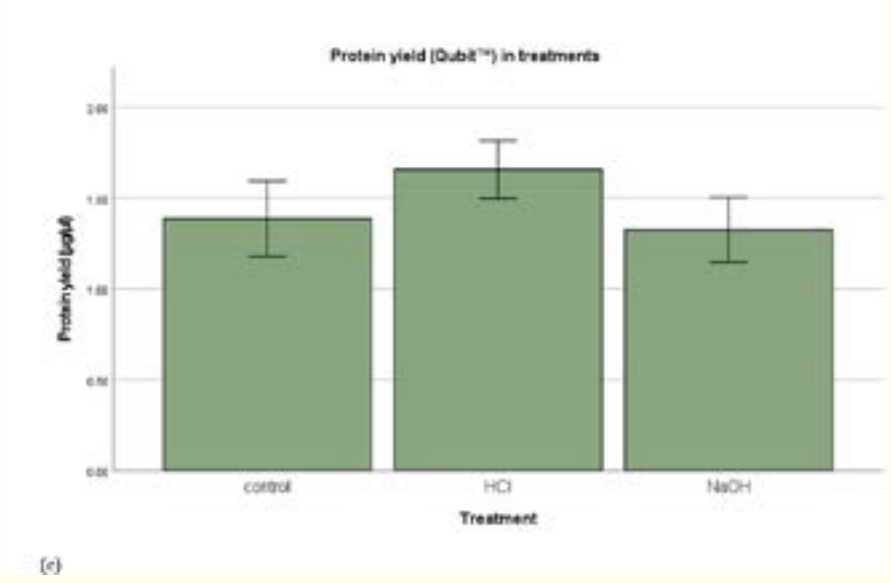


Figure 5: *DNA, RNA, and protein yields in the treatment groups (Control, NaOH, and HCl). (a) The control group showed significantly higher DNA yield compared to NaOH, and the HCl group showed significantly higher DNA yield compared to NaOH. (b) RNA yield was significantly higher in the control group compared to NaOH, and in the HCl group compared to NaOH. A significance level of #p < 0.05 indicates a difference between the control and NaOH groups, while *p < 0.05 denotes a significant difference between the HCl and NaOH groups. (c) No significant differences in protein yield were observed between the treatment groups. Error bars represent the standard error (SE).*

No significant differences were observed in the amount of biomolecules recovered, with treatment times ranging from 30 minutes to 2 hours, for both HCl and NaOH (data not shown).

Regarding quality assessment, no samples were inhibited. Out of the 30 samples, only 6 showed a DI above the threshold, which corresponded to 4 teeth.

STR analysis

The Promega PowerPlex® Fusion 6C System was used to obtain complete DNA profiles for all 23 autosomal markers and amelogenin, except for two cases where incomplete profiles were observed. These incomplete profiles corresponded to pulp samples from two distinct teeth treated with HCl and NaOH at different time intervals. Y-STR markers were excluded from the analysis for comparative purposes, as some samples were from female donors. The electropherogram confirms successful DNA profiling for both dentin and pulp samples from teeth treated with NaOH and HCl, as represented in the yellow channel (Figure 6).

Regarding the quality assessment parameters, total peak height (TPH), peak height ratio (PHR), and possible dropout were assessed. The total TPH for the 24 markers was calculated for each sample, and the average TPH for all samples under the same treatment was determined. The highest value was obtained for the control samples, with a mean of 222,360.11 ± 66,348.72, followed by the HCl-treated samples, with a mean of 108,339.79 ± 15,902.92, and the lowest for the NaOH-treated samples, with a mean of 64,866.00 ± 13,372.37. Significant differences were observed between the mean TPH of the control samples and both the HCl-treated (Mann-Whitney p = 0.027) and

