

**COMPARATIVE STUDY OF DNA EXTRACTION
METHODS FOR RECOVERING DEGRADED DNA
FROM CHEWING GUM UNDER VARIED
ENVIRONMENTAL CONDITIONS**

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UNIVERSITI SAINS MALAYSIA

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by

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LIST OF ABBREVIATIONS

A230	Absorbance at 230 nm
A260	Absorbance at 260 nm
A280	Absorbance at 280 nm
BME	β -Mercaptoethanol
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide Triphosphates
dsDNA	Double-Stranded DNA
EDTA	Ethylenediaminetetraacetic Acid
EtBr	Ethidium Bromide
HCl	Hydrochloric Acid
HID	Human Identification/DNA Unit
IPC	Internal PCR Control
JEPeM	Human Research Ethics Committee of Universiti Sains Malaysia
NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
PCR	Polymerase Chain Reaction
qPCR	Quantitative Polymerase Chain Reaction
SDS	Sodium Dodecyl Sulfate
ssDNA	Single-Stranded DNA
TBE	Tris-Borate-EDTA Buffer
T _m	Melting Temperature
Tris-HCl	Tris Hydrochloride
UV	Ultraviolet Light
Mg ²⁺	Magnesium ion

LIST OF SYMBOLS

~	Approximately
bp	Base pair
°C	Degrees Celsius
g	Gram
μL	Microliter
μM	Micromolar
mL	Milliliter
M	Molar
n	Sample size
ng	Nanogram
nm	Nanometre
%	Percentage
®	Registered trademark
™	Trademark
V	Volt

ABSTRAK

Gula-gula getah merupakan sumber DNA forensik yang berpotensi kerana tempoh sentuhannya yang lama dengan air liur serta kerap ditemui di tempat kejadian jenayah. Walau bagaimanapun, DNA yang terdapat pada gula-gula getah sering terdedah kepada faktor persekitaran yang boleh menjejaskan integritinya. Kajian ini bertujuan untuk menilai pemulihan dan penguatan DNA terdegradasi daripada sampel gula-gula getah yang terdedah kepada pelbagai keadaan persekitaran. Sampel gula-gula getah dikumpulkan daripada peserta manusia dan didedahkan kepada keadaan persekitaran yang berbeza sepanjang beberapa selang masa bagi mensimulasikan senario forensik yang realistik. Pengekstrakan DNA dilakukan menggunakan kaedah resin Chelex® 100. DNA yang diekstrak dinilai menggunakan elektroforesis gel agarosa, analisis spektrofotometri, tindak balas rantai polimerase (PCR) konvensional, dan PCR masa nyata (qPCR) bagi menilai kehadiran DNA, corak degradasi, serta kejayaan penguatan DNA. Elektroforesis gel agarosa menunjukkan tahap degradasi DNA yang berbeza-beza antara sampel, dengan peningkatan fragmentasi diperhatikan dalam sampel yang terdedah untuk tempoh yang lebih lama. PCR konvensional yang menyasarkan amplicon pendek khusus manusia menunjukkan penguatan berjaya bagi beberapa sampel, menandakan kehadiran DNA yang masih boleh dikuatkan walaupun selepas pendedahan persekitaran. Analisis qPCR seterusnya mengesahkan pengesanan DNA manusia dalam beberapa sampel, walaupun kecekapan penguatan berbeza bergantung kepada keadaan dan tempoh pendedahan. Keputusan kajian ini menunjukkan bahawa gula-gula getah mampu mengekalkan DNA manusia yang boleh dikesan dan dikuatkan selepas pendedahan persekitaran, walaupun kualiti DNA dipengaruhi oleh faktor masa dan persekitaran. Kajian ini menyediakan data empirikal mengenai ketahanan dan degradasi DNA dalam sampel gula-gula getah serta

menyokong kerelevanan forensik gula-gula getah sebagai sumber DNA surih. Dapatan ini menyumbang kepada literatur forensik yang terhad mengenai substrat biologi bukan konvensional dan memberikan pemahaman terhadap cabaran pemulihan DNA daripada bukti air liur yang terdedah kepada persekitaran

