AN ASSESSMENT OF THE ROLE OF SALIVA, SALIVARY PROTEINS/IONS AND ACQUIRED ENAMEL PELLICLE ON EROSION

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AN ASSESSMENT OF THE ROLE OF SALIVA, SALIVARY PROTEINS/IONS AND ACQUIRED ENAMEL PELLICLE ON EROSION

THESIS SUBMITTED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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2017

ABSTRACT:

There is a growing interest in the relative contribution of ions and proteins in acquired enamel pellicle (AEP) to protect against erosion and erosive tooth wear, but not enough is understood as yet.

The effect of immersion of human enamel specimens in whole mouth saliva (WMS), artificial saliva (AS) and deionised water (DW) for three time periods [30 minutes (1), 60 minutes (2), 24 hour+30 minutes (3)] on erosion was assessed *in vitro* (n=90). Significantly less step height formation and greater surface microhardness change (SMHC) was observed for WMS3 [3.80 (0.59) µm and 249.4 (29.6) KHN respectively] compared with AS3 [6.34 (0.55) µm and 181.87 (20.48) KHN respectively] and DW3 [8.80 (1.28) µm and 148.82 (25.68) KHN respectively] ($P<0.0001$).

The effect of AEP proteins was further investigated *in vitro* (n=80) after 24 hour immersion in either WMS, parotid saliva (PS), AS and DW followed by five or one cycles of erosion. WMS group had significantly ($p<0.0001$) less step height [4.16 (0.9) µm] than PS group [6.41 (0.3) µm] after five erosion cycles ($p<0.0001$). Concentration of total protein and mucin5b and albumin were higher in WMS derived AEP, compared with PS derived AEP and increased after 5 cycles, suggesting protection through physical barrier, diffusion barrier and lubrication. After one cycle erosion, there was a lower SMHC in specimens immersed in PS [85.19 (6.07) KHN ($p<0.0001$)], compared with those immersed in WMS [98.68 (8.5) KHN], suggesting protection through buffering and calcium homeostasis as PS AEP is richer in CA VI and statherin.

Finally, same protein variables were also measured in *in vivo* film (F) and AEP (P) from eroded (E) and non-eroded (N) tooth surfaces in erosive wear patients

(n=29). The total protein on EP [0.41mg/mL (0.03)] was significantly lower than that on NP [0.61 mg/mL (0.11) $p< 0.05$]. The amount of statherin was also significantly lower on EP [84.1 (221.8, 20.0) ng] compared to that from NP in the same subjects [97.1(755.6, 30.0) ng] ($p=0.002$).

The overall findings in this thesis imply that proteins in AEP have a major contribution in protection against erosion and erosive tooth wear *in vitro* and *in vivo*.

ACKNOWLEDGEMENT

My sincere thanks and gratitude to:

Dr. Rebecca Moazzez, my first supervisor, for her tireless support throughout my PhD studies and contribution to make this thesis possible. For being an abundant source of knowledge, kind and available throughout the project for support and encouragement. I am lucky to have a supervisor who has cared so much for my work and helped me develop my academic career at all levels.

Professor David Bartlett, my second supervisor, with whom I have been proud to be associated who has supervised my research work and continuously evaluated my progress and provided me with invaluable advice and moral support.

Dr. Guy Carpenter, without whom the work of salivary research would not be possible. For his patient guidance and scientific advice in saliva analysis who always inspired me with his confidence in my research.

Professor Gordon Proctor, for his scientific advice on salivary experiments.

Dr. Mathew German, from Newcastle dental School, for his support in the use and analysis of the Atomic Force Microscopy.

Manoharan Andiappan, for his help and guidance with the statistical analysis.

Mr Peter Pilecki, for his help in the lab and proof reading my thesis**.**

My fellow PhD student and friends, **Dr. Saoirse O'Toole**, **Dr. Miten Mistry**, who have kindly assisted in proof reading my thesis and **Dr. Francesca Mullan and Dr. Petros Mylonas**, who have shared the frustrations and hopes.

Finally, I dedicate this work to my mother, siblings, wife and son who have endured all difficult and unpleasant times during my studies.

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LIST OF ABBREVIATIONS

AEP: Acquired Enamel Pellicle

AFM: Atomic Force Microscopy

AS: Artificial Saliva

BCA: Bicinchoninic Acid

BEWE: Basic Erosive Wear Examination

BSA: Bovine serum albumin

CMC: Carboxymethyl Cellulose

CA VI: Carbonic Anhydrase VI

DTT: DL-Dithiothreitol

DW: Deionised Water

ECL: enhanced chemiluminescence

FITC: Fluorescein isothiocyanate

HAP: Hydroxyapatite

ICP-MS: Inductively coupled plasma – mass spectrometry

PIS: Patient Information Sheet

PRPs: Proline-rich proteins

PS: Parotid Saliva

SMHC: Surface microhardness change

SNCP: Surafec Non-Contacting Profilometry

SDS-PAGE: Sodium dodecyl sulfate Polyacrylamide gel electrophoresis

SDS: Sodium dodecyl-sulphate

TTBS: Tris Tween-20 Buffer Saline

WMS: Whole Mouth Saliva

PREFACE

This thesis aims to investigate the role of AEP and salivary proteins/ions in an *in vitro* erosion model as well as an *in vivo* study. In Chapter one, the literature relating to dental erosion and salivary proteins is reviewed. This includes an overview of the current concepts surrounding erosion, natural saliva [whole mouth saliva (WMS) and parotid saliva (PS)] and artificial saliva (AS), acquired enamel pellicle (AEP) formation and harvesting as well as the role of proteins and ions of AEP in erosion and erosive tooth wear.

Chapter 2 describes the common materials and methods used in all experiments within this thesis as well as the common techniques used in erosion and protein analysis. This Chapter also includes detail about training of the author prior to carrying out the experiments.

An *in vitro* investigation into the protective effect of different solutions (WMS, AS and DW) and the effect of maturation of AEP against prolonged exposure to acid is described in Chapter 3.

Chapter 4 and 5 further investigate the role of proteins and ions in protection against five cycle and one cycle erosion by analysing WMS, PS, AS and distilled water (DW) in *in vitro* studies.

An *in vivo* investigation into proteins in AEP from eroded and non-eroded tooth surfaces in the same subjects is described in Chapter 6. This Chapter highlights the potential role of total protein concentration, statherin, carbonic anhydrase VI (CA VI), albumin and mucin5b in protection against *in vivo* erosive tooth wear. Chapter 7 provides a general discussion and evaluation of the findings reported within this thesis with an overview summary of the thesis. Chapter 8 suggests recommendations for future work.

Chapter 1: Review of the literature

1.1 Enamel structure

1.1.1 Enamel minerals

Dental enamel is a highly mineralised tissue comprised of long, thin hydroxyapatite crystallites arranged in parallel arrays (Baldassarri *et al.*, 2008) forming unique hierarchical levels from the microscale down to the nanoscale hydroxyapatite (HAP) fibers (Gwinnett, 1967). The nano-hydroxyapatite crystallites, are composed of 96 wt % substituted carbonated hydroxyapatite (HAP) [$Ca_{10-x}(PO_4)_{6-x}(CO_3)_x(OH)_2$] which have a width of approximately 68 nm, thickness of 26 nm and length that can be 2 mm extending from EDJ to surface. The properties of enamel, such as porosity, would influence the movement of minerals and acids into and out of enamel which in turn may influence enamel susceptibility to acid challenege. The outrmost layer of the enamel is prismless layer which has less pores and formed from either striae of Retzius (incremental lines) or an extension of the underlyning prism layer, giving three structural patterns; step-, band-, and island-like shapes (Kodaka *et al.*, 1991). This structure may make the enamel surface more resistant to physical and chemical insults including such as acid challenege and tooth brushing than the subsurface enamel structures (Carvalho and Lussi, 2015). Also, the tightly packed hydroxyapatite crystal of enamel rods is formed of calcium phosphate with which many salivary proteins tend to interact on the enamel surface (Habelitz *et al.*, 2001; Zimmerman *et al.*, 2013). This organic structure within the enamel may influence its hardness and elastic properties and thus its resistance to acid challenges (Lubarsky *et al.*, 2014; Baumann *et al.*, 2015).

1.1.2 Enamel Proteins

Between the enamel rods, there is also approximately 1% organic matrix present after enamel maturation. This organic matrix is initially formed from many proteins including amelogenin, collagen and enamelin as the major enamel proteins (Bartlett, 2013). These proteins influence the crystal growth and final properties of mature enamel such as surface, structural and mechanical characteristics are believed to be influenced by the organic matrix–mineral interactions during amelogenesis (Baldassarri et al., 2008; Deshpande et al., 2010; Bartlett, 2013; Robinson, 2014; Baumann et al., 2015). Amelogenin is an enamel protein that is rich in proline, leucine, histidine and glutamyl amino acids, and synthesised by the ameloblast cells and becomes mineralised to form the mature enamel (Finchman et al., 1997). It has been shown that during enamel development amelogenine subjects for proteolytic transformation that leads to reducing the binding affinity of amelogenine for hydroxyapatite, a process that has been linked to the development of amelogenesis imperfecta (Finchman et al., 1999).

Enamelin, a 32 kilodaltons (kDa) molecular weight, is hydrophilic and acidic enamel protein and may be important in the process of enamel demineralisation. This is because the subunits of enamelin are cross-linked by calcium ions (Ca^{2+}), mediating the structural changes of this protein which may influence the rate of enamel demineralisation (Fan et al., 2008; Lubarsky et al., 2014). These enamel proteins cover the enamel crystals within the prisms and have recently been shown to limit the progression of erosive tooth wear (Lubarsky et al., 2014; Baumann et al., 2015).

The above characteristics of the enamel structure may provide important information on the role of enamel proteins in erosive tooth wear (Cuy et al., 2002; Ge et al., 2005).

1.2 Tooth wear

Tooth wear is a prevailing and increasing dental condition affecting an increasing number of the population (Jaeggi and Lussi, 2014) as a result of constantly changing lifestyles (Johansson *et al.*, 2012). It is a complex problem that results from overlapping and multifactorial aetiologies. It can be defined as the progressive loss of enamel and dentine as a result of erosion, attrition or abrasion or a combination of these (Bartlett and Dugmore, 2008). Although the clinical appearance of tooth wear may suggest a predominant contributory factor, the lack of clinical detection techniques have made it difficult for clinicians to distinguish its main cause (Huysmans *et al.*, 2011). Clinically, tooth wear can be considered to be pathological if it results in problems with appearance and function (Barbour and Rees, 2005; Bartlett, 2005; Ganss *et al.*, 2006).

1.2.1 Abrasion

Abrasion is defined as the loss of hard dental tissues due to physical contact with foreign objects other than the teeth (Imfeld, 1996). A number of studies have investigated abrasion in the laboratory (Attin *et al.*, 2001; Parry *et al.*, 2008) using tooth brushing as an abrasion-wear model (Wiegand and Schlueter, 2014). Enamel can be readily removed by abrasion when preceded by an acidic challenge (Jaeggi and Lussi, 1999; Addy, 2005; Laurance-Young *et al.*, 2011).

1.2.2 Attrition

Attrition is a form of wear which occurs due to tooth-to-tooth contact during bruxism, grinding or clenching (Bartlett and Smith, 2000; El Wazani *et al.*, 2012). As this thesis focuses on the erosive component of erosive tooth wear, the abrasion and attrition processes were not extensively reviewed.

1.2.3 Erosion

Erosion is defined as a condition where dental hard structures are subjected to partial and/or progressive loss of minerals (demineralisation) caused by the dissolution of the constituent hydroxyapatite crystals due to intrinsic or extrinsic chemical exposure without microbial involvement (Imfeld, 1996; Buzalaf *et al*., 2012). The aetiology of erosion can be classified into extrinsic and intrinsic causes. Extrinsic sources of acid include intake of acidic foods and drinks. These include citrus fruits such as lemons and oranges as well as carbonated drinks, sports drinks, fruit juices and other fruit products, most of which contain citric acid. The above causes are influenced by behavioural factors. These include the drinking pattern, consumption frequency of acidic foods and drinks, and the type and method of ingestion of acidic foods and drinks (Millward *et al.*, 1997; Amaechi and Higham, 2005).

Intrinsic sources of acids are often related to health disorders that involve vomiting or regurgitation such as gastro-oesophageal reflux disease (GORD), bulimia nervosa, anorexia nervosa, alcoholism and pregnancy (Amaechi and Higham, 2005; Moazzez *et al.*, 2005; Zero and Lussi, 2005). The intrinsic acid of interest to erosive wear is hydrochloric acid (HCl) in gastric juice (Moazzez *et al.*, 2005).

1.3 Erosive wear

Erosive tooth wear is a term used recently to describe the wear of teeth primarily as a result of erosion but in combination wih attrition and abrasion (Lussi and Carvalho, 2014). Erosive tooth wear can result either through repeated acidic insults or through softening of the enamel surface by an acid followed by mechanical removal of tooth tissue through attrition and abrasion (Eisenburger *et al*., 2000; Wiegand *et al.*,2007).

1.3.1 Stages of erosive tooth wear development

Erosive tooth wear may develop in two stages, an early stage and an advanced stage. The early stage is difficult to be diagnosed clinically but was defined by Koulourides (1968) as the initial loss of structural and mechanical strength of the tooth surface without bulk tissue loss (Koulourides, 1968). This process was termed ‘softening’ which may occur when the crystal minerals leach out. The advanced stage includes the loss of both inorganic and organic components. There is little known about the structural differences associated with the two stages and whether the damaged tooth can be repaired at any point. Given the fact that the early stage (initial stage) of this process is caused by mineral loss, one can speculate that this stage may be reversible by replacing the lost minerals with chemical ions such as calcium, phosphate and fluoride. These minerals can come from saliva and/or oral healthcare products, remineralising the softened enamel surface (Cardoso *et al*., 2009). Cardoso *et a*l. (2009) demonstrated that the time of exposure to saliva in the oral cavity might result in an increase in mineral content and hardness due to the incorporation of calcium, phosphate and fluoride (Cardoso *et al.*, 2009). On the other hand, advanced erosive tooth wear appears to be destructive and

may, ultimately, lead to complete tissue loss and subsequent exposure of dentine. This can occur either by prolonged and/or frequent acidic challenges or by acid exposure associated with or followed by abrasion and/or attrition (Attin *et al.*, 2003; Barbour and Rees, 2004; Addy, 2005).

1.3.2 Clinical aspects of erosive tooth wear:

1.3.2.1: Clinical significance:

Erosive wear has become a prevalent oral health problem affecting an increasing number of the population worldwide (Ganss *et al*., 2001; Jaeggi *et al*., 2014). It can lead to many clinical concerns such as poor appearance, loss of function, pain and discomfort (Daly *et al.*, 2011).

1.3.2.2 Basic Erosive Wear Examination (BEWE)

BEWE is a simple tooth wear index designed for recording erosive tooth wear in general practice (Bartlett *et al.*, 2008). This index uses four grading scores (0-3) to indicate the severity of tooth wear on the teeth: 0 (no surface loss), 1 (initial loss of enamel surface texture), 2 (hard tissue loss < 50% of the surface area) and 3 (hard tissue loss > 50% of the surface area) and sums the maximum score in each sextant to give a total score (Bartlett *et al.*, 2008). BEWE does not assess dentine exposure separately from enamel but dentine involvement is included in scores 2 and 3 (Olley *et al.*, 2013).

1.3.2.3 Biology and chemistry of erosive tooth wear

Erosive tooth wear in the mouth is influenced by many factors, mainly, biological and chemical (Bartlett, 2005; Zero and Lussi, 2005; Lussi and Jaeggi, 2008; Buzalaf *et al.*, 2012). Saliva is one of the most important

biological factors protecting teeth against erosive tooth wear through many mechanisms (Zero and Lussi, 2005; Lussi and Jaeggi, 2008; Buzalaf *et al.*, 2012).The functions of saliva in erosive wear can be summarised in neutralisation, clearance, forming a physical barrier on tooth surfaces and possibly remineralisation. The role of saliva in erosive wear will be further explored in section 1.4. Other biological factors include the structure of teeth and their position, oral soft tissues, dental anatomy and occlusion (Zero and Lussi, 2005; Piangprach *et al.*, 2009; Hellwig *et al.*, 2013).

The chemistry of erosive tooth wear relates to many factors including the pH and buffering capacity of acids, titratable acidity, type of acid, the presence of chelating agents, and the concentration of the supplemented ions such as phosphate (PO_4^{3-}), calcium (Ca^{2+}) and fluoride (F^-) (Shellis *et al.*, 2014). Some erosive acids such as citric acid have been found to remove calcium ions from the hydroxyapatite by the citrate group (chelation), whereas other acids such as oxalic acid and polyacrylic acid work differently where the carboxylic group of acids bonds to the hydroxyapatite of enamel crystal to compensate for the released PO_4^{3-} (Yoshida *et al.*, 2001). One driving force for prevention of dissolution of the tooth mineral is the saturation of solutions by calcium and phosphate. These minerals can be added to erosive drinks to retard the progression of erosive wear caused by influencing the concentration gradient of minerals around the enamel surface (Lussi and Jaeggi., 2006; Barbour and Lussi., 2014).

1.3.2.4 In-situ erosive tooth wear studies

Previous *in-situ* studies have used soft/hard acrylic splints prepared from impressions taken from the mouth, containing prepared and sterilised enamel

specimens attached to the splint before they are worn by the subjects (Hannig *et al.*, 2003; Carpenter *et al.*, 2014). AEP is then formed on the enamel specimens inside the mouth. Commonly available soft drinks such as blackcurrant, orange juice and cola drinks have been used for *in-situ* studies (West *et al.*, 2003; Hara *et al.*, 2006; Rios *et al.*, 2006; Hannig *et al.*, 2009; West *et al.*, 2015). Other studies have used hydrochloric acid (HCl), a strong inorganic acid, and citric acid, a weak organic acid to mimic gastric and dietary acids respectively, applied to enamel specimens *ex-vivo* (Wiegand *et al.*, 2008; Attin *et al*., 2012).

1.3.3 Laboratory models in erosion and erosive tooth wear

A variety of laboratory methods have been used to study erosion and erosive tooth wear. As different studies frequently use different experimental designs, comparisons between outcomes is limited (Shellis *et al.*, 2011; Young and Tenuta, 2011). The following sections will explain elements of experiment design requiring consideration in *in vitro* studies of erosion.

1.3.3.1 Erosive challenges in in vitro models

The erosive challenges used in any *in vitro* erosion and erosive tooth wear study should reflect the aim and the research question of the study. One can either choose pure acid or commercially available products. A wide range of acidic challenges have been used *in vitro*. Inorganic acids such as hydrochloric acid (HCl) and phosphoric acid (H_3PO_4); citric acid ($C_6H_8O_7$), a weak organic acid; or orange juice are usually used (West *et al*., 2001). A standard acid challenge used is 1.0 % citric acid with a pH range of 2.14-3.75, for 10 minutes at 22 ± 1°C (Shellis *et al.*, 2011). In some studies, specimens are cycled in the

acid with or without other solutions. For example, the specimens can be treated with human or artificial saliva prior to or after immersion in the acid for a specific time. This is often considered as one erosion cycle but can sometimes be repeated several times, often five times, to simulate drinking of orange juice at breakfast, midday, lunch, late afternoon and dinner (Amaechi *et al.*, 1999).

1.3.3.2 Specimen preparation and substrates in erosion studies

Human or bovine enamel and dentine as well as pure hydroxyapatite have been used in various *in vitro* erosion studies (Hannig *et al.*, 2004; Carpenter *et al.*, 2014). While hydroxyapatite may be preferred to be used for standardisation, human or bovine enamel are often used because they are more relevant to the clinical situation. In this respect, bovine enamel specimens have the advantage of easy resourcing and being more uniform, reducing the intra and interspecimen variability, but they have been reported to be more susceptible to acid challenge compared with human enamel (Meurman and Frank, 1991). In a review, Yassen et al. (2011) concluded that both human and bovine enamel have similar calcium and carbonate content and calcium/phosphate ratio, but they differ in the distribution of calcium, with more uniform distribution and higher protein content observed in bovine enamel. Physically, bovine teeth are larger and flatter compared to human teeth, which makes preparation of bovine teeth easier for laboratory studies (Yassen *et al*., 2011). Although bovine and human enamel have similar microstructures (Laurance-Young *et al.*, 2011), human enamel specimens often reflect more relevant results to the clinical situation than bovine specimens. However, one should acknowledge the cost and difficulties

associated with resourcing human teeth as well as the ethical limitations which can result in difficulty obtaining the appropriate sample size. Another disadvantage often cited for the use of human enamel is that they may contain variable amounts of fluoride from extrinsic sources such as toothpastes and mouth rinses which can lead to intra and inter-specimen variability (Mellberg, 1992).

1.3.3.3 Polishing enamel specimens:

In the majotity of *in vitro* erosion studies, enamel and bovine specimens are polished flat using standardised protocols. Some studies use unpolished tooth surfaces to simulate the clinical situation but this reduces the laboratory techniques which can be used to assess erosive tooth wear. For example, the natural curvature of the tooth leads to inaccurate measurements with profilometry and microhardness testing (Austin *et al.*, 2011). Different methods of grinding, then polishing of the samples have been used. These include grinding stones, abrasive disks, suspensions and silicon carbide, diamond pads, diamond and aluminium oxide films, and polishing cloths. Silicon carbide papers are most commonly used in the preparation of enamel surfaces (Hanning *et al*., 2004; Wetton et al., 2006; Hellwig *et al*., 2013). The silicon carbide papers of grit size up to 4000 are often used to polish the enamel surfaces, removing any surface defects or artefacts (Wang *et al.*, 2012). Silicon carbide papers with different grits have also been used where paper grits of 80, 180 and 600 are used to remove the superficial prismatic enamel whereas 1200, 2500 and 4000 are used to polish the enamel surfaces, removing any surface defects or artefacts are most commonly used in the preparation of enamel (Rodriguez and Bartlett, 2010; Austin *et al*., 2011).

1.3.3.4 Stirring of erosive solution in in vitro models

The amount of erosion in *in vitro* erosion studies is highly influenced by the agitation rate of the erosive challenge (Eisenburger and Addy, 2003; Barbour and Rees, 2005; Shellis *et al.*, 2005). In some *in vitro* studies, the erosive challenge was unstirred (Cheng *et al.*, 2009; LEVY *et al.*, 2012) or slightly agitated (West *et al.*, 2000) whereas in some other studies it was stirred at various velocities (Lussi *et al.*, 2000; Shellis *et al.*, 2005). An increase in flow rate has been demonstrated by many studies to increase erosion depth (Finke *et al.*, 2000; Eisenburger *et al.*, 2003; Shellis *et al.*, 2005) which may be related to the rate of the clearance of dissolution products from the eroded surface and the degree of saturation and diffusion of the erosive solution with respect to the enamel mineral.

Investigations on enamel have shown that increasing the speed of the agitation increased the amount of erosion (Eisenburger and Addy, 2003; Shellis *et al.*, 2005; Attin *et al.*, 2013). The principle behind this may be that the high speed of solution physically removes the dissolved tissues at a faster rate. Also, fresh ions in the dissolving solution may be replaced constantly by the stirring, leading to increased dissolution. Flow rate can be maintained in *in vitro* studies by using various apparatus including a chamber through which the erosive solution is pumped at a known rate (Attin *et al.*, 2003) or a calibrated stirrer (Hemingway *et al.*, 2008). A stuart mini gyro rocker is also available which provides a 3D gyratory motion. This apparatus allows movement of the tilt angle to any position by hand to optimise mixing allowing both speed and time to be digitally selected and adjusted (Stuart, 2017).

1.3.3.5 Exposure time, volume and temperature variables in in vitro models:

In vitro models of erosion studies are influenced by many factors including the time of exposure to erosive solution, total volume of solution and temperature which have been investigated by many studies (Amaechi *et al.*, 1999; Eisenburger and Addy, 2003; Shellis *et al.*, 2005). Different exposure times to demineralising solutions have been reported in the literature, varying from a short period of 1 minute exposure (Cheaib and Lussi, 2011), a medium exposure of 2 hours (Eisenburger and Addy, 2003) to a long time exposure of 12-24 hour (Amaechi *et al.*, 1999; Martins *et al.*, 2013). In previous studies (Eisenburger *et al.*, 2000; Eisenburger *et al.*, 2001), a period of between 1-2 hour acid exposure was demonstrated to be the period where the rate of enamel loss at the surface equals the rate of acid penetration into the underlying tissues. The effect of varying temperatures of citric acid on enamel were also studied by Amaechi *et al*. (1999), West *et al*. (2000) and Eisenburger and Addy (2003) who demonstrated that a higher temperature of acidic challenge lead to a non-linear increase in erosion depth (Amaechi *et al*.,1999; West *et al*., 2000; Eisenburger and Addy, 2003). However, direct comparison between these studies is difficult since different erosion models were used (Amaechi *et al*., 1999; West *et al*., 2000; Eisenburger and Addy, 2003). Amaechi *et al*. (1999) immersed specimens in orange juice and continuously stirred for 5 minutes, six times daily. Eisenburger and Addy (2003) used 0.3% citric acid adjusted to pH 3.2, agitated at 270 rpm and immersed the specimens for 2 hours, whereas West *et al*. (2000) used 0.3% citric acid with

an unadjusted pH, with gentle agitation and immersed the specimens for 10 minutes (West *et al*., 2000; Eisenburger and Addy, 2003).

Therefore, the temperature and agitation rate of the erosive challenge should be controlled. According to the literature, the temperature of the erosive challenge has to be either the body temperature (37 °C) or room temperature (22 ± 1) (Amaechi *et al*., 1999; Eisenburger and Addy, 2003; Shellis *et al*., 2011).

1.3.3.6 Treatment of enamel specimens after erosion cycling:

Immediately after the completion of the erosion cycles, methods of treatment of specimens can influence the outcomes of experiments. Most studies have reported rinsing specimens in water after exposure to acidic solutions. Some of these studies left specimens to dry in air after being washed (Barbour et al., 2004; Hemingway *et al.*, 2006) whereas other studies dried specimens using paper towels (Rodriguez and Bartlett, 2010). There are many benefits of washing enamel specimens with water after acidic exposure. These include the removal of any acid traces and/or calcium and phosphate ions as well as stopping the dissolution process (Barbour and Rees, 2004). Despite these benefits, there are a number of problems associated with washing and drying specimens after exposure to acid solution. These might include the development of mineral precipitation in the form of surface artefacts. This may occur as a result of the sudden rise in pH at the solid/liquid interface since enamel specimens are often exposed to water immediately after acidic solution (Boyde *et al*., 1978). Boyde *et al*. (1978) also pointed out that drying of softened enamel surface might lead to surface artefacts on the softened enamel due to the surface tension which affects the previously eroded enamel

crystals (Boyde *et al*., 1978). However, these problems may be resolved in *in vitro* studies where WMS is used after the acid exposure. As in the oral cavity, the use of WMS in *in vitro* models allows a progressive rather than a sudden change of pH after acid intake (Millward *et al.*, 1997).

1.4 Saliva

1.4.1 Natural saliva

The term natural saliva will be used throughout this thesis to refer to human saliva. Natural saliva is a non-Newtonian, hypotonic fluid of complex mixture composed of water (99.5 %), inorganic and organic components. It is regarded as non-Newtonian because its viscosity decreases when its shear rate increases allowing it to spread over the hard and soft oral tissues (Carpenter, 2013). Electrolytes in saliva include sodium, potassium, calcium, magnesium, bicarbonate, and phosphates whereas organic components are composed of proteins, including enzymes and antibodies (Edgar, 1992; Humphrey and Williamson, 2001). Taste, chewing and smell are the important stimulators of salivary secretion (Carpenter, 2013), citric acid being the greatest stimulator as compared to salt, sweet and bitter substances (Hodson and Linden, 2006). Saliva secretion is controlled by the parasympthatic and sympthatic parts of the autonomic nervous system. The parasympathetic activity controls the secretion of water and electrolytes, whereas protein synthesis and exocytosis are mainly controlled by the sympathetic activity (Jensen *et al.*, 1991; Nederfors *et al.*, 1994; Humphrey and Williamson, 2001). According to the source, natural saliva can either be whole mouth saliva (WMS) or glandular

saliva, separated from a single gland as will be explained in the following sections.

1.4.1.1 Human whole mouth saliva (HWMS)

Human whole mouth saliva (HWMS) will be referred to as whole mouth saliva (WMS) throughout this thesis. WMS is a complex mixture derived from all major salivary glands (parotid, submandibular and sublingual) as well as minor salivary glands (labial, buccal, palatal and von Ebner's glands at the tongue base) and gingival crevicular fluid (GCF) (Edgar, 1992). The varied composition and functions of WMS are summarised in Table 1. The complexity and diversity in WMS are attributed to different variables such as glandular source, stimulation nature (e.g. chemical or mechanical), diet, age, gender, health status, medications and time of day for collection (Humphery et al., 2001; Greabu *et al*., 2009; Edgar and Dawes, 2012). Due to such variations, collection of saliva for research and/or diagnosis should be standardised. In this regard, WMS can be collected at rest or as stimulated saliva. Different methods can be used for collecting resting saliva such as suctioning, swabbing with filterpapers, draining or spitting. The draining method can be achieved by allowing saliva to drip off the lower lip whereas the spitting method is where a person expectorates into a test tube (Navazesh and Christensen 1982; Navazesh 1993). Saliva can be stimulated by sucking an acidic candy or chewing a piece of gum (Turner and Sugiya, 2002; Jensdottir *et al*., 2005). Considering the high subject variation, other stimuli such as the gustatory response to flavoured food and citric acid as well as masticatory stimuli from tasteless parafilm have also been reported to cause an increase in the flow rate of saliva (Navazesh and Christensen 1982; Amerongen *et al*., 1987;

Stokes and Davies, 2007). Secretions of the parotid and submandibular/sublingual glands contribute to more than 90 % of the WMS (Payment *et al.*, 2001).

In a recent systematic review of the reported proteomic data, 4,833 proteins uniquely found in saliva were reported once or twice but a total of 1,515 proteins remained in the salivary proteome when using a stringent cutoff for inclusion of three independent experimental identification (Schweigel *et al*., 2016). Amongst these proteins, mucin5b and mucin7, high molecular salivary proteins, are the primary mucins present (Dawes, 2008). WMS contains serous and mucous secretions with similar proteins to that of parotid except for the presence of cystatins and mucins that are not expressed by the parotid glands (Jensen *et al.*, 1992; Proctor *et al.*, 2005). Also, basic proline-rich proteins (PRPs) appear to be exclusive to the parotid glands (Jensen *et al*., 1992; Carpenter, 2013). The majority of proteins in resting WMS are derived from submandibular and sublingual glands whereas stimulated WMS contains approximately two thirds parotid-derived proteins and one third from submandibular and sublingual glands (Amerongen *et al.*, 1987; Rantonen and Meurman, 1998; Carpenter, 2013). One important calcium binding salivary protein that is derived from parotid saliva is statherin. Statherin is found in higher levels in stimulated WMS and tends to decrease in resting saliva, whereas the opposite is the case for mucins (Rayment *et al*., 2001). Total protein and mucin5b have been found to be significantly higher in resting WMS as compared to stimulated WMS (Payment *et al*., 2001). The viscosity and rheological properties of the WMS is influenced by many factors including whether it is stimulated or not (Gittings *et al.*, 2015) and the interactions of high

molecular salivary mucins with small salivary proteins such as amylase, PRPs, statherins, and histatins (Iontcheva *et al.*, 1997).

WMS has a pH value of 6 to 7 with mean daily saliva production ranging from 0.5 to 0.6 litre. Although this varies from person to person, the normal resting salivary flow rate ranges between 0.1 to 0.35 mL/min with a mean of 0.3 mL/min, levels below 0.1 mL/min are regarded as a hyposalivation. Stimulated flow rate of WMS ranges from 3 to 7 mL/min and levels below 0.7 mL/min is regarded as hyposalivation (Humphery and Willaimson, 2001; Edgar *et al*., 2012).

Functions of WMS	Composition and conditions								
Cleansing: Clears food and aids swallowing	**Inorganic components**			**Organic components**			**Other variables**		
Lubrication: Mucins coat hard and soft tissue which helps alleviating mechanical, thermal and chemical irritation and tooth wear and assisting smooth air flow, shear resistance, speech and swallowing.		Unstimulated (mmol/L) (mM)	Stimulated (mmol/L) (mM)		Unstimulated (mg/L)	Stimulated (mg/L)		Unstimulated	Stimulated
Mineralization: Saliva is an ion reservoir that is saturated with calcium and phosphate. Salivary proteins facilitate remineralisation of the teeth and prevent precipitation of calcium phosphate salts.	Sodium	5.76	20.67	Total protein	1630	1200- 1600	Flow Rate	0.1- 0.4 (2) ml/min	1.5 – 7 ml/min (6,2)
	Potassium	19.47	13.62	IgA	76.1	40			
	Calcium	1.06-1.36 (1,2)	0.61- 1.2 (2,9)	Mucin5b	830	460			
	Magnesium	0.20	0.15	Mucin7	440	330	pH	6 - 7	6.5 - 7.6
	Chloride	16.40	18.09	Amylase (U/ml)	317	100 - 390			
	Bicarbonate	5.47	60.0 (3)	Albumin	10 - 51.2	30-190	Water	99.55 %	99.53%
				Statherin	56 – 215 (4,5)	-------			

***Table 1**: An overview of the general WMS functions and compositions at rest and after stimulation (after Edgar et al.,2012 with some modifications as referenced)*

1. **Anderson *et al*., (2001)**
2. **Jager *et al*., (2011)**
3. **Hara and Zero, (2014)**
4. **Hay *et al*.. (1984)**
5. **Ferguson (1999) in Shah *et al*., (2011)**
6. **Cheaib and Lussi (2013)**

Table continued

Table continued

Functions of WMS	Composition and conditions								
Buffer: CA6 helps to neutralise acidic food and drinks and raise the plaque pH reducing demineralisation effect.	**Inorganic components**			**Organic components**			**Other variables**		
Antimicrobial action: IgA, Lysozyme, Lactoferrin and Myeloperoxidase have anti-microbial mechanisms against oral microflora		Unstimulated (mmol/L) (mM)	Stimulated (mmol/L) (mM)		Unstimulated (mg/L)	Stimulated (mg/L)		Unstimulated	Stimulated
Agglutination: agglutinins in saliva aggregate bacteria, resulting in accelerated clearance of bacterial cells. Examples are mucins and parotid saliva glycoproteins	Phosphate	4.62- 5.96 (1)	2.70- 3.7 (2,7)	Lysozyme	28.9-200 (8)	23.5 – 100			
Pellicle formation: Thin (500 nm) protective diffusion barrier formed on enamel from salivary and other proteins	Thiocyanate	0.70	0.34	Lactoferrin	8.4-24 (8)	5.5			
	Iodide	------	0.0138	Lactoperoxidase	13 (8)	-----			
	Fluoride	0.00137	0.00116	PRPs	5-18 (9)	-----			
				CA VI	30 (10)	----			
				Cystatins	180 (11)	32.4			
				Histatins	25–304 (8,12)	0.22			
				Urea (mmol/L)	3.57	2.65			

Table 1: An overview of the general WMS functions and compositions at rest and after stimulation (after Edgar *et al*.,2012 with some modifications as referenced)

7. **Ferguson (1999) in Shah *et al*., (2011)**
8. **Svendsen *et al*., (2006)**
9. **Kousvelari *et al*.. (1980)**
10. **(Kohavi *et al*., 1997)**
11. **Shomers *et al*.. (1982)**
12. **Jensen *et al*.. (1994)**

1.4.1.2. Human parotid saliva (PS)

Human parotid saliva (PS) will be referred to as parotid saliva (PS) throughout this thesis. Parotid saliva is secreted by the serous acinar cells of the parotid glands and consists of predominantly small molecular proteins such as glycosylated PRPs, statherin and histatins (histidine-rich proteins) as well as amylase rich proteins (Jensen *et a*l., 1992; Veerman *et al*., 1996; Humphery and Willaimson, 2001; Carpenter, 2013). Such salivary proteins have been demonstrated to be the first proteins to adhere to the enamel components (Hay, 1973; Siqueira and Oppenheim, 2009). This is because these proteins possess phosphate groups, which attract calcium and phosphate ions to the enamel surface and may also be attributed to their small molecular weight. These proteins are characterised by having a high level of certain amino acids such as glutamate, glycine, aspartate, histidine and alanine that possibly contribute to the interaction of salivary proteins to enamel crystals to form the AEP.

1.4.2 Artificial saliva (AS)

For *in vitro* studies of erosion, WMS is commonly replaced by artificial saliva (AS), reducing the effects of acids by dilution and buffering mechanisms. WMS is often replaced due to issues relating to its collection, storage and degradation (Leung and Darvell, 1997; Schipper *et al.*, 2007; Hara *et al.*, 2008). Ideally, AS formulations should be able to simulate the lubrication, adhesion, remineralising and protective effects of WMS. Several studies have assessed the potential use of artificial formulations in remineralising a softened erosive lesion (Amaechi and Higham, 2001; Eisenburger *et al.*, 2001; Austin

et al., 2011; Ionta *et al.*, 2014; Batista *et al.*, 2016). There are still contradictory results amongst researchers whether AS can have a protective effect on the erosion process in *in vitro* studies. Batista *et al*. (2016) compared the effects of different AS formulations and *in vitro* WMS with *in-situ* WMS in reducing the dental erosion on bovine enamel (Batista *et al*., 2016). The authors subjected enamel and dentine specimens for microhardness and calcium analysis and demonstrated that the microhardness loss did not differ between the three groups (*in-situ*, *in vitro* WMS experiments and AS formulations) whereas calcium loss was significantly reduced in the *in-situ* experiment compared to AS formulations and *in vitro* WMS (Batista *et al.*, 2016). Table 2 summarises the four different formulations for the AS that has been commonly used in *in vitro* studies of erosive tooth wear.

The composition of the artificial saliva formula	**Authors**
2 mg/l $C_6H_8O_6$; 30 mg/l $C_6H_{12}O_6$; 580 mg/l NaCl; 170 mg/l $CaCl_2$; 1,270 mg/l KCl; 160 mg/l NaSCN; 330 mg/l KH_2PO_4; 200 mg/l CH_4N_2O; 340 mg/l Na_2HPO_4; 2,700 mg/l Mucin, 1,000 ml deionized water. pH 6.4	Klimek *et al*. (1982); Attin *et al*., 2000; Wiegand et al., 2008a; Magalhaes *et al*, 2012.
2,000 mg/l Methyl-p-hydroxybenzoate; 10,000 mg/l sodium carboxymethyl cellulose; 624.73 mg/l KCl,; 58.96 mg/l $MgCl_2{\cdot}6H_2O$; 166.13 mg/l $CaCl_2{\cdot}2H_2O$; 804.712 mg/l K_2HPO4; 326.620 mg/l KH_2PO_4; 0.022 mg/l fluoride, pH 6.75	Amaechi *et al*. (1998a and 1998b); Amaechi and Higham, (2001);
77.686 mg/l $CaCl_2$, 19.04 mg/l MgCl2, 544.360 mg/l KH_2PO_4, 2,236.50 mg/l KCl; 4,766.20 mg/l $C_8H_{18}N_2O_4S$ HEPES; pH 7.0	Eisenburger *et a*l., (2001a and 2001b); Fowler *et al*.,(2009); Austin *et al*., (2011)
11,182.50 mg/l KCl; 60.12 mg/l Ca(NO 3)2 4H $_2$ O; 0.066 mg/l NaF; 160.19 mg/l Na_2HPO_4 $2H_2O$; 12,114.00 $H_2NC(CH_2OH)3$ (TRIS); 1,000 ml deionized water; pH 7.0	Vieira *et al.*, (2005); Magalhaes *et al.*, 2010b; Barbosa et al, (2012); Kato *et al.,* (2012)

Table 2*: Artificial saliva formulations commonly used in in vitro erosive tooth wear.*

Klimek *et al*. (1982) reported the first AS formulation used for *in vitro* studies. This contained minerals added to glucose, ascorbic acid, mucin and urea and was used to cultivate an artificial salivary plaque (Klimek *et al.*, 1982). Other AS formulations have also been used. The use of AS has the advantage of consistent amounts of components as compared with the individual variability and difficulties in collection that are associated with WMS. However, the current commercially available AS formulations have only managed to mimic the inorganic and some of the rheological properties of WMS but still lacking the protein content (Tschoppe *et al.*, 2009).

A number of saliva substitutes have been developed and become commercially available for use in severe xerostemia conditions. These include Artisial which is widely used in France, Glandosane and Saliva medac which are commonly used in central Europe and Great Britain and Oralube in Australia (Meyer-Luecke *et al*, 2002). Although there have been controversial reports in the literature on the best recommended saliva substitutes, there is a general agreement that fluoridated saliva substitutes, which contain mucins as well as calcium and phosphates should ideally be recommended to patients with extensive xerostomia (Meyer-Lueckela and Tschoppe, 2010).

A number of previous *in vitro* erosion experiments have found that AS can achieve remineralisation (Amaechi and Higham, 2001; Eisenburger *et al.*, 2001; Meyer-Lueckel *et al.*, 2010; Ionta *et al.*, 2014). In addition, AS formulations with polymer and mineral contents in combination with fluoride gel or mouthrinses have been found to yield a reminerlising effect to eroded enamel (Urquhart and Fowler, 2005; Tschoppe *et al*., 2009; Meyer-Lueckela and Tschoppe, 2010). Not all AS formulations have positive effects on erosive

tooth wear as some formulations have been reported to have a negative effect. It has been demonstrated that the removal of the organic matrix from the AS may be detrimental for the remineralisation process and can significantly increase the progression rate of the dental erosion lesion (Hara *et al.*, 2005 and 2008). Other authors have shown that some of the remineralising solutions have a demineralising effect on enamel surfaces due to their low pH values (Tschoppe *et al.*, 2009; Meyer-Lueckel *et al.*, 2010). Furthermore, some studies have shown that the addition of mucin or carboxymethyl cellulose (CMC) into AS may cause a decrease in its remineralising effect (Hara *et al.*, 2008; Ionta *et al.,* 2014; Batista *et al.*, 2016). Hara *et al*. (2008) have related the reduction in the remineralising effect of the AS to the CMC component. Ionta *et al.* (2014) also reported that CMC could form complexes with calcium and/or phosphate ions, resulting in the unavailability of these ions for lesion rehardening. CMC increases the viscosity of AS, possibly decreasing the rate of diffusion of the minerals into the initial erosive lesion (Ionta *et al.*, 2014). Batista *et al*. (2016) found that AS containing CMC showed a lower protective effect after the second erosion cycle compared to the first cycle. It may be that the CMC component of the AS binds to the calcium and/or phosphate ions, reducing their availablability for remineralising (Batista *et al*., 2016). From reviewing the literature on the available AS formulations, there is, however some evidence that these formulations are still far away from WMS as an ideal substitute has not been formulated yet for two main reasons. First, the structure-function relationships requirements for WMS are quite complex. Also, there is still a lack of clear understanding of the function of all organic components of WMS in the oral cavity. Therefore, more work is needed to

improve the properties of AS formulations such as surface tension, viscoelasticity, shear and mucoadhesion to mimic that of WMS.

1.4.3 Structure and composition of AEP

Some of the WMS proteins contribute to the formation of a thin protein film on oral hard and soft tissues known as acquired pellicle. In this thesis, the term 'Acquired Enamel Pellicle (AEP) will be used to refer to the pellicle formed onto human enamel surfaces. The term AEP was first used to refer to the enamel pellicle by Dawes (1963). Previous proteomic studies have reported between 50-89 proteins in the AEP (Siqueira *et al.*, 2007; Lee *et al.,* 2013; Schweigel *et al*., 2016).

AEP is a thin biofilm layer free of bacteria formed onto tooth enamel surfaces (Ash *et al.*, 2014) by selective adsorption of proteins in WMS. It is composed of glycoproteins, proteins, lipids and several enzymes (Hannig *et al.*, 2005; Hannig and Joiner, 2006). Formation of AEP is believed to start moments after brushing teeth and equilibrium saturation reaches maximum after a time period between 30 minutes and 2 hour (Lamkin *et al*., 1996; Hannig and Balz, 1999; hannig, 1999; Ash *et al*., 2014). Other studies also observed continuous maturation of AEP at longer immersion periods ranging between 24 hour and several days (Amerongen *et al.,* 1987; Hannig *et al*., 1999; Hannig *et al*., 2004). Typically, the AEP layer is characterised by an electron dense basal layer ranging between 10 and 40 nm, and an outer, loosely arranged granular and globular layer (50–500 nm) where thickness depends on the site of the intra-oral location (Amaechi *et al.*, 1999; Hannig and Joiner, 2006). This may be influenced by which glandular source of saliva contributes more to the formation of AEP. The protein composition of *in vitro* formed AEP vary

between WMS and PS (Jensen *et al*., 1992; Ash *et al*., 2014). Ash *et al*, (2014) demonstrated the formation of a more viscous and diffuse AEP from WMS as opposed to the more elastic and compact AEP from PS which may be attributed to the different protein composition. They also suggested that AEP from WMS reached a plateau after 20 minutes adsorption whereas the proteins from PS continually adsorb to the surface even after 120 minutes (Ash *et al*., 2014).

Although 3000 different proteins have been identified in WMS proteom (Schweigel et al., 2016), only a few have been detected and well-studied in AEP (Yao *et al*., 2001; Li *et al*., 2003, Yao *et al*., 2003; Lussi, 2006; Hannig and Joiner, 2006; Siqueira et al., 2007, Lee *et al*., 2013). These include mucins (mucin5b and mucin7), albumin, amylase, CA VI, statherin, histatins, cystatins, PRPs, S100, lysozomes, lactoferrin and IgA (Schupbach *et al.*, 2001; Kosoric *at al.*, 2007; Cheaib and Lussi, 2011 and 2013; Algarni *et al*., 2015). These proteins are believed to have a high binding affinity to enamel and have been suggested to play an important protective function against enamel loss (Lamkin and Oppenheim, 1993; Lamkin *et al.*, 1996; Schupbach *et al.*, 2001; Hannig and Joiner *,* 2006). Mucin5b and mucin7 contribute substantially to the formation of AEP and they show selective binding to hydroxyapatite (Tabak *et al*., 1985; Al-Hashimi and Levine, 1989; Siqueira *et al*., 2007b). Mucins provide the AEP with its viscoelastic and gel-forming properties (Amerongen *et al*.,1987). Statherin is another main contributor to the formation of AEP (Li *et al.*, 2004; Xiao *et al*., 2015). Together with other small molecular proteins, statherin may initiate the formation of AEP, followed by cross-linking and

aggregation of a loose thick layer formed from large molecular proteins such as mucins (Hannig *et al.*, 2004; Siqueira *et al*., 2007; Yao *et al.*, 2001).

It has been found that the amino acid profiles of the 15-minutes AEP only contained traces of proline and arginine as compared to the greater thickness of 2 hour *in vivo* AEP which identified 78 natural peptides with molecular weights < 5 KDa containing 8–33 amino acid residues (Siqueira and Oppenheim, 2009). Enzymes such as transglutaminase, glucosyltransferases, amylase, CA VI, and fructosyltransferase, peroxidase and lysozyme have also been reported to be in the AEP (Hannig *et al.*, 2005). These salivary enzymes and proteins are synthesised in different glands and have different structural properties. Such differences depends on where they are synthesised as well as on the process of their biosynthesis. All salivary proteins are subject to constant modifications and alterations starting from their site of synthesis within the individual salivary glands to their final destination in the oral cavity. These processes include glycosylation, phosphorylation, acylation, deamination, sulfation and proteolysis (Helmerhorst and Oppenheim, 2007; Thomadaki *et al.*, 2011). For example, mucins are the most commonly glycosylated salivary proteins, whereas small molecular proteins such as statherin, cystatins and PRPs are non-glycosylated (Helmerhorst and Oppenheim, 2007). One interesting modification is the phosphorylation of statherin, cystatins S and SA-III, histatin and acidic PRPs at the amino acid serine residues which render them carrying a negative charge. This phenomenon is important for the homeostasis role of AEP since phosphorylation enhances the adherence of proteins to the enamel surface (Helmerhorst and Oppenheim, 2007). Helmerhorst and Oppenheim, (2007)

reported that salivary proteins of molecular weight less than 40 kDa are subjected to proteolytic activity before being secreted into the oral cavity (Helmerhorst and Oppenheim, 2007). Some individual salivary proteins have been found to be subjected to proteolytic activities in the oral cavity which tends to degrade them, compromising their function (Li *et al.*, 2004; Hannig *et al.,* 2005; Helmerhorst *et al*., 2006). Helmerhorst *et al*. (2006) reported that statherin, histatins, acidic PRPs and basic non-glycosylated PRPs were the most susceptible salivary proteins for proteolytic degradation (Helmerhorst *et al*., 2006).

Some researchers have looked into the modification of AEP structure with individual proteins to optimise the protective function of AEP against erosive tooth wear. Cheaib and Lussi (2011) combined casein and mucin to treat enamel samples before three citric acid cycles which resulted in a significant reduction in human enamel softening. The compositional differences of AEP are closely related to its protective role against erosive tooth wear. Carpenter *et al.* (2014) compared the levels of total proteins, statherin and calcium in the AEP between thirty participants with and thirty without erosive tooth wear. The total proteins concentration and amount of statherin in AEP collected from the erosive tooth wear group were significantly lower than that from the participants without erosive tooth wear. They also compared the amount of mucin5b and CA VI in unstimulated WMS between the individuals with and without erosive tooth wear. However due to the variability between subjects differences in mucin5b and CA VI were not found.

1.4.3.1 Differences between in vitro and in vivo AEP

Having outlined the general structure and composition of AEP, it is important to indicate that many studies have shown that *in vitro* AEP has a different composition compared with *in vivo* AEP (Carlén *et al.*, 1998; Lindh *et al.*, 2002, Yao *et al.*, 2001 and 2003). The unique features of the oral environment such as the dynamics of salivary flow, enzymatic activities, thickness of the AEP, mineral surface properties and health and age of patients may account for these differences between *in vitro* and *in vivo* AEP formation (Yao *et al.*, 2001, Hannig and Hannig, 2003). In addition, *in vitro* studies generally use ground and polished enamel surfaces which differ in enamel mineral content compared to the outer natural enamel layer (Ganss *et al*., 2000; Carvalho *et al.*, 2015). Calcium and phosphate ions of enamel crystals interact with the charged molecules of some salivary proteins which means that the type of enamel surface layer can influence the type of proteins adsorbed to the AEP (Hannig and Hannig, 2009). A limited number of studies have been carried out investigating the compositional differences of *in vivo* AEP. These studies investigated the protein composition of AEP formed on permanent teeth (Vitorino *et al.*, 2008; Siqueira and Oppenheim, 2009; Siqueira *et al*., 2012; Carpenter *at al*., 2014; Carvalho *at al*., 2016) as well as on deciduous teeth (Zimmerman *et al.*, 2013; Carvalho *at al*., 2016). These studies were limited by the large variations between subjects within each group making comparisons difficult. Examples of these variations include the inter-subject variability in saliva and tooth structure, and perhaps most importantly, regional differences in salivary proteins which may be caused by local topography and/or tribology.

1.4.3.2 Adsorption of AEP onto enamel surfaces

As indicated above, AEP is defined as the selective adsorption of proteins from WMS onto enamel surfaces. A number of studies have investigated the behaviour of AEP when adsorped to/desorbed from enamel surfaces using a variety of techniques including optical techniques such as ellipsometry and reflectometry (Lindh *et al.*, 1999; Kawasaki *et al.*, 2003). Protein adsorption/desorption to and from the enamel surfaces is an important aspect of studying and understanding the composition and formation of AEP. For example, hydrophobic surfaces have a high affinity to higher amounts of salivary proteins as compared to hydrophilic samples (Lindh *et al.*, 1999). The quality of adsorbed AEP depends on the type of underlying substrate to which AEP is attached with more proteins adsorbed to rough surfaces (Carlen *et al.*, 2001; Cárdenas *et al*., 2008). The adsorption of proteins onto oral structures and their structural changes after adsorption are also influenced by many chemical forces of different strengths and durability. These forces can either be long or short-term interaction forces. The long-term forces include Van der Waals forces and Coulomb forces. The short-term forces include hydrophobic interactions, electrostatic interactions, ionic interactions, covalent bonds, hydrogenic bonds and Lewis acid–base interactions (Vassilakos *et al.*, 1993).

1.4.4 Role of the AEP in erosion and erosive tooth wear

Despite there being a growing interest amongst researchers in the role of salivary proteins and AEP on enamel erosion and erosive tooth wear (Amaechi *et al.*, 1999, Wetton *et al.*, 2006 and 2007; Hellwig *et al.*, 2013), the literature is still limited in certain aspects. These include whether salivary proteins, ions or both offer the protection against erosion. Also, it is still unclear

what the roles of specific salivary proteins are against erosion and erosive tooth wear. AEP serves many functions against erosion and erosive tooth wear. It can act as a lubrication membrane on the soft and hard oral tissues. This property has mainly been attributed to the function of mucins (Amerongen and Veerman, 2002; Wickström and Svensäter, 2008; Siqueira *et al.*, 2012). It can act as a diffusion barrier, reducing the direct contact between acids and the tooth surface as well as protecting against abrasion and attrition (Carlen *et al.*, 1998; Hannig *et al.*, 2004; Vukosavljevic *et al.*, 2014). This property is often attributed to the mucin and albumin contents of AEP (Carpenter, 2013; Hemingway *et al.*, 2008). In this, the protons (H^+ ions) originating from the acidic source cannot reach the tooth surface if AEP is present unless protons diffuse through the AEP or, in the case of severe acid attack, after the removal of AEP (Hannig and Hannig, 2014). Interestingly, part of *in vivo* AEP remains in place even after severe erosive challenges indicating that some proteins remain in place which may have the potential to have anti-acid properties (Hannig *et al.*, 2006; Zimmerman *et al.*, 2013). Furthermore, AEP acts as a neutraliser of protons originating from acids. The enzyme CA VI found in AEP catalyses the reaction between the free hydrogen ions from the acid and the bicarbonate ions within the AEP (Leinonen *et al.*, 1999). This causes the pH at the enamel surface to rise and return to normal pH levels. AEP may also acts as a selectively permeable membrane which becomes supersaturated with calcium and phosphate ions, trafficking the passage of minerals such as calcium, phosphate, hydrogen bicarbonate and fluoride as well as delaying the dissolution rate of tooth minerals (Carlen *et al.*, 1998; Hannig *et al.*, 2004; White *et al.*, 2011). This notion supports the idea that AEP acts as a perm-

selective barrier of ions in and out of the enamel surface (Carlen *et al.*, 1998; Hannig *et al.*, 2005). Most importantly, AEP can act as a reservoir zone that is rich in minerals that can potentially remineralise the demineralised tooth tissue (Proctor *et al*., 2005). This property is often attributed to the small molecular proteins present in AEP such as statherin and PRPs. These proteins are suggested to adhere quickly and strongly to the enamel crystals (Hay *et al.*, 1979; Zimmerman *et al*., 2013). These proteins possess phosphate groups, which electrostatically interact with calcium and phosphate ions within the enamel surface. This potentially plays a role in the regulation of calcium phosphate homeostasis (Kosoric *et al*., 2007). For this reason, AEP is believed to act as a reservoir of fluoride compounds such as CaF at a low pH level which leads to the formation of fluorohydroxyapatite reducing enamel solubility (Tschoppe and Meyer-Lueckel, 2012; Kensche *et al.*, 2016). In addition, AEP has been reported to act as a network on the enamel surface allowing ingredients and particles of oral hygiene products to pass to the enamel surface (Kensche *et al.,* 2016).

A number of studies have claimed that the protein components of the AEP may be responsible for its protective role against enamel softening (Vissink, 1985; Kielbassa *et al*., 2005; Kirkman, 2007; Kosoric *et al.*, 2007; Cheaib and Lussi, 2011; Hellwig *et al.*, 2013; Moazzez *et al*., 2013; Carpenter *et al*., 2014). One study has confirmed that the protective effect of AEP against erosion is a combined function of both proteins and minerals (Martins *et al.*, 2013), whereas another recent study has demonstrated that salivary proteins depleted from ions provided the best protection against erosion (Baumann *et al.*, 2016). The role of salivary minerals without proteins in protection against

erosion has also been investigated by some studies using artificial saliva which has shown a hardening effect on enamel (Amaechi and Higham, 2001; Eisenburger *et al.*, 2001; Karlinsey *et al.*, 2012).

The protective effect of *in vitro* and *in vivo* AEP against erosion and erosive tooth wear is time dependant and thickness of AEP is an important factor (Amerongen *et al*.,1987; Featherstone *et al*., 1993). Amaechi *et al*. (1999) demonstrated that the degree of protection offered by the *in vivo* AEP is proportionally related to its thickness which varied within the dental arches. An investigation of the resistance of 6 hour *in-situ* AEP against 10 seconds of *ex-vivo* tooth brushing with toothpaste slurry demonstrated that AEP is reduced to 1–30 nm residual basal layer without any adhering globular layers (Hannig and Joiner, 2006). Interestingly, it has been shown that the basal structure of AEP survives relatively severe acid exposures and that brushing removes only a part of it (Hannig and Balz, 1999 and 2001; Joiner *et al.*, 2008).

1.4.5 Targeted proteins in the AEP:

The exact salivary components and mechanism of AEP responsible for its protective function against erosion and erosive tooth wear are not as yet known due to the complex interaction of many factors (Gibson and Beeley, 1994; Sreebny, 2000; Jager *et al.*, 2011; Hellwig *et al.*, 2013). There are however, specific salivary proteins that are believed to be the most abundant proteins in the AEP and substantially contribute to the AEP formation on enamel crystals (Ca´rdenas *et al.*, 2007; Hannig and Joiner, 2006, Siqueira *et al.*, 2012). These include mucins, amylase, human serum albumin, CA VI, statherin, histatins, cystatins and PRPs. The next section will review these four

individual salivary proteins that are believed to have different mechanisms of actions against erosion.

1.4.5.1 Mucins

Mucins are high molecular weights glycoproteins and more than 50 % of their weight is carbohydrates (4:1 carbohydrates to protein ratio) in the form of oligosaccharide chains linked to threonine and/or serine amino acids. In the literature, mucins have been suggested to form more between 7 to 27 % of the total prteins in WMS. Five human salivary mucins have been identified to date. These are mucin1, mucin4, mucin5b, mucin7 and mucin19 (Iontcheva *et al.*, 2000; Linden *et al.*,2008; Frenkel and Ribbeck, 2015). Salivary mucins are secreted from submandibular and sublingual glands and numerous minor salivary glands which are scattered all over the oral cavity (seormucus glands) (Shomers *et al.*, 1982; Amerogen *et al*., 1987 and 1995). These mucins share similar characteristics such as the O-glycosylation, glycosylated regions rich in repeats of the three amino acids: proline, threonine and serine (PTS) which were referred to as PTS regions (Shomers *et al.*, 1982; Feiler *et al.*, 2007). Mucins have the ability to concentrate other proteins at the enamel surface such as amylase, histatins and lysozyme which in turn increase their retention time. This helps mucins provide antibacterial activity and form a physically protective barrier on enamel surfaces against erosive tooth wear (Amerongen *et al.*,1995; Linden *et al*, 2008; Cheaib and Lussi,2011). In this thesis, mucin5b and mucin7 will only be reviewed as they are the key salivary mucins found in saliva and AEP (Ca´rdenas *et al.*, 2007; Wickstro¨m and Svensa¨ter, 2008; Gibbins *et al*., 2013; Frenkel and Ribbeck, 2015). Mucin5b and mucin7 are structurally distinct species of great importance in hydration and lubrication of

the oral cavity due to their molecular properties particularly carbohydrate portion of the molecule (Tabak, 1995).

I. Mucin5b:

Mucin5b, formerly known as MG1, is a multimeric, oligomeric, multifunctional, higher molecular weight protein which has gel-forming properties and may physically form a protective barrier from acids and pathogens (Lindh *et al*., 2002; Raynal *et al.*, 2003; Linden et al, 2008; Frenkel and Ribbeck, 2015). Mucin5b has a molecular weight of about 1350 KDa (Vitorino *et al.*, 2007) and encoded by the MUC5B gene (Nielsen *et al.*, 1997; Wickstro¨m *et al.*, 1998), producing unique, large and diverse oligosaccharides units. It contains 15 % protein, 78 % carbohydrate, and 7 % sulfate (Iontcheva *et al.*, 2000) and is organized into cysteine-rich N- and C-terminal regions with a central tandem-repeat domain. The N-terminal region contains 1000 amino acids while the C-terminal region contains 805 amino acids (Offner *et al.*, 1998). The central tandem-repeat region is composed of 3570 amino acids (Desseyn *et al.*, 1997), contains two serine and threonine- rich non-tandem-repeat subdomains, seven cysteine-rich subdomains, and five tandem-repeat subdomains containing from 5 to 10 imperfect 29-amino-acid-residue repeats (Iontcheva *et al*., 2000).

