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Forensic Exhibit Packaging: Paper or Plastic, the Potential for DNA Degradation

Corinne Waite

A thesis submitted to the University of Huddersfield in
partial fulfilment of the requirements for the degree of
MSc by Research

January 2014

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Table of Contents

Copyright Statement.....	2
List of Tables and Figures.....	5
Abbreviations.....	6
Glossary of Terms.....	7
Abstract.....	8
Introduction	9
Current Forensic Practice.....	9
Previous Studies – Packaging and Transfer.....	10
Previous Studies – DNA Recovery Techniques.....	12
Previous Studies – Quantification Methods	15
Previous Studies – Storage.....	23
Previous Studies – Process Review	24
Contamination	27
DNA Structure and Degradation	29
Summary	32
Experimental	33
Sample Selection:.....	33
Sample Preparation:	33
Protocols:	36
DNA Purification from Buccal Swabs (Spin Protocol)[51]	36
Investigator® Quantiplex Kit – Quantification of DNA using the Rotor-Gene Q[52]	38
Instrument - Qiagen® Rotor-Gene® Q	39
Contamination	42
Results and Discussion	43
Contamination	55
Standards and Statistics	56
Summary	58
Future Work.....	60
Conclusion.....	61
Reagents.....	62
References	63

Appendix 1	66
Statistical Analysis of Results - Week 1 - 4.....	66
Statistical Analysis of Results - Week 5 – 8	67
Appendix 2	69
Week 4 packaging results for saliva in plastic.....	69
Week 4 packaging results for saliva in paper.....	70
Appendix 3	71
Comparison of results for weeks 1 to 5 with error bars.	71
Week 5 packaging results for saliva in plastic.....	72
Week 5 packaging results for saliva in paper.....	72
Appendix 4	73
Comparison of results for weeks 1 to 6 with error bars.	73
Appendix 5	74
Comparison of results for weeks 1 to 7 with error bars.	74
Week 7 packaging results for saliva in plastic.....	75
Week 7 packaging results for saliva in paper.....	75

Word count: 13,411

List of Tables and Figures

Table 1. Total number of saliva samples prepared for paper and plastic packaging	35
Table 2. DNA standards required for quantification of unknown (values quoted from manual) [52] .	38
Table 3. Sample position in wells on the Rotorgene	39
Table 4. Determining the optimal quantity of DNA obtained from saliva for this study.....	43
Table 5. Results for day zero	44
Table 6. Week 1 packaging results for saliva	45
Table 7. Week 2 packaging results for saliva	46
Table 8. Week 3 packaging results for saliva	47
Table 9. Week 4 packaging results for saliva	48
Table 10. Week 6 packaging results for saliva in plastic	49
Table 11. Week 6 packaging results for saliva in paper	50
Table 12. Week 8 packaging results for saliva in plastic	52
Table 13. Week 8 packaging results for saliva in paper	52
Table 14. Week 8 packaging results for saliva in plastic	53
Table 15. Week 8 packaging results for saliva in paper	53
Table 16. The overall reduction of DNA concentration over total of 8 weeks	54
Figure 1. An example of a laminar flow cabinet[36]	28
Figure 2. An example of the DNA structure	30
Figure 3. Investigator kit mechanism[52]	41
Figure 4. An example of a standard curve used during the saliva studies.....	56

Abbreviations

CV	Coefficient of Variation
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme Linked Immunosorbent Assay
HID	Human Identification
HPLC	High performance liquid chromatography
HRM	High Resolution Melt
ISO	International Organisation for Standardisation
NTC	No template control
PCR	Polymerase Chain Reaction
PM	Primer Mix
PBS	Phosphate Buffered Saline
PCR-STR	Polymerase Chain Reaction – Short Tandem Repeat
RFLP	Restriction Fragment Length Polymorphism
RM	Reaction Mix
RDS%	Relative Standard Deviation %
SD	Standard Deviation
STR	Short Tandem Repeat
SWGDM	Scientific Working Group of DNA Analysis Methods
UKAS	United Kingdom Accreditation Service
UV	Ultra Violet

Glossary of Terms

Buccal Swabs – A non-invasive way to collect cells from the inside of the cheek for DNA analysis.

CV – A statistical measure to assess the dispersion of values around the mean in order to compare the degree of variation.

Cycle Threshold – The point at which the threshold line passes through the amplification curve and provides a relative measure of the concentration.

DNA – A source of genetic information. More detail provided in the introduction.

Extraction Blank – During extraction the reagent mix is used with no sample. Water is added instead of sample in order to determine if contamination is present during extraction. If no amplification occurs it shows that contamination is not present at all or not in sufficient quantities to be detected during the cycles used.

NTC – This control is used during preparation of the reagents used for quantification. The reagents are added to the tube with no sample and buffer added instead in order to determine if contamination is present during preparation. If no amplification occurs it shows that contamination is not present at all or not in sufficient quantities to be detected during the cycles used.

PCR - The polymerase chain reaction is an enzymatic process that allows a piece of DNA to be amplified without affecting other areas of the strand.

Quantitative real-time PCR – A technique based upon traditional PCR where the quantity of DNA is calculated in 'real-time' during the process rather than at the end of the process. The quantity of DNA in a sample causes an increase in the intensity of fluorescence during PCR which is measured during each cycle of PCR. This results in the concentration of DNA to be quantified.

RSD% - Is the absolute value of the CV expressed as a percentage to express the precision of the values.

SD – A statistical value that expresses the difference between values used to create the mean and the mean value itself.

Abstract

A vital part of a forensic science investigation is the recovery of DNA from a crime scene. Body fluids such as blood or saliva are most commonly left at the scene of a crime and are frequently found in only minute quantities. In order to protect the recovery of this critical evidence it is required to be packaged in such a way that further degradation of the sample is not a possibility. In the United Kingdom, DNA evidence is packaged in plastic whereas other countries package DNA evidence in paper. This difference is due to the belief that DNA can degrade in plastic packaging. Evidence of a documented study that proves or disproves this theory has not been determined so therefore a study was carried out in order to establish which packaging would provide the most suitable option for DNA evidence. This study investigated different drying times prior to saliva samples being stored in either paper or plastic packaging and then determining the DNA quantities present over a set period of time using quantitative real-time PCR. The results indicated that if a wet saliva sample was to be immediately placed in plastic packaging and analysed within one week then this would provide the largest concentration of DNA. If this sample was to be analysed eight weeks after collection, the sample concentration would reduce when compared with a sample packaged immediately in paper packaging. Even with this reduction the sample immediately packaged in plastic presented a consistently higher DNA concentration when compared with all of the other samples. It was determined that if the wet saliva sample was allowed to dry for six hours and then packaged in plastic packaging, this presents the lowest degree of DNA loss.

Introduction

Current Forensic Practice

Crime scene investigation teams around the world have different DNA evidence recovery techniques and methods for body fluids. If this evidence is not properly recognised, documented, collected and preserved, it becomes useless in a criminal investigation [1]. In the United States of America, police and investigators are instructed to ensure that evidence which may contain DNA should not be placed directly in plastic bags as plastic bags will retain damaging moisture [2]. In Australia they also say that biological material should be placed in paper packaging as it allows the items to breathe whereas placement in a plastic bag may result in bacterial action and encourage the growth of mould, even samples packaged in paper should be air dried first [3]. In the United Kingdom DNA evidence can be packaged in polythene bags with an adhesive seal or tubes which are then placed into polythene bags for small items. Larger items or items that have the potential to rot or decay can be placed into paper bags.

The Biological Evidence Preservation Handbook provides guidelines for packaging biological evidence following a number of studies conducted to determine best practice [4]. It states that the majority of wet biological evidence should be dried once it has been collected so that it can be correctly stored. If the wet sample is unable to be dried straight away then the sample can be temporarily stored in a metal can or glass jar and placed under refrigeration at 2°C to 8°C out of direct sunlight. It states that plastic bags should not be used for the long term storage of wet samples and samples should be dried correctly before storage due to the possibility of bacterial or mould growth but plastic bags can be used for

temporary storage. Although an optimum drying time is not stated in this document, they do recommend that evidence should be dried out of direct sunlight in a temperature and humidity controlled environment with the relative humidity below 60% and the temperature between 15.5°C and 24°C. Once the evidence has been dried it is advised to place the samples into paper packaging or other breathable packaging. Breathable packaging is important due to its prevention of condensation build up which would damage DNA through bacterial growth with oxygen providing a protective barrier [5]. Once the sample is dry and packaged correctly it should then be stored in one of the following conditions depending upon the type of evidence; room temperature, temperature and humidity controlled, refrigerated or frozen at or below 10°C. Buccal swabs are best stored in a temperature controlled environment both short and long term but can also be stored for twenty four hours at room temperature.

Previous Studies – Packaging and Transfer

There have been no published studies found that suggest the effects of packaging types on the DNA concentration obtained from body fluids have been investigated. DNA evidence is a vital part of nearly all forensic investigations therefore it is pivotal to an investigation that it is collected in packaging that will not compromise the quantity or quality of the DNA. This study was established in order to determine if the United Kingdom were jeopardising the integrity of their DNA evidence by using the wrong packaging type and drying time.

Research into the transfer of DNA within the packaging has been investigated by Goray *et al.* [6], which showed that a significant quantity of DNA is frequently transferred from the exhibit to the inside of the packaging as well as transferring from one area of the exhibit to another [6]. The study utilised a range of packaging types, different sources of DNA; skin

deposits, blood and saliva and many other variables but they did not look at the potential for loss in DNA quantity as being caused by the packaging itself. They used the Chelex extraction method and quantified the DNA using Quantifiler™ Human DNA Quantification kit along with the Applied Biosystems Prism™ 7500 real-time PCR system. The study concluded that they demonstrated crime scene exhibits, during transit can potentially transfer DNA from the exhibit material to the inside of the packaging and also from one area on the exhibit to another part or even to another exhibit that may be within the same package.

Warshauer *et al.*[7] also conducted an investigation into the transfer of saliva derived DNA. They examined the primary, secondary and tertiary transfer during routine human behaviours and they evaluated the effects of drying time, moisture and surface composition. Warshauer *et al.* agreed with findings from a number of studies [8-12] that a smooth nonporous surface as the primary substrate and the presence of moisture increased the efficiency of transfer as well as the contributor being a 'good shedder' of DNA [7]. They used the QIAamp® DNA Mini kit for the extraction process and then they used the Quantifiler™ Human DNA Quantification kit along with the Applied Biosystems® Prism™ 7500 real-time PCR system as per the Goray *et al.* study.

Warshauer *et al.*[7] also used AmpFISTR® Identifiler® Plus PCR Amplification kit with an Applied Biosystems® GeneAmp® 9700 PCR thermal cycler in order to amplify the DNA ready for capillary electrophoresis on the Applied Biosystems® 3130xl genetic analyser with POP-4™ polymer. POP-4™ polymer is suitable for denaturing fragment analysis application, for example microsatellite and SNP genotyping analysis [13]. POP-6™ and POP-7™ is also available to use on this instrument with POP-6™ being for standard and rapid sequencing and POP-7™ being used for DNA sequencing and fragment analysis. The study concluded

that with each transfer event, a loss of DNA was evident and that when saliva is the original DNA source, the majority of the remaining DNA mixture after transfer is the originators DNA. The results of the tertiary transfer indicated that if moisture was present during the initial DNA deposit from the primary source, it played a larger role in the following transfer stages, this was more so than if moisture was present in any of the other transfer stages [7]. The method design discussed evaluated the effects of packaging DNA which involved the use of filter paper. The saliva samples were placed directly onto filter paper which was later used in the DNA extraction step. It was therefore important that the filter paper would not interfere or inhibit the extraction and quantification process.

Previous Studies – DNA Recovery Techniques

Sewell *et al.*[14] investigated the factors that could affect the DNA profile recovered from fingerprints when deposited onto various paper samples before and after fingerprint enhancement. They found that the DNeasy® Plant Mini Kit improved the recovery from paper by over 150% when compared with the QIAamp® Mini Kit. They deposited fingerprints on a number of different paper types which included Whatman® filter paper, A4 office paper (80 gsm), glossy magazine paper, A4 white card (240 gsm) and newspaper which they then stored in plastic tamper evident bags at 4°C. They used the Microcon® Ultracell YM-100 protocol to concentrate the extracted samples and then quantified them using the Quantifiler® Human Quantification Kit on the Applied Biosystems® Prism 7000 real-time PCR system. It was established that the office paper and white card interfered with the extraction of DNA and resulted in poor quality profiles but the filter paper, magazine and newspaper all allowed recovery of transferred DNA [14]. The results generated during this research provided evidence that the use of filter paper should not

interfere with this study and would provide a good substrate for the saliva to be placed on during the eight weeks.

The DNA obtained from saliva has become a crucial part in many forensic investigations whether it be a sexual assault, a robbery where a balaclava or helmet was used, an assault where someone had spat, a cigarette butt or drink container found at the scene. Abaz *et al.*[15] investigated the variables that have the potential to impact on the recovery of DNA from common drink containers. They looked at the types of drink containers, the effect of the contents of the drink container, person to person variation and the time factor that would represent sample recovery in a real life scenario. They utilised a modified Chelex® 100 extraction method and Phadebas® was used to assess α -amylase activity. They also used the ACES™ 2.0+ Human DNA Quantification System and AmpFISTR® SGM Plus™ multiplex for amplification and fragment analysis completed on the Applied Biosystems® Prism 377 Gene Sequencer.

It was concluded that time was the least significant variable in the study (24 and 48 hour period was evaluated). A person to person variability was evident from the results of the DNA concentration and α -amylase activity. Alcoholic drinks affected the α -amylase activity, DNA concentration and the profiles obtained when compared with non-alcoholic drinks. The type of drink container also affected the DNA concentration with cans providing a higher concentration than bottles. Overall, depending on the person and what they were drinking, it could potentially reduce the likelihood of obtaining a profile during a forensic investigation [15].

Brownlow *et al.*[16] compared the collection of DNA using the nylon flocked swab versus the traditional cotton swabs that have been used by the Metropolitan Police Service for

some time to retrieve DNA for forensic profiling. The study looked at how the swabs performed when extracted using three different extraction methods; QIAcube®, BioRobot® EZ1 and manually processed QIAamp® DNA Investigator Kit. The DNA was quantified using Quantifiler® Human DNA Quantification Kit on the Applied Biosystems® 7500 real-time PCR system. The samples were also amplified using AmpFISTR® SGM Plus Kit and using the Applied Biosystems® 3130xl genetic analyser to generate a profile. They determined that there was not enough evidence produced in the study to be able to recommend if the Metropolitan Police should start to use the nylon flocked swabs rather than the cotton swabs but they did establish that the extraction method had a significant effect on the results. The cotton swab combined with the spin-column extraction was shown to be the most effective in terms of recovered DNA whereas the nylon flocked swab combined with the BioRobot® EZ1 was the least effective [16].

Nunes *et al.*[17] investigated the use of less invasive methods for obtaining DNA samples, such as saliva collection, even though it produces a lower amount of DNA when compared with blood collection, it is becoming more widely used as it is more convenient for the donor and the person taking the sample. The study aimed to determine if the storage time of eight months would decrease the quality of DNA in the sample. The Oragene™ DNA Self Collection Kit was used to collect the saliva samples. The donors were requested to refrain from eating or drinking for at least 30 minutes prior to collection and upon arrival they were asked to perform a mouthwash with water and wait at least 15 minutes before the sample was taken. For the saliva collection they were asked to rub their tongue against the inside of the mouth for 15 seconds and then provide a quantity of saliva into a vial. They were then processed with an eight month interval between two aliquots of the same sample.

Spectrophotometry and TaqMan® with HRM assay and RFLP PCR were used for the quantitative and qualitative evaluations. The study concluded that after eight months, a sample stored in Oragene™ solution at room temperature did not affect the quality or quantity of DNA extracted from the sample. They also determined that the collection of over 2.2mL of saliva did not provide a higher overall yield of DNA per kit [17].

Another study that used Oragene™ DNA Self Collection kits was completed by Abraham *et al.*[18] and they looked at saliva samples being a viable alternative to blood samples as a source of DNA for high throughput genotyping. They completed a comparison between saliva extracted DNA and blood derived DNA by using both Applied Biosystems TaqMan™ and Illumina Beadchip™ genome arrays. They used the Oragene™ kit to collect and extract the saliva samples and DNA from EDTA blood samples was extracted by Gen-Probe. The results showed that the total DNA yield from saliva was lower than that obtained from blood. They also found that protein contamination and DNA fragmentation measures were greater in saliva than in blood. They concluded that genotyping quality was comparable on both TaqMan™ and Illumina™ beadchip arrays. With saliva collection being less invasive compared with blood collection, the collection of saliva derived DNA would be beneficial to clinical trials and could reduce costs for collection [18].

Previous Studies – Quantification Methods

A comparison of five quantification methods for DNA was carried out by Nielsen *et al.*[19]. They used six commercially available preparations for human genomic DNA and quantified them by UV spectrometry, SYBR-Green dye staining, slot blot hybridization with the probe D17Z1, Quantifiler™ Human DNA Quantification Kit and RB1 rt-PCR. The results indicated that the DNA quantified by UV spectrometry and Quantifiler™ were similar and were close

to the expected DNA concentration stated by the manufacturers. They later determined that a calibration problem with the Quantifiler™ Human DNA standard may have occurred when used with the Quantifiler™ Human DNA Quantification Kit. A higher DNA quantity was seen and determined to be due to degraded DNA as single stranded DNA absorbs 20-30% more of the UV light than double stranded DNA would at 260nm. They also determined that contaminants in the UV light range of 200-230nm with a maximum of six units at 210nm may have interfered with the measurements that were produced at 260nm. This presence of UV absorbing contaminants shows that UV measurements of DNA concentrations should not be relied upon without ensuring that the quality and condition of DNA is such that these interferences would not occur [19].

UV spectroscopy for the quantification of DNA is inadequate in forensic science due to poor sample specificity. This method means that the analyst is unable to determine if the sample contains contaminants, bubbles or background noise as well as the method requiring a large amount of sample which is not often available in forensic science. Most cases have minimal quantities of DNA to perform analysis on and it is required that profiling is carried out in duplicate with the option of a third back-up sample. Using this information, it was decided that the use of UV spectrometry as the quantification method was not satisfactory for the forensic study of plastic versus paper packaging.