ABSTRACT

Chewing gum is a potential source of forensic DNA due to its prolonged contact with saliva and frequent recovery at crime scenes. However, DNA deposited on chewing gum is often exposed to environmental factors that may compromise its integrity. This study aimed to evaluate the recovery and amplification of degraded DNA from chewing gum samples subjected to varied environmental conditions. Chewing gum samples were collected from human participants and exposed to different environmental conditions over multiple time intervals to simulate realistic forensic scenarios. DNA extraction was performed using the Chelex® 100 resin method. The recovered DNA was assessed using agarose gel electrophoresis, spectrophotometric analysis, conventional polymerase chain reaction (PCR), and quantitative polymerase chain reaction (qPCR) to evaluate DNA presence, degradation patterns, and amplification success. Agarose gel electrophoresis revealed varying degrees of DNA degradation across samples, with increased fragmentation observed in samples exposed for longer durations. Conventional PCR targeting short human-specific amplicons demonstrated successful amplification in selected samples, indicating the presence of amplifiable DNA despite environmental exposure. qPCR analysis further confirmed the detection of human DNA in multiple samples, although amplification efficiency varied depending on exposure conditions and duration. The results indicate that chewing gum can retain detectable and amplifiable human DNA following environmental exposure, although DNA quality is influenced by time and environmental factors. This study provides empirical data on DNA persistence and degradation in chewing gum samples and supports the forensic relevance of chewing gum as a source of trace DNA. The findings contribute to the limited body of forensic literature on unconventional

biological substrates and offer insight into the challenges of DNA recovery from environmentally exposed salivary evidence.

CHAPTER 1

INTRODUCTION

1.1 Background of study

DNA profiling is one of the most important and reliable method used in forensic investigations to identify individuals from biological evidence found at crime scenes. Thou it is a powerful tool; the success of an analysis depends on the quality and quantity of the DNA recovered. Saliva commonly deposited on everyday items such as chewing gums, represent rich DNA source due to its epithelial cell content but chewed gum often subjected to diverse environmental factors making it challenging or impossible to conduct DNA profiling (Sabnani & Sundaragiri, 2025).

Saliva serves as a cornerstone of forensic biology due to its rich composition of epithelial cells, microbial signatures, and genetic markers, which are frequently recovered from violent crime scenes. Recent studies emphasize that while saliva is often invisible to the naked eye, its preservation within complex substrates like chewing gum significantly enhances its evidentiary value, as the gum matrix protects DNA from rapid dehydration (Thornbury et al., 2021). However, the reliability of this evidence is heavily dictated by environmental stressors; research by Poetsch et al. (2022) and Khorwal et al. (2024) demonstrates that high temperatures and UV exposure accelerate DNA fragmentation and strand breaks, potentially compromising PCR amplification. Consequently, understanding the interplay between the protective nature of the substrate and the degradative effects of the environment is critical for successful forensic profiling.

The technical challenge of recovering high-quality DNA from chewing gum has led to various comparative studies on extraction methodologies. While commercial kits like QIAamp are noted for producing high-purity DNA suitable for sensitive downstream applications, traditional methods such as phenol-chloroform extraction often yield higher total DNA concentrations from degraded or aged samples (Rosenbaum et al., 2019). Despite these advancements, the sticky and inhibitory nature of chewing gum remains a hurdle, particularly when samples have been exposed to fluctuating outdoor conditions. Building upon these findings, this study aims to evaluate the efficiency of different extraction techniques in recovering viable human DNA from chewing gum across varying environmental durations and temperatures.

1.2 Problem statement

In forensic investigations, chewed gums are one of the common sources of biological evidence that can be recovered from crime scenes. However, their DNA stability under various environmental conditions and over time is not well established in local forensics context due to the challenges posed by environmental degradation making a successful DNA profiling less guaranteed. Variables like time, humidity, sunlight exposure and temperature can affect the integrity of DNA, leading to low yield and fragmentation. This degradation can render the DNA unsuitable for established forensic techniques, for instance Short Tandem Repeat (STR) analysis, thus hindering the individual identification (Bhoyar et al., 2024). While previous studies have examined the persistence of DNA on certain substrates, there is limited research on the comparative performance of different extraction methods on chewing gum samples exposed to varied environmental conditions over four-week period.

1.3 Research questions

- i. Which DNA extraction method produces the highest DNA yield from chewing gum samples?
- ii. How does different environmental exposure affect DNA yield and quality?
- iii. What is the rate of DNA degradation over different time intervals?

1.4 Objectives

1.4.1 General objective

To determine how extraction methods and storage environments influence DNA yield from chewing gum over four weeks.

1.4.2 Specific objectives

- i. To identify the most suitable extraction method and storage condition for maximizing DNA recovery from chewing gum evidence.
- ii. To compare the efficiency of DNA recovery from chewed gum under controlled (refrigerated) and uncontrolled (room temperature and sunlight) environmental conditions over a four-week period.
- iii. To evaluate the rate of DNA degradation across different time intervals in order to identify the practical time window during which DNA can still be reliably recovered for forensic analysis.

1.5 Significant of study

This research aims to address the gap in understanding the optimal DNA extraction approach for chewing gum samples subjected to environmental stress. By comparing three extraction techniques (silica-based column, Chelex 100 and modified Chelex 100

method), this research seeks to determine which method provides the highest DNA yield and purity across different environmental conditions (room temperature, refrigeration, and outdoor exposure) and time intervals. The findings will provide empirical data on the persistence and degradation of DNA from saliva traces on chewing gum exposed to different environmental conditions over time.