Mucin5b was detected in 2 hour *in vivo* AEP (Al-Hashimi and Levine, 1989). It serves many functions in the oral cavity. These include maintaining oral mucosa integrity and contributing to the AEP structure through selective binding to hydroxyapatite and enamel surfaces (Hannig *et al*., 2005). Mucin5b is heavily glycosylated and has gel-forming properties, therefore, it contributes substantially to the lubrication and physical barrier properties of AEP

(Wickstro¨m and Svensa¨ter, 2008; Siqueira *et al.*, 2012). It also acts as a source of nutrients for microbes as well as a retention site for other protective salivary proteins (Iontcheva *et al.*, 1997; Wickstro¨m and Svensa¨ter, 2008).

II. Mucin7:

Mucin7 has a smaller molecular weight (200 to 250 KDa) than that of mucin5b (Tabak, 1990). Mucin7 is a small, monomeric protein of 357-amino-acid backbone that is exclusively found in salivary secretions and is encoded by the MUC7 gene (Bobek *et al.*, 1993). Mucin7 is unable to form a polymer due to the absence of a terminal cysteine rich domain in its structure (Frenkel and Ribbeck, 2015). It is believed that mucin7 serves many oral functions. It is present in concentrated levels creating a protective immune reservoir within the bound mucosal pellicle (Wickström and Svensäter, 2008; Gibbins *et al.*, 2014) and could also further aid in the immune functions of the AEP by forming a heterotypic complex with IgA and lactoferrin (Biesbrock *et al*., 1991; Soares *et al.*, 2012 in Gibbins *et al*., 2013). Furthermore, mucin7 agglutinate various oral bacterial species, facilitating their removal (Frenkel and Ribbeck, 2015).

1.4.5.2 Human Serum Albumin

Gingival crevicular fluid is the major source of salivary albumin where albumin enters the oral cavity from the bloodstream (Rantonen and Meurman, 2000). With regard to the AEP, human serum albumin has been found to have a high affinity to hydroxyapatite (Rathman *et al.*, 1989) and is abundant in 60 minutes formed AEP on hydroxyapatite (Carlen *et al*., 1998). The role of human serum albumin in the oral cavity is still unclear but it has been suggested that it substantially contributes to the protective properties of the AEP against erosive demineralisation of the enamel surface (Jager *et al*., 2011). It has also been

suggested that human serum albumin tends to change its structure leading to a strong affinity to adhere to hydroxyapatite at pH 6.0 but this binding tends to decrease at higher pH value and it also contributes to the physical barrier property of AEP, reducing the dissolution rate of hydroxyapatite in citric acid (Rathman *et al.*, 1989; Hemingway *et al*., 2008; Rabe *et al*., 2011). In addition, albumin is also believed to bind to calcium ions in the enamel crystals (Hemingway *et al.*, 2008) but such affinity for hydroxyapatite was reported to be low (Carlen *et al*., 1998). Human serum albumin can be detected reliably with western blot technique. The expected band size on gel is based entirely on amino acid sequence or the size of the protein. However, there are other factors which may influence the observed size of the protein in an actual western blot. These include protein post translation modification such as post translational cleavage where a larger pro-form of the protein is cleaved into a smaller active form which decreases its size in the gel or post-translational modification where a protein becomes glycosylated (N or O linked sites), phosphorylated or ubiquitinated increasing its size in the gel. The second factor could be the overall net protein charge determined by the amino acid composition which may affect the migration speed through the negatively charged (Sodium dodecyl sulfate) SDS of the gel. Thirdly, multimeric proteins can be formed as a result of trimerization or dimerization but the use of reducing conditions such as DL-Dithiothreitol (DTT) will help to eliminate these interactions (Chen, 1967; Sigma, 2017).

Human serum albumin undergoes three different post-translational modifications; oxidation, glycation, and S-nitrosylation. Modifications usually occur on the surface of the globular protein, and do not significantly affect

conformation. However, modification strongly affects binding of fatty acids and drug molecules.

Fatty acid free human serum albumin are used to solubilise lipids in tissue culture due to their free hydrophobic region. They are also used as blocking agents in western blots or enzyme-linked immunosorbent assay (ELISA) applications. Globulin free albumins are suitable for use in applications where no other proteins should be present (Chen, 1967; Sigma, 2017).

1.4.5.3 Carbonic anhydrase VI (CA VI)

From human saliva, CA VI enzyme was first purified by Murakami and Sly (1987). As was demonstrated by immunohistochemical studies, it is secreted from the serous acinar cells of the parotid and submandibular glands (Parkkila *et* al., 1990 in Kivelä *et al*., 1999). CA VI, known as gustin or salivary CA VI, is a 42 KDa zinc-metalloprotein that constitutes 3 % of the parotid saliva proteins (Thatcher *et al*., 1998). This human salivary enzyme was found to be larger than the cytosolic isoenzymes CA I, CA II, and CA III (Mr 29 KDa) which are derived from human tissue sources (Murakami and Sly, 1987). CA VI is encoded by a gene located on chromosome 1 and structurally has three potential N-linked glycosylation sites and two cysteine residues (Cys25 and Cys207) (Murakami and Sly, 1987; Aldred *et al.*, 1991). Cys207 was reported to form a disulphide bond (Fernley *et al*.,1988 in Kivela *et al.*, 1999). CA VI molecule has two complex N-linked oligosaccharide chains but has no O-linked oligosaccharides which is sialic acid residue. This was confirmed by the ability of endo-β-N-acetylglucosaminidase F to cleave CA VI and the cleaved protein is not affected by Neuraminidase (Murakami and Sly, 1987).

In terms of its function in the oral cavity, CA VI has been identified in the WMS (Vitorino et al., 2004) and is involved in the saliva buffer system in the oral cavity (Kivelä *et al.*, 1999; Cheaib and Lussi, 2011). To do this, CA VI catalyses the reversible reaction of CO_2 and H_2O to HCO_3 and H^+ which helps maintain a high bicarbonate level in saliva. CA IV was detected in AEP using immunostaining antibody techniques formed *in-situ* (Leinonen *et al*., 1999) and proteomic analysis (Delecrode et al, 2015). It is believed that CA VI influences the saliva buffer system and oral homeostasis (Van Nieuw Amerongen *et al.*, 2004; Dawes, 2008). It is also of interest to mention that CA VI has been reported to be the oral enzyme with the highest turnover rate (3.6/ min) (Hannig *et al*., 2005). In a previous study (Algarni *et al.*, 2015), CA VI has also been found in higher relative abundance on bovine AEP after immersing in fluoride and stannous for 2 minute followed by 2 hour in WMS. This may have some impact on erosion prevention via acid neutralisation (Kimoto *et al.*, 2006) even though CA VI level was not significantly different in the WMS of participants with erosive tooth wear as compared to healthy subjects (Zwier *et al.*, 2013; Carpenter *et al.*, 2014).

1.4.5.4 Statherin

The word statherin is originated from the Greek word ‘‘statheropio’’ which means to stablise’’ (Li *et al.*, 2004). Statherin is a 43-residue phosphopeptide of unique composition with a high degree of structural and charge asymmetry, secreted mainly from human parotid salivary glands but also present in submandibular and sublingual saliva (Hay, 1973; Schlesinger and Hay, 1977; Hay *et al.*, 1989; Li *et al.*, 2004). Tyrosine, glutamine, and proline amino acids are dominant components in the structure of statherin with one amino acid

lysine (Schlesinger and Hay, 1977; Sevendsen *et al.*, 2008). One third of the statherin molecule is an N-terminal hydrophilic, phosphorylated head which carries most of its charge (10 of the 12 charges) on 13 amino acids. The C-terminal tail is hydrophobic and constitutes two thirds of the statherin structure (Hay and Moreno, 1989). These structural differences in statherin give it the amphipathic property. The affinity of statherin to hydroxyapatite is attributed to the negatively charged phosphorylated serines (Johnsson *et al.*, 1993). The interaction of statherin with the enamel surface can also be attributed to the binding energy of the amino acid arginine particularly noted for exhibiting the strongest hydrogen bond and strongest van der Waals interaction (Makrodimitris *et al.*, 2007). This interaction take place between the amino acid and Ca^{2+} of the enamel surface crystals (Furedi-Milhofer *et al.*, 1994). These ions would be strongly attached to the surface, and the energy required to remove/hydrate these ions would be higher than if the peptide is absent (Christoffersen and Christoffersen, 1981). Some of the available Ca^{2+} may form complexes with protein, and only the ‘‘free’’ Ca^{2+} is able to influence the demineralisation process (Anderson *et al.*, 2001).

The concentration of statherin in saliva is varied between individuals and different ranges have been reported by different authors. Hay *et al.* (1984) reported a statherin range between 16–147 mg/L (30 umol/L) whereas a range of 9-233 mg/L (Jensen *et al.*, 1994), 17.7 to 208.2 mg/L (Li *et al.*, 2004) and 54-256 mg/L (Shah *et al.*, 2011) have also been reported.

Statherin has been found to be abundant in AEP (Hay *et al.*, 1984, Hay and Moreno, 1989; Schupbach *et al*., 2001; Hannig *et al.*, 2004; Proctor *et al.*, 2005) as well as bonded with calcium around enamel surfaces which may help

modify the rate of enamel dissolution and remineralisation (Hay and Bowen, 1999; Nieuw Amerongen, 2004; Li *et al.*, 2004; Proctor *et al.*, 2005). Statherin is a multifunctional molecule that possesses a high affinity for calcium phosphate minerals which allows it to act as a precursor of AEP. In the oral cavity, statherin serves many oral functions. It is believed that statherin controls the homeostasis of Ca^{2+} in the oral environment (Kosoric *et al.*, 2007; Xiao *et al.*, 2015). It also biologically functions to inhibit the spontaneous precipitation of calcium phosphate on tooth surfaces as well as the growth of calcium phosphate minerals of enamel crystal from supersaturated solutions of calcium phosphate minerals. The N terminus of statherin is highly charged, the glutamic acids of which have been shown to be important in the recognition of hydroxyapatite (Raj *et al.*, 1992). Moreover, statherin and its C-terminal fragments inhibit the growth of anaerobic bacteria from the oral cavity (Kochanska *et al.*, 2000). Furthermore, while statherin functions as a boundary lubricant on the enamel surface, it has been found that statherin promotes selective initial bacterial colonization such as Actinomyces viscosus and Fusobacterium nucleatum determining the initial microbial colonisation of tooth surfaces (Hay, 1983; Gibbons and Hay, 1988; Hay and Moreno, 1989; Douglas *et al.*, 1991; Xie *et al*, 1991). In addition, statherin may function in the transport of calcium and phosphate during secretion in the salivary glands (Schlesinger and Hay, 1977; Bennick *et al*., 1981). Statherin, along with other small molecular proteins such as acidic proline-rich proteins (PRPs), histidine-rich polypeptides (histatins), and cystatins in order to maintain the supersaturated state of saliva with respect to enamel crystal. This process is important in enamel remineralisation as it contributes to the recalcification and

stabilisation of the tooth enamel as well as inhibit the formation of mineral accretions.

1.4.6 Variables in the use of WMS for *in vitro* AEP formation:

In vitro AEP for erosion studies are commonly formed using collected saliva that is frozen and subsequently thawed (Hall *et al.*, 1999; Hellwig *et al.*, 2013). Saliva collection methods should ideally be standardised owing to the variability in the components of saliva between individuals and time of collection, however this standardisation is lacking in the literature at present (Schipper *et al*., 2007).

1.4.6.1 Fresh versus frozen WMS

The collection and use of fresh WMS has been reported in some studies (Hall *et al.*, 1999; Wetton *et al.*, 2007; Faller *et al.*, 2011; Batista *et al.*, 2016). Collection of fresh WMS on a daily basis is not always practical (Wetton *et al.*, 2006; Shellis *et al.*, 2011). A number of other studies have used protocols for collection of WMS followed by immediately freezing at -80 °C and allowing it to thaw prior to use (Nekrashevych and Stösser, 2003; Creanor *et al.*, 2011; Wang *et al.*, 2011; Brevik *et al.*, 2013; Hellwig *et al.*, 2013). One study investigated the protective effect of fresh and frozen WMS against erosion, and found that AEP formed from fresh WMS did not show a significant difference in protection against erosion compared to AEP formed from frozen WMS (Hemingway *et al*., 2010). In this regard, when collecting WMS, short-term storage of samples on ice is recommended (Thomadaki *et al.*, 2011) whereas freezing at −80°C is better preferred for long-term storage (Schipper *et al.*, 2007).

1.4.6.2 The length of the AEP formation

There is no consensus in the literature on the length of time needed for formation of *in vitro* and/or *in vivo* protective AEP against erosion. Some studies suggest that 60 minutes of AEP formation can offer maximum protection against erosion with no improved protection at longer immersion times (Amaechi *et al.*, 1999; Wetton *et al.*, 2006; Hannig *et al.*, 2003). Hannig *et al*, (2004) showed that 3 minutes *in-situ* AEP formation was comparable to a protective AEP formed after 2 hour in *in- situ* (Hannig *et al.,* 2004). Long immersion periods have also been studied and one study demonstrated that 24 hour of AEP formation showed although the same protective effect as 7 days, AEP formed over a shorter period is less resistant to dissolution (Hannig *et al.*, 1999). This contrasts with two other studies which suggested that AEP formed after several days provided greater protection against demineralisation (Amerongen *et al.*, 1987; Hannig *et al*., 2004). Amerongen *et al*. (1987) reported that protection against erosion was improved with AEP formation time up to 3 days (Amerongen *et al.*, 1987). Amerongen *et al*. (1987) investigated the protective role of mucins against 1% citric acid erosion for 1 minute by comparing WMS, PS, mixed sublingual and submandibular (SL-SM), SL-SM without mucins and isolated human whole salivary mucins (HWSM). They concluded that HWSM provided better protection than WMS and PS. WMS showed better protection than PS with full protection being achieved after 3 days of incubation. Featherstone *et al*. (1993) also observed a linear relationship between increasing time of AEP formation up to 7 days and reduction in mineral loss (Featherstone *et a*l., 1993). Featherstone *et al*. (1993) compared the protective role of clarified (centrifuged), dialysed and fractioned

WMS against acid resistance to human enamel and concluded the importance of a combined specific non-dialysable salivary proteins (>3500 MW) and lipids in such protection. Some laboratory models used long-term cycling procedures immersing enamel specimens in WMS over days during which WMS was changed daily (Hall *et al*., 1999; Wetton *et al*., 2007; Hara *et al.*, 2008; Cheaib and Lussi, 2011; Faller *et al.*, 2011; Karlinsey *et al.*, 2012). When not in a cycle enamel specimens were stored under different conditions. In other studies, the laboratory cycling procedures took one day (Eisenburger *et al.*, 2001; Nekrashevych and Stosser, 2003; Wetton *et al*., 2006; Creanor *et al.*, 2011; Hellwig *et al*., 2013; Brevik *et al*., 2013). It would be more practical if *in vitro* salivary-erosion experiments are conducted over short periods since teeth in the oral cavity tend to be exposed to saliva and acid within a short span of time.

1.4.6.3 Multiple versus single AEP formation

In terms of the number of times that enamel specimens are immersed in WMS, some studies have immersed specimens in the collected WMS for only one single time within the test interval (Nekrashevych and Stösser, 2003; Creanor *et al.*, 2011; Brevik *et al*., 2013), whereas others have used various protocols for multiple immersion times of specimens in WMS as shown in Table 3.

Authors	Length of AEP formation (minutes)	Number of exposure per day	Length of experiment (days)	Interim storage If applicable
Hall *et al.*, (1999)	5	2	14	Saliva changed daily
Wetton *et al*., (2006)	2 60 120 240	12	1	Not applicable
Wetton *et al.*, (2007)	120	2	6	Stored on damp tissue paper in a sealed jar
Hara *et al.*, (2008)	30	3	3	Not specified
Creanor *et al.,* 2011	1 10	6	6	Artificial saliva
Cheaib and Lussi, (2011)	120	1	3	Stored in a humidity chamber at 4°C
Faller *et al.,* (2011)	110	4	5	Stored in pooled saliva (gently stirred).
Hellwig *et al*., (2013)	2	3	1	Stored in saliva for 8 hour
Batista *et al.*, (2016)	120	1	1	Not applicable
Baumann *et al.*, (2016)	60	4	1	Not applicable

Table3: *Summary of some AEP formation protocols*

1.4.6.4. Rinsing in deionised water (DW) after WMS exposure

In some erosion studies the specimens were only rinsed in deionised water (DW) after a complete experiment which can be several cycles (Hannig and Balz, 1999; Nekrashevch and Stösser, 2003; Jager *et al*., 2011), whereas other studies reported that rinsing the specimens with DW took place after each cycle of immersion in both WMS and acid (Wetton et al 2006 and 2007;

Cheaib and Lussi, 2011; Hellwig *et al.*, 2013). The latter protocol is advantageous as rinsing with water at the end of each erosion cycle would help remove any residual saliva or acid from the specimens. The lack of standardisation on rinsing after WMS exposure makes it difficult to compare studies.

1.4.6.5 Stimulated versus unstimulated WMS

Some studies have used stimulated saliva (Hall *et al.*, 1999; Amaechi and Higham, 2001; Schupbach *et al*., 2001; Hara *et al*., 2008; Bruvo *et al*., 2009; Creanor *et al*., 2011; Hellwig *et al.*, 2013) whereas other studies used unstimulated saliva (Wetton *et al*., 2006 and 2007; Jager *et al.*, 2011; Zwier *et al.*, 2013). The properties of saliva are influenced by whether it is stimulated or not (Humphery and Willaimson, 2001; Carpenter, 2013) which makes standardisation crucial if comparison between studies is to be performed.

1.4.6.6 Time of WMS collection

There have been disagreements between studies on the time of WMS collection and different studies have collected WMS at various times of the day. For example, in one study saliva was regularly collected at 15:30–15:40 p.m. (Brevik *et al.*, 2013) whereas others have reported early morning collection between 8.30 am to 11 am (Wetton *et al*., 2007). It is important that WMS is collected at the same time for all study participants. However, sometimes this cannot be achieved due to practical reasons.

1.4.6.7 Pooled versus individual WMS

The WMS used for the *in vitro* AEP formation can either be from one individual or pooled from different individuals. Again there is variation in the literature

and some studies used WMS collected from the same person (Wetton *et al.*, 2007) whereas others have used pooled saliva collected from different participants (Faller *et al.*, 2011; Creanor *et al.*, 2011; Hellwig *et al.*, 2013). The advantage of using pooled natural saliva is that saliva from only one donor could lead to bias due to variabilities within each individual.

1.4.7 Background on harvesting *in vivo* AEP

Different methods have been reported for *in vivo* AEP collection. For studying the role of an *in vivo* AEP against erosive tooth wear, AEP collection techniques and methods are also crucial. There are some difficulties that can be encountered when harvesting *in vivo* AEP. One difficulty could be the surface area of each sampling site that needs to be uniform and standardised. Sampling a larger surface area would invariably lead to a greater amount of AEP collected and greater amount of protein. Without controlling for this variable, it would be impossible to accurately compare the AEP composition between individuals or on tooth surfaces within same individuals without possible bias. Another difficulty can be the small amounts of proteins that can be collected from the tooth surfaces. Another obstacle can be the collection accuracy of AEP as a separate entity from salivary film. For solving these problems, many collection techniques have been adopted and applied. Sönju *et al*. (1997) introduced the first method for *in vivo* AEP collection by mechanically scaling the tooth surfaces (Sønju *et al.*, 1997). The same group of researchers used glass wool connected to a suction device to collect *in vivo* AEP (Sönju and Rölla, 1973). Alternatively, a hydrophilic polyvinylidene difluoride (PVDF) membrane and scaling technique were found to be more effective in eluting *in vivo* pellicle (Al-Hashimi and Levine, 1989; Sønju *et al.*,

1997; Yao *et al.*, 2001). Also, filterpapers, Whatman paper and/or polyurethane sponges soaked in 2% SDS have been used to collect *in vivo* AEP by mechanically scrubbing tooth surfaces (Embery *et al.*, 1986; Carlen *et al.*, 1998; Hannig *et al.*, 2005). Furthermore, a new technique was developed using polyvinylidene fluoride (PVDF) membranes held with cotton pliers (Yao *et al.*, 2001) and was found to be efficient and welcomed by the subjects compared with mechanical scaling (Lendenmann *et al.*, 2000). A more recent study by Svendsen *et al*. (2008) has used filter pellets soaked in SDS of different concentrations to collect *in vivo* AEP from the coronal two thirds of all buccal surfaces of the upper and lower teeth, excluding restorations (Svendsen *et al*., 2008). A combined use of mechanical rubbing with chemical surfactants as well as pumicing teeth surfaces before rinsing teeth with water, then isolating teeth with cotton rolls have been reported to improve the efficiency of AEP removal and collection (Yao *et al*., 2001; Li *et al.*, 2004; Hannig *et al*., 2005). In addition to SDS, other chemical agents such as sodium hypochlorite and sodium phosphate buffer have been reported to be used for AEP removal (Hay, 1967; Hannig and Balz, 1999; Hannig *et al.*, 2005).

Each method of AEP collection has advantages and disadvantages. For example, using mechanical rubbing alone can only partially remove the outer globular layer of the AEP but not the basal layer (Hannig *et al.*, 2005). In the same way, using only chemical means does not completely remove the AEP from the enamel surfaces (Hannig *et al.*, 2005). The advantage of soaking papers in SDS when collecting AEP is that SDS, a negatively charged detergent, is expected to form complexes with the proteins which are readily adsorbed to the negatively charged enamel surfaces (Arnebrant and

Simonsson, 1991). This property allows the SDS-protein complexes to electrostatically be repelled away from the enamel surfaces (Svendsen *et al*., 2008). However, SDS should only be used at a critical concentration (0.5 %) to avoid interference with the SDS buffer in the 2D gel during protein separation (Svendsen *et al.*, 2008).

In conclusion, an important factor when choosing a collection method for AEP is that it should yield a complete removal of AEP from the tooth surfaces and complete recovery of the AEP from the collection means such as filterpapers. This can only be accomplished by mechanically-assisted chemical treatments.

1.5 Measurements of *in vitro* erosive tooth wear

As indicated in section 1.3.2, the process of erosive tooth wear involves two stages which can be a reversible softening of the dental surfaces or irreversible tissue loss. Therefore, the currently available *in vitro* techniques for tooth wear measurements fall into two categories: quantitative and qualitative techniques. Examples of quantitative techniques are surface profilometry, microhardness, atomic force microscopy, microradiography and whereas qualitative methods include scanning electron microscopy, optical coherence and atomic force microscopy. Based on the literature review, there is not a single ideal technique that can be used for assessing *in vitro* erosive wear (Schlüter *et al.*, 2011). This section will provide a summary of the literature review on the most commonly used *in vitro* techniques for assessing erosive wear.

1.5.1 Surface profilometry

Surface profilometry is a measurement tool used to assess the surface profile and morphology of worn dental surfaces by tracking the worn areas against reference areas from different angles. Surface profilometry measurement can be achieved with surface non-contacting profilometry (SNCP) or a contact stylus profiometry (CSP) (Attin *et al*., 2009). Both types are composed of a detector to collect the data points reflected from the specimen surface and a stage where the specimen is held in place during the measurement process. Contact stylus profilometers were the first surface measurement techniques to be applied to tooth wear research and the technique is still widely used by some groups. CSP typically consists of a stylus made of metal or diamond (20–100 μm) that physically contacts the surface being measured at a rate of around 10 mm/min with loaded force of few millinewtons (Rodriguez *et al*. 2009; Schlueter *et al*., 2011). CSP is slower than SNCP as it comes into contact with the specimen surface whilst scanning, which in turn can pose the disadvantage of damaging the specimen surface (Rodriguez *et al.,* 2009).

1.5.1.1 Surface non-contact Profilometry (SNCP)

SNCP is considered the 'gold standard' for *in vitro* studies (Paepegaey *et al*., 2013) detecting the loss of dental hard tissues (step height) between an intact reference area and an experimental area that has been subjected to wear (Schlueter *et al.*, 2005; Paepegaey *et al*., 2013). When compared to CSP, SNCP is often preferred in dental erosion measurements due to its greater accuracy which is attributed to the diameter of its measuring tip. CSP has a stylus radius of 2 μm whereas SNCP has a sensor radius of 0.2 μm (Schlüter *et al.*, 2011).

The SNCP uses a laser light of 7 µm spot size emitted from its source and directed onto the specimen surface to scan over the reference and eroded area of an enamel specimens with 3 mm X 3 mm X/y area. The principles on which the SNCP works is summarised in (Figure 1). A light source produces laser light that is amplified by stimulated emission of radiation and is monochromatic. Thi laser light is then transmitted through an optic cable to a chromatic lens in the sensor head which disperses the white light into different wavelengths before hitting the exposed specimens at different distances from the lens. On the targeted surface, areas on the specimen that are closer to the sensor are exposed to the blue end of the spectrum of chromatic focus points and those further from the sensor are exposed to the red end of the spectrum (Austin *et al*., 2011).

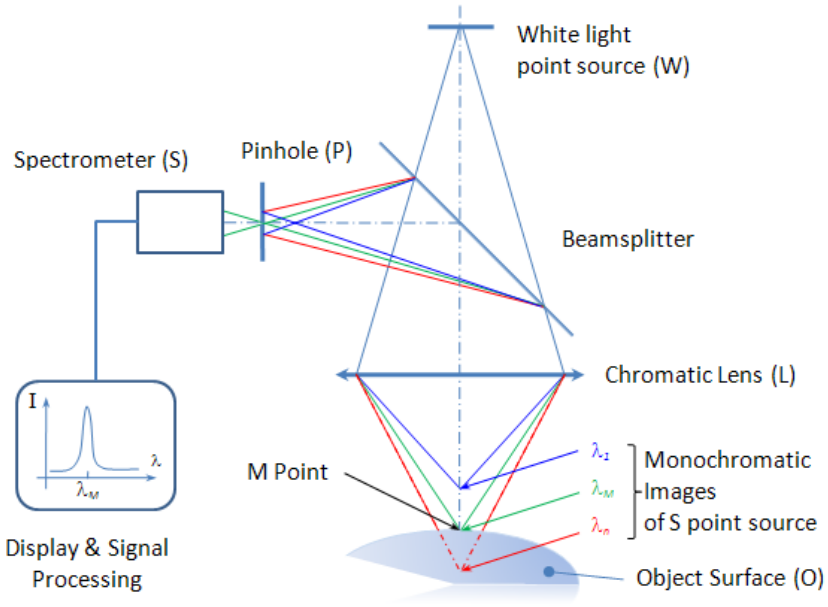


Figure 1: *Schematic representation of the SNCP measurements (after Austin, 2011; Mistry, 2016)*

The light reflected back from a surface becomes more focused and monochromatic rather than polychromatic which can only be used for a

measurement. This focused light returns back through an optical cable to a spectrometer which contains a charge coupled device (CCD) sensor. The reflected light is then analysed in the spectrometer by detecting the position of spectral changes of the reflected light received on the CCD which is correspondent to the wavelength of the reflected light, allowing accurate measurements of distances and heights of a surface. Data analysis is performed using Taicaan XYRIS (Boddies).

Using SNCP, the amount of tissue loss is quantified as the height from the reference area to the bottom of the worn area and is measured by extracting single line step height profiles using surface analysis software. Three software packages are available for analysing the data: Proscan 2000 (proscan application software v2.0.17), MicroProf (Mark III) and Taicaan XYRIS (Boddies). To increase accuracy, mean step height for the entire worn area can be calculated from a series of single line profiles. SNCP cannot measure the small surfaces, subsurface demineralisation or surface softening of erosive lesions. Additionally, its accuracy is affected by colour and transparency (Rodriguez and Bartlett, 2011).

There are advantages and disadvantages for using the SNCP in the profilomtric measurements of dental tissue loss. SNCP can provide a better full 3D representation of the entire specimen on dental tissue as well as volume change utilising the imaging software (Paepegaey *et al.*, 2013). It can also provide information about the surface roughness, volume loss or gain and waviness. Roughness describes the finest measureable detail of the surface texture with roughness average (Ra) as the most common parameter used to express roughness (Austin *et al.*, 2011).

SNCP has the disadvantage of requiring sufficient surface loss as otherwise the amount of tissue loss cannot be detected by the profilometer. This makes profilometry unsuitable for detection of early stages of erosion (Hara and Zero, 2008). Scanning the entire surface also takes more time. In addition, the presence of cracks or defects in the specimen surface can undermine the profilomteric measurments therefore detailed flattening of the specimens surface is an essential criterion for the SNCP measurements (Attin *et al.*, 2009). Acceptable lowest range of surface profile measurements are within 0.3-0.5 µm at 2 different regions (Attin, 2006). Detection of losses below 1 µm is generally difficult (Attin, 2006); only a surface loss of at least 1 µm can be reliably detected (Attin, 2006).

1.5.2 Hardness testing:

Hardness is defined as the resistance of a material to permanent indentation caused by a diamond indenter loaded at a known force and duration. The two types of hardness tests used commonly reported as used in erosive wear studies are nanoindentation and microhardness.

1.5.2.1 Microhardness

Microhardness tests have been reported in previous studies of dental erosion (Meredith *et al.*, 1996; Hannig and Balz, 1999; Hara *et al.*, 2006; Joiner *et al.*, 2008; Cardoso *et al.*, 2009; Hellwig *et al.*, 2013). The hardware of the microhardness machine consists of a stage, diamond indenter, variable loads, timer and a micrometer for measurement of the indentation (x500) and a microscope to view the specimen. Microhardness testing, like nanoindentation, is used to measure the mechanical properties of the enamel

surface before and after an erosive challenge but on a larger scale. This gives information about the resistance of the softened substrate to the penetration of the indenter. The nanoindenter is less intrusive (150-500 nm), detecting very early erosion whereas the measurement of microhardness reach micrometres (1.5 µm and 5 µm). Despite this difference, both hardness tests, like chemical analyses, are suitable for measuring early erosive wear (Schlueter *et al.*, 2011, Jager *et al*., 2012).

A Knoop (Lussi *et al.*, 1995; Lussi and Hellwig, 2001; Cheaib and Lussi, 2011; Hellwig *et al.*, 2013; Baumann *et al.*, 2015; Carvalho *et al.*, 2016) or a Vickers diamond indenter (Attin *et al.*, 1997; Baldassarri *et al.*, 2008; Baumann *et al.*, 2016) which are rhomboidal and tetra pyramidal, respectively are often used giving either a Knoop hardness number (KHN) or Vickers hardness number (VHN). The change in indent length (Δl) is determined for each specimen and the change in indent depth (Δd) is calculated from the equation: $\Delta d = 0.032772\ \Delta L$. The hardness values are calculated from the length of the indentation and the applied load. The hardness values obtained are useful indicators of a material's properties and expected service behaviour. According to the American Society for the Testing of Materials (ASTM) the Knoop Hardness Number (KHN) can be calculated using the following formula:

$$\mathrm{KHN} = \frac{\mathrm{F}}{\mathrm{C_P\ L^2}}$$

Where F is the load in kg, L is the length of the long diagonal in mm and C_P is a constant (0.070279). There are some concerns with using surface microhardness as testing method in erosive tooth wear. The technique requires a smooth, planar surface which may destroy the specimen's structure. This is important as the hardness of enamel surface decreases away from the

surface regardless of the erosive challenge (Carvalho and Lussi, 2015). This is particularily important when the prepared enamel surface is exposed to an erosive challenge as softened enamel surface plateaus at certain erosive levels where reading microhardness values becomes inconclusive (Barbour *et al.*, 2003; Hara and Zero, 2008; Venasakulchai *et al.*, 2010). Therefore, it is recommended that baseline microhardness values of enamel specimens are taken prior to testing in order to ensure that hardness values fall within the acceptable range (Lussi *et al.*, 2011). This range has been reported to be 272 to 440 KHN (Meredith *et al.*, 1996) or 280-390 KHN (Lussi *et al*., 2011).

Microhardness testing has the advantage of being simple to use, and has relatively low cost of analysis. However, it is time consuming and does not provide information about the chemical composition of the demineralised tissue. Additionally, it is more sensitive to changes in the most superficial layer of an erosive lesion and indentation borders are hard to detect. These surface hardness changes have been reported in an *in-situ* study to be equivalent to 90 seconds of acid exposure (Attin *et al.*, 2001).

Unlike Vickers indentation, the Knoop indenter is considered more suitable for testing enamel and dentine for two reasons. First, the Knoop indenter has an elongated nature that increases measurement accuracies reducing the plastic deformation. Secondly, subsurface cracking and crazing is reduced due to the shallower indentation of the relatively blunt Knoop indenter (Waters, 1979; Sirdeshmukh *et al.*, 2006). Additionally, the Knoop diamond indenter creates an indentation of 1.5 µm in depth as opposed to 5 µm for the Vickers indentation, making it more sensitive to surface changes and better for studies

of the properties of the outermost layer of an erosive lesion than Vickers indentation (Schlueter *et al.*, 2011).

1.5.2.2 Nanoindentation

Nanoindentation is used to measure the hardness and elastic modulus of enamel quantifying its mechanical characteristics before and after erosion. It can be used to detect enamel dissolution at small timescales comparable to the oral dwell-time of a single 'mouthful' of a beverage (White *et al.*, 2010). Its principle is based on a formed indentation of known dimension by pressing a diamond tip of a 3-sided pyramidal berkovich indenter under known load and duration. This creates an indentation between 100-200 nm of maximally 1 µm in length under loads of 0.25–50 mN (Kinney *et al.*, 1996; Mahoney *et al.*, 2003). Given the small scale of nanoindentation, it is a more suitable technique than microindentation in the early stages of enamel erosion. Nanoindentation uses the SI unit of Pascals (Nm^{-2}) (Barbour and Rees, 2004).

1.5.3 Atomic force microscopy (AFM):

AFM is a powerful tool used for imaging surfaces at nanometer scales. This is achieved by placing a pointed probe with a tip attached to a cantilever spring of different stiffness degrees in contact with a sample surface and measuring the minute deflections of the cantilever as the probe is moved laterally along the surface (Binnig *et al.*, 1986; Vukosavljevic *et al.*, 2014). The cantilever is often made of silicon nitride and covered with a very thin aluminium layer to make it very reflective. The tip has a 10 nm diameter so any space less than 10 nm between two atomic particles would lead to the tip not reaching to the bottom of the eroded area. This is known as a tip artefacts. A diode laser beam

is reflected from the back of the cantilever and is incident on a four-segment photodiode. The position of the laser on the photodiode reflects the deflection of the cantilever as the tip moves. A map is then built up of the surface of the specimen.

For nanohardness measurements of enamel, AFM can be used to control the exact area of prism that is required to be indented (Cheng *et al.*, 2009). AFM microscopy can also be used during *in vitro* experiments involving enamel deproteination to ensure that the organised structures of hydroxyapatite are not affected and that only proteins are removed from enamel (Lubarsky *et al.*, 2014). In the field of protein studies, AFM can be applied to explore the structure-characterisation of proteins at the molecular scale under physiological conditions (Hansma and Hoh, 1994; Karrasch *et al.*, 1994; Fotiadis *et al.*, 2002; Cárdenas *et al.*, 2007; Lindh *et al.*, 2007). This facilitates the study of conformational changes in proteins upon their adsorption (Holland and Marchant, 2000; Dufrêne, 2003; Toscano and Santore, 2006).

When comparing the use of AFM with SEM in the erosive tooth wear studies, AFM provides significantly higher resolution than SEM. In addition, AFM imaging of native specimens, including those in solutions, is possible without the need for sample sputtering with heavy metals as is the case with SEM. Further to that, roughness measurements can be made with AFM even though some studies demonstrated that AFM underestimates the roughness values compared to SEM due to the high steepness of the enamel crystallites (Vitkov *et al.*, 2008).

1.5.4 Inductively coupled plasma- Mass Spectrometry (ICP-MS)

In the chemical sciences, inductively coupled plasma – mass spectrometry (ICP-MS) is an established method which is used to analyse very small concentrations (part per million) of elements by processing the sample with an inductively coupled plasma followed by analysis with a mass spectrometer. The ICP-MS analysis requires that samples are in liquid form and must not be turbid as they have to pass through very small jets. In addition, the samples must be provided in water or a dilute acid solution.

The liquid samples are introduced via a nebuliser and spray chamber into the induction system which uses a high temperature argon plasma with an average core temperature of 6000-10000 K to generate positively charged ions with ionisation efficiency approaching 100%. When injected into the system, the samples are transformed from a liquid aerosol to solid particles, then into a gas before becoming atoms and ions as it reaches the analytical zone of the plasma. The properties of production, conveying and detection give the ICP-MS powerful trace detection characteristics via the fundamental basis of atomic emission. In this, its plasma carries enough energy to excite an electron from the outermost shell to generate positively charged ions. Another difficulty often encountered when using ICP-MS to analyse very small samples is determining the detection limit of the system as well as solving background signals as the lower the background, the lower one can see the sample signal. The background is represented by the signal/noise (s/n) ratio. Ideally one would monitor the ^{40}Ca isotope, as this is by far the most intense

of the Ca isotopes. Unfortunately, there is an enormous (about 6-8 orders higher) background signal present from the plasma ^{40}Argon (Ar).

With regard to the use of this method in dental research for elemental analysis, Hitomi *et al*. (2013) used it to analyse mineral content in the solution coming out of the erosive cycle (Hitomi *et al.*, 2013). Carpenter *et al*. (2014) and Khambe *et al*. (2014) also used this technique to analyse mineral content in AEP (Carpenter *et al.*, 2014; Khambe *et al.*, 2014).

1.5.5 Scanning electron microscopy (SEM):

SEM is widely used in research of erosive tooth wear for qualitative assessment of the surface but not for erosion depth. It uses an electron beam to produce an image of the surface to visualise the surface changes and estimate the elemental compositions of eroded surfaces (Attin *et al.*, 2009). One disadvantage of it is the irreversible destruction of the specimen due to desiccation and sputtering processes associated with the technique. It has been used in many studies to investigate the qualitative change of enamel surfaces. Many studies have used SEM to study the effect of erosive challenges on enamel surfaces in the presence or absence of AEP (Meurman and Frank, 1991; Eisenburger *et al.*, 2004; Nekrashevych *et al.*, 2004). Meurman and Frank (1991) used SEM to study the effect of AEP in protection against an acidic cola beverage on bovine enamel. They demonstrated that the gross prism dissolution of the underlying tissues was protected by the AEP as compared to specimens that were not covered by AEP. Coupled with profilometric analysis, Nekrashevych *et al*, (2004) used SEM to confirm the changes on enamel surfaces after *in vitro* 0.1% and 1.0% citric acid challenges in the presence and absence of AEP (Nekrashevych *et al*., 2004). Eisenburger

et al. (2004) also used SEM to develop techniques to avoid the artefacts developed on enamel surfaces as a result of drying and mineral precipitation (Eisenburger *et al*., 2004). They used SEM to examine the surface of enamel specimens after being titrated and/or rinsed followed by air dried and/ or freeze-dried. They found that when specimens are immediately rinsed in water or dried in air after they have been immersed in acidic challenge, the outer region of softened enamel becomes more susceptible to physical forces.

1.5.6 Scanning microradiography (SMR):

This method is a non-destructive subsurface visualisation of worn teeth quantifying mineral loss based on the attenuation of X-ray irradiation transmitting through dental hard tissues (Attin, 2009). Transverse microradiography (TMR) was first developed to analyse the mineral loss of transversely sectioned enamel blocks with maximum depth of 500 µm. This was then further developed to longitudinal microradiography (LMR) which can scan hard dental tissues without the need of transverse sectioned specimens even though it is less sensitive and requires thicker sections of dental tissues than TMR. Although microradigraphy techniques have widely been used in dental caries research they have recently been introduced into dental erosion research with good correlation with profilometry in the analysis of enamel demineralisation (Hall *et al*., 1997; Ganss *et al.*, 2005). Using SMR for analysing dental hard tissues, loss of minerals in the form of maximum depth of erosion (µm of mineral) can be calculated by a microdensitometric method (Amaechi *et al.*, 1999; Hall *et al.*, 1999; Kielbassa *et al.*, 2005). Measurements of specimens are taken at 22 points 400 µm apart, along two parallel lines 1.0 mm apart, for 30 seconds at each scanning position for a total period of 3

weeks. The projected mineral mass per unit area (g cm^{-2}) at each point (mHAp) is calculated using the mass attenuation coefficient for HAp (μm) of 4.69 cm^2 g $^{-1}$) 1 for AgKa X-ray, as previously described (Anderson and Elliott, 2000). The number of AgKa X-ray transmitted photons at each point is usually 50,000 giving the error in the mass value due to photon statistics of ± 0.5% (Kosoric *et al.*, 2007).

1.5.7 Chemical analysis of mineral loss:

These methods are based on quantifying the concentration of minerals such as calcium and/or phosphate released into solutions. These methods are used for indirect analysis of erosive tooth wear which include ion-selective electrode and atomic absorption spectrophotometry (Hara and Zero, 2008; Ganss *et al.*, 2009) and also more recently inductively coupled plasma mass spectroscopy as described above. Scanning electron microscopy – energy dispersive x-ray spectroscopy has also been used for elemental analysis of the surface of the enamel (Hegde and Moany, 2012).

Although these methods can be used for detecting small amounts of dissolved minerals in acidic solutions, they possess some limitations. The ion-selective electrode works in a specific pH and also forms complexes with saliva and citric acid, whereas atomic absorption spectrophotometry requires intensive solution preparation. Additionally, both methods do not provide information on mineral gain or physical and morphological changes of enamel surfaces (Attin *et al.*, 2005; Schlüter *et al.*, 2011).

1.5.8 Optical coherence tomography (OCT):

OCT is used for cross-sectional imaging of internal dental microstructures by measuring the time delay of optical backscattered light in a cross-sectional plane or three-dimensional images through the tissue up to 2 to 3 mm deep (Fujimoto and Drexler, 2008). For enamel, it allows the measurement of its thickness through visualising its optical properties by measuring the back reflected light. OCT also allows high resolution, reflectivity and absorbance of demineralised dental tissues by cross-sectional, high quality 3D imaging using near-infrared light (NIR) (Schlueter *et al.*, 2011). The bandwidth of the light used in OCT provides an axial image resolution ranging from 1 μm to 15 μm, 10 to 100 which is finer than the standard ultrasound (Fujimoto *et al*., 2000) and enables the visualisation of the detailed shpe of the tissue. Although this method has been used to quantify *in vivo* erosive wear, the change within the enamel is often insufficient to accurately measure early erosion (Chew *et al.*, 2014; Austin *et al.*, 2017).

1.5.9 Confocal laser scanning microscopy (CLSM):

CLSM is non-destructive technique used to scan the enamel surface with a laser light in order to measure surface texture of polished and unpolished enamel samples during demineralisation and remineralisation. This is achieved through the production of high resolution 3D images from tissue-emitting fluorescent signal and optical sections through 3D specimens (Schlueter *et al.*, 2011). CLSM has the advantages of non-destructive examination and no need for specimen drying minimising the risk of technical artefacts (Carvalho *et al.*, 2008).

1.6 Protein analysis methods

This section describes the techniques used within this thesis to analyse proteins content in the *in vitro* and *in vivo* AEP. A number of methods have been described in the literature to analyse protein samples in saliva and/or AEP. These methods apply different principles and the selection of each technique depends on the information that is required to be obtained from the study. These techniques can be classified into three categories: separation, quantification and identification of proteins as will be described in this section.

1.6.1 Bicinchoninic acid assay (BCA)

This method is widely used for quantitation of total protein in biological samples and is based on change in colour intensity of the formed protein complexes between the protein and the BCA reagents. This method is based on the principle of reducing Cu^{+2} by the amino acids cystine, tyrosine, and tryptophan as well as universal peptide back bone present in the protein sample, forming a purple colour by bicinchoninic acid. This method uses BCA reagents with bovine serum albumin (BSA) protein as a standard protein of known concentration (2 mg/mL) (Pierce Chemical, Rockford, Ill., USA). In addition to the sample solutions, standard solutions are often prepared for this process. A spectrophotometer employing a UV-visible light is used to measure the density of solutions or the intensity of transmitted light, known as the absorbance, of all solutions at a specific wavelength. The spectrophotometer is composed of four main parts; light source, filter, detector and reading meter. These components are arranged so that liquid in a cuvette can be placed between the spectrometer beam and the photometer. It measures the fraction of an incident beam of light which is transmitted by a sample at a particular

wavelength. According to the Beer-Lambert law, the UV wavelength absorbance depends on extinction coefficient, concentration of the measured substance and path length. Beer-Lambert (ABS) = ε CL where C is the concentration of the absorbing species, ε is the decadic molar absorptivity and L is the path length of the light through the absorbing species. Cuvettes, made of quartz and plastic, are often used for holding the sample solutions.

The use of BCA assay for analysing the total protein concentration in natural saliva and/or AEP has been reported (Walsh *et al.*, 2004; Kratz *et al.*, 2013; Carpenter *et al.*, 2014; Baumann *et al.*, 2016). This is a simple and cheap analytical method of the salivary protein concentration and has the advantage of being suitable for analysing any AEP sample formed on any substrate, independent from its surface properties (Kratz *et al*., 2013). There are however more accurate techniques for determining protein concentrations such as the use of high performance liquid chromatography with carbon18 column for amino acid analysis (Chaeib and Lussi, 2013), though expensive and time consuming. Consistent and accurate measurement of the proteins concentration in saliva and AEP depends on the measurement technique and the protein standard used (Chaeib and Lussi, 2013).

1.6.2 Sodium dodecyl sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS–PAGE is a fast, inexpensive technique used for qualitative separation and identification of proteins in complex biological samples such as proteins and DNA (Gallagher, 2007). TruPAGE™ Precast gels, used to run the samples, are designed to provide precise SDS-PAGE protein separation consistently with every consecutive experiment. TruPAGE gels are uniquely

formulated with Triethanolamine Tricine (TEA-Tricine) and often have 15 lanes that lead to pores and are used for loading biological samples. The TEA-Tricine provides a running environment of neutral pH which helps prevent protein modification during the course of the experiment. These gels are cast between a pair of glass plates by polymerising a solution of acrylamide monomers into polyacrylamide chains, cross-linked into a semisolid matrix by compounds such as bisacrylamides (Lodish *et al.*, 2000). Manipulating the concentrations of polyacrylamide and the cross-linking agent are believed to control the pore size of a gel (Lodish *et al.*, 2000).

Proteins in biological samples are found as folded up and complex three dimensional shapes of different sizes. Such protein structure is organised in four different levels: primary, secondary, tertiary and quaternary. There are two common problems with separation these proteins using SDS-PAGE. One is that the ratio of size to charge is different for each protein since each has a different number of amino acids and therefore would carry a different charge according to the type of amino acids as well as the pH of their environment. Another problem is the different shape of different proteins which would affect how they move through the gel pores due to their folded up and complex three dimensional shapes. To solve these problems, an anionic SDS detergent (sodium dodcyl sulfate) is used to denature the proteins samples so that secondary and tertiary protein structures are destroyed. Additionally, SDS binds the proteins and thereby covers their chemical charges, leading to equally negatively charged proteins. Therefore separation happens solely by the size of the polypeptide chains in the polyacrylamide gel.

SeeBlue Plus2 protein standard is often used in the precast gel to estimate the molecular weight of proteins in the range of 4-100 kDa. It consists of 10 Pre-stained protein bands (8 blue and 2 contrasting colour) in the range of 4-250 kDa. SeeBlue Plus2 is only used for estimating the molecular weight of small proteins. For estimating the molecular weights of large proteins, other alternative protein standards should be used. Novex sharp Prestained protein standard is often used as a standard for proteins of molecular weights greater than 100kDa, whereas HiMark Prestained protein standard is used to estimate the molecular weight greater than 250kDa.

This method has been used for the recovering and separation of the protein fractions of the AEP collected from the enamel surface (Hannig *et al.*, 2005; Svendsen *et al.*, 2008; Ash *et al.*, 2014) and the proteins from the mucosal oral pellicle (Gibbins *et al.*, 2014). It is worth noting that SDS-PAGE remains a qualitative application for protein separation and a precise quantification might require another application.

1.6.3 Western blot

Immunoblotting is a powerful method used for transferring, identifying and quantifying proteins in a given biological sample. This method involves protein electrophoresis using SDS-PAGE and it then transfers proteins from an SDS-PAGE gel to a solid membrane, usually a polyvinyl dichloride (PVD) or nitrocellulose. The type of membrane and gel as well as the molecular mass of the proteins being transferred greatly influence the efficiency of the transfer (Kurien and Scofield, 2006). This transfer is an exact replica of the gel followed by immunodetection that allows the detection and characterisation of a multitude of proteins, especially those proteins that are of low abundance

(Kurien and Scofield, 2003). Many studies have used this technique to study the structures of *in vitro* and *in vivo* formed AEP (Al-Hashimi and Levine, 1989; Carlen *et al.*, 1998; Yao *et al.*, 2001; Yao *et al.*, 2003; Carpenter *et al.*, 2014). Using this method, the transferred proteins are labelled by blotting them with a primary antibody of interest followed by a suitable secondary antibody conjugated with horseradish peroxidase (HRP). The benefit of HRP is to catalyse the emission of light to allow the detection of proteins on the PVD membrane (Thorpe *et al.*, 1985). The intensity of the emitted light depends on the amount of detected proteins but this is often low and of short duration. For this reason, the intensity of the emitted light is often enhanced by immersing the PVD membrane in a western blotting substarte known as enhanced chemiluminescent (ECL) substarte that increases the light emission by 1000 fold (Thorpe *et al.*, 1985).

Western blot is an inexpensive and useful technique for protein detection and quantification but can be associated with some problems as it involves many steps (Taylor *et al.*, 2013). These problems include the unexpected appearance of protein bands, undetected bands, nonspecific background and contaminated patches on the blots (Mahmood and Yang, 2012). These problems can be solved by many approaches ranging from optimising the concentration of the antibodies used by testing them with a dilution series of the study samples, using fresh buffer solutions, avoiding air bubbles, applying correct voltage to proper washing and even agitation during incubation.

The most debatable problem is the reliability of western blot to quantify protein in biological samples. Chemiluminescent western blot data compares the protein levels in biological samples but the challenge is that whether such data

can measure an absolute quantity of protein. For absolute quantificiation of protein in samples, purified protein standards of known concentration can be used as a positive control to allow reliable quantitative data (Taylor *et al.*, 2013). Purified standards allow production of a linear detection signal across the concentration range of samples to work out the protein concentration in the samples. Avoiding these problems and carrying out correct, standardised and validated technique with sophisticated software for data analysis allows western blot to provide accurate and reliable quantitative data on protein quantification (Taylor *et al.*, 2013).

1.6.3.1 Fluorescein isothiocyanate (FITC)

Flouorescein isothiocyanate (FITC) is a simple, inexpensive and widely used method in immunochemistry for labelling proteins in biological samples as it reacts with some amino acids yielding the fluorescein thiocarbamoyl (FTC) (Twining, 1984; Rath *et al.*, 1998). In the case of immunoblotted membranes, the fluorescent component of the stain (FITC) is used to visualise the presence of proteins on the blotted membrane. It has been used for visualization of the full positively stained layer under the confocal laser scanning microscope (Amaechi *et al.*, 1999). FITC is conjugated mainly to the amino acids cysteine and/or lysine residue of the protein chains. FITC labels the α-amino group of the amino acid cysteine and/or Lysine and also the terminal amino group in proteins (Svendsen *et al.*, 2008).

1.6.3.2 Image analysis technique

The presence of proteins on the immunoblotting membranes can be assessed and quantified using a photographic quantification to quantify the light intensity

of the chemiluminescent reaction. In most luminescent assays the exposure times can be optimised to prevent pixel saturation. The amounts of proteins on the blotted nitrocellulose membranes can be quantified using tools of ImageLab software version 4.1 (Bio-Rad Laboratories Ltd., Hertfordshire, UK) to select and determine the background-subtracted density of the protein bands (Bio-Rad Laboratories, 2011; Taylor et al, 2013). This method is based on a film and high sensitive detection technology which is CCD high-resolution that provides information on the amounts of proteins from complex protein mixtures or homogenates as well as information on the presence or absence, size, and modification or degradation states of target proteins (Taylor *et al.*, 2013). The system is usually controlled by Image Lab™ software to optimize performance for fast, integrated, and automated image capture and analysis of various samples (Bio-Rad Laboratories, 2011).

1.6.4 Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA is one of the most commonly used analytical techniques in the immunological assays (Paulie and Perlmann, 2003; Lequin, 2005). It is widely used for quantitative and qualitative assessment of antigen–antibody interactions or any antigenically active molecule. Such interactions can be amplified and visualised by using enzyme-conjugated reagents which can allow for antigen–antibody interaction. The enzyme-conjugate can either be an enzyme-linked anti-immunoglobulin antibody or a secondary antibody to the specifically bound antigen. Commonly used ones are alkaline phosphatase, horseradish peroxidase and β-galactosidase (Paulie and Perlmann, 2003).

The ELISA technique has been used in the quantification of protein concentration in natural saliva and AEP. Chaeib and Lussi, (2013) used ELISA to measure the concentration of albumin and IgA in whole mouth saliva to better understand its buffering system. ELISA and western blot are both sensitive methods for analysing proteins but western blot can have more specificity as it is performed after an electrophoresis step but it requires higher skills and longer steps than ELISA.

1.6.5 Proteomics

Proteomics is defined as the large-scale analysis of proteins with the goal of systematic analysis of the much larger number of proteins expressed in a given sample. For complex protein samples, mass spectrometry (MS) is used which is becoming possible due to the presence of gene and genome sequence databases. The proteomics analysis has also become a reachable technique in protein analysis due to the technical and conceptual advances in many areas, most notably the discovery and development of protein ionisation methods. A mass spectrometer consists of a number of items including a mass analyser that measures the mass-to-charge ratio (m/z) of the ionized analytes, an ion source and a detector that registers the number of ions at each m/z value. The mass analyser is central to the proteomic technology since it provides the system with high sensitivity, resolution, mass accuracy and the ability to generate information-rich ion mass spectra from peptide fragments (tandem mass or MS/MS spectra) (Aebersold and Mann, 2003).

The use of quantitative proteome analysis for the identification of proteins/peptides within AEP has been reported (Siqueira *et al.*, 2007; Siqueira and Oppenheim, 2009; Delecrode *et al.*, 2015). A number of

softwares have been reported to process the obtained MS/MS spectra. These include proteome Discoverer (ThermoScientific;, San Jose, CA, USA, V 1.4), SIEVE software (Version 2.0, Thermo Scientific, San Jose, CA, USA) and SEQUEST (Bioworks Browser 3.2, Thermo-Finnigan, San Jose, CA) (Siqueira *et al*., 2007; Delecrode *et al*., 2015). Mass spectrometry data can then be uploaded into protein search engines for characterisation and quantitation of proteins. This allows filtering the protein data at various confidence levels. For instance, Mascot applies a 95% probability CI in the MOWSE scoring algorithm that is an identification threshold. This threshold is calculated as described on the Matrix Science website: “Given an absolute probability that a match is random, and knowing the size of the sequence database being searched, it becomes possible to provide an objective measure of the significance of a result (Perkins *et al*., 1999). A commonly accepted threshold is that an event is significant if it would be expected to occur at random with a frequency of less than 5%” (Perkins *et al*., 1999). Therefore, any protein that is above this identity threshold is deemed significant.

1.7 Summary and aims of the research

There is some evidence suggesting that saliva and AEP have a protective role against erosion and erosive tooth wear (Buzalaf *et al*. 2012; Vukosavljevic *et al.*, 2014). However the exact mechanisms by which this protection is offered need to be further investigated both *in vitro* and also *in vivo*. It is unclear whether the main protective effects are due to the action of the proteins or ions within saliva and AEP or both to varying degrees. The overall aim of this PhD is to investigate the role of ions and proteins in AEP in protection against erosion and erosive tooth wear.

This investigation shall take place in three parts.

1. Laboratory studies investigating the protective effect of WMS, PS and AS against one cycle (referred to as early erosion for the rest of the thesis) and five cycle (referred to as advanced erosion for the rest of the thesis) erosion.
2. Investigation of total protein and four specific salivary proteins: mucin5b, albumin, CA VI and statherin, as well as calcium and phosphorus in *in vitro* AEP in laboratory studies representing early and advanced erosion.
3. Investigation of total protein and four specific salivary proteins: mucin5b, albumin, CA VI and statherin in *in vivo* film and AEP on eroded and non-eroded tooth surfaces in participants with erosive tooth wear.

Objectives of research

1. To develop a laboratory protocol using natural saliva in an *in vitro* erosion model.
2. To compare WMS, PS, AS and DW in protection against advanced and early erosion in an *in vitro* model using non-contacting profilometer and microhardness testing.
3. To compare the total protein and four specific salivary proteins: mucin5b, albumin, CA VI and statherin between AEP from WMS and PS in an *in vitro* erosion model using BCA assay and SDS-PAGE.
4. To compare the amount of calcium and phosphorus between AEP from WMS and PS in an *in vitro* erosion model using ICP-MS.

5. To compare AS without and with human serum albumin of varying concentrations in protection against erosion using non-contacting profilometer and microhardness testing.
6. To compare the total protein and four specific salivary proteins: mucin5b, albumin, CA VI and statherin between salivary film and AEP from eroded and non-eroded teeth surfaces in participants with erosive tooth wear using BCA assay and SDS-PAGE.

The null hypotheses of the research

1. There is no difference between WMS, PS, AS and DW in protection against advanced and early erosion *in vitro*.
2. There is no difference in the concentration of total proteins, amount of four key salivary proteins: mucin5b, albumin, CA VI, statherin and the concentration of calcium and phosphorus in *in vitro* AEP from WMS and PS in advanced and early *in vitro*.
3. There is no difference in the concentration of total proteins and amount of four key salivary proteins: mucin5b, albumin, CA VI and statherin in *in vivo* salivary film and AEP between eroded and non-eroded tooth surfaces in participants with erosive tooth wear.

Chapter 2: General methods, materials, training and development of techniques

2.1 Enamel specimens preparation

2.1.1 Tooth collection:

Extracted, caries free, permanent human molar teeth were collected from the oral surgery department at Guy's hospital. Teeth were collected after gaining consent from patients in accordance with the approved guidelines and regulations of the the National Research Ethics Committee, London (REC ref: 12/LO/1836). The teeth were collected after giving a patient information sheet (PIS) and obtaining written consent from the patient. Details of the PIS and consent form are shown in appendix I and II respectively. The collected teeth were disinfected by storage in sodium hypochlorite solution for at least 72 hours at 4 º C prior to use.

2.1.2 Tooth sectioning

The buccal and lingual surfaces of the collected teeth were sectioned with a 4 inch diamond coated saw blade (Diamond wafering blade XL 12205, Benetec Ltd, London, UK) using a cutting machine (Buehler Isomet GmbH, Düsseldorf, Germany) at a speed of 500rpm with a force of 0.98 N using previously developed protocols (Austin *et al*., 2011). Enamel specimens were embedded in a metal tube filled with impression compound (Kerr, Peterborough, UK) (Figure 2). Firstly, the root was removed at the cementoenamel junction and then the buccal and lingual surfaces were sectioned, with the cut starting at the cusp. All enamel sections were stored dry.