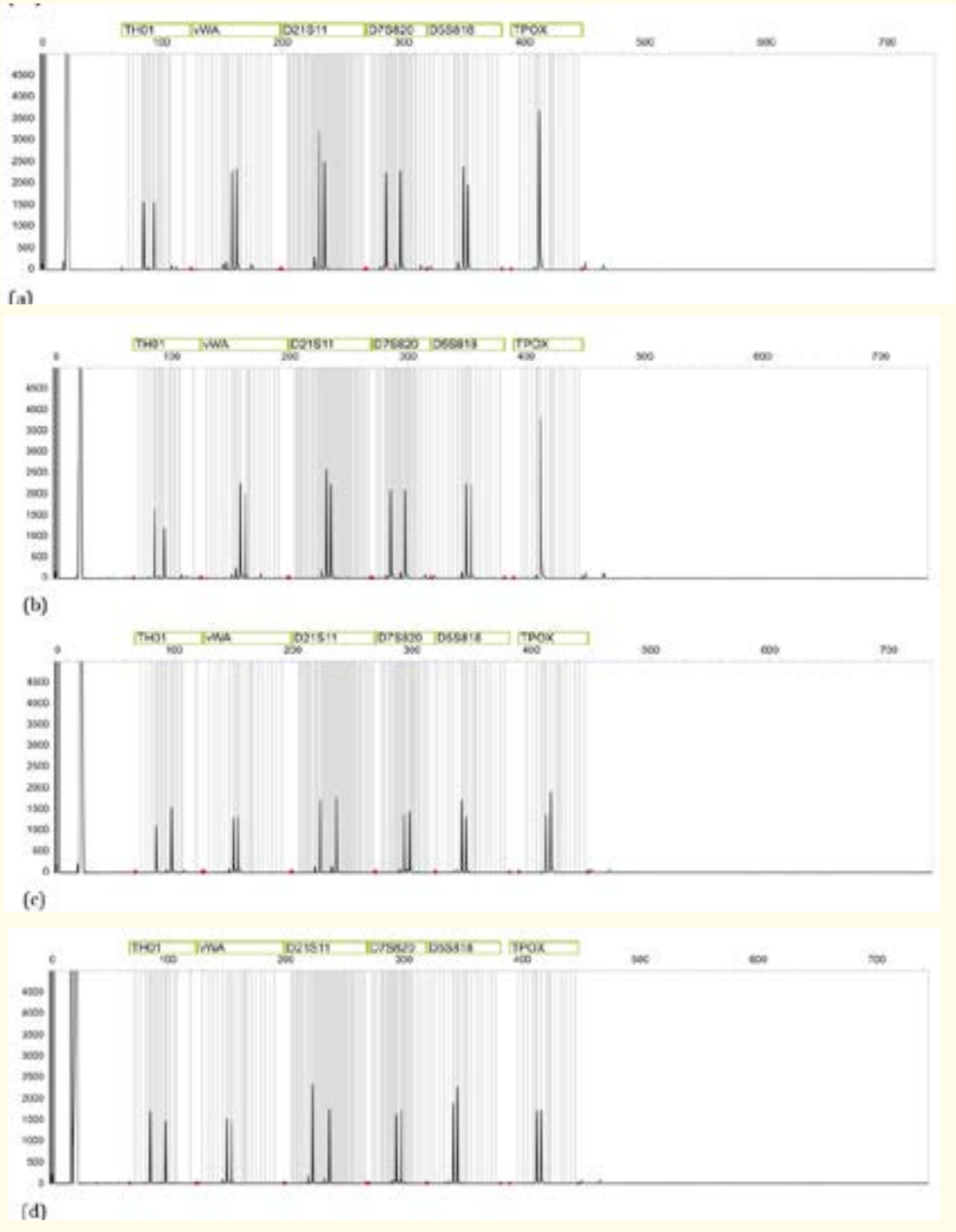


Figure 6: *Electropherogram profiles (yellow channel) of pulp and dentin from treated teeth. (a) Pulp from tooth 7 treated with HCl. (b) Dentin from tooth 7 treated with HCl. (c) Pulp from tooth 8 treated with NaOH. (d) Dentin from tooth 8 treated with NaOH. Allele calls were removed for privacy.*

NaOH-treated samples (Mann-Whitney p = 0.003). Furthermore, significant differences were found between the two treatment groups (NaOH and HCl, Mann-Whitney p = 0.021) (Figure 7).