A large number of papers have focused on quantification methods, Nicklas et al.[20] focused on a review of the classic techniques and the newer quantification methods. They reviewed ultraviolet and fluorescence spectroscopy, the use of PicoGreen and OliGreen, RFLP, gel electrophoresis, slot blot, colorimetric assay, AluQuant Human DNA Quantification System, real-time PCR (Taqman, SYBR Green) and others. It was concluded that initially the methods

concentrated on quantifying the total DNA using UV or dyes without being able to determine the species of origin, whereas with the developments in technology it is possible to assess human specific DNA so that precise quantification can take place [20]. Since this paper was written technology has continued to develop.

Tringali *et al.*[21] looked at the real-time fluorescence probe system in more detail. They viewed the real-time PCR system as a highly sensitive, specific, cost effective, fast and flexible assay that can even perform when using poor quality DNA. They used saliva, hair, bone, urine, blood, seminal liquid tissues, stamps and cigarettes as the source of DNA and carried out extraction using a number of different extraction methods. They concluded that real-time PCR allowed for the accurate quantification of nucleic acid during the PCR reaction without the requirement of post-PCR analysis. Analysis time was reduced by around 50% when compared with the QuantiBlot analysis method and can perform on poor quality DNA samples [21].

Swango *et al.*[22] specifically assessed a quantitative PCR assay to determine the degree of degradation in DNA samples. They used TaqMan® along with the Applied Biosystems Prism® 7000 real-time PCR system and the AmpFISTR® Identifiler™ PCR amplification kit was used for the STR genotyping along with the Applied Biosystems GeneAmp® 9700 PCR thermocycler and Applied Biosystems Prism® 3100 genetic analyser. They concluded that the real-time PCR target sequence length was extremely important when quantifying degraded DNA for STR genotyping. They looked at a number of samples with varying degrees of degradation and found that they could provide a good estimation of the degree of degradation present in the sample. Along with an internal control for PCR inhibition they provided a valuable tool for post-extraction sample assessment [22].

Another study a year later looked at absolute quantification of forensic samples using quantitative real-time PCR methods. Schulz *et al.*[23] used two different quantitative real-time PCR techniques to amplify the same target sequence that only differed in their amplicon length. The samples were extracted using the M48 BioRobot® and the QIAamp® DNA Mini Kit and they were profiled using multiplex kits (SEfiler, Identifiler, AB: PowerPlex 16 and Y, Promega). They were analysed on the Applied Biosystems® Prism™ 3100 genetic analyser. For quantification, TaqMan® Universal PCR Mastermix was used with either the Quantifiler™ Human DNA quantification Kit or a custom made Telomerase assay. It was determined that the Quantifiler™ Human DNA quantification kit was more sensitive and efficient than the Telomerase assay [23].

Another study produced in 2009 looked at an automated system for extraction, quantification and STR amplification of forensic evidence samples. Stray *et al.*[24] investigated the use of an automated system due to the lengthy process involved in DNA analysis. With the steps including extraction of DNA, quantification of human DNA in the extract, possible sample clean up requirements, amplification of products using multiplex STR systems, separation of products and data analysis it is creating pressure on laboratories to complete the quantity of forensic casework that is being generated. By introducing automated processes into the laboratory it increases the resource available as it essentially acts as an additional employee and allows analysts to concentrate on other areas such as data analysis or report writing. The paper looked at the use of the HID EVolution™ Combination System to process blood stained paper, cotton fabric and denim, dried blood spiked with known PCR inhibitors, saliva on cotton swabs and semen stains. They used the AmpFISTR® Identifiler® Kit to assess the STR profiles. They concluded that the HID

EVolution™ Combination System achieved automation of extraction of DNA with the PrepFiler™ Automated DNA Extraction Kit, quantitative real-time PCR using the Quantifiler® kit and PCR setup for STR amplification using the AmpFISTR® PCR Amplification Kit. The amplified products were successfully analysed on the Applied Biosystems® 3130xl genetic analyser and they found that the DNA quantity and STR profile were complete, reproducible and equivalent to the manual method [24].

If an automated system has been validated along with the method, it is understandable about the benefits that it would bring to a working laboratory especially in a forensic laboratory. It would remove the possibility of human error during sample preparation, if the reagents were contaminant free it would also reduce the risk of analyst contamination and cross-contamination. It is extremely important that before an instrument or new method is utilised within a forensic testing laboratory, it has undergone a stringent validation protocol to ensure that it is reliable and the results can be trusted.

Green *et al.*[25] produced a developmental validation study of the Quantifiler™ Real-Time PCR Kit for the quantification of human nuclear DNA samples. They used the official guidelines from the Scientific Working Group of DNA Analysis Methods (SWGDM) to perform the developmental validation study and tested the kits (Quantifiler® Human Quantification kit and Quantifiler® Y kit) for performance criteria such as sensitivity, species specificity, stability, precision and accuracy. They used UV absorbance, dye intercalation and slot blot hybridization methods to assess the accuracy of the Quantifiler® Kit assays. They concluded that the assays were reliable and robust and that they produced quantification results that were consistent with other DNA quantification methods. The assays also have a unique characteristic in that they have the ability to separately detect

male human DNA in mixed samples. They could also provide more information about PCR inhibitors which are present in the analysed sample which would benefit laboratories carrying out forensic DNA analysis [25]. Instrument and method validation is a lengthy process and usually involves samples to be processed by different analysts, in different laboratories and on a number of different instruments to ensure result accuracy and precision.

In 2011 a review of the Investigator® Quantiplex Kit was produced by Pasquale et al. [26] that looked at the increasing requirement for fast and accurate results due to the forensic workload in most laboratories. Due to multiplex assays for the identification of human DNA being complex and with the requirement for a defined range of template input, in order to ensure that genotyping can be completed successfully in the very first attempt it is important to be able to assess the potential presence of any PCR inhibitors and be able to complete accurate quantification of sample even at low concentrations. Over the last few years quantitative real-time PCR has become the standard method for the quantification of DNA in any forensic casework samples. Due to the increase in forensic casework and the requirement for DNA analysis there was a necessity to increase the accuracy and reduce the time it was taking for the results to be obtained. The Investigator® Quantiplex Kit provided them with fast and accurate results and when they combined this kit with the Rotor-Gene Q real-time PCR instrument it meant that results for up to 72 samples were produced with 48 minutes. The Investigator® Quantiplex Kit gives sensitivity to 0.3 pg/μl and accurate quantification in the linear range of the standard curve of 4.9 pg/μl. They even found that it was possible to combine the Investigator® Quantiplex Kit with the QIAgility instrument that

allowed the automation of routine procedures such as quantification preparation and STR setup [26].

The Investigator® Quantiplex Kit combined with the Rotor-Gene Q real-time PCR instrument was selected for use within this study of plastic versus paper packaging. Klein [27] carried out a review of real-time PCR and discussed the applications and limitations of the technology. The cheapest method of real-time PCR does not require an additional fluorescence-labelled oligonucleotide making this the easiest method but it means that non-specific PCR products are also detected. Although the method is straight forward it means that additional work is required to optimize the PCR conditions and melt curve analysis is required to differentiate between products. Other methods include a fluorescence labelled oligonucleotide which only fluoresces when the probe is cleaved or during hybridisation of oligonucleotides to the amplicon. The method of using Scorpion primers, which was discussed above, allows for rapid assays with short equilibration times due to the fluorescent labelled tail that hybridizes to an amplified target.

Advantages of using real-time PCR are that post-PCR steps are not required so the potential for contamination is reduced; the technique is sensitive and produces high precision in the results [28]. It is also a rapid process and requires minimal sample for quantification. Many of the earlier methods for measuring quantities of DNA, for example, gel electrophoresis, Northern and Southern hybridizations, HPLC and PCR-ELISA, have a number of limitations. They could be time consuming, insufficiently sensitive, labour intensive or non-quantitative [29]. The sensitivity in real-time PCR allows for small samples to be analysed and relatively quickly when compared with previous methods. The chances of cross contamination are also reduced due to the reaction being completed in a closed vessel that requires no post-

PCR manipulation [29]. As long as the instrument is correctly operated and the samples prepared correctly and accurately the results should be of high quality.

Although there are a number of advantages to real-time PCR, there are also several limitations. One of the limitations has been identified as the possibility of false negative results and it was concluded that although time is saved during processing, it requires time to check the validity of the results as sample preparation and the quality of the standards can have a large impact on the accuracy and precision of the results [27]. Real-time PCR is also susceptible to PCR inhibition which can affect the sensitivity of the assay or cause false-negative results [29]. Inhibitors that can be found in body fluids are haemoglobin and urea but also organic and phenolic inhibitors could be present [30]. Inhibitors can be an issue with any source so as long as steps are taken to remove or reduce inhibitors where possible this will reduce the effect seen during real-time PCR.

An interesting paper that has been produced recently presents a rapid extraction method that could dramatically speed up the extraction process. Kalyanasundaram *et al.*[31] reported a novel extraction method for human genomic DNA from buccal swabs and saliva samples. The DNA is attracted onto a gold-coated microchip using an electric field and capillary action while the captured DNA is eluted by thermal heating at 70°. They compared the results obtained with QIAamp® DNA Mini Kit and they achieved an equivalent quantity of DNA using fewer extraction steps. This method of extraction is environmentally friendly as a significant reduction in reagents could complete the DNA extraction. It was also much less time consuming as it took less than ten minutes to complete the DNA extraction from small volume saliva samples [31].

Previous Studies – Storage

Ng *et al.*[32] investigated the effects of storage conditions on the extraction of PCR quality DNA from saliva samples. They looked at different storage conditions in order to determine if they would impact on the ability to extract genomic DNA in a sufficient quantity and quality for PCR. They used the following conditions; saliva washed with PBS and extracted the same day of collection, washing and centrifugation to form a pellet then stored at -70°C for one week, storage of whole saliva at 4°C for seven days followed by washing and extraction, storage at 4°C for seven days followed by washing and pellet formation which was then stored at -70°C for one month, storage at -70°C for one month followed by washing and extraction. The DNA quantity and quality was assessed using spectrophotometry at 260-280nm. The results indicated that the storage conditions still allowed for PCR to generate single specific product of the correct size from all of the samples involved in the study. Storage at 4°C for seven days followed by washing and pellet formation which was then stored at -70°C for one month and storage at -70°C for one month followed by washing and extraction produced the weakest amplification and the sample that was only washed with PBS and extracted on the same day provided the best result. They concluded that although some conditions affected samples more than others, it is still possible to retrieve genomic DNA from saliva even when it has been stored in less than optimal conditions [32]. The method choice within this paper could potentially raise the question of precision and accuracy due to the use of UV spectrophotometry to assess purity and agarose gel electrophoresis to quantify the PCR bands produced. Interferences such as UV absorbing contaminants during spectrophotometry as discussed earlier in this introduction could potentially generate an erroneous result. Poor separation or unclear bands in agarose gel electrophoresis could also potentially add to the degree of error.

With sample storage being critical in the reduction of DNA degradation, it is often a topic for investigation. Lee et al.[33] investigated a novel room temperature DNA storage medium; SampleMatrix™ that was used to protect and stabilize samples. They prepared samples that had a varying amount of DNA in SampleMatrix™ and stored them from one day to one year under different conditions from an ambient laboratory environment to a number of freeze-thaw cycles. They determined that there were no substantial differences between the quality of samples. For low concentration samples or samples requiring long term storage then SampleMatrix™ provided an advantage over typical freezer storage with no detectable inhibition [33]. This would be very valuable for forensic casework as the storage for cold case samples would need to be optimum.

A comparative study of forensic saliva stains by Balitzki *et al.*[34] placed 120 buccal swabs into optimal storage conditions and 40 buccal swabs into degrading conditions for storage. The experimental period of 173 or 367 days was used and the α -amylase activity was detected using the Phadebas® assay and the DNA was quantified using the Human Quantifiler® Kit. They determined that only one sample out of the 160 samples showed no DNA present and no α -amylase activity. All of the other samples were positive for α -amylase activity but 13 of the samples showed no DNA and 16 other samples provided very low DNA quantities. From this they concluded that α -amylase is very stable in saliva even in degrading conditions and is not affected by storage time [34].

Previous Studies – Process Review

Caddy *et al.*[35] documented a review in 2008 about low template DNA analysis due to doubts that had been expressed about the use of the analysis in legal proceedings. The group looked at the profiling techniques that were being used to produce DNA profiles from

samples that were unable to generate useable results from SGM Plus®, assess the validity of the profiling techniques, comment on the interpretation of results, provide advice on the production of formal technical standards and provide recommendations.

The review involved three laboratories and a number of personnel from other areas associated with forensic science, for example the United Kingdom Accreditation Service (UKAS) who can assess organisations and services against set standards and award certification for laboratory processes, quality processes and other areas. SGM Plus® examines ten short tandem repeats plus a gender marker using 1ng of DNA amplified using 28 PCR cycles which is a validated method. The use of 28 PCR cycles should not generate a full DNA profile if low level contamination was to occur even with the incorporation of anti-contamination procedures. DNA extraction was completed either manually or using an automated process with either the Qiagen kit or the phenol/chloroform method.

Caddy *et al.*[35] determined that the personnel in the laboratories had undergone rigorous training and anti-contamination precautions were in place such as cleaning regimes, pressurised laboratories and UV irradiation of work areas. They observed that contamination was monitored regularly and corrective actions were put into place should contamination be determined. The analysis of DNA was completed by PCR and capillary electrophoresis. Real-time PCR has now allowed for the quantification of samples using a minimal amount of the sample so that full analysis can be carried out in duplicate on the remaining product. Quantification has previously been an issue in cases where the starting quantity was low; even though it is a useful tool for reducing the possibility of over amplifying the PCR products and allows for an estimate of inhibition. One method to increase PCR product was to increase the number of PCR cycles from 28 to 34 but this can

be detrimental as it can cause difficulties in allocating the second peak during profiling. Another method is to clean the sample post PCR (28 cycles) and analyse using optimised capillary electrophoresis to produce a profile similar to the one obtained using 34 PCR cycles.

Caddy *et al.* went on to explain the importance of method validation and that once a method has been validated it should continue to be open to review and optimisation. An example was when inhibition was preventing samples to be profiled and the solution was to dilute the sample so that the inhibitor was diluted which meant that the laboratory had to re-test 5000 samples using this change to the method. It was determined that the laboratories had completed in house validation studies on the methods associated with low template DNA analysis and although this was accepted by UKAS as part of the laboratory accreditation (ISO 17025), it was felt that inter-laboratory studies should have been carried out in order to confirm repeatability. It was determined that technical standards for extraction, quantification and interpretation needed to be agreed by not only the providers but the users as well such as the police and the criminal justice system. Also a clear protocol needed to be created for the interpretation of results so that statistics did not cause confusion in court. The aspects reviewed in this paper were areas that were criticised during the case of the Omagh Bombing. The validity of the methods were brought into question as well as contamination issues [35]. This shows how vital it is to ensure that methods have been validated correctly and that anti-contamination procedures are in place and monitored.

Contamination

Contamination can be extremely problematic due to the sensitivity of the techniques used; the smallest levels of background DNA have the potential to be detected and interfere with results, especially in this study where the DNA quantities were extremely small and levels of degradation were being assessed. As PCR can amplify very small amounts of DNA, it also means that any unwanted DNA molecules present in the environment can also be amplified. Contamination can be very costly in terms of wasted research time and reagents; also in the forensic field it could potentially affect people's lives. The main sources of DNA contamination are on the bench surfaces, laboratory equipment, pipettes, and airborne particles such as debris from hair and skin or microbes along with contaminated solutions.

Preparation of samples in a laminar flow cabinet helps to reduce contamination due to a constant air flow blowing out of the cabinet. The air draws through filters at the top of the cabinet to remove contamination particles and passes through the cabinet and out of the bottom working area to help keep particles at bay.



Figure 1. An example of a laminar flow cabinet[36]

Working in this type of cabinet helps to stop cross contamination from other experiments taking place in the laboratory at the same time. The use of filter tips can be very beneficial as it stops the reagents being contaminated. The filter stops the exchange of aerosols between the barrel of the pipette and the reagent being pipetted; it is possible that contamination can enter the pipette barrel and it can be passed between reagents.

Cleaning the laboratory and environmental monitoring are also extremely important to reduce contamination levels. Regular deep cleaning as well as surface cleaning before and after sample preparation helps to keep contamination to a minimum. The deep clean

should include washing down all surfaces high and low as well as include a variety of cleaning steps. For example, using a general surface cleaner to initially clean down all surfaces to remove any dirt and then once this has dried use alcohol wipes on the surfaces followed by a bleach solution or specialised product for DNA (Microsol). This must have thoroughly dried prior to any sample preparation as it could interfere with the samples. A daily clean down can also be completed with the alcohol wipes and the Microsol/ bleach solution. Environmental monitoring determines if DNA contamination is present and the location of it. If this is done regularly in different areas of the laboratory it can be very useful. For example, pipettes, work surfaces, laboratory coats, refrigerator, centrifuge and other instruments can all be swabbed and analysed. Determining the areas of contamination can help to create preventative measures.

DNA Structure and Degradation

DNA is a linear polymer made up of four different types of nucleotide subunits that are linked together by phosphodiester bonds. DNA is made up of nucleotides with each one containing a five carbon sugar, one of four nitrogen containing bases attached to the sugar and a phosphate group. The nucleotides in DNA contain deoxyribose which binds with phosphate at the three prime (3') and five prime (5') carbon ends with the genetic code always starting at the 5' end. The bases present in DNA are adenine (A), guanine (G), cytosine (C) and thymine (T) which are attached to a sugar phosphate backbone [37]. Adenine and guanine are the larger of the bases and are purines whereas cytosine and thymine are pyrimidines. DNA is a double stranded helix with each of the strands running in the opposite direction. The strands of DNA are hydrogen bonded through the bases; either A to T or C to G. This hydrogen bonding is what twists the DNA structure into a helix [38].