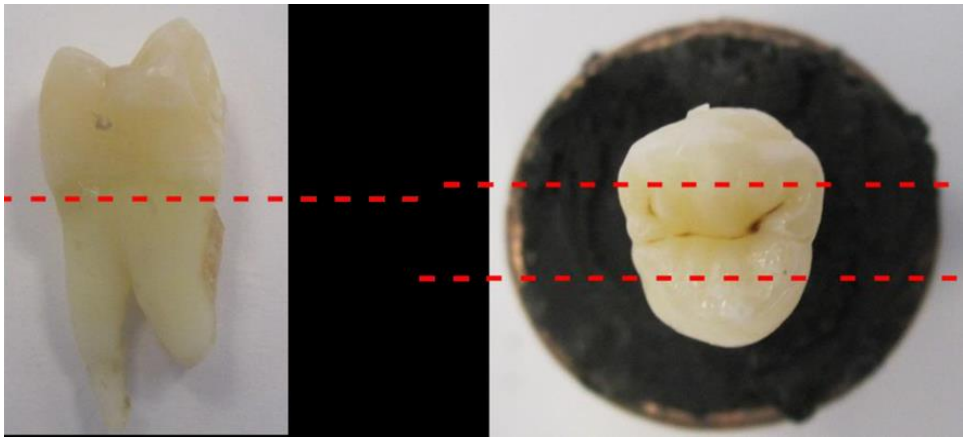


Figure 2: *An image of a tooth embedded in impression compound during sectioning*

2.1.3 Power calculation:

Statistical advice was obtained prior to each *in vitro* study in order to determine the sample size required by a suitable power calculation. The power calculation for comparing the mean step height and surface microhardness in all *in vitro* studies within this thesis was carried out using Gpower version 3.1.5. based on ANOVA and paired t test as well as on previous studies (Nekrashevycha and Stösserb, 2003; Martins *et al*., 2013; Mistry *et al*., 2015; O'Toole *et al*., 2015). For *in vitro* protein analysis studies, a power calculation for comparing the mean protein levels between WMS group and PS group was carried out based on paired t test as well as on previous studies (Martin *et al*., 2013).

2.1.4 Embedding and mounting

Enamel sections were embedded using a custom-made silicone mould as shown in Figure 3.

Figure 3: *An image of the silicone mould used to create specimens*

Silicone duplicating material (Metrodent Ltd UK, Sussex, UK) was used to fabricate the mould. Six blank, unpolished (8 × 21.5 × 24 mm) blocks were placed into a rectangular container and were attached with a very small amount of beading wax to avoid any unwanted movements during pouring of the silicone material. The silicone duplicating material was mixed following the manufacturer's instructions and then slowly poured into the container taking care that air bubbles were not formed. After the silicone material had set, the mould and six blocks were carefully removed from the container creating the base. A mould lid was created using the same rectangular container filled with soft putty (Dentsply Ltd, Surrey, England). Once set, the formed lid was carefully removed. Holes were then created in the centre of the lid using a metal bar (4 mm diameter) to allow the removal of excess acrylic during later sample mounting (Figure 4).

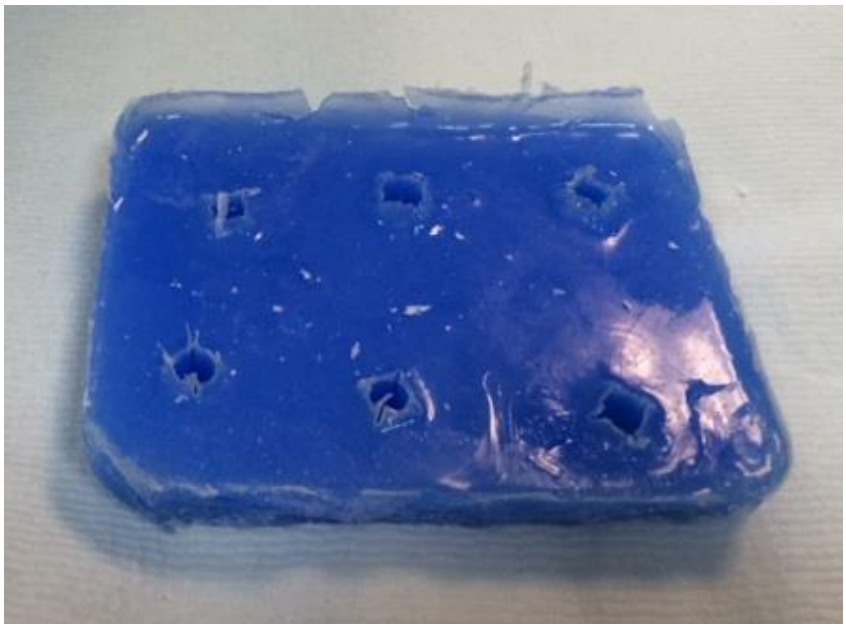


***Figure 4**: The mould lid with holes for excess removal during acrylic mounting*.

Sectioned enamel surfaces were embedded in cold cure acrylic resin using the custom-made silicone mould. No lubrication was required since the acrylic resin did not adhere to the silicone mould. The acrylic resin used was Stellon Q-20 (Dentsply Ltd, Surrey, UK). The mixing ratio of powder polymer/monomer was approximately 1:1. The enamel specimens were placed facing down inside the mould before the acrylic resin was poured on them. An excessive amount of acrylic was left over each specimens before the lid was placed on top and was compressed with a light force using a metal block with 600 g of weights. This force applied on the lid allowed the removal of any excess resin through the lid holes formerly created with a 4 mm metal bar. After the resin material had set, the lid was removed and the specimens were taken out and immersed in DW. The mould was then cleaned carefully and re-used almost immediately to mount more enamel specimens. The enamel sections mounted

in cold cure acrylic resin are shown in Figure 5 which will be referred to as'' enamel specimens'' throughout this thesis.

Figure 5: *Photograph of a mounted enamel specimen before grinding/ polishing procedure.*

2.1.5 Grinding/Polishing

Enamel specimens were ground and polished using a water-cooled rotating polishing machine (Meta-Serv 3000 Grinder-Polisher, Buehler, Lake Bluff, Illinois, USA) with a semi-automated polishing head (Vector LC Power Head, Buehler, Lake Bluff, Illinois, USA) under constant water irrigation Figure 6.

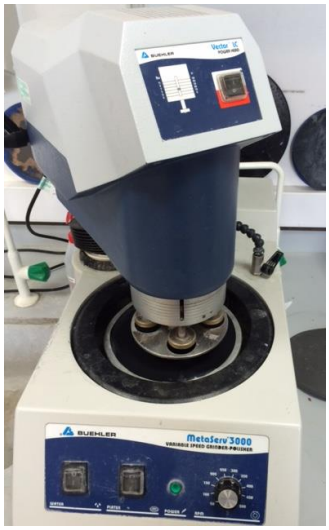


Figure 6*: An image of the grinding and polishing Buehler lapping machine.*

Federation of European Producers of Abrasives (FEPA) standard silicon carbide sandpaper (SiC-Paper, Struers A/S, Copenhagen, Denmark) was used applying previously published regimes (Rodriguez and Bartlett 2010; Austin *et al.*, 2011). Progressively abrasive grit silicon carbide papers were used starting at 80 grit, followed by 180, 600, 1200, 2500, and 4000 grit (SiC-Paper, Struers A/S, Copenhagen, Denmark). Custom-made jigs made from cold cure acrylic resin to fit the power head held the samples in place. A force of 10 N was applied to the centre of the specimen and a speed of 300 rpm was applied. An initial flattened area on the enamel was created by polishing the surface at 80 grit for approximately 4 seconds. At this stage, the specimens were dried with clean absorbent paper until free from visible moisture which then individually, visually inspected checking that an area of enamel (~2 x 3 mm) had been exposed. If there was not any exposed enamel, the specimen was then ground again for 3 more seconds and re-checked, until there was exposed enamel. Specimens were then polished with silicon carbide papers to provide a surface large and flat enough for analysis. After which a progressively abrasive grit was used as follows: 80 (4 seconds), 180 (8 seconds), 600 (15 seconds), 1200 (25 seconds), 2500 (35 seconds) and 4000 (45 seconds) grits to produce a flat, highly polished enamel surface of approximately 3 x 3 mm wide as displayed in Figure 7. This procedure removed approximately 400 µm of enamel as will be described in the following section 2.1.6. Specimens were ground/polished in batches and the silicon carbide disks replaced after polishing 8 specimens. Specimens were stored in DW baths in between polishing sequences. All polished specimens were then immersed in 80 mL of DW and ultrasonicated (Nusonics GP-70,

T310,Germany) at 70 Hz for 15 min followed by a 2-min water rinse. After the 4000 grit level polishing and ultrasonication, the specimens were placed horizontally in a weighing boat with the enamel surface facing upward and were allowed to dry naturally for at least 12 hours.

Figure 7: *Photograph of polished enamel specimen*

2.1.6 Measurement of the amount of enamel removed

In order to assess how much enamel the polishing procedure removed, the thickness of the specimens before polishing and after the polishing procedure (after 4000 grit) were repeatedly checked using digital callipers (Duratool D00325, Farnell Company, Leeds, England). In the development of the protocol for polishing, initially 10 specimens were put through the polishing procedure and the amount of enamel removed was measured for all specimens. Fifty specimens were prepared for the training and development of *in vitro* models within this thesis as described in section 2.6. Thirty out of fifty polished enamel specimens were randomly selected and assessed for the amount of enamel removed. This was accomplished in order to standardise the polishing procedure and to ensure that nearly the same amount of enamel was removed for all specimens (Mistry, 2016).

2.1.7 Non-eroded reference area

After polished specimens were left to air dry for at least 12 hours, they were then taped with poly (vinyl chloride) (PVC) adhesive tape (RS Components Ltd, Corby, UK) to a window approximately 2 X 3 mm wide with a reference area 1 mm wide on either side to create two intact reference areas (Figure 8). Strips of the adhesive tape were produced by cutting the tape with a scalpel and a ruler against a clean glass block. After applying the tape strips on the enamel surface, the width of the exposed enamel window was checked with a ruler to create approximately the same enamel windows.

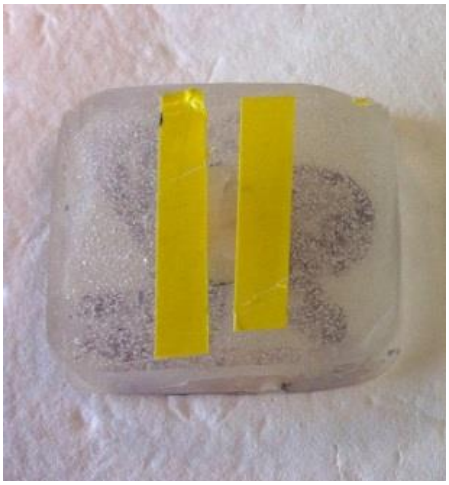


Figure 8: *A photograph of polished and taped enamel specimen*

2.1.8 Specimens randomisation:

Prepared, polished and taped specimens were then numbered for identification by an independent researcher and randomised by another independent researcher using SPSS random sample generator.

2.2 Preparation of solutions

2.2.1 Natural saliva collection

2.2.1.1 Whole Mouth Saliva (WMS)

Saliva collection from healthy volunteers was approved by the National Research Ethics Committee, Northampton (REC ref: 14/EM/0183). Volunteers were given the PIS and written informed consent was obtained. Details of the PIS and consent form are shown in appendices III and IV respectively. The participants were asked to abstain from eating and drinking for at least one hour prior to saliva sample collection. Stimulated saliva was collected by asking the participants to chew on a piece of standardised paraffin wax on both sides of the mouth. Participants were asked to expectorate saliva immediately after starting to chew the paraffin wax and continue for 5 minutes. Saliva was collected into a standard 20-mL sterile polypropylene universal tube. The collection tubes were pre-weighed empty and re-weighed after saliva collection.

The flow rate, pH and buffering capacity of the collected saliva were immediately measured to ensure that they fell within the normal range. The salivary flow rate was determined using the following formula:

$$\text{Salivary Flow rate (ml/min)} = \frac{\text{Weight of tube with saliva – Weight of pre-weighed tube with no saliva}}{\text{Time of collection (mins)}}$$

A pH meter (Oakton pH 510 bench top meter, Eutech Instruments Pte Ltd, Singapore) was used to measure the pH. A pH value between 6- 7.5 was considered to be within the normal range (Humphrey and Williamson et al, 2001; Edgar et al, 2012). The buffering capacity of the saliva was also

immediately measured using a specified saliva kit by GC Company (GC America INC, 3737 W. 127th Street Alsip, IL 60803). The saliva kit contained disposable pipettes, pH indicator strips and a colour chart explaining the interpretation of results and conversion table of points. One drop of saliva was placed on each of the three test pads which began to change colour where the final result of colour change was calculated after 2 minutes. Adding the points as a final colour change on each test pad indicated very low (0-5 points, red colour), low (6-9 points, yellow colour) and normal/high (10-12 points, green colour) salivary buffer capacity (Ericson and Bartthall, 1989; GC America INC, 2014).

Although saliva was collected from different individuals, the flow rate and buffering capacity were determined by calculating the mean of individual flow rate and buffering capacity. All collected saliva samples was anonymised and stored in a freezer at -80 °C until before use. The collected saliva samples were defrosted at -4 Cº prior to use and thawed saliva was mixed vigorously with a vortex mixer (Bibby Scientific Limited, Staffordshire, UK) to re-suspend precipitation of proteins on thawing to avoid the loss of a specific proteins of less than 14 kDa such as statherin and/or histatin (Francis *et al.*, 2000). Once all the studies were completed, any remaining saliva was discarded according to the protocol submitted to the Ethics Committee. The collection, storage and disposal of saliva samples was conducted in accordance with the Human Tissue Act (2004).

2.2.1.2 Parotid Saliva (PS)

The participants were asked to abstain from eating and drinking for at least one hour prior to saliva sample collection. Saliva from parotid glands was

collected through a number of steps. First, the orifice of the parotid gland was located, then the area was dried with gauze for better vision. The parotid collector, a Lashley cup (Figure 9) (Granton medical Ltd, Code: 17140, Sheffield, UK), was placed on the mucosa so that the inner ring surrounded the duct orifice. The cup was held on the mucosa by suction from the outer ring by pulling back on the syringe and allowing the pressure to come to equilibrium. The syringe was rested on the patient's shoulder or sometimes held by the researcher. A medium binder clip was then attached to the tygon tubing going from the collector to the syringe to lock the air in the tubing. The suction created was sufficient so that the cup was in place without occluding the inner chamber of the parotid collector with excess tissue. Saliva from the parotid gland then flowed passively into the inner ring and through the attached tubing. The subjects had to avoid unnecessary movement of their head or jaw to prevent dislodging the cup. The flowing saliva was collected into an ice-cooled pre-weighed and pre-labeled container. A maximum of 5 minutes was allowed for saliva to appear in the clear portion of the tubing. The parotid saliva secretion was then stimulated using 2 drops of citric acid 2 % solution (Guy's hospital pharmacy, London, UK) every 30 seconds applied to the posterior lateral surface of the tongue bilaterally. Once saliva flow was observed, an additional 2 minutes was allowed for the saliva to reach the end of the tubing. When the saliva began to exit the tygon tube, a 10-minute collection period began. The collected saliva was weighed, measured, stored and discarded in the same way as the whole mouth saliva as described above in section 2.2.1.1.

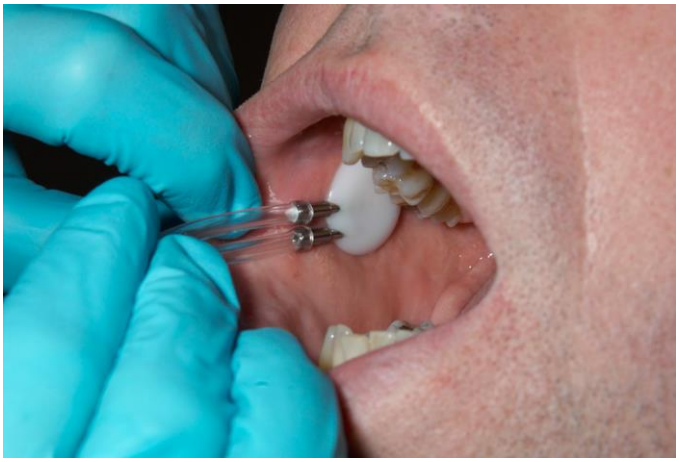


***Figure 9**: Lashley cup showing the outer and inner rings placed on the mucosa with the inner ring surrounding the duct orifice of the parotid gland.*

2.2.2 Artificial saliva preparation

The artificial saliva was prepared according to the protocol used by Eisenburger *et al.* (2001b). It contained the following ingredients in DW: $CaCl_2$ x $2H_2O$ 0.7 mmol/L; $MgCl_2$ 0.2 mmol/L; KH_2PO_4 4.0 mmol/L; HEPES buffer (acid form) 20.0 mmol/L; KCl 30.0 mmol/L. The required quantities of the ingredients were measured using an electronic analytical scale (Mettler Toledo, XS105 Dual Range Analytical Balance, Fisher Scientific UK Ltd, Loughborough, UK). Solid ingredients in grams were added to 1 L of DW to prepare the artificial saliva solution. Initially, 500 mL of DW was added to a 1 L- volumetric flask. The weighed solid ingredients were added into the flask immediately after weighing. After all ingredients were added to the flask, the solution was continually stirred for 30 minutes with a magnetic stirrer (Fisher Scientific, Magnetic hotplate stirrer, USA) to allow components to be dissolved in the DW. The volume was then increased to 1 L by adding DW using a graduated measuring cylinder while the solution was continuously stirred. The pH of the prepared solution was adjusted to 7.0 by adding sodium hydroxide

(NaHO) and using a pH meter (Oakton pH 510 bench top meter, Eutech Instruments Pte Ltd, Singapore) and was always used within 24 hour of its preparation.

2.2.3 Acid solution

The solid form of citric acid (Sigma Aldrich, Lots# MKBF1347V, Saint Louis, MO 63103, USA) was used to prepare the citric acid solution to be used as the erosive challenge for all *in vitro* studies within this thesis. The citric acid solution was prepared by the addition of the acidic solid form into DW. Three grams of solid citric acid was added to 1 L of DW to prepare a citric acid solution of 0.3 % 0.02 M. The pH of citric acid was adjusted to 3.2 with sodium hydroxide (NaHO) using a pH meter (Oakton pH 510 bench top meter, Eutech Instruments Pte Ltd, Singapore) and was always used within 24 hour of its preparation. Solids were weighed using an electronic analytical scale (Mettler Toledo, XS105 Dual Range Analytical Balance, Fisher Scientific UK Ltd, Loughborough, UK) and liquids were measured using a graduated measuring cylinder.

Titratable acidity of the prepared acidic solution was measured every time the solution was prepared. It was calculated by measuring the volume of 0.05 M NaOH solution required to raise the pH of 10 mL of the acidic solution to pH 7 using the same calibrated pH meter as above. The solution was continually stirred with a magnetic stirrer (Fisher Scientific, Magnetic hotplate stirrer, USA) whilst the NaOH was added and the pH probe was fully immersed in the acidic solution. After the addition of NaOH, the solution was stirred for 2 minutes and then the pH reading was observed. Initially, 5 mL of NaOH was added, but as the pH approached pH 7, smaller quantities (≤ 1mL) were added. The

experiment was stopped after two readings for NaOH were within 0.5 mL of each other. To calculate the mmol/L the following equation was used:

$$mmol/L = (C_{base} \times V_{base}) / V_{sample\ (acid)}$$

Where C_{base} is the concentration of the base in mol/L, V_{base} is the volume of base required to raise the solution to the end point pH in Litters and $V_{sample\ (acid)}$ is the volume of the acid sample that was titrated in Litters.

2.2.4 Sodium dodecyl-sulphate (SDS)

The powder form of SDS (Sigma Aldrich, Saint Louis, USA) was used to prepare a 0.5% SDS solution to be used for mechanically assisted elution of *in vitro* and *in vivo* AEP throughout this thesis (Svendsen *et al*.,2008). The SDS solution was prepared by weighing 0.5 g of SDS powder using an electronic analytical scale (Fisher Scientific UK Ltd, Loughborough, UK) and then added to 70 mL DW in 100 mL conical flask to prepare a SDS solution of 0.5 %. The solution was continually stirred for 30 minutes with a magnetic stirrer to allow the solid SDS components to be dissolved in the DW. The volume was then increased to 100 mL by adding DW using a graduated measuring cylinder while the solution was continuously stirred with an orbital shaker (Bibby Scientific, Staffordshire, UK).

2.3 *In vitro* erosive tooth wear model

2.3.1 Filterpaper development:

Sialostrips have been used in previous studies for *in vivo* AEP elution (Carpenter *et al.*, 2014). Due to difficulties in obtaining sialostrips, filterpapers were used to elute *in vitro* and *in vivo* AEP throughout this thesis. Filterpapers

were prepared by cutting consistently sized rectangular filterpapers of 21 cm length and 3 mm width using new scissors. A new ruler was used to choose the correct measurements. Filterpapers had a surface area of 63 mm^2 and were handled with gloved hands at all times. The cut filterpapers were then sterilised by the sterilisation services at Guy's and St. Thomas' NHS (Guy's Belimed steriliser No. 2, cycle number 10215). This cycle was a standard 134 C˚with a hold time of 3 minutes.

The efficacy of sterilised filterpapers to elute AEP against non-sterilised filterpapers was assessed using SDS-PAGE and immunoblotting technique against albumin antibody. Albumin antibody was used for the development of filterpapers because it has been well validated previously as an antibody free from protein contamination which could produce a clear protein band on SDS-PAGE. WMS samples were directly pipetted onto the filterpapers (FTP). Two different volumes [1 µl (n=3) and 2 µl (n=3)] of WMS were used on filterpapers before and after sterilisation (3 filterpapers each) as shown in Table 4.

Whole mouth saliva (WMS) sample (1 µL)						**Whole mouth saliva (WMS) sample (2 µL)**						**Stdandard**
Non-sterilised			Sterilised			Non-sterilised			Sterilised			
Filterpaper (FTP)	Filterpaper (FTP)	Filterpaper (FTP)	Filterpaper (FTP)	Filterpaper (FTP)	Filterpaper (FTP)	Filterpaper (FTP)	Filterpaper (FTP)	Filterpaper (FTP)	Filterpaper (FTP)	Filterpaper (FTP)	Filterpaper (FTP)	

Table 4: *WMS samples used for the development of filterpapers before and after sterilisation.*

The result of immunoblotting is shown in Figure 10. As can be seen from Figure 10, the filterpapers after sterilisation revealed more clear bands than filterpapers before sterilisation. This may suggest that using sterilised filterpapers may provide better recovery of protein than non-sterilised filterpapers.

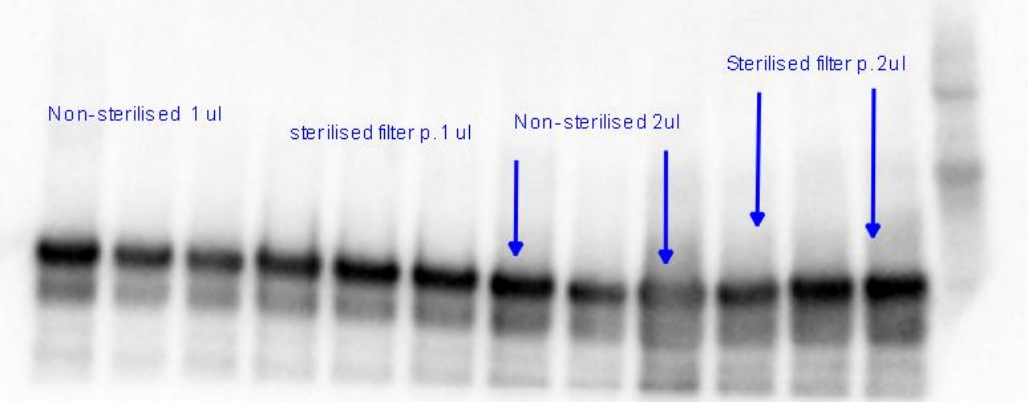


Figure 10*: WMS samples immunoblotted against albumin antibody before and after sterilisation for development of the filterpapers for protein elution.*

Following from these development results, the efficacy of sterilised filterpapers against sterilised sialostrips in eluting AEP from enamel specimens in an *in vitro* model were also assessed using SDS-PAGE followed by fluorescein isothiocyanate (FITC) labelling technique. WMS samples were directly pipetted into the filterpapers and sialostrips.

Two different volumes [(1 µl (n=2) and 2 µl(n=2)] of WMS were pipetted on sterilised filterpapers (FTP: n=2) and sialostrips (SP: n=2) as shown in Table 5 and Figure 11. Both filterpapers and sialostrips had a surface area of 63 mm^2 and were able to absorb up to 3 µl of WMS.

WMS sample (1 µl)				WMS sample (2 µl)				Standard
Filterpape (FTP)	Filterpape (FTP)	Sialostrips (SP)	Sialostrips (SP)	Filterpape (FTP)	Filterpape (FTP)	Sialostrips (SP)	Sialostrips (SP)	

Table 5: WMS samples used in the development of filterpapers for protein elution against sialostrips.

Figure 11 is a fluorescein isothiocyanate (FITC) image of eluted proteins using filterpapers against sialostrips. It shows that the use of sterilised filterpapers for protein elution from enamel tooth surfaces produced more abundant protein bands compared to sterilised sialostrips. This indicated the suitability of filterpapers to be used as an alternative means to sialostrips for the elution of *in vitro* and *in vivo* AEP samples throughout this thesis.

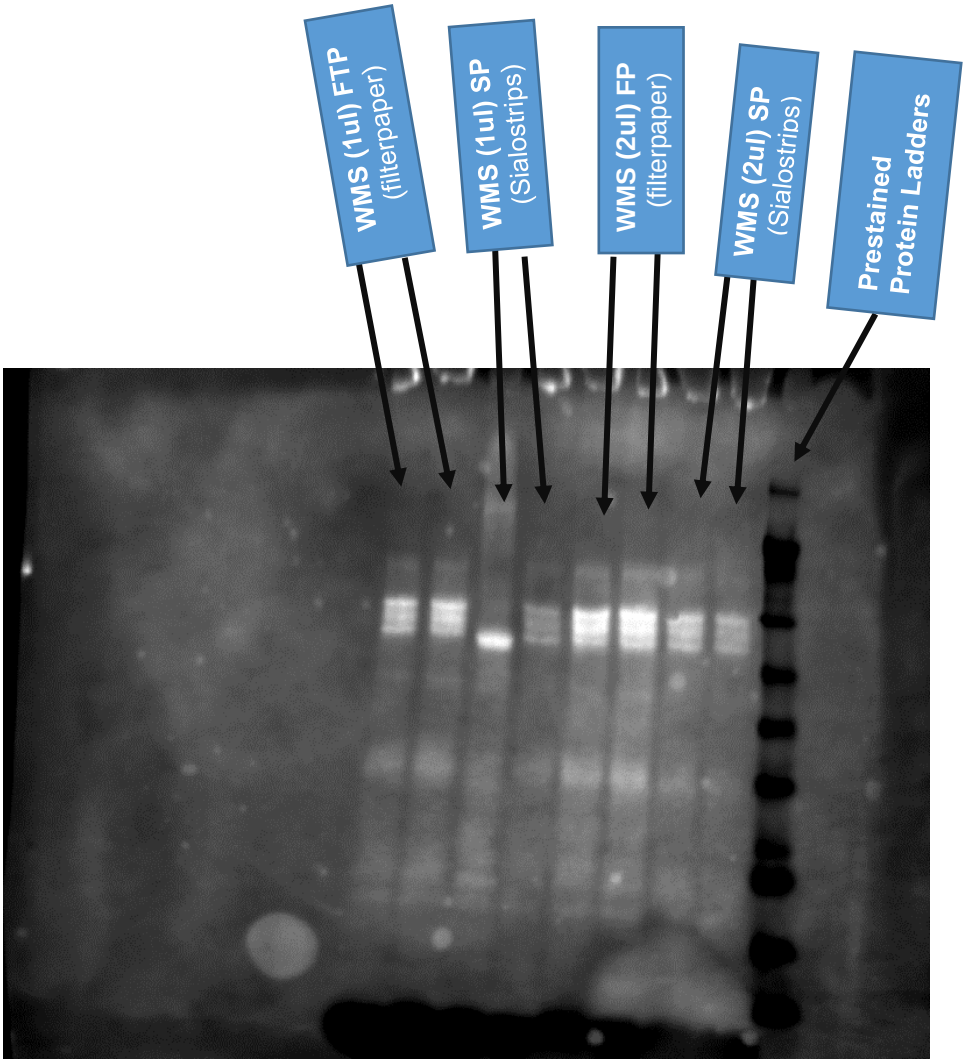


Figure 11: *Fluorescein isothiocyanate (FITC) image for the development of filterpapers against sialostrips for protein elution showing that filterpapers provided more abundant protein bands compared to sialostrips indicating their suitability in eluting in vitro and in vivo AEP from enamel tooth surfaces.*

2.3.2 In vitro AEP formation

In vitro AEP was formed by immersing the enamel specimens in natural saliva for specific times under specific conditions according to the objectives of each study as has previously been described in the literature (Meurman and Frank, 1991; Hall *et al*., 1999; Wetton *et al.*, 2006 and 2007; Hellwig *et al.,* 2013).

The frozen, stored natural saliva was defrosted at room temperature and vigorously vortexed before use as described in 2.2.1.1. Prepared enamel

specimens were immersed in plastic containers containing natural saliva (8 mL per specimen) for the required time according to each study protocol. Specimens were stirred at 60 rpm with an orbital shaker (Bibby Scientific, Staffordshire, UK). When immersed for 24 hour in saliva, specimens were stored un-agitated overnight at 22 °C±1. All specimens were taken out of the container with saliva immediately at once using specialised handles as described below in section 2.3.41. After the formation of AEP, enamel specimens were then either immersed immediately in the erosion cycle (One cycle and five cycles) or as in the case of AEP elution, AEP samples were immediately eluted before allowing the enamel specimens to dry.

2.3.3 *In vitro* AEP harvest and recovery:

The *in vitro* AEP was eluted from enamel specimens using sterilised filterpapers (VWR International Ltd, Leicestershire, England) of standardised size (21 mm length X 3 mm width). Approximately 5 mm length of the filterpaper was soaked in in 3 µL SDS (0.5 % w/v) sodium dodecyl sulphate (SDS) sample buffer (Novex, Thermo Fisher Scientific Inc, UK) which was freshly made each morning as described in section 2.2.4. The soaked filterpaper was then mechanically rubbed against standaridsed tooth surface (2 x 3 mm) for 15 seconds to elute the *in vitro* formed AEP from the enamel surfaces using previously published protocols (Svendsen *et al*., 2008; Carpenter *et al*., 2014). Two tubes (0.2 mL small tube and 1.5 mL Eppendorf tube) were used for the recovery of AEP from the filterpapers as shown in Figure 12. Filterpapers carrying the AEP were then suspended in a small 0.2 mL tube which in turn was placed in a 1.5 mL microcentrifuge tube by using a fine forceps to carefully secure the tail of the filterpaper and the rim of the a

0.2 mL tube along the rim of the 1.5 mL tube and closing the lid, thus holding the 0.2 mL tube and filterpaper in place (Figure 12).

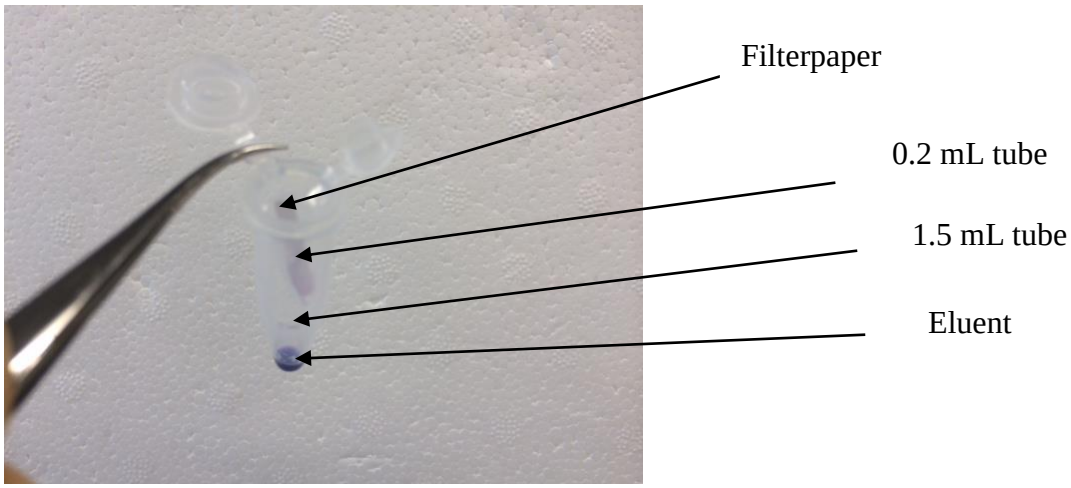


***Figure 12**: AEP eluent was recovered from a small test tube (0.2 mL) placed within an outer microcentrifuge tube (1.5 mL).*

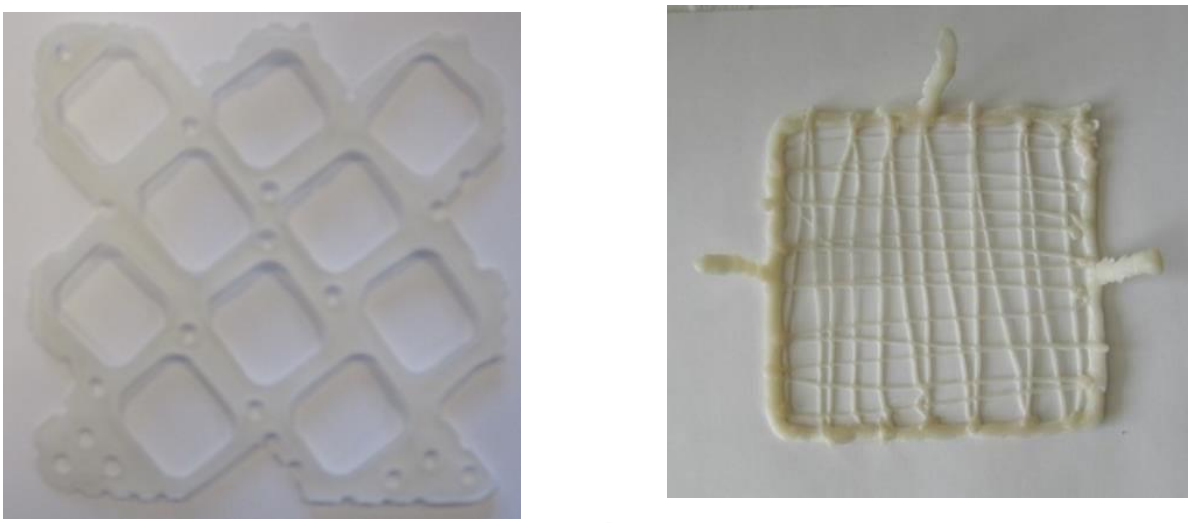
The bottom of the 0.2 mL tube was then perforated and the adsorbed proteins on each filterpaper were recovered by adding a 15 µL 0.5 % SDS, 5 µL of lithium dodecyl sulfate (LDS) buffer (1:4) (Novex, Thermo Fisher Scientific Inc, UK) which were applied directly onto the filterpaper portion where the AEP was formerly eluted from the tooth surfaces. The AEP eluent was recovered in the outer microcentrifuge tube by placing the outer tube containing the small tube in a table-top centrifuge for 8 minutes at 8000 rpm. Dithiothreitol ($C_4H_{10}O_2S_2$, 1.8 µL, 0.5 mM) reducing agent (1:10) (DTT, Sigma-Aldrich, GMbH, BCBN 4033V, MW 154.25, Steinheim, Germany) was then added to the eluent. Protein samples were vortexed for 1 minutes with a vortex mixer (Bibby Scientific Limited, Staffordshire, UK) and were then heat denatured at 100 º C for 5 minutes. Each of the recovered *in vivo* AEP samples was

collected in the universal 1.5 mL Eppendorf tube and numbered and randomised by another independent researcher using SPSS random sample generator. The numbered samples were immediately frozen at -20 º C until analysis. Analysis was performed by an investigator blinded to the sample type.

2.3.4 *In vitro* erosion cycle procedure:

2.3.4.1 Development of the immersion and removal model

When specimens were immersed in saliva, acidic solution, removed and/or rinsed with DW, it was necessary that they were immersed and removed at the same time for standardisation. A net made of composite and base holder made of acrylic resin were developed to hold the enamel specimens whilst immersed into or removed from solutions (Figure 13).



a) b)

Figure 13*. Images of a) acrylic resin base and b) bis-acrylic composite net*

2.3.4.2 Cycling procedure

One cycle erosion (early erosion)

After the specimens were immersed in solution for the specific time according to the objectives of each study, they were then exposed to citric acid erosion. The acid erosion consisted of 80 mL 0.3 % citric acid (Sigma Aldrich, Saint Louis, MO 63103, USA), 0.02 M, pH=3.2, at 22 °C ± 1, agitated with an orbital shaker (Bibby Scientific, Staffordshire, UK) at 60 rpm, followed by 2-minute rinse in 80 mL of DW, again under agitation with the orbital shaker set at 60 rpm for a final 2 minutes (Figure 14).

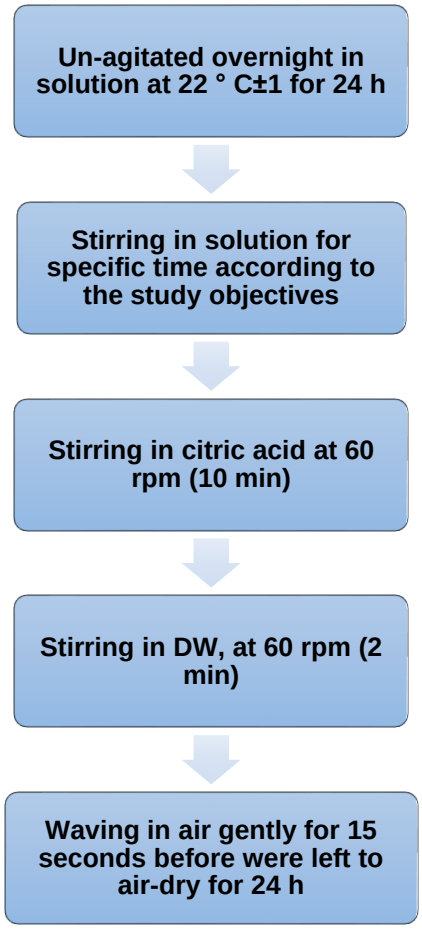


***Figure 14**: A flow chart of the generic one cycle erosion model (early erosion)*

When immersed for 24 hour in solution, specimens were stored un-agitated overnight at 22 ° C ± 1. This was classified as an early erosion model throughout this thesis. After the completion of one erosion cycle, specimens were dried by waving in air gently for 15 seconds and then left to air-dry for 24 hour before the tape was carefully removed and the profilometric and microhardness measurements were taken (Mistry, 2016). For protein analysis, AEP samples were eluted immediately after the one cycle erosion before allowing the specimens to dry.

Five cycle erosion (advanced erosion)

The one cycle of immersion of the specimens in solution followed by the 10 minute acid erosion was repeated 5 times for each group as shown in Figure 15. This was classified as an advanced erosion model throughout this thesis. Once again, after the completion of five erosion cycles, specimens were dried by waving in air gently for 15 seconds then left to air-dry for 24 hour dry before the tape was removed and the profilometric and microhardness measurements were taken. For protein analysis, AEP was eluted immediately after the five cycle erosion before allowing the specimens to dry.

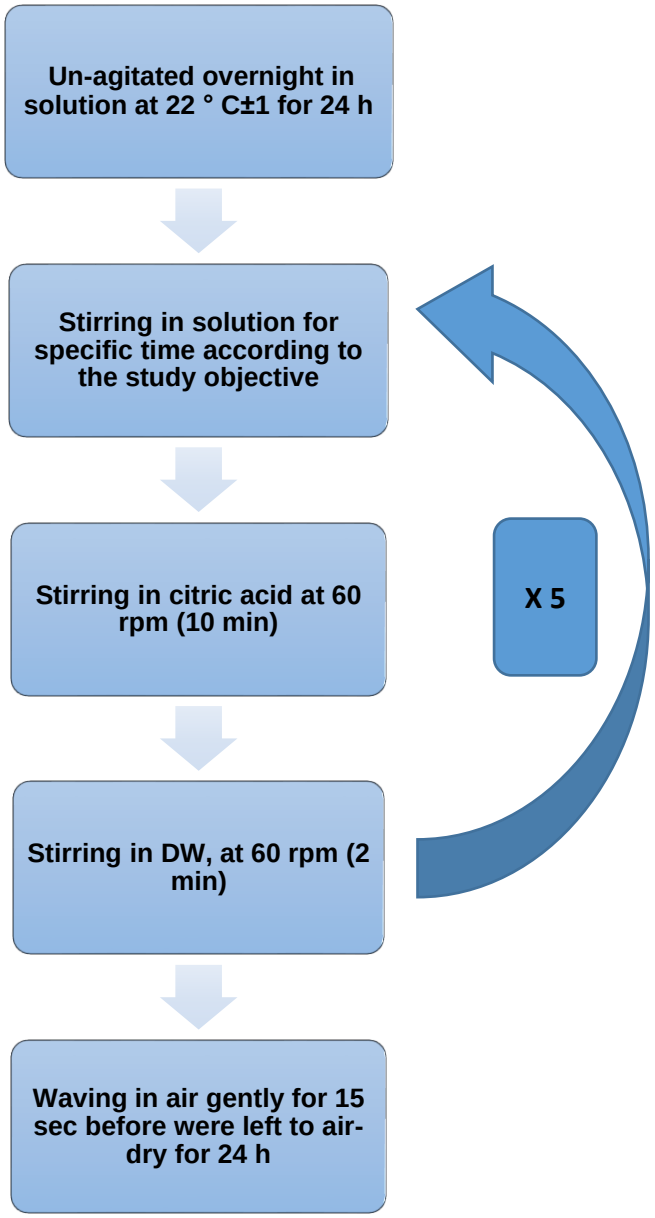


Figure 15: *A flow chart of the generic five erosion cycles model (advanced erosion)*

2.4 Measurements used in this thesis:

2.4.1 Surface non-contacting profilometer (SNCP)

Enamel specimens were air-dried for 24 hours after which the tape was removed for profilometric measurements. Step height was measured using a surface non-contacting white light profilometer (SNCP) (Taicaan XYRIS 2000, Taicaan™ Technologies Ltd., Southampton, UK) as shown in (Figure 16). The hardware of the SNCP consists of a polychromatic light source, an optical stylus sensor (sensor head), a spectrometer, a charge coupled device (CCD) sensor and a highly precise motion controller. The specifications are detailed in Table 16. The profilometer has gauge of 350 µm z axis distance over which the sensor can operate. When the specimen surface is brought to the centre of the sensor gauge range, the distance between the surface of the lens and the object surface is 12.7 mm. The light source was focused onto the specimen and the sensor head adjusted manually until the focused wavelength of light was in the area between 175 µm and 183 µm away from the surface (the middle of the sensors range). Once focused, a preview scan was taken which determined the area to be scanned, at medium precision with 12 lines, producing a basic overview of the surface profile. Once the reference and worn areas had been identified the specimens were scanned with full resolution, moved at a maximum speed of 25 mm/s of the X/y stage and a 10 x 10 µm x/y spacing using previously validated protocols (Austin *et al.*,2011; Mistry, 2016). The white light scanned across the surface of the specimen line by line in a raster pattern with a single line profile of data points recorded on X axis, creating a set of multiple parallel profile measurements, 10 µm apart from each other. Ten randomly selected step height measurements were taken from

each specimen and averaged to give a mean surface profile value. The amount of lost tissue was quantified as the height from the non-eroded reference area to the bottom of the worn area using surface analysis software (Boddies 2D v1.4 TaiCaan Technologies Ltd., Southampton, UK).

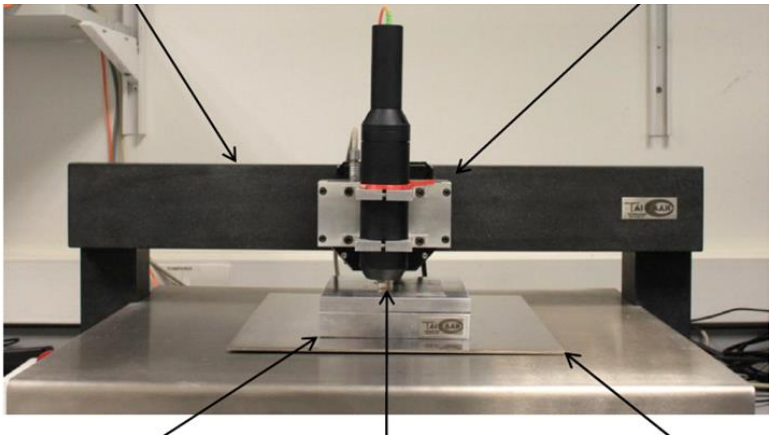


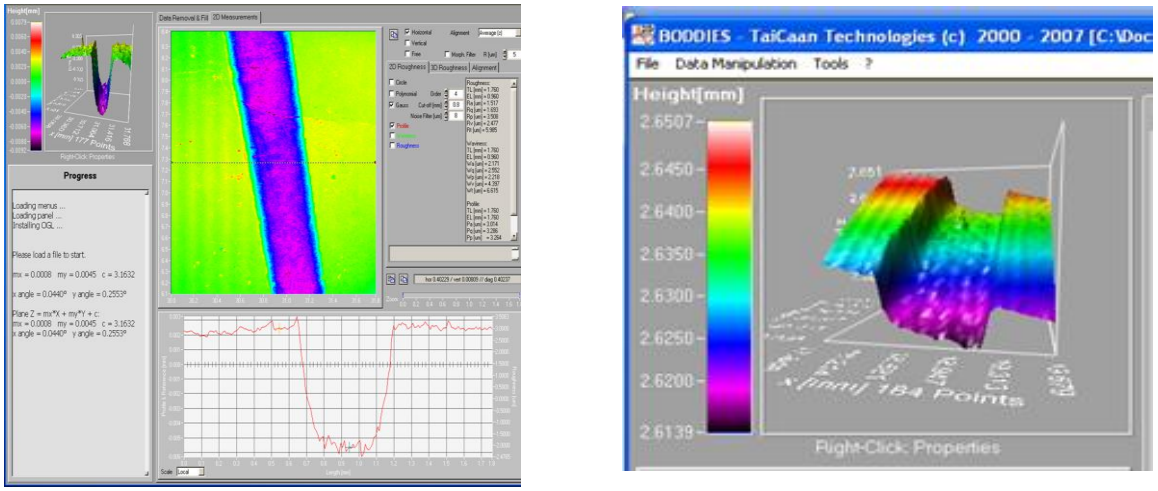
***Figure 16**: Image of the surface non-contacting profilometry.*

Light Source	Spot size	Vertical resolution	Gauge Range	Stand-off distance
Halogen	7 µm	10 nm	350 µm	12.7 mm

***Table 6:** The specifications of surface non-contacting profilometry (Austin et al., 2011).*

When the whole area of the specimen had been scanned, a file consisting of a cloud of individual data points in the ASCII format was saved as a '.tai' file extension. The data were collected from the deflected laser beam by the spectrometer and signals were determined by a charge coupled device (CCD) sensor as described in section 1.6.1. The software packages used for analysing the data was Taicaan XYRIS (Boddies 2D v1.4 TaiCaan Technologies Ltd., Southampton, UK) producing an image as shown in Figure

17 a. The yellow/green areas are the reference areas and the purple area in the centre is the eroded area. The total scanned area was chosen using STAGES™ software (Taicaan™ Technologies Ltd., Southampton, UK) and a 3D image of the eroded area is shown in Figure 17b.



a) b)

***Figure 17**:a) Step height caused after erosive challenge in vitro being analysed using BODDIES software. b) Representative 3D image of enamel specimen analysed after five cycle 10-minutes erosion using Taicaan XYRIS (Boddies) surface analysis software.*

2.4.2 Surface Microhardness (SMH)

Specimens were air-dried for 24 h after which the tape was removed for surface microhardness (SMH) measurments. SMH was measured at baseline (SMHb) and after immersion in solutions (SMHe) using a Knoop microhardness tester (Duramin-5Hardness Tester, Struers Inc., Rotherham, UK) as shown in (Figure 18).

Figure 18: *An image of the Knoop microhardness tester*

The surface microhardness (SMH) value of each specimen was determined by the average of five indentations with their long axis parallel to the vertical borders of the window, 100 µm intervals from each other, under a load of 100 g (981 mN) and a dwell time of 10 seconds. The SMH value of each indentation was determined by specialised software (Duramin-5Hardness Tester, Struers Inc., Rotherham, UK) through measuring the length of each indentation with an optical analysis system calculating hardness in Knoop units (KHN) (Figure 19). Typical Knoop SMH values for enamel range between 272 to 440 KHN (Meredith et al, 1996; Austin, 2011). Therefore, specimens with initial average SMH values range between 272 KHN and 400 KHN were only selected for the studies within this thesis. The surface microhardness change (SMHC) of each specimen was then calculated by subtracting the mean surface microhardness value after erosion (SMHe) from the mean surface microhardness value before erosion (SMHb) using the formula:

SMHC = (SMHb – SMHe).

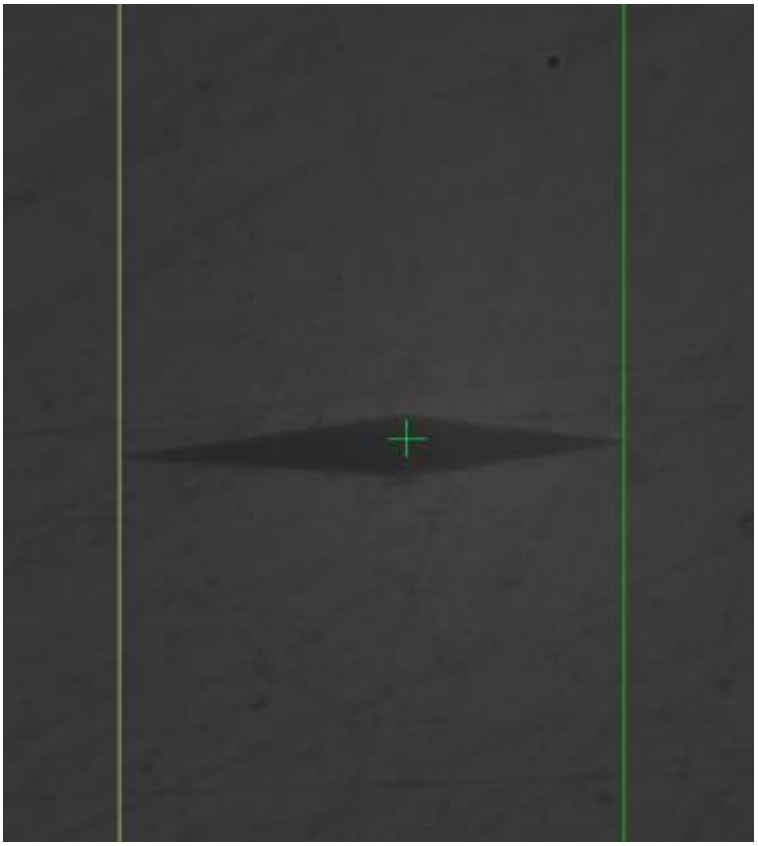


***Figure 19**: Image of an indentation on the sound enamel surface*

2.4.3 Atomic Force Microscopy (AFM):

Atomic force microscopy (Nanowizard 3, JPK Ltd, Cambs., UK) assessment within this thesis was performed by an investigator at the Centre for Oral Health Research, School of Dental Sciences, Newcastle University. AFM was used to analyse a random selection of the specimens (Nanowizard 3, JPK Ltd, Cambs., UK). Images of the specimen surfaces were undertaken in Quantitative Imaging™ mode (QI) in air with Si_3N_4 high spring-constant cantilevers (ACTA, AppNano, USA) calibrated using the dedicated JPK software spring constant measurement procedure. QI mode was used because it allowed the simultaneous measurement of surface topography and mechanical properties by conducting nanoindentation measurements at each pixel. The AFM was equipped with a piezoelectric scanner and cantilever

probe of 10 µm sharp tip. The specimen surfaces were probed using the sharp tip attached to the flexible cantilever. The tip was moved in such way that the tip moves back and forth across the specimens and tracks the surface features. Two areas of 50 × 50 μm^2 on each specimen, one eroded and one non-eroded, were scanned at a rate of 0.9 Hz with a resolution of 256 × 256 pixels (256 lines/specimen) with a range of 15 µm in the z-direction. The 'top to bottom' acquisition time for each image was 9 minutes. From the QI images three roughness parameters: number average roughness (Ra), root-mean square roughness (Rq) and peak-to-valley roughness (Rt) were measured. In addition, stiffness was measured at each pixel.

2.4.4 Bicinchoninic acid assay (BCA)

This method was used to quantify the protein amount in the eluted AEP samples by measuring the absorbance value of the eluted samples. The AEP samples were prepared into microtiter plates (96-wells, Fisher Scientific, Leicestershire). Two 200 µL samples of BSA standard solution were pipetted into two top wells of the first two plate columns using a dropping pipette. A 100 µL of DW was then pipetted into seven wells down the plate in the first two columns. Two-fold serial dilution was made by transferring 100 µL of BSA from the top well down the plate until reaching the second to last row of the first two columns. This was left as water blank sample or zero which was used as a reference. All wells in the first columns were at a final volume of 100 µL. The AEP samples were diluted in water at 1:10 in duplicate to a final volume of 100 µL. The BSA working reagent was then prepared according to manufacturer's instructions (50:1; A:B) in a way that 200 µl was pipetted into each well

including the standard solution wells, blank solutions wells and AEP samples wells making a total volumes of 300 µL.

A spectrophotometer employing a UV-visible light (BioRad laboratories Ltd, Hemel Hempstead, UK) was used to measure the density of solutions or the intensity of transmitted light, known as the absorbance, of all samples including standard solutions, blank solution and AEP samples at 562 nm. A calibration curve was constructed by plotting the line-of-best-fit of the absorbance scores of BSA and blank. Next, the intensity of the transmitted lights for the standard solution was measured before calculating the absorbance of the unknown solution. The calibration curve was used to determine the concentration of the unknown AEP samples.

2.4.5 Sodium dodecyl sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

This method was used to separate the proteins in the AEP samples within this thesis. As explained in section 1.6.2, proteins in biological samples are present in different sizes and charges with complex three dimentional structures. Polyacrylamide gel used in SDS-PAGE technique is made up of pores and serves as a porous medium that acts as a molecular sieve to separate proteins based on their mass but not their charges. This is because SDS-PAGE uses the ionic detergent SDS buffer which denatures and binds to proteins,making them uniformly negatively charged. The set-up of the SDS-PAGE system is shown in Figure 20. This process of separation using SDS-PAGE involves the following steps: solution preparation, apparatus set up, protein loading and separation as will be described in the next sections.

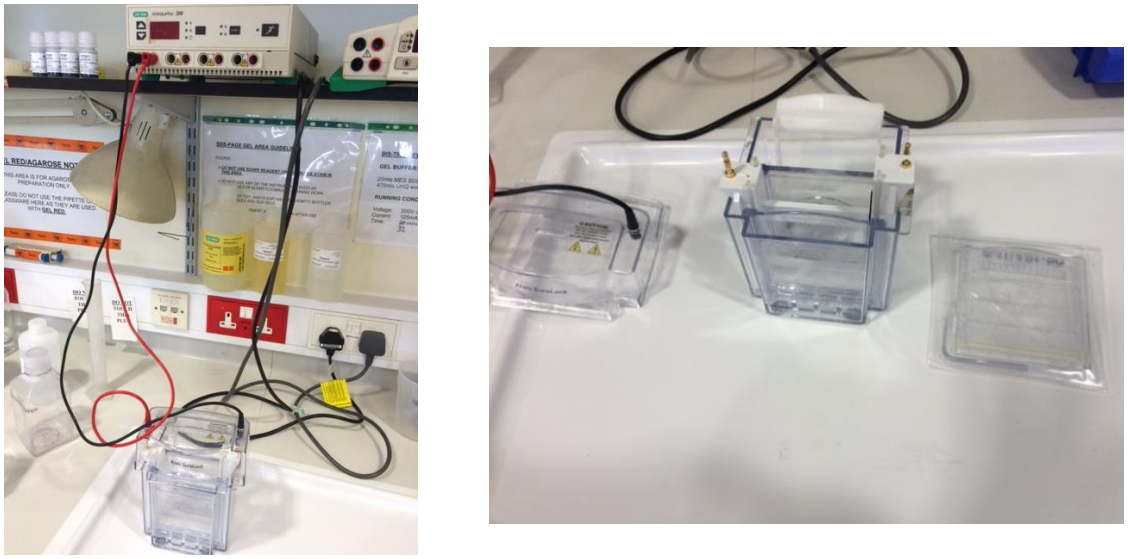


Figure 20: *Illustration of SDS electrophoresis setup*

2.4.5.1 Solutions preparation

To perform the SDS-PAGE, a number of solutions were prepared as will be explained in the following sections.

SDS Running buffer:

The running SDS (sodium dodecylsulfate) buffer (Novex Tris-Glycine SDS (20X) 500 ml, Carlsbad, California, USA) was prepared according to the manufacturer's instructions. It was used on the Novex Tris-Glycine gels to assist protein separation in their denatured state. This buffer was used to maintain an alkaline pH where SDS denatured and unfolded the proteins by binding the hydrophobic portions of the protein creating SDS-protein complexes of negatively net charge. The equal negative charge causes proteins to repel, breaking up some of their complex structures. This allowed proteins to migrate through the gel pores during separation process based on their size rather than their charges. 25 mL of a pre-mixed running buffer was

added into a 500 mL-beaker. 475 mL of sterile DW was added to make the volume to 500 mL. The liquids were measured using a graduated measuring cylinder.

Transfer buffer:

Transfer buffer was prepared according to the manufacturer's instructions. 25 mL of a pre-mixed running buffer was added into a 500 mL-beaker. 475 mL of sterile DW was added to make the volume to 500 mL. The liquids were measured using a graduated measuring cylinder.

Tris Tween Buffer solution (TTBS):

TTBS was prepared according to the manufacturer's instructions. It was prepared by adding 2.43 g of Trizma base, 2-Amino-2-(hydroxymethyl)-1, 3-propanediol (Sigma-Aldrich, GMbH, Steinheim, Germany), 9.0 g of sodium chloride (NaCl) into a 1-L laboratory flask. 700 mL of DW was added to the flask which was placed on a magnetic stirrer (Fisher Scientific, Magnetic hotplate stirrer, USA). The solution was stirred for 15 minutes before the volume was adjusted to 1000 mL. The solution was stirred for an additional 5 minutes until the components were fully dissolved. 1 mL of Tween 20 (Sigma-Aldrich, GMbH, Steinheim, Germany) was then added whilst stirring. Solids were weighed using an electronic analytical scale (Mettler Toledo, XS105 Dual Range Analytical Balance, Fisher Scientific UK Ltd, Loughborough, UK) and liquids were measured using a graduated measuring cylinder. The pH of the solution was adjusted to 7.6 using 32 % hydrochloric acid (HCL) solution (Fisher Scientific UK, Loughborough, UK) using a pH meter (Oakton pH 510 bench top meter, Eutech Instruments Pte Ltd, Singapore). TTBS was used for

washing nitrocellulose membranes and to prepare the antibody working solutions as will be described in sections 2.4.5.3 and 2.4.5.4.

DL-Dithiothreitol (DTT)

DTT, 1, 4-Dimercapto-2, 3-butanediol, was prepared according to the manufacturer's instructions. 1 M DTT stock solution was prepared by adding 1.54 g of Dithiothreitol (DTT, Sigma-Aldrich, GMbH, Steinheim, Germany) to 10 mL of sterile DW. The solid was allowed to dissolve completely in the sterile DW before 1 mL of the solution was aliquoted into a 2 mL tube and stored at -20°C. Stocks were kept up to six months. DDT solid was weighed using an electronic analytical scale (Mettler Toledo, XS105 Dual Range Analytical Balance, Fisher Scientific UK Ltd, Loughborough, UK) and the sterile DW was measured using a graduated measuring cylinder. DTT was used in the preparation of the protein-containing samples for SDS-PAGE. DTT was used as a strong reducing agent for reduction of protein disulfide (SH) covalent bonds between cysteine residues which can not be interrupted by SDS. DTT assist denaturing the tertiary and quaternary structure of any remaining proteins in the sample to disassociate into individual polypeptide subunits.

2.4.5.2 SDS-PAGE apparatus

SDS–PAGE was used for separating different proteins in the AEP samples of the *in vitro* and *in vivo* studies within this thesis. As shown in Figure 20, the SDS-PAGE apparatus was composed of a number of items including the inner and outer chamber, dummy glass plate, clamping plastic plate and electrical power supply. All items were washed thoroughly and the two glass plates of the inner chamber were assembled. The precasted gel (Invitrogen NuPAGE, Fisher Scientific, Paisley, UK), described in section 1.7.2, was used for the

protein separation procedure. The gel cast was unpacked and its disposable comb was gently removed and all lanes were rinsed with dH_2O. The tape on the bottom of the precast gel was removed before the precast gel was inserted into the inner chamber. The inner chamber was fixed into the casting frames of the outer chamber and was clamped with the clamping plastic plate against the casting frames of the outer chamber. The SDS buffer was prepared as above and shaken well before it was used to fill both chambers. The 15 lanes of each SDS-PAGE gel were loaded with protein samples and purified standards as is explained in the next section (2.4.5.3). After the gel lanes were loaded, they were checked visually to ensure they had been loaded evenly. The lid of the outer chamber was then attached to the positive (Anode) and negative electrodes (Cathode) of the inner chamber and was connected into an external electrical power supply set at a voltage of 200 V constant and current 125 mA for a dwell time of 32 minutes.