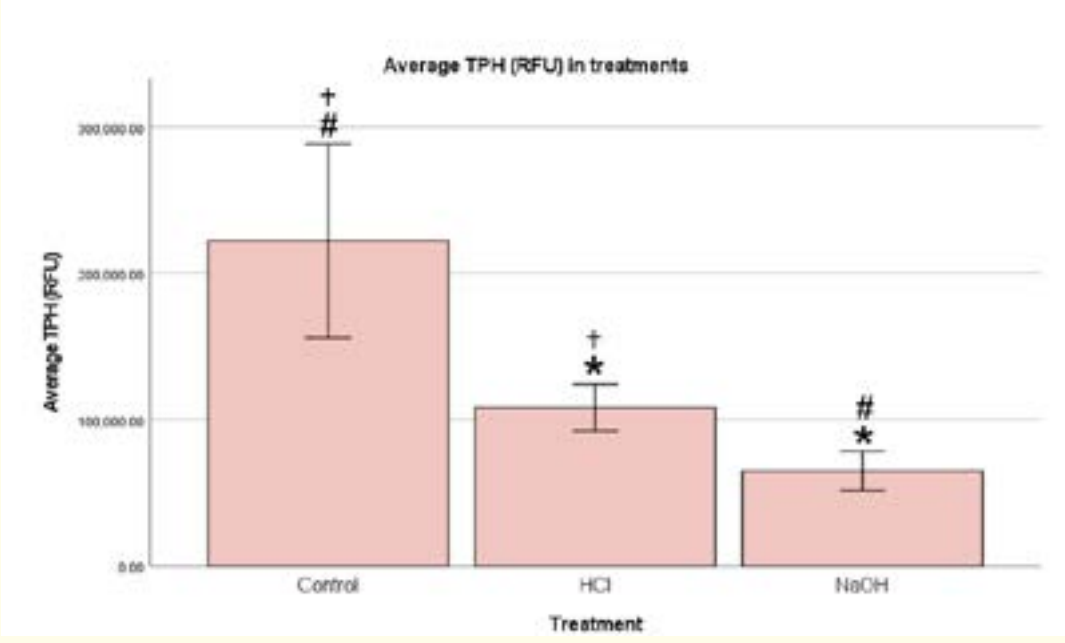


Figure 7: *Average Total TPH (in RFU) for each treatment group. Significant differences in TPH were observed between the control group and both HCl-treated and NaOH-treated samples, as well as between the two treatment groups. A significance level of #p < 0.05 indicates a difference between the control and NaOH groups, while †p < 0.05 indicates significant differences between the control and HCl group, and *p < 0.05 denotes a significant difference between the NaOH and HCl groups. Error bars represent the standard error (SE).*

The PHR analysis was also conducted, with the calculation based on the ratio of the largest peak to the smallest peak. The average PHR for the markers was calculated for control samples and those treated with HCl and NaOH. The mean PHR for the control samples was 0.91 ± 0.005, for the HCl-treated group was 0.90 ± 0.005, and for the NaOH-treated group was 0.82 ± 0.011. Similar to the TPH averages, significant differences in PHR were observed between the control and HCl-treated groups (Mann-Whitney, p = 0.068), between the control and NaOH-treated groups (Mann-Whitney, p < 0.001), and between the NaOH and HCl-treated groups (Mann-Whitney, p = 0.001).

Possible dropout was assessed in the heterozygous samples. Peaks below the threshold (<150 RFU) were detected only in the NaOH-treated samples, accounting for 20 out of 264 alleles.

Discussion

High-quality and quantity DNA, RNA, and protein were successfully extracted simultaneously from both control and treated samples using the Quick-DNA/RNA Miniprep Plus Kit (Zymo Research).

Teeth possess characteristics that make them highly valuable for human identification in challenging scenarios, primarily due to their low porosity, high mineral content, and the protective environment within the jaw [23]. For this study, third molars were selected based

on their preferred location in the posterior region of the dental arch, which offers protection from external damage, as well as previous findings indicating that they yield higher quantities of DNA [24].

It is essential to distinguish whether whole teeth or individual tissues are employed in a study, as the whole tooth is composed of multiple layers with distinct structures, which may impact the recovery of genetic material [37]. Additionally, the source of the samples must be considered, as this study utilized two types of teeth: those from living humans and those from a modern skeletal collection. Notably, efficient simultaneous extraction was achieved from both sample types. Structurally, teeth are composed of both hard and soft tissues, including enamel, cementum, dentin, and pulp, each differing in their proportions of inorganic material, organic material, and water. Enamel, which covers the crown, is the hardest tissue in the human body, with the highest inorganic content (95%). Cementum, which covers the root, has a lower inorganic fraction (65%) and a hardness more comparable to bone. Dentin lies beneath these layers, with an intermediate mineral content (75%), while the centrally located pulp is highly vascularized and contains various cell types, including odontoblasts and mesenchymal stem cells [38,39]. This study focused on the analysis of two distinct dental tissues: pulp and dentin.