By quantifying DNA concentration, purity and amplification success, this study will fill critical knowledge gap by providing insights and understanding of the rate of DNA degradation and reliable DNA recovery after the relative impact of environmental factors. Furthermore, the result from this study will help forensic laboratories establish standardized protocols for handling chewing gum as biological evidence and provide a clear practical time window during which forensic laboratories can reasonably expect to obtain a complete and reliable DNA profile. This is significant particularly for country within tropical climates such as Malaysia where heat and humidity accelerate DNA degradation.

Ultimately, this research is scientifically relevant, practically significant and feasible therefore making it plausible to contribute directly to the efficiency and effectiveness of forensic investigations, particularly in cases where evidence has been exposed to the elements for an extended period. In addition, it may help improving forensic DNA analysis accuracy and reliability, supporting better case outcomes and advancing forensic science practices in Malaysia.

CHAPTER 2

LITERATURE REVIEW

2.1 Trace DNA

2.1.1 DNA in saliva evidence

Saliva is known to be one of the crucial biological evidence in forensic investigations due to its abundant DNA and biological markers. Saliva contains secretion from the salivary gland, GCF, epithelial cells and microbes, is often found at crime scenes especially in violent cases such as rape and homicides (Sabnani & Sundaragiri, 2025). The retrieving of saliva is a challenging process as saliva stain often appear as invisible to the naked eye therefore swabbing technique is the most common sample collection method used to collect saliva fluids.

As various biochemical and genetic information can be found in saliva, it provides wide range of information in forensic contexts such as DNA and Genetic Information, Blood Group Typing, Microbial Signature, Lifestyle and Behavioral Clues (Singhet al., 2025). Moreover, though detection of saliva is popular in DNA profiling, it is also has been use in identification of drug abuse such as amphetamines, barbiturates and opioids. Therefore, the evolution in saliva detection is significant to enhance the evidentiary value of saliva and its reliability in forensic investigations.

2.1.2 Chewing gum as source of DNA evidence

Chewing gum can be a valuable source of DNA evidence in forensic investigation as it often contains the saliva from the person who chewed, thus making it possible to retrieve human DNA for identification purposes. In addition, saliva dries

much slower in chewed gum as saliva cells are trapped in the gum making it having a significant forensic value (Thornbury, et al., 2021). While it is possible to extract DNA from chewed gum, it can be challenging to do so as chewing gum is a unique substrate that is soft and sticky.

DNA content of chewing gum can be influenced by the time since chewing, environmental exposure and method of collection. DNA yield in chewing gum initially decreased as chewing begin, then recovered during and after chewing (Thornbury, et al., 2021). Environmental factors like drying time and substrate can also impact DNA preservation and transfer. Moreover, substance in chewing gum can inhibit PCR amplification, thus lowering the quality of DNA profiles. Proper extraction protocols and inhibitor-tolerant analysis kits are essential for reliable results (Sanga, et al., 2015).

2.2 DNA degradation and environmental effect

Stability of saliva DNA is significantly affected by environmental conditions. Temperature, pH, humidity, microbial activity, and light exposure are the primary environmental drivers accelerating DNA decay, impacting the persistence and detectability of DNA in forensic context (Poetsch, et al., 2022).

2.2.1 Temperature

Temperature often associated with the degradation of saliva DNA. High temperatures accelerate the rate of DNA degradation in saliva. For example, the half-life of DNA in saliva at 37°C is much shorter than at room temperature, with protein-bound DNA being more stable than naked DNA (Wang, et al., 2016). On the otherhand, research consistently shows that both refrigeration (4°C) and freezing (-20°C, -80°C)

maintain DNA quality for extended periods, making these methods standard in forensic and biomedical applications. Saliva DNA remains stable for short-term storage (up to 1 week), with only minor decreases in yield and PCR performance (Finnis, et al., 2023).

2.2.2 Sunlight or UV exposure

Sunlight, especially UV radiation, causes DNA strand breaks and cross-linking, further accelerating degradation. UV exposure can also reduce the detectability of both human and bacterial DNA markers in saliva (Khorwal, et al., 2024). For example, UV exposure make bacterial DNA-based saliva identification difficult in a dose–dependent manner. However, protein-based saliva assays that used salivary α -amylase as an indication were unaffected by UV irradiation (Ohta & Sakurada, 2019).

2.3 DNA Recovery and extraction technique

DNA recovery from a chewing gum sample can be tricky due to its sticky nature. Numerous studies have investigated the most effective methods for enhancing DNA recovery from complex samples. Eychner et al. (2017) found that the QIAamp DNA extraction kit was the most effective approach to get high-quality DNA from whole pieces of chewed gum. The DNA QI method worked best for gum samples that had been outside. Conversely, a comparative study conducted by Van Biesen et al. (2025) assessing various extraction methods, including Chelex 100 resin and the QIAamp kit, determined that Chelex 100 produced the highest DNA concentration when utilised on dried blood spot samples. However, despite its higher yield, the DNA purity ratios ($A_{260}/_{280} = 1.7\text{--}2.0$; $A_{260}/_{230} = 2.0\text{--}2.2$) obtained using Chelex 100 were lower than

those from the QIAamp kit, indicating possible co-extraction of impurities.