2.4.5.3 Protein loading and separation

In each SDS-PAGE gel, 8 lanes were occupied by the AEP samples and the other 4 lanes were occupied by a mixture of the four purified proteins for standards of known concentration. In each gel, prepared protein samples (15 µl each) were loaded carefully to the precast gel using loading pipettes. The mixture of the purified standards was prepared from mucin5b (156 µg/mL), albumin (1 µg/mL), CA VI (140 µg /mL) and statherin (382 µg/mL) as described in section 2.5.2.5.

2.4.6 Western blot

2.4.6.1 Protein Transfer

Western blotting was used to transfer proteins from the SDS-PAGE gels onto a nitrocellulose membrane. The western blot apparatus composed of a number of items: two metal plates that make up the inner chamber, a casting frame of the outer chamber, a clamping plastic plate and six spongy pads. The set of the inner chamber and the casting frame of the outer chamber were washed thoroughly. Two filterpapers (VWR International Ltd, Leicestershire, England) and a nitrocellulose membrane (Bio-Rad Laboratories Ltd. Hertfordshire, England) were prepared by cutting equal size pieces using a pair of well washed scissors. The cut filterpapers and nitrocellulose membranes were soaked in the transfer buffer. The gel was removed from the gel cast using an opening key and was also soaked in the transfer buffer. The gel and membrane were sandwiched between the spongy pads, filterpapers and two plates of the inner chamber in a specific order as shown in Figure 21.

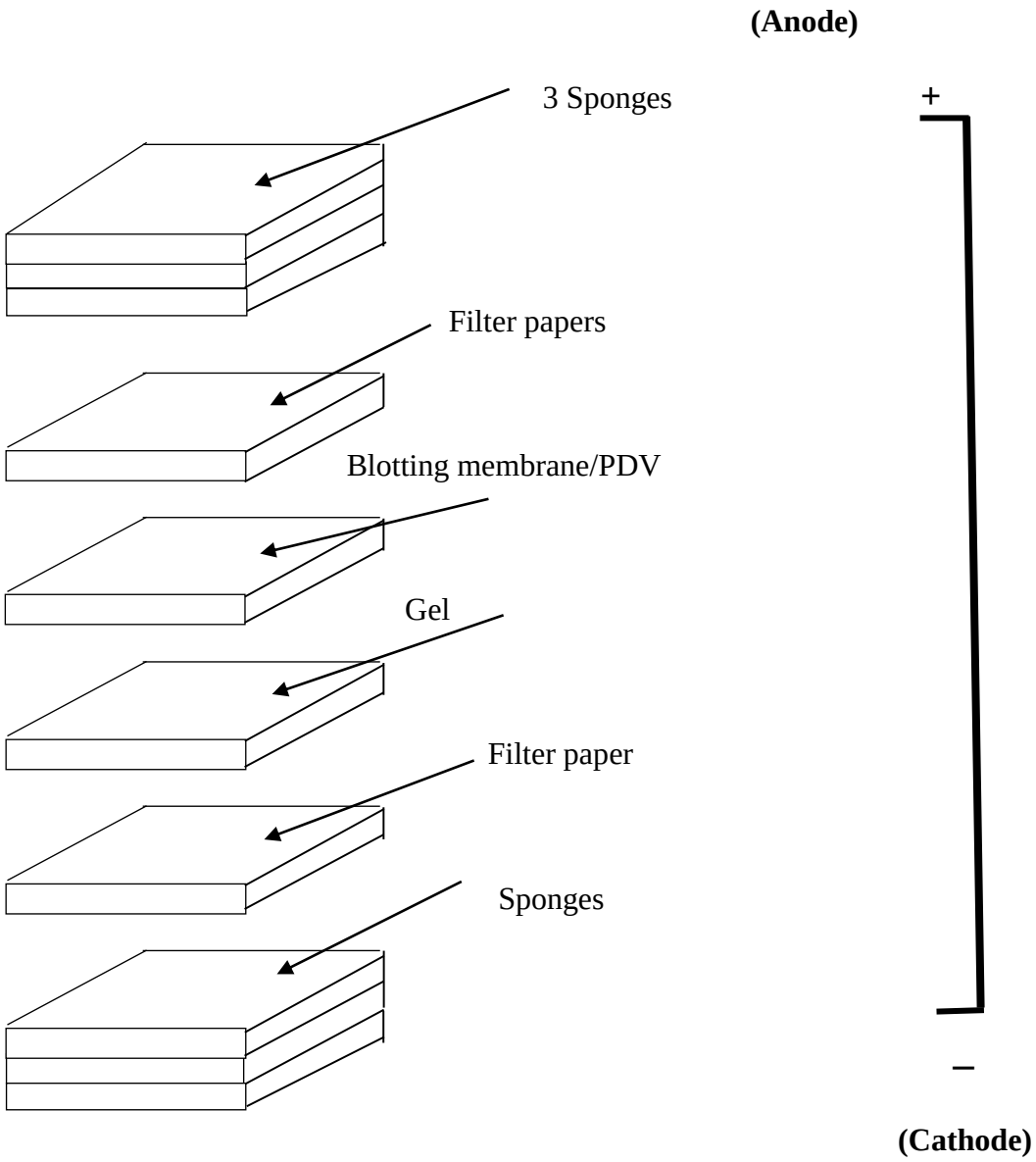


Figure 21: *Illustration of the sandawitch technique of western blot*

Step by step details of the western blot protocol is shown in appendix VII. The two plates of the inner chamber were then assembled tightly against the casting frames of the outer chamber. The lid of the outer chamber was then attached to the positive (anode) and negative electrodes (cathode) of the inner chamber before they were clamped with the clamping plastic plate and was connected into an external electrical power supply set at a voltage of 30 V

constant and a current of 160 mA for a dwell time of 60 minutes. Next, nitrocellulose membrane was then taken out and was immediately incubated in a fluorescein isothiocyanate preparation as shown in the next section.

2.4.6.2 Fluorescein isothiocyanate (FITC)

FITC stain was prepared by adding 1 mg of fluorescein isothiocyanate powder, one mL anhydrous dimethyl sulfoxide (DMSO) and 50 mL of bicarbonate buffer. The carbonate buffer was prepared by adding 5.3 g of sodium bicarbonate ($NaHCO_3$) in 500 mL of DW and adjusted to a pH value of 9.6. The fluorescent component of the stain (FITC) was used for visualisation and confirm the presence of proteins on the nitrocellulose membranes. The nitrocellulose membranes contained proteins was incubated in the prepared FITC solution for 20 minutes and was continually stirred with an orbital shaker (Bibby Scientific, Staffordshire, UK). The nitrocellulose membranes were then taken out and was exposed onto ChemiDoc MP imaging analysis (Bio-Rad) as will be described in 2.4.6.4.

2.4.6.3 Immunoblotting and immunodetection

The identification of specific antibodies was achieved after the separation and blotting of proteins as explained above. Proteins contained in the nitrocellulose membranes were immunolabelled to assess the presence, the quality and quantity of proteins transferred onto the nitrocellulose membranes. Using a sterile razor, each nitrocellulose membrane was cut transversely into four sections corresponding to the specific protein of interest: mucin5b, albumin, CA VI and statherin. The cut nitrocellulose membranes were then blocked in TTBS (pH 7.6) for 1 hour before membranes were probed with the primary

antibodies for 1 hour at room temperature (± 22 ºC) to identify the presence of the protein of interest. Specific primary antibodies (mono- or polyclonal) bind to "their" band of proteins which had previously transferred into the nitrocellulose membranes. Unspecifically binding antibodies were removed by washing the cut nitrocellulose membranes with detergent-containing buffers (TTBS). Additionally, unspecific binding was carried out by incubating the blot in skim milk before the addition of specific antibodies.

Next, nitrocellulose membranes washed in TTBS for 15 minutes (5 minutes X 3 times) and was then incubated with the required secondary antibody for 1 hour at room temperature (± 22 ºC). A final 15-minute wash in TTBS was completed before the membranes was developed with the western blotting enhanced chemiluminescent (ECL) substarte as described in section 1.6.3. The primary antibody was applied first, which was then recognised by a secondary antibody conjugated with HRP. Detailed steps of the immunoblotting protocol is shown in appendix VII. The developed nitrocellulose membrane was then exposed onto ChemiDoc MP imaging analysis (Bio-Rad) as is shown in 2.4.6.4.

2.4.6.4 Imaging analysis:

The densitometric chemiluminescence western blot data were then analysed using chemiDoc MP imaging analysis (Bio-Rad Laboratories Ltd., Hertfordshire, UK). It was used to quantify the light intensity of the chemiluminescent reaction and exposure times optimised to prevent pixel saturation (Figure 22). The amounts of proteins on the blotted nitrocellulose

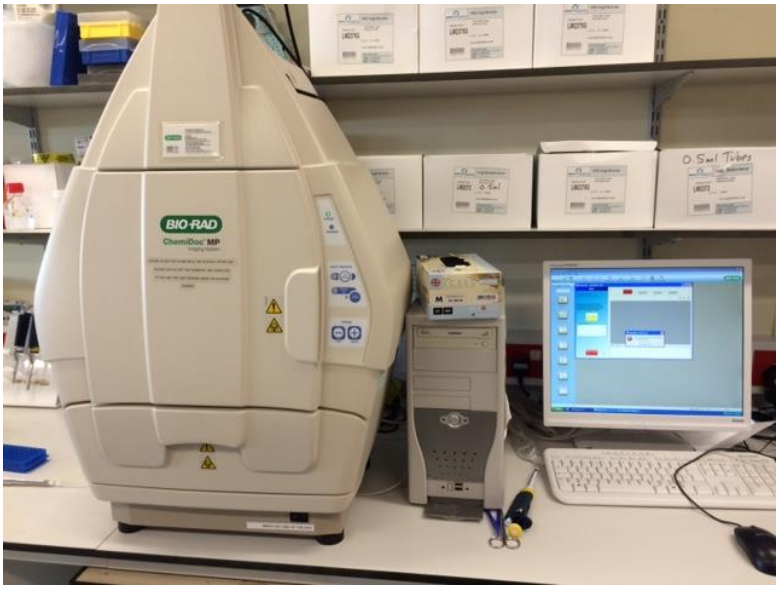


***Figure 22**: ChemiDoc MP imaging system set up*

membranes were quantified using tools of ImageLab software version 4.1 (Bio-Rad Laboratories Ltd., Hertfordshire, UK) to select and determine the background-subtracted density of the bands in all the gels using purified protein standards of known concentration. It applies white light conversion screen and the silver stain (visible stain) application. In each gel, the readings for volume intensities and absolute quantities of the four standard proteins were obtained and used to generate a calibration curve using the linear formula. This formula was used to calculate the amount of each protein in the *in vitro* and *in vivo* protein samples. This allowed accurate and normalised quantitation of the target proteins by means of densitometric analysis. The proteins bands of the purified standards on different SDS-PAGE gels were used to assess reproducibility. The coefficients of variation (CV) for volume intensities of standards bands on different SDS-PAGE gels was also used as reported in previous studies (Reed *et al.*, 2002; Pramanik *et al.*, 2010). The CV is defined as the standard deviation of the repeated measurements of the same sample divided by the mean, with the result often reported as a

percentage (Reed *et al*., 2002). A twofold difference in measurements of the same sample was reported to be widely regarded as the upper limit on acceptable variability (Reed *et al.*, 2002).

2.4.7 Inductively coupled plasma- Mass Spectroscopy (ICP-MS)

The ICP-MS system used in the studies within this thesis (Nexion 350D, Perkin Elmer, Waltham, USA) had a plasma torch through which the sample usually travels leading to the sample being dried, vaporised, atomised and ionised. In this thesis, ICP-MS was used to analyse the amount of calcium (Ca^{2+}) and phosphorus (P) ions present in the *in vitro* AEP samples [WMS (n=10) and from PS (n=10)] (Chapter 4 and 5). One mL of each sample (1:1000 dilution) needed to be provided, ideally in a falcon type tube to fit the autosampler and racks used in the system. All samples were sent off for analysis which was carried out by the Mass Spectrometry Facility, King's College London, Franklin-Wilkins Building, 150 Stamford St, London, UK. One difficulty with using this method to analyse the amounts of Ca^{2+} and P was the large dilution factor compared to the small size of AEP sample relative to potential sources of Ca^{2+} and P in the 1 mL sample. To solve this, a number of additional experimental blank samples (SDS and DW with no AEP samples) were included in the analysis to help work out if there was any significant background Ca^{2+} and P in the experimental system.

2.4.8 Proteomics

Two samples of *in vitro* AEP, one from WMS and one from PS, were prepared by eluting the *in vitro* AEP from two enamel surfaces, one enamel specimen each.

The *in vitro* AEP was eluted from the enamel surfaces using 0.5 % SDS and filterpapers as described in section 2.3.3. These two samples were sent off for proteomic analysis which was carried out by the Centre of Excellence for Mass Spectrometry, King's College London, Institute of Psychiatry, Psychology and Neuroscience.

Liquid-chromatography- MS systems (LC-MS) LC/MS/MS

Liquid chromatography coupled to mass spectrometry was used for the proteomic analysis in many steps as follows:

Stacking Gel Preparation

An SDS-PAGE stacking gel (4% stack/20% resolve) was prepared for 30 minutes (150 volts; 35 mA; 5 watts). AEP samples were added until the protein contents of the samples just entered the 20 % gel phase. Proteins were fixed in a solution of 7 % acetic acid/ 40 % methanol for 30 minutes then stained overnight with colloidal Coomassie brilliant blue. After destaining with 2 % acetic acid and 25% methanol the gel was washed with water and the protein bands excised.

Enzymatic Digestion

In-gel reduction, alkylation and digestion with trypsin were performed on gel band samples prior to subsequent analysis by mass spectrometry. Cysteine residues were reduced with dithiothreitol and derivatised by treatment with iodoacetamide to form stable carbamidomethyl derivatives. Trypsin digestion

was carried out overnight at room temperature after initial incubation at 37 º C for 2 hour.

LC-MS/MS

Peptides were extracted from the gel pieces by a series of acetonitrile and aqueous washes. The peptides extract was pooled with the initial supernatant and lyophilised. Each sample was then resuspended in 10 μL of 50 mM ammonium bicarbonate [$(NH_4)HCO_3$] and analysed by LC/MS/MS. Chromatographic separations was performed using an EASY NanoLC system (ThermoFisherScientific, UK). Peptides were resolved by reversed phase chromatography on a 75 μm C18 column using a three step linear gradient of acetonitrile in 0.1 % formic acid. The gradient was then delivered to elute the peptides at a flow rate of 300 nL/min over 120 minutes. The eluate was ionised by electrospray ionisation using an Orbitrap Velos Pro (ThermoFisherScientific, UK) operating under Xcalibur v2.2. The instrument was programmed to acquire an automated data-dependent switching mode, selecting precursor ions based on their intensity for sequencing by collision-induced fragmentation using a Top20 CID method. The MS/MS analysis was conducted using collision energy profiles that were chosen based on the mass-to-charge ratio (m/z) and the charge state of the peptide.

Database Searching and gel band analysis

Raw mass spectrometry data were processed into peak list files using Proteome Discoverer (ThermoScientific, San Jose, CA, USA, V 1.4) as shown in Figure 23.

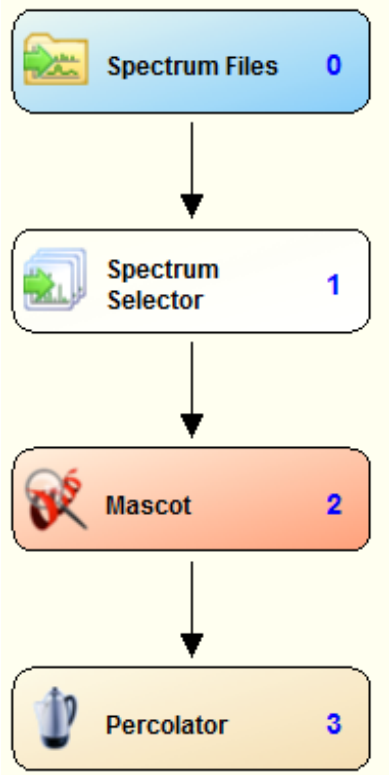
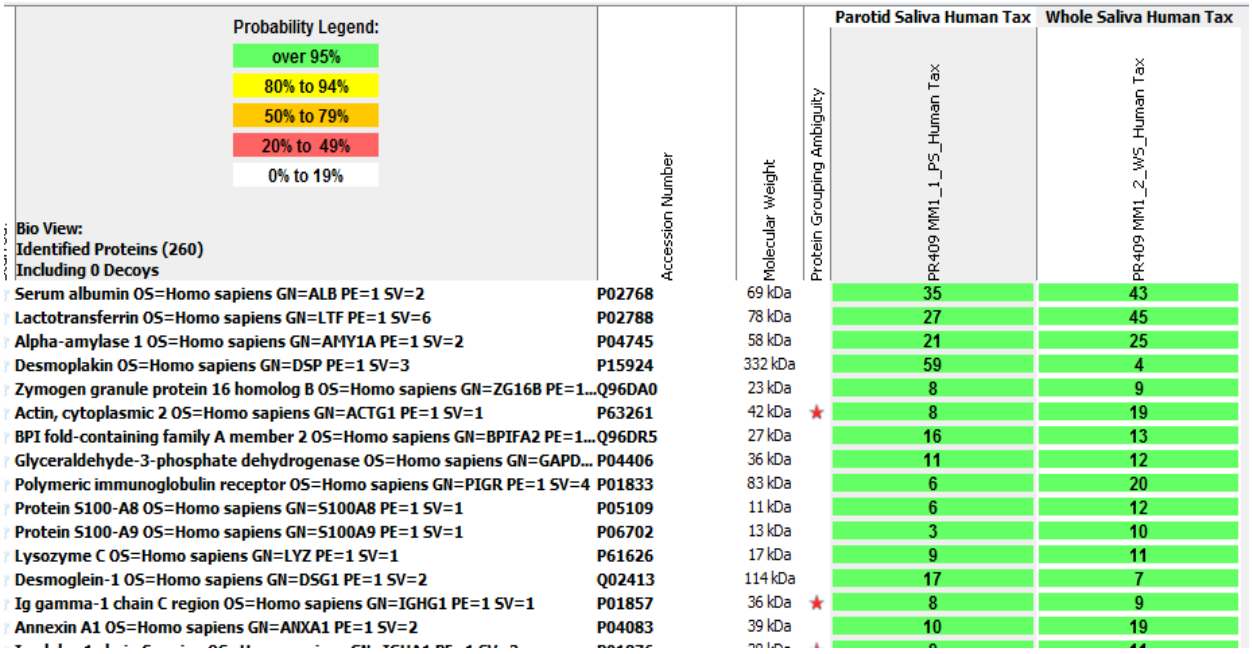


***Figure 23**: Proteome discoverer nodal workflow for raw data processing*

The Uniprot database selecting Human Taxonomy (HT) was used to search the samples in order to detect proteins from the host origin of the sample. Database generated files were uploaded into Scaffold 4 (v4.4.5) software (www.proteomesoftware.com) to create a .sfd file (PR409 MM1_1_1_2 HT 06012016; Figure 24).



Probability Legend:
over 95%
80% to 94%
50% to 79%
20% to 49%
0% to 19%

Bio View:
Identified Proteins (260)
Including 0 Decoys

	Accession Number	Molecular Weight	Protein Grouping Ambiguity	Parotid Saliva Human Tax	Whole Saliva Human Tax
				PR409 MM1_1_PS_Human Tax	PR409 MM1_2_WS_Human Tax
Serum albumin OS=Homo sapiens GN=ALB PE=1 SV=2	P02768	69 kDa		35	43
Lactotransferrin OS=Homo sapiens GN=LTF PE=1 SV=6	P02788	78 kDa		27	45
Alpha-amylase 1 OS=Homo sapiens GN=AMY1A PE=1 SV=2	P04745	58 kDa		21	25
Desmoplakin OS=Homo sapiens GN=DSP PE=1 SV=3	P15924	332 kDa		59	4
Zymogen granule protein 16 homolog B OS=Homo sapiens GN=ZG16B PE=1...	Q96DA0	23 kDa		8	9
Actin, cytoplasmic 2 OS=Homo sapiens GN=ACTG1 PE=1 SV=1	P63261	42 kDa	★	8	19
BPI fold-containing family A member 2 OS=Homo sapiens GN=BPIFA2 PE=1...	Q96DR5	27 kDa		16	13
Glyceraldehyde-3-phosphate dehydrogenase OS=Homo sapiens GN=GAPD...	P04406	36 kDa		11	12
Polymeric immunoglobulin receptor OS=Homo sapiens GN=PIGR PE=1 SV=4	P01833	83 kDa		6	20
Protein S100-A8 OS=Homo sapiens GN=S100A8 PE=1 SV=1	P05109	11 kDa		6	12
Protein S100-A9 OS=Homo sapiens GN=S100A9 PE=1 SV=1	P06702	13 kDa		3	10
Lysozyme C OS=Homo sapiens GN=LYZ PE=1 SV=1	P61626	17 kDa		9	11
Desmoglein-1 OS=Homo sapiens GN=DSG1 PE=1 SV=2	Q02413	114 kDa		17	7
Ig gamma-1 chain C region OS=Homo sapiens GN=IGHG1 PE=1 SV=1	P01857	36 kDa	★	8	9
Annexin A1 OS=Homo sapiens GN=ANXA1 PE=1 SV=2	P04083	39 kDa		10	19

***Figure 24**: Scaffold sample view representing protein identifications from the two gel bands following database searching against the Human portion of the Uniprot database.*

Scaffold allowed statistical filtering of the data at the protein and peptide level. These filters were applied to the data at various confidence levels for protein identification with a minimum of three peptides, and also a lower stringency of one peptide (Table 7).

High stringency filters of 95 % confidence interval (CI) for minimum protein and 0 % CI for peptide values was applied. Any protein that was above this identity threshold was deemed significant. The protein identifications from the individual database searches were visualised in Figure 23. Number of protein identifications was reported with a minimum of three peptides (bold numbers; Table 7). High levels of human keratin proteins, which were thought to be contaminating, were detected. These proteins were removed from the Scaffold data file.

Filter Stringency	PS (3pep/1pep)	WMS (3pep/1pep)
99%	88/128	133/178
95%	**88**/138	**133**/221
80%	92/158	133/235
50%	94/223	135/267
20%	95/590	135/319

Table 7*: Qualitative protein assignments from sequence data following LC/MS/MS analysis.*

2.5 Investigated AEP proteins, antibodies and purified standards.

2.5.1 AEP proteins investigated in this thesis

Four targeted proteins in *in vitro* and *in vivo* AEP were investigated in this thesis. These were mucin5b, human serum albumin, CA VI and statherin. Human serum albumin will be referred to as albumin throughout this thesis.

2.5.2 Primary and secondary antibodies

Protein antibodies used for the studies within this thesis were: mucin5b, albumin, CA VI and statherin antibodies. Information of these antibodies is listed in (Table 8). They were used in the protein analysis studies to identify the proteins of interest in the *in vitro* and *in vivo* AEP samples. Upon delivery, aliquots of 0.2 mL were taken to avoid the repeatitive freeze / thaw cycle and they were stored at -20°C until use during experiments. The immunoblotting

of nitrocellulose membrane with the primary antibodies was followed by blotting the membrane with the appropriate secondary antibodies both diluted in TTBS as described in section 2.4.6. The secondary antibodies against mucin5b (Polyclonal antimouse, Dako UK Ltd, Cambridgeshire, UK) diluted 1:1000, albumin (Polyclonal antimouse, Dako UK Ltd, Cambridgeshire, UK) diluted 1:1000, CA VI (Polycolonal rabbit anti-Goat, Dako UK Ltd, Cambridgeshire, UK) diluted 1:2000 and statherin (peroxidase-conjugated donkey anti-Sheep, Jackson ImmunoResearch laboratories, West Grove, USA) diluted 1:2000 were also used to assess the presence of four investigated proteins in the AEP samples.

Primary antibodies	Mucin5b	Albumin	Carbonic anhydrase VI (CA VI)	Statherin
Product name and source	Polyclonal Anti-Mucin5b, antibody produced in mouse, clone 8C11, ascites fluid.	Monoclonal Anti-Albumin, antibody produced in mouse, HSA-11, ascites fluid.	Polyclonal Goat IgG	Anti-Statherin antibody, Sheep polyclonal to Statherin
Lot number	--------	071M4813	XUX016071	ab97950
Concentration	1 µg/uL	31.2 mg/mL	0.5 µg/mL 0.5 µg/mL	0.5 µg/mL
Wester blotting dilution	1:1000 dilution	1:5000 dilution	1:1000	1:1000
Molecular Weight (KDa)	590	70	42	7
Manufacturer	GENTAUR Ltd. 1910 kampenhout, Belgium	Sigma-Aldrich,Saint Louis,MO 63103 USA	R&D Systems UK Abingdon, OX14 3NB, UK.	Abcam, Cambridge, UK

***Table 8**: List and information of primary antibodies used in this PhD*

2.5.3 Purified protein standards

Table 9 gives background information on the purified protein standards used for the studies within this thesis. This information includes their concentration and molecular weight. Upon preparation, aliquots of 0.2 mL were taken to avoid the repeatitive freeze/thaw cycle and were stored at -20°C until needed.

Protein standard	**Mucin5b ***	**Albumin**	**Carbonic anhydrase VI**	**Statherin**
Product name	Kind gift from Claes Wickström*	Purified human serum albumin	Native, human milk	Purified in our laboratory from parotid saliva (Details are in section 2.5.3.4)
Concentration for wester blotting	156 µg/ml	1 µg/mL	140 µg /mL	382 µg/mL
Lot number	------	XA60312-P	1004001	------
Molecular Weight (KDa)	600- 2100	70	42	7
Manufacturer	Purified in the laboratory using density-gradient centrifugation technique (Wickstro¨m and Svensa¨ter, 2008).	Alpha Diagnostic IntL. Inc, San Antonio, Texas 78244 USA	Jena Bioscience D-07749 Jena Germany	Prepared in our laboratories as described previously (Proctor *et al*., 2005)

Table 9*: List and information of purified protein of standards used in this PhD.*

*Kind gift from the department of Oral Biology, Faculty of Odontology, Malmö University, Malmö, Sweden.

2.5.3.1 Mucin5b

Purified mucin5b was provided as a kind gift from the department of Oral Biology, Faculty of Odontology, Malmö University, Malmö, Sweden. Mucin5b was purified as has been described in previous studies (WICKSTRÖM *et al.*, 1998; Wickström and Svensäter, 2008) using density-gradient centrifugation techniques with caesium chloride. Gel chromatography with a dissociative solution was tried but using this method had drawbacks such as the relatively small volumes that can be put on the column and the possible contaminants. High molecular mucins such as mucin5b can be isolated from submandibular and sublingual glands as well as from WMS by an ultracentrifugation procedure (Amerogen *et al.*, 1987). A range from 12 mg to 28 mg dry weight mucin can be isolated from 100 mL clarified WMS using ultracentrifugation procedure (Amerogen *et al*., 1987).

2.5.3.2 Albumin:

Human serum albumin (albumin) was provided as a 1 g powder (Sigma-Aldrich, Gillingham, UK). It was used for the quantification of the albumin protein present in the *in vitro* and *in vivo* protein samples of the studies within this thesis. An albumin solution was prepared by adding albumin powder to DW at 1 µg/mL concentrations. One milligram of albumin powder was measured using an electronic analytical scale (Mettler Toledo, XS105 Dual Range Analytical Balance, Fisher Scientific UK Ltd, Loughborough, UK). The weighed amount was added to 1 L of DW to prepare the albumin protein solution. Initially, 500 mL of DW was added to a 1 L volumetric flask. The weighed albumin powder was added into the flask immediately after weighing. After the weighed albumin powder was added to the flask, the solution was

continually stirred for 30 minutes with a magnetic stirrer (Fisher Scientific, Magnetic hotplate stirrer, USA) to allow the components to be dissolved in the DW. The volume was then increased to 1 L by adding DW using a graduated measuring cylinder while the solution was continuously stirred for at least 2 hour until fully dissolved.

2.5.3.3 Carbonic anhydrase VI (CA VI)

The product was supplied dry as CA VI full length protein (Jena Bioscience, D-07749 Jena, Germany). This was used as a positive control in SDS-PAGE and western blot for the quantification of the CA VI protein present in the *in vitro* and *in vivo* protein samples of the studies within this thesis.

The purified CA VI protein used within this thesis was a novel CA VI which was purified from human saliva with inhibitor affinity chromatography followed by ion-exchange chromatography (Jena Bioscience, D-07749 Jena, Germany). The molecular weight was determined to be 42 kDa on SDS-PAGE. Each molecule of the salivary enzyme had two N-linked oligosaccharide chains which were cleaved by endo-beta-N-acetylglucosaminidase F but not by endo-beta-N-acetylglucosaminidase H, indicating that the oligosaccharides are of a complex type. Some other human CA VI full length proteins appears at 60 kDa because they possess a Tag for the purification process of the protein.

2.5.3.4 Statherin:

Statherin standard was prepared by the author of this thesis. Fresh human parotid saliva was collected from four healthy individuals (S1- S4) at King's College London as described in section 2.2.1.2. Statherin was purified using two methods. The first method of purification was achieved by fractioning

parotid saliva using hydroxyapatite (HAP) adsorption techniques (Jensen *et al*., 1991). In this method, HAP powder (Sigma-Aldrich Company Ltd., UK) was incubated in parotid saliva at two concentrations: saliva ratios of 1 mg/mL and 5 mg/mL at room temperature 22 ± 1 ° C. The mixture was centrifuged at 2,000 g for 15 minutes at 4 ° C. The sediment was washed 3 times with 0.1 M NaCl (pH 7.5) by centrifugation. The supernatant from each centrifugation, containing unadsorbed and/or weakly adsorbed proteins, was pooled. The final sediment containing tightly adsorbed proteins, was redissolved in 0.2 M EDTA (pH 7.5) overnight at 25 ° C. The desorbed proteins were collected and kept in a 1.5 mL universal tube at 4 ° C.

The second method of statherin purification was the air-saliva interface technique adopted from previous studies (Proctor *et al.*, 2005; Harvey *et al.*, 2011). In this method, statherin was purified from the film formed at the air interface with parotid saliva after 1 hour. In this, 10 mL of parotid saliva was collected from each individual and was distributed into 10 petri dishes 35 mm, 1 mL each. Fresh parotid saliva was left in petri dishes for 1 hour until the statherin layer was seen on the saliva-air interface. The residual saliva underneath the film was pipetted to leave only the statherin layer in place in the dishes. Three- 100 µL washes of DW were added to each dish to wash off the residual of the protein from the statherin layer. All water was sucked each time and statherin layer was left in place. A 100 µL wash of 10 mM EDTA was then added to solubilise the statherin layer which was then separated and added into a 1.5 mL universal tube in order to be used for the studies within this thesis. The four collected statherin samples from (S1-S4) subjects were then loaded to a SDS gel (Novex, Thermo Fisher Scientific Inc, UK) with equal

volumes, 15 µL each. The gel was stained with Coomassie Brilliant Blue and antibody detection to test for identity and purity.

Figure 25 shows the Coomassie Brilliant Blue image that resulted from SDS-PAGE of the purified statherin from four different individuals. Lanes 1-4 show the statherin samples purified using the air-saliva film technique, whereas lanes 5-6 show the statherin samples purified using the HAP adsorption technique. As can be seen from Figure 25, the fractioning of parotid saliva using HAP adsorption technique resulted in less purified statherin than the air-saliva interface film technique.

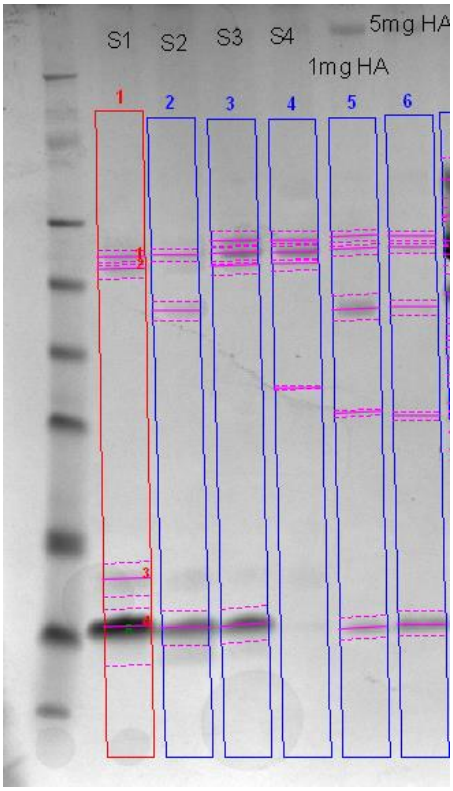


Figure 25: *Coomassie Brilliant Blue-stained image of purified statherin using air-saliva interface film and HAP adsorption techniques.*

As statherin sample (S1) purified using the air-saliva interface film technique showed the most abundant statherin content, it was selected to be used for

the studies within this thesis (Figure 25: band 4 of lane 1). As the image indicates, the statherin was not 100 % pure due to the presence of other protein bands (band 1-3), therefore, the percentage of its purity was quantified using tools of ImageLab software version 4.1 as described in 2.4.6. As shown in Table 10, the purified statherin constitutes 79.5 % of the total protein in the sample. The total protein in the purified protein sample from subject (S1) was estimated using BCA assay as was described in section 2.4.4.

Band No	Volume (Int)	Relative quantity	Band %
1	37	0.062418	**5.0**
2	27	0.045463	**3.6**
3	91	0.150369	**12.0**
4	608	1	**79.5**

***Table 10**: Quantifying the percentage of the purified statherin (band 4) in the total purified protein sample using tools of ImageLab software version 4.1.*

Figure 26 shows the standard curve used to calculate the total protein concentration using the following linear formula: **y = 2.9518x + 0.0704.** The standard curve was constructed using the absorbance values (Y-axis) against the concentration of total protein (X-axis) of the purified protein sample.

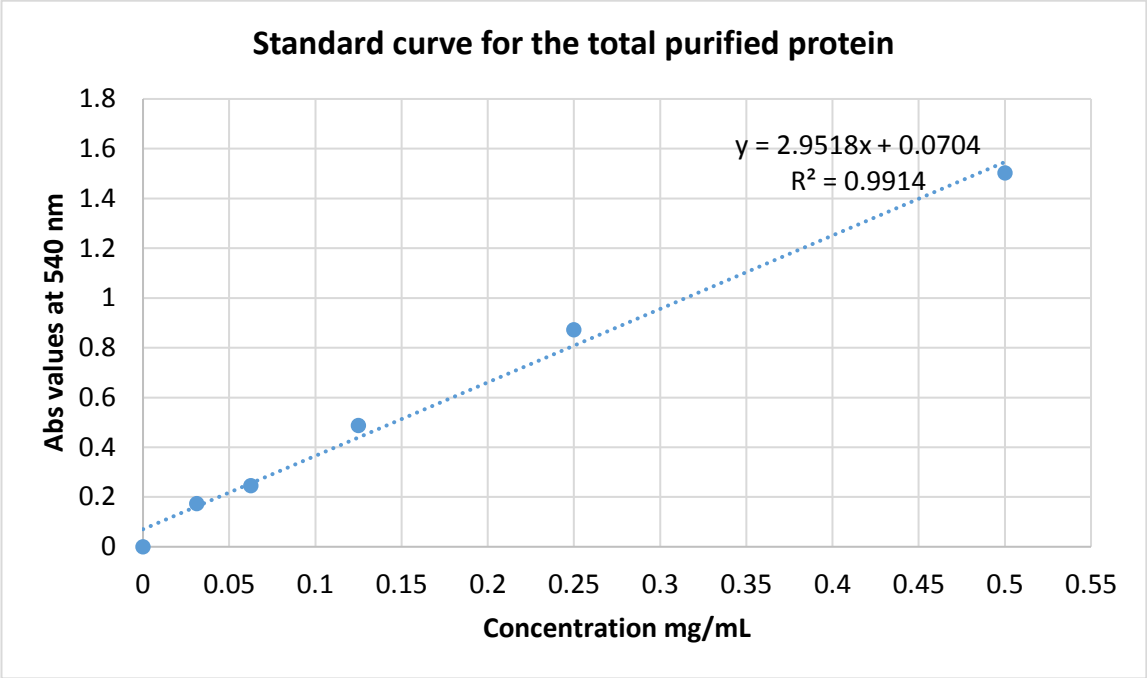


Figure 26: *The standard curve used for calculating the concentration of the total protein in the purified sample from freshly collected parotid saliva.*

The total protein concentration was calculated using the standard curve linear formula: Y = 2.9518x + 0.0704 where Y was the absorbance value and X was the total protein concentration (mg/mL). Y was calculated as 1.49 and therefore, X was 0.481 mg/mL (480.0 µg/mL). Therefore, the concentration of statherin in the purified sample was calculated as 79.5 % of the total protein as follows: X= (480.0 * 79.5)/100 = 382 µg/ml.

2.5.3.5 Preparation of the purified standards mixture:

A 300 µL mixture of the above four purified protein standards of known concentration was prepared to be used for all the protein studies within this thesis (Chapter 4,5 and 6). Each purified standard was defrosted at -4 Cº in

0.2 mL aliquots prior to use. Specific amount of each purified standard was defrosted and pipetted onto 1.5 mL tube. The volume of purified proteins used in the mixture were: mucin5b (10 µL), albumin (10 µL), CA VI (5 µL) and statherin (5 µL) to make a 30 µL mixture of the purified standards which was optimised to give a linear standard curve as will be described in section 2.6.2. This was duplicated ten times to produce a 300 µL to generate a sufficient amount of the purified standard mixture to be used for all the studies within this thesis. The concentrations of the purified standards used in the mixture were as shown in Table 9. These were mucin5b (156 µg/mL), albumin (1 µg/mL), CA VI (140 µg /mL) and statherin (382 µg/mL).

2.6 Training and development of *in vitro* models

2.6.1 Training and development of the erosion and saliva models used in this research

2.6.1.1 Introduction:

It is important to mention that the design of the laboratory model and the particular erosive cycles chosen influence the outcome. Although unfortunately detailed information is often omitted in the literature, most researchers provide the basic information required including the length of the experiment, number of cycles, pH and type of acid, time of immersion and the amount of the solution. In addition, preparation and measuring specimens in erosive wear studies requires sufficient training and practice owing to the highly sophisticated techniques required. In this thesis, some of these variables and requirements for the studies of erosive tooth wear were considered for validation and were mastered before commencing the actual studies.

A number of issues in erosion models required training and development. Firstly the method of mounting the enamel specimens needed to be developed to allow grinding and polishing. Enamel specimens are usually mounted in a supportive material to hold the specimens in place in order to allow grinding and polishing of the enamel specimens creating flat surfaces which is required by the erosion measurement techniques. In this regard, enamel specimens can be mounted in acrylic resin or self-curing bis-acryl composite which are two commonly used materials. As bis-acryl composite is costly, the two materials were compared and assessed for differences whilst carrying SMH

testing. This was to ensure that both materials were as equally effective at holding the specimens in securely.

In addition, training and calibration on the methods used for SMH testing and SNCP equipment were necessary prior to commencing the studies as described in sections 2.4.1 and 2.4.2 respectively. Validating the use of WMS for the *in vitro* model was also necessary.

2.6.1.2 Aims and objectives:

The aim of this section was to train the author of this thesis in SMH and SNCP measurment skills and to develop an *in vitro* erosion and saliva model.

The objectives were:

1. To compare two mounting materials; acrylic resin and a self-curing bis-acryl composite when subjected to SMH measurements.
2. To compare the microhardness and profilomtery measurements between two investigators, the author of this thesis and a senior investigator.
3. To assess an *in vitro* erosion protocol suitable for measurements using SNCP.
4. To compare the method of quantifying the step height between a manual extraction method and the imageJ software.

2.6.1.3 Material and methods

III. Comparison of the method of enamel specimens mounting and training:

Two mounting materials were investigated; acrylic resin (Dentsply Ltd, Surrey, UK) and self-curing bis-acryl composite (Protemp™4, 3M ESPE, Seefeld,

Germany) for their ability to secure the enamel specimens in place whilst subjected to SMH measurements. Acrylic resin was used to mount the enamel specimens as was described in section 2.1.4 above. The self-curing bis-acryl composite was used to mount enamel specimens using an aluminium cast (Syndicad Ingenieurbüro, München, Germany) as shown in (Figure 27) below and as was described in previous work (Austin *et al.*, 2011).

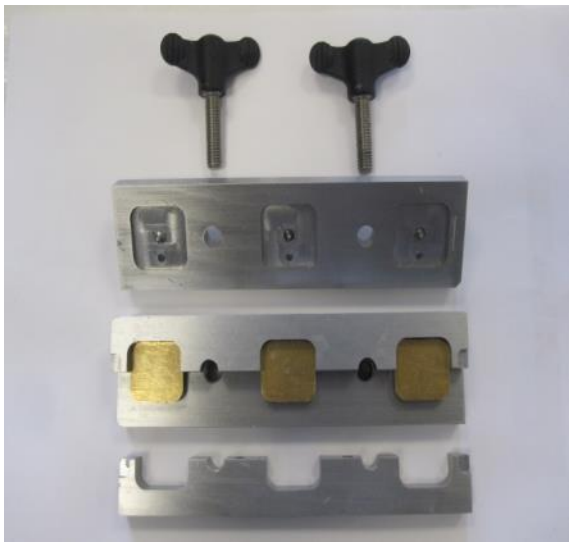


***Figure 27**: The aluminium cast, used to mount enamel specimens using self-curing bis-acryl composite*

The aluminium cast was dissembled and lubricated using Vaseline followed by a silicone mould release spray (DAS Silicone Mould Release aerosol, Electrolube). The cast was re-assembled to create a base with 3 wells for the enamel sections, which were then positioned with the buccal/lingual surface facing into the mould and the longest side of the enamel specimens parallel to the shortest side of the cast. The bis-acrylic composite was supplied as a two-paste cartridge system. The bis-acrylic composite was packed into the metal spaces on the enamel specimens. The metal spaces were filled before the bis-

acrylic composite set. Once all spaces were filled, the lid of the cast was reassembled with the body and tightly screwed.

Specimen preparation and testing:

Based on the power calculation as described in section 2.1.3, ten specimens were prepared for this study from human molar sound teeth. Sixteen specimens were prepared by sectioning eight buccal and eight lingual surfaces. Initial surface microhardness values (SMH) measured. The specimens with a SMH value between 272 KHN and 400 KHN were selected (Meredith *et al*., 1996; Austin *et al*., 2011; Lussi *et al*., 2011). Ten specimens were selected and eight were rejected. All specimens were prepared as described in section 2.1. Specimens were mounted in two different materials to produce two groups: acrylic resin and self-curing bis-acryl composite, 5 specimens each.

SMH measurements were taken as described in section 2.4.2 and they were carried out by two different PhD investigators; the author of this thesis and a senior investigator. The mean SMH values of enamel specimens mounted in two different materials was calculated and compared.

Statistical analysis:

Paired t test was used to compare the mean SMH between the two groups and within each group.

Results:

The result of this experiment is shown in Figure 28. There were no significant differences between the mean baseline surface microhardness values (SMHb) of the acrylic resin group and self-curing bis-acryl composite for both investigators ($p>0.05$). For the acrylic resin group, the SMHb assessed by the

author of this thesis was [344.2(18.0) and that by the senior investigator was [357.88 (4.7)] and there was no statistical differences between the values of the two groups. For the self-curing bis-acryl composite group, the SMHb assessed by the author of this thesis was [356.12(17.4) and that produced by the senior investigator was [361.26(14.6). Within groups, the mean SMHb values of the acrylic resin group was not statistically different from that of self-curing bis-acryl composite ($p>0.05$).

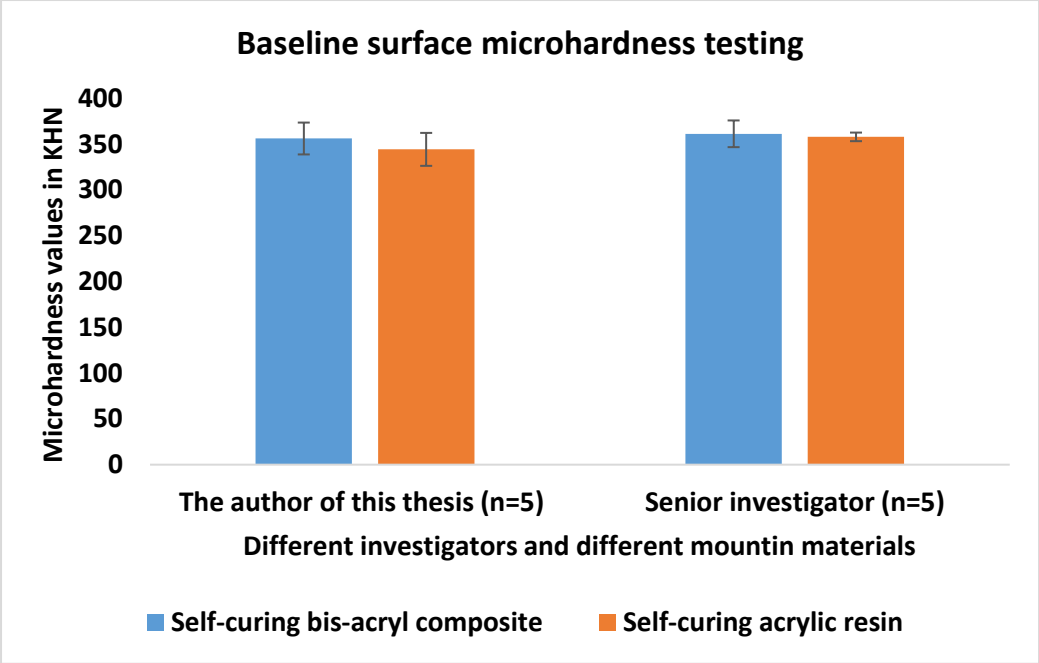


Figure 28: *Baseline surface microhardness values(SMHb) in KHN for enamel samples mounted in two different mounting materials carried out by the author of this thesis compared to a senior investigator.*

IV. Training the author of this thesis in the measurement of SMH and SNCP testing

Specimen preparation and testing:

Based on the results from Gpower version 3.1.5, ten specimens were prepared for this study from human molar sound teeth. Twelve specimens were prepared by sectioning six buccal and six lingual surfaces. Initial surface microhardness values (SMH) of 272 KHN and 400 KHN were selected (Meredith *et al*., 1996; Austin *et al.*, 2011; Lussi *et al*., 2011). Only ten specimens were selected for this study and two specimens were rejected. All specimens were prepared and mounted as described in section 2.1. Specimens were allocated to two different investigators, 5 specimens each. Specimens were immersed in DW for 60 minutes prior to exposure to a 10-minutes erosion cycle. The erosion cycle consisted of 80 mL 0.3 % citric acid (Sigma Aldrich), 0.02 M, pH=3.2, at 22°C ± 1, agitated with an orbital shaker (Bibby Scientific, Staffordshire, UK) at 60 rpm, followed by 2-minutes rinse in 80 mL of DW, again under agitation with an orbital shaker set at 60 rpm for a final 2 minutes. The cycle of immersion of the specimens in DW and citric acid was repeated 5 times for each subgroup. Profilometric measurement data were obtained as was explained in section 2.4.1. SMH was measured and data was obtained as was explained in section 2.4.2.

Statistical analysis:

Paired t test was used to compare the mean SMHC and step height between the two investigators.

Results:

Figures (29 a) shows the mean [standard deviation (SD)] step height for the two investigators. It shows that the erosive challenge produced a step height of 12.3 µm (01.4) by the author of this thesis and a step height of 11.25 (0.75) by the senior investigator and there was no statistically significant difference between the two investigators.

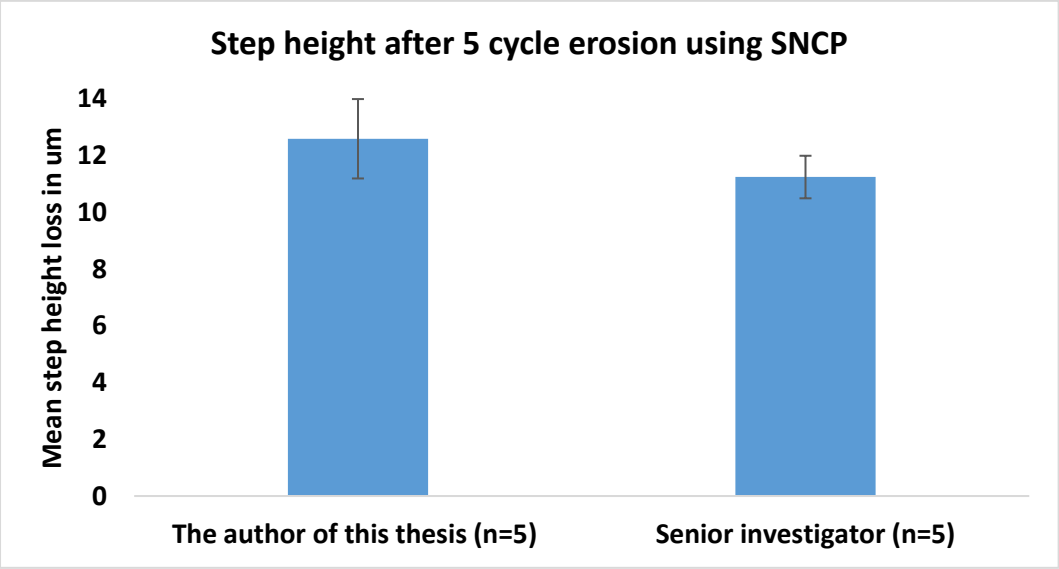


Figure (29 a): *Mean (SD) step height loss (µm) after five cycle erosion carried out manually by the author of this thesis and a senior investigator.*

Figure (29 b) shows the mean (SD) baseline surface microhardness values (SMHb) (baseline: before receiving any treatment) and the mean (SD) surface microhardness change (SMHC) after five cycle erosion for the two investigators. The mean (SD) SMH value produced by the author of this thesis [344.24 (17.9)] was not significantly different from that of the senior investigator [357.88 (4.7)] ($p<0.05$). There was also no significant difference in the mean (SD) SMHC produced by the author of this thesis [135 (10.4)] compared to that produced by senior investigator [155.6 (19.6)] ($p<0.05$).

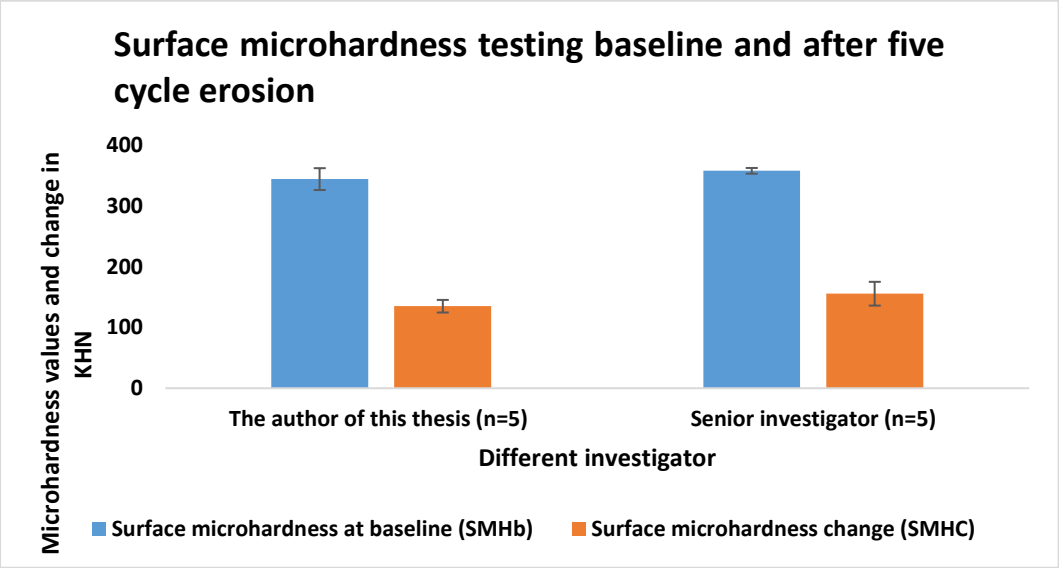


***Figure (29 b)**: Mean (SD) surface microhardness at baseline (SMHb) values (before erosion) and surface microhardness change (SMHC) after five erosion cycle (10 min each) in Knoop microhardness units (KHN) by the author of this PhD compared to a senior investigator.*

III. Development of *in vitro* erosion immersion time and saliva protocol

Specimen preparation and testing:

Based on the power calculation as described in section 2.1.3, thirty specimens were prepared for this study from human molar sound teeth. Thirty four specimens were prepared by sectioning seventeen buccal and seventeen lingual surfaces. Initial surface microhardness values (SMH) measured. The specimens with a SMH value between 272 KHN and 400 KHN were selected (Meredith *et al*., 1996; Austin *et al*., 2011; Lussi *et al*., 2011). Thirty specimens were selected and four were rejected. All specimens were prepared and mounted as described in section 2.1. Specimens were randomly assigned to 3 groups: WMS, AS and DW, 10 specimens each. All specimens were

immersed in the corresponding solution for 60 minutes prior to exposure to the erosion cycle. From each group of ten specimens, 5 specimens were exposed to a 5 minute (n=15) erosion cycle and and 5 specimens to a 10 minutes erosion cycle (n=15). The erosion cycle consisted of 80 mL 0.3 % citric acid as described above.

Statistical analysis:

Two-way ANOVA was used to compare the mean step height between the three groups at two different erosion timings. Paired t test was used to compare the mean step height between the 5 minutes and 10 minutes immersion of each group.

Results:

Figure 30 shows the mean (SD) step height of the three groups:, WMS, AS and DW at two different erosion immersion timings (5 minutes and 10 minutes erosion).

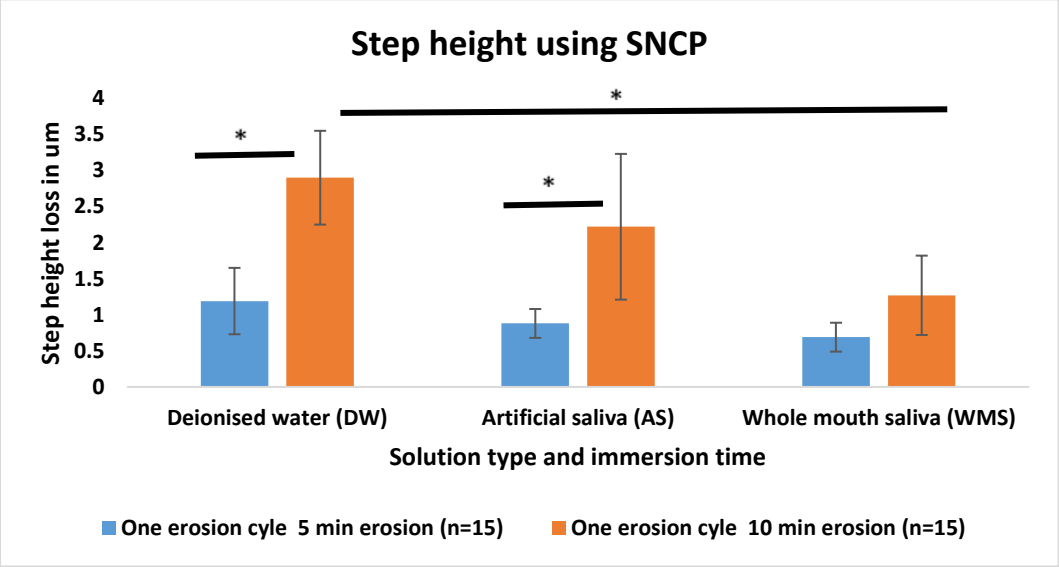


Figure 30: *Mean (SD) step height of enamel samples of six groups (n=5) exposed to 5 minutes and 10 minutes erosive challenge after 30 minutes immersion in three different solutions.*

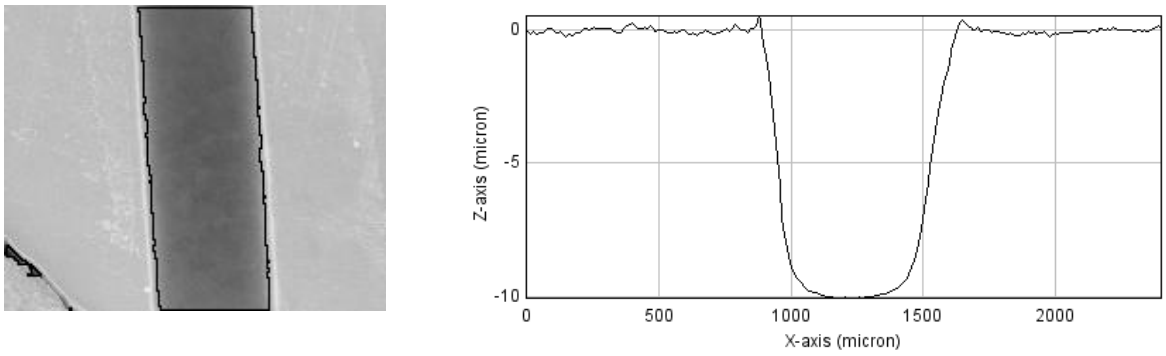
At 5 minutes erosion, there were no significant differences between the mean step height of the three types of solutions ($p>0.05$). At 10 minutes erosion, only WMS had significantly lower step height value than DW ($p<0.05$). When comparing within groups, 5 minutes erosion demonstrated significantly lower step height than 10 minutes erosion for only AS and DW groups ($p<0.05$) but was not significant for WMS group ($p>0.05$).

IV. Comparison of the step height using manual extraction method and the ImageJ programme

Specimen preparation and testing:

Based on the power calculation as described in section 2.1.3, thirty specimens were prepared for this study from human molar sound teeth. Thirty eight specimens were prepared by sectioning ninteen buccal and ninteen lingual surfaces. Initial surface microhardness values (SMH) measured. The specimens with a SMH value between 272 KHN and 400 KHN were selected (Meredith *et al*., 1996; Austin *et al*., 2011; Lussi *et al*., 2011). Thirty specimens were selected and eight were rejected. All specimens were prepared as described in section 2.1. and measured for step height manually and using ImageJ software. The manual step height was calculated as described in section 2.4.1. Specimens were randomly assigned to 2 main experimental groups: one cycle (n=15) and five cycles group (n=15). In each experimental group, specimens (n=15) were divided into 3 subgroups: WMS, AS and DW, 5 specimens each. For one cycle erosion, all specimens in each group were immersed in the corresponding solution for 60 minutes prior to exposure to 10-minute erosion cycle. The erosion cycle consisted of 80 mL 0.3 % citric acid as described above. For the five cycles erosion, the cycle of immersion of the

specimens in solution and citric acid was repeated 5 times for each group. The step height was measured using ImageJ macro to calculate mean step heights. The macro converted the 3D data set into a greyscale image in which each pixel represented a data point and whose grey-scale value represented the z height (the lighter grey values means a higher z height) (Austin *et al*., 2011; Mistry, 2016). Therefore the worn area was represented by the dark values whereas the light values represented the reference area (Figure 31).



A) B)

Figure 31*: A 32-bit grey scale image (a) and profile representation (b) of a scanned sample by the ImageJ macro to calculate the mean step height* (Austin *et al*., 2011; Mistry, 2016).

The z value (µm) for the reference and worn areas were averaged. The mean value of the reference areas was subtracted from the worn and this difference was the mean step height for the specimen.

Statistical analysis:

Paired t test was used to compare the mean step height between the manual method and imageJ for each subgroup. Two-way ANOVA was used to compare the mean step height (manual and imageJ) within the three subgroups.

Results:

Figure 32 shows the mean (SD) step height of the one cycle and five cycles erosion quantified manually and by ImageJ software for three different groups: WMS, AS and DW.