The unique characteristics of the pulp contribute to the differential DNA recovery observed between these tissues, with previous studies showing that DNA recovery from pulp is higher than from dentin [24,26,40]. Consistent with these findings, the current study demonstrated significantly greater DNA yields from pulp compared to dentin. However, dentin offers a practical advantage, as it allows for the extraction of larger sample volumes—typically five to seven 200 mg Eppendorf tubes per tooth, compared to a single pulp sample. Therefore, the selection of dental tissue for DNA analysis should balance both DNA yield and the availability of sample material. In the initial phase of this study, which focused on incubation effects, the ability to work with dentin was particularly beneficial. The larger sample volume allowed for comparisons across different incubation times while maintaining tissue consistency, enabling a more accurate evaluation of extraction efficiency.

The Quick-DNA/RNA Miniprep Plus Kit (Zymo Research) employs silica column-based technology. Unlike the traditional phenol/chloroform/isoamyl alcohol method, this technique does not require a precipitation step. Furthermore, avoiding this method may help minimize contamination from external proteins and reduce toxicity for the operator [41]. It is also important to note that many researchers incorporate a decalcification step prior to extraction [42,43]. However, in this study, decalcification was considered unnecessary, as cementum and enamel were removed, and previous research has demonstrated that high-efficiency DNA extraction can be achieved without this step [24,44].

Few studies have explored simultaneous DNA and RNA extraction in forensic sciences, and, in contrast to the present study, most of them focused on body fluid identification [3,5]. Schweighardt., *et al*. (2015) [3] compare four commercial silica column-based extraction kits using blood samples, identifying Zymo Research ZR-Duet™ as the most effective for both DNA and RNA recovery. Loureiro., *et al*. [5] investigate dual DNA and RNA extraction in semen, comparing the commercial ExtractME RNA and DNA Kit with an in-house method [45]. They conclude that while the commercial kit yields superior results, the in-house method could achieve better outcomes if further optimized. Furthermore, for simultaneous DNA, RNA, and protein extraction, Mathieson and Thomas (2013) [4] compare silica column kits using rat liver tissues, finding that each kit is more effective for extracting a specific biomolecule, with All Prep (Qiagen) being better for RNA and Triple Prep (GE Healthcare) more suitable for DNA and proteins. However, a direct comparison with the current study is not possible, as the aforementioned studies focus on different tissues or fluids, none of which include hard tissues, and employ varying quantification methods. This highlights the necessity for continued research in this area to address these gaps.

In the initial phase, it was found that longer incubation times (24 hours) yielded significantly higher DNA concentrations. According to the Quick-DNA/RNA Miniprep Plus Kit protocol (Zymo Research), Proteinase K treatment is optional, with incubation times ranging from 2 to 5 hours, and may require optimization. When comparing incubation times, DNA concentrations after 24 hours were notably higher than those obtained after 2 or 5 hours. Although the identification of human remains often requires rapid processing, a 24-hour incubation period could enhance efficiency and minimize the need for re-extractions in many cases.

In the second phase, which focused on comparing treatments, the greatest impact was observed with the alkali treatment, where significantly lower DNA and RNA concentrations were obtained compared to the HCl treatment and controls. The primary objective of these treatments was to simulate the effects on samples under challenging conditions, such as those buried in a different type of soil, as well as to examine the potential impact on identification in criminal cases where the perpetrator may have used household chemicals intending to destroy evidence. Both the sodium hydroxide and hydrochloric acid solutions employed in this study are commercially available.

Studies correlating chemical treatments with varying pH levels and DNA yield are few. Specifically, research assessing DNA recovery after treatment presents significant challenges for comparison due to differences in experimental variables that may influence DNA yield. Sowmya., *et al*. (2013) [21] investigated DNA recovery from teeth submerged in an acidic solution for two hours, reporting high DNA concentrations. However, their quantification method—Nanodrop spectrophotometry—limits direct comparison due to its lower specificity. Additionally, their extraction method relied on phenol-chloroform, differing from the approach used in this study. Similarly, Al-Owaidi., *et al*. (2020) [20] extracted DNA from teeth treated with HCl and NaOH using phenol-chloroform. Their study employed different chemical concentrations and a longer exposure time (24 hours). While NaOH-treated samples yielded DNA concentrations similar to those in this study, HCl-treated samples yielded lower amounts of DNA compared to those in the present research. Yet again, methodological differences hinder direct comparison. In their study, Snedeker., *et al*. (2025) (Snedeker, Houston, and Hughes) analyzed multiple human tissues, including bones, teeth, soft tissues, nails, and hair, to examine the relationship between chemical treatment and DNA recovery. Although they applied the same chemical concentrations as this study, their exposure period was significantly longer (28 days). Contrary to the present study, they found that HCl was more detrimental to DNA than treatment with NaOH. Specifically for teeth, their day 1 results showed lower DNA concentrations in both HCl- and NaOH-treated samples compared to those in this study. This difference may be attributed to their use of a different extraction protocol, which involved prior demineralization followed by the AFDIL protocol (Automated Forensic DNA Indices Logic) [46]. It is important to note that in the aforementioned studies, dentin and pulp were not separated for analysis when working with teeth, which may also contribute to the observed differences.