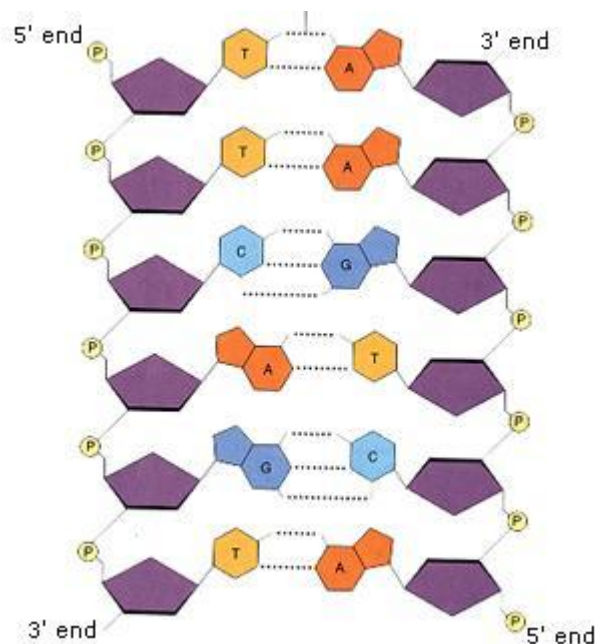


Figure 2. An example of the DNA structure

With forensic DNA samples often being found in less than optimal conditions and of small quantities, DNA degradation is a potential issue for analysts. DNA degradation is often caused by environmental factors such as UV light, humidity, temperature, bacterial decomposition and mould [5, 39, 40]. The effects of these factors are that the DNA strand may split into smaller fragments or become chemically modified [41]. This can often result in partial profiles, artifacts and a 'drop-out' of heavier alleles (described later) [42]. Although typing for high molecular weight DNA becomes difficult, research has shown that it is still possible for results to be obtained for relatively light molecular weight DNA [43, 44]. Using sensitive, PCR-STR amplification kits it allows the degraded DNA to still be forensically analysed. During PCR it is also possible that there might be a failure in amplification due to a degradation of the DNA sample. Using commercially available PCR-STR amplification kits tailored for use with low quality and quantity samples, analysts many still be able to forensically analyse the degraded DNA sample. Although these kits have been designed

with low-level samples in mind, difficulties may still arise during the amplification process. If very low quantities of DNA are being used it is possible to increase the number of cycles used in PCR but this can also cause other issues – known as stochastic effects. These stochastic effects include phenomena such as amplifying extraneous DNA types – commonly referred to as “allelic drop-in”, the loss of DNA types – “allelic drop-out”, and peak height imbalance at heterozygous loci [45]. If one of the two alleles in a heterozygote sample fails to amplify correctly it could lead to the incorrect genotyping of the forensic sample. This issue may be further problematic if the PCR products have different denaturation or annealing temperatures or if inhibitors remain in the sample after purification [41]. Prevention of DNA degradation is usually achieved through freezing but this is not always an option and the sample may have already degraded [46].

In gel electrophoresis, indications of degradation can be seen by a ‘smear’ of the banding [41, 47]. Studies have shown that some qPCR methods are a sensitive enough assay to be able to quantify the extent of DNA degradation using different kits to achieve similar results [48]. One study showed that neither a fluorescent dye assay or UV spectrophotometric assay methods of quantification on highly degraded DNA provide consistent and accurate results [47]. Fragment analysis using capillary electrophoresis would be one way to assess how quickly and under what circumstances DNA breaks down, allowing analysts to look at the base pairs of the samples. Real-time PCR would also show this using the amplification data as the smaller fragments present a curve at lower cycles than the larger fragments.

The sample is not the only place where degradation can occur; the standards used for quantitative analysis also have the potential to degrade [49]. Research has shown that it is important to store them correctly in order to prevent nucleic acid decay to ensure that

reliable results can be obtained. Although DNA degradation can be an issue, research has shown that DNA can still be analysed from ancient samples using the low molecular weight fragments [50]. Advancements in technology have allowed for the analysis of difficult forensic samples that previously, may not have yielded any result.

Summary

The research discussed has investigated various aspects of saliva derived DNA collection, extraction and quantification that are relevant to this study. Using the information provided in these papers, basic techniques for alpha amylase detection were decided not to be used as the starting material was known to be saliva. Other basic techniques such as gel electrophoresis were also ruled out as it was felt that they did not provide a new skill. The research provided a valuable selection of commercially available kits and techniques to choose from for this study.

The research showed that the potential effects of packaging types on the quantity of saliva samples has not yet been documented or evaluated. Due to this, the investigation detailed in this paper took place.

Experimental

Sample Selection:

A saliva donor was asked to refrain from eating or drinking for 30 minutes prior to providing a sample. The saliva from the donor was placed directly into a sterile 50ml collection tube and mixed to ensure a homogenous solution. A pipette was used to place three different quantities of saliva onto three pieces of filter paper; 10 μ l, 15 μ l and 20 μ l. These samples were extracted using the QIAamp® DNA mini and blood mini kit protocol (DNA purification from Buccal swabs) detailed below and then quantified using the Investigator® Quantiplex method also detailed below. From the results the 20 μ l sample quantity was selected for use as this provided the highest yield of DNA (10 μ l = 0.975ng/ μ l, 15 μ l = 0.992ng/ μ l, 20 μ l = 1.212ng/ μ l).

This packaging study had the potential for starting sample variation due to the variation in quantity of buccal epithelial cells present in the saliva sample. If they were not evenly spread within the saliva mixture then the sample would not have been homogenous when it was pipetted onto the filter papers. A variation with the number of these cells in each 20 μ l saliva sample would have interfered with the investigation. Only one donor was used due to the number of replicates that required extracting on the same day (50 extractions).

Sample Preparation:

Saliva was collected in a sterile collection tube from the donor over a twenty-four hour period due to the quantity required in total. The saliva donor was asked to refrain from

eating or drinking for thirty minutes prior to providing each contribution. The sample was vortexed in order to form a homogenous solution prior to pipetting.

Thirty plastic bags and thirty paper bags were labelled in preparation for the samples to be added. Sixty pieces of filter paper were labelled and divided into eight sections using a pencil. Each section represented a sample to be removed each week of the eight week study.

A pipette was used to add 20µl of saliva to each section of the filter paper. The first twelve completed filter papers were packaged immediately; six were placed straight into plastic bags and six into paper bags that were labelled as zero hours along with the sample number 1-6. The next twelve samples were allowed to open air dry at room temperature and packaged one hour after pipetting; six were placed into plastic bags and six into paper bags that were labelled as one hour along with the sample number 1-6.

Twelve more filter papers were allowed to open air dry at room temperature for six hours prior to packaging; six were placed into plastic bags and six into paper bags that were labelled as six hours along with the sample number 1-6. A further twelve samples were given twelve hours to open air dry at room temperature before six were placed into plastic bags and six into paper bags that were labelled as twelve hours along with the sample number 1-6. The final twelve samples were dried for twenty-four hours at room temperature prior to six being placed into plastic bags and six into paper bags that were labelled as twenty-four hours along with the sample number 1-6.

Sample Description	Number of Replicates
Saliva dried for 0 hours packaged in plastic	6
Saliva dried for 0 hours packaged in paper	6
Saliva dried for 1 hour packaged in plastic	6
Saliva dried for 1 hours packaged in paper	6
Saliva dried for 6 hours packaged in plastic	6
Saliva dried for 6 hours packaged in paper	6
Saliva dried for 12 hours packaged in plastic	6
Saliva dried for 12 hours packaged in paper	6
Saliva dried for 24 hours packaged in plastic	6
Saliva dried for 24 hours packaged in paper	6

Table 1. Total number of saliva samples prepared for paper and plastic packaging

The pipetting of saliva was occasionally problematic as some areas of the sample were more viscous than others which may have produced errors with the controlled starting quantity. After drying it was evident that the saliva stains were not visible on the filter paper. This meant that larger quantities of filter paper had to be removed for extraction and therefore could not guarantee that the entire area of the saliva stain had been removed. Alternatively too much of the paper could have been removed which could affect extraction by retaining a larger quantity of the extract liquor. This would have meant that a proportion of the extract would have been discarded.

Replicate 6 was surplus to the requirements and was produced as a contingency should extra samples be required during the study. After all of the samples were prepared and packaged, a sample was removed from replicate 6 of each package type and drying time.

This was removed by using a sterile scalpel and was used as control for day zero of the study. The samples were stored inside a cardboard box at room temperature (15-25°C) for the duration of the study. Seven days after this sample was collected a sample from each of the filter papers 1-5 was collected, extracted and quantified using the protocols below. This was repeated every seven days for a total of eight weeks providing 400 samples for extraction and quantification.

Protocols:

DNA Purification from Buccal Swabs (Spin Protocol)[51]

The original method design was to incorporate the use of the QIAcube but with the research discussed in the introduction indicating that the manually processed QIAamp method was more effective and that the QIAcube takes approximately one hour to extract twelve samples versus fifty samples that could be manually extracted in three hours, it was decided that the manual QIAamp extraction method would be used. All of the steps were carried out at room temperature (15-25°C) and the reagents were equilibrated at room temperature. The heat block was pre-heated to 56°C. Firstly the filter paper was placed in a 2ml microcentrifuge tube and 400µl of PBS was added to the sample. This was followed by the addition of 20µl of QIAGEN® Proteinase K and 400µl of Buffer AL. This was then mixed immediately by vortexing for 15 seconds.

The sample was then incubated at 56°C for ten minutes followed by centrifugation to remove the drops from inside the microcentrifuge lid. This is so that when the lid is opened droplets do not spray from the tube and cause contamination or reduce the quantity of product. Next 400µl of ethanol (96-100%) was added to the sample and mixed using the vortex. The sample was then briefly centrifuged once again in order to remove the drops

from the inside of the lid. Using the mixture obtained so far, 700µl was carefully added to the QIAamp® Mini spin column that is seated in a 2ml collection tube without wetting the rim. The cap was closed and it was centrifuged at 8000 rpm for one minute. The QIAamp® Mini spin column was then placed inside a new 2ml collection tube and the tube containing the filtrate was discarded. In order to maximise the product, any of the remaining mixture from the microcentrifuge tube and ensuring that the filter paper was 'squeezed' the remnants (up to 700µl) were again added into the QIAamp® Mini spin column. The cap was closed and it was centrifuged at 8000 rpm for one minute. The QIAamp® Mini spin column was then placed inside a new 2ml collection tube and the tube containing the filtrate was discarded. The QIAamp® Mini spin column was then carefully opened and 500µl of Buffer AW1 was added without wetting the rim. The cap was closed and it was then centrifuged at 800 rpm for one minute. The QIAamp® Mini spin column was then placed in another clean 2 ml collection tube and the collection tube containing the filtrate was discarded. Once again the QIAamp® Mini spin column was then carefully opened and 500µl of Buffer AW2 was added without wetting the rim. The cap was closed and it was centrifuged at 14,000 rpm for three minutes. The QIAamp® Mini spin tube was then placed in a new 2ml collection tube and the tube containing the filtrate was discarded. The new tube and spin tube were then centrifuged at 14,000 rpm for one minute in order to eliminate the chance of possible Buffer AW2 being carried over into the sample. The QIAamp® Mini spin column was then placed in a clean 1.5ml microcentrifuge tube with a lid and the collection tube containing the filtrate was discarded. The QIAamp® Mini spin column was carefully opened and 150µl of Buffer AE was added. This was allowed to incubate at room temperature for one minute and was then centrifuged at 8000 rpm for one minute.

The handbook states that one buccal swab typically yields 0.5-3.5µg of DNA in 150µl of buffer (3-23ng/ µl). In this study that would be considerably less due to filter paper being used and the sample being pipetted. Buccal swabs produce a higher yield as they allow the donor to rub the swab on the inside of the cheek in order to remove skin cells, the filter paper technique does not allow for cell removal in this way.

Investigator® Quantiplex Kit – Quantification of DNA using the Rotor-Gene Q[52]

All reagents were mixed thoroughly prior to use and allowed to equilibrate to room temperature. Firstly fresh serial dilutions of the Control DNA Z1 were created as detailed in the table below which was recommended in the user manual. Each dilution was mixed thoroughly prior to removing the aliquot for the next dilution and a new pipette tip was used for each dilution in order to avoid cross contamination.

Serial dilution of Control DNA Z1	Control DNA Z1	QuantiTect Nucleic Acid Dilution Buffer
20 ng/µl	Undiluted DNA	-
5 ng/µl	10 µl	30 µl
1.25 ng/µl	10 µl	30 µl
0.3125 ng/µl	10 µl	30 µl
0.078125 ng/µl	10 µl	30 µl
0.01953125 ng/µl	10 µl	30 µl
0.0048828125 ng/µl	10 µl	30 µl

Table 2. DNA standards required for quantification of unknown (values quoted from manual) [52]

The master mix was then prepared by using 11.5µl of the Reaction mix (RM) and 11.5µl of the Primer mix (PM) to produce 23µl of master mix per sample and mixed thoroughly. Once

mixed, 23µl of the master mix was then placed into each of the Q strip tubes required. The first 14 strip tubes were for the duplicate Control DNA Z1 serial dilutions, to ensure that the final quantity was 25µl; 2µl of each control was added to each tube in duplicate so that the well contents were set up as follows:

1	20	2	20	3	5	4	5
5	1.25	6	1.25	7	0.3125	8	0.3125
9	0.0781	10	0.0781	11	0.0195	12	0.0195
13	0.0049	14	0.0049	15	NTC	16	NTC
17-72				Unknown Sample			

Table 3. Sample position in wells on the Rotorgene

In tubes 15 and 16 was 2µl of QuantiTect Nucleic Acid Dilution Buffer in order to produce NTC tubes. Then 2µl of the unknown sample was added to the remaining tubes and mixed thoroughly. The PCR tubes were then capped and placed in the 72-well rotor in the Rotor-Gene Q cycler with the locking ring attached. No positions were left empty, blank tubes were used to ensure that the rotor was balanced. The software was then opened and the on screen steps were followed in order to set up and run the plate.

Instrument - Qiagen® Rotor-Gene® Q

The Investigator Quantiplex Kit was created by Qiagen® in order to quantify human genomic DNA by utilising quantitative real-time PCR on the Rotor-Gene® Q instrument. The kit was designed to assess the quantity of DNA to enable DNA profiling analysis and also assess the quality of DNA; for example if the sample contains inhibitors and therefore requires a

further purification step. It provides sensitivity down to $< 1 \text{ pg}/\mu\text{l}$ with accurate quantification below $4.9 \text{ pg}/\mu\text{l}$ (if the standard curve shows linearity)[52].

The reagents were created to specifically target a 146 bp region that is present on several autosomes of the human genome and allow it to be detected on the Rotor-Gene® Q. The target region was validated in an external study and was selected due to the high degree of sensitivity and reliability within different populations and individuals. The kit also uses an internal amplification control mechanism that is used to test successful amplification and detects PCR inhibitors as a 200 bp internal control in the yellow channel on the instrument[52].

Detection of amplification is completed by using fast PCR chemistry (the run takes around 50 minutes to complete) and Scorpion primers. These primers are bifunctional molecules that contain a PCR primer that is covalently attached to the probe (see figure 3 below). The probe contains a fluorophore that interacts with a quencher that reduces fluorescence. The fluorophore and the probe are separated during PCR. When the probe binds to the PCR products, an increase of fluorescence is detected by the instrument and is recorded which is directly proportional to the amount of amplification product [52].

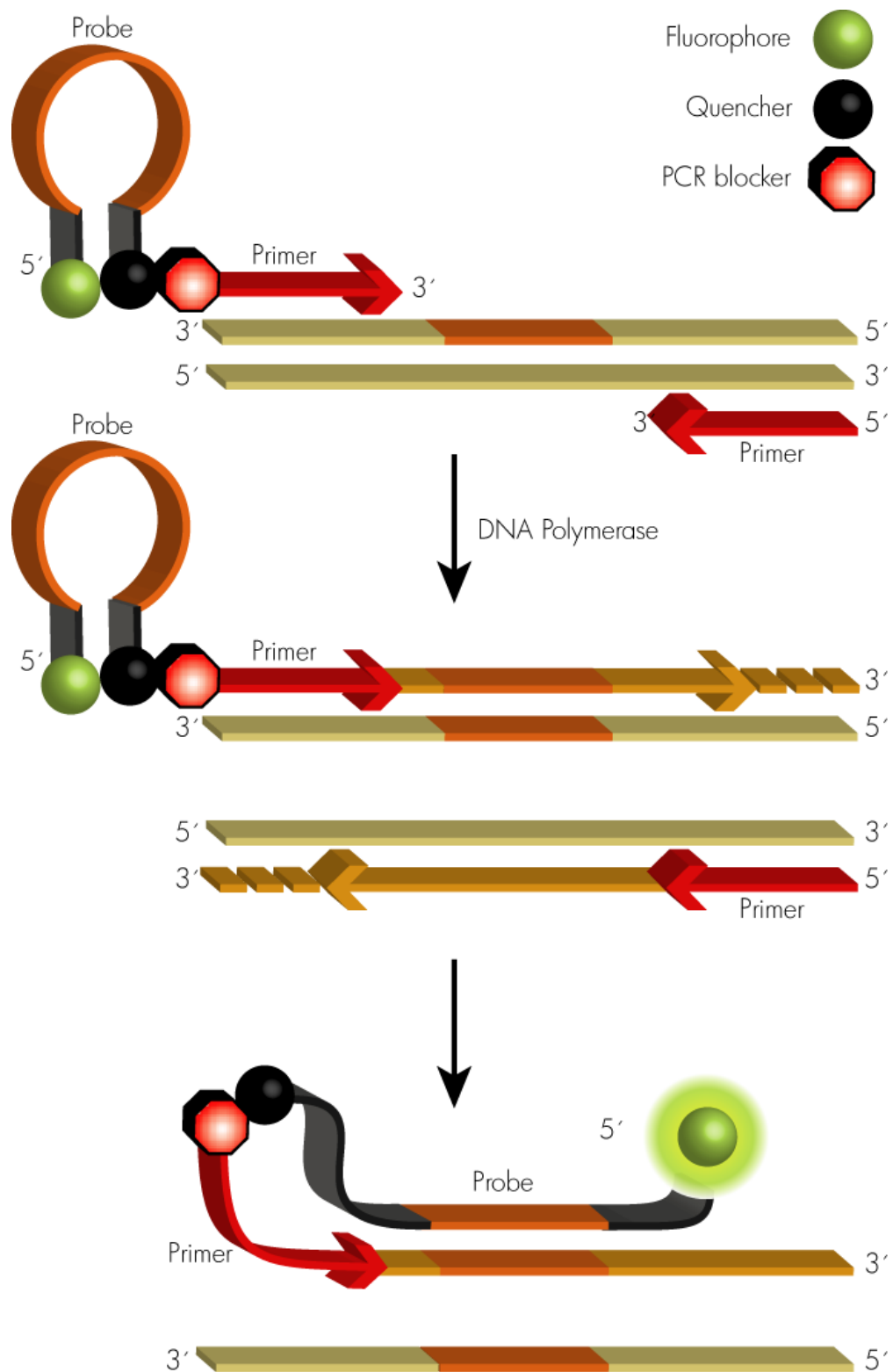


Figure 3. Investigator kit mechanism[52]

Contamination

During DNA extraction, extraction blanks were used in order to detect if contamination was present in any of the reagents used or was entering the samples via pipettes, tips or general laboratory practice. During quantification, no template controls (NTC) were used in duplicate in each quantification run so that contamination could be detected. The NTC uses all of the reagents for quantification but does not contain any of the positive controls or samples so should therefore show no DNA unless the reagents or strip tubes have been contaminated. In some cases during preparation other researchers were carrying out DNA extraction at the same time or the samples had been extracted directly before being prepared for quantification in the same laboratory so this was documented as a potential source of contamination. Potentially an automated instrument for sample preparation during the quantification step could have reduced contamination as analyst interference would have been minimal.