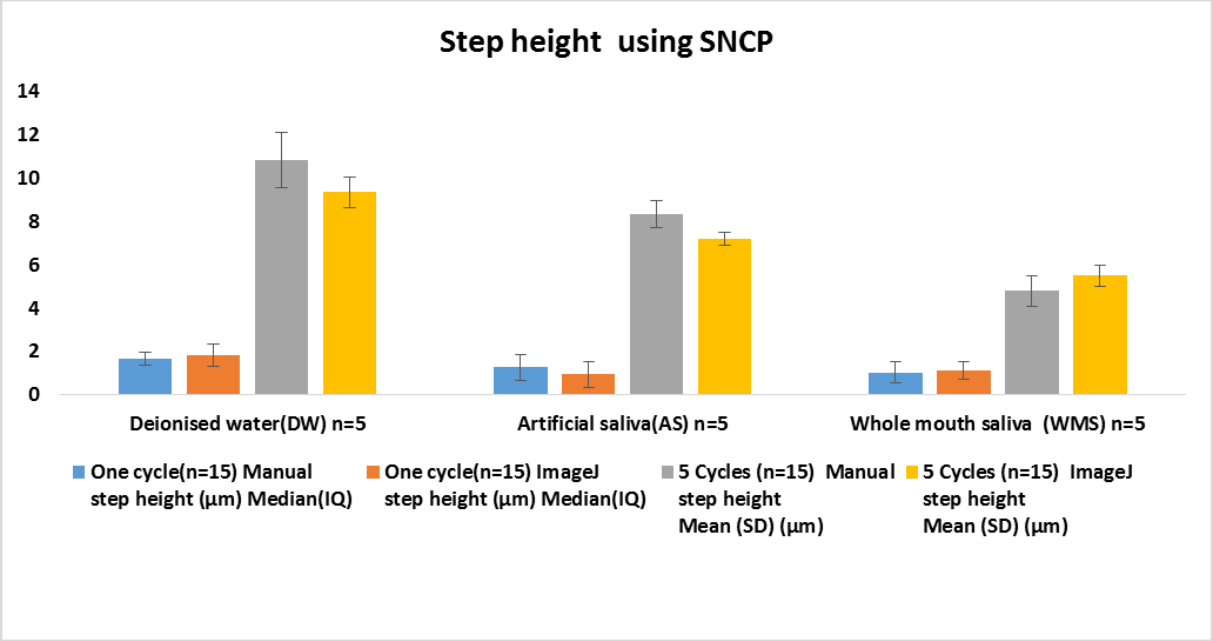


***Figure 32**: Mean (SD) step height and median (IQR) (manual and ImageJ) of three different groups according to the corresponding solution after one cycle and five cycles erosion (10 minutes acid challenge). Similar shapes in the table denote significant differences between groups in rows*

For one cycle erosion, there was no statistical differences between the step height of the three groups (WMS, AS, DW). The mean step height of WMS and DW groups for both manual [1.24 (0.8) and 1.53 (1.1) respectively p= 0.06] and ImageJ [1.13 (0.9) and 1.33 (1.0) respectively p=0.1] also showed no significant difference. When comparing manual to ImageJ data across the three groups, both methods showed no significant difference between the mean step height after one cycle erosion (p>0.5). For five cycle erosion, for

both manual and ImageJ, specimens in WMS had statistically lower step height [4.8 (1.19) and 5.5 (0.5) respectively] than AS [8.34 (0.9) and 7.2 (0.8) respectively] and DW [10.82 (1.28) and 9.36 (0.7) respectively] groups ($p<0.0001$). No significant differences were observed between AS and DW groups for both manual and ImageJ. When comparing manual to ImageJ data between the three groups, both methods showed no significant difference between the mean step height of the three groups after five cycles ($p>0.5$).

2.6.2 Training and optimisation of protein quantification methods used in this research

2.6.2.1 Introduction:

There have been methods described for measuring proteins present in saliva and AEP samples. In this thesis, quantification of proteins on *in vitro* and *in vivo* enamel surfaces was required. Problems often faced in protein quantification are reliability, reproducibility and uncertainty which may make it difficult to compare measurements between the outcomes of such research studies. The uncertainty in quantification of proteins in biological research is one of the main concerns in protein analysis (Taylor *et al.*, 2013; Lee *et al.*, 2015). The uncertainty of measurement is often linked to two other important concepts that are, precision and accuracy. Precision involves two measurements conditions, repeatability and reproducibility (Barwick and Prichard, 2011). Repeatability refers to the measurements carried out on the same sample over a short period of time by the same investigator under the same conditions, whereas reproducibility refers to carrying out measurements on the same samples

by different investigators under different conditions and repeated at different times. Accuracy of a measurement system refers to the closeness of a measured value to a standard or known value of the object being measured. Therefore, when assessing reliability of measurement results, it is the precision that one should take into consideration rather than the accuracy (Barwick and Prichard, 2011). In the protein analysis reported within this thesis, repeatability and reproducibility of protein standards were taken into consideration to assess the reliability of quantifying the proteins in the AEP samples.

2.6.2.2 Aims

The aims of this section were:

- To train the author of this thesis in various apsects of protein analysis methods.
- To optimise the volumes of the four purified protein standards: mucin5b, albumin, CA VI and statherin to produce a standard curve that can be used to quantify the amount of the corresponding proteins within AEP samples.

2.6.2.3 Materials and methods:

The author of the thesis was trained in the use of SDS-PAGE technique to sparate, transfter and quantify proteins. This was done by repeating various experiments under the guidance of a senior expert until the methods and measurements were optimised. These included the running various saliva and AEP samples through SDS-PAGE gels and visualisation of the proteins using FITC and Coomassie Blue stains. The training also involved quantification of

proteins using chemiluminescent densitometric analysis with chemiDoc MP imaging technique.

Once the training was completed, the amounts of four purified proteins were optimised to be: [mucin5b (10 µL), albumin (10 µL), CA VI (5 µL) and statherin (5 µL)]. These volumes were mixed to make a mixture of 30 µL to be used in SDS-PAGE to quantify the amount of proteins in the *in vitro* and *in vivo* AEP. Four different volumes of the purified standard mixtures were loaded into the the SDS-PAGE gels (n=3) alongside other samples. These volumes were 15 µL/lane 1, 7.5 µL/lane 2, 3.8 µL/lane 3 and 1.5 µL/lane 4. The final optimisation was done as follows:

10 mL aliquot of stimulated WMS was collected from two healthy volunteers at King's college London Dental Institute (Northampton, REC ref: 14/EM/0183) to be used as a positive control as described in section 2.2.1.1. Enamel specimens (n=4) were prepared and mounted in acrylic resin as described in section 2.1. *In vitro* AEP was formed for 2 hours on two prepared enamel specimens (n=2) as explained in sections 2.3.1 and was harvested as explained in sections 2.3.3. Another two enamel specimens without AEP (n=2) were also used as a negative control. Different volumes of four samples were loaded to each SDS-PAGE gel (n=3) as shown in Table 11.

Mixed Stdandards	**Eluted AEP samples**	**Enamel specimens without AEP (negative control)**	**WMS (Positive control)**
4 volumes (15 µL,7.5 µL,3.8 µL,1.5 µL)	3 enamel specimens (10 µL each)	3 enamel specimens (10 µL each)	3 volumes (5 µL,1 µL,0.5 µL)

Table 11*: Different volumes of samples loaded to each SDS-PAGE gel (n=3) for optimisation the volumes of the purified protein standards.*

These four samples were WMS as a positive control (3 different volumes), enamel specimens without AEP as a negative control (3 volumes, 10 µL each), and eluted AEP from 3 enamel specimens (10 µL each). AEP was eluted and harvested as described in section 2.3. The four purified standards were prepared as described in section 2.5.2. SDS-PAGE and western blot were used to separate and transfer protein fractions in the samples respectively as described in section 2.4.5. and 2.4.6. Antibodies against four specific salivary proteins were used: mucin5b, albumin, CA VI and statherin. Image lab was used to analyse data as described in 2.4.6.4. The volume intensities (total number of pixels) of all samples were worked out using tools of ImageLab software version 4.1 (Bio-Rad). The known concentration of the four purified standards were used to quantify the concentration of the four proteins in the corresponding saliva and AEP samples. The standard curves of purified proteins of standards were generated from the mean (SD) volume intensities (n=3) (Y-axis) against the absolute quantities (X-axis) of the corresponding purified proteins of the standards. This was used to generate a linear calibration curve of the best fit line with a linear equation. The linear equation is A = slope * C + intercept where A (Y-axis) against concentration C (X-axis). The linear relationship (R^2) value is often given indicating the overall relationship between different concentration points of each standard.

This formula was used to calculate the amount of each protein in the saliva and AEP samples. Standards bands on different SDS-PAGE gels (n=3) were used to assess reproducibility. The repeatability of the purified protein of standards to quantify the four proteins in the AEP samples was also assessed

using the coefficients of variation (CV) for the standard volume intensities on different gels (n=3) (Reed *et al.*, 2002; Pramanik *et al.*, 2010).

2.6.2.4 Results:

Mucin5b:

Figure 33 shows an image of the SDS-PAGE and western blot of the four different samples: purified protein standards, AEP, enamel specimens without AEP (negative control) and WMS (positive control) blotted against mucin5b antibody.

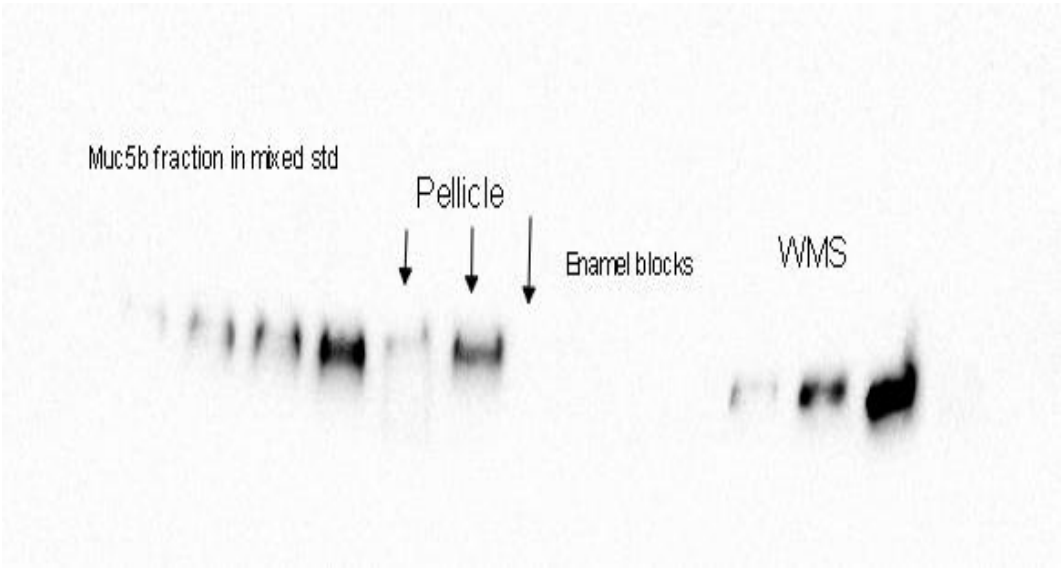


***Figure 33**: SDS-PAGE and western blot of the samples: purified protein standards, AEP, enamel blocks without AEP (negative control) and WMS (positive control) blotted against mucin5b antibody to work out the amount of mucin5b.*

Table 12 shows the volume intensities (n=3) and absolute quantity of the purified mucin5b standard from which the absolute quantities of mucin5b in WMS and AEP samples were calculated.

Sample type	Volume of standards and samples used (µL)	Volume(Ints) Mean (SD) n=3	Repeatability % 0f the protein standards	Absolute quantity of mucin5b (ng)
Purified mucin5b	15	410 (35.8)	28	780.00
Purified mucin5b	7.5	305 (60.9)	32	520.00
Purified mucin5b	3.8	238 (74.3)	19	260.00
Purified mucin5b	1.5	89 (44.3)	23.1	52.00
Whole mouth saliva (WMS)	5	515		989.35
Whole mouth saliva (WMS)	1	350		609.21
Whole mouth saliva (WMS)	0.5	201		265.92
AEP	10	198		259.01
AEP	10	311		519.35
AEP	10	10		-
Enamel block without AEP	10	-		-
Enamel block without AEP	10	-		-
Enamel block without AEP	10	-		-

Table 12: *The volume intensities (n=3) for the purified mucin5b standard at different volumes from which the volume intensities and concentration of the four samples were calculated.*

Figure 34 shows the mucin5b standard curve (R^2 = 0.96) generated from the volume intensities (n=3) and absolute quantities of the purified mucin5b standard which was used to estimate mucin5b concentrations in the samples (WMS and AEP). It can be seen from the figures that the purified mucin5b used in this study was optimised in a way that the data points between a high

volume (15 µL) and low volume (1.5 µL) provided a suitable curve range to calculate very little mucin5b in the samples whilst producing a gradual change of intensities.

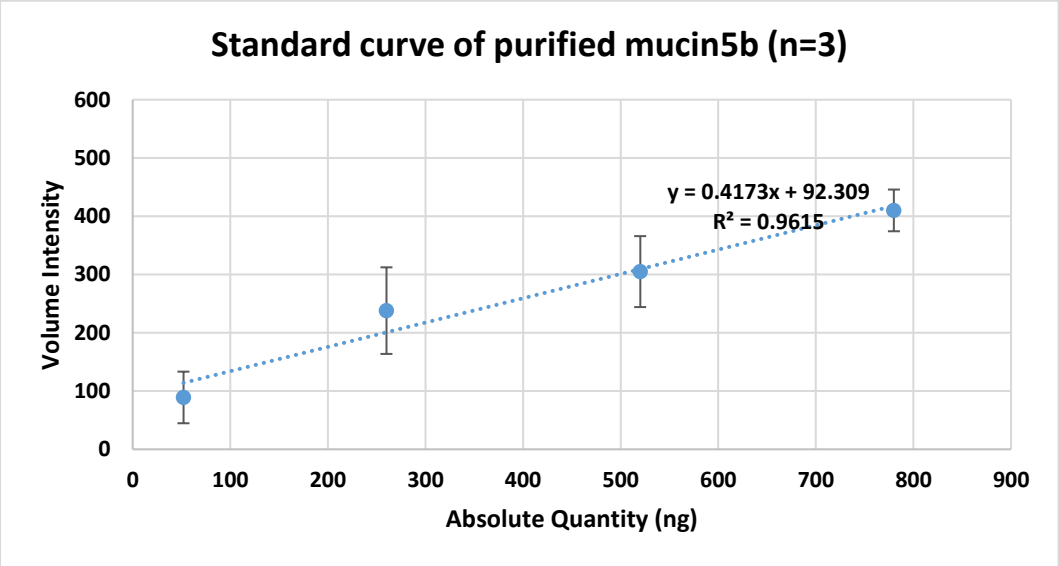


Figure 34: *Standard curve of the mucin5b standard constructed from the mean (SD) volume intensities (n=3) data against absolute quantity in nanogram.*

Albumin:

Figure 35 shows an image of the SDS-PAGE and western blot of the same four different samples as above but blotted against albumin. Table 13 shows the volume intensities and absolute quantities of the purified albumin standard from which the absolute quantities of albumin in samples (purified albumin, AEP and whole mouth saliva) were calculated. Figure 36 shows the albumin standard curve (R^2 = 0.98) generated from the volume intensities and absolute quantities of the purified albumin standard which was used to estimate albumin concentrations in the samples (WMS and AEP). It can be seen that the mean volume intensities and concentrations of albumin standard were optimised in a way that the data points of the proteins samples lie between the three

standard data points. It can be seen from the figures that the purified albumin used in this study was optimised in a way that the data points between a high volume (15 µL) and low volume (1.5 µL) provided a suitable curve range to calculate very little albumin in the samples whilst producing a gradual change of intensities.

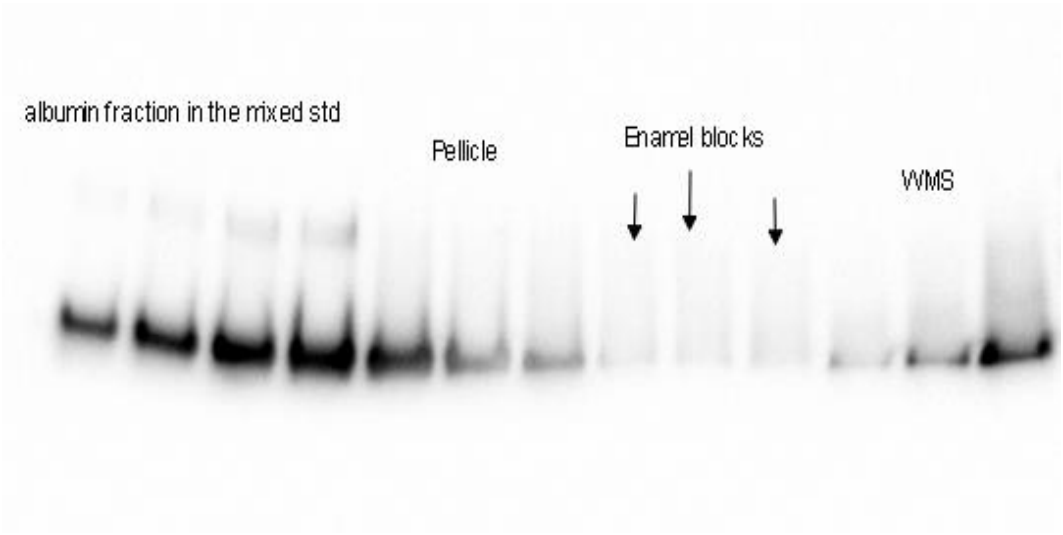


***Figure 35**: SDS-PAGE and western blot of the samples: purified standards, AEP, enamel blocks without AEP (negative control) and WMS (positive control) blotted against albumin antibody to work out the amount of albumin.*

Sample type	Volume of standards and samples used (µL)	Volume(Ints) Mean (SD) n=3	Repeatability % 0f the protein standards	Absolute quantity of albumin (ng)
Purified albumin	15	470 (32.1)	10.1	5
Purified albumin	10	381 (35.5)	16	3.33
Purified albumin	5	203 (33.12)	21.3	1.67
Purified albumin	1	126 (36.7)	11.9	0.33
Whole mouth saliva (WMS)	5	343 (44.1)		3.20
Whole mouth saliva (WMS)	1	209 (27.2)		1.50
Whole mouth saliva (WMS)	0.5	156 (26.0)		0.82
AEP	10	286		2.47
AEP	10	198		1.35
AEP	10	118		0.33
Enamel block without AEP	10	-	-	-
Enamel block without AEP	10	-		-
Enamel block without AEP	10	-	-	-

Table 13: *The volume intensities (n=3) for the purified albumin standard at different volumes from which the volume intensities and concentration of the four samples were calculated.*

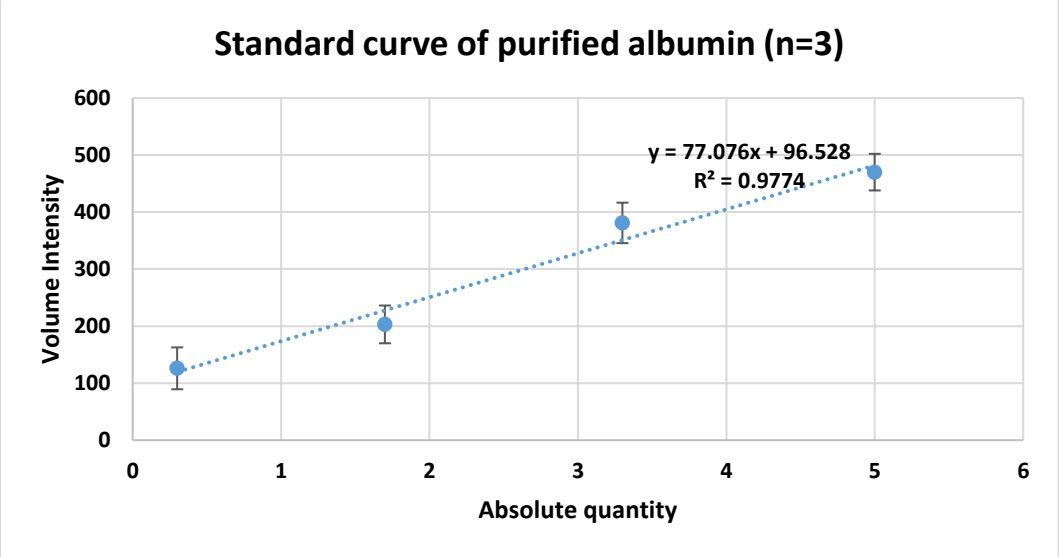


Figure 36: *Standard curve of the albumin standard constructed from the volume intensities (n=3) data against absolute quantities in nanogram.*

Carbonic anhydrase VI (CA VI):

Figure 37 shows an image of the SDS-PAGE and western blot of the same four different samples as above but blotted against CA VI antibody.

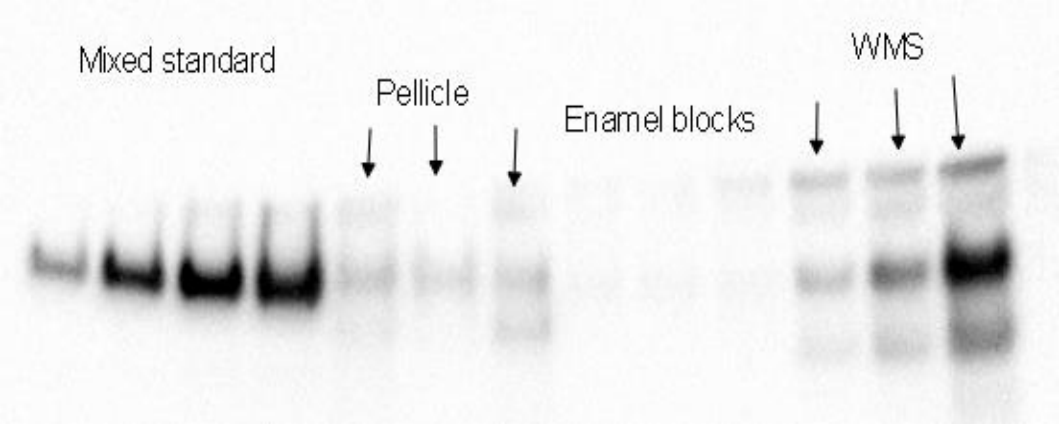


Figure 37*: SDS-PAGE and western blot of the samples: purified standards, AEP, enamel blocks without AEP (negative control) and WMS (positive control) blotted against CA VI antibody to work out the amount of CA VI.*

Table 14 shows the volume intensities and absolute quantities of the purified albumin standard from which the absolute quantities of CA VI in samples (purified CA VI, AEP and WMS) were calculated.

Sample type	Volume of standards and samples used (µL)	Volume(Ints) Mean (SD) n=3	Repeatability % Of the purified standard	Absolute quantity of CA VI (ng)
Purified CA VI	15	490 (43.1)	8.7	350.0
Purified CA VI	10	414 (32.1)	7.8	233.33
Purified CA VI	5	315 (55.4)	17.6	116.66
Purified CA VI	1	165 (39.1)	23.7	23.33
Whole mouth saliva (WMS)	5	405 (45.2)		239.15
Whole mouth saliva (WMS)	1	311 (31.1)		146.10
Whole mouth saliva (WMS)	0.5	138 (25.4)		71.11
AEP	10	111.2		39.50
AEP	10	92.1		31.10
AEP	10	98.1		32.59
Enamel block without AEP	10	-		
Enamel block without AEP	10	-		-
Enamel block without AEP	10	-		

Table 14: *The volume intensities (n=3) for the purified CA VI standard at different volumes from which the volume intensities and concentration of the samples were calculated.*

Figure 38 shows the CA VI standard curve (R^2 = 0.96) generated from the volume intensities and absolute quantities of the purified CA VI standard which was used to estimate CA VI concentrations in the samples (WMS and AEP). It can be seen from the figures that the purified CA VI used in this study was optimised in a way that the data points between a high volume (15 µL) and low

volume (1.5 µL) provided a suitable curve range to calculate very little CA VI in the samples whilst producing a gradual change of intensities.

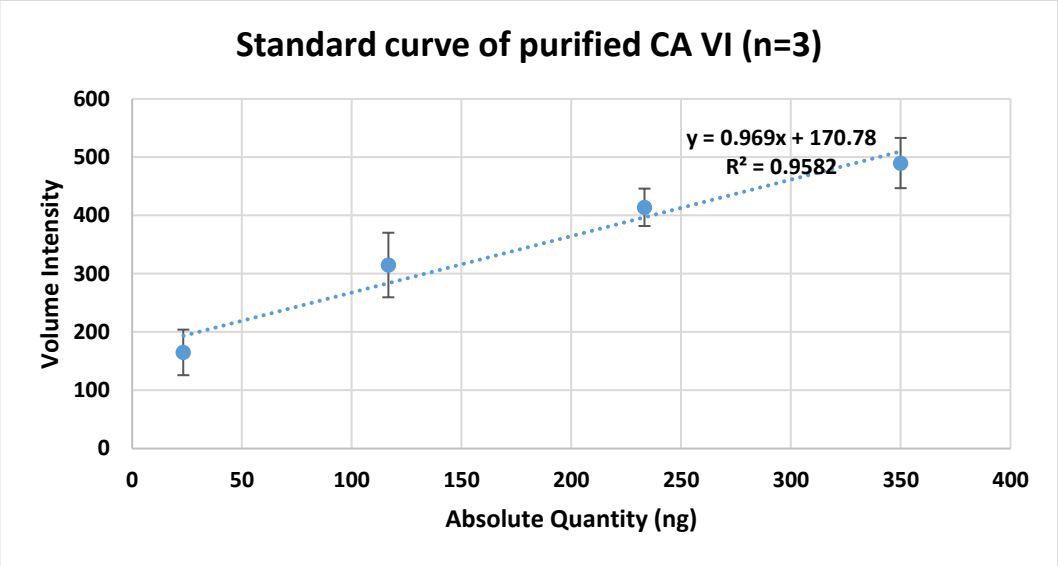


Figure 38: *CA VI standard curve constructed from the volume intensities (n=3) data against absolute quantity in nanogram.*

Statherin:

Figure 39 shows an image of the SDS-PAGE and western blot of the four different samples: purified protein standards, AEP, enamel blocks without AEP (negative control) and WMS (positive control) blotted against statherin antibody.

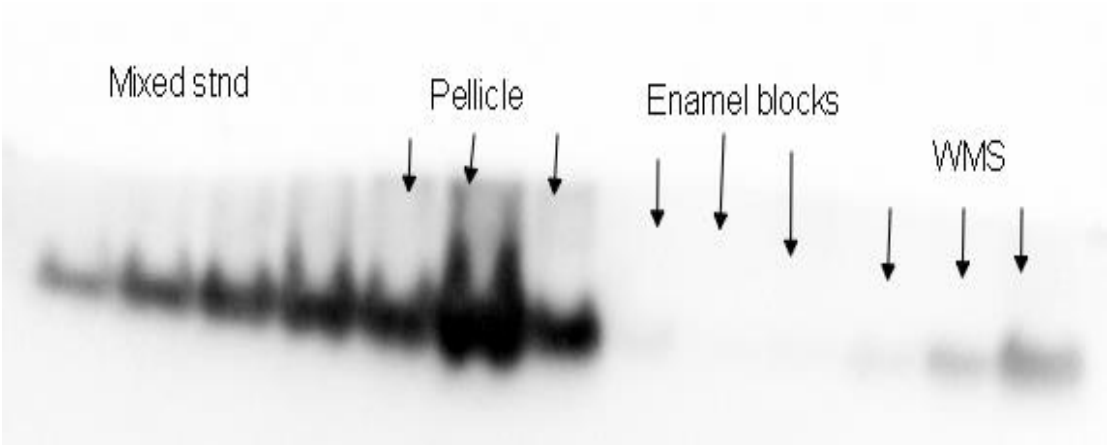


Figure 39: *SDS-PAGE and western blot of the samples: purified standards, AEP, enamel blocks without AEP (negative control) and WMS (positive control) blotted against statherin antibody to work out the amount of statherin.*

Table 14 shows the volume intensities and absolute quantities of the purified statherin standard from which the absolute quantities of statherin in samples (WMS and AEP) were calculated. Figure 40 shows the statherin standard curve (R^2 = 0.96) generated from the volume intensities and absolute quantities of the purified statherin standard which was used to estimate statherin concentrations in the samples (WMS and AEP). It can be seen from the figures that the purified statherin used in this study was optimised in a way that the data points between a high volume (15 µL) and low volume (1.5 µL) provided a suitable curve range to calculate very little statherin in the samples whilst producing a gradual change of intensities.

Sample type	Volume of standards and samples used (ul)	Volume(Ints) Mean (SD) n=3	Repeatability % Of the purified standard	Absolute quantity of statherin (ng)
Purified statherin	15	351(48.9)	13.9	781.25
Purified statherin	10	219 (39.0)	17.8	520.83
Purified statherin	5	173 (23.8)	13.8	260.41
Purified statherin	1	94 (26.1)	14.5	52.08
Whole mouth saliva (WMS)	5	74		13.19
Whole mouth saliva (WMS)	1	31		7.17
Whole mouth saliva (WMS)	0.5	9		3.93
AEP	10	316		711.6276
AEP	10	595		1,516.85
AEP	10	238		486.51
Enamel block without AEP	10	-	-	-
Enamel block without AEP	10	-		-
Enamel block without AEP	10	-	-	-

Table 15: *The volume intensities (n=3) for the purified statherin standard at different volumes from which the volume intensities and concentration of AEP and WMS samples were calculated.*

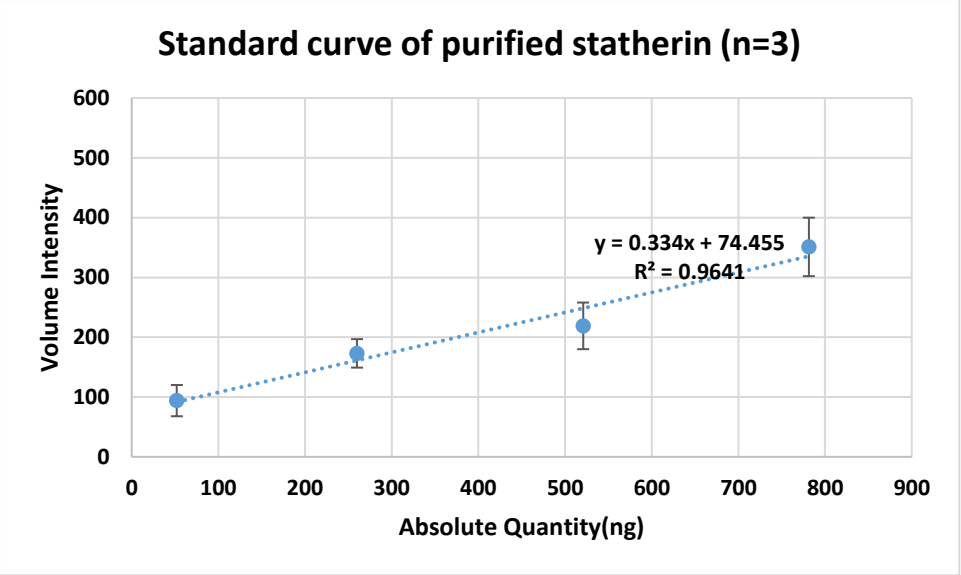


***Figure 40**: Statherin standard curve constructed from the volume intensities (n=3) data against absolute quantity in nanogram.*

2.6.3 Discussion:

This section of the thesis had two main aims. Firstly to train the author of this thesis in performing accurate SMH, SNCP measurements and protein analysis as well as develop the *in vitro* erosion models and natural saliva and AEP protocols used in the thesis. The skills of the author in carrying out specimen preparation and precision in measurements were compared to that of a senior investigator. No statistically significant differences were found between the mean SNCP and SMHC measurements obtained by the two investigators and therefore the author was adequately trained to carry out the rest of the experiments and obtain the required measurements. There was no statistically significant differences between the mean baseline SMH of two mounting materials: acrylic resin and self-curing bis-acryl composite for both investigators. Therefore acrylic resin (Dentsply Ltd, Surry, UK) was chosen as the material for mounting enamel specimens throught out the thesis. The time

in which the enamel specimens should be immersed in the erosive solution was also assessed. Specimens were immersed in citric acid for either 5 minutes or 10 minutes. The results suggested that a 5-minute erosion cycle demonstrated significantly lower step height than 10 minutes erosion for only AS and DW groups but was not significant for WMS group therefore, a 10-minute erosion cycle was selected so that detectable and measurable step height could be gained. The 60 minutes immersion time in WMS, AS and DW prior to exposure to the erosion cycle was selected as many previous studies have reported 60 minutes AEP formation to offer protection against erosion (Amaechi *et al*., 1999; Wetton *et al.*, 2006; Hannig *et al*., 2003). The results of one cycle erosion suggested that only specimens immersed in WMS had significantly lower step height than DW after 10 minutes erosion but there was no statistical difference in protection between WMS and AS. This may warrant using longer immersion period in WMS to offer greater protection. Moreover, the validity of manually extracted step height was compared to ImageJ in extracting data. ImageJ used a custom-made macro, which automatically selected the reference and worn areas by converting the data points into a 2D greyscale image, where the grey value of each pixel represents the height of that data point. The surface was levelled and step height automatically calculated for each available profile based of the grey values of each pixel. ImageJ has, however, the disadvantage to discriminate between the eroded areas of small step height vlaues as compared to the reference areas and may fail to calculate the step height correctly without human manipulation and interference. On the other hand, the manual step height measurement is prone to human bias as the operator has to physically choose the reference and worn

areas. However, the high level of agreement suggested by provious studies (Austin *et al*., 2011; Mistry, 2016) as well as by the results above shows that this is still a valid method to use, as long as it is the same operator doing the measurements. The results of these training studies showed that manually extracted step height and ImageJ data were consistent and followed the same pattern with no statistical differences between the mean manual and ImageJ step height of WMS, AS and DW groups after one cycle or five cycle erosion. Furthermore, early erosion models were also investigated which lead to mean step height reaching a very low value than may be correctly detected by ImageJ. Therefore, manual measurements were used for all step height measurements within this thesis. The results of the protein analysis suggested that the volumes of the purified proteins standards used in this study were optimised to give a linear standard curve. Therefore, the volume of purified proteins: mucin5b (10 µL), albumin (10 µL), CA VI (5 µL) and statherin (5 µL) will be used to make the purified standards mixture in this thesis. Also, the volumes of this mixture will be loaded in the SDS-PAGE in the following folds: 15µL/lane 1, 7.5 µL/lane 2, 3.8 µL/lane 3 and 1.5 µL/lane 4.

Chapter 3: *In vitro* assessment of the effect of AEP on erosion

3.1 Introduction

The protective role of proteins and minerals present in saliva and AEP in the demineralisation/remineralisation processes of erosion has been investigated *in vitro* using WMS and AS (Wetton *et al.*, 2006; Baumann *et al.*, 2016). Some studies have attributed the protective effect of saliva to the protein components, which contribute substantially to the formation of the AEP (Kielbassa *et al.*, 2005; Hannig and Joiner, 2006; Cheaib and Lussi, 2011; Buzalaf *et al.*, 2012; Hellwig *et al.*, 2013; Baumann *et al.*, 2016), Whereas others have reported that the minerals have an important role (Eisenburger, 2009; Zwier *et al.*, 2013; Ionta *et al.*, 2014). Recent studies have differing views on which components of saliva provide the most protection against erosive tooth wear. Martins *et al.* (2013) demonstrated that although saliva (WMS and PS) without minerals (dialysed) can provide protection against enamel demineralisation compared to non-coated specimens, the mineral compnents of saliva (undialysed saliva) enhanced the protecttive effect (Martins *et al.*, 2013). In contrast, Baumann *et al*, (2016) demonstrated the opposite, suggesting that saliva containing only salivary proteins but no ions provided significantly better protection than WMS, AS and dialysed saliva containing salivary proteins and ions (Baumann *et al*., 2016).

AS has been used *in vitro* to mimic the role of WMS and currently found in a number of AS formulations as detailed in section 1.4.2 (Gibson and Beeley, 1994; Amaechi *et al.*, 1998; Amaechi and Higham, 2001; Ionta *et al.*, 2014; Batista *et al.*, 2016). Several studies have assessed the potential use of AS

formulations in remineralising a softened *in vitro* erosive lesion (Amaechi and Higham, 2001; Ionta *et al.*, 2014; Batista *et al.*, 2016).

Despite previous work (Featherstone *et al*., 1993; Martins *et al*., 2013; Baumann *et al.*, 2016), it is still unclear as to whether the protective function of saliva and AEP is mainly derived from proteins or minerals or a combination of both to varying degrees. This chapter aims to further investigate this by comparing WMS, AS and DW. It also assesses the effect of AEP maturation on the protection against erosion.

3.2 Aims, objectives and hypothesis

The aim of this *in vitro* study was to compare the effect of immersion of human enamel specimens in whole mouth saliva (WMS), artificial saliva (AS) and deionised water (DW) for varying time periods prior to an erosive challenge.

The objective was that:

1- To measure the step height, SMHC and surface roughnes of enamel surfaces after immersion in WMS, AS, and DW at three immersion times: 30 minutes, 60 minutes and 24 hour followed by 30 minutes (24 hour+30 minutes) followed by five erosion cycles.

The null hypothesis was that:

1- Immersion of enamel specimens in WMS, AS or DW at various immersion times: 30 minutes, 60 minutes and 24 hour followed by 30 minutes (24 hour+30 minutes) followed by five erosion cycles will not produce significantly different step height, SMHC or surface roughness measurements.

3.3 Materials and methods

3.3.1 Specimen preparation

Based on the power calculation as described in section 2.1.3, a sample size of 90 was required yielding 95% power at 5% level with an effect size of 0.6. One hundred and six human enamel specimens were prepared (London, REC ref: 12/LO/1836) for this study by sectioning fifty three buccal and fifty three lingual surfaces from fifty three human molar extracted sound teeth. Initial surface microhardness values (SMH) between 272 KHN and 400 KHN were selected (Meredith *et al.*, 1996; Austin *et al.*, 2011; Lussi *et al.*, 2011). Ninty specimens were selected for this study and sixteen were rejected. Enamel surfaces were then placed into a custom-made silicone mould (8 × 21.5 × 24 mm) and embedded in cold cure acrylic resin as described in section 2.1.4. Enamel specimens were ground and polished to provide a highly polished, flat surface 3 x 3 mm in size as explained in section 2.1.5. All prepared specimens were then taped with PVC adhesive tape to create a window of exposed enamel approximately 2 X 3 mm with two intact reference areas 1 mm wide on either side as explained in section 2.1.7. and they were numbered and randomised as described in section 2.1.8.

3.3.2 Study methodology

The study consisted of three groups according to the three solutions used, 30 specimens per group: WMS, AS and DW. The WMS and AS were prepared as described in section 2.2. Thawed WMS was mixed vigorously prior to use to re-suspend precipitation of proteins and avoid loss of specific proteins less than 14 kDa (Francis *et al.*, 2000). Within each group, specimens were

randomly allocated by an independent investigator using SPSS random sample generator to 3 subgroups: 30 minutes immersion in the allocated solution (Subgroup 1, n=10), 60 minutes immersion (Subgroup 2, n=10) and (24 hour immersion+30 minutes) (Subgroup 3, n=10) prior to the erosion cycle as described in section 2.3.4.2. When immersed for 24 hour in solution, specimens were stored un-agitated overnight at 22 ° C ± 1. The cycle of immersion the specimens in either 30 minutes or 60 minutes in the corresponding solution followed by the erosion cycle was repeated 5 times for each subgroup as shown in Figure 41.

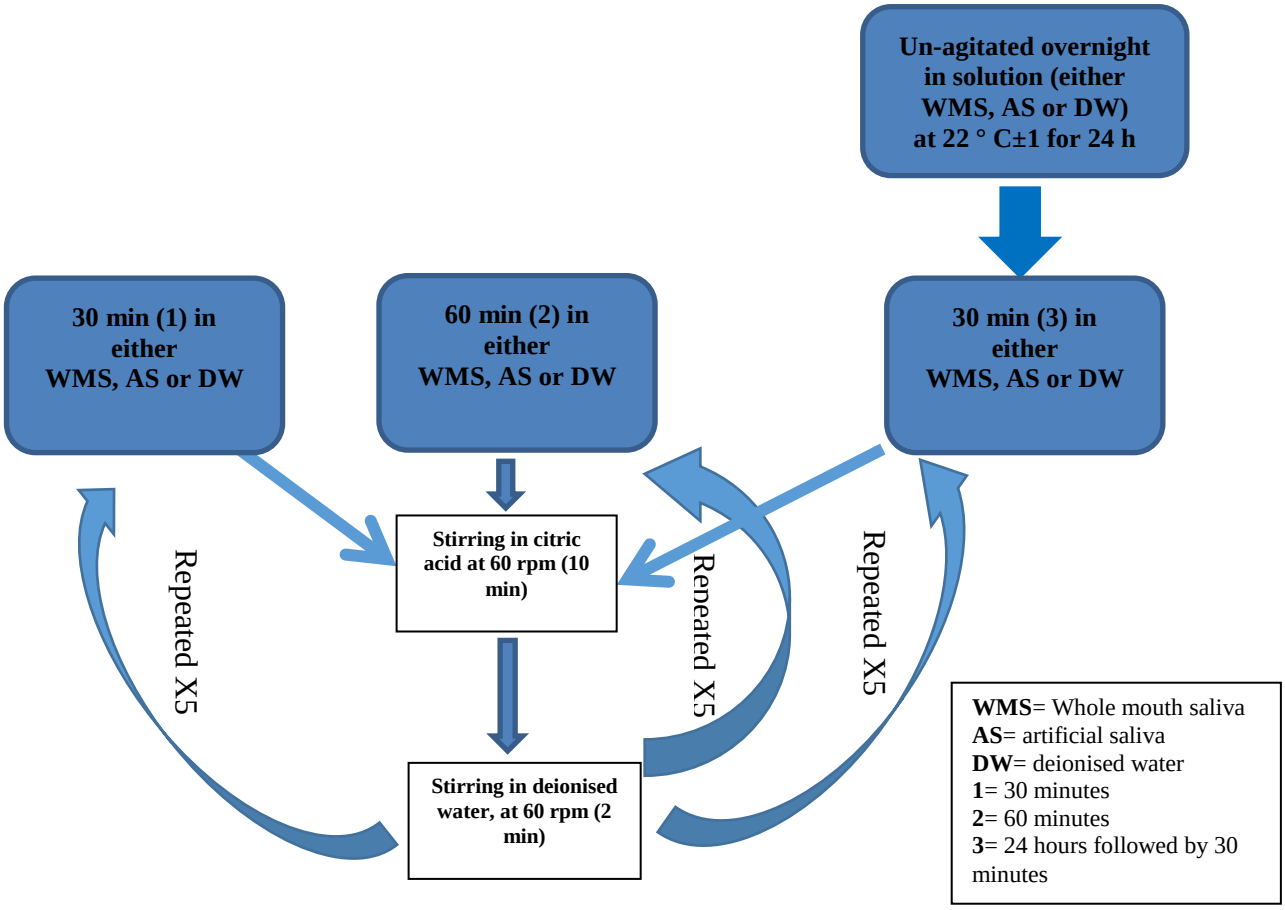


Figure 41: *A flowchart representation of the AEP formation and erosion cycle protocol.*

When experiments were finished, specimens were air-dried for 24 hours after which the tape was removed and profilometric measurement, microhardness and AFM data were obtained.

3.3.3 Testing

3.3.3.1 Profilometric measurements

Profilometric measurement after the fifth erosion cycle were obtained using surface non-contacting profilometer (SNCP) as explained in section 2.4.1. Each surface profile was taken from within the first 1/3 of the taped zone on one side across the exposed window to just within the first 1/3 of the taped zone on the opposite side. Ten randomly selected step height measurements were taken from each specimen and averaged to give a mean surface profile value. Measurments of step height were taken manually rather using imageJ software due to the high level of agreeement between the two methods as described in section 2.6.1.4.

3.3.3.2 Surface microhardness (SMH) measurements

Surface microhardness at baseline (SMHb):

The surface microhardness (SMH) values before immersion in acid [Surface microhardness baseline values: (SMHb)] were measured for the nine experimental groups, 10 specimens each, as described in section 2.4.2. The SMH value of each specimen was determined by the average of five indentations made at the exposed window under a load of 100 g and a dwell time of 10 seconds. The SMH value of each indentation was determined by specialised software (Duramin-5Hardness Tester, Struers Inc., Rotherham,

UK) through measuring the length of each indentation with an optical analysis system calculating hardness in Knoop units (KHN).

Surface microhardness change (SMHC) after five cycles erosion:

SMH values were repeated after the experiment using the method explained above. The surface microhardness change (SMHC) of each specimen was then calculated by subtracting the mean SMH value after five erosion cycles (SMHe) from the mean SMH value before erosion (SMHb) using the formula: SMHC = (SMHb – SMHe).

3.3.3.3 Atomic Force Microscopy (AFM) analysis:

AFM analysis was undertaken in Quantitative Imaging™ mode (QI) in air with Si_3N_4 high spring-constant cantilevers (ACTA, AppNano, USA) calibrated using the dedicated JPK software spring constant measurement procedure. QI mode allows the simultaneous measurement surface topography and mechanical properties by conducting nanoindentation measurements at each pixel. Only specimens from subgroups (3) were subjected for the AFM analysis (WMS3,AS3,DW3). Three specimens from each group (n=9) were selected randomly by an independent investigator using SPSS random sample generator. They were observed after five erosion cycles on which two areas per specimen of 50 × 50 μm^2 were analysed, one eroded and one non-eroded. All images were obtained at a rate of 0.9 Hz with a resolution of 256 × 256 pixels (256 lines/sample), using a maximum contact force of 2.5 N. From the QI images, the number average area roughness (Sa) was measured at each pixel. In addition, stiffness was also measured at each pixel.

3.3.4 Statistical analysis

Data obtained from the profilometry, microhardness and roughness were analysed using SPSS (version 22.0, IBM, Portsmouth, UK). The measured outcomes were analysed using descriptive quantitative methods to summarise the study characteristics of the various subgroups. Shapiro-Wilk and Kolmogorov-Smirnov tests were used to assess the normality distributions of data. Data were also visually assessed using histogram, Q-Q plots and Box and Whisker Plots. Data were normally distributed and were described using means and standard deviations. Two way ANOVA test was used to establish if significant statistical differences existed between the means of all groups. The mean difference was considered to be significant at a P value < 0.05. Post Hoc Bonferroni test was used to determine which means were significantly different from others.

3.4 Results

3.4.1 Step height (µm)

Table 16 and Figure 42 show the mean (SD) step height for the nine experimental groups after five cycles of erosion. These were three WMS groups (WMS1, WMS2, WMS3), three AS groups (AS1, AS2, AS3) and three DW groups (DW1, DW2, DW3). The mean step height (SD) of WMS groups were [WMS1: 5.91 (1.09) µm, WMS2: 6.33 (0.95) µm, WMS3: 3.80 (0.59) µm]. The mean step height (SD) of the corresponding AS groups were [AS1: 6.02 (0.55) µm; AS2: 6.72 (1.05) µm; AS3: 6.34 (0.55) µm]. The mean step height (SD) of DW groups were [DW1: 8.61 (0.58) µm; DW2: 8.24 (0.98) µm; DW3: 8.80 (1.28) µm].

WMS groups had significantly lower step heights for all three groups (WMS1,WMS2,WMS3) than their counterpart DW groups (DW1,DW2,DW3) ($P < 0.0001$). This was also true for AS groups (AS1,AS2,AS3) which had significantly lower step height values than their correspondent DW groups ($P < 0.0001$). When comparing WMS groups with AS groups, there was only a significant difference in the step height between the specimens immersed in the solutions for 24 hours followed by a further 30 minutes (WMS3 and AS3 respectively $P<0.0001$). Within subgroups, significant differences were observed only in natural saliva groups. WMS3 group had significantly lower step height [3.80 (0.59) µm] than WMS1: 5.91 (1.09) and WMS2: 6.33 (0.94) µm] ($P<0.0001$).

Solution type (n=10)	**Step height (µm) Mean (SD)**
Whole mouth saliva (WMS1) (30 minutes)	5.91 **(**1.09)●ǂ
Whole mouth saliva (WMS2) (60 minutes)	6.33 **(**0.95)○Π
Whole mouth saliva (WMS3) (24 hour + 30 minutes)	3.80 **(** 0.59)◊ǂΠ
Artificial saliva (AS1) (30 minutes)	6.02 **(** 0.55)□
Artificial saliva (AS2) (60 minutes)	6.72 **(**1.05)¥
Artificial saliva (AS3) (24 hour + 30 minutes)	6.34 **(**0.55)◊
Deionised water (DW1) (30 minutes)	8.61 **(** 0.58)□ ●
Deionised water (DW2) (60 minutes)	8.24 **(**0.98)¥ ○
Deionised water (DW3) (24 hour + 30 minutes)	8.80 **(**1.28) ◊×

Table 16 *Mean (SD) step height (µm) of enamel surfaces for nine subgroups after three immersion times (1, 2 & 3) in three different solutions (WMS,AS,DW) (0.3%, pH 3.2, citric acid, 10 min). Similar shapes in the table denote significant differences between groups in rows (○, ◊, □, ¥, ●, Π, ×)*

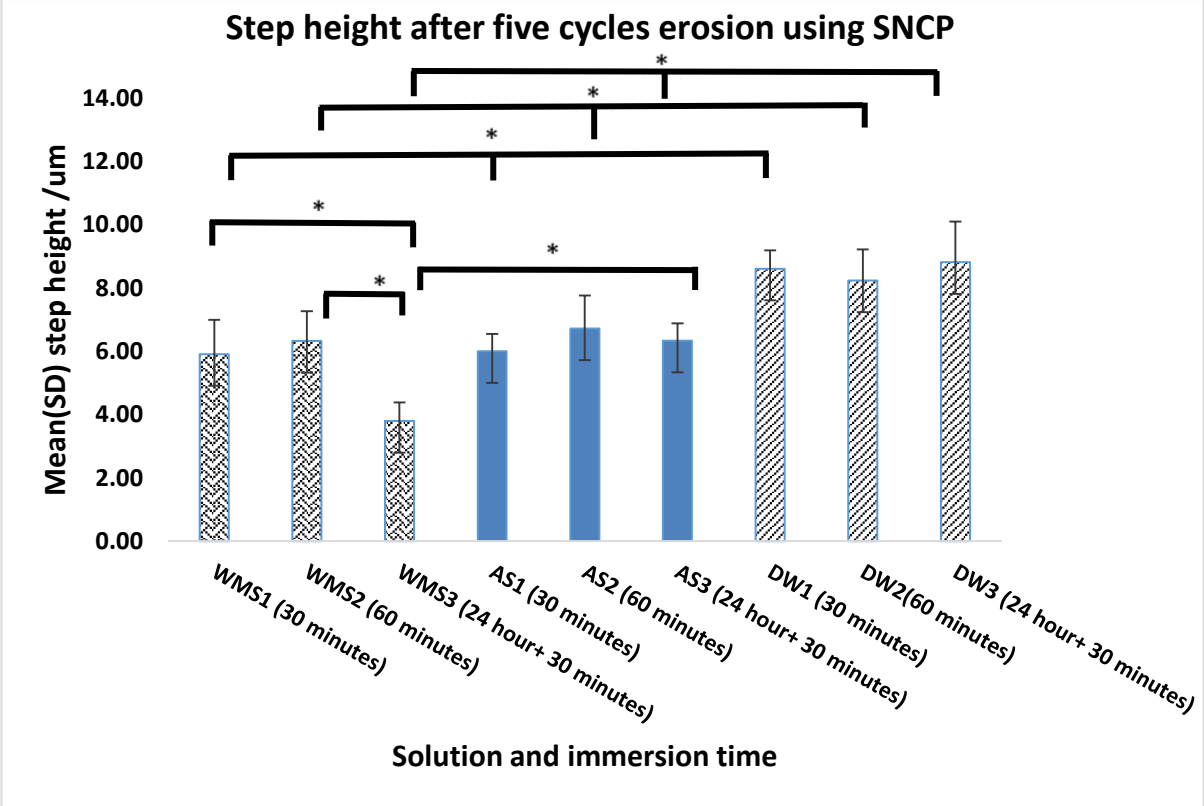


Figure 42 *Mean (SD) step height (µm) (manual) for enamel surfaces of nine subgroups after three immersion times (1, 2 & 3) in three different solutions (WMS,AS, DW). Asterisks denote statistical significance*

3.4.2 Surface microhardness (SMH) measurements

Surface microhardness at baseline (SMHb):

Table 17 shows the SMHb values of nine experimental groups before immersion in either WMS, AS or DW. These were three WMS groups (WMS1, WMS2, WMS3), three AS groups (AS1, AS2, AS3) and three DW groups (DW1, DW2, DW3). This shows that the mean SMH values of the nine groups ranged between 321.79 (12.49) KHN and 341.44 (8.12) KHN. There were no significant differences between the mean SMH values of all groups ($p > 0.05$).

Solution type (n=10)	surface microhardness at baseline (SMHb) Mean (SD)
Whole mouth saliva (WMS1) (30 minutes)	341.44 (8.12)
Whole mouth saliva (WMS2) (60 minutes)	328.98 (31.02)
Whole mouth saliva (WMS3) (24 hour + 30 minutes)	315.72 (11.74)
Artificial saliva (AS1) (30 minutes)	335.85 (13.29)
Artificial saliva (AS2) (60 minutes)	324.09 (21.88)
Artificial saliva (AS3) (24 hour + 30 minutes)	321.79 (10.49)
Deionised water (DW1) (30 minutes)	336.71 (11.00)
Deionised water (DW2) (60 minutes)	337.31 (15.99)
Deionised water (DW3) (24 hour + 30 minutes)	329.00 (19.31)

Table 17: *Mean (SD) baseline surface microhardness (SMHb) in Knoop microhardness units (KHN) after specimens were polished and before receiving any treatments. No significant differences between the groups.*

Table 18 and Figure 43 show the mean (SD) SMHC for the nine experimental groups after the five cycles of erosion. The mean (SD) SMHC of WMS groups were [WMS1:213.52 (13.53); WMS2:207.09 (20.01); WMS3: 249.40 (19.56)]. The mean (SD) SMHC of AS groups were [AS1: 205.05 (15.95); AS2: 191.41 (17.56); AS3: 181.87 (20.48)]. The mean (SD) SMHC of DW groups were [DW1: 177.34 (19.98); DW2: 186.10 (15.95); DW3: 167.12 (15.68)].

DW3 group had a significantly lower SMHC than WMS3 and AS3 groups respectively ($p < 0.0001$)]. The same pattern was observed for specimens immersed in solutions for 30 minutes, where significantly lower SMHC was observed for DW group [DW1: 177.34 (19.98)] compared to both WMS and

AS groups [WMS1: 213.52 (13.53) and AS1: 205.05 (15.95) respectively (p< 0.0001)]. However, when comparing specimens immersed in WMS with those immersed in AS, there was only a significant SMHC difference between WMS3 and AS3 groups (P<0.0001). Within subgroups, only specimens immersed in WMS experienced significant differences. Specimens immersed for 30 minutes in WMS [WMS1: 213.52 (13.53)] and those immersed in 60 minutes [WMS2: 207.09 (20.01)] showed significantly lower SMHC than those immersed for 24 hours followed by 30 minutes [WMS3: 249.40 (19.56) P<0.0001].

Solution type (n=10)	**Surface microhardness change (SMHC) Mean (SD)**
Whole mouth saliva (WMS1) (30 minutes)	213.52 (13.53) ¥×
Whole mouth saliva (WMS2) (60 minutes)	207.09 (20.01) Π
Whole mouth saliva (WMS3) (24 hour + 30 minutes)	249.4 (19.56) Δ Π¥
Artificial saliva (AS1) (30 minutes)	205.05 **(**15.95) ●
Artificial saliva (AS2) (60 minutes)	191.41 **(**17.56)
Artificial saliva (AS3) (24 hour + 30 minutes)	181.87 (20.48) Δ
Deionised water (DW1) (30 minutes)	177.34 **(**19.98)●×
Deionised water (DW2) (60 minutes)	186.10 (15.95)
Deionised water (DW3) (24 hour + 30 minutes)	167.12 (15.68) Δ

Table 18: *Mean (SD) surface microhardness change (SMHC) of enamel surfaces after five cycles erosion in Knoop microhardness units (KHN). Similar shapes in the table denote significant differences between the groups (¥, ●, Δ, Π, ×)*

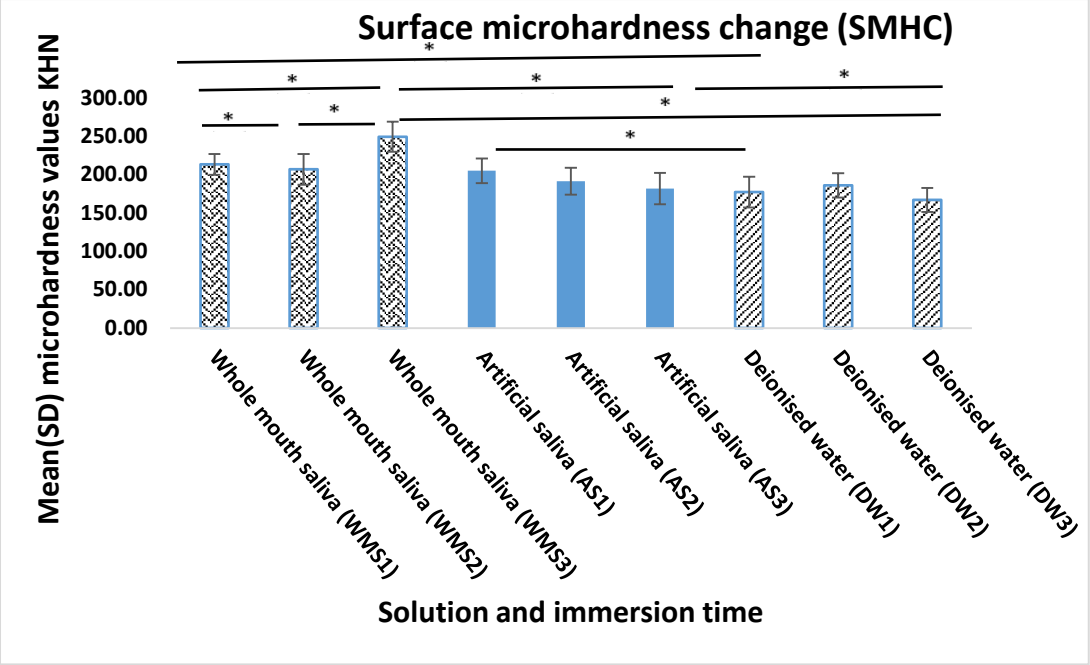


Figure 43: *Mean (SD) surface microhardness change (SMHC) of enamel surfaces after five erosion cycles in Knoop microhardness units (KHN). Asterisks denote statistical significance.*

3.4.3 Atomic Force Microscopy (AFM) analysis

Typical AFM micrographs, together with example 2D profiles, for three selected specimens from the three different groups are shown in Figure 44. The roughness values of the three parameters are summarised in Table 19. The topography images for the all non-eroded areas appeared similar, exhibiting surface scratches typical of a mechanically polished surface but with features in general within the 100-200 nm range. The specimens immersed in WMS appeared to have deeper scratches but there were no significant differences between any of the non-eroded surfaces for any of the roughness parameters ($P>0.45$ for all parameters). The eroded surfaces all exhibited a markedly different appearance to the non-eroded areas, with significantly greater roughness ($P<0.001$ for all parameters). The appearance of the eroded areas was also different depending on the solution the specimens had

been treated with. The specimens that had been stored in DW exhibited the characteristic lock-and-key appearance of enamel prisms indicative of an eroded enamel surface. Specimens immersed in AS exhibited very different surface topography, characterised by a much narrower height range and less well defined prism-like structures compared to those stored in DW. Finally, the specimens immersed in WMS appeared to have very rough surfaces, characterised by steep peaks and sharp valleys, potentially showing the early stages of erosive wear with some prism-like structures beginning to appear.