The burial environment plays a crucial role in the preservation of human remains [8]. The complex interaction between soil pH, soil type, local climate, and microbial activity significantly influences cadaveric decomposition [47]. While research on the impact of soil pH on genetic material recovery from hard tissues is scarce, there is more available data concerning the effects on bones, with fewer studies specifically addressing teeth. According to Dent., *et al*. (2004) [48], bones are generally better preserved in neutral or alkaline soils; however, the decomposition process can alter soil pH, with factors such as the release of NH_4^+ and acetic acid production contributing to a decline in pH [49,50]. Ali., *et al*. (2024) [51] found that slightly alkaline soils (pH 8.2), low salt concentration, and low moisture levels helped preserve DNA. However, other studies suggest that both extreme pH conditions (acidic and alkaline) present challenges in DNA recovery [6]. In contrast to Ali's findings, this study determined that an alkaline pH significantly hindered DNA recovery, underscoring the detrimental impact of basic environments on the preservation of genetic material. Interestingly, studies on racemization rates in dentin align with this conclusion. Racemization, the process by which L-amino acids convert into their D-amino enantiomers over time, can be used for age estimation of skeletal remains. Research suggests that this process occurs more rapidly in alkaline conditions, leading to an overestimation of age when exposed to such environments [52]. In this case, higher pH environments appear to have a more significant impact on the preservation of genetic material than acidic conditions, further supporting the negative impact of alkaline environments on genetic material preservation.

Regarding STR profiling, successful amplification of complete DNA profiles was observed across all incubation times (2, 5, 12, and 24 hours). Notably, the 24-hour incubation not only resulted in full amplification but also yielded higher DNA concentrations, as mentioned earlier. This makes it preferable for cases requiring reanalysis of samples. In the second phase, regarding the treatments, complete profiles were successfully obtained for 39 out of 41 samples (including controls). As demonstrated in the profiles, even under what could be considered the most unfavorable condition—dentin treated with NaOH—high-quality profiles were still achieved.

The quality of the samples obtained in the human quantification process was satisfactory. None of the samples from either phase exhibited inhibition, an important factor when selecting the most efficient DNA extraction method [53]. The proportion of degraded samples

was minimal in both cases, and several of these samples were found to originate from the same tooth. This suggests that the issue was more likely related to the nature of the sample rather than the extraction process itself. Furthermore, in some cases, it was only possible to obtain dentin from the tooth. Previous studies have indicated that the amount of pulp in the tooth decreases with age [24]; hence, the samples may belong to elderly people, albeit could not be verified due to a lack of comparable data.

Regarding the quality parameters related to TPH and PHR in the treatments, it was observed that the average TPH was lower in the NaOH treatment, which also aligned with the quantification results. The TPH yielded high-quality profiles with values above 50,000 RFU according to previous studies [35]. Given that the average TPH value was lower for the alkali treatment, a detailed analysis was performed to identify the markers contributing to this difference. The markers exhibiting the poorest performance were those with higher molecular weight (CSF1PO, D22S1045, PENTA D, SE33). This finding aligns with the expectation that STR loci amplifying larger fragments may be more susceptible to DNA degradation in compromised samples, particularly those treated with NaOH (Figure 8).