Results and Discussion

The first results that were looked at were the three different quantities of saliva placed onto filter paper (10 μ l, 15 μ l and 20 μ l) in order to determine the most suitable quantity to use for the study. Different quantities were reviewed as there was a possibility that the DNA concentration would not be homogenous due to epithelial cells not being evenly distributed and possibly more concentrated in the more viscous areas of saliva.

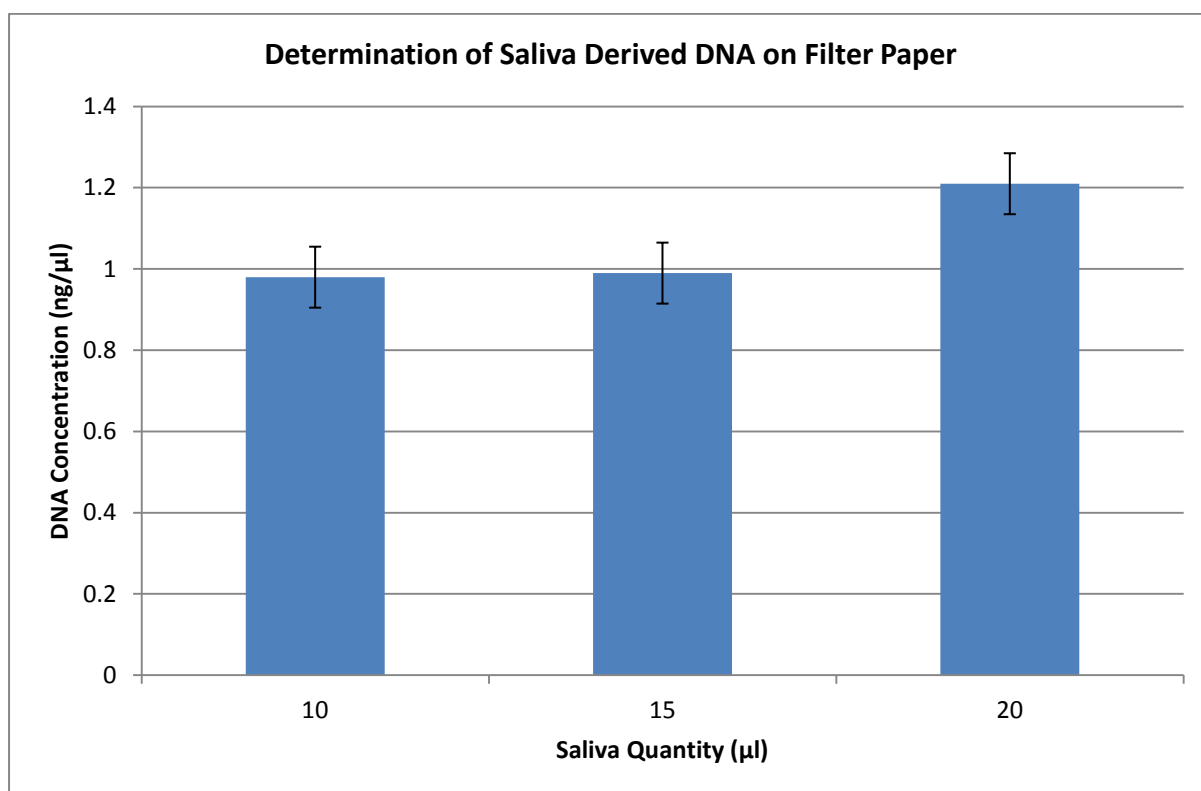


Table 4. Determining the optimal quantity of DNA obtained from saliva for this study

The 10 μ l sample produced 0.98ng/ μ l, 15 μ l produced 0.99ng/ μ l and 20 μ l produced 1.21ng/ μ l. The 20 μ l quantity was used as it produced a higher starting quantity although all of the quantities represented a typical forensic DNA sample; this one was selected as it had the potential to provide a quantity of DNA over the eight week study regardless of

degradation. The error bars on the graph represent one standard deviation throughout this study.

0 hours plastic	1.10ng/ μ l	0 hours paper	0.48ng/ μ l
1 hour plastic	0.14ng/ μ l	1 hour paper	0.52ng/ μ l
6 hours plastic	0.59ng/ μ l	6 hours paper	0.30ng/ μ l
12 hours plastic	0.40ng/ μ l	12 hours paper	1.10ng/ μ l
24 hours plastic	0.54ng/ μ l	24 hours paper	0.92ng/ μ l

Table 5. Results for day zero

These results were only produced from single samples and are not very representative of the replicate samples. If this study was to be continued or repeated it would be more beneficial to run these samples in multiples in the same way as the rest of the samples in the study. What can be seen from these results is that there is a large amount of variation in the starting material when it should have been a homogenous mixture of saliva that presented the same DNA concentration. The viscous areas of the saliva could have potentially caused pipetting errors as the quantity might have varied if the pipette could not aliquot correctly. There was also a possibility that cells from inside the mouth may not have been evenly distributed and this could have caused a variation of DNA concentration between samples. Another issue during the study was that after drying it was evident that the saliva stains were not visible on the filter paper. This meant that larger quantities of filter paper had to be removed for extraction and therefore could not guarantee that the entire area of the saliva stain had been removed. Alternatively too much of the paper could have been removed which could affect extraction by retaining a larger quantity of the extract liquor. This would have meant that a proportion of the extract would have been discarded. Pre-cut filter paper pieces could have eliminated this issue. It was decided that

these results would not be used as part of the data compared in graphical form due to the lack of replicates.

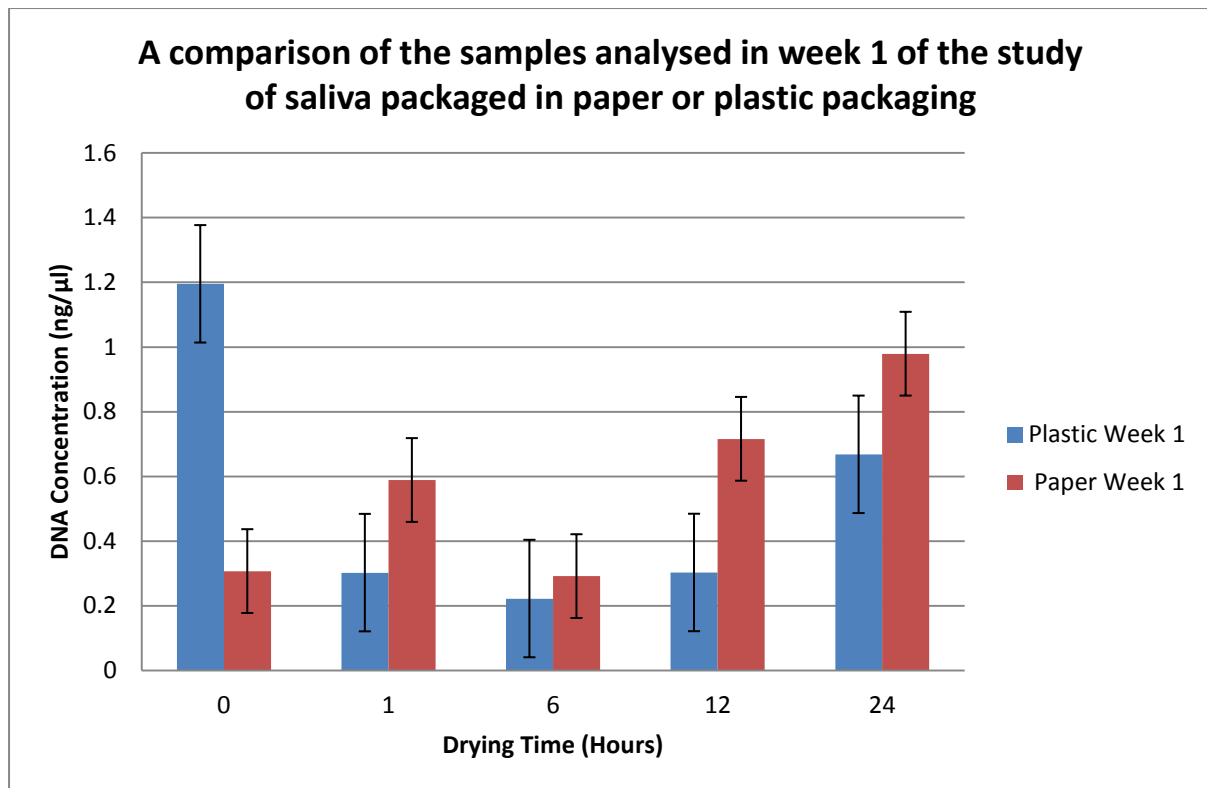


Table 6. Week 1 packaging results for saliva

Table 6 indicates that the largest concentration of DNA was recovered from the plastic packaging that was packaged immediately. When this was sampled on week one the filter paper was seen to be still damp. This could be a reason for the larger concentration; it could be that when the sample dries a reduced amount of DNA can be extracted from the filter paper. The paper packaging appears to have been more suitable after the samples were allowed to dry.

Standard error bars have been used on the graphs to provide an indication of the accuracy of the results by using the number of replicates used to generate the mean; an increase in

the number of replicates used in the study would decrease the size of the error bars. The error bars indicate that there is room for improvement on the data in this study.

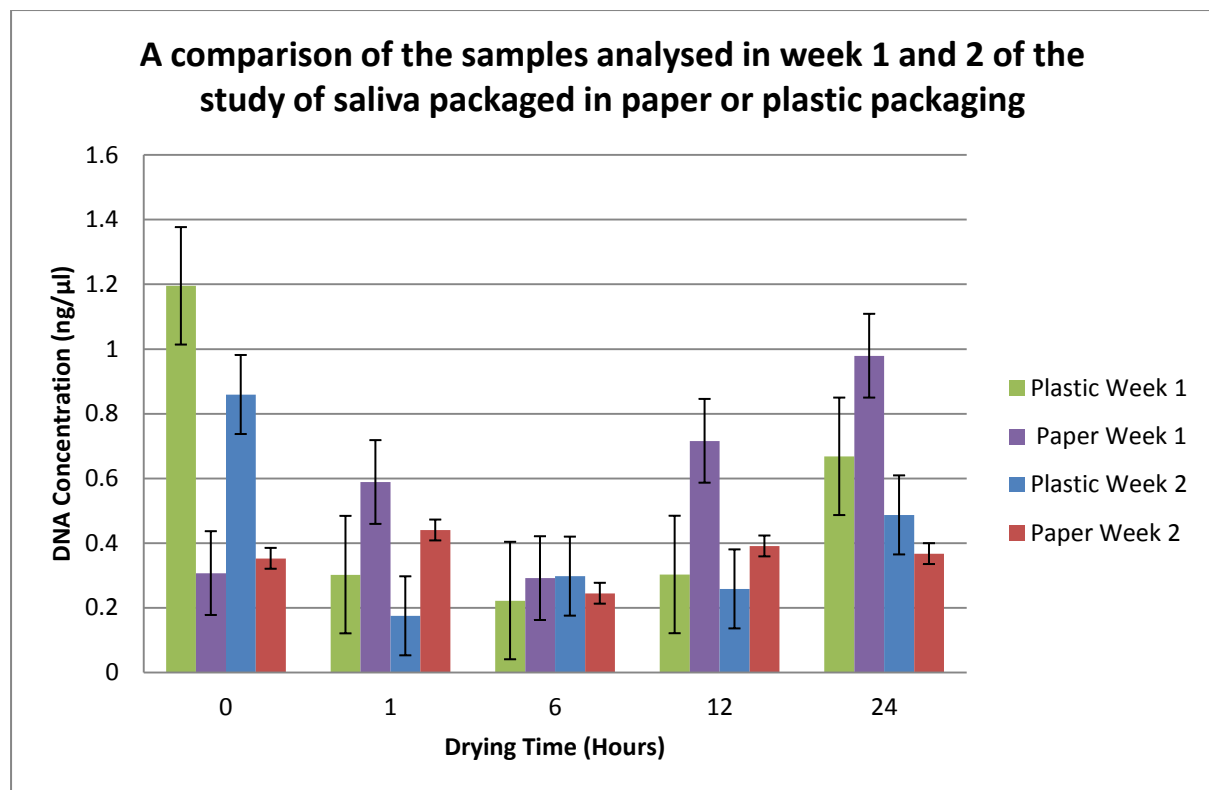


Table 7. Week 2 packaging results for saliva

The data in table 7 suggests that a reduction in DNA concentration of the plastic zero hour sample occurred. When this sample was removed the filter paper had dried during the second week which could account for this reduction. It is also a possibility that transfer of DNA from the filter paper to the inside of the packaging could have occurred. A reduction in the DNA concentration for the plastic packaging could also be seen in the 4 hour, 12 hour and 24 hour samples. The six hour plastic sample showed a slight increase in DNA concentration which could have potentially been caused by contamination or the inconsistency of the starting material. The paper packaging showed a decrease in DNA quantity in the 1 hour, 6 hour, 12 hour and 24 hour samples. The zero hour samples showed a slight increase in DNA concentration. The error bars indicate that the data

obtained in week two has less error in the paper samples when compared with the plastic samples and all of the week one samples.

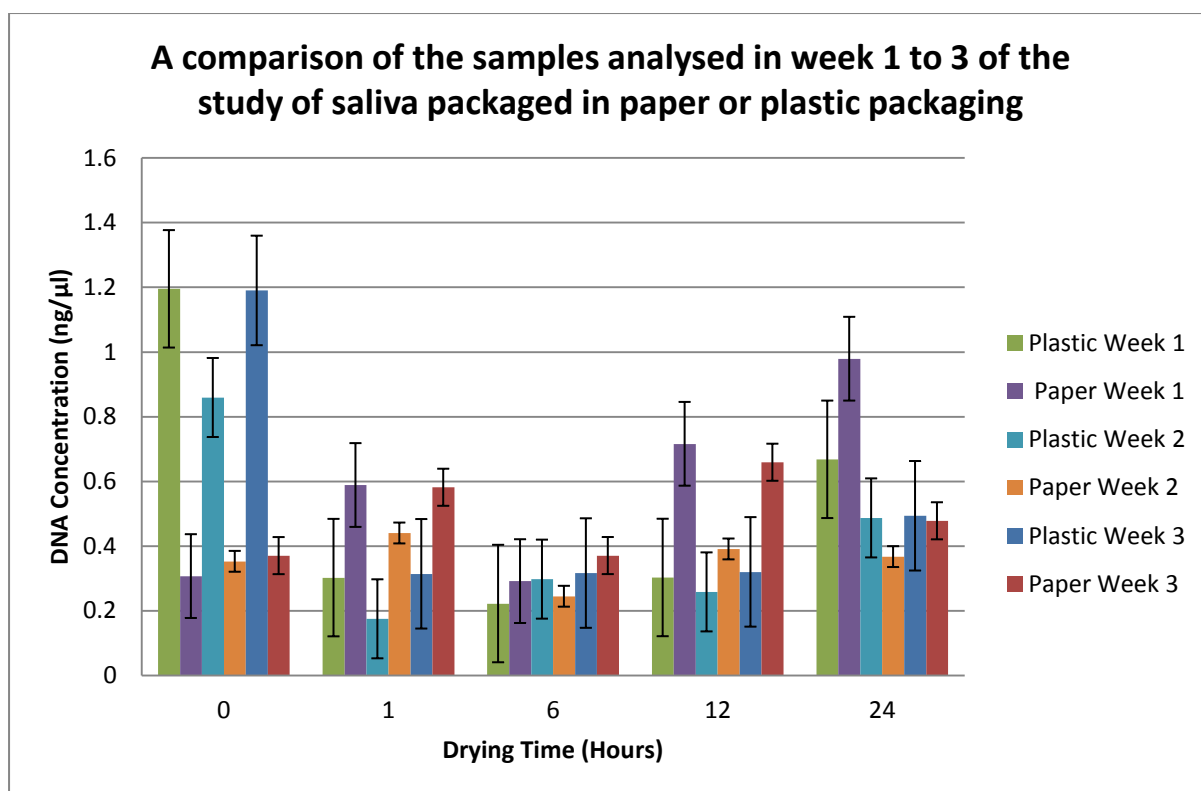


Table 8. Week 3 packaging results for saliva

All of the results from week three show an increase in the DNA concentration compared with week two. With such sensitive techniques and using low quantities of DNA these results show how vital accuracy and anti-contamination procedures are. Again it is possible that contamination played a part in the increase in these results. The extraction blank showed no results for DNA but the NTC determined that DNA was present during the quantification preparation stage. This suggests that all of the samples may have been contaminated which would account for the increase in DNA quantity when compared with the previous week. It is also a possibility that starting sample variation contributed to this increase.