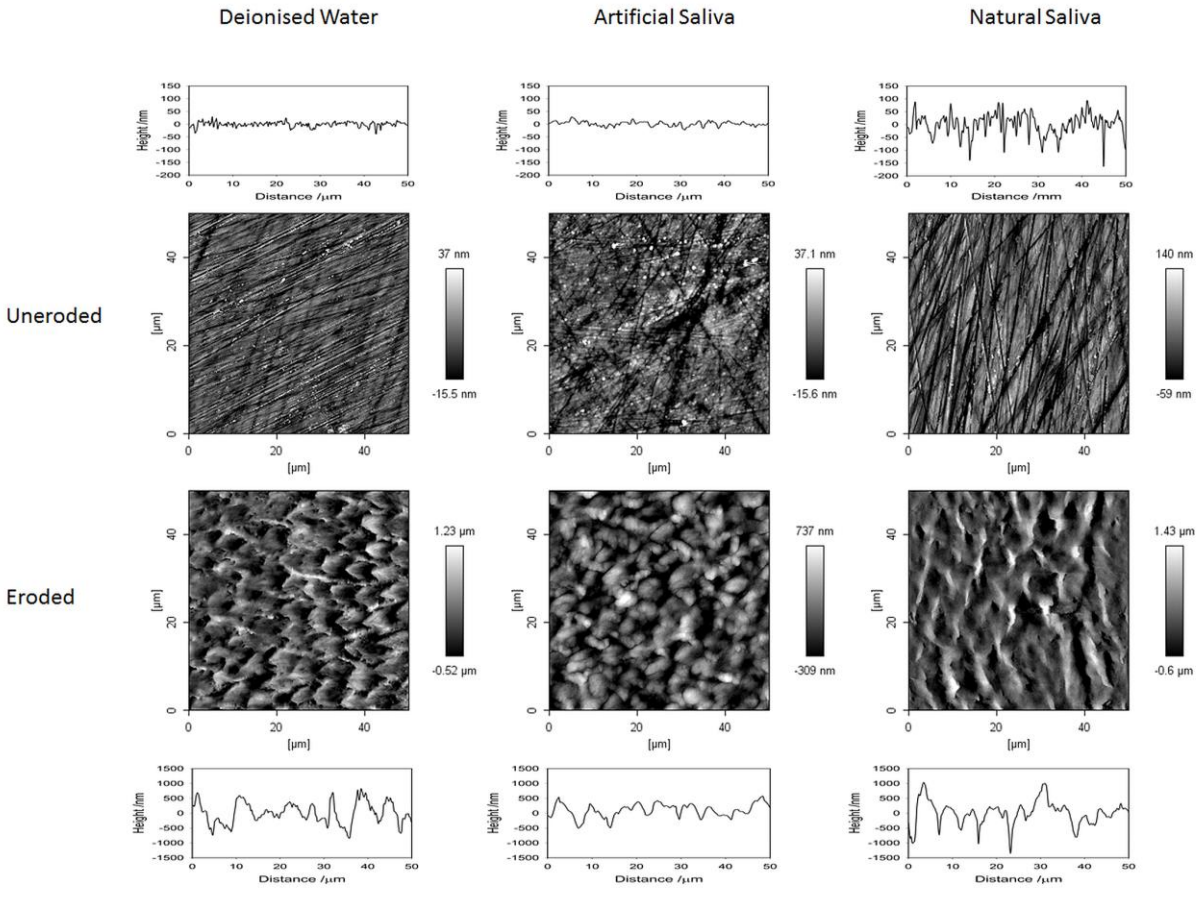


***Figure 44**. Typical AFM micrographs and 2D line profiles for non-eroded and eroded areas for all three storage solutions.*

	Number average roughness (Ra) /nm Mean (SD)		Root-mean square roughness (Rq)/ nm Mean (SD)		Peak-to-valley roughness (Rt) /µm Mean (SD)	
	Non-eroded	**Eroded**	**Non-eroded**	**Eroded**	**Non-eroded**	**Eroded**
Whole mouth Saliva (WMS3)	36 (3.0)[a]	382 (18)	47 (5)[c]	489 (27)	0.54 (0.11)[e]	4.00 (0.55)
Artificial Saliva (AS3)	11 (2)[a]	253 (68)[b]	17 (4)[c]	319 (81)[d]	0.35 (0.08)[e]	2.39 (0.43)[f]
Deionised Water (DW3)	8 (2)[a]	243 (18)[b]	11 (2)[c]	303 (35)[d]	0.18 (0.02)[e]	2.12 (0.40)[f]

Table 19*: Summary of the AFM measured mean (SD) roughness parameters for three different groups of enamel specimens according to the solutions used (WMS,AS,DW). Enamel specimens were immersed for 24 h in the corresponding solution followed by 10-minute citric acid and was repeated five times. Eroded and non-eroded surfaces were scanned for roughness. Columns with same superscript letter are not significantly different (P>0.05).*

These differences in appearance were mirrored in the roughness data for the eroded areas, with the WMS specimens having a significantly higher roughness than specimens stored in the other two solutions (AS and DW, $P<0.001$) with no significant difference between roughness for specimens immersed in either AS or DW.

Typical examples of force-distance curves extracted from the QI images are shown in Figure 45. All of the curves show a typical load-unload profile, in which the force increases from 0 to 2.5 N upon contact and then after the maximum force is reached there is a period in which the force creeps upwards until the unload cycle begins after which the force is unloaded until the AFM probe detaches from the surface and the force returns to 0 N. For specimens immersed in WMS and AS, the shape of the force-distance curves were similar exhibiting a rapid increase in force upon contact and rapid decrease of force upon unloading. For the enamel specimens immersed in DW, while the non-eroded areas showed identical behaviour to that described above, the eroded areas showed a markedly different behaviour, in which, upon contact the force increased at a much slower rate, as shown by the shallower gradient of the force-distance curve after contact and the smaller increase in force prior to commencement of the unload phase. The gradient of the unload curve, however, was approximately the same as that exhibited by the other specimens.

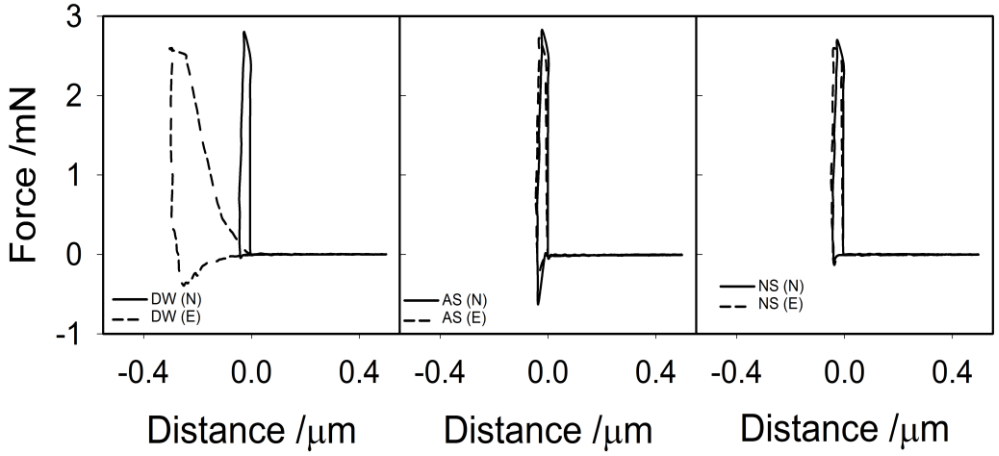


***Figure 45**. Typical force-distance load-unload curves for each specimen type where DW is deionised water, AS is artificial saliva and WMS is natural saliva. Curves measured on the non-eroded regions are shown by a solid black line and denoted (N), while those measured on eroded regions are shown by a broken line and denoted E.*

3.5 Discussion

In this laboratory study, immersion of enamel specimens in WMS, AS and DW for various time periods resulted in differences in step height, surface microhardness change and AFM results and therefore the null hypotheses were rejected. Immersion of the specimens in WMS for (24 hours+30 minutes) prior to acid exposure offered the best protection against step height formation [3.80 (0.59) µm] but interestingly resulted in greater microhardness change [(249.4 (19.56) KNH] leaving a softer surface compared to AS [6.34 (0.55) µm and 181.87 (20.48) KHN respectively] and DW [8.80 (1.28) µm and 167.12 (15.68) KHN]. While the reduction in step height formation is a clear sign of enamel protection, softness of enamel surface (i.e. greater microhardness change) may or may not be. One hypothesis could be that the softer surface has resulted from a slower rate of the erosion process leaving a surface that has the potential to be remineralised.The findings of this study also suggest that the protective potential of *in vitro* formed AEP may be time-dependant. WMS3 showed the best protection, compared with WMS 2 and WMS1.

Broadly, the DW groups showed the greatest step height formation and least surface microhardness change compared to WMS and AS groups. One would expect that DW would not provide protection of enamel from erosion as it lacks minerals and proteins as opposed to natural and artificial saliva. The mineral components of artificial saliva provided some protection against erosive tooth wear which supports previous findings (Dawes and Dong, 1995; Amaechi and Higham, 2001; Hara *et al.*, 2006). Using transverse microradiography, Amaechi *et al*. (2001) investigated the remineralisation effect of natural and artificial saliva on bovine enamel samples after 1 hour immersion in orange

juice (Amaechi *et al., 2001*). They observed significant remineralisation using mean mineral loss and lesion depth analysis following exposure to AS as compared to DW. Ganss *et al.* (2001) also reported that a layer of minerals can be formed on the enamel surface that would be dissolved when acid attacks enamel, reducing the erosion of underlying enamel surface (Ganss *et al.*, 2001). The mineral content was determined using longitudinal microradiography and presented as cumulative mineral loss (μm) from eroded enamel over a 5-day demineralisation/remineralisation cycle. Other studies using immersion in AS for longer than 1 hour have shown rehardening of eroded surfaces (Hara *et al*., 2008; Amaechi *et al.*, 2001; Eisenburger *et al.*, 2001; Wang *et al.*, 2012). Using a combined profilometric measurements with ultrasonication, Eisenburger *et al.* (2001) showed that when enamel specimens were exposed to AS for 24 hours following a 0.3% citric acid at pH 3.2 for 2 hour erosion cycle, complete rehardening of enamel surfaces was observed by measuring softened surface depth before and after ultrasonication (Eisenburger *et al.*, 2001). Featherstone *et al.* (1993) examined the acid resistance to enamel between WMS as compared to a mineral solution containing the same concentrations of calcium and phosphate present in the original WMS (Featherstone *et al.*, 1993). Their findings also agree with our results that long term immersion in WMS provide better protection against subsequent demineralisation as compared to the mineral solution.

Immersion of enamel specimens in WMS provided better protection than AS only when specimens were immersed in WMS for (24 hour+30 minutes). There are many rationales that might explain such significant reduction in step height

formation and greater microhardness change presented in this study. This is clearly important as it demonstrates significant differences at and below the surface of the enamel if pre-treated with (24 hour+30 minutes) WMS (proteins and ions) compared to AS (ions) and DW (neither proteins nor ions). The presence of the AEP appears to change the dissolution process by which protons destroy the crystal matrix and supporting organic structures to a process of softening. Softened enamel may be more susceptible to abrasion from the soft tissues, mucosa and opposing teeth as well as extrinsic abrasion by toothbrushes and toothpastes. Although our experimental model is designed to examine the demineralisation side of dental erosion rather than remineralisation, the AEP is possibly modifying the ion exchanges that occur during acidic challenges. Clearly the AEP is not working as a barrier to the proteins as considerable softening occurred in all groups, and especially the WMS3 group suggesting protons had permeated the AEP. Thus it is more likely that the AEP modifies ion movements (protons in, calcium and phosphate out), helping to maintain a high calcium concentration adjacent to the tooth. Another explanation may be that the prolonged immersion time in WMS increase the binding sites of AEP for more salivary proteins to join, facilitating greater protein–protein interactions and enhancing the uptake rate of additional proteins (Hannig and Joiner, 2006; Gibbins *et al.*, 2014). Furthermore, mature AEP has a more protective role against erosion as opposed to early formed AEP and that maturity is closely related to mucin heterotypic complexes (Iontcheva *et al.*, 1997; Hannig and Hannig, 2009). Iontcheva *et al.* (1997) demonstrated that complexes of different proteins are formed between mucins and other small molecular proteins (Iontcheva *et*

al.,1997) which may contribute to formation of a thick, protective AEP. Another possible explanation for the protection of (24 hour+30 minutes) formed AEP may be that the organic components from WMS may have contributed to filling the pores on the enamel surface created by the erosive challenge (Hannig *et al.*, 2009) or self-assembled on the enamel surface to facilitate remineralisation (Kirkham *et al.*, 2007). In addition, a mixed layer of organic and inorganic salivary components may have been formed which in turn is dissolved before the subsurface enamel was completely exposed to acid. With regard to the greater microhardness change, it may be that the organic components cause a decrease in remineralising effect, creating a porous eroded subsurface (Ionta *et al*., 2014; Hara *et al*., 2008; Dawes *et al.*, 1995).

AFM images confirm the results from SNCP and SMH that the mechanism of erosion damage is different between enamel surfaces immersed in different solutions. Although the data in the literature on surface texture are still contradictory, it is generally understood that erosive challenges increase enamel roughness to a certain degree before smoothing of the surface takes place (Las Casas *et al*., 2008). In this study, AFM images of enamel specimens immersed in DW exhibited the characteristic scallop-shell surface indicative of erosive tooth wear of both prismatic and inter-prismatic enamel (Parkinson *et al.*, 2010). The specimens immersed in WMS appeared to show the early stages of erosive tooth wear, with potentially some prism-like structures appearing, however, this was significantly less clear than the specimens immersed in DW. These surfaces were found to be the roughest of all specimens, with erosion progressing and the prism-core material was being lost first while the peripheral tissue remained relatively undamaged, leading to

the sharp peak-and-troughs appearance seen in the 2D profile (Figure 44). This behaviour has been reported previously (Parkinson *et al.*, 2010) and is typical of the type 1 etching pattern first proposed by Silverstone *et al.* (1975) (Silverstone *et al.*, 1975). The specimens immersed in AS showed a different behaviour, which although different to the non-eroded surfaces, was considerably flatter than either of the specimens immersed in WMS or DW and the prism-like structures were far harder to define. This seems to show a difference in the mechanism of erosion when specimens have been immersed in AS compared to WMS, something that to our knowledge has never been shown before.

A range of time periods have been used in previous laboratory studies as described in section 1.4.6.2. In this study three time periods were chosen to investigate the degree of protection from erosive tooth wear offered by a short immersion (30 and 60 minutes) and longer immersion (24 hour+ 30 minutes) in the three different solutions: WMS, AS and DW. 30 minutes was added to 24 hour immersion in order to easily compare it to the 30 minutes only group and to assess the different protection provided by the additional 24 hour immersion. Ten minutes was chosen as the erosion time applied throughout this thesis based on the assumption of the average time it take to drink a glass of orange juice. Additionally, it has been reported to take a minimum of 10 minutes to create enough erosion to distinguish between eroded and non-eroded surfaces using some measuring techniques such as the white light profilometer (Azzopardi *et al.*, 2004). SNCP and SMH were used in this study to provide a broad range of information on the surface change of eroded enamel surfaces. SNCP is considered as the ‘gold standard’ technique for *in*

vitro tooth wear measurements (Schlueter *et al.*, 2005). SMH is used for measurement of specimens subjected to a short immersion in acid referred to as early erosion. Early surface softening can be detected using SMH but profilometric tissue loss cannot be measured accurately (Barbour *et al.*, 2003; Hara and Zero 2008). SMH measurments have been found to be inaccurate after severe *in vitro* erosion. (Rakhmatullina *et al*., 2013). Therefore, SMH was used in this study to provide information on the surface softening of eroded specimens. SNCP on the other hand is more suitable for measurement of bulk tissue loss. The white laser light profilometer used in this thesis has of a spot size of 7 µm which is not suitable for capturing very detailed surface changes and therefore not suitable for accurate measurements in early erosion. Other profilometers with a smaller spot size have been developed that can detect changes of 2 µm and above. This is a potential limitation in this study, however combinig the two techniques aimed to address these limitations. SNCP and SMH also have the challenge of requiring enamel surfaces to be polished flat which render enamel specimens more susceptible to erosion due to possible loss of minerals (Ganss *et al*., 2000). SMH measurments are subjective and can be influenced by many factors such as the presence of smear layer and AEP on the enamel surfaces.

The results of step height measurements in this study for enamel specimens without saliva treatment (control groups) were similar to previously published results which showed that a mean step height of 8.2 µm was obtained after five 10-minutes citric acid (0.3%; 0.2 M) erosion cycles (Mistry, 2016). Our step height results for control group ranged between 8.24 (0.98) µm and 8.82 (1.28) µm. We also chose to use QI mode to obtain the AFM images for two reasons.

Firstly, like other oscillating contact modes the force imparted by the probe on the surface is lower, reducing the potential for surface damage during imaging. Clear differences were observed in the behaviour exhibited by the acid-exposed specimens immersed in DW compared to all NS and AS groups. However, all attempts to quantify the difference in stiffness using AFM failed. A number of methods to derive stiffness or reduced elastic modulus have been proposed for AFM force-distance curves, which rely on either contact mechanics analysis of the initial contact region of the loading curve or the elastic part of the unloading curve (Butt *et al.*, 2005). However, these methods rely on the stiffness of the AFM probe being greater than the stiffness of the substrate. Careful analysis of the gradient of the elastic part of the unloading curves showed them all to have a stiffness within the range of 30-60 N/m (data not shown), which is approximately the same as the spring constant of the probes used to image the specimens. This suggests that the probes used in this study were less stiff than the enamel substrate, precluding any meaningful stiffness measurements in this study. Further, the almost immediate increase in load upon contact with the surface clearly seen in the majority of the loading curves is also indicative of the substrate being stiffer than the probe. Interestingly, for the specimens immersed in DW, it seems that the stiffness of the eroded enamel had reduced to a level below that of the probe. This suggests that future analysis, using stiffer probes may reveal further information regarding the mechanical properties of eroded enamel surfaces.

Application of the findings of *in vitro* studies, such as the present study, to the *in vivo* situation should be interpreted with caution. It is difficult to say whether the significant difference in the protection from erosion offered by the *in vitro*

24 hour formed AEP is directly relevant to the clinical situation. The changes and degradation of pooled saliva due to the collection, storage, and cycling such as CO_2 evaporation may have altered the salivary protective properties (Hall *et al.*, 1999; Schipper *et al.*, 2007; Zwier *et al.*, 2013). This study has used ground and polished enamel surfaces which differ in enamel mineral content compared to the outer natural enamel layer (Ganss *et al*., 2000; Carvalho *et al*., 2015). The quality of *in vitro* AEP in this study may be compromised since the charged molecules of some salivary proteins interact with the calcium and phosphate ions of enamel crystals which can influence the type of proteins adsorbed to the AEP (Hannig and Hannig, 2009). In addition, the polished enamel tooth surfaces can have different susceptibility to erosive wear as compared to unpolished natural enamel surfaces. Ganss *et al*.(2000) reported an increased erosive tooth wear for polished enamel specimens compared to unpolished enamel which they attributed to the lower surface mineral content and underlying pores (Ganss *et al*., 2000).

3. 6 Conclusions

Immersion of enamel specimens in WMS (containing proteins and minerals) offered better protection against erosion compared with AS (containing minerals only) and DW (containing no proteins or minerals) manifested by a lower step height. AS in turn offered better protection than DW. Increasing immersion times of enamel specimens in WMS demonstrated increasing protective effects.

Overall aims of Chapter 4 and 5:

In light of the results and conclusions in Chapter 3 that (24 hour+30 minutes) immersion in WMS offered the best protection, (24 hour+30 minutes) formed AEP model was used in all subsequent *in vitro* studies within this thesis. (24 hour+30 minutes) immersion will be referred to as 24 hour immersion within this thesis. The five cycle erosion model used in Chapter 3 represents a longer exposure to acid and will be referred to as advanced erosion in this thesis. Also in Chapter 3, it was demonstrated that a combination of salivary proteins and ions (WMS) offered better protection against erosion compared to salivary ions only (AS). The next two Chapters further investigate the role of some specific salivary proteins against a longer exposure to acid (advanced erosion) using the 24 hour model. Chapter 4 compares the protection of enamel surfaces offered by WMS formed AEP, PS formed AEP, AS and DW against advanced erosion. As WMS and PS contain different protein compositions, comparison between the two could provide some insight into identification of salivary proteins that may play a role in protection against erosion.

Chapter 5 uses a similar model to Chapter 4, but compares the protection of enamel surfaces offered by WMS formed AEP, PS formed AEP, AS and DW against a shorter exposure to acid which uses only one cycle erosion and will be referred to as early erosion within this thesis. It is possible that different types of saliva offer different levels of protection when subjected to either early or advanced erosion.

Chapter 4: Do salivary proteins mediate greatest protection against *in vitro* advanced erosion?

Overall aims of Chapter 4

In this Chapter, 24 hour *in vitro* model is used to compare the protective effect of WMS and PS against a five cycle erosion model representing *in vitro* advanced erosion. Chapter 4 is composed of four sections (4.1,4.2,4.3,4.4). Section 4.1, compares the protection level between WMS, PS, AS and DW against advanced erosion using SNCP and SMH techniques. Section 4.2 is divided into three subsections looking at three aspects of the *in vitro* AEP. The first aspect compares the amount of total proteins between AEP from WMS and PS before and after advanced erosion. The second aspect compares the amount of four specific proteins: mucin5b, albumin, CA VI and statherin in the *in vitro* AEP from WMS and PS before and after advanced erosion. The third aspect compares the concentration of calcium and phosphorus between AEP from WMS and PS before and after advanced erosion. Section 4.3, discusses sections 4.1 and section 4.2. Finally section 4.4, aims to evaluate the effect of combining AS with human serum albumin at varying concentrations on protection against advanced erosion.

Section 4.1: Does the protective effect of whole mouth saliva against *in vitro* advanced erosion differ from that of parotid saliva?

4.1.1 Introduction

Our data on advanced erosive tooth wear (Chapter 3) demonstrated that 24 hour immersion of enamel specimens in WMS offers better protection against a five cycle erosion model representing *in vitro* advanced erosion than AS leaving behind a softer, rougher surface but less step height formation as compared with AS and DW. The sources of organic and inorganic salivary constituents are from major and minor salivary glands as described in section 1.4.1.1. The interfacial behaviour between WMS and enamel surface is a dynamic process that offers protective functions against erosion in many ways as mentioned in section (1.4.4). The intriguing question that remains is which components or combination of components of WMS offer this protection.

Natural saliva can be collected as WMS as described in section (2.2.1.1) or from a single salivary gland such as submandibular, sublingual or parotid gland (as described in 2.2.1.2) which are different in structure and composition. Two previous studies have compared the protection against advanced erosion between WMS and PS (Amerongen *et al.*, 1987; Martins *et al.*, 2013). Amerongen *et al.*, (1987) has demonstrated that WMS provided better protection against enamel demineralisation than PS, whereas no significant difference between WMS and PS was found by Martins *et al.* (2013). One of the most important functions of both WMS and PS in relation to erosion is the formation of AEP. The source of saliva appears to play an important role in the quality of the AEP (Wetton *et al.*, 2007; Martins *et al.*,

2013; Ash *et al.*, 2014). Owing to the structural and compositional complexity of WMS as well as the instantaneous interaction between salivary proteins and minerals, it is difficult to determine the exact components of AEP that have a role in the protection of the enamel surface against erosive tooth wear. It is still unknown as to which AEP protein/s is/are responsible for protection against erosive tooth wear. Our hypotheses for this section were that proteins (WMS and PS) could provide extra protection against erosive tooth wear as compared to salivary ions (AS),or no proteins or ions (DW) and that proteins collected from all glands (WMS) would differ in protection against advanced erosion than proteins derived from parotid gland only (PS).

4.1.2 Aims, objectives and hypotheses

The aim of this *in vitro* study was to compare the level of protection between WMS, PS, AS and DW against advanced erosion.

The objective was

1. To compare the step height and SMHC of enamel surfaces when immersed in WMS, PS, AS and DW for 24 hours followed by five erosion cycles.

The null hypothesis was:

1. Immersion of enamel specimens in WMS, PS, AS and DW for 24 hours followed by five erosion cycles will not produce significantly different step height and SMHC after five erosion cycles.

4.1.3 Materials and methods

4.1.3.1 Specimen preparation

Based on a power calculation, a sample size of 40 was required yielding 80 % power at 5 % level with an effect size of 0.31 (Nekrashevycha and Stösserb, 2003; Martins *et al*., 2013; Mistry *et al.*, 2015; O'Tool *et al.*, 2015). Forty six enamel specimens were prepared by sectioning twenty three buccal and twenty three lingual surfaces from twenty three human molar extracted sound teeth. Initial surface microhardness values (SMH) measured. The specimens with a SMH value between 272 KHN and 400 KHN were selected (Meredith *et al*., 1996; Austin *et al*., 2011; Lussi *et al*., 2011). Forty specimens were selected for this study and six were rejected. Enamel surfaces were then embedded in cold cure acrylic resin as described in section 2.1.4. Enamel specimens were ground and polished to provide a highly polished, flat surface 3 x 3 mm in size as explained in section 2.1.5. All prepared specimens were then taped as explained in section 2.1.7. and they were then numbered and randomised as described in section 2.1.8.

4.1.3.2 Study methodology

The study consisted of four groups according to the four solutions used, 10 specimens per group: WMS, PS, AS and DW groups. The WMS, PS and AS were prepared as described in section 2.2. Thawed WMS and PS were mixed vigorously prior to use to re-suspend precipitation of proteins and avoid loss of specific proteins less than 14 kDa (Francis *et al.*, 2000). Specimens were randomly allocated by an independent investigator using SPSS random sample generator to 4 groups: WMS, PS, AS and DW. Specimens of each

group were exposed to five cycle erosive modle as described in section 2.3.4.2. Specimens were immersed in the corresponding solution (either WMS, PS, AS or DW) for 24 hour followed by a further 30 minutes prior to exposure to a 10-minute citric acid followed by 2-minute water rinse. The cycle of immersion for 30 minutes in the corresponding solution followed by the erosion cycle was repeated 5 times for each group as shown in Figure 46. When immersed for 24 h in solution, specimens were stored un-agitated overnight at 22 ° C ± 1. The erosion cycle consisted of 80 mL 0.3% citric acid, pH=3.2, at 22 °C ± 1, agitated with an orbital shaker (Stuart Scientific, orbital shaker) at 60 rpm followed by 2-minute in 100 mL of DW rinse, again, under agitation with an orbital shaker set at 60 rpm for a final 2 minutes.

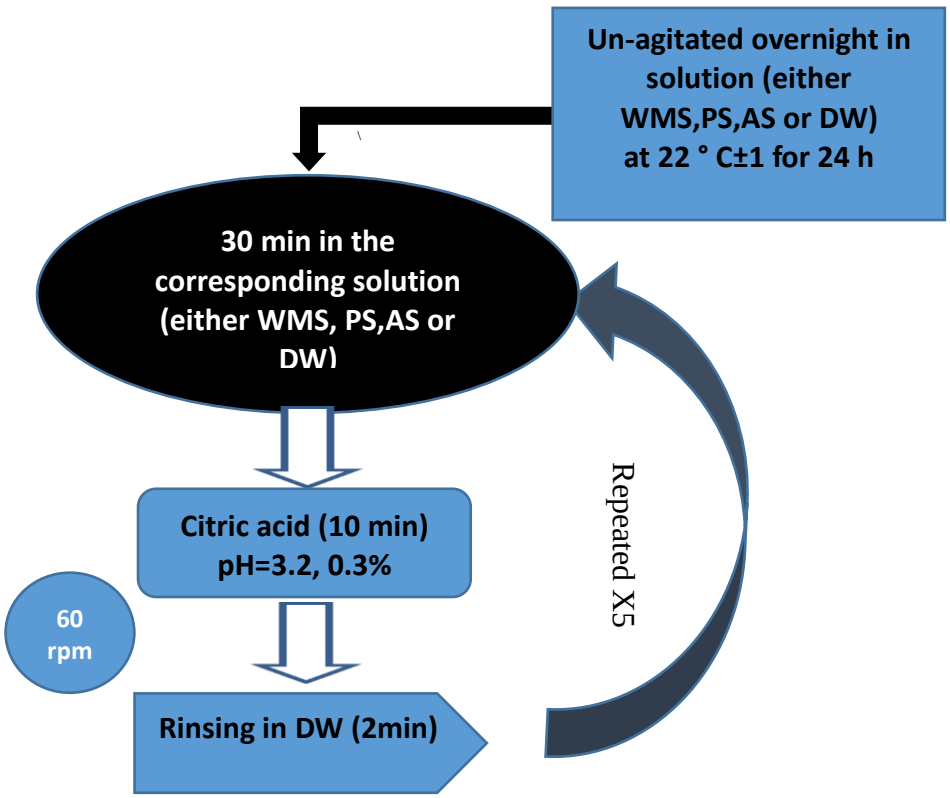


Figure 46: *A flowchart representation of the AEP formation and five erosion cycles protocol.*

4.1.3.3 Testing

Profilometric measurements

Profilometric measurement data after five erosion cycles were obtained using SNCP as explained in section 2.4.1. Each surface profile was taken from within the first 1/3 of the taped zone on one side across the exposed window to just within the first 1/3 of the taped zone on the opposite side. Ten randomly selected step height measurements were taken from each specimen and averaged to give a mean surface profile value.

Surface microhardness (SMH) measurements

Surface microhardness at baseline (SMHb):

The surface microhardness (SMH) values before immersion in WMS, PS, AS, and DW (surface microhardness at baseline: SMHb) were measured for the four experimental groups as described in section 2.4.2. The SMH values of each specimen was determined by the average of five indentations made at the exposed window under a load of 100 g and a dwell time of 10 seconds. The SMH value of each indentation was determined by specialised software (Duramin-5Hardness Tester, Struers Inc., Rotherham, UK) through measuring the length of each indentation with an optical analysis system calculating hardness in Knoop units (KHN).

Surface microhardness (SMH) after five cycles erosion:

Surafce microhardness (SMH) values were repeated after the experiment using the method explained above. The surface microhardness change (SMHC) of each specimen was then calculated by subtracting the mean SMH

value after five erosion cycles (SMHe) from the mean SMH value before erosion (SMHb) using the formula: SMHC = (SMHb – SMHe).

4.1.3.4 Statistical analysis:

Data obtained from the profilometry and microhardness were analysed using SPSS stata version 12.0 (StataCorp LP, Texas 77845-4512, USA). The measured outcomes were analysed using descriptive quantitative methods to summarise the study characteristics of the various subgroups. Shapiro-Wilk and Kolmogorov-Smirnov tests were used to assess the normality distributions of data. Data were also visually assessed using histogram, Q-Q plots and Box and Whisker Plots. Data were normally distributed and were described using means and standard deviations. Linear regression models were used to test the significant difference between solutions (WMS,PS,AS,DW) with respect to step height and SMHC. The initial model included the interaction between cycles and solutions along with the main effects. The mean difference was considered to be significant at a P value < 0.05. If the interaction between solutions was significant, then further post hoc Bonferroni test analyses were carried out to find out which solution was statistically significant in relation to step height and microhardness change.

4.1.4 Results:

Step height

Table 20 and Figure 47 show the results of the mean (SD) step height of the enamel specimens after five cycles of erosion. The mean (SD) step height for WMS group was [4.14 (0.9) µm], PS group was [6.42 (0.3) µm], AS group was [7.47 (1.0) µm], DW group was [10.89 (1.3) µm]. WMS group [4.14 (0.9) µm] and PS group [6.42 (0.3) µm] had significantly lower step height than AS group [7.47 (1.0) µm] and DW group [10.89 (1.3) µm]. WMS group showed significantly lower step height than PS group ($p < 0.0001$). Significant differences were observed between all groups ($p < 0.0001$).

Solution Type (n=10)	**Step height (µm) Mean (SD)**
Whole mouth saliva (WMS)	4.14 (0.9)$^{\beta}$
Parotid Saliva (PS)	6.42 (0.3)$^{\beta}$
Artificial Saliva (AS)	7.47 (1.0)$^{\beta}$
Deionised water (DW)	10.89 (1.3)$^{\beta}$

Table 20: *Mean (SD) step height of enamel specimens for four groups after five erosion cycles. Significant differences were observed between all groups (p< 0.0001). Same symbols indicate significant differences.*

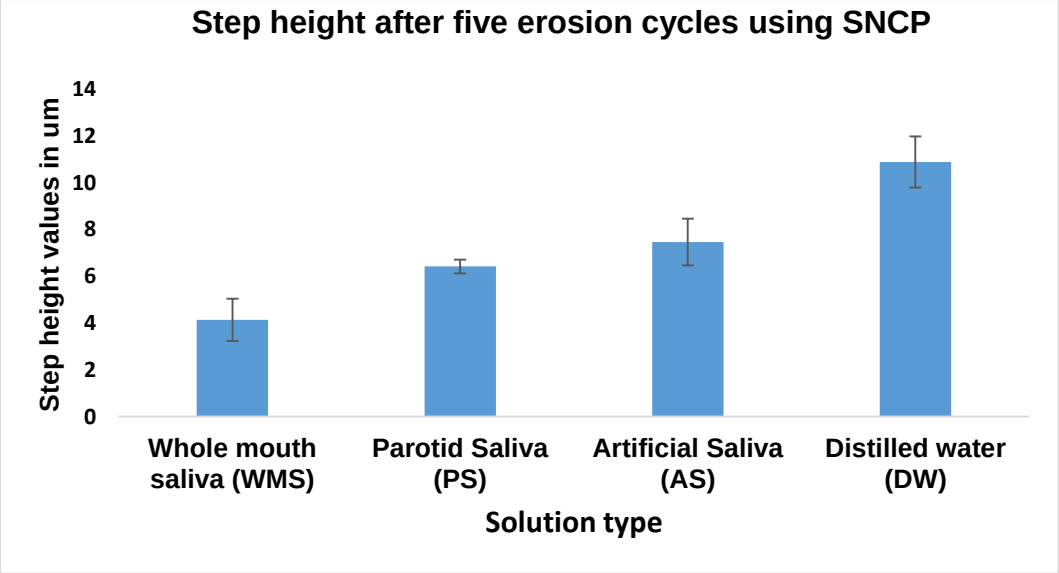


***Figure 47**: Mean (SD) step height (µm) for enamel surfaces for four groups after the five cycles erosion using white optical light profilometer. Significant differences between all groups (p<0.0001).*

Surface microhardness change (SMHC)

Surface microhardness (SMH) at baseline

Table 21 shows the surface microhardness at baseline (SMHb) values before immersion in WMS, PS, AS, and DW and before erosion cycle. This shows that the average of SMH values ranged between [352.21 (8.87) KHN] and [357.40 (10.52) KHN]. There were no significant differences between the mean SMH values of the four groups (p>0.05).

Solution type (n=10)	Surface microhardness at baseline (SMHb) Mean (SD)
Whole mouth saliva (WMS)	355.22 (16.70)
Parotid saliva (PS)	352.21 (8.87)
Artificial saliva (AS)	357.40 (10.52)
Deionised water (DW)	354.72 (17.52)

Table 21: *Mean (SD) surface microhardness at baseline (SMHb) of enamel surfaces in Knoop microhardness units (KHN) after samples were polished and before immersed in solutions. No significant differences between the groups.*

Table 22 and Figure 48 show the results of the mean (SD) surface microhardness change (SMHC) of enamel specimens for four experimental groups after five cycles of erosion. The mean SMHC for WMS group was [224.11 (25.2) KHN], PS group was [208.16 (17.3) KHN], AS group was [194.0 (12.8) KHN] and DW group was [155.34 (18.4) KHN]. DW group [155.34 (18.4) KHN] had significantly lower SMHC than WMS group ($p<0.0001$), PS group ($p<0.0001$) and AS group ($p=0.002$). When comparing WMS, PS and AS groups, only AS group had significantly lower SMHC than WMS group ($p=0.012$). PS group had no significant difference in SMHC with either the WMS group ($p=0.18$) or the AS group ($p=0.23$).

Solution Type (n=10)	Surface microhardness change (SMHC) Mean(SD)
Whole mouth saliva (WMS)	224.11 (25.2)Δβ
Parotid Saliva (PS)	208.16 (17.3)ο
Artificial Saliva (AS)	194.0 (12.8)¥β
Deionised water (DW)	155.34 (18.4)Δο¥

***Table 22**: Mean (SD) surface microhardness change (SMHC) of enamel surfaces of four groups after five cycle erosion in Knoop microhardness units (KHN). Similar shapes in the table denote significant differences between the groups.*

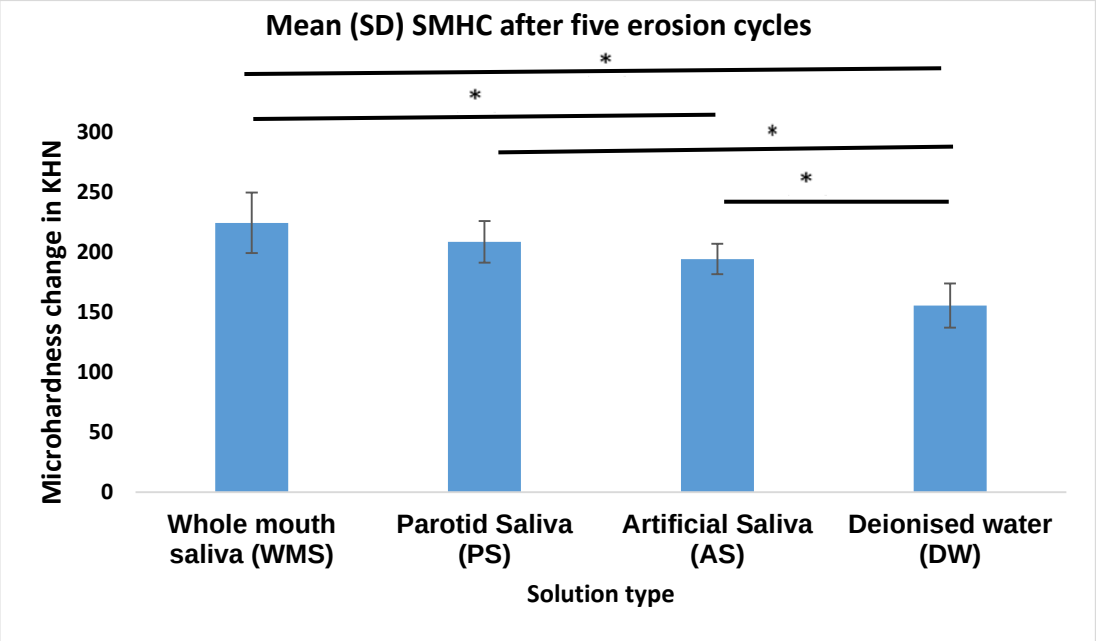


***Figure 48**: Mean (SD) surface microhardness change (SMHC) of enamel surfaces in Knoop microhardness units (KHN) for four groups after the fifth erosion cycle.*

Section 4.2: The role of proteins derived from whole mouth and parotid saliva on advanced erosion: An *in vitro* study.

4.2.1 Introduction

Following from section 4.1 which demonstrated significant differences in the protective effect of WMS compared to PS against advanced erosion, this section aims to compare the protein contents between WMS and PS. Section 4.2 compares the amount of total protein as well as four specific proteins present in the *in vitro* AEP derived from WMS and PS. The *in vivo* formation of AEP is a complex process and the intra oral dynamics of WMS lead to constant changes in the type of proteins adsorbed and incorporated into the AEP (Cheaib and Lussi, 2011). AEP has two layers: a basal, thin, dense inner protein layer and an outer thicker layer (Hannig and Joiner, 2006). The basal layer of AEP has been suggested to play an important role against erosive tooth wear (Hannig and Balz, 2001; Hannig *et al.*, 2005). The protein components of the AEP have been studied by several authors (Carlen *et al.*, 1998; Leinonen *et al.*, 1999; Yao *et al.*, 2001; Li *et al.*, 2003; Yao *et al.*, 2003; Vitorino *et al.*, 2004; Cárdenas *et al.*, 2007; Siqueira *et al.*, 2007) and a number of key proteins have been identified including mucin5b, albumin, CA VI and statherin. These proteins serve many functions in AEP, including lubrication and physical coating by mucins (Amerongen *et al.*, 1987; WICKSTRÖM *et al.*, 1998), provision of a diffusion barrier by albumin (Hemingway *et al.*, 2008) and acid neutralisation by CA VI (Leinonen *et al.*, 1999). Statherin is believed to initiate the formation of AEP playing an important role in calcium homeostasis (Li *et al.*, 2004; Kosoric *et al.*, 2007). These proteins are delivered into AEP from different salivary glands as well as from non-exocrine sources

such as gingival cervicular fluid as in the case of albumin. Until now, the role of the proteins within AEP that play a protective function against erosive tooth wear has not been well understood. Due to the small amounts of protein available in AEP, a targeted approach was used in this section to measure four key salivary proteins: mucin5b, albumin, CA VI and statherin. Therefore, this section explores the amount of total protein and the protection levels of these four specific proteins present in AEP from WMS and PS against advanced erosion. Another important physiological factor of interest in the role of AEP against erosive tooth wear is the synergy between salivary proteins and salivary ions that are often overlooked in AEP studies. Little is known about the calcium and phosphorus amounts retained in the remaining AEP after erosion. To the author's knowledge, there is only one study which looked at the concentration of calcium in the AEP (Carpenter *et al.*, 2014). Section 4.2 also looks at the levels of calcium and phosphorus ions in AEP before and after advanced erosion.

4.2.2 Aims, objectives and hypotheses

The aim of this *in vitro* study was to measure the total protein and four specific salivary proteins present in AEP after 24 hour immersion in WMS and PS (before erosion) and after advanced erosion (five erosion cycles).

The objectives were:

1. To compare the amount of total protein in *in vitro* AEP after 24 hours immersion in WMS or PS (before erosion) and after five erosion cycles using BCA assay.

2. To compare the amount of mucin5b, albumin, CA VI and statherin in *in vitro* AEP after 24 hours immersion in WMS or PS (before erosion) and after five erosion cycles using SDS-PAGE and immunoblotting.
3. To compare the amount of calcium and phosphorus ions in *in vitro* AEP after 24 hours immersion in WMS or PS (before erosion) and after five erosion cycles using inductively coupled plasma mass spectroscopy (ICP-MS).

The null hypotheses were:

1. The concentration of total protein in *in vitro* AEP derived from WMS would not differ from that in *in vitro* AEP derived from PS before and after five erosion cycles.
2. The amount of mucin5b, albumin, CA VI and statherin in *in vitro* AEP derived from WMS would not differ from that in AEP derived from PS before and after five erosion cycles.
3. The concentration of calcium and phosphorus in *in vitro* AEP derived from WMS would not differ from that in *in vitro* AEP derived from PS before and after five erosion cycles

4.2.3 Materials and methods

4.2.3.1 Specimen preparation

Based on the power calculation as described in section 2.1.3, twenty enamel specimens were prepared for this study yielding an effect size of 0.6 and 80 % power at 5 % level. Twenty two enamel specimens were prepared by sectioning eleven buccal and eleven lingual surfaces from eleven human extracted molar sound teeth. Initial surface microhardness values (SMH)

measured. The specimens with a SMH value between 272 KHN and 400 KHN were selected (Meredith *et al*., 1996; Austin *et al*., 2011; Lussi *et al*., 2011). Twenty specimens were selected and two were rejected. Enamel surfaces were mounted in cold cure acrylic resin as described in section 2.1.4. Enamel specimens were ground and polished as explained in section 2.1.5. All prepared specimens were then taped with PVC adhesive tape as explained in section 2.1.7. and they were randomised and numbered as described in section 2.1.8.

4.2.3.2 Study methodology

The study consisted of 2 experimental groups according to the two solutions used, 10 specimens per group: WMS and PS groups. The WMS and PS were prepared as described in section 2.2. Thawed WMS and PS were mixed vigorously prior to use to re-suspend precipitation of proteins and avoid loss of specific proteins less than 14 kDa (Francis *et al*., 2000). Within each group, specimens were randomly allocated by an independent investigator using SPSS random sample generator to 2 subgroups, 5 specimens each: control: no erosion (n=5) and five cycles erosion (n=5). The AEP was eluted for the control group after 24 hour immersion in the corrsponding saliva followed by 2 minute rinse in DW prior to acid erosion (control) as shown in (Figure 50). For the five cycles erosion group, AEP was eluted after five erosion cycles as shown in (Figure 49). Details on the design of both groups and how AEP is eluted in each group are explained in the following sections.

Five cycles erosion

In the five cycle erosion group, enamel specimens were exposed to five cycles erosion model as described in section 2.3.4.2 and as shown in Figure 49.

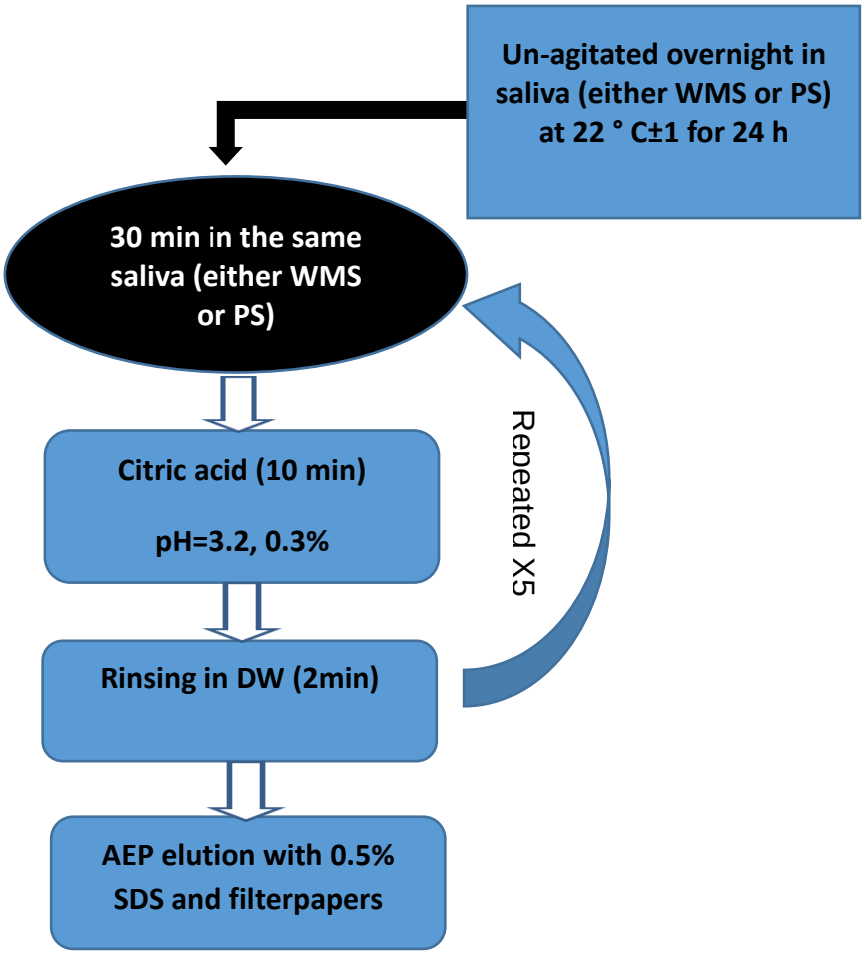


Figure 49: *A flowchart representation of the 24 hours in vitro AEP formation and elution protocol after five erosion cycles.*

Specimens were immersed in either WMS (n=5) or PS (n=5) for 24 hour followed by a further 30 minutes prior to exposure to a 10-minute citric acid followed by 2-minute water rinse. When immersed for 24 hour in WMS or PS, specimens were stored un-agitated overnight at 22 °C ± 1. The cycle of immersion of the specimens in 30 minutes in the corresponding saliva followed

by the erosion cycle was repeated 5 times for each subgroup. The AEP was then eluted after the completion of the fifth erosion cycle using 0.5 % SDS and filterpapers and then recovered as detailed in section 2.3.

Control group:

In order to assess the amount of proteins in the *in vitro* AEP before erosion cycles, AEP was eluted after enamel specimens were immersed in either WMS (n=5) or PS (n=5) for 24 hour followed by 2 minutes immersion in DW Figure 50.

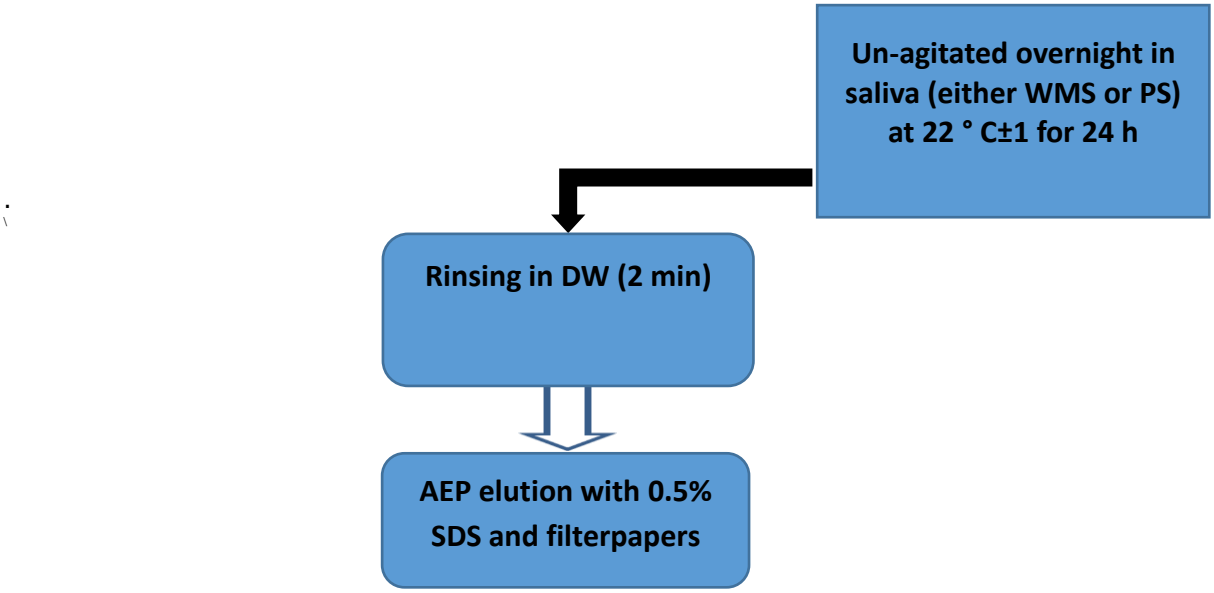


Figure 50*: A flowchart representation of the 24 hours in vitro AEP formation protocol before erosion cycle (control group).*

This served as the control group where AEP was eluted prior to acid erosion. *In vitro* AEP was eluted using 0.5 % SDS and filterpapers and then recovered as detailed in section 2.3.2.

4.2.3.3 Testing

The eluted *in vitro* AEP were then recovered from the filterpapers using the same procedure as described in section 2.3.3. The protein contents was

analysed for total protein, four specific proteins and calcium and phosphorus concentration.

4.2.3.3.1 Total protein analysis

Part (1µL) of each in vitro recovered AEP samples from WMS (n=10) and PS (n=10) were prepared for the analysis of total protein concentration. Each sample was diluted in DW at 1/100 to a final volume of 100 µL. Prepared *in vitro* AEP samples were placed into microtiter plates (96-wells, Fisher Scientific, Leicestershire). The concentration of total protein of each AEP sample was measured using the bicinchoninic acid (BCA) assay (Pierce Chemical, Rockford, Ill., USA) and bovine serum albumin (BSA) standard as a reference. The concentration of total protein was measured spectrophotometrically employing a UV-visible spectrophotometer (BioRad laboratories Ltd, Hemel Hempstead, UK) determining the optical density at a wavelength of 562 nm as explained in section 2.4.4. All samples were analysed in duplicate.

4.2.3.3.2 Specific protein analysis

Protein separation:

Qualitative differences between the *in vitro* recovered AEP samples from WMS (n=10) and from PS (n=10) were analysed using SDS–PAGE. The prepared protein fractions were loaded and run equally (15 µL each) through the precast gels and were separated consistently as described in section 2.4.5.3.

Protein transfer and immunoblotting

After the separation of proteins, western blotting was completed according to the manufacturer's instructions and used to transfer proteins onto a nitrocellulose membrane as explained in section 2.4.6.1. Protein bands of the proteins of interest were cut transversely from the nitrocellulose membranes with a sterile razor. Immunoblotting was used to examine the presence of four proteins of interest in the AEP: mucin5b, albumin, CA VI and statherin as described in section 2.4.6.3. At room temperature, the nitrocellulose membranes were blocked in TTBS for 1 hour before membranes were probed with primary antibodies as described in section 2.4.6.3. The nitrocellulose membranes were then washed in TTBS for 15 minutes (5 minutes X 3 times) and then followed by incubation with the required secondary antibody. Details of the primary and secondary antibodies used were given in section 2.5.2. A final 15-minutes wash in TTBS was completed before the membranes were developed with ECL substrate and were imaged as described in the next section.

Imaging analysis:

The presence of proteins on the blotted and developed nitrocellulose membrane was assessed using photographic quantification of the staining intensity of proteins as was explained in section 2.4.6.4. ChemiDoc MP imaging analysis (Bio-Rad) was used to quantify the light intensity of the chemiluminescent reaction and exposure times optimised to prevent pixel saturation. The amounts of proteins on the blotted nitrocellulose membranes were quantified using tools of ImageLab software version 4.1 (Bio-Rad Laboratories Ltd., Hertfordshire, UK) to select and determine the background-subtracted density of the bands in all the gels (n=3) using purified protein

standards of known concentration. The standard curves of purified proteins were generated from the mean (SD) volume intensities (n=3) against the absolute quantities of the corresponding purified standard. This was used to generate a calibration curve using a linear formula. This formula was used to calculate the amount of each protein in the *in vitro* AEP samples. The bands of standard proteins on different SDS-PAGE gels (n=3) were used to assess reproducibility.

4.2.3.3.3 Calcium and phosphorus analysis

The *in vitro* AEP samples were eluted from enamel surfaces after 24 hours immersion in WMS or PS (control) and after five erosion cycles as described in 4.2.3.2. Part of each prepared *in vitro* AEP sample from WMS (n=10) and from PS (n=10) was further diluted in DW (1:1000 dilution) to provide a 1 mL sample. Each diluted AEP sample was then subjected to calcium and phosphorus analysis using inductively coupled plasma mass spectroscopy (ICP-MS) (SCIEX ICP mass spectrometer, ELAN DRC 6100; PerkinElmer, Waltham, Mass., USA) as explained in section 2.4.7.

4.2.3.4 Statistical analysis

Data obtained from the protein analysis test were analysed using Stata 12.0. The total and four specific protein as well as calcium and phosphorus data did not follow normal distribution and hence they were log transformed to achieve normality. Therefore, data were described using mean and standard deviation as well as median and interquartile range. Total protein, mucin5b, albumin, calcium and phosphorus were log transformed to attain normality and log transformed values were used for the analysis. CA VI and statherin were

square root transformed and the transformed values were used for the analysis. Linear regression models were used to find out the effect of saliva type (WMS and PS) as well as the erosion condition (before erosion and after five cycles). The initial model included interaction between groups and saliva. If the interaction was statistically significant, the post hoc Boneferroni analysis testing the linear combinations of groups and saliva was used to find out which group and saliva were statistically significant. All p values were to be adjusted for multiple testing. If the interaction effect was not statistically significant, then the final model included only the main effects of groups and saliva.

4.2.4 Results

4.2.4.1 Total protein

Table 23 and Figure 51 show the total protein concentration (SD) in the *in vitro* AEP samples derived from WMS and PS before erosion (control) and after five cycles of erosion.

The means (SD) concentration of total proteins in AEP from WMS before erosion was [1.65 (0.16) g/L] and after five erosion cycles was [0.38 (0.10) g/L]. The mean (SD) concentration of total protein in AEP from PS before erosion was [0.67 (0.12) g/L] and after five erosion cycles was [0.15 (0.05) g/L].

Saliva type and erosion condition	Concentration of total proteins (g/L)	
	Mean (SD)	**Median (IQR)**
Whole mouth saliva at control (WMSC)	1.65 (0.16)μπ	1.66 (0.19)
Whole mouth saliva after five cycle erosion (WMSEV)	0.38 (0.10)μΔ	0.37 (0.18)
Parotid saliva control (PSC)	0.67 (0.12)#π	0.65 (0.07)
Parotid saliva after five cycle erosion (PSEV)	0.15 (0.05)#Δ	0.12 (0.11)

Table 23*: Mean (SD) and median (interquartile range) concentration of total protein (g/L) in vitro salivary AEP formed on enamel specimens immersed in either WMS or PS for 24 h. AEP were then eluted before or after five cycles erosion using 0.5% SDS and quantified using BCA assay. Same symbols in the table indicate significant differences (p<0.0001). (WMSC: whole mouth saliva at control; WMSEV: whole mouth saliva after 5 erosion cycles; PSC: parotid saliva at control; PSEV: parotid saliva after 5 erosion cycles)*

Generally, AEP from PS had significantly lower concentration of total protein than AEP from WMS in all groups (before and after erosion) (p<001). For AEP from WMS, the concentration of total protein after five erosion cycles [0.38 (0.10) g/L] was significantly lower than that in AEP before erosion [1.65 (0.16) g/L] (p<0.000). For AEP from PS, the total protein concentration in AEP after five erosion cycles [0.15 (0.05) g/L] showed significantly lower concentration than that before erosion [0.67 (0.12) g/L] (p<0.000).

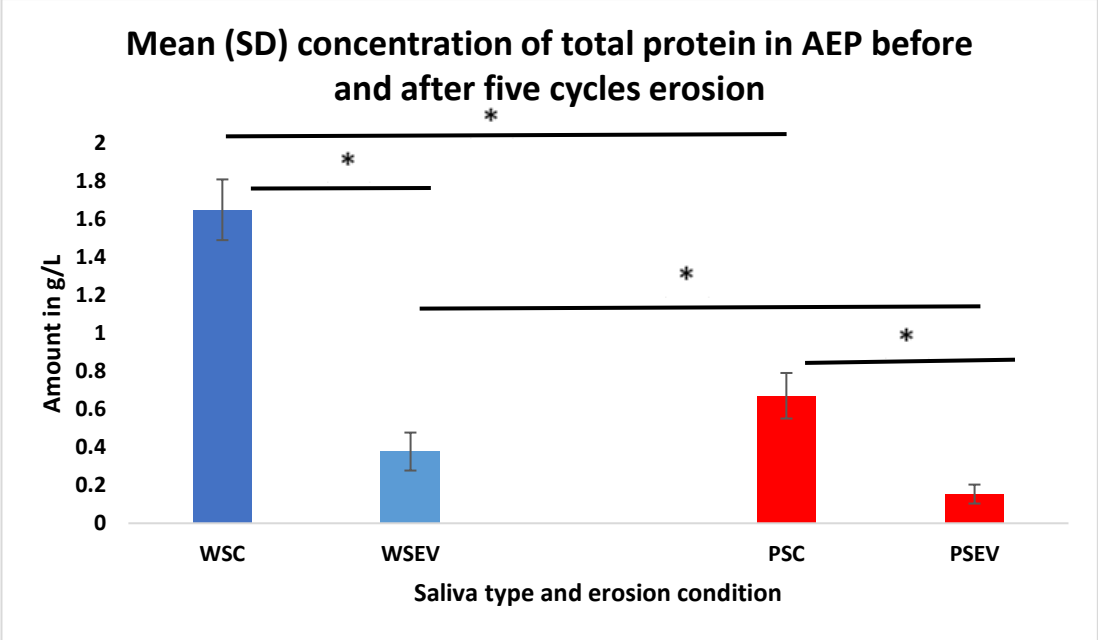
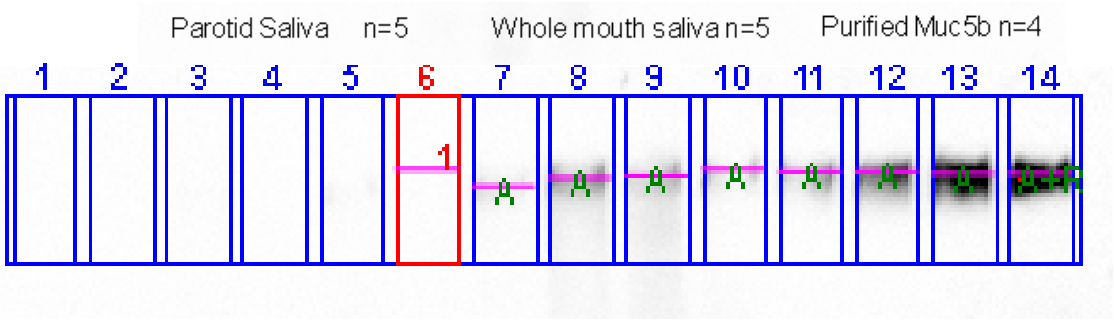


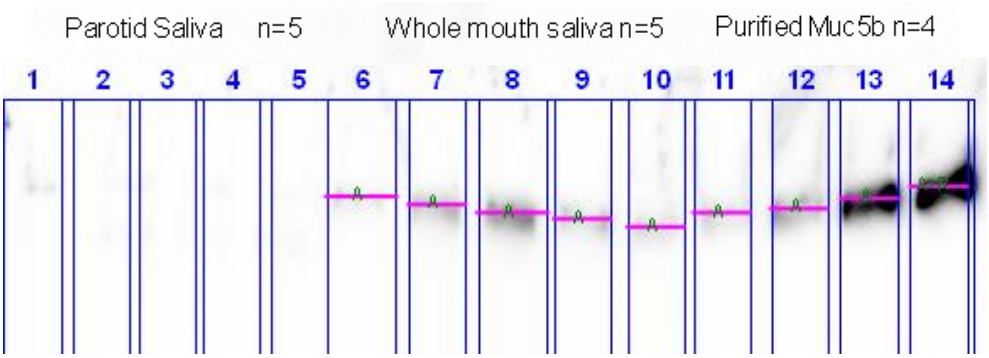
***Figure 51**: Mean (SD) amount of total protein (µg/ul) in vitro AEP formed on enamel specimens immersed in either WMS or PS for 24 h. AEP were then eluted before or after five cycles erosion using 0.5% SDS and quantified using BCA assay. Asterisk shapes in the figure indicate significant differences (p<0.0001). (WSC: whole mouth saliva at control; WSEV: whole mouth saliva after 5 cycles erosion; PSC: parotid saliva at control; PSEV: parotid saliva after 5 cycles erosion)*

4.2.4.2 Specific proteins

Figure 52 (I,II,III,IV,V) shows images of SDS-PAGE and western blots of the four specific proteins investigated before and after five erosion cycles. Western blots of AEP samples from WMS (n=10) and PS (n=10) before erosion and after five cycle erosion were probed with antibodies against mucin5b, albumin, CA IV and statherin.