2.3.1 Chelex 100 resin

The Chelex 100 method for DNA extraction is widely used due to its simplicity, cost-effectiveness, and ability to produce DNA suitable for PCR amplification. In DNA extraction from complex sample, its efficiency and limitations, along with advancements involving enhancing agents like digestion buffer and DTT (dithiothreitol), are significant.

Chelex 100 is a chelating resin that has a high affinity for polyvalent metal ions. Chelex 100 resin is a weak cation exchange medium. It effectively binds divalent metal ions like magnesium (Mg^{2+}), which are essential for metallo-nuclease function. High temperatures can result in the release of DNA into solution, as well as facilitate the binding of Chelex resin to magnesium ions. Magnesium ions act as cofactors for deoxyribonucleases, facilitating their activation. Chelex 100 resin suppresses nuclease activity in complex samples and stabilizes them for PCR applications. It has long been used for quick and easy DNA or RNA extraction from various sample types, such as blood, insects, bacteria, and viruses (Hu, et al., 2022).

Chelex 100 resin is known for its boiling method during the extraction of the DNA. A model for the extraction process is that the boiling treatment is useful for releasing DNA from low numbers of cells, and that Chelex protects the DNA from the effects of the boiling. It is not certain what the role of Chelex is in DNA preparation for PCR but the DNA treated by boiling without Chelex becomes inactive in PCR (Walsh, et al., 2013).

Forensic cases often deal with various types of samples thus sample preparation such as pretreatment with proteinase K and DTT is often required. Adding Proteinase K in Chelex 100 extraction method produce higher purity of DNA as Chelex 100 resin often subjected to a higher frequency of contamination (Núñez, et al., 2021). The combination has been applied successfully in forensic labs to optimize DNA recovery from mixed samples, although some protocols still rely on Chelex 100 alone for simplicity and speed (González, et al., 2004).

A bunch of study has been conducted to improve the quality of DNA extraction of Chelex 100 method by adding purification steps such as washing and precipitating to the extracted sample.

2.3.2 Silica-based method

Silica-based membranes are widely used for DNA extraction due to their ability to selectively bind nucleic acids in the presence of chaotropic agents, enabling efficient purification through binding, washing, and elution steps. Silica membrane methods generally yield higher DNA amounts compared to silica bead methods, though they may co-extract more inhibitors, which can affect PCR success, especially in challenging samples such as bones (Rothe & Nagy, 2016). Silica-based commercial kits remain a gold standard for DNA extraction in forensic investigations due to their robustness, reproducibility, and adaptability to diverse sample types (Connon, 2023).

Similarly, sample pretreatment is also necessary in silica-based method to improve DNA recovery especially from complex sample. Silica-coated paramagnetic

beads have also been used to improve DNA recovery from difficult samples by combining demineralization and extraction steps, highlighting the importance of buffer conditions such as pH and additives like DTT for optimal yields (Dukes, et al., 2012).

Commercial silica-based DNA extraction kits are widely used in forensic and molecular biology applications due to their efficiency, reliability, and compatibility with various sample types such as blood, saliva, semen, and forensic evidence. Popular kits include Promegas DNA IQ System and Qiagen QIAamp DNA Blood Mini Kit and Investigator Kit, which use silica-packed columns or silica-coated paramagnetic resins to bind DNA in the presence of chaotropic salts, enabling effective purification (Connon, 2023). These kits generally provide high-quality DNA suitable for downstream applications like PCR and sequencing, outperforming traditional organic extraction methods in purity and ease of use (Şengüven, et al., 2014). Some silica-based protocols have been optimized for automation and high-throughput processing, offering rapid and cost-effective solutions for large-scale genotyping and forensic casework (Connon, 2023).

However, the challenges for this method remain in recovering very low DNA concentrations and complex sample (Vutukuru, et al., 2025). One major issue is low recovery efficiency from samples with very small amounts of DNA, as many commercial silica protocols require higher input DNA and often lose over 70% of DNA during elution unless harsh buffers like heated formamide or NaOH are used, which are impractical for routine use (Katevatis, et al., 2017). In forensic and ancient DNA contexts, silica methods can co-extract inhibitors along with DNA which can interfere with downstream PCR amplification; silica membrane methods tend to yield higher

DNA amounts but also more inhibitors compared to silica bead methods (Rothe & Nagy, 2016). Despite these challenges, silica-based extraction remains a preferred method due to its balance of yield, purity, and compatibility with automation, but ongoing improvements in buffer chemistry and elution strategies are needed to maximize DNA recovery from difficult samples (Katevatis, et al., 2017).