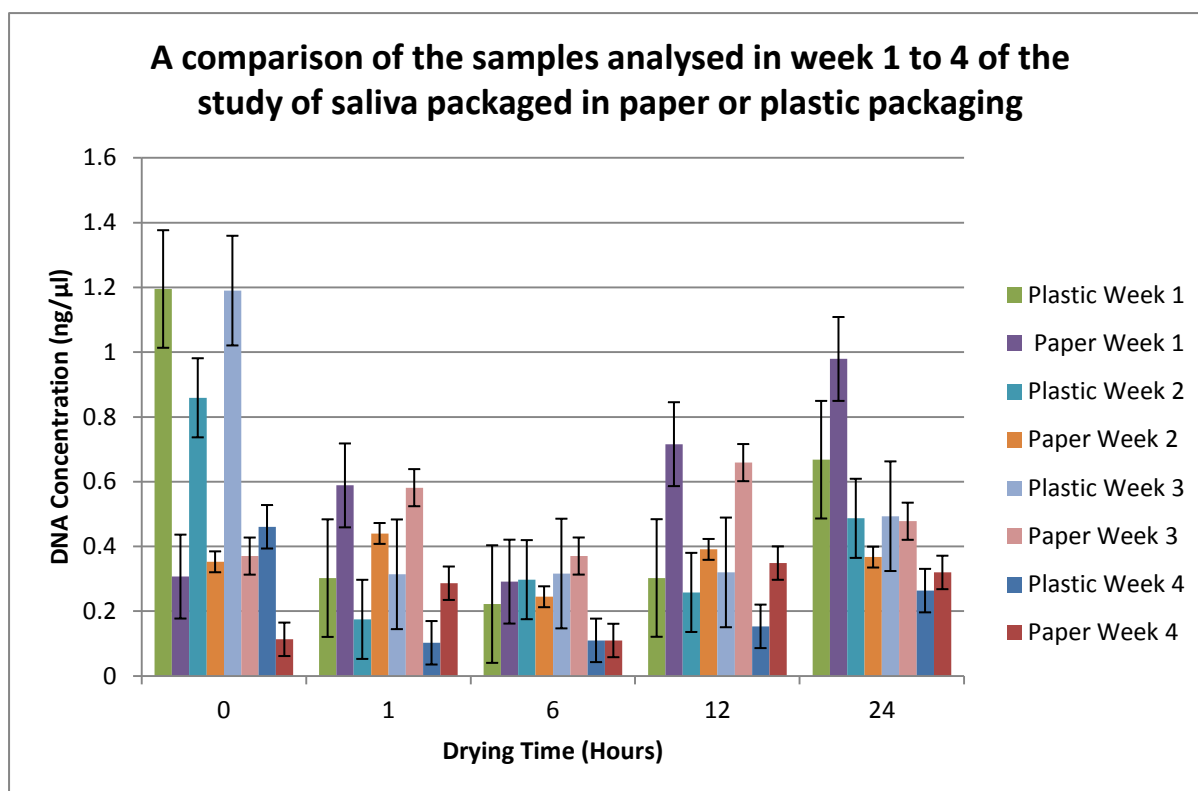


Table 9. Week 4 packaging results for saliva

It can be determined from table 9 (and appendix 2) that all of the samples showed a decrease in DNA concentration during week four of the study. The largest DNA concentration reduction was in the plastic packaging zero hour samples. This reduction could potentially be due to sample degradation caused by bacteria growing in the moist conditions over the first week of the study. The DNA concentration contained in the plastic packaging from the zero hour samples is still at a higher quantity than the samples that were allowed to dry prior to being packaged in plastic. It is also a higher concentration than any of the samples packaged in paper and samples during week four.

The results from week five have once again seen an increase in the DNA concentration of the samples when compared with the results from week four (appendix 3). The saliva sample that was packaged immediately after sampling in plastic packaging has consistently shown a larger DNA concentration than all of the other samples during the study so far.

Although the quantity has decreased from the first week of sampling, the week five samples continued to show a larger quantity of DNA than the other samples. Potentially this could be due to the inconsistency of the starting material or possibly that it is more effective to package a wet DNA sample rather than a dry one. In order to fully determine this, the quality of the DNA packaged in plastic would need to be established through capillary electrophoresis. The error bars in the graph in appendix 3 indicate that the error related to the results in week five are an improvement on some of the other weeks, especially weeks one and two.

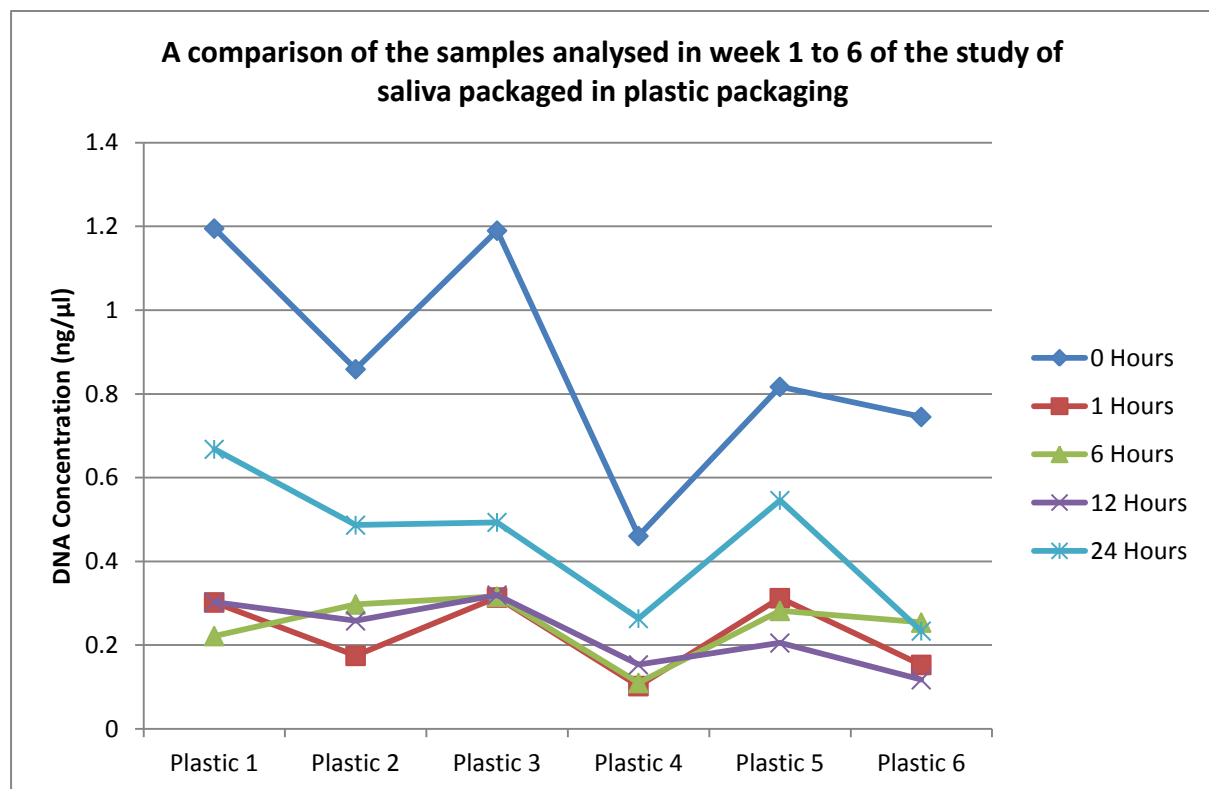


Table 10. Week 6 packaging results for saliva in plastic

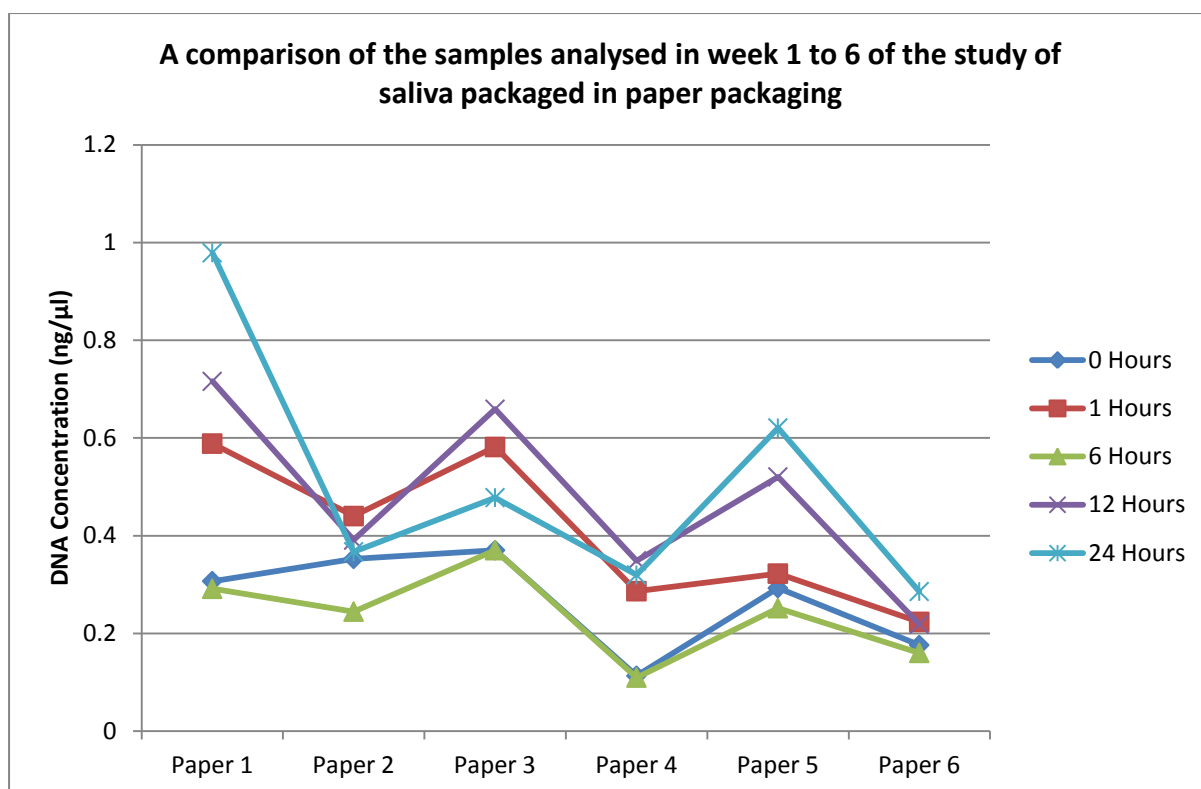


Table 11. Week 6 packaging results for saliva in paper

The results for week six have seen an overall decrease in DNA concentration when compared with the results gained from week five (with an additional graph in appendix 4). The sample obtained from the plastic packaging that was packaged immediately has again shown that the DNA concentration is higher than all of the other samples. All of the other samples packaged in both plastic and paper over all of the drying time ranges have been determined to be closer to the same region in terms of DNA concentration (0.12 - 0.25 ng/μl). During week six a number of samples provided no result during quantification which indicates that either no DNA was present in the sample or an error was made during preparation of reagents and sample addition.

The concentration of DNA that was determined in the saliva samples removed during week seven of the packaging study can be seen in the graphs in appendix 5.

A decrease in DNA concentration was determined for every sample apart from the paper packaged 12 and 24 hour drying time samples; they increased in DNA concentration in week seven when compared with week six. It is a possibility that contamination during sampling, extraction or quantification could have affected these two results. Another possibility for the increase in DNA concentration that has been seen in different weeks of the study is that the DNA could be transferring to the inside of the packaging and each time it was removed it could have either transferred from the filter paper to the inside of the packaging or transferred from the inside of the packaging back onto the remaining filter paper. This has been demonstrated in the study discussed in the introduction and could have been the cause of the increases evident in this study. The error bars produced on the graph in appendix 5 indicate that a large amount of error is associated with the samples during week 7. The differences between samples may also have been caused by the transfer of sample from the filter paper to the packaging and from the packaging to the filter paper.

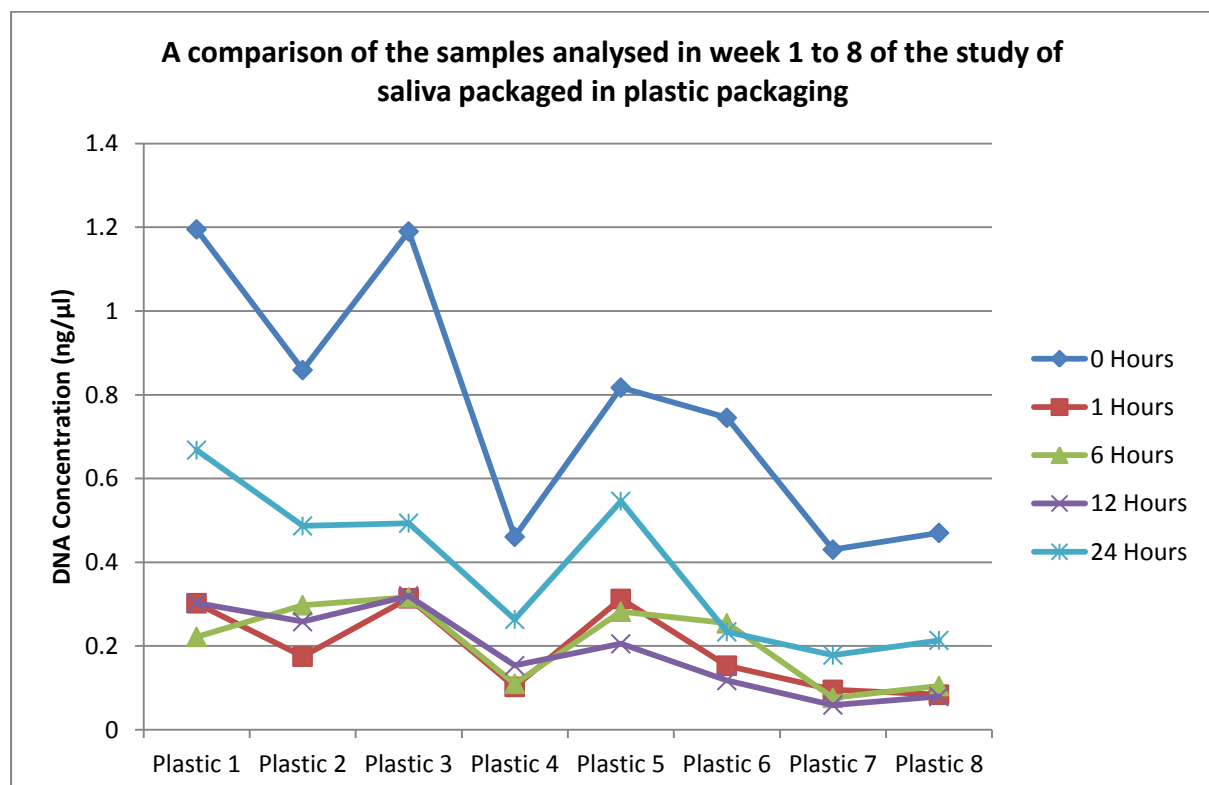


Table 12. Week 8 packaging results for saliva in plastic

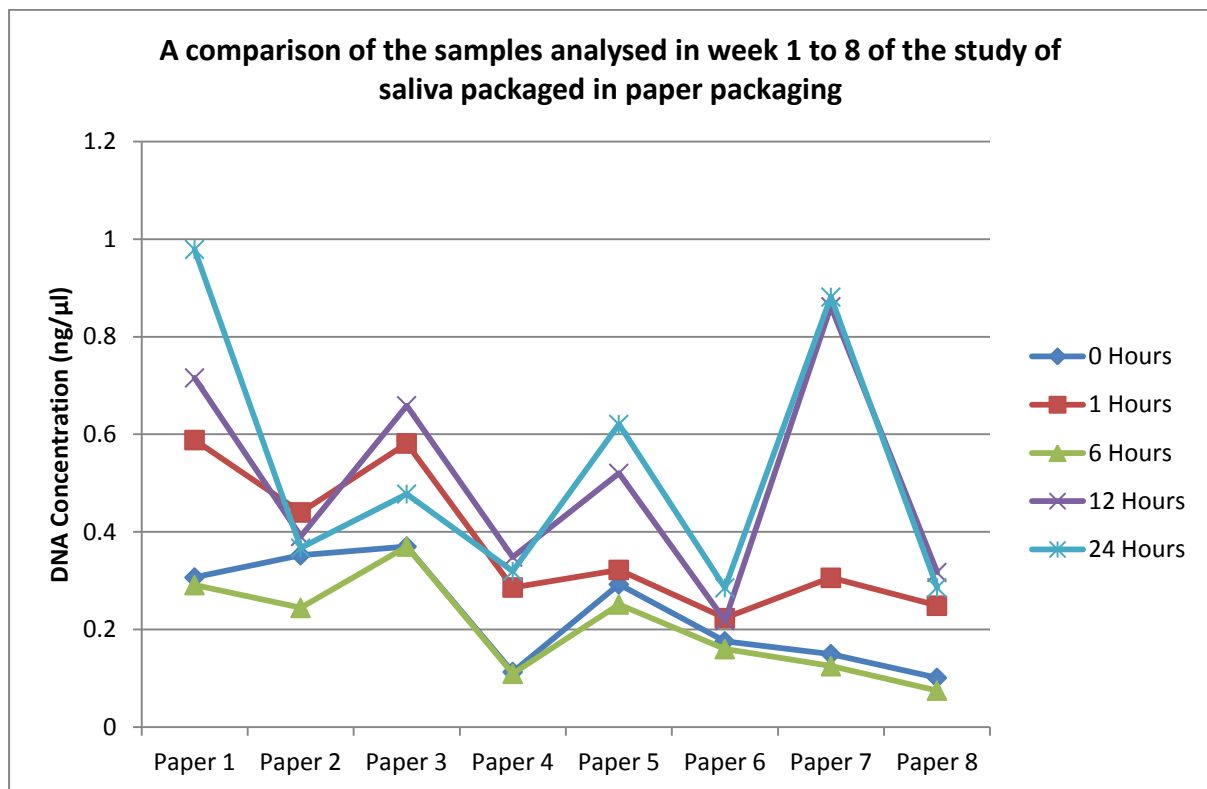


Table 13. Week 8 packaging results for saliva in paper

During week eight, each of the saliva samples packaged in paper presented a decrease in DNA concentration when compared with the samples analysed during week seven. The saliva samples packaged in plastic only showed a decrease in DNA concentration in the one hour drying time sample; the remaining plastic packaging samples presented an increase in DNA concentration. As previously discussed there could be a number of reasons for this increase; contamination during sampling, extraction or quantification, inconsistency of the starting material in terms of DNA concentration and packaging transfer. The graphs below show that overall during the eight weeks of the study, the DNA concentration of the samples decreased in all of the samples regardless of drying time or packaging type.