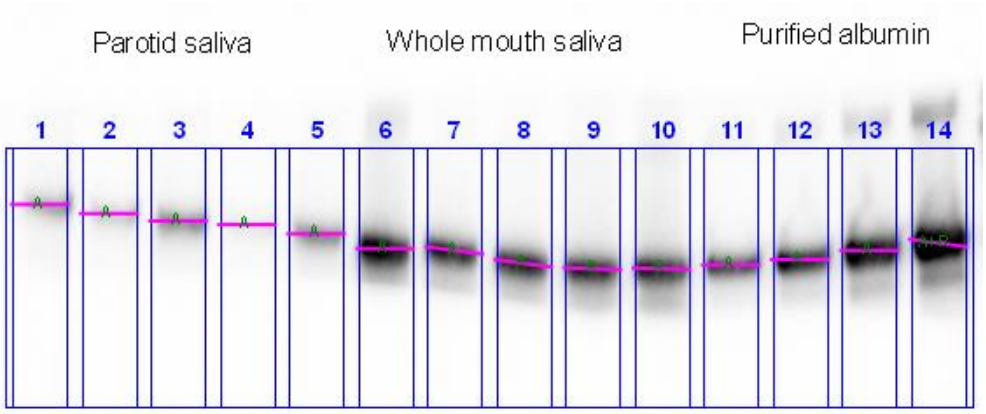


a) SDS-PAGE and western blots of AEP samples before erosion from WMS (n=5) and PS (n=5) probed with mucinb5 antibody

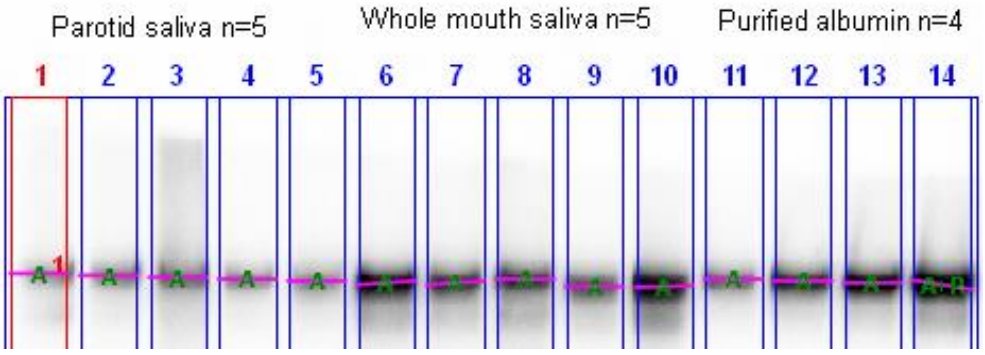


b) SDS-PAGE and western blots of AEP samples after five cycles erosion from WMS (n=5) and PS (n=5) probed with mucinb5 antibody

Figure 52 (I): *SDS-PAGE and western blots of in vitro AEP samples from WMS (n=5) and PS (n=5) and purified proteins of standards (n=4). All samples were immunoblotted against mucin5b (a: before erosion (control); b: after five cycles erosion).*

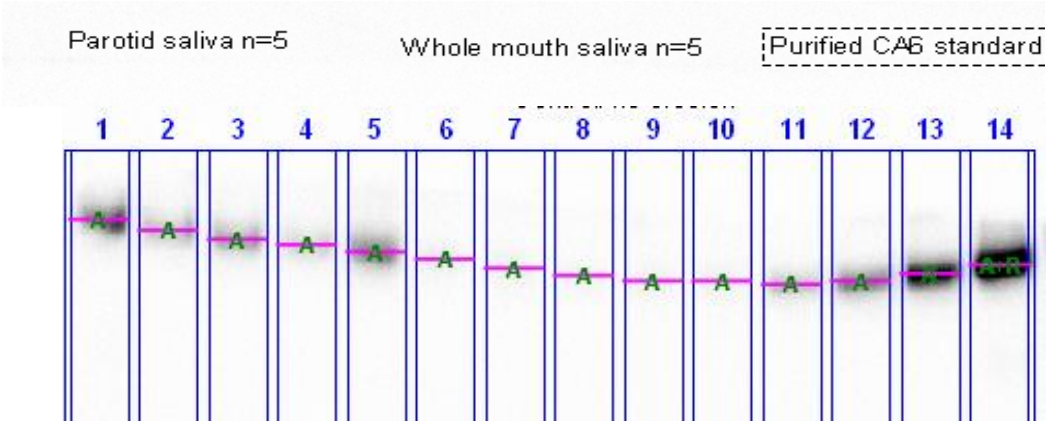


a) SDS-PAGE and western blots of AEP samples before erosion from WMS (n=5) and PS (n=5) probed with albumin antibodies

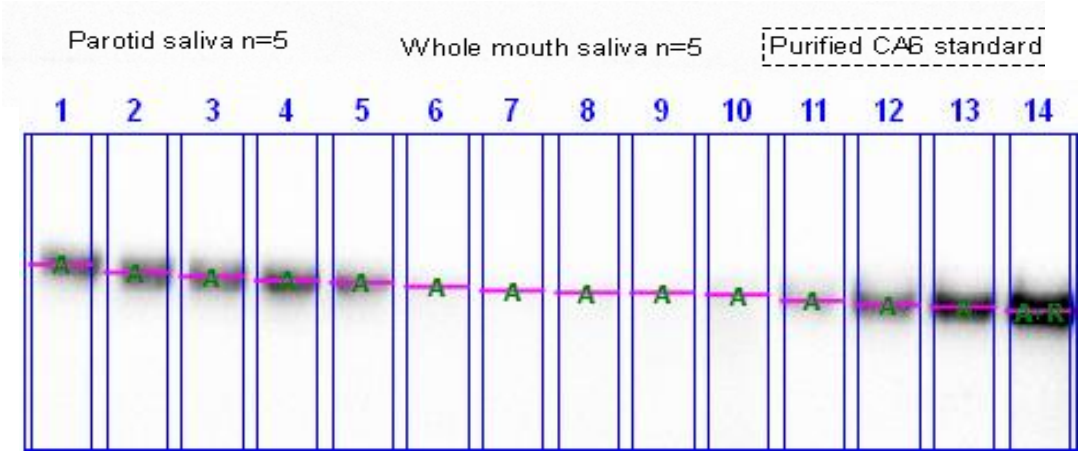


b) SDS-PAGE and western blots of AEP samples after five cycles erosion from WMS (n=5) and PS (n=5) probed with albumin antibodies

Figure 52 (II): *SDS-PAGE and western blots of AEP samples from WMS (n=5) and PS (n=5) and purified proteins of standards (n=4). All samples were immunoblotted against albumin antibody (a: before erosion (control); b: five cycles erosion).*

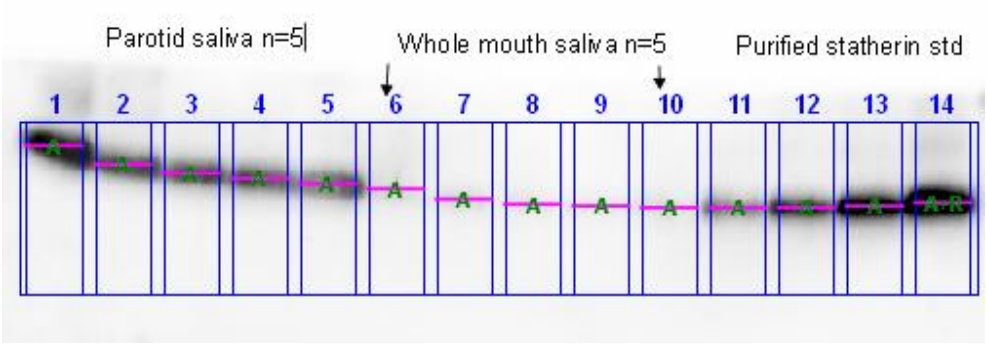


a) SDS-PAGE and western blot of AEP samples before erosion from WMS (n=5) and PS (n=5) probed with CA VI antibody.

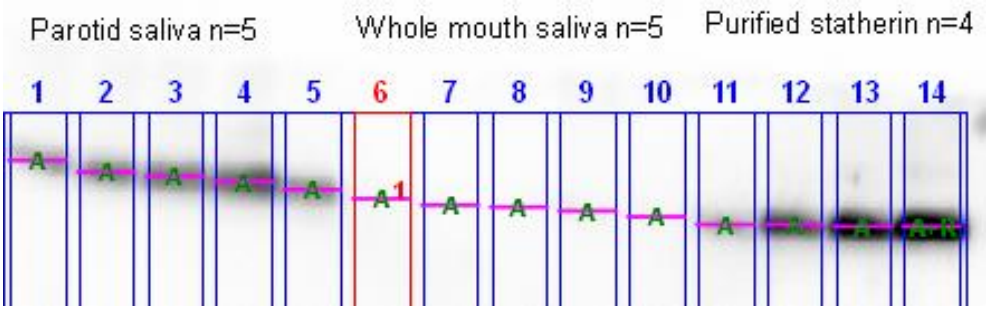


b) SDS-PAGE and western blots of AEP samples after five cycles erosion from WMS (n=5) and PS (n=5) probed with CA VI antibody.

***Figure 52 (III)**: SDS-PAGE and western blots of AEP samples from WMS (n=5) and PS (n=5) and purified proteins of standards (n=4). All samples were immunoblotted against CA IV antibody (a: before erosion (control); b: five cycle erosion).*



a) SDS-PAGE and western blots of AEP samples before erosion from WMS (n=5) and PS (n=5) probed with statherin antibodies.



b) SDS-PAGE and western blots of AEP samples after five cycles erosion from WMS (n=5) and PS (n=5) probed with statherin antibodies.

***Figure 52 (IV)**: SDS-PAGE and western blots of AEP samples from WMS (n=5) and PS (n=5) and purified proteins of standards (n=4). All samples were immunoblotted against statherin antibody (a: before erosion (control); b: after five cycle erosion).*

Figures 53 (a,b,c,d) shows the standard curves of the four purified proteins standards generated from the mean (SD) volume intensities of all gels (n=3) against the absolute quantities of the purified protein standards. This was used to generate a calibration curve using a linear formula which was used to

calculate the amount of the corresponding protein in the *in vitro* AEP samples. It can be seen from the figures that the purified proteins used in this study were optimised in a way that the data points between a high volume (15 µL) of purified proteins and low volume (1 µL) provided a suitable curve range to calculate very little proteins in the *in vitro* AEP samples whilst producing a gradual change of intensities.

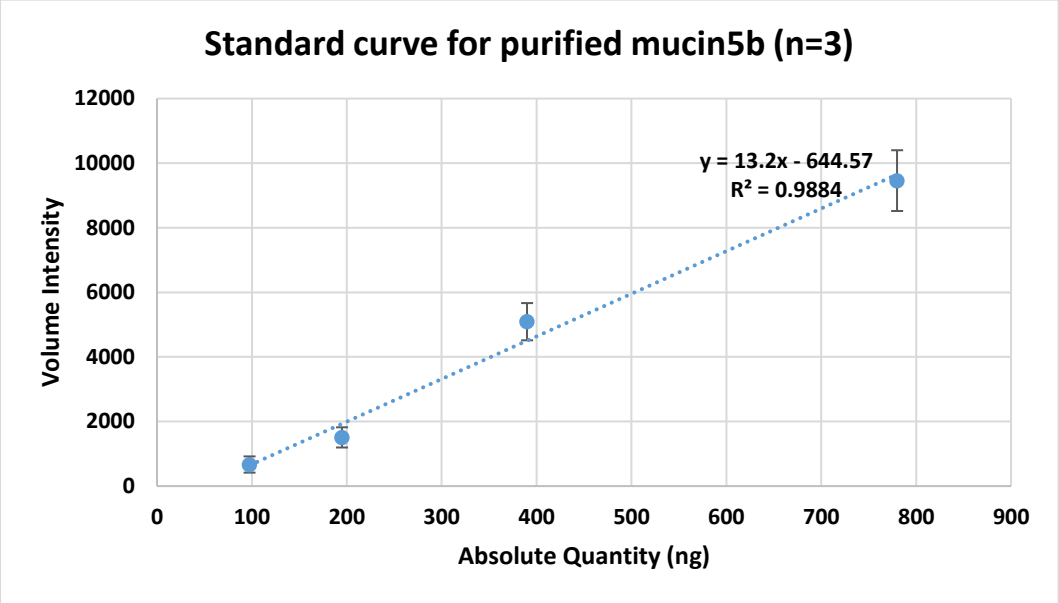


Figure 53 (a) : *Standard curve of the purified mucin5b generated from the mean (SD) volume intensities against the absolute quantity in nanogram (n=3) and was used to quantify the absolute quantity of proteins in the AEP samples.*

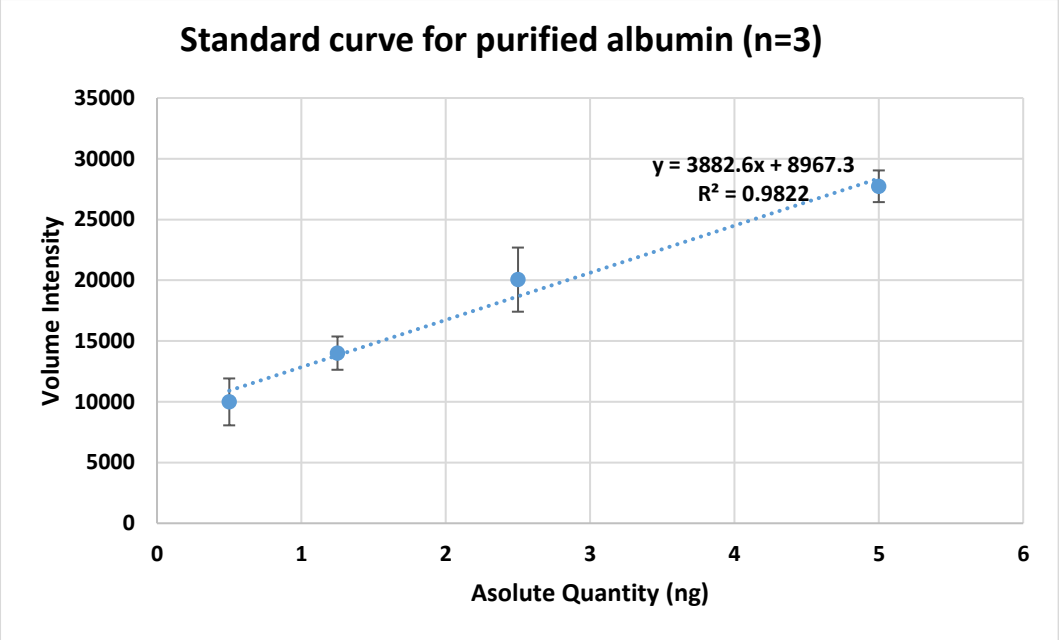


Figure 53 (b) : *Standard curve of the purified albumin generated from the mean (SD) volume intensities against the absolute quantity in nanogram (n=3) and was used to quantify the absolute quantity of proteins in the AEP samples*

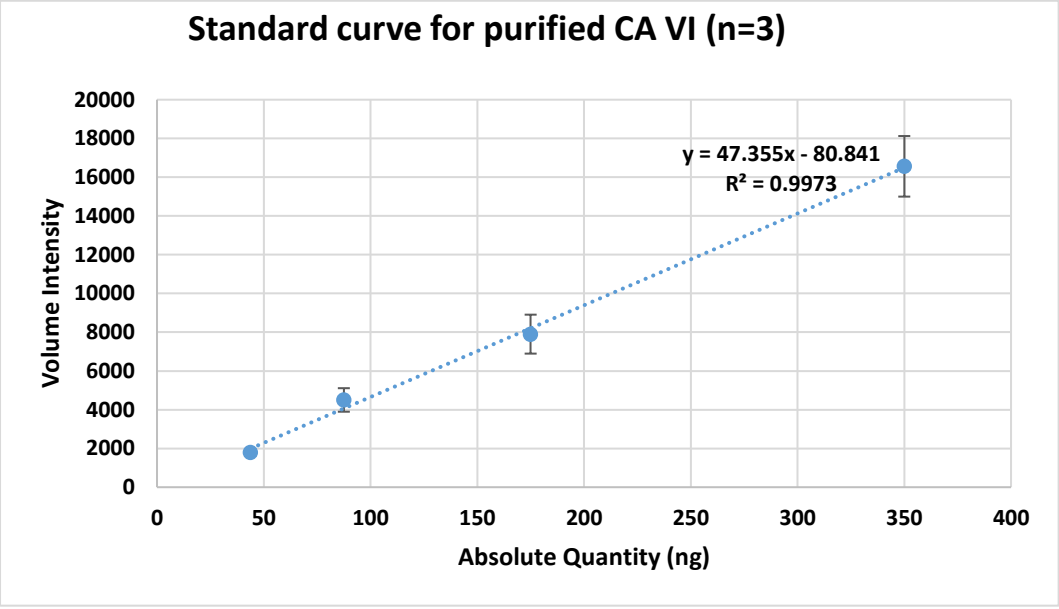


Figure 53 (c): *Standard curve of the purified CA VI generated from the mean (SD) volume intensities against the absolute quantity in nanogram (n=3) and was used to quantify the absolute quantity of proteins in the AEP samples*

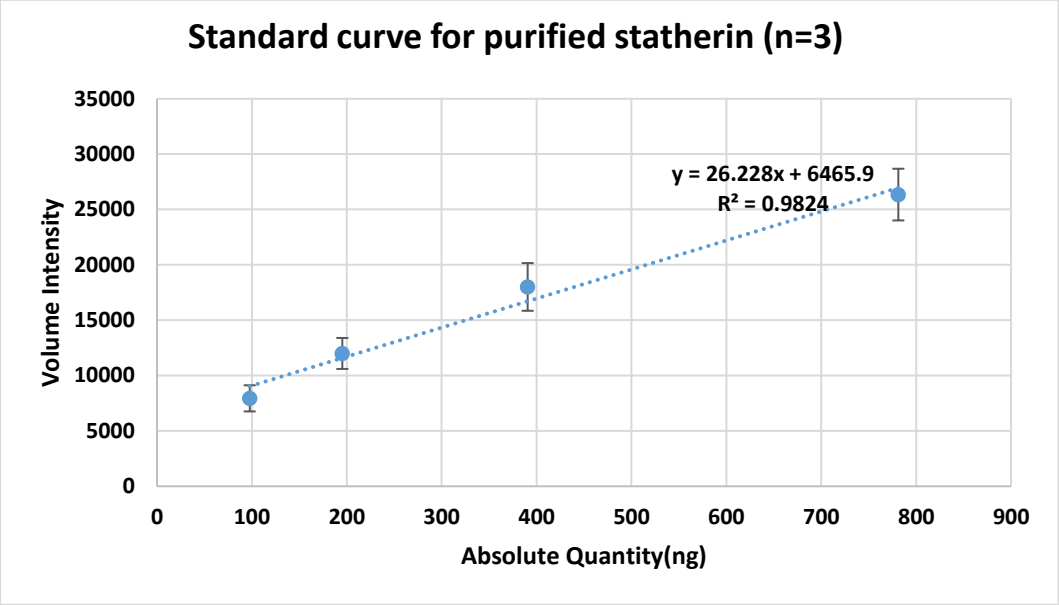


Figure 53 (d) : *Standard curve of the purified statherin generated from the mean (SD) volume intensities against the absolute quantity in nanogram (n=3) and was used to quantify the absolute quantity of proteins in the AEP samples.*

Table 24 shows the mean (SD) and median (IQR) amount of the four specific proteins *in vitro* AEP from WMS and PS after 24 hour immersion in the corresponding solution [before erosion (control)] and after five cycles erosion (EV). Figure 54 shows the mean (SD) amount of mucin5b, CA VI and statherin after 24 hour immersion in the corresponding solution [before erosion (control)] and after five cycles erosion. As the amount of albumin (ng) before and after five erosion cycles was very small compared to the amount of the other three proteins, albumin was presented in a separate figure (Figure 55). In the AEP from WMS before and after five cycles erosion, the mean (SD) amount of mucin5b was [57.5 (33.3) ng and 121.5 (19.9) ng respectively], albumin was [1.4 (0.8) ng and 1.9 (0.8) ng respectively], CA VI was [6.3 (2.3) ng and 0.14 (0.1) ng respectively] and statherin was [19.4 (6.3) ng and 0.20 (0.04) ng

respectively]. In the AEP from PS before and after erosion, the mean (SD) amount of albumin was [0.3 (0.2) ng and 0.3 (0.1) ng respectively], CA VI was [60.7 (22.5) ng and 92.3 (17.1) ng respectively] and statherin was [210.4 (25.8) ng and 180.6 (23.4) ng respectively]. Mucin5b was not detected in the AEP from PS.

In all groups and conditions, the amount of mucin5b and albumin were significantly more dominant in AEP from WMS compared to PS ($p<0.0001$) whereas the amount of CA VI and statherin were significantly more dominant in PS ($p < 0.0001$).

The amount of mucin5b in AEP from WMS before erosion [57.5 (33.3) ng] significantly increased to [121.5 (19.9) ng $P< 0.0001$] after five cycles erosion. The amount of albumin in AEP from WMS before erosion [1.4 (0.74) ng] increased after five cycles erosion [1.9 (0.8) ng] but this was not significantly different ($p>0.05$).

The amount of CA VI in AEP from WMS before erosion [6.3 (2.3) ng] significantly decreased after five erosion cycles [(0.14 (0.09) ng $p<0.0001$]. The amount of CA VI in AEP from PS before erosion [60.7 (22.6) ng] increased significantly after five cycles erosion [92.3 (19.15) ng $p<0.0001$].

For statherin, its amount in AEP from WMS before erosion [19.4 (6.3) ng] significantly decreased by nearly twenty folds after five cycles erosion [0.2 (0.04) ng $P<0.0001$]. The amount of statherin in AEP from PS before erosion [210.4 (25.9) ng] decreased after five cycles erosion [180.6 (23.5) ng] but this was not significant ($P>0.05$).

Saliva type and erosion condition	Mucin5b amount of protein (ng)		Albumin amount of protein (ng)		CA VI amount of protein (ng)		Statherin amount of protein(ng)	
	Mean (SD)	Median (IQR)	Mean (SD	Median (IQR)	Mean (SD	Median (IQR)	Mean (SD)	Median (IQR)
Whole mouth saliva Control (no acid exposure) (WMSC)	57.5 (33.3)β	38.0 (46.0)	1.4 (0.8) α	1.2 (0.1)	6.3 (2.3)€	6.6 (3.7)	19.4 (6.3)¥	21.0 (9.0)
Whole mouth saliva Five cycle erosion (WMSEV)	121.5 (19.9)β	119.0 (21.0)	1.9 (0.8)Δ	1.3 (1.5)	0.14 (0.1)€	0.10 (0.01)	0.20 (0.04)¥∑	0.10 (0.01)
Parotid saliva Control (no acid exposure) (PSC)			0.3 (0.2) α	0.3 (0.3)	60.7 (22.5)€	61.2 (27.0)	210.4 (25.8)¥	205.2 (7.6)
Parotid saliva Five cycle erosion (PSEV)			0.3 (0.1)Δ	0.3 (0.1)	92.3 (17.1)€	94.9 (5.2)	180.6 (23.4)∑	170.0 (23.1)

Table 24*: Mean (SD) and median (interquartile range) amount of proteins (nanogram) in vitro AEP formed on enamel specimens immersed in WMS (n=10) or PS (n=10) for 24 hour. The AEP was then eluted before(control) or after five cycles erosion using 0.5% SDS and quantified using ImageLab software. Same symbols in the table indicate significant differences (p<0.0001). (WMSC= whole mouth saliva at control; WMSEV: whole mouth saliva after 5 erosion cycles; PSC: parotid saliva at control; PSEV: parotid saliva after 5 erosion cycles).*

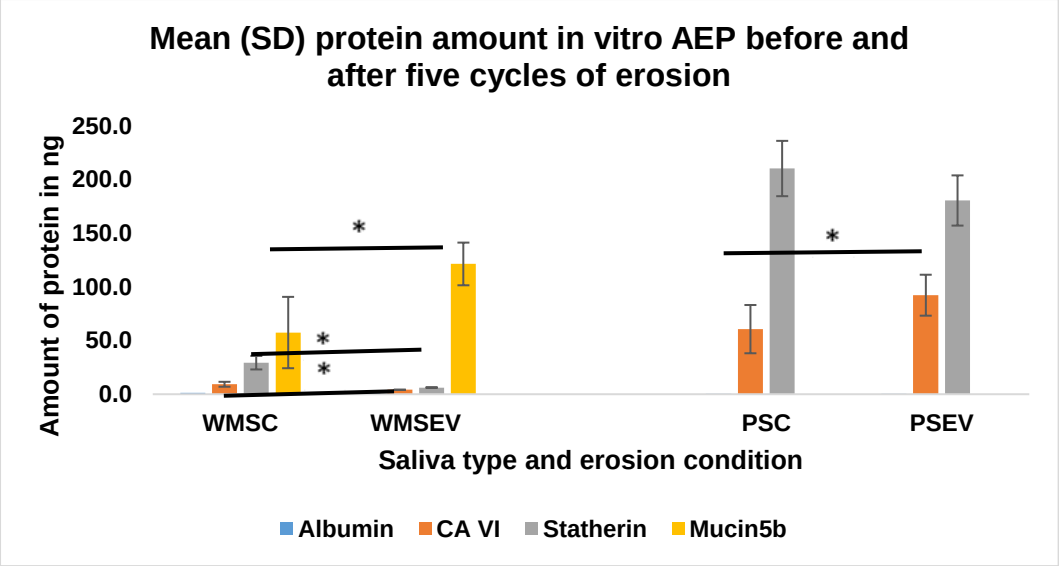


Figure 54: *Mean (SD) amount of proteins (nanogram) in vitro AEP formed on enamel specimens immersed in WMS (n=10) or PS (n=10) for 24 hour. The AEP was then eluted before(control) or after five cycles erosion using 0.5% SDS and quantified using ImageLab software. Asterisk shapes in the figure indicate significant differences (p<0.0001). (WMSC: whole mouth saliva at control; WMSEV: whole mouth saliva after 5 erosion cycles; PSC: parotid saliva at control; PSEV: parotid saliva after 5 erosion cycles)*

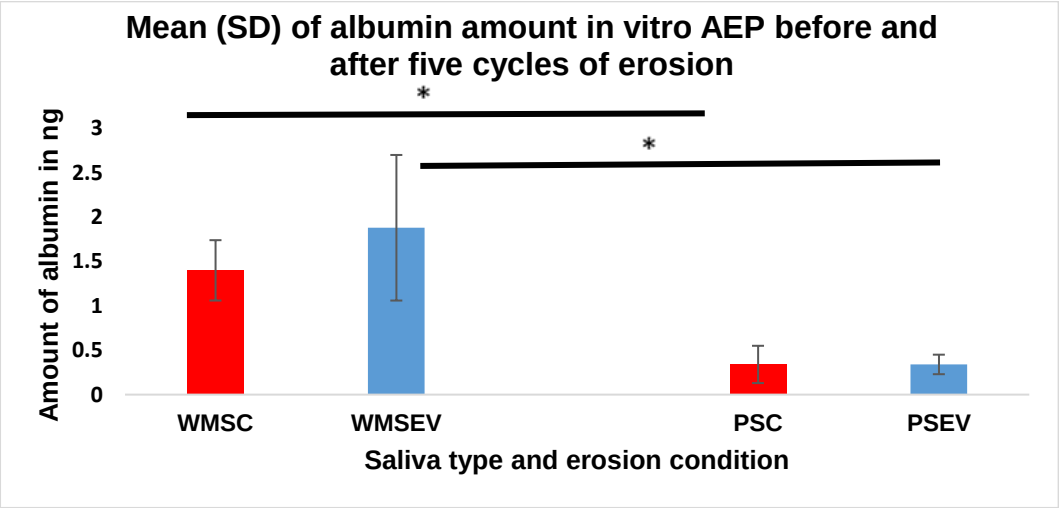


Figure 55*: Mean (SD) amount of albumin (nanogram) in vitro AEP formed on enamel specimens immersed in WMS (n=10) or PS (n=10) for 24 hour. The AEP was then eluted before or after five erosion cycles erosion using 0.5% SDS and quantified using ImageLab software. Same symbols in the table indicate significant differences (p<0.0001). (WMSC= whole mouth saliva at control; WMSEV: whole mouth saliva after 5 cycles erosion; PSC: parotid saliva at control; PSEV: parotid saliva after 5 cycles erosion).*

4.2.4.3 Calcium and phosphorus analysis

Table 25 and Figure 56 show the mean (SD) concentration of calcium and phosphorus (nM/mm^2) in the AEP from WMS and PS after 24 hour immersion in the corresponding solution [before erosion, control (C)] and after five erosion cycles (EV). In the AEP from WMS at control, the mean (SD) concentration of calcium and phosphorus was [0.06 (0.07) nM/mm^2 and 0.14 (0.05) nM/mm^2 respectively] whereas after five cycles erosion was [0.15 (0.10) nM/mm^2 and 0.20 (0.06) nM/mm^2 respectively]. In the AEP from PS at control, the mean (SD) concentration of calcium and phosphorus was [0.03 (0.02) nM/mm^2 and 0.17 (0.10) nM/mm^2 respectively] whereas after five erosion cycles was [0.08 (0.10) nM/mm^2 and 0.25 (0.12) nM/mm^2 respectively]. When comparing the AEP from WMS and PS before erosion, there was neither significant difference in the concentration of calcium ($P=0.21$) nor in that of phosphorus ($p=0.41$). After five cycles of erosion, AEP from WMS also experienced no significant differences in the concentration of calcium and phosphorus compared to that from PS ($p=0.41$). When comparing within groups, there was no significant difference between the concentration of calcium and phosphorus in the AEP from WMS before erosion [0.06 (0.08) nM/mm^2 ($p=0.21$) and 0.14 (0.05) nM/mm^2 ($p=0.11$) respectively] and that after five erosion cycles [0.15 (0.10) nM/mm^2 and 0.22 (0.10) nM/mm^2 $p>0.05$]. For the AEP from PS, the concentration of calcium and phosphorus before erosion [0.03 (0.02) nM/mm^2 and 0.17 (0.10) nM/mm^2 respectively] also were not significantly different from that after five erosion cycles [0.08 (0.10) nM/mm^2 ($P=0.71$) and 0.25 (0.12) nM/mm^2 ($P=0.11$) respectively ($P>0.05$)].

Saliva type and erosion condition	Calcium concentration (nM/mm^2)		Phosphorus concentration (nM/mm^2)	
	Mean (SD)	**Median (IQR)**	**Mean (SD)**	**Median (IQR)**
Whole mouth saliva Control (n=5) (no acid exposure) (WMSC)	0.06 (0.06)	0.03 (0.08)	0.14 (0.05)	0.12 (0.07)
Whole mouth saliva Five cycles erosion (n=5) (WMSEV)	0.15 (0.10)	0.16 (0.11)	0.20 (0.06)	0.22 (0.10)
Parotid saliva Control (n=5) (no acid exposure) (PSC)	0.03 (0.02)	0.03 (0.02)	0.17 (0.10)	0.13 (0.03)
Parotid saliva Five cycles erosion (n=5) (PSEV)	0.08 (0.10)	0.05 (0.05)	0.25 (0.12)	0.20 (0.05)

Table 25*: Mean (SD) and median (interquartile range) amount of calcium and phosphorus (nM/mm2) in vitro AEP formed on enamel specimens immersed in WMS (n=10) or PS (n=10) for 24 hour. The AEP was then eluted before or after five cycles erosion using 0.5% SDS and quantified using ICP-MS. No significant differences were observed between groups (P > 0.05). (WMSC: whole mouth saliva at control; WMSEV: whole mouth saliva after five cycles erosion; PSC: parotid saliva at control; PSEV: parotid saliva after five cycles erosion).*

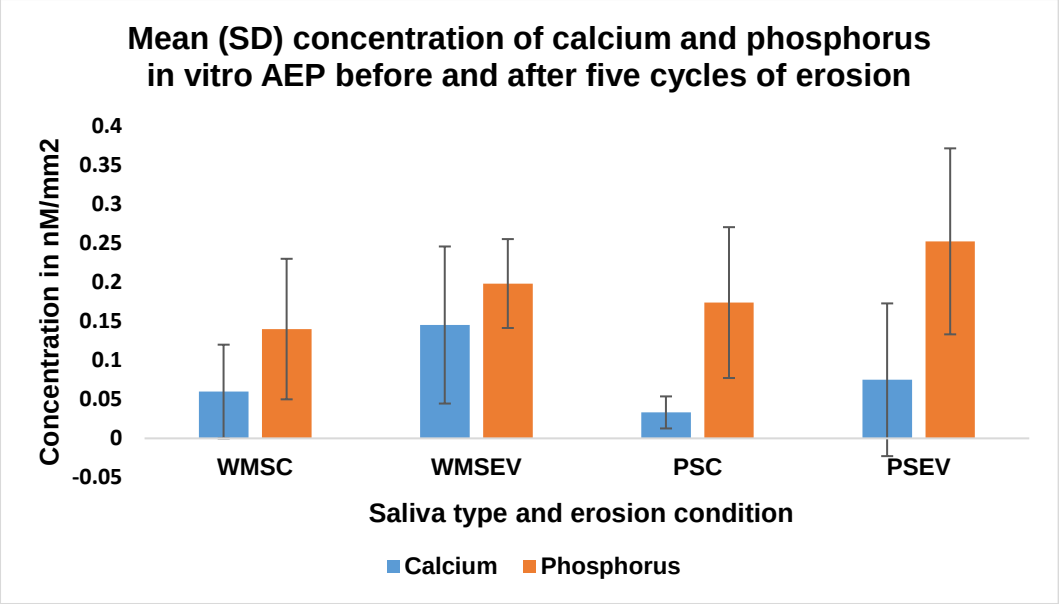


Figure 56 : *Mean (SD) and median (interquartile range) amount of calcium and phosphorus (nM/mm2) in vitro AEP formed on enamel specimens immersed in WMS (n=10) or PS (n=10) for 24 hour. The AEP was then eluted before or after one cycle erosion using 0.5% SDS and quantified using ICP-MS. No significant differences were observed between groups (P > 0.05). (WMSC: whole mouth saliva at control; WMSEV: whole mouth saliva after five erosion cycles; PSC: parotid saliva at control; PSEV: parotid saliva after five erosion cycles).*

Section 4.3: Discussions of sections (4.1 and 4.2) (Advanced erosion)

In this laboratory study representing *in vitro* advanced erosion, immersion of enamel samples in WMS, PS, AS and DW resulted in significant differences in step height. WMS resulted in the lowest stepheight and hence offered the most protection followed by PS, AS and DW. However such differences were not detected by SMH measurement. This was somewhat expected, as white light SNCP measures advanced erosion accurately, whereas results for SMH are more unpredictable for advanced erosion and more reliable for early erosion. In section 4.2, it was shown that the concentration of total protein in AEP from WMS was significantly higher than that in AEP from PS and both reduced after five erosion cycles compared to control (after 24 hour immersion in the corresponding solution with no acid challenge). Mucin5b and albumin were also more dominant in AEP from WMS, whereas CA VI and statherin were dominant in PS. The amount of mucin5b in AEP from WMS at control increased significantly after five cycles of erosion, whereas the amount CA VI and statherin decreased significantly and there was no change in the amount of albumin. In the AEP from PS, only the amount of CA VI increased significantly after five erosion. No changes were observed in the calcium (Ca^{2+}) and phosphorus (P) concentrations in AEP from both WMS and PS after five erosion cycles. Combining the step height results and proteins analysis results, the concentration of total protein and the presence of mucin5b and albumin may play a role in the protection against advanced erosion *in vitro*, as shown by their higher concentration of total protein and dominance of mucin5b and albumin in AEP from WMS which offered more protection. It has been

reported that AEP from WMS is more viscous and diffuse compared to AEP from PS which is more elastic and compact (Ash *et al.*, 2014). Vissink *et al*., (1985) added mucin of high concentrations (30 g/L) to saliva substitutes, demonstrating that mucin had a rehardening role against demineralised enamel as compared to saliva substitutes without mucin (control) (Vissink *et al.*, 1985). In the same way, addition of gastric human mucin (2.7 g/L) to a remineralising solution lead to mineral gains as compared to mucin-free mineralising solutions due to perhaps calcium deposition into the lesion area (Meyer-Lueckel *et al.*, 2004). Another observation in our results was that statherin and CA VI were not abundant in the AEP from WMS. This can be explained by the high proteolytic effect of WMS enzymes as opposed to the PS which has a weak proteolytic activity that makes it's salivary proteins less susceptible to proteolysis (Martins *et al.*, 2013; Zimmerman *et al.*, 2013). WMS is derived from all salivary glands, whereas PS is derived from an individual gland, the parotid gland, which means that the protein contents of both types of saliva would be different.

The four individual proteins were selected based on their different protective mechanisms against erosion. These included the physical barrier and lubrication of mucin5b (Amerongen *et al.*, 1987), the diffusion barrier of albumin (Hemingway *et al*., 2008), the buffer capacity or acid neutralisation of CA VI (Leinonen *et al.*, 1999) and calcium binding mechanism of statherin (Kosoric *et al*., 2007).

Amerongen *et al.* (1987) and Martins *et al*. (2013) also compared the protective effects of WMS and PS using different measurement techniques to those used in this thesis. Martins *et al.* (2013) measured the amount of calcium

and phosphate released from enamel specimens after the demineralisation period and found that WMS and PS provided an effective protection against 12 days enamel demineralisation (Martins *et al.*, 2013). A previous study examined the protective effect of WMS compared with a mineral solution containing the same concentrations of calcium and phosphate present in the original WMS (Featherstone *et al.*, 1993). They demonstrated that the calcium and phosphate solution did not provide resistance against subsequent demineralisation. This is in agreement with our results on advanced erosive tooth wear which indicated that WMS and PS provided significantly greater protection against erosive tooth wear than AS.

The results of the present study for Ca^{2+} and P were inconclusive as no changes were detected. This could mean that all dissolved minerals from the enamel crystal were not retained in the AEP due to the repeated erosive cycles and the minerlas leached into the erosive solution. Analysis of the mineral contents in the erosive solution could have been done but this technique is still an indirect measurement of erosion and re-precipitation of the minerals can occur (Shellis et al. 2011).

A limitation of this study is that a solution containing proteins only without ions was not compared to the other solutions which either contained ions only (AS) or ions and proteins (WMS and PS). Martins *et al,* (2013) showed that the ionic composition of saliva, independently of the type of saliva sample (WMS or PS), can further improve the reduction of enamel demineralisation as compared to proteins without ions (dialyzed samples) (Martins *et al.*, 2013). However, a recent study disagreed with the results of Martins *et al.* (2013) demonstrating that proteins alone (WMS depleted from all ions) can provide better protection

against erosive tooth wear than WMS (proteins and ions) or AS (ions only) (Baumann *et al.*, 2016). Baumann *et al*, (2016) suggested that the binding sites on calcium and phosphate-binding proteins can be occupied by the calcium and phosphates present in saliva, negatively affecting the protective role of the salivary pellicle (Baumann *et al.*, 2016).

It is difficult to directly apply the results of this *in vitro* study to the *in vivo* clinical situation and whether in fact the proteins chosen for this study are the most significant ones. Other small proteins are also known to be abundant in saliva such as PRPs which accounts for up to 70 % in PS (Beeley *et al.*, 1991). PRPs were not included in this study due to the unavailability of commercial antibodies for PRPs which would be necessary for visualising PRPs using western blotting techniques (Gibbins *et al.*, 2013). The composition of AEP may also be affected by the bacterial involvement (Hannig and Hannig, 2009). Hence, although the results provide some insight into the role of proteins in erosion further work is needed to clarify their role further.

4.4 Conclusions

- WMS provided better protection against advanced erosion than PS.
- Total protein, mucin5b and albumin were more prevalent in *in vitro* AEP from WMS after advanced erosion and offered more protection than AEP from PS, whereas CA VI and statherin were prevalent in in vitro AEP from PS.
- The mechanism of the protection against advanced erosion could be via provision of a physical barrier, diffusion barrier and lubrication in this in vitro study.

Section 4.4: Does human serum albumin mediate protection against advanced erosion?

4.4.1 Introduction

Following from section 4.2 which concluded that albumin is likely to be involved in the protection against advanced erosion, it was decided to investigate this further. Several studies have investigated the effect of individual proteins against erosion *in vitro* and *in-situ* (Barbour *et al.*, 2008; Hemingway *et al.*, 2010; White *et al.*, 2011; Jager *et al.*, 2012). Barbour *et al.*, (2008) demonstrated that casein protein reduced the dissolution of hydroxyapatite when exposed to 0.3 % citric acid, and further reduction was observed when adding 5 and 10 mM calcium ions in the form of calcium chloride dihydrate ($CaCl_2$). In another study, the AEP was also modified with DW containing casein and mucin, which also resulted in a significant reduction in enamel softening after three 1-minute erosion cycles as compared to single protein treatment (mucin or casein) and a control group (DW with no protein) (Cheaib and Lussi, 2011). Another group of researchers examined the combined casein, casein phosphopeptide (CPP) and glycomacropeptide (GMP) with and without fluoride as anti-erosive agents using surface nanoindentation measurments and non-contact optical profilomtery (White *et al.*, 2011). They found that all proteins reduced the tissue loss and only casein and fluoride reduced enamel softening (White *et al.*, 2011). Along with casein, ovalbumin, a protein found in egg white that has similar properties to human serum albumin, was also found to reduce the erosion of hydroxyapatite placed in *in vitro* acidic solutions once adsorbed to the hydroxyapatite surface in the form of AEP (Hemingway *et al.*, 2008). The same group of researchers studied the

effect of casein and ovalbumin combination on *in vitro* enamel erosion. They suggested that these proteins increased the resistance properties of the AEP to ion-diffusion, thus increasing the ability of the AEP to prevent erosion of the underlying enamel. A reduction in enamel demineralisation has also been observed with high albumin concentrations (Arends *et al.*, 1986). In addition, crystal growth inhibition was demonstrated by the application of albumin which was found to bind to hydroxyapatite (Garnett and Dieppe, 1990; Robinson *et al.*, 1992).

However, findings in the literature on the role of albumin in protection against enamel and hydroxyapatite demineralisation have proved to be contradictory. Using microradiograph, albumin added to a demineralising solution did not offer significant protection against enamel demineralisation (Kielbassa *et al.*, 2005).

Based on the reviewed literature, it was speculated that albumin may promote the diffusion of calcium into the eroded pores. An albumin molecule in human serum has up to 19 calcium binding sites which can be fully utilised in normal physiological conditions (Klinger *et al.*, 1997). This binding property may be applied to enamel crystals which contain calcium ions. As albumin contributes to the formation of AEP and has been found to be dominant in the protective AEP (section 4.2), it is of interest to investigate its individual role against enamel erosion. This part of the thesis aims to investigate the role of human serum albumin added into AS in protection against advanced erosion caused by citric acid as a function of concentration.

4.4.2 Aim, objectives and hypotheses

The aim of this section was to assess the effect of human serum albumin with varying concentrations added to artificial saliva (AS) in protection against *in vitro* advanced erosion.

The objective was to:

1. To compare the step height and SMHC of enamel surfaces when immersed in AS to that of AS with high, medium and low albumin concentration for 24 hours followed by five erosion cycles.

The null hypothesis was that:

1. There will be no differences in the step height and SMHC of enamel surfaces when immersed in AS with varying albumin concentrations or AS without albumin for 24 hours followed by five cycles of erosion.

4.4.3 Materials and methods

4.4.3.1 Specimen preparation

Based on the results of the power calculation using Gpower verion 3.1.7, a sample size of 60 (10 per solution) was required for comparing six different solutions (Nekrashevycha and Stösserb, 2003; Martins *et al.*, 2013; Mistry *et al.*, 2015; O'Toole *et al.*, 2015). Sixty eight enamel specimens were prepared from human extracted molar teeth. Thirty four buccal and thirty four lingual surfaces were sectioned from thirty four human molar sound teeth. Initial surface microhardness values (SMH) measured. The specimens with a SMH value between 272 KHN and 400 KHN were selected (Meredith *et al*., 1996; Austin *et al*., 2011; Lussi *et al*., 2011). Sixty specimens were selected for this study and eight specimens were rejected. Enamel surfaces were embedded

in cold cure acrylic resin as described in section 2.1.4. Enamel specimens were ground and polished to provide a highly polished, flat surface 3 x 3 mm in size as explained in section 2.1.5. All prepared specimens were then taped as explained in section 2.1.7 and they were numbered and randomised as explained in section 2.1.8.

4.4.3.2 Study methodology

The study consisted of 6 groups according to the solution used 10 specimens per group. These groups were WMS, three different concentrations of human serum albumin (albumin) added to AS: high [0.2 % ; 2 g/L (High albumin (Alb3)], medium [0.02 %; 0.2 g/l (medium albumin (Alb2)] and low [0.002%; 0.02 g/L (Low albumin (Alb1)], AS and DW. The WMS and AS were prepared as described in section 2.2. Thawed WMS was mixed vigorously prior to use to re-suspend precipitation of proteins and avoid loss of specific proteins less than 14 kDa (Francis *et al.*, 2000). The albumin solution was prepared as explained in the next section (4.4.3.2.1). Within each group, specimens were randomly allocated by an independent investigator.

Specimens from each group were exposed for five cycles erosion as described in section 2.3.4.2. Specimens were immersed in the corresponding solution (either WMS, Alb3,Alb2, Alb1, AS or DW) for 24 hour followed by a further 30 minutes prior to exposure to a 10-minute citric acid followed by 2-minute water rinse. The cycle of immersion for 30 minutes in the corresponding solution followed by the citric acid erosion was repeated 5 times for each group as shown in Figure 57. When the experiments were completed, specimens were air-dried for 24 hours after which the tape was removed and profilometric measurement and surface microhardness data were obtained.

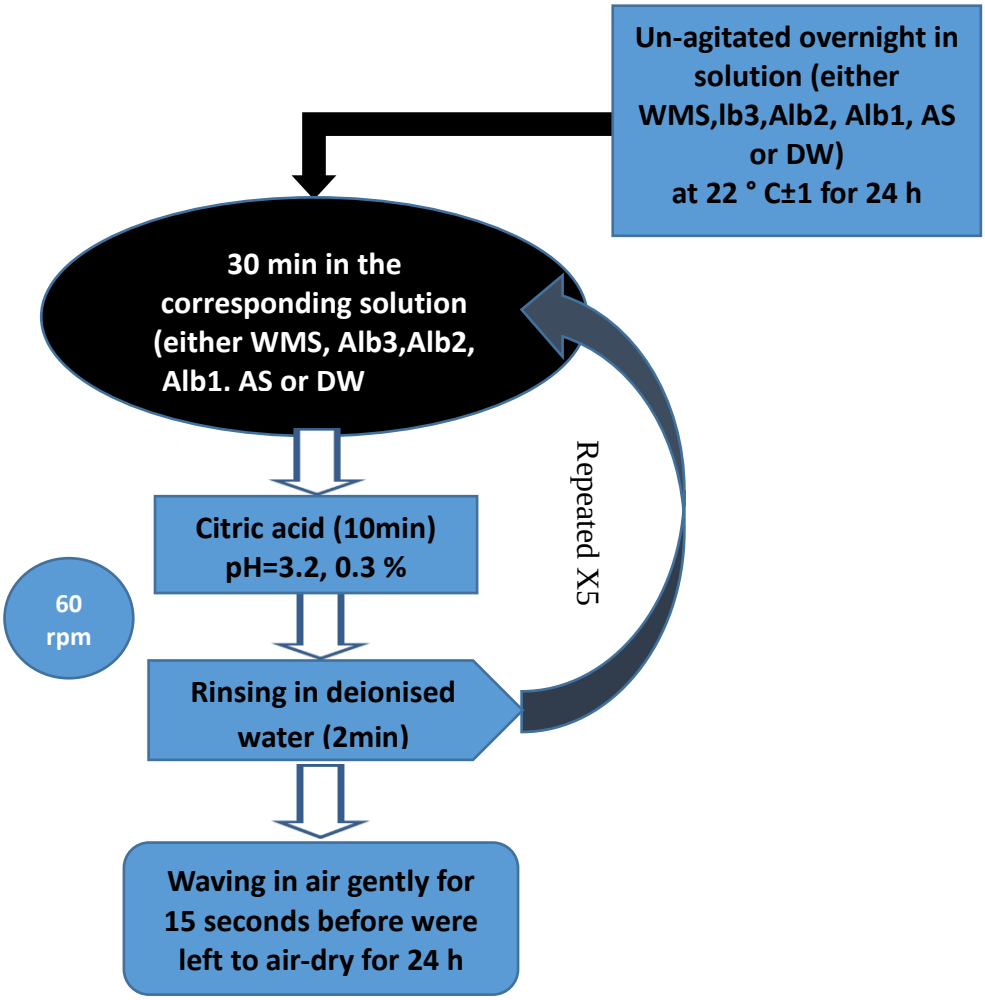


***Figure 57**: The in vitro model of 24 hour AEP formation and five erosion cycles*

4.4.3.2.1 Albumin solution preparation:

Fresh AS solution was prepared as described in section 2.2.2. Human serum albumin was provided as a 10 g powder (Sigma-Aldrich, Gillingham, UK). It was used to be added into an artificial saliva solution at three different concentrations (low: 0.002 %; medium: 0.02 %; high: 0.2 %). The required albumin powder was measured using an electronic analytical scale (Mettler Toledo, XS105 Dual Range Analytical Balance, Fisher Scientific UK Ltd, Loughborough, UK). The weighed amount was added to 1 L of freshly

prepared artificial saliva. Initially, 500 mL of artificial saliva was added to a 1 L- volumetric flask. 2 g of albumin powder was weighed and was added into the flask immediately after weighing. After the weighed albumin powder was added to the flask, the solution was continually stirred for 30 minutes with a magnetic stirir (Fisher Scientific, Magnetic hotplate stirrer USAr) to allow components to be dissolved in the artificial saliva. The volume was then increased to 1 L by adding artificial saliva using a graduated measuring cylinder while the solution was continuously stirred for at least 2 hour until fully dissolved. This prepared an albumin solution of 0.2% concentration (2 g/L). The 0.02 % albumin solution was prepared by weighing another 2 g of albumin powder and was dissolved in 100 mL of AS in a 1 L- volumetric flask. The volume was increased to 1 L by adding AS using a graduated measuring cylinder as above. The 0.002 % albumin solution was prepared by adding 10 mL of the 0.2 albumin solution to a 1 L- volumetric flask 1 L and the volume was then increased to 1 L by adding AS using a graduated measuring cylinder as above.

4.4.3.3 Testing

Profilometric measurements

Profilometric measurements after the fifth erosion cycle were obtained using surface non-contacting profilometer (SNCP) as explained in section 2.4.1. Each surface profile was taken from within the first 1/3 of the taped zone on one side across the exposed window to just within the first 1/3 of the taped zone on the opposite side. Ten randomly selected step height measurements

were taken from each specimen and averaged to give a mean surface profile value.

Surface microhardness (SMH) measurements

Surface microhardness (SMH) at baseline (SMHb):

The surface microhardness (SMH) values before immersion of enamel specimens in either WMS, AS with the three different concentrations of albumin (Alb3, Alb2, Alb1), AS alone or DW [surface microhardness at baseline (SMHb)] were measured for the six experimental groups as described in section 2.4.2. The SMH values of each specimen was determined by the average of five indentations made at the exposed window under a load of 100 g and a dwell time of 10 seconds. The SMH value of each indentation was determined by specialised software (Duramin-5Hardness Tester, Struers Inc., Rotherham, UK) through measuring the length of each indentation with an optical analysis system calculating hardness in Knoop units (KHN).

Surface microhardness(SMH) measurements after five cycles erosion:

Surface microhardness (SMH) values were repeated after the experiment using the method explained above. The surface microhardness change (SMHC) of each specimen was then calculated by subtracting the mean SMH value after five cycles of erosion (SMHe) from the mean SMH value before erosion (SMHb) using the formula: SMHC = (SMHb – SMHe).

4.4.3.4 Statistical analysis

Data obtained from the profilometry and microhardness tests were analysed using SPSS (version 22.0, IBM, Portsmouth, UK). The measured outcomes were analysed using descriptive quantitative methods to summarise the study characteristics of the various subgroups. Shapiro-Wilk and Kolmogorov-

Smirnov tests were used to assess the normality distributions of data. Data were also visually assessed using histogram, Q-Q plots and Box and Whisker Plots. Data were normally distributed and were described using mean and standard deviation. Two way ANOVA test was then used to establish if significant statistical differences existed between the means of groups. The mean difference was considered to be significant at a P value < 0.05. Post Hoc Bonferroni test was used to determine which means were significantly different from others.

4.4.4 Results:

Step height

Table 26 and Figure 58 show the mean (SD) step height for the six groups. These groups were WMS, AS with three different albumin concentrations: high (Alb3), medium (Alb2), low (Alb1), AS alone and DW. The mean (SD) step height of WMS was [4.2 (0.6) µm], Alb3 was [6.7 (0.1) µm, Alb2 was [7.2 (0.9) µm, Alb1 was [8.0 (1.0) µm], AS was [7.44 (1.05) µm, DW was [10.9 (9.0) µm]. No significant difference was observed between albumin groups [high (Alb3), medium (Alb2) and low (Alb1) albumin concentration ($p>0.05$)]. Albumin groups had no significant difference in their step height with that of AS without albumin (AS) ($p>0.05$). All groups had significantly lower step height than DW group $p<0.0001$. When comparing WMS [4.2 (0.6) µm], albumin groups and AS, WMS group showed significantly lower step height ($p<0.0001$) than AS and all albumin groups (Alb3, Alb2, Alb1).

Solution type (n=10)	Step height (µm) Mean (SD)
Whole mouth saliva (WMS)	4.2 (0.6) ¥πΔβ
Artificial saliva+ High albumin (Alb3)	6.7 (0.1) β
Artificial saliva+ Medium albumin (Alb2)	7.2 (0.9) Δ
Artificial saliva+ Low albumin (Alb1)	8.0 (1.0) π
Artificial saliva (AS)	7.44 (1.1) ¥
Deionised water (DW)	10.9 (9.0) ¥πΔβ

***Table 26**: Mean (SD) step height (µm) of enamel surfaces for six groups according to the six different solutions (WMS, Alb3, Alb2, Alb1, AS, DW) after five erosion cycles (0.3%, pH 3.2, citric acid, 10 min). Similar shapes in the table denote significant differences between the groups.*

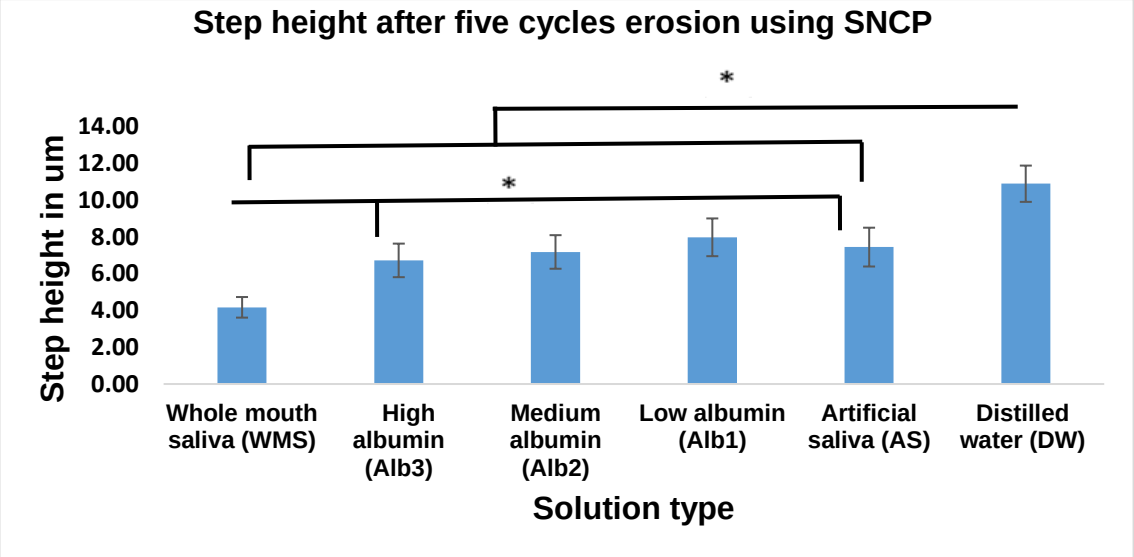


***Figure 58**: Mean (SD) step height (µm) of enamel surfaces for six groups according to the six different solutions (WMS, Alb3, Alb2, Alb1, AS, DW) after the five cycles erosion (0.3%, pH 3.2, citric acid, 10 min). Asterisks indicate significant differences between groups.*

Surface microhardness change (SMHC)

Surface microhardness (SMH) at baseline:

Table 27 shows the surface microhardness (SMH) values before immersion of enamel specimens in either WMS, Alb3, Alb2, Alb1, AS or DW, and before the erosion cycle (Surface microhardness at baseline: SMHb). This shows that the average of SMH values ranged between 344.20 (31.90) KHN and 371.12 (23.96) KHN. There were no significant differences between the mean SMH values of all groups (p> 0.05).

Solution Type (n=10)	**Surface microhardness(KHNb) Mean (SD)**
Whole mouth saliva (WMS)	355.22 (16.70)
Artificial saliva+ High albumin (Alb3)	371.12 (23.96)
Artificial saliva+ Medium albumin (Alb2)	346.02 (24.77)
Artificial saliva+ Low albumin (Alb1)	361.86 (18.62)
Artificial saliva (AS)	344.20 (31.90)
Deionised water (DW)	354.72 (17.52)

Table 27: *Mean (SD) surface microhardness at baseline (SMHb) of enamel surfaces in Knoop microhardness units (KHN) after specimens were polished and before receiving any treatments. No significant differences between the groups.*

Table 28 and Figure 59 show the mean (SD) surface microhardness change (SMHC) of the enamel surfaces after the experiment for the six groups as described above in the study methodology. The mean (SD) of WMS group was [224.10 (29.3) KHN], Alb3 group was [198.2 (44.2) KHN], Alb2 group was

[191.78 (33.97) KHN], Alb1 group was [173.8 (50.0) KHN], AS group was [160.2 (24.4) KHN] and DW group was [123.9 (16.6) KHN].

No significant differences were observed between the albumin groups [high (Alb3), medium (Alb2), low (Alb1) albumin concentration]. Also, Albumin groups had no significant difference in their SMHC with that of AS without albumin (AS) ($p>0.0001$). Only DW group had significantly lower SMHC than WMS group ($p=0.023$). The SMHC of DW group was not significantly different from that of Alb3, Alb2, Alb1 and AS.