2.4 DNA quantification

2.4.1 Agarose gel electrophoresis

One of the common methods use in quantifying DNA is agarose gel electrophoresis. Agarose gel electrophoresis is a commonly used laboratory technique for isolating and sizing DNA or RNA fragments based on length. It involves preparing a gel made from agarose, a polysaccharide extracted from seaweed, which forms a porous matrix through which nucleic acids migrate when an electric field is applied (Lee, et al., 2012). DNA and RNA molecules, which carry a uniform negative charge due to their phosphate backbone, move toward the positive electrode, with smaller fragments traveling faster and farther through the gel pores than larger ones . The concentration of agarose in the gel can be adjusted to optimize the separation of different fragment sizes, typically ranging from 0.5 kb to 25 kb for DNA (Green & Sambrook, 2019). After electrophoresis, nucleic acids are visualized by staining the gel with fluorescent dyes such as ethidium bromide or SYBR Green, which intercalate into the DNA and fluoresce under UV light. This technique is fundamental for analyzing PCR products, restriction enzyme digests, plasmid DNA, and is often a first step before further analyses like Southern or Northern blotting (Lee, et al., 2012).

In forensic science, agarose gel electrophoresis is commonly used to assess DNA quality, quantity, and degradation in forensic samples. It separates DNA fragments by size, allowing visualization of intact versus degraded DNA, which is crucial for evaluating sample suitability for further forensic analysis (Elkins K. , 2013). This technique can detect DNA fragmentation patterns that help estimate postmortem intervals, although more sensitive methods like comet assay may provide more precise degradation assessments (Mohamed, et al., 2020). In forensic identity testing, agarose gel electrophoresis is used to analyze restriction fragment length polymorphisms, but the presence of intercalating dyes like ethidium bromide can affect DNA mobility and complicate interpretation. The method is valued for its simplicity, rapid execution, and ability to recover DNA bands for downstream applications, making it a foundational tool in forensic DNA analysis (Green & Sambrook, 2019). Overall, agarose gel electrophoresis provides a reliable first step in forensic DNA evaluation, especially for quantifying and visualizing DNA extracted from crime scene samples.

2.4.2 Spectrophotometry

Spectrophotometry is an analytical method that determines a substance's concentration and characteristics by measuring the amount of light it absorbs or transmits at various wavelengths. It detects variations in light intensity caused by molecules in the sample absorbing, reflecting, or scattering light (Ramkumar, 2020). Spectrophotometry is sensitive, quick, economical, and repeatable. This technique is frequently used for identifying and quantifying compounds in a variety of fields, including biology, food science, and pharmaceuticals (Christodoulou, et al., 2022). Derivative techniques can also improve spectrophotometry by resolving overlapping

spectral bands and improving the analysis of complex mixtures without first separating them. The method, which frequently uses standard curves for quantification and depends on the relationship between absorbance and concentration, necessitates careful calibration and validation for accurate results (Ramkumar, 2020).

DeNovix® DS-11+ Series instrument is a UV spectrophotometer/fluorometer developed for nucleic acid quantification. It detects absorbance at specified wavelengths (usually 260 nm for nucleic acids) to determine DNA content and purity, allowing for speedy and reliable quantification in applications such as whole genome sequencing and quantitative PCR. Compared to other platforms such as NanoDrop (also UV spectrophotometry) and Qubit (fluorometry), DeNovix® shows very high correlation with NanoDrop in DNA concentration measurements, generally reporting about twice the DNA concentration compared to Qubit for pure DNA samples. DeNovix®s purity ratios (A260/280 and A260/230) are compatible with other spectrophotometric procedures, and the readings stay stable even after multiple freeze-thaw cycles (Versmessen, et al., 2024).

In addition, DeNovix® is commonly used for DNA quantification of forensic samples. IT is widely used for assessing DNA purity through absorbance ratios (A260/280 and A260/230), which is important for forensic DNA quality evaluation. Forensic samples often vary in quality and purity thus combining spectrophotometric methods like NanoDrop or DeNovix® with fluorometric or PCR-based techniques can improve accuracy in DNA qualification for downstream applications such as sequencing or profiling (Simbolo, et al., 2013).

2.4.3 Polymerase Chain Reaction (PCR)

Polymerase Chain Reaction (PCR) is a molecular biology technique used to amplify specific DNA sequences, enabling detection and analysis of small amounts of DNA. It involves repeated cycles of denaturation, annealing of primers to target DNA, and extension by DNA polymerase to create millions of copies of the target sequence (Kralik & Ricchi, 2017). Quantitative PCR (qPCR), also known as real-time PCR, builds on this by monitoring the amplification process in real time using fluorescent markers, allowing both detection and quantification of DNA during the exponential phase of PCR. qPCR can provide absolute quantification by comparing to standards or relative quantification by comparing gene expression levels between samples, making it widely used in diagnostics, gene expression studies, and microbial quantification (Harshitha & Arunraj, 2021). Key considerations in qPCR include primer design, reaction efficiency, and data interpretation to ensure accurate and reproducible results. While qPCR requires calibration curves and can be affected by inhibitors, it offers rapid, sensitive, and specific quantification compared to conventional PCR (Valasek & Repa, 2005).