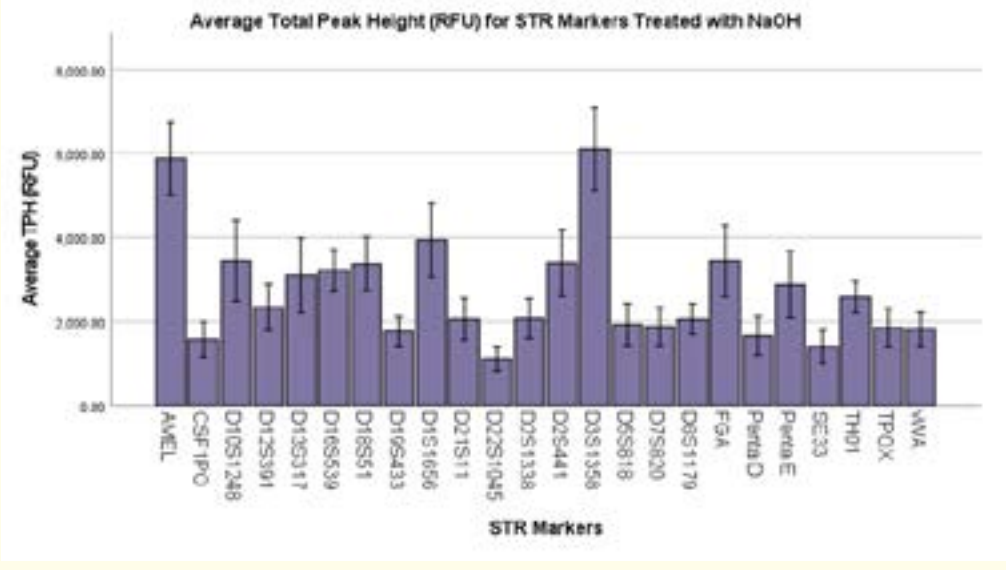


Figure 8: *Comparison of total peak height (TPH) values for 24 STR markers treated with NaOH. The x-axis represents markers with high and low molecular weight, while the y-axis shows the relative fluorescence units (RFU) of TPH. Error bars represent the standard error (SE).*

Moreover, the average PHR ranged from 82% to 91%, with the lowest value for the NaOH treatment and the highest for the control. This suggests that the heterozygous balance was adequate, which improves as the heterozygous peaks approach 100% [54]. Possible dropouts were observed solely in the samples treated with alkali. Since NaOH treatment appears to have the most detrimental effect on the teeth, it is reasonable to expect that dropouts would be primarily observed in these specific samples.

Overall, the results demonstrated that the simultaneous quantification of DNA, RNA, and proteins was robust. Human DNA quantification was effectively performed using *PowerQuant® Kit*, while RNA and protein quantification was achieved with fluorescence-based Qubit™ Fluorometer. If needed, future studies could further assess the quality of RNA and proteins using electrophoresis, RT-qPCR (Reverse Transcription-Quantitative Polymerase Chain Reaction) for RNA, and mass spectrometry for proteins.

It is important to consider that this study was conducted under controlled conditions, where samples were directly immersed in a household solution. Snedeker., *et al*. (2025) [11], suggest that smaller, isolated fragments tend to have faster chemical absorption, which could accelerate degradation and potentially lead to different results when varying amounts of tissue or immersion volumes are applied. However, despite these accelerated conditions, our study successfully achieved high-quality quantification, further validating the robustness of the method employed. To enhance the applicability of these findings, it would be valuable to replicate this study with extended immersion times or by placing teeth in different types of soil. Additionally, studying teeth samples buried in a body farm would introduce more environmental and realistic variables while maintaining a controlled environment for the decomposition study [55]. Expanding the sample pool to include different types of teeth would help determine whether the same results can be achieved regardless of tooth type. Future investigations could also further explore the impact of other challenging conditions, such as burned samples or extreme cases that hinder the extraction process and, ultimately, human identification.

Conclusion

In this two-phase study, high quantities of DNA, RNA, and proteins were simultaneously extracted and successfully recovered from teeth using the Quick-DNA/RNA Miniprep Plus Kit (Zymo Research). Additionally, successful human identification was achieved through traditional STR DNA analysis**.** Furthermore, a qualitative analysis of human DNA samples was performed through human quantification and STR analysis. The results demonstrated that samples incubated for 24 hours improved the extraction efficiency for DNA, RNA, and proteins. Moreover, the study found that treatment with sodium hydroxide (Instant Power® Crystal Lye Drain Opener) had the most significant effect on the recovery of biomolecules.

To date, there is limited scientific data regarding the environmental impact on the simultaneous recovery of DNA, RNA, and proteins for human identification. However, the combined extraction method employed in this study could prove highly advantageous, as it maximizes the amount of biological material available, which could be used for different forensic applications. To our knowledge, this study is the first to demonstrate the efficient simultaneous extraction of DNA, RNA, and proteins from human remains under varying environmental conditions, providing valuable insights for human identification and time-since-death estimation.

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Conflict of Interest Statement

All authors declare that they have no conflicts of interest.

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