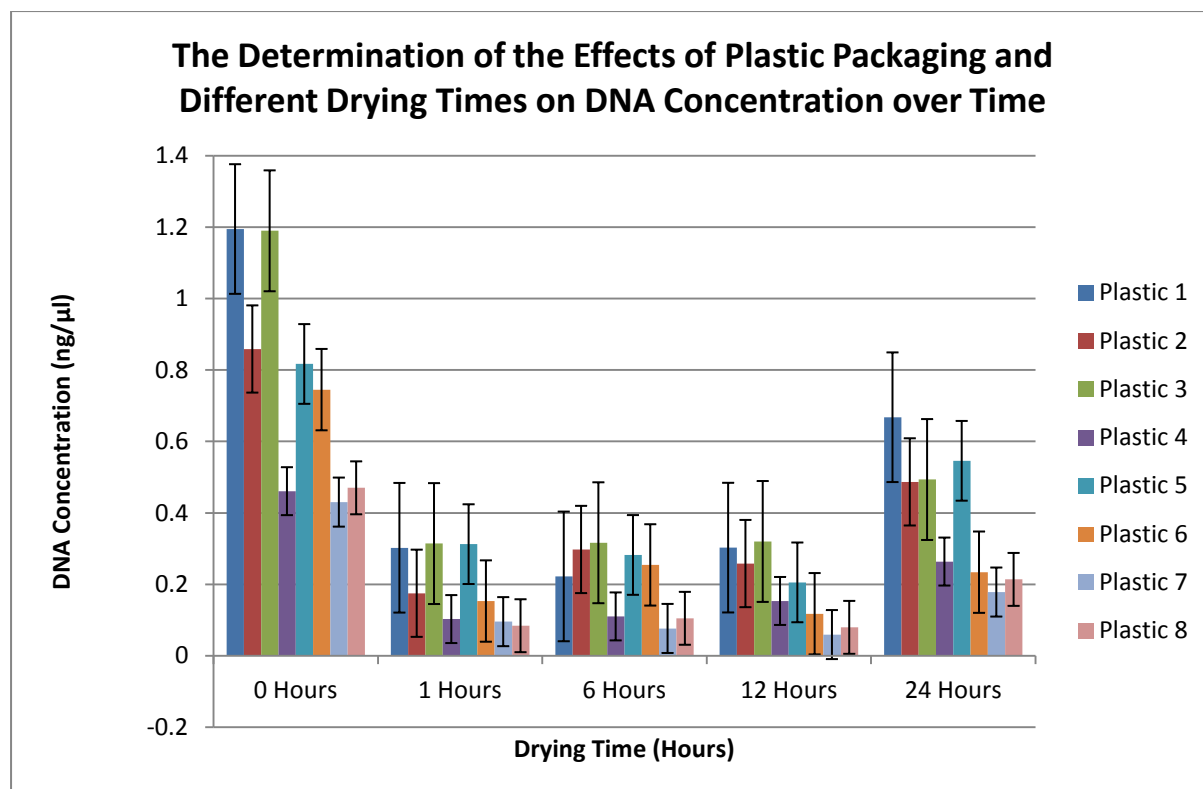


Table 14. Week 8 packaging results for saliva in plastic

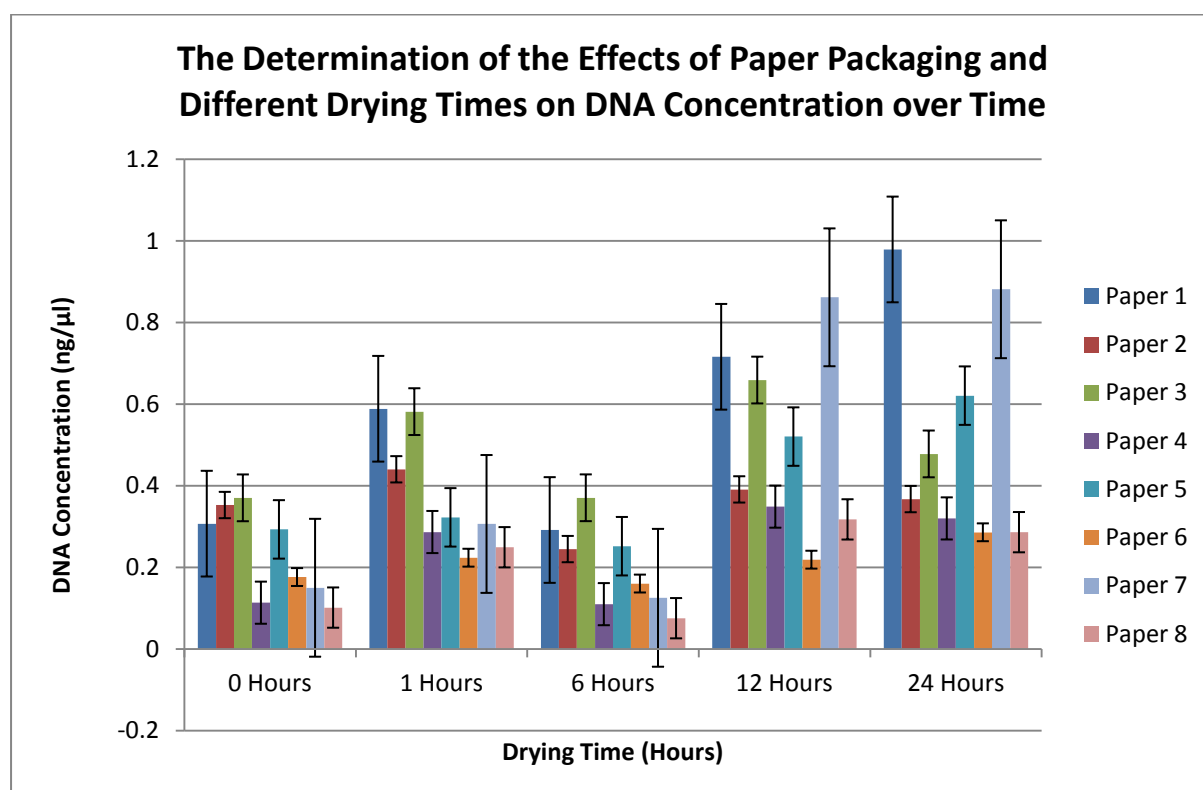


Table 15. Week 8 packaging results for saliva in paper

As illustrated in the graph below, the largest overall reduction of DNA concentration from week one to eight was seen in the plastic packaging that was packaged immediately after sampling. This potentially confirms the theory that plastic traps moisture and the sample degrades due to it encouraging the growth of bacteria. Although the growth of mould was not evident, potentially if the study had continued it might have been seen. Even with this reduction, the largest DNA quantity recovered at the end of the study was from the plastic packaging at zero hours.

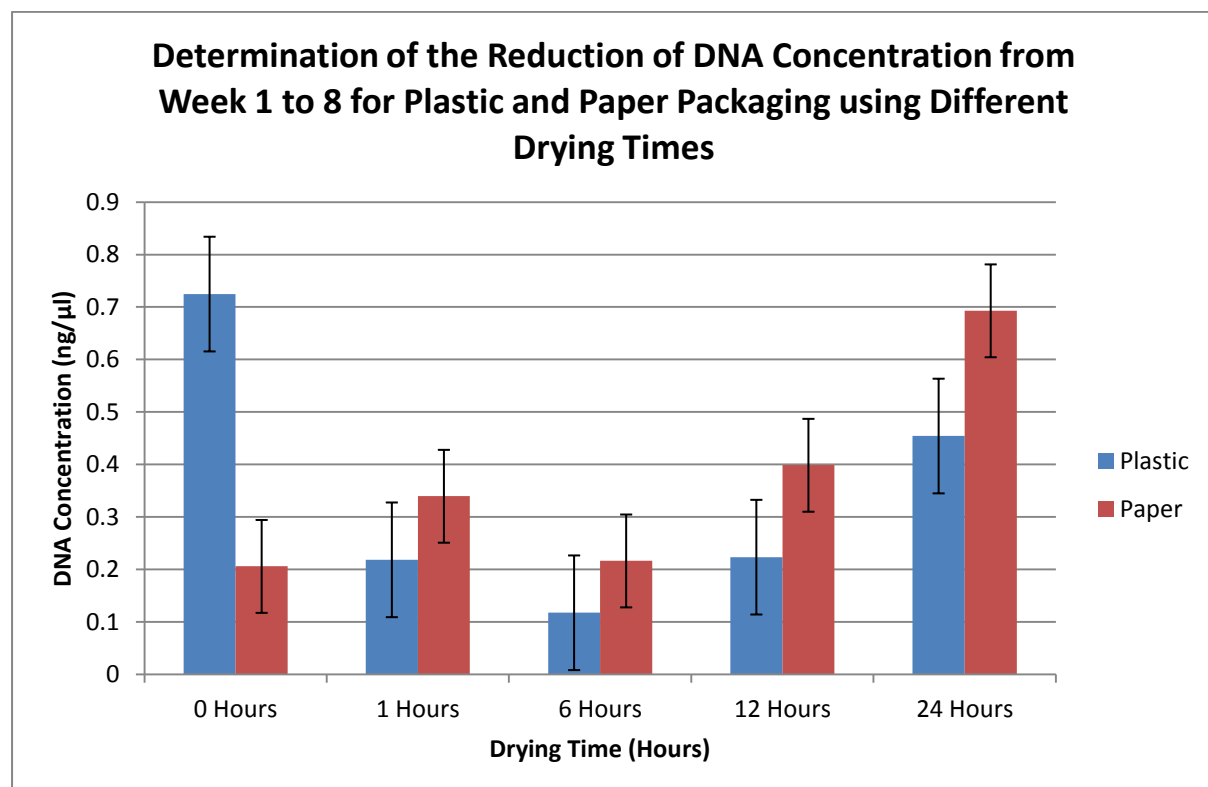


Table 16. The overall reduction of DNA concentration over total of 8 weeks

The smallest reduction in DNA concentration from week one to eight was seen in the plastic packaging that was allowed six hours for the sample to dry. This study indicates that six hours of drying time is the optimum time and placing this sample in plastic packaging provides a minimum loss of DNA. The paper samples that were packaged immediately and at six hours after drying presented the next amount of minimal DNA loss. The saliva sample

that was allowed 24 hours to dry was determined to have the second largest amount of DNA loss although this might not be feasible in a forensic laboratory. Overall during this study the largest decrease of DNA concentration was with the samples packaged in paper packaging when compared with the samples packaged in plastic.

Contamination

During this study, in terms of contamination, only week eight presented DNA in the extraction blank with the previous weeks providing no value for DNA concentration. This suggests that the anti-contamination procedures used were more effective during the earlier weeks and that there is still room for improvement to ensure that the risk of contamination is as low as possible. The NTC's used during quantification showed that weeks four and six were the only weeks that provided no value for DNA contamination during quantification. All of the other weeks showed that DNA was present in a low quantity with an average of 8.14×10^{-4} ng/ μ l. As the extraction blanks were free from DNA but some of the NTC's showed DNA present it appears that the contamination occurred during the preparation stage. If this study was to be repeated, extra anti-contamination procedures would be required to ensure that contamination does not occur during this stage as this level of contamination makes the results unreliable. The plastic packaging and paper packaging that was used for the study was also swabbed for DNA in order to determine if they were sterile and DNA free. The results determined that both were free from DNA contamination.

The strip tubes also had the potential to be contaminated as they arrived in a large quantity supplied in one bag. Each time the bag was opened it increased the chances of contamination occurring as analysts were in contact with the tubes more frequently. One

way to reduce this possibility of contamination would be to transfer smaller quantities of tubes and lids into smaller sterile containers. This would need to be done following good laboratory practice and ensure that sterile gloves are worn and the contact between the tubes and lids are limited. By doing this, if a set of tubes is determined to be contaminated only a small quantity will need to be disposed of which will also have a smaller impact on the cost compared with having to dispose of the large bag. Also when using the tubes it reduces the risk of contamination if clean sterile gloves were worn when handling the tubes and when they were removed from the packaging they should be 'poured' out rather than putting a hand in to remove them.

Standards and Statistics

The linear regression for the standards that were created for the quantification step were measured in order to ensure the accuracy of the DNA concentration. Each week gave a value of 0.99 and above apart from week eight which was 0.98. An example of one of the graphs can be seen in figure 4, which indicates that the standards were prepared with a degree of accuracy and precision.

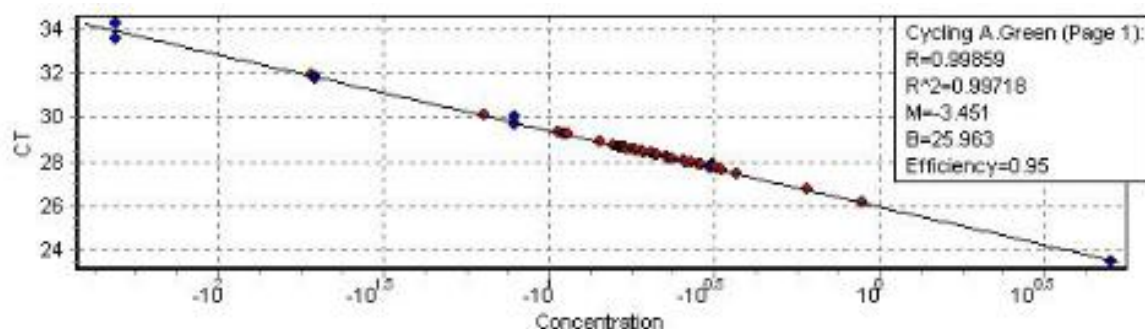


Figure 4. An example of a standard curve used during the saliva studies.

The statistical analysis in appendix one shows the mean, standard deviation, coefficient of variation and relative standard deviation (%). Overall this analysis shows that variation

between replicates could be seen in different degrees. It can be determined that from this statistical analysis that the results are not reliable and would need to be repeated to gain a better understanding of the effects of packaging on DNA.

For example, the statistical analysis of the results from week one show that there is a large variation between each of the replicates in the sample set. These values are not ideal and could potentially be due to the saliva sample that was used for the study as previously discussed.

The statistical analysis entered into appendix one, for week three, indicates that the standard deviation for the zero and one hour paper samples are lower than the plastic but the 6, 12 and 24 hour plastic samples are lower than the paper values. The fluctuations in these values so far in the study show that the each of the replicates from a sample set varies between each other and therefore does not provide confidence in the results and their meaning.

The standard deviation for week six also showed an improvement and therefore the results are much closer together than any other week in the study. If all of the results had presented this type of results during statistical analysis, the results obtained during this study would have been much more beneficial and a larger degree of certainty might have been able to be applied.

The issues that could have caused these variations in data sets, as discussed throughout this section could have been the saliva sample variation, contamination and the possibility of DNA transfer onto the inside of the packaging. For further statistical analysis to be

completed more samples would need to be analysed using changes suggested in future work.

Summary

The findings from this study indicate that samples that are packaged wet in plastic may degrade quicker than if it had been dried and then placed into plastic packaging or if it had been placed directly into paper packaging. This would support the recommendations from Schaffter [2] and Horswell [3]. The differences that were seen from week to week could support the research by Goray *et al.*[6] as the variation in DNA quantity could have been caused by transfer from sample to packaging and then packaging to sample each time it was removed for sampling. The results for the sample that was packaged immediately into plastic packaging could potentially support the work of Warshauer *et al.*[7] by potentially transferring more readily to the inside of the packaging with it being a smooth non-porous surface and the sample was wet.

If the research in this study was confirmed, potentially the way crime scene investigators package DNA samples could change, as overall the use of paper packaging determined minimal reduction in DNA loss. Alternatively, if samples were to be packaged wet in plastic packaging and tested within one week of recovery, this would produce the maximum yield. This could have a huge impact on cases where low template DNA is a possibility.

In relation to current forensic practices, as there appears to be no standard drying time set for samples although it would not be feasible to dry all samples for 24 hours prior to packaging, in some cases sample may have to be dried for this amount of time if they are extremely wet, for example an item of clothing found outside in the rain. Therefore the effect of all potential drying times should be reviewed.

As discussed in the introduction, wet samples should be stored in the refrigerator if they are unable to be dried immediately. This would also be interesting to involve in the study as well as other types of storage types once the sample has been dried. As this study only looked at the effect of room temperature storage, other variable such as the refrigerator and freezer would be required to be investigated before a full conclusion and recommendation could be made for the storage of sample during and after drying.

Future Work

For the continuation of this project the sample preparation would require altering so that the starting quality and quantity of DNA was constant. This would improve the accuracy of the results.

If small filter papers were used it would eliminate the requirement for cutting out the samples. This would reduce the chance of cutting in the wrong place as once the saliva had dried on the filter paper the full area it had spread across could not be seen. The saliva never spread out of the triangle area's that were drawn onto the filter paper but it meant that the size of the paper that had to be placed into the eppendorf was quite large and a sizeable quantity of reagent was retained by the filter paper. This could have been another contributing factor to the starting sample variation.

Previous research has shown that there is a large potential for DNA to transfer to the inside of the packaging, therefore it might be valuable to swab the inside of the packaging at the end of the study in order to quantify the transfer as this could account for sample loss or variation between each week.

It would also be recommended to carry out a study on different body fluids as the most suitable packaging for saliva may not be the most effective packaging for blood. Blood can easily degrade as it can be damaged by chemicals and putrefaction, caused by bacterial growth in warm and wet conditions. At the end of the study, capillary electrophoresis could be utilised to determine if the quality of the DNA was still adequate to obtain a profile.

Also other variable for storage of wet and dried samples should be explored further.