In forensic science, PCR amplification is a fundamental technique used to amplify trace amounts of DNA from crime scene samples, enabling DNA profiling even from degraded or low-quantity specimens. It involves cycles of DNA denaturation, primer annealing, and extension to generate millions of copies of specific DNA regions, which are then analyzed for identification purposes (McDonald, et al., 2024). qPCR, enhances this by quantifying DNA during amplification, providing critical information on DNA quantity, degradation status, and the presence of inhibitors before downstream analysis like STR profiling. Forensic qPCR assays often target multicopy loci to

increase sensitivity and can include internal controls to detect PCR inhibitors, improving the reliability of DNA quantification in challenging forensic samples (Jäger, 2024). qPCR also supports forensic applications such as body fluid identification and simultaneous quantification of human and microbial DNA, aiding in case interpretation and sample prioritization (McDonald, et al., 2024).

CHAPTER 3

METHODOLOGY

3.1 General Laboratory Safety in DNA Analysis

All laboratory procedures during this study followed stringent safety standards and guidelines. Throughout the experiments, laboratory personnel used protective gear such as lab coats, disposable gloves, and surgical face masks. Upon using, all materials and apparatus were fully sterilized with autoclaving procedures, and all instruments were calibrated on a regular basis. To reduce cross-contamination and maintain public health standards, different phases of DNA analysis, such as genomic DNA extraction, polymerase chain reaction (PCR) amplification, and gel electrophoresis, were carried out in separate designated areas of the laboratory, including pre- and post-PCR rooms.

3.2 Laboratory Facilities and Workstations

Sample collection, sample preparation, DNA extraction, purification, and gel electrophoresis procedures were conducted out in the Forensic Science Laboratories. PCR was performed at the Human Identification Unit/DNA (HID) Laboratory, and DNA quantification was conducted in the Immunology Laboratory. All laboratory involved in this study were located in the School of Health Sciences, Health Campus, Universiti Sains Malaysia.

3.3 Ethical Approval

The research involved human DNA samples and was carried out in complete accordance with institutional policy and ethical guidelines. The Human Research Ethics Committee (JEPeM) of Universiti Sains Malaysia (USM/JEPeM/KK/25111028;

Appendix A) approved the study before it began. Before collecting chewing gum samples, all participants provided informed consent (Appendix B).

3.4 Materials

3.4.1 Chemical and Reagents

All the chemicals, reagents, commercial kits and consumables used in this study were listed in the Appendix C.

3.4.2 Instruments and Apparatus

All instruments and apparatus used in this study were listed in Appendix D.

3.4.3 Chemical and Reagent Preparations

3.4.3(a) 70% Ethanol

To prepare 500 mL of 70% ethanol, the required volume of absolute ethanol was calculated using the equation $M_1V_1 = M_2V_2$. Accordingly, 350 mL of absolute ethanol was measured and added to 150 mL of deionized water. The solution was mixed and stored in a 500 mL spray bottle for future use.

3.4.3(b) Tris-hydrochloride, Tris-HCl (pH8.0)

To prepare Tris-HCl (pH8.0), 12.11 g of 121.1g/mol Tris base was weighted into a 250mL beaker and approximately 80mL of deionized water were added into the same beaker. The solution then was stirred well. Next, the pH of the Tris base solution was measured and the pH was then adjusted to 8.0 by gradually adding 1 M HCl solution, with the pH monitored continuously using a pH meter throughout the adjustment

process. Once the desired pH of 8.0 was achieved, the solution was transferred into a 250mL Schott Duran® bottle.

3.4.3 (b) Lysis buffer

The lysis buffer as prepared following the protocol published by Köchl et al. (2004). To prepared the lysis buffer, 20 mL of 0.5M EDTA (pH 8.0), 2mL of Tris-HCl (pH8.0), 4mL of 5M NaCl and 10mL of 20% v/v SDS were added into a 200mL volumetric flask. Then, deionized water was added until it reached the line. The volumetric flask was inverted to allow the mixture uniformly mix. This buffer act as the working stock. Next, 49.5 mL of the working stock was measured and added into a 50mL beaker. In a fume hood, 0.5 mL 1% v/v β -mercaptoethanol (BME) was measured and tranfer into the beaker containing the buffer. The buffer was stirred well and was transfer into a 50mL Falcon Tube. The lysis was stored in the refrigerator as BME can lose effectiveness over time if exposed to air or light (Pavani et al., 2021). The lysis buffer with pre-added 1% v/v BME will be aliquote in small amount to minimize thawing the buffer. The remaining lysis buffer was transferred into a 200 mL Schott Duran® bottle and stored for future use.

3.4.3(c) 10% Chelex®100

Prior to preparing a 10% Chelex 100 solution in a final volume of 50 mL, the necessary mass of Chelex 100 resin was calculated. The mass of 50 mL was determined using the following formula: $\text{Mass of Chelex 100 resin} = 10 \text{ g}/100 \text{ mL} \times 50 \text{ mL} = 5 \text{ g}$. An analytical balance was used to weigh 5 g of Chelex 100 resin based on the computation. After that, a clean 50 mL Falcon Tube was filled with approximately 40 mL of deionized water. To avoid clumping, the resin was added gradually while being

stirred. After the resin was completely distributed, more deionized water was added to bring the volume up to 50 mL. To guarantee uniform suspension, the mixture was carefully mixed.

3.4.3(d) 0.5X Tris-Borate-EDTA Buffer (TBE Buffer)

To prepare 0.5X TBE buffer, the required volume of 10X TBE buffer was calculated using the equation $M_1V_1 = M_2V_2$. The 10X TBE buffer was readily available (preprepared) in the lab.

M_1 = Initial concentration of TBE buffer (10X)

V_1 = Volume of 10X TBE buffer to be used (? mL)

M_2 = Final concentration of TBE buffer (0.5X)

V_2 = Final total volume of 0.5X TBE buffer (1000 mL)

$$(10X) (V_1) = (0.5X)(1000 \text{ ml})$$

$$V_1 = \frac{0.5 \times 1000}{10}$$

$$V_1 = 50 \text{ ml}$$

Therefore, 0.5X TBE buffer was prepared by diluting 50 mL of 10X TBE buffer with 950 mL of deionized water and mixing thoroughly. The solution was mixed and stored in a 1 L Schott Duran® bottle for future use.

3.5 Sampling

3.5.1 Subject Criteria

Participant consent was the primary eligibility requirement for sample collection in this study. Samples were collected from healthy adults aged 18 years or above who were born in Malaysia. The participant must have no active oral infections, open sores or recent dental procedures for the past 2 weeks and was not under the influence at the time of testing. Individual that has recent antibiotic use (past 2 weeks) or known immunosuppressive conditions was excluded from this study.

3.5.2 Participants Recruitment

The research sample collection will be advertised to USM health campus students via email and WhatsApp groups. In addition, sample recruitment posters will also be distributed to all USM departments and throughout the campus to be display on the noticeboard (Appendix B). Written informed consent will be provided to all volunteers before sampling.

3.5.3 Sampling Procedure

The sampling procedure will be performed by the researcher herself to ensure proper sample collection while wearing gloves and a mask to prevent contamination. The samples were collected from six (6) students of Universiti Sains Malaysia. Participants were asked to rinse their mouth with water before chewing the gum (Ghemrawi, et al., 2024). Each participant was provided with an individually wrapped, unused piece of chewing gum and was asked to chew the gum for 2 to 3 minutes to ensure the saliva transfer. The chewed gum then be expelled directly into a sterile

collection tube using sterile tweezers to avoid any contamination. The tube then was sealed instantly and labelled accordingly.

The samples were labelled to be “Extraction Method—Time Interval—Environment Condition” : Silica-based column (1), Chelex 100 resin (2) and Modified Chelex 100 sample (3)—for extraction technique— day 1 (1), day 3 (3), day 5(5), day 7(7), day 14 (14) and day 28 (28) — for time interval— indoor condition (A), Outdoor condition (B) and refrigerated at 4°C (C)— for environmental exposure condition. For example, gum sample that was extracted using silica-based column (1) after been exposed for 3 days (3) at outdoor condition (B), the sample will be label as “1-3B”.

3.6 Sample Preparation

3.6.1 Sample exposure to different environment

After the samples were collected, the samples were exposed to 3 different environments— indoor (~30°C), outdoor and refrigerated (4°C). For indoor condition, the collected samples were exposed in a room at Desasiswa Murni USMKK. For outdoor condition, the samples were exposed openly to direct sunlight on a tree trunk near the laboratory building and the samples exposed at refrigerated condition were stored in a refrigerator in Forensic Science Laboratory.

3.6.2 sample collection on 6-time intervals

The samples were collected after been exposed at 6 different time intervals— day 1 (1), day 3 (3), day 5 (5), day 7 (7), day 14 (14) and day 28 (28)— imitating the early, intermediate and extended periods of evidence exposure in real forensic casework (Hemant S. et al., 2024). The samples were collected using sterile metal tweezer and

was transferred into a 2.0 ml microcentrifuge tube. The samples were labelled properly before proceeding to the next procedure.