Conclusion

This study determined that if a wet saliva sample is immediately packaged in plastic packaging it may present a high degree of degradation in terms of the DNA quantity. This could be due to the transfer of DNA to the packaging, in the study conducted by Warshauer *et al.* they concluded that a wet saliva sample presented a higher degree of transfer when compared with other samples. During this study, it appeared that the optimum drying time for samples to be placed into plastic packaging was six hours in order to minimise the reduction in DNA concentration. The optimum sample drying time for a sample that is to be placed into paper packaging is zero as paper packaging still allows the sample to breathe so it continues to dry within the packaging. It is less efficient to allow 24 hours drying time for samples as a decrease in DNA concentration was evident. Even with the plastic packaging showing the largest DNA concentration reduction at zero drying time, the highest DNA quantity was recovered from this sample. Further work is required in order to confirm these findings as the statistical analysis showed that the results are not reliable. If improvements were to be made to the study as per the project continuation recommendations and the DNA quality was determined to be satisfactory, it is possible that if DNA evidence was to be immediately packaged in plastic packaging and analysed within one week of recovery, then the quantity of DNA would be at its optimum.

Reagents

QIAamp® DNA Mini Kit

Quantiplex® Investigator Kit

1 X PBS

100% Ethanol

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Appendix 1

Statistical Analysis of Results - Week 1 - 4

	Plastic 1	Paper 1	Plastic 2	Paper 2	Plastic 3	Paper 3	Plastic 4	Paper 4
0 Hours	1.163349	0.201774	0.858956	0.319468	1.507219	0.377773	0.604849	0.166194
	1.264684	0.211408	0.602492	0.17072	0.943211	0.282324	0.601975	9.33E-02
	1.067151	0.406098	0.775576	0.213564	1.202984	0.2578	0.374752	9.25E-02
	1.021365	0.440769	0.716672	0.630577	0.921325	0.491866	0.226022	0.127501
	1.458986	0.274708	1.341724	0.428742	1.375736	0.44139	0.496432	8.66E-02
Mean	1.195107	0.306951	0.859084	0.352614	1.190095	0.370231	0.460806	0.113225
SD	0.174671	0.110643	0.285498	0.184783	0.259029	0.100354	0.161613	0.033705
CV	0.146156	0.360459	0.332328	0.524036	0.217654	0.271059	0.350718	0.297678
RSD %	14.61555	36.04587	33.23278	52.40362	21.76544	27.10591	35.07176	29.76781
1 Hour	0.522148	0.425489	0.171936	0.454623	0.414129	0.471113	0.167715	0.288039
	0.329671	0.721163	0.348267	0.479643	0.481719	0.555823	9.50E-02	0.595652
	0.212422	0.856699	0.161962	0.250284	0.392741	0.604891	0.11319	0.22828
	0.263027	0.431811	9.31E-02	0.440181	0.135202	0.451144	4.12E-02	0.217979
	0.183912	0.507576	9.89E-02	0.576186	0.146335	0.824772	9.55E-02	0.102227
Mean	0.302236	0.588548	0.174828	0.440183	0.314025	0.581549	0.102528	0.286435
SD	0.134832	0.191835	0.103324	0.118663	0.161583	0.149637	0.045359	0.185485
CV	0.446114	0.325947	0.591007	0.269577	0.514553	0.257309	0.442405	0.647562
RSD %	44.61144	32.59466	59.10067	26.9577	51.45532	25.73085	44.24051	64.75621
6 Hours	0.140606	0.284063	0.141207	0.168044	0.28991	0.281013	8.34E-02	6.62E-02
	0.227544	0.246576	0.265028	0.245573	0.282039	0.567991	6.94E-02	0.130953
	0.288744	0.266988	0.445861	0.237718	0.252942	0.456379	0.144756	0.111931
	0.204201	0.45261	0.404799	0.338985	0.383008	0.33566	0.101384	0.111008
	0.249046	0.206658	0.230535	0.232869	0.373742	0.210906	0.151127	0.12803
Mean	0.222028	0.291379	0.297486	0.244638	0.316328	0.37039	0.110006	0.109626
SD	0.055134	0.094642	0.125986	0.061146	0.058383	0.142364	0.036511	0.025913
CV	0.248318	0.324809	0.423504	0.249944	0.184563	0.384362	0.331897	0.236376
RSD %	24.83179	32.48085	42.35041	24.99441	18.45633	38.43615	33.18967	23.63764
12 Hours	0.22381	0.735976	0.29238	0.341634	0.450323	0.47245	9.31E-02	0.281145
	0.316156	0.820596	0.164203	0.569627	0.256603	0.660158	0.379437	0.470803
	0.544143	0.629372	0.323516	0.470614	0.318132	0.951841	0.132974	0.350436
	0.283611	0.898679	0.335126	0.274225	0.270796	0.536698	7.77E-02	0.356954
	0.145394	0.494986	0.175034	0.298262	0.303601	0.673519	8.31E-02	0.284012
Mean	0.302623	0.715922	0.258052	0.390872	0.319891	0.658933	0.153277	0.34867
SD	0.149841	0.158952	0.082317	0.125419	0.076969	0.184205	0.128266	0.07702
CV	0.49514	0.222024	0.318993	0.32087	0.24061	0.279551	0.836825	0.220897

RSD %	49.51401	22.20236	31.8993	32.08702	24.06098	27.95507	83.6825	22.08975
24 Hours	0.882701	0.692564	0.649925	0.320347	0.435798	0.30787	0.285132	0.274814
	0.880592	0.88605	0.440685	0.360435	0.513638	0.623031	0.346253	0.299535
	0.537567	1.010632	0.492326	0.423087	0.421827	0.500705	0.21241	0.436082
	0.503444	1.312714	0.482286	0.313526	0.50713	0.471113	0.245539	0.369895
	0.535428	0.993257	0.368867	0.418559	0.588707	0.486619	0.228895	0.218476
Mean	0.667946	0.979043	0.486818	0.367191	0.49342	0.477868	0.263646	0.319761
SD	0.195549	0.225419	0.103314	0.052161	0.067309	0.112489	0.053493	0.084752
CV	0.292762	0.230244	0.212224	0.142053	0.136414	0.235398	0.202899	0.26505
RSD %	29.27622	23.02436	21.2224	14.20531	13.64138	23.53983	20.28986	26.50499

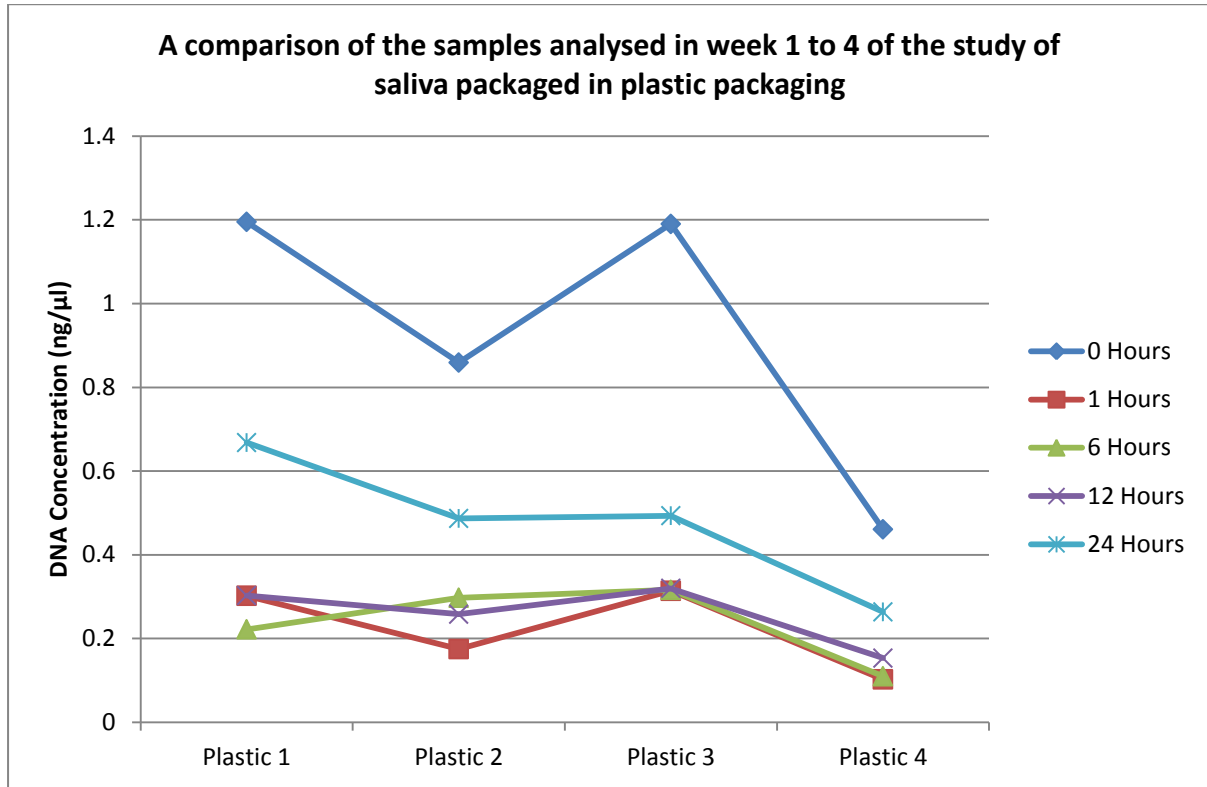
Statistical Analysis of Results - Week 5 – 8

	Plastic 5	Paper 5	Plastic 6	Paper 6	Plastic 7	Paper 7	Plastic 8	Paper 8
0 Hours	1.133256	0.179172		0.178913	0.320855	0.14475	0.396776	7.03E-02
	0.640673	0.257807		0.180196	0.583165	0.107382	0.440872	8.74E-02
	0.726105	0.240201	0.888443	0.106139	0.482577	0.223393	0.50582	9.83E-02
	0.591747	0.49565		0.259654	0.2533	0.195751	0.347558	0.143465
	0.993826	0.291428	0.602051	0.155545	0.511008	7.77E-02	0.65985	0.106887
Mean	0.817121	0.292851	0.745247	0.176089	0.430181	0.149805	0.470175	0.101243
SD	0.235254	0.12047	0.202509	0.055504	0.13777	0.060298	0.120963	0.027279
CV	0.287905	0.41137	0.271735	0.315206	0.32026	0.402513	0.257273	0.269435
RSD %	28.79055	41.13701	27.17346	31.5206	32.02596	40.25126	25.72726	26.94352
1 Hour	0.332499	0.197063	0.212856	0.287779	0.183627	0.920071	0.166604	0.129299
	0.366243	0.251302		0.168033	7.56E-02	0.106138	1.00E-01	0.624498
	0.500909	0.170466	0.19278	0.255918	0.130797	0.141277	7.61E-02	0.191193
	6.81E-02	0.674517	6.33E-02	0.203315	5.28E-02	0.168246	3.23E-02	0.176035
	0.294248	0.3184	0.143214	0.202509	3.36E-02	0.195412	4.46E-02	0.124481
Mean	0.312393	0.32235	0.153029	0.223511	0.095288	0.306229	0.08393	0.249101
SD	0.157217	0.204848	0.066615	0.047716	0.061363	0.344734	0.0533	0.211836
CV	0.503266	0.635483	0.435312	0.213484	0.643982	1.125741	0.635054	0.850399
RSD %	50.32659	63.54833	43.53115	21.34841	64.39824	112.5741	63.50541	85.03994
6 Hours	0.241628	0.175202	0.330357	0.112073	4.61E-02	7.47E-02	0.066951	4.92E-02
	0.130422	0.326943	0.235959	0.183409	6.97E-02	0.099639	0.121129	0.115431
	0.381401	0.228571	0.282907	0.11079	6.99E-02	0.30953	9.80E-02	8.62E-02
	0.351232	0.330168	0.113281	0.164894	7.02E-02	0.084962	0.124955	6.65E-02
	0.306086	0.197429	0.308732	0.229627	0.125495	5.72E-02	0.112243	5.88E-02
Mean	0.282154	0.251663	0.254247	0.160159	0.076284	0.125191	0.10465	0.075225
SD	0.099824	0.072717	0.086297	0.050345	0.029388	0.104204	0.023491	0.026271
CV	0.353793	0.288946	0.339421	0.314343	0.385247	0.832363	0.224475	0.349226
RSD %	35.37932	28.8946	33.94206	31.43426	38.52473	83.23631	22.44748	34.92262

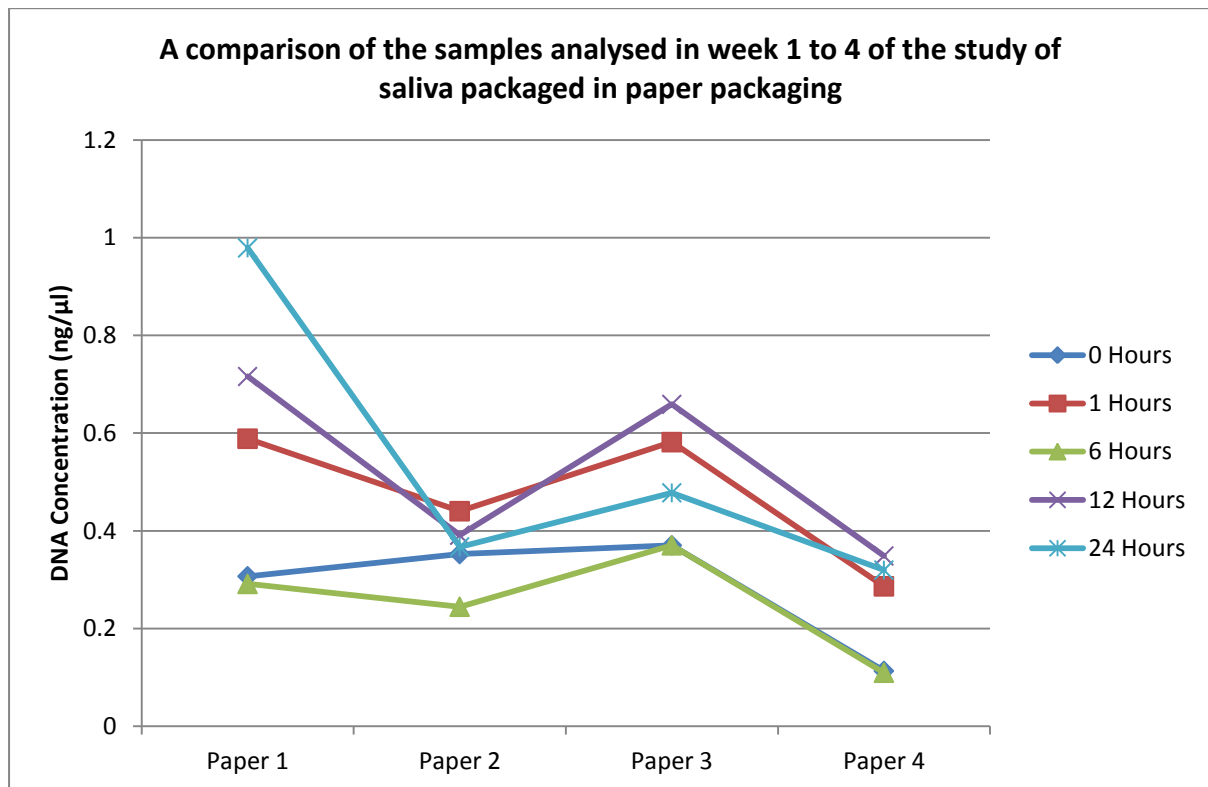
12 Hours	0.25411	0.318753			7.34E-02	0.818507	9.83E-02	0.191966
	0.158589	0.566969			6.20E-02	0.982764	6.73E-02	0.346405
	0.295668	0.517598	1.92E-02	0.227811	5.04E-02	0.86673	0.041274	0.259869
	0.150744	0.642574	0.170114	0.209461	5.63E-02	0.88299	0.126117	0.283655
	0.167338	0.556052	0.163233		5.36E-02	0.757606	6.34E-02	0.50498
Mean	0.20529	0.520389	0.117503	0.218636	0.059116	0.861719	0.079278	0.317375
SD	0.065476	0.121486	0.085235	0.012976	0.009021	0.083421	0.033154	0.118543
CV	0.318942	0.233452	0.72538	0.059348	0.152593	0.096808	0.418202	0.373511
RSD %	31.89422	23.34519	72.53803	5.934803	15.25934	9.680774	41.82017	37.35115
24 Hours	0.488361	0.558425	0.269957		0.208572	1.210492	0.176789	9.40E-02
	0.563098	0.551438	0.25801	0.189479	0.269155	1.039118	0.100729	0.459134
	0.350193	1.030368	0.255664	0.367085	0.114188	0.492606	0.311535	0.44762
	0.557702	0.499982	0.172461	0.317244	0.107089	0.771054	0.231117	0.189746
	0.769718	0.463342	0.213068	0.269315	0.192099	0.893998	0.247997	0.239721
Mean	0.545814	0.620711	0.233832	0.285781	0.178221	0.881454	0.213634	0.28605
SD	0.151774	0.232292	0.040509	0.075599	0.068086	0.272338	0.079335	0.161521
CV	0.278069	0.374235	0.173238	0.264535	0.382031	0.308965	0.37136	0.564659
RSD %	27.80688	37.42346	17.32382	26.4535	38.20309	30.8965	37.13598	56.46585