3.6.3 Sample homogenisation

Each sample were freeze dried with liquid nitrogen and crushed into powder for homogenisation using a pestle and mortar. The samples were transfer into 1.5ml microcentrifuge tube and weighted. Each sample was able to be transferred into 2 tubes. Each tube (with samples) was weighted to be approximately 0.2000g to 0.2300 grams per tube.

3.6.4 Sample digestion

To digest the samples, 500µL of the prepared digestion buffer and 25µL proteinase K were added into each tube. The samples then were mixed vigorously by vortex and were incubated overnight at 56°C. Then, the samples were vortexed again and centrifuge at 12000 X g for 5 mins. The sample will be separated into 2 layers — precipitate and supernatant— and the supernatant, containing the lysis DNA, was carefully transferred to a new, sterile 1.5 mL microcentrifuge tube and stored at -20°C for future use.

3.7 Genomic DNA Extraction

3.7.1 Extraction Using Silica-based column

1st Base PrimeWay Genomic DNA Purification Kit, which utilized silica-based membrane technology in the form of a convenient spin column (1st Base, 2021), was used to extract DNA from 18 gum samples. Firstly, 200 µL of AL Buffer was added to a 2.0 mL microcentrifuge tube containing the sample lysate and was mixed thoroughly

by vortexing to obtain a uniform suspension. The sample was incubated at 70 °C for 10 minutes, then centrifuged at $11,000 \times g$ for 5 minutes. Next, 210 μL of absolute ethanol was added to the tube and mixed by vortexing. The prepared lysate was transferred to a PrimeWay Genomic DNA Purification Column inserted in a collection tube, then centrifuged at $11,000 \times g$ for 1 minute. The collection tube then was discarded, then the purification column was inserted into a new collection tube. A volume of 500 μL of Wash Buffer I (with ethanol added) was added to the column, and the tube was centrifuged at $11,000 \times g$ for 1 minute. The flow-through was discarded, and the purification column was placed back into the collection tube. Subsequently, 600 μL of Wash Buffer II (with ethanol added) was added to the column and then centrifuged at $11,000 \times g$ for 1 minutes. The flow-thru in the collection tube was emptied, and the column was centrifuged again at $11,000 \times g$ for 1 minute. The purification column was centrifuged again at $11,000 \times g$ for 1 minute to remove ethanol residual. The collection tube containing the flow-through solution was discarded, and the column was transferred to a sterile 1.5 mL microcentrifuge tube. 100 μL of Elution Buffer was added to the center of the purification column membrane to elute genomic DNA. The sample was let stand at room temperature for 2 minutes and then centrifuged at $11,000 \times g$ for 1 minute. The extracted DNA was either used in downstream applications immediately or stored at -20 °C until further processing.

3.7.2 Extraction Using 10% Chelex

The remaining 18 gum samples were extracted using the Chelex method, adapted from the original protocol by Suenaga and Nakamura (2005), with modifications introduced based on the publication by Köchl et al. (2004). Initially, 200 μL of 10% Chelex 100 was added into a 2.0 mL microcentrifuge tubes containing the

sample lysate and were thoroughly mixed by vortex. Then, the mixture was incubated at 56°C for 45 minutes with regular vortexing performed every 15 minutes. Subsequently, the samples were vortexed and then incubated at 95°C for another 10 minutes. Then, the tubes were centrifuged at 13,000 x g for 7 minutes. Next, 200 µL of the supernatant, containing the extracted DNA, was carefully transferred to a new, sterile 1.5 mL microcentrifuge tube and stored at -20°C for future use.

3.8 Genomic DNA Purification

Genomic DNA extracted from gum samples using the Chelex 100 was purified using the 1st Base PrimeWay Genomic DNA Purification Kit. Firstly, 100 µL of the supernatant was transferred into a purification column pre-inserted in a collection tube. Subsequently, 200 µL of AL Buffer and 200 µL of absolute ethanol were added into the same column and the column was centrifuged at 11,000 × g for 1 minute. The collection tube then was discarded, then the purification column was inserted into a new collection tube. A volume of 500 µL of Wash Buffer I (with ethanol added) was added to the column, and the tube was centrifuged at 11,000 × g for 1 minute. The flow-through was discarded, and the purification column was placed back into the collection tube. Subsequently, 600 µL of Wash Buffer II (with ethanol added) was added to the column and then centrifuged at 11,000 × g for 1 minutes. The flow-thru in the collection tube was emptied, and the column was centrifuged again at 11,000 × g for 1 minute. The purification column was centrifuged again at 11,000 × g for 1 minute to remove ethanol residual. The collection tube containing the flow-through solution was discarded, and the column was transferred to a sterile 1.5 mL microcentrifuge tube. 80 µL of Elution Buffer was added to the center of the purification column membrane to elute genomic DNA. The sample was let stand at room temperature for 2 minutes and then centrifuged