Appendix 2

Week 4 packaging results for saliva in plastic

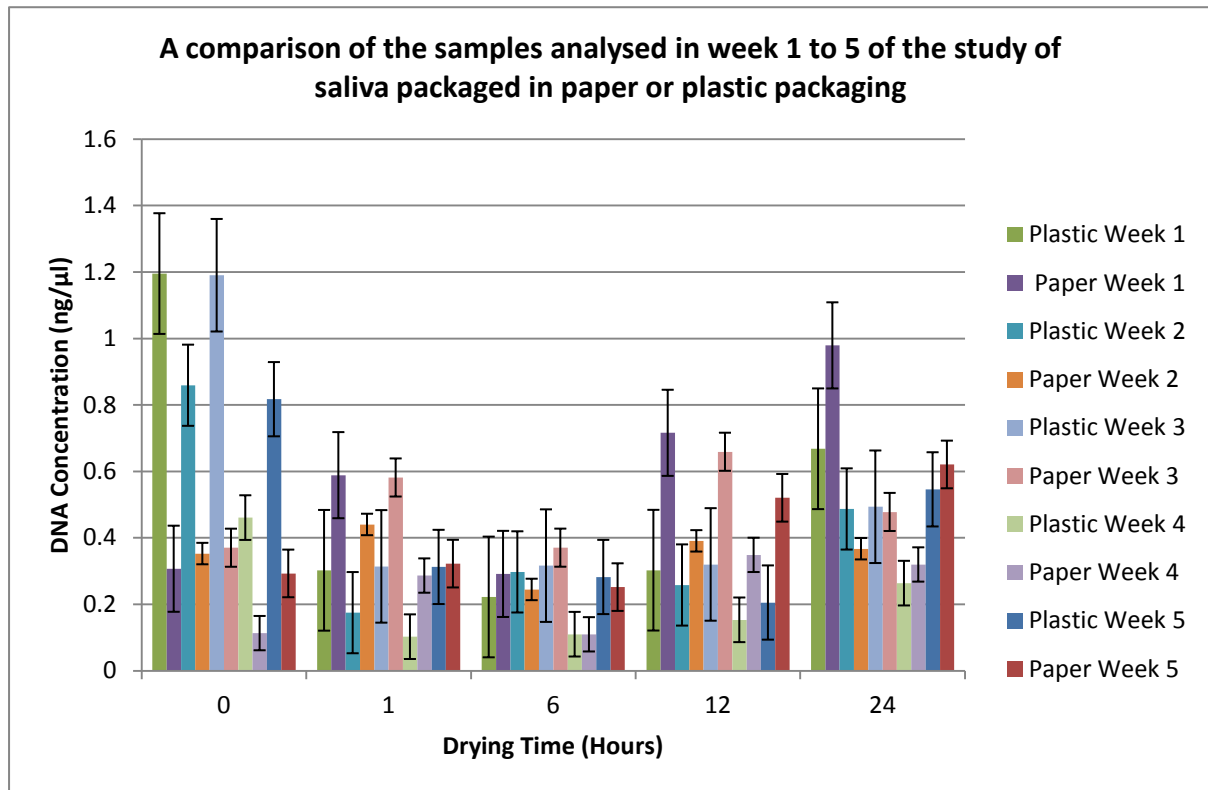


Week 4 packaging results for saliva in paper

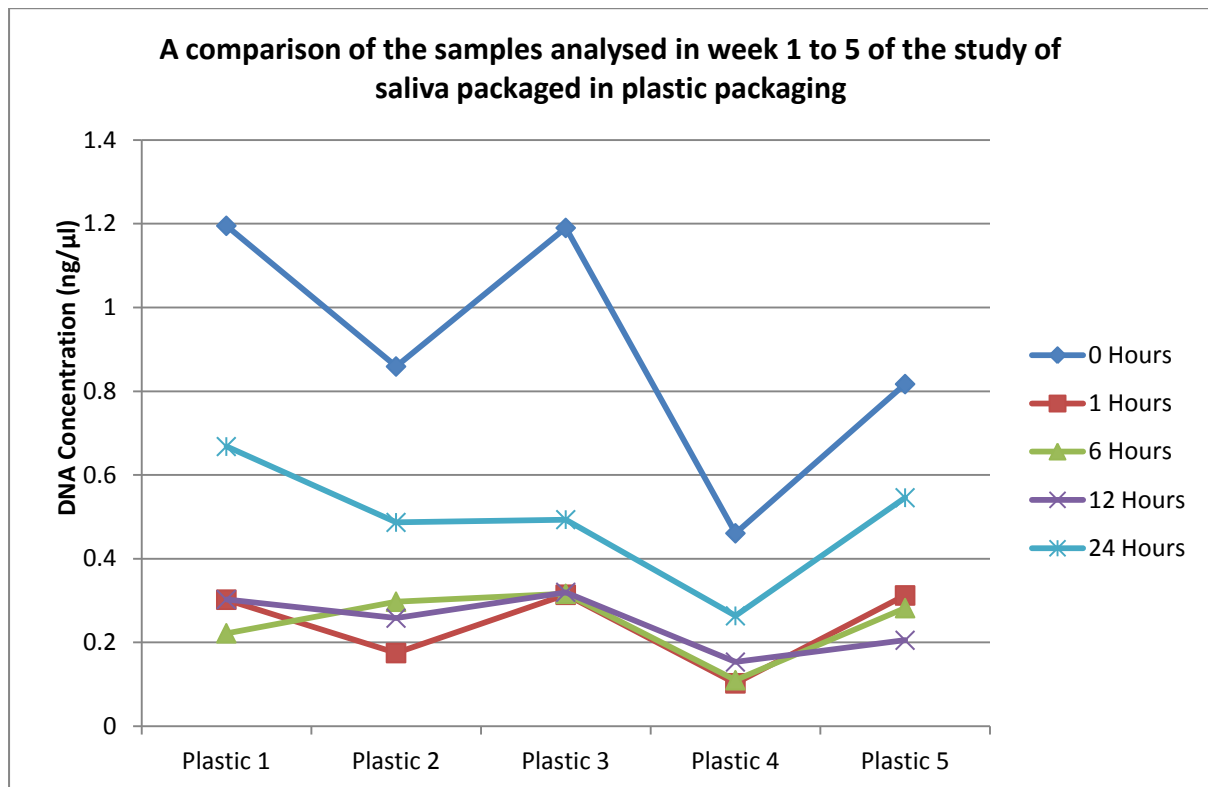


Appendix 3

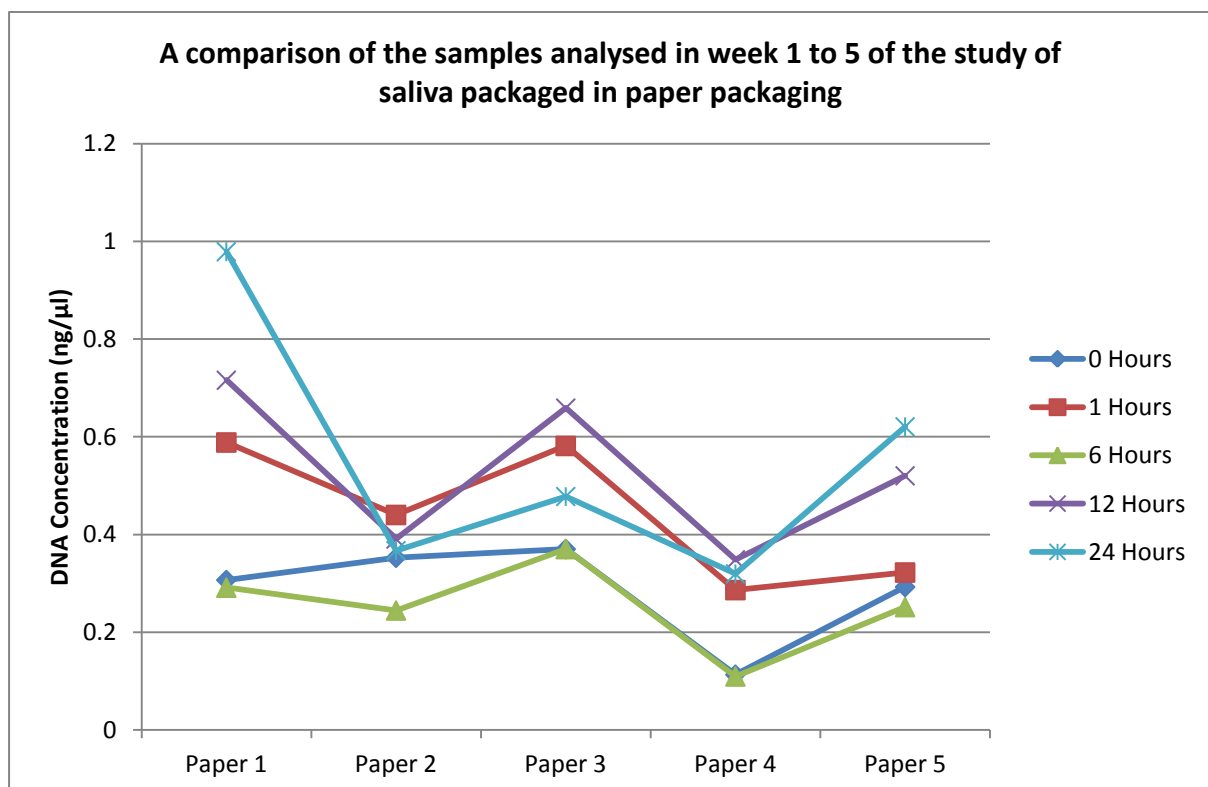
Comparison of results for weeks 1 to 5 with error bars.



Week 5 packaging results for saliva in plastic

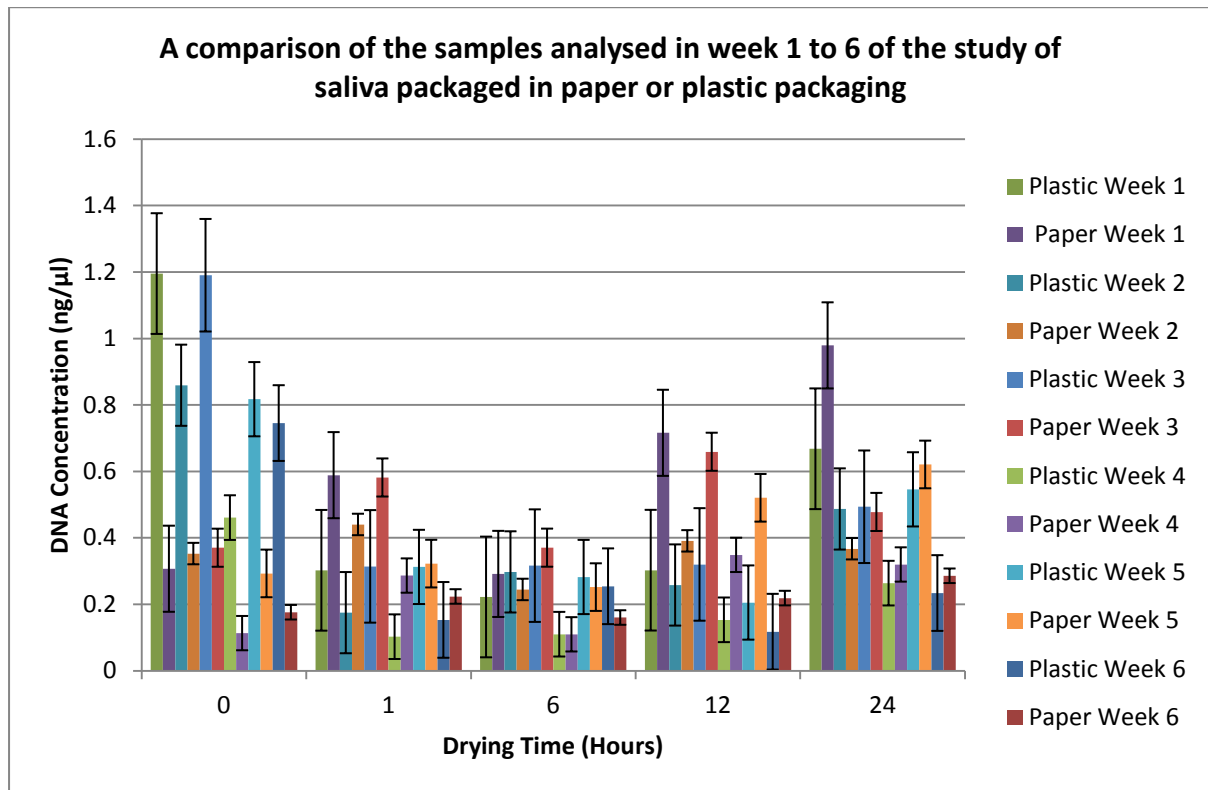


Week 5 packaging results for saliva in paper



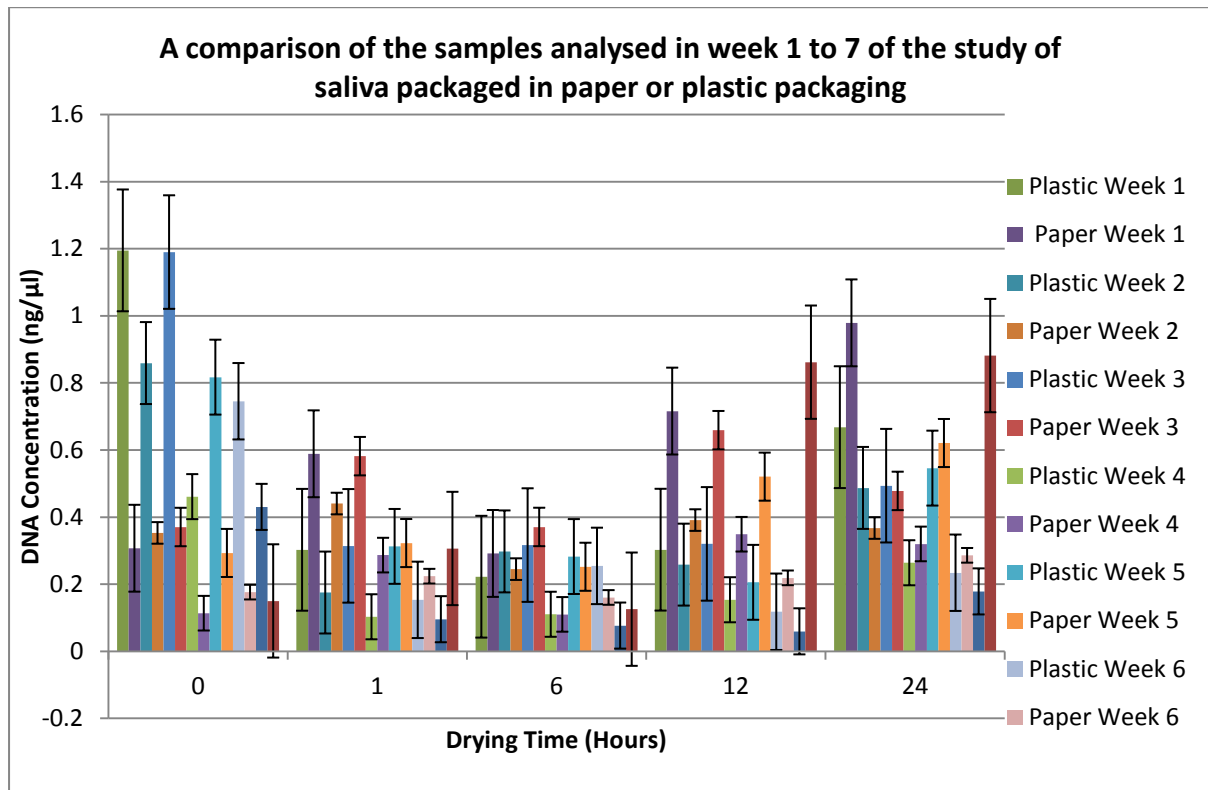
Appendix 4

Comparison of results for weeks 1 to 6 with error bars.

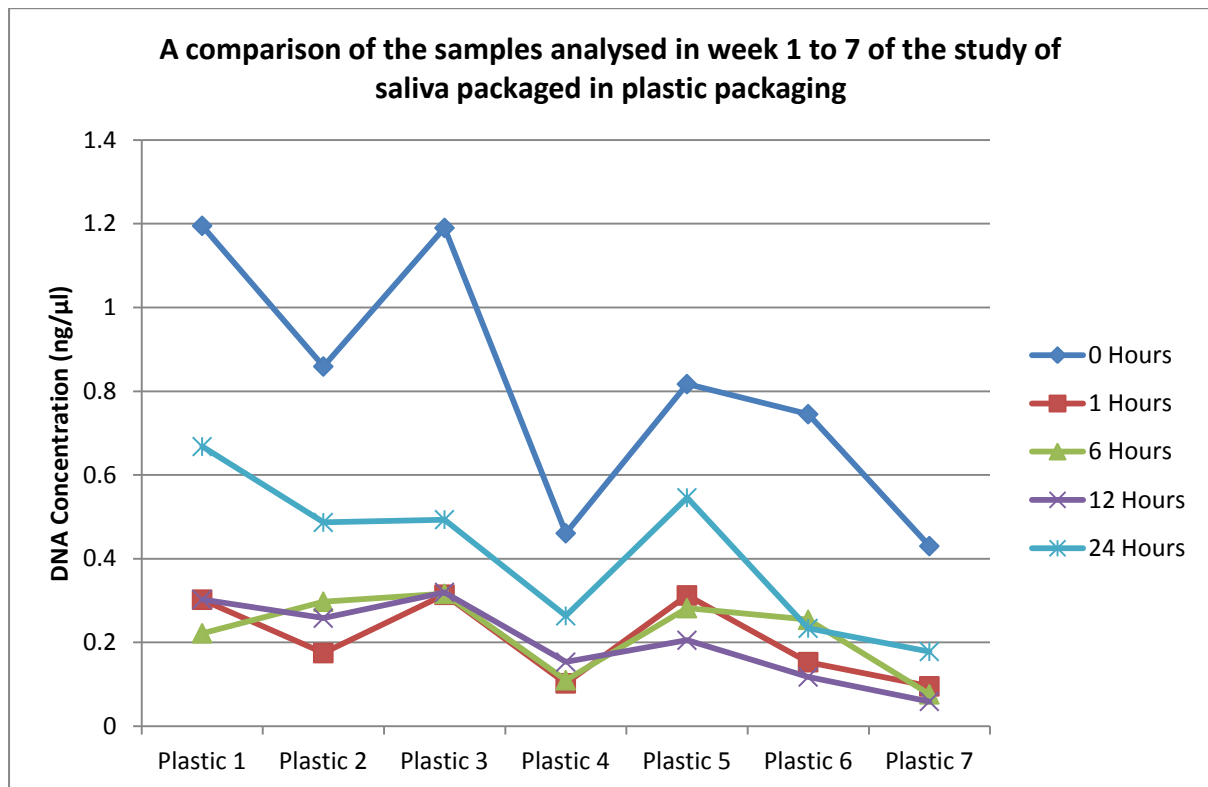


Appendix 5

Comparison of results for weeks 1 to 7 with error bars.



Week 7 packaging results for saliva in plastic



Week 7 packaging results for saliva in paper