Solution type (n=10)	**Surface microhardness change (SMHC) Mean(SD)**
Whole mouth saliva (WMS)	224.10 (29.3)*
Artificial saliva+ High albumin (Alb3)	198.2 (44.2)
Artificial saliva+ Medium albumin (Alb2)	191.78 (33.97)
Artificial saliva+ Low Albumin low (Alb1)	173.8 (50.0)
Artificial saliva (AS)	160.2 (24.4)
Deionised water (DW)	123.9 (16.6)*

Table 28: *Mean (SD) surface microhardness change (SMHC) of for six groups according to the six different solutions used (WMS, Alb3, Alb2, Alb1, AS,DW) after five erosion cycles. Same signs indicate significant differences between groups.*

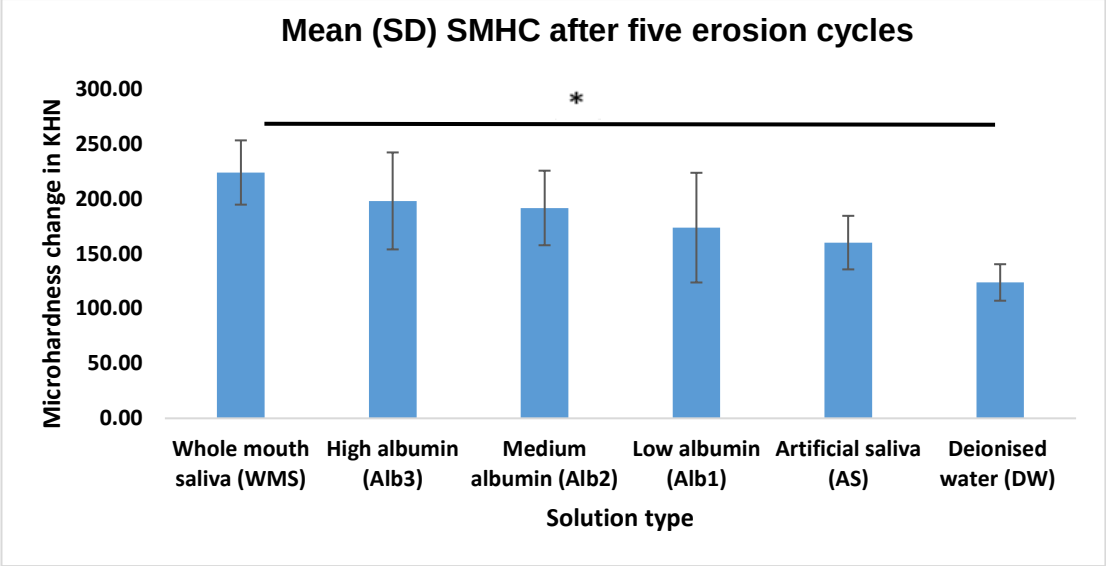


Figure 59: *Mean (SD) surface microhardness change (SMHC) for six groups according to the six different solutions (WMS, Alb3, Alb2, Alb1, AS, DW) after five cycles erosion. Asterisks indicate significant differences between groups.*

4.4.5 Discussion:

This laboratory study did not show any significant differences between the protective effects of AS and AS solutions with added human serum albumin of various concentrations. The results however did show a better protective effect offered by WMS compared to DW, AS and AS containing a single protein. This may suggest that it is not albumin that offers the protection against advanced erosion.

To the author's knowledge, only one study has added an individual protein (mucin) to artificial saliva solution (Hara *et al.*, 2008). Hara *et al*, (2008) investigated the effect of human saliva substitutes in an erosion–abrasion cycling model by comparing WMS, artificial saliva and mucin (AS+M), AS, DW (negative control).

They found that (AS + mucin) showed results similar to WMS and the authors recommended its use as a suitable substitute for the erosion–abrasion cycling model studied. Mucin was not used in the study due to the fact that mucin has

been studied more widely than albumin in the literature, and due to the limited time available for this PhD only albumin was investigated. Also, there was limited availability of mucin. An erosion only model was used in the current study which differ from Hara *et al,* (2008).

Other studies have added proteins to the erosive solution, such as citric acid, which could act as a buffer rather than assess the function of the protein binding into the enamel crystal. We hypothesised that the addition of albumin into artificial saliva could affect the enamel surfaces by coating their surfaces with an albumin layer preventing the erosive challenge from reaching the enamel crystals or may be buffering the erosive solution when interacting with the enamel surfaces. A number of previous studies have reported the ability of albumin to adhere to enamel surface modulating the protection against enamel erosion (Arends *et al.*, 1986; Kawasaki *et al.*, 2003; Hemingway *et al.*, 2008). In the present study, the enamel dissolution was measured using SNCP and SMH to assess the amount of tissue loss and surface microhardness change of enamel specimens respectively.

There are four theories that might explain how albumin has worked during this study. First, enamel adhered albumin may have been removed by water rinsing since ovalbumin, a similar protein, was previously reported to have this property (van der Linden and Sagis, 2001). Secondly, it may be that albumin as an individual protein did not statistically impact on the function of the AEP (Cheaib and Lussi, 2011). A number of studies concluded that only protein-protein interaction significantly improved the erosion-inhibiting properties of the AEP (Yin *et al*., 2006; Cheaib and Lussi). It may be that albumin molecules in the AS did not interact with enamel surface due to the absence of other

salivary proteins. Protein-protein interactions are important for more enamel-interacting AEP with additional binding sites that could enhance the selective enamel-adsorption of proteins (Yin *et al.*, 2006; Cheaib and Lussi, 2011). A third possible theory could be that in our study albumin was added to artificial saliva rather than to the citric acid solution. The absence of citrate ions in the albumin solution rendered albumin molecules unable to adhere to the enamel surface. Ovalbumin, of similar biological structure as albumin, has been reported to have some interaction with the citrate ion when added to citric acid solution, promoting the adsorption of ovalbumin (Hemingway *et al.*, 2008). A fourth possibility is that the presence of minerals in saliva actually seems to hinder the role of proteins in protection against erosion (Baumann *et al.*, 2016). This may be due to the ions present in saliva occupying the binding sites of calcium and phosphate binding proteins reducing the affinity of proteins to the ions on the enamel crystal (Baumann *et al.*, 2016). Our result concluded that the addition of albumin to the artificial saliva in the concentration range of 0.2% to 0.002% did not offer any protection against advanced enamel erosion.

4.4.6 Conclusion:

Human serum albumin added to artificial saliva (AS) in the concentration range of 0.2% to 0.002% did not offer extra protection against *in vitro* prolonged erosion compared to artificial saliva without albumin.

Chapter 5: Do salivary proteins mediate greatest protection against *in vitro* early erosion?

Overall aims of Chapter 5

Chapter 5 is composed of three sections and uses the the 24 hour *in vitro* formed AEP model used in Chapter 4. However, in this Chapter an *in vitro* one cycle erosion model was used representing early erosion within this thesis. The first section (5.1) compares the level of protection between WMS, PS, AS and DW on early erosion using SNCP and SMH techniques. The second section (5.2) is divided into four subsections looking at four aspects of the *in vitro* AEP. The first aspect compares the concentration of total protein between AEP from WMS and PS against early erosion. The second aspect compares at the amount of specific four proteins: mucin5b, albumin, CA VI and statherin between *in vitro* AEP from WMS and PS against early erosion. The third aspect of section 5.2 explores the calcium and phosphorus ions released from enamel after 24 hours immersion in either WMS or PS followed by one cycle of erosion. Unlike section (4.2), section (5.2) has also a fourth subsection which explores the large-scale characterisation of the entire protein profile of AEP derived from WMS and PS after 24 immersion in either WMS or PS using proteomics. In addition, Chapter 5 has a third section (5.3) which discusses sections 5.1 and section 5.2.

Section 5.1: Does the protective effect of whole mouth saliva against *in vitro* early erosion differ from that of parotid saliva?

5.1.1 Introduction:

In Chapter 4 it was shown that WMS provided better protection against advanced erosion (five erosion cycles) than PS in terms of less step height formation. This section assesses the same parameters but using a less aggressive erosion (early erosion). The protection of 24-formed AEP against enamel surfaces was observed to be different between early and advanced citric acid erosion (Nekrashevycha and Stösser, 2003). If systematic investigation of different factors in laboratory erosion studies are to be achieved, single cycle erosion models are useful in order to predict the erosive potential of substances or methods (Shellis *et al*. 2011, Young and Tenuta 2011). Other studies investigating the relationship between early erosion and several salivary parameters have shown that both the loss of AEP and susceptibility of HAP to early erosion (2 minutes citric acid exposure) to be associated with several salivary parameters (Jager *et al.*, 2011). Jager *et al*, (2011) investigated a number of salivary factors in relation to early erosion such as total protein and concentration of calcium, phosphorus and albumin. In this section, the salivary variation between WMS and PS are compared using early erosion model mimicking a short acidic drinking cycle. It is possible that the protective effect of WMS and PS against erosion would differ under varying erosive conditions which may be influenced through diffrent mechanisms and dynamics.

5.1.2 Aim, objectives and hypotheses

The aim of this *in vitro* study was to assess the protection levels between WMS, PS, AS and DW against early erosion.

The objective was:

1- To compare the step height and SMHC of enamel surfaces when immersed in WMS, PS, AS and DW for 24 hours followed by one erosion cycle.

The null hypothesis was that:

1- Immersion of enamel specimens in WMS, PS, AS or DW for 24 hours followed by one erosion cycle will not produce significantly different step height or SMHC measurements.

5.1.3 Materials and methods

5.1.3.1 Specimen preparation

Based on the power calculation, a sample size of 40 was required for this study yielding 80 % power at 5 % level with an effect size of 0.31 (Nekrashevych and Stösser, 2003; Martins *et al.*, 2013; Mistry *et al.*, 2015; O'Toole *et al.*, 2015). Forty two human enamel specimens were prepared by sectioning twenty one buccal and twenty one lingual surfaces from forty human extracted molar sound teeth. Initial surface microhardness values (SMH) measured. The specimens with a SMH value between 272 KHN and 400 KHN were selected (Meredith *et al.*, 1996; Austin *et al.*, 2011; Lussi *et al.*, 2011). Forty specimens were selected and two specimens were rejected. Enamel surfaces were embedded in cold cure acrylic resin as described in section 2.1.4. Enamel specimens were ground and polished as explained in section 2.1.5. They were

then taped as explained in section 2.1.7 and were randomised and numbered as described in section 2.1.8.

5.1.3.2 Study methodology

The study consisted of four groups according to the four solutions used (n=40). Specimens were randomly allocated by an independent investigator using SPSS random sample generator to the 4 groups, 10 specimens per group: WMS, PS, AS and DW groups. The WMS, PS and AS were prepared as described in section 2.2. Thawed WMS and PS were mixed vigorously prior to use to re-suspend precipitation of proteins and avoid loss of specific proteins less than 14 kDa (Francis *et al*., 2000). Enamel specimens of each group were exposed to one cycle erosion as described in section 2.3.4.2. Specimens were immersed in the corresponding solution (either WMS, PS, AS or DW) for 24 hours followed by a further 30 minutes prior to exposure to a 10-minute erosion cycle (Figure 60).

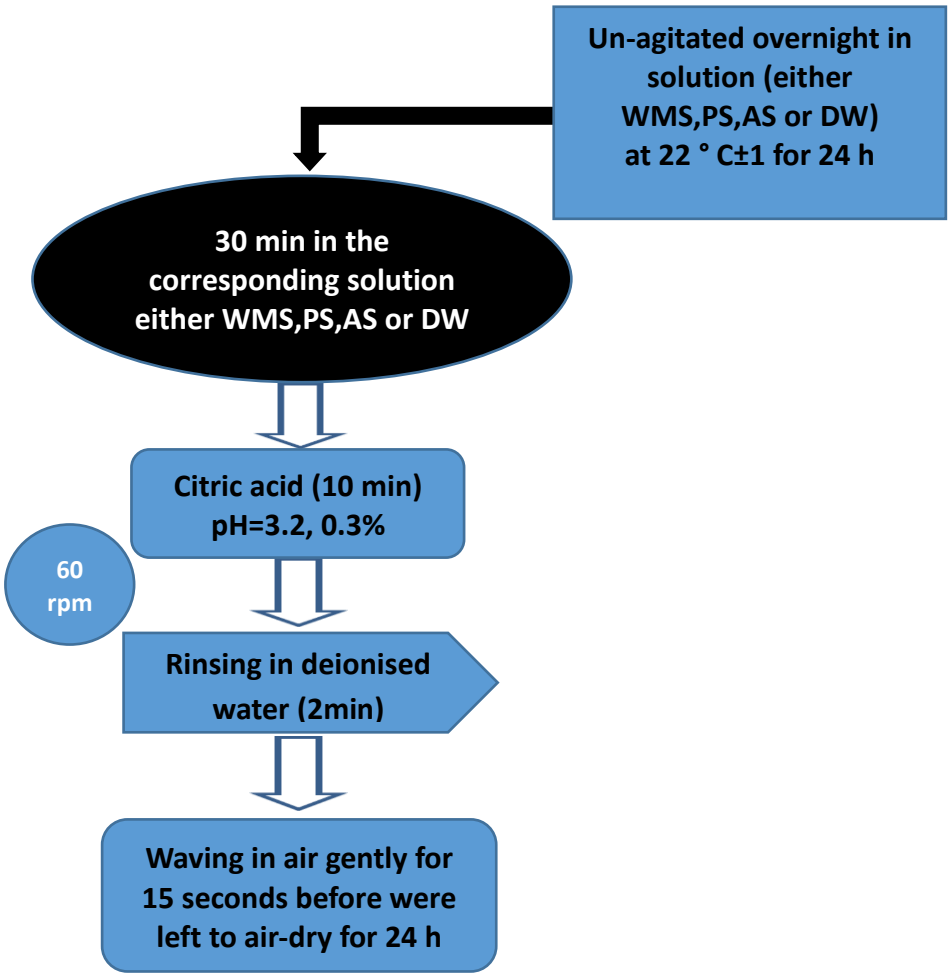


Figure 60: *A flowchart representation of the AEP formation and one erosion cycle (early erosion) protocol.*

5.1.3.3 Testing

Profilometric measurements

Profilometric measurement data after one cycle erosion were obtained using SNCP as explained in section 2.4.1. Each surface profile was taken from within the first 1/3 of the taped zone on one side across the exposed window to just within the first 1/3 of the taped zone on the opposite side. Ten randomly selected step height measurements were taken from each specimen and averaged to give a mean surface profile value.

Surface microhardness (SMH) measurements

Surface microhardness at baseline (SMHb):
The surface microhardness (SMH) baseline values before immersion in WMS, PS, AS or DW (Surface microhardness baseline values: SMHb) were measured for the four experimental groups, 10 specimens each, as described in section 2.4.2. The SMH values of each specimen was determined by the average of five indentations made at the exposed window under a load of 100 g and a dwell time of 10 seconds. The SMH value of each indentation was determined by specialised software (Duramin-5Hardness Tester, Struers Inc., Rotherham, UK) through measuring the length of each indentation with an optical analysis system calculating hardness in Knoop units (KHN).

Surface microhardness change (SMHC) after one cycle erosion:
Surface microhardness (SMH) values were repeated after the experiment using the method explained above. The surface microhardness change (SMHC) of each specimen was then calculated by subtracting the mean surface microhardness value after one cycle erosion (SMHe) from the mean surface microhardness value at baseline (SMHb) using the formula: SMHC = (SMHb – SMHe).

5.1.3.4 Statistical analysis:

Descriptive statistics were used to summarise the step height and SMHC. Shapiro-Wilk and Kolmogorov-Smirnov tests were used to assess the normality distributions of data. Data were normally distributed and were described using mean and standard deviation. Linear regression models were used to test the significant differences between solutions (WMS,PS,AS,DW) with respect to step height and SMHC. The initial model included the interaction between one cycle erosion and solutions along with the main

effects. The mean difference was considered to be significant at a P value < 0.05. If the interaction between solutions was significant, then further post hoc Bonferroni test analyses were carried out to find out which solution was statistically significant in relation to step height and SMHC. All the analyses were carried out using stata version 12.0 (StataCorp LP, Texas 77845-4512, USA).

5.1.4 Results:

Step height

Table 29 and Figure 61 show the results of the mean (SD) step height after one erosion cycle for four experimental groups. The mean step height (SD) for WMS group was [1.13 (0.1)µm], PS group was [1.39 (0.3) µm], AS group was [1.43 (0.3) µm], DW group was [2.29 (0.5) µm]. WMS, PS and AS groups had significantly lower mean step height ($p<0.05$) than DW group. When comparing WMS, PS and AS, the differences between their mean step height values were not significant.

Solution type (n=10)	**Step height (µm) Mean (SD)**
Whole mouth saliva (WS)	1.13 (0.1)€
Parotid Saliva (PS)	1.39 (0.3)©
Artificial Saliva (AS)	1.43 (0.3)Δ
Deionised water (DW)	2.29 (0.5)€©Δ

***Table 29**: Mean (SD) step height (µm) of enamel surfaces for four groups according to the solution used (WMS, PS,AS, DW) after one cycle erosion (0.3%, pH 3.2, citric acid,10 min). Similar shapes in the table denote significant differences between the groups.*

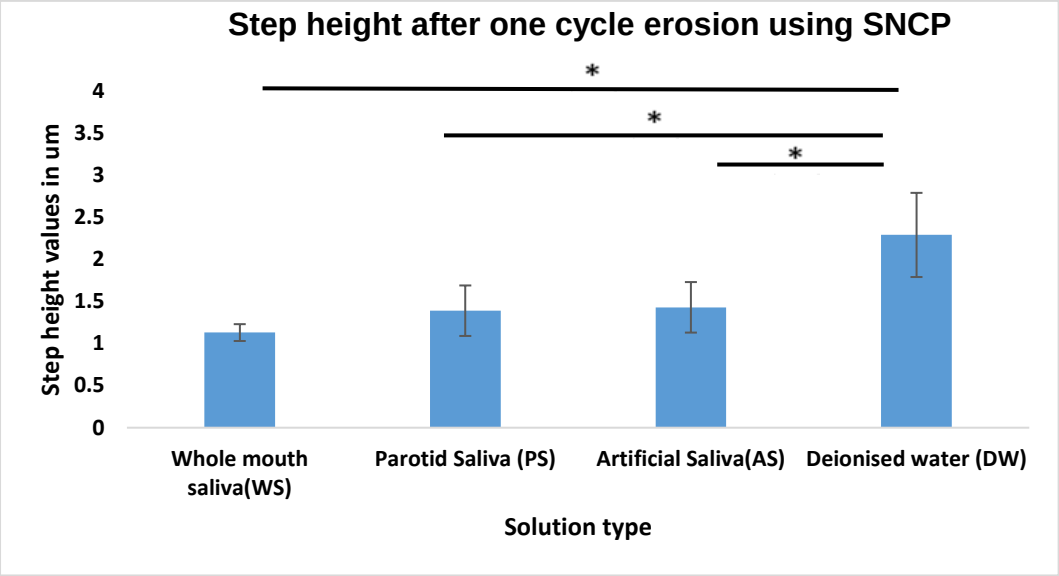


Figure 61: *Mean (SD) step height (µm) or four groups according to the solutions used (WMS, PS,AS, DW) after one cycle erosion (0.3%, pH 3.2, citric acid, 10 min). Asterisks indicate statistical significance.*

Surface microhardness (SMH) measurements

Surface microhardness at baseline (SMHb)

Table 30 shows the surface microhardness (SMH) values before immersion in all four solutions (WMS,PS,AS,DW) and before erosion cycle (Surface microhardness at baseline: SMHb) of the four experimental groups. The mean SMH baseline values of the four groups ranged between 350.65 (14.78) KHN and 366.43 (12.19) KHN. There were no significant differences between the mean microhardness values of all groups.

Solution type (n=10)	surface microhardness at baseline (SMHb) Mean (SD)
Whole mouth saliva (WMS)	364.63 (11.23)
Parotid saliva (PS)	350.65 (14.78)
Artificial saliva (AS)	353.3 (12.7)
Deionised water (DW)	366.43 (12.19)

Table 30: *Mean (SD) surface microhardness baseline (SMHb) in Knoop microhardness units (KHN) after specimens were polished and before immersed in solutions. No significant differences between the groups.*

Surface microhardness after one cycle erosion (SMHe):

Table 31 and Figure 62 show the results of the mean (SD) surface microhardness change (SMHC) after one cycle erosion for the four groups. The mean SMHC for WMS group was [98.68 (8.5) KHN], PS group was [85.19 (6.07) KHN], AS group was [63.97 (12.95) KHN] and DW group was [60.45 (11.3) KHN]. DW group [60.45 (11.3) KHN] had significantly lower SMHC value than that of WMS group [98.68 (8.5) KHN (p=0.002)] and PS group [85.19 (6.07) KHN (p=0.04)] but was not statistically significant than that of AS group [63.97 (12.95) KHN (p>0.05)]. AS (p= 0.004) and PS (p=0.025) groups had significantly lower SMHC value than that of WMS group. The SMHC value of AS group was not significantly different than that of PS group (p= 0.074).

Solution type (n=10)	Surface microhardness change (SMHC) Mean (SD)
Whole mouth saliva (WMS)	98.68 (8.54)β?μ
Parotid Saliva (PS)	85.19 (6.07)∑μ
Artificial Saliva (AS)	63.97 (12.96)?
Deionised water (DW)	60.45 (11.34)β∑

Table 31: *Mean (SD) microhardness change(MHC) of four groups based on four solutions (WMS, PS,AS and DW) after one cycle erosion (0.3%, pH 3.2, citric acid, 10 min). Similar shapes in the table denote significant differences between the groups.*

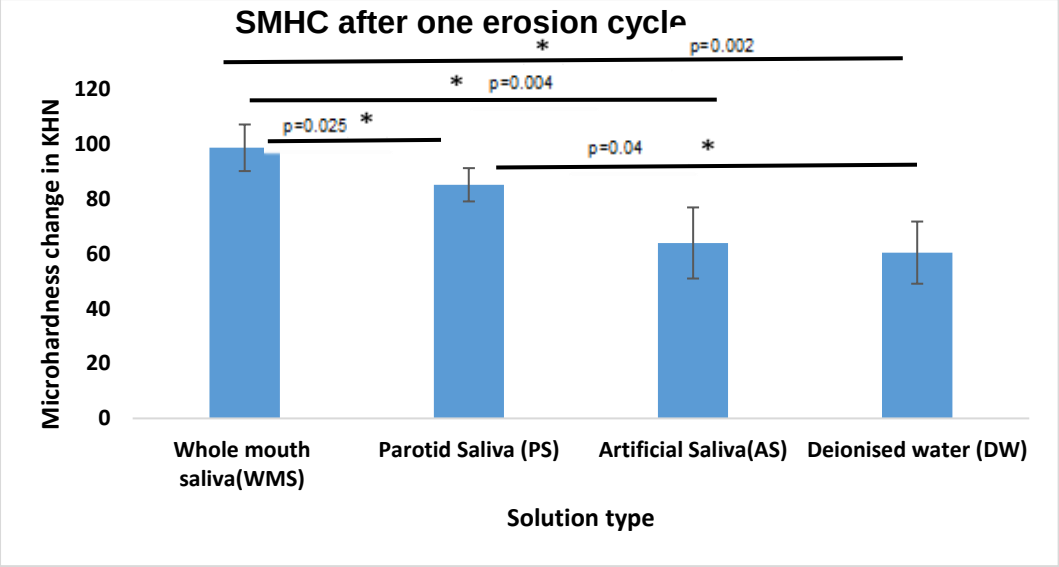


Figure 62*: Mean (SD) surface microhardness change (SMHC) of four groups based on four solutions (WMS, PS,AS, DW) after one cycle erosion (0.3%, pH 3.2, citric acid, 10 min). Asterisks indicate statistical significance.*

Section 5.2: The role of proteins derived from whole mouth and parotid saliva on early erosion: An *in vitro* study.

5.2.1 Introduction

In the light of the results from chapter 4 section (4.2), the concentration of total protein and amount of four specific salivary proteins appeared to be significantly different between AEP from WMS and AEP from PS before and after advanced erosion. Given these differences, it would be of interest to assess whether such differences exist against less aggressive erosion (early erosion). Undesratnding the processes involved in early erosion and the protective mechanisms are of great interest. This is because early erosion involves initial partial softening of enamel surface rather than enamel loss (Shellis *et al*., 2011; Lussi and Ganss, 2014). Identifying which proteins remains in the AEP at this stage of erosion can increase our understanding of the processes involved and hence assist in prevention of demineralisation and promoting remineralisation to avoid loss of tooth tissue.

AEP consist of two layers; an outer thick, globular layer and a thin, inner layer. It is may be possible that during early stages of erosion only part of the outer thick layer of the AEP is dissolved and the basal, inner protein layer of the AEP remains intact (Hannig *et al*., 2005; Hannig and Joiner, 2006). The inner AEP layer is rich of statherin, histatins and PRPs that are possibly function in protecting against early erosion.

5.2.2 Aim, objectives and hypotheses

The aim of this *in vitro* study was to measure the total protein and four specific salivary proteins present in AEP after 24 hour immersion in WMS and PS (before erosion) and after early erosion (one erosion cycle).

The objectives were:

1. To compare the amount of total protein in *in vitro* AEP after 24 hours immersion in WMS or PS (before erosion) and after one erosion cycle using BCA assay.
2. To compare the amount of mucin5b, albumin, CA VI and statherin in *in vitro* AEP after 24 hours immersion in WMS or PS (before erosion) and after one erosion cycle using SDS-PAGE and immunoblotting.
3. To compare the amount of calcium and phosphorus ions in *in vitro* AEP after 24 hours immersion in WMS or PS (before erosion) and after one erosion cycle using inductively coupled plasma mass spectroscopy (ICP-MS).
4. Identify the proteome of the *in vitro* AEP after 24 hours immersion in WMS or PS (before erosion) using liquid chromatography–mass spectrometry (LC/MS/MS).

The null hypotheses were that:

1. The concentration of total protein in *in vitro* AEP derived from WMS would not differ from that in AEP derived from PS before and after one erosion cycle.
2. The amount of mucin5b, albumin, CA VI and statherin in *in vitro* AEP derived from WMS would not differ from that in AEP derived from PS before and after one erosion cycle.

3. The concentration of calcium and phosphorus in AEP derived from WMS would not differ from that in *in vitro* AEP derived from PS before and after one erosion cycle.
4. The proteome of 24 hours *in vitro* formed AEP derived from WMS would not differ from that in AEP derived from PS.

5.2.3 Materials and methods

5.2.3.1 Specimen preparation

Based on the power calculation as described in section 2.1.3, twenty enamel specimens were prepared for this study yielding an effect size of 0.6 and 80% power at 5% level. Twenty two enamel specimens were prepared by sectioning eleven buccal and eleven lingual surfaces from eleven human extracted molar sound teeth. Initial surface microhardness values (SMH) were measured. The specimens with a SMH value between 272 KHN and 400 KHN were selected. Twenty specimens were selected and two were rejected. Enamel surfaces were mounted in cold cure acrylic resin, ground, polished, taped and randomised as explained in section 2.1.

5.2.3.2 Study methodology

The study consisted of 2 experimental groups according to the two solutions used, 10 specimens per group: WMS and PS. The WMS and PS were prepared and thawed as described in section 2.2. Within each group, specimens were randomly allocated by an independent investigator using SPSS random sample generator to produce 2 subgroups, 5 specimens each: control: no erosion (n=5) and one cycle erosion (n=5). The AEP was eluted for the control group after 24 hour immersion in the corrsponding saliva followed

by 2 minute rinse in DW prior to acid erosion (control) as shown in Figure 63. For the one cycle erosion group, AEP was eluted after one erosion cycle as shown in Figure 63. Details on the design of both groups and how AEP is eluted in each group are explained in the following sections.

One cycle erosion

In the one cycle erosion group, enamel specimens were immersed in either WMS (n=5) or PS (n=5) for 24 hours followed by a further 30 minutes prior to exposure to a 10-minute citric acid followed by 2-minute water rinse as shown in Figure 63.

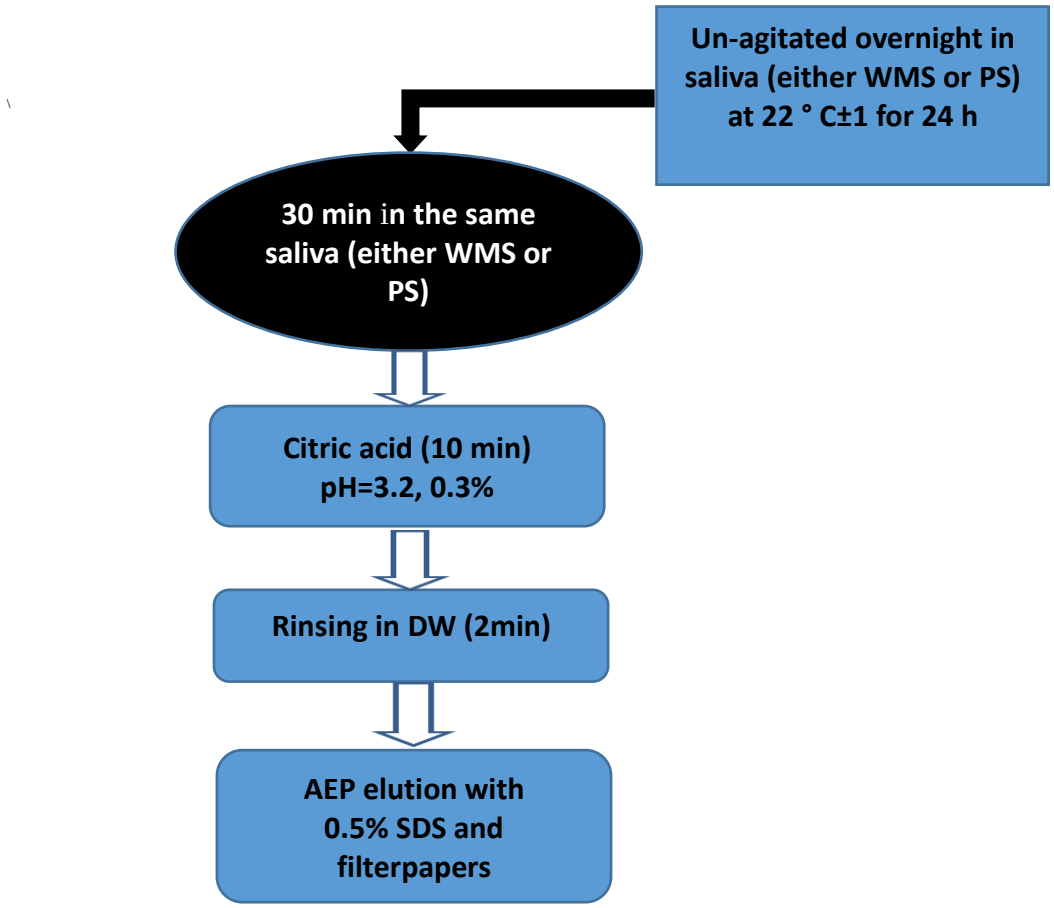


***Figure 63**: A flowchart representation of the 24 hours in vitro AEP formation and elution protocol after one erosion cycle.*

The erosion cycle consisted of 80 mL 0.3% citric acid, pH=3.2, at 22 °C ± 1, agitated with an orbital shaker (Stuart Scientific, orbital shaker) at 60 rpm followed by 2-minute in 100 mL of DW rinse, again, under agitation with an orbital shaker set at 60 rpm for a final 2 minutes. AEP was then eluted after the completion of the erosion cycle using 0.5% SDS and filterpapers and then recovered as detailed in section 2.3.3.

Control group:

In order to assess the amount of proteins in the AEP before one cycle erosion, the AEP was eluted after enamel specimens were immersed in either WMS (n=5) or PS (n=5) for 24 hours followed by 2 minutes immersion in DW Figure 64. This was served as the control group where AEP was eluted prior to acid erosion. AEP was eluted using 0.5 % SDS and filterpapers and then recovered as detailed in section 2.3.3.

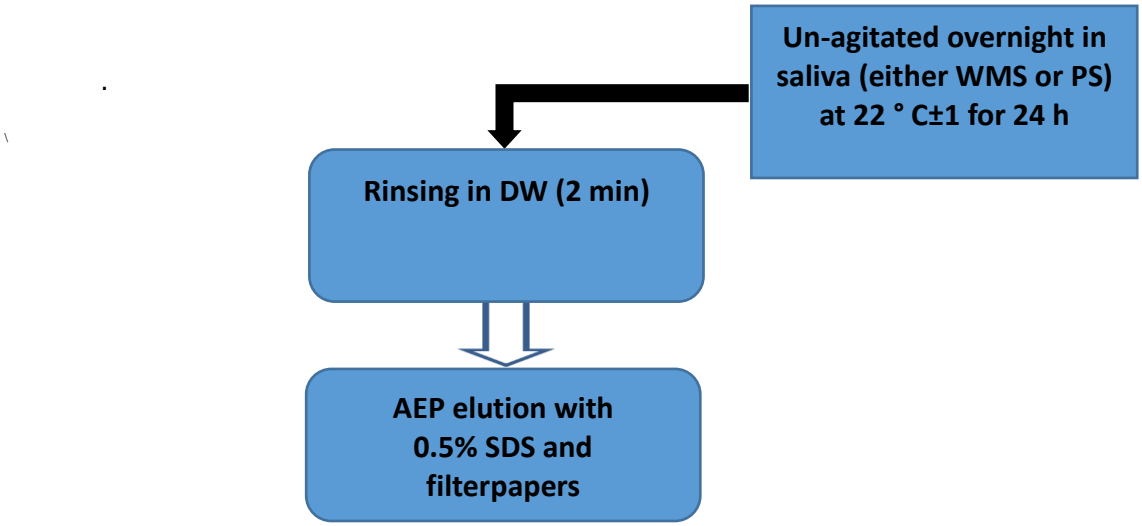


Figure 64: *A flowchart representation of the 24 hour AEP formation and elution protocol before erosion cycle (control group).*

5.2.3.3 Testing

The eluted *in vitro* AEP were then recovered from the filterpapers using the same procedure as described in section 2.3.3. The protein contents was analysed for total protein, four specific proteins, calcium and phosphorus concentration and proteomics.

5.2.3.1.3 Total protein analysis

Part (1µL) of each *in vitro* recovered AEP samples from WMS (n=10) and PS (n=10) were prepared for the analysis of total protein concentration. Each sample was diluted in DW at 1/100 dilution to a final volume of 100 µL. Prepared *in vitro* AEP samples were then placed into microtiter plates (96-wells, Fisher Scientific, Leicestershire). The amount of total protein of each AEP sample was measured using the bicinchoninic acid (BCA) assay (Pierce Chemical, Rockford, Ill., USA) and bovine serum albumin protein standard (BSA). The amount of total protein was measured spectrophotometrically employing a UV-visible spectrophotometer (BioRad laboratories Ltd, Hemel Hempstead, UK) determining the optical density at a wavelength of 562 nm as explained in 2.4.4. All samples were analysed in duplicate.

5.2.3.3.2 Specific protein analysis

Protein separation:

Qualitative differences between the *in vitro* recovered AEP samples derived from WMS and PS were analysed by SDS–PAGE. Prepared protein fractions were loaded and run equally (15 µL each) through precast gels and were separated consistently as described in section 2.4.5.2.

Protein transfer and immunoblotting

After the separation of proteins, western blotting was completed according to the manufacturer's instructions and used to transfer proteins onto a nitrocellulose membrane as explained in section 2.4.6.1. Protein bands of the proteins of interest were cut transversely from the nitrocellulose membranes with a sterile razor. Immunoblotting was used to examine the presence of four proteins of interest in the AEP: mucin5b, albumin, CA VI and statherin as described in section 2.4.6.3. At room temperature, the nitrocellulose membranes were blocked in TTBS for 1 hour before membranes were probed with primary antibodies as described in section 2.4.6.3. The nitrocellulose membranes were then washed in TTBS for 15 minutes (5 minutes X 3 times) and then followed by incubation with the required secondary antibody. Details of the primary and secondary antibodies used was given in section 2.5.2. A final 15-minutes wash in TTBS was completed before the membranes were developed in ECL substarte and were imaged as described in the next section.

Imaging analysis:

The presence of proteins on the blotted and developed nitrocellulose membranes in the AEP samples was assessed using photographic quantification of the staining intensity as was explained in section 2.4.6.4. ChemiDoc MP imaging analysis (Bio-Rad) was used to quantify the light intensity of the chemiluminescent reaction and exposure times optimised to prevent pixel saturation. The amounts of proteins on the blotted nitrocellulose membranes were quantified using tools of ImageLab software version 4.1 (Bio-Rad Laboratories Ltd., Hertfordshire, UK) to select and determine the background-subtracted density of the bands in all the gels (n=3) using purified

protein of standards of known concentration. The standard curves of purified proteins were generated from the mean (SD) volume intensities (n=3) against the absolute quantities of the corresponding purified standard. This was used to generate a calibration curve using a linear formula. This formula was used to calculate the amount of each protein in the AEP samples. The bands of standard proteins on different SDS-PAGE gels (n=3) were used to assess reproducibility.

5.2.3.3.3 Calcium and phosphorus analysis

The *in vitro* AEP samples were eluted from enamel surfaces after 24 hours immersion in WMS or PS (control) and after one erosion cycle as described in 5.2.3.2. Part (1 µL) of each prepared *in vitro* AEP sample from WMS (n=10) and from PS (n=10) was further diluted in DW (1:1000 dilution) to provide 1 mL sample. Each diluted AEP sample was then subjected to calcium and phosphorus analysis using inductively coupled plasma mass spectroscopy (ICP-MS) (SCIEX ICP mass spectrometer, ELAN DRC 6100; PerkinElmer, Waltham, Mass., USA) as was explained in 2.4.7.

5.2.3.3.4 Proteomic analysis

Two samples of *in vitro* AEP, one from WMS and one from PS, were prepared by eluting the *in vitro* AEP from two enamel surfaces, one enamel specimen each. One enamel surface was immersed for 24 hours in WMS and one in PS. The two AEP samples were eluted from the enamel surfaces using 0.5 % SDS and filterpapers. The *in vitro* AEP samples were harvested and recovered as described in section 2.3.3. The AEP sample from of WMS [WMS 40 µL (MM1_2)] and PS [PS 40 uL (MM1_1)] were prepared in eppendorf tubes at

volumes mentioned above, 40 µL each. These two AEP samples were used for the proteomic analysis which was carried out by the Centre of Excellence for Mass Spectrometry, King's College London, Institute of Psychiatry, Psychology and Neuroscience. The proteomic analysis at the Centre of Excellence for Mass Spectrometry was performed through several steps as shown in appendix VIII. First, sample buffer was added to each tube and heated at 96 ºC for 10 minutes prior to centrifugation at 14,000 rpm. The final step of protein analysis was that boiled protein samples were loaded in to an SDS 4%/20% stacking gel into a single band (Figure 65). Each sample was loaded into two lanes as the volume was too large for a single lane. The bands were excised and pooled for each individual sample prior to enzymatic digestion and LC/MS/MS analysis as explained in section 2.4.8.

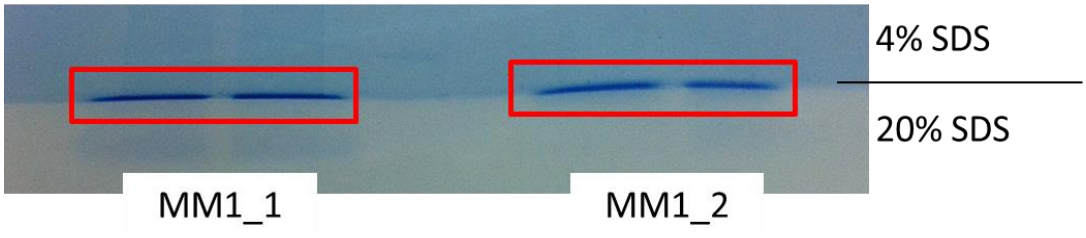


Figure 65 : *SDS-PAGE stacking gel containing proteins from WMS (MM1_2) and PS(MM1_1). The in vitro AEP formed on enamel specimens by immersion in either WMS or PS for 24 hours. AEP samples were then eluted using 0.5 % SDS. Whole protein sample 'stacked' into one band at the interface between the high and low percentage gels. Gel bands were excised and pooled prior to enzymatic digestion and LC/MS/MS analysis.*

5.2.3.4 Statistical analysis

Data obtained from the protein analysis tests were analysed using Stata 12.0 (StataCorp LP, Texas 77845-4512, USA). The calcium and phosphorus as well as total protein and four specific protein data did not follow normal

distribution and hence they were transformed to achieve normality. Therefore, data were described using mean and standard deviations as well as median and interquartile ranges. Calcium, phosphorus, total protein, mucin5b and albumin were log transformed to attain normality and log transformed values were used for the analysis. CA VI and statherin were square root transformed and the transformed values were used for the analysis. Linear regression models were used to find out the effect of two different conditions [before erosion (control) and one cycle erosion] and type of saliva (WMS and PS). The initial model included interaction between groups and saliva. If the interaction was statistically significant, the post hoc analysis testing the linear combinations of groups and saliva was used to find out which group and saliva were statistically significant. All such p values were to be adjusted for multiple testing. If the interaction effect was not statistically significant, then the final model included only the main effects of groups and saliva.

5.2.4 Results

5.2.4.1 Total protein

Table 32 and Figure 66 show the mean (SD) concentration of total protein in AEP from WMS and PS after 24 hours immersion in solution [before erosion, control (C)] and after one erosion cycle (E1). The mean (SD) concentration of total protein in *in vitro* AEP from WMS before erosion was [1.65 (0.16) g/L] and after one erosion cycle was [0.44 (0.13) g/L]. The mean (SD) concentration of total protein in *in vitro* AEP from PS before erosion was [0.67 (0.12) g/L] and after one erosion cycle was [0.27 (0.07) g/L].

Generally, *in vitro* AEP from PS before and after erosion had significantly lower concentration of total protein than that in *in vitro* AEP from WMS (p<001). For AEP from WMS, the total protein concentration after one cycle erosion [0.44 (0.13) µg/µL] was significantly lower than that before erosion [1.65 (0.16) µg/µL] (p<0.0001). For AEP from PS, the concentration of total protein after one erosion cycle [0.27 (0.07) µg/µL] was significantly lower than that before erosion [0.67 (0.12) µg µL] (p<0.0001).

Saliva type and erosion condition	**Concentration of total proteins (g/L)**	
	Mean (SD)	**Median (IQR)**
Whole mouth saliva at control (WMSC)	1.65 (0.16)πµ	1.66 (0.19)
Whole mouth saliva after one cycle erosion (WMSE1)	0.44 (0.13)π#	0.41 (0.13)
Parotid saliva at control (PSC)	0.67 (0.12)ϕµ	0.65 (0.07)
Parotid saliva after one cycle erosion (PSE1)	0.27 (0.07)ϕ#	0.27 (0.03)

Table 32*: Mean (SD) and median (interquartile range) concentration of total protein (g/L) in vitro AEP formed on enamel specimens after 24 hours immersion in WMS or PS. AEP samples were then eluted before (C) or after erosion (E1) using 0.5% SDS and quantified using BCA assay. Same symbols in the table indicate significant differences (p<0.0001). (WSC: whole mouth saliva at control; WSEI: whole mouth saliva after one cycle erosion; PSC: parotid saliva at control; PSEI: parotid saliva after one cycle erosion).*

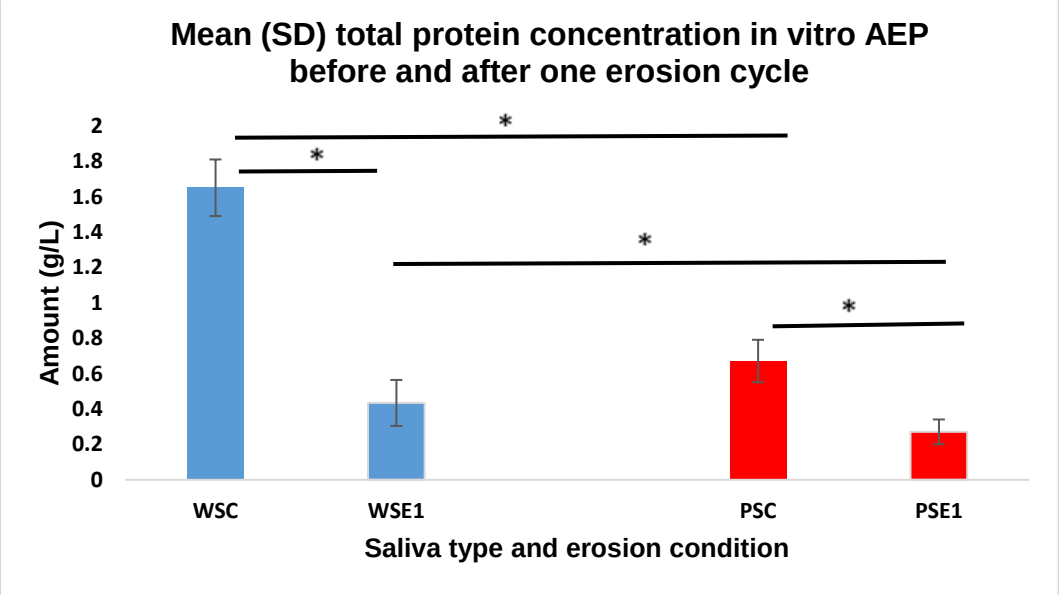
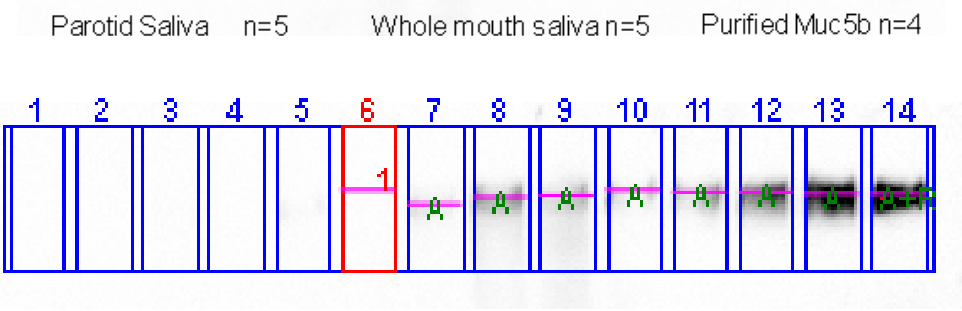


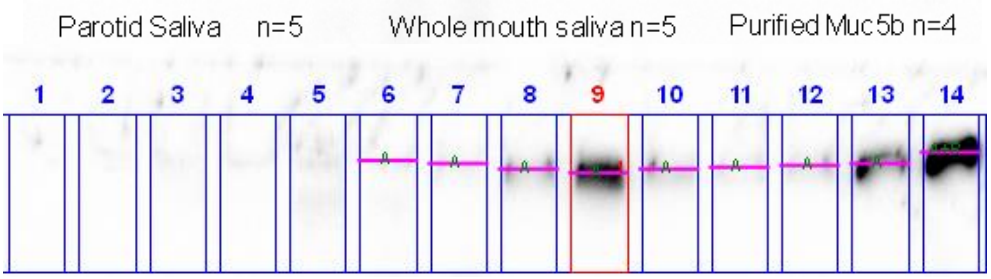
***Figure 66**: Mean (SD) concentration of total protein (g/ L) in vitro AEP formed on enamel samples immersed in WMS or PS for 24 hour. AEP were then eluted before or after erosion using 0.5% SDS and quantified using BCA assay. Asterisk shapes in the graph indicate significant differences (p<0.0001). (WSC: whole mouth saliva at control; WSEI: whole mouth saliva after one erosion cycle; PSC: parotid saliva at control; PSEI: parotid saliva after one erosion cycle).*

5.2.4.2 Specific proteins

Figures 67 (I,II,III, IV) shows images of SDS-PAGE and western blots of the four proteins investigated in this study. Western blots of *in vitro* AEP samples from WMS (n=10) and PS (n=10) before erosion and after one cycle erosion were probed with antibodies against mucin5b, albumin, CA IV and statherin.

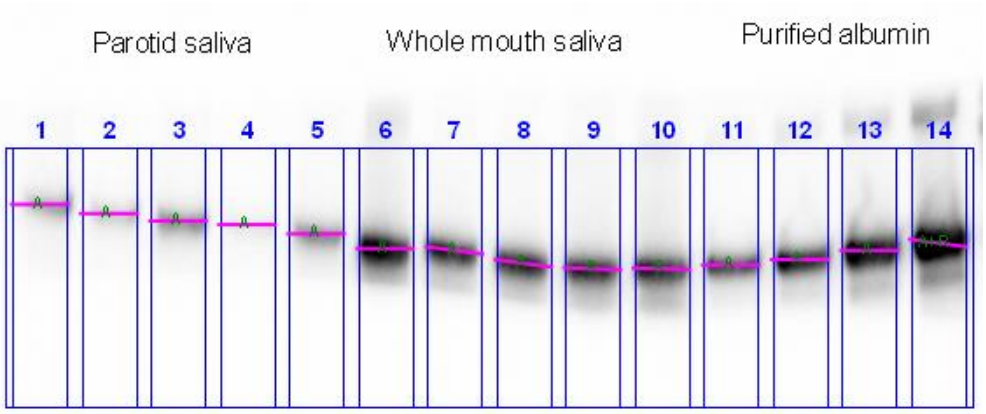


I (a) SDS-PAGE and western blot before erosion from WMS (n=5) and PS (n=5) probed with mucinb5 antibody

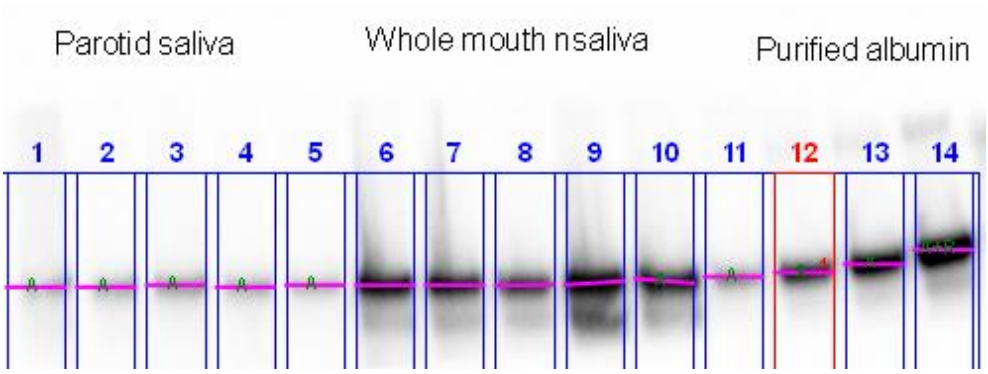


I (b) SDS-PAGE and western blot after one cycle erosion from WMS (n=5) and PS (n=5) probed with mucinb5 antibody

Figure 67 (I): *SDS-PAGE and western blots of in vitro AEP samples from WMS (n=5) and PS (n=5) and purified protein of standards (n=4). All samples were immunoblotted against mucin5b[I (a): before erosion (control);I(b): after one cycle].*

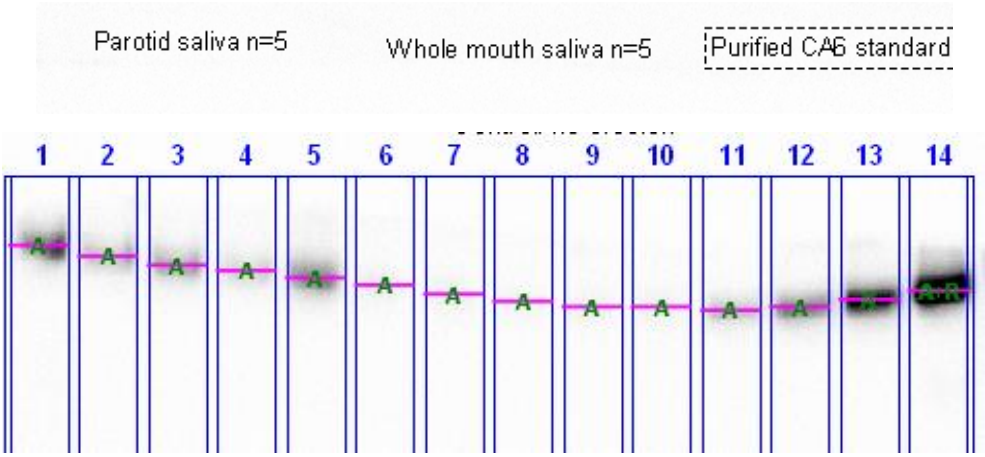


II (a) SDS-PAGE and western blot before erosion from WMS (n=5) and PS (n=5) probed with albumin antibody

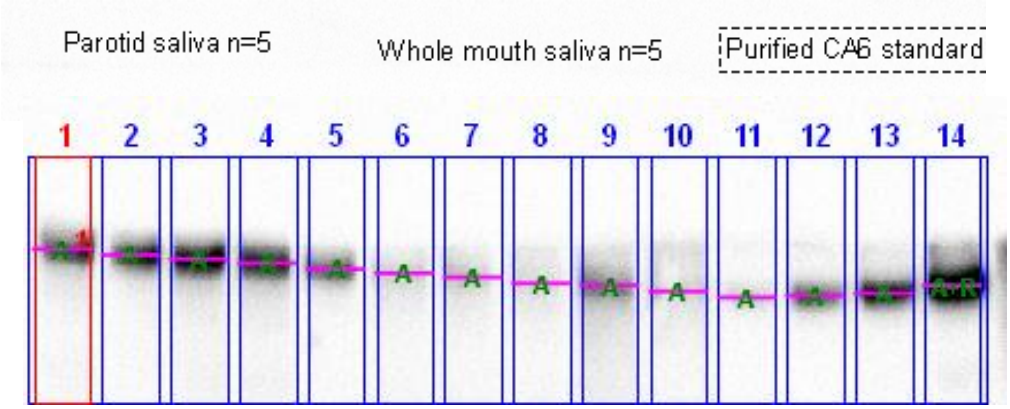


II (b) SDS-PAGE and western blot after one cycle erosion from WMS (n=5) and PS (n=5) probed with albumin antibody

Figure 67 (II): *SDS-PAGE and western blots of in vitro AEP samples from WMS (n=5) and PS (n=5) and purified protein of standards (n=4). All samples were immunoblotted against albumin antibody [II(a): before erosion (control); II(b): after one cycle).*

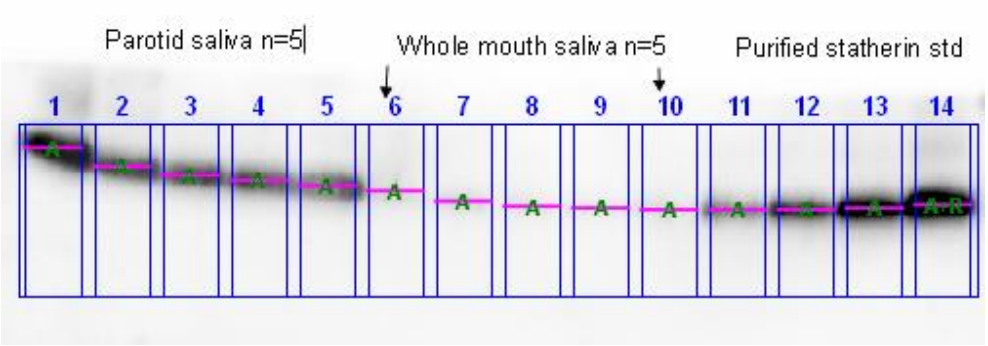


III (a) SDS-PAGE and western blot before erosion from WMS (n=5) and PS (n=5) probed with CA VI antibody.

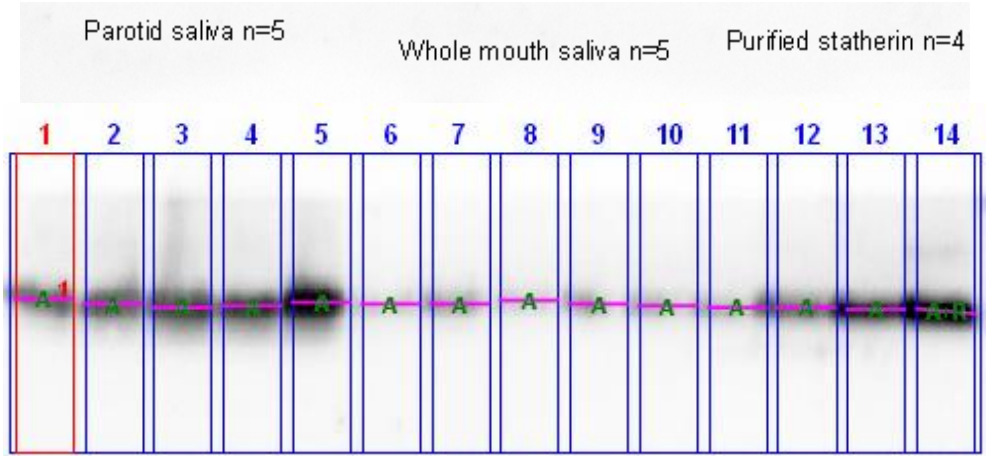


III (b) SDS-PAGE and western blot after one cycle erosion from WMS (n=5) and PS (n=5) probed with CA VI antibody.

***Figure 67 (III)**: SDS-PAGE and western blots of in vitro AEP samples from WMS (n=5) and PS (n=5) and purified proteins of standards (n=4). All samples were immunoblotted against CA IV antibody (a: before erosion (control); b: after one cycle).*



IV (a) SDS-PAGE and western blot before erosion from WMS (n=5) and PS (n=5) probed with statherin antibody



IV (b) SDS-PAGE and western blot after one cycle erosion from WMS (n=5) and PS (n=5) probed with statherin antibody

***Figure 67 (IV)**: SDS-PAGE and western blot of in vitro AEP samples from WMS (n=5) and PS (n=5) and purified proteins of standards (n=4). All samples were immunoblotted against statherin antibody [IV(a): before erosion (control); IV(b): after one cycle).*

Figures 68 (a,b,c,d) shows the standard curves of the purified proteins of standards generated from the mean (SD) volume intensities against the absolute quantities of the purified proteins of standards in all gels (n=3). This was used to generate a calibration curve using a linear formula which was

used to calculate the amount of the corresponding protein in the *in vitro* AEP samples. It can be seen from the figures that the purified proteins used in this study were optimised in a way that the data points between a high volume (15 µL) of purified proteins and low volume (1 µL) provided a suitable curve range to calculate very little proteins in the *in vitro* AEP samples whilst producing a gradual change of intensities.

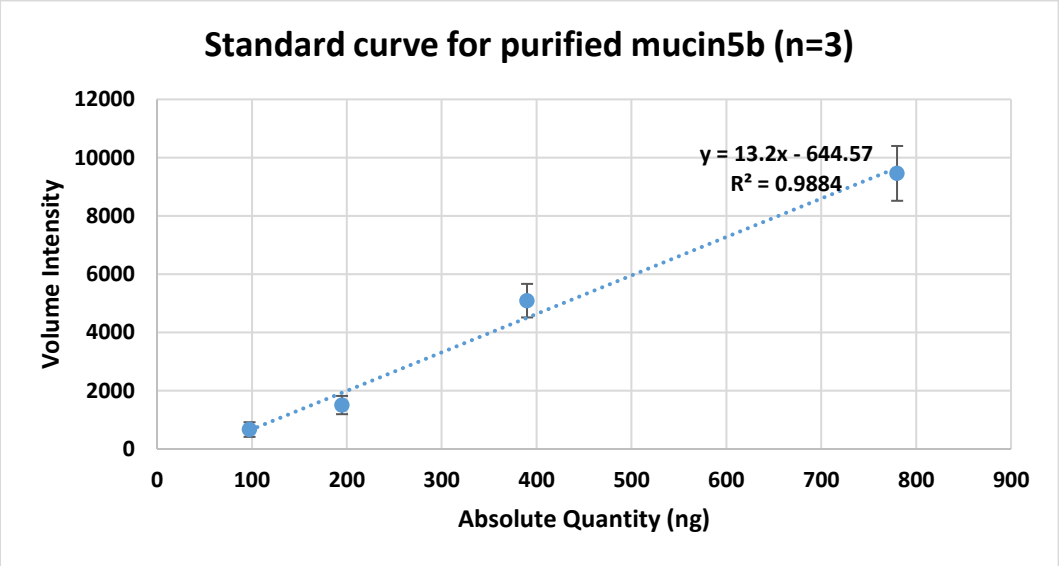


Figure 68 (a) : *Standard curve of the purified mucin5b generated from volume intensities mean (SD) against the absolute quantity (ng) (n=3) and was used to quantify the absolute quantity of mucin5b in the in vitro AEP samples.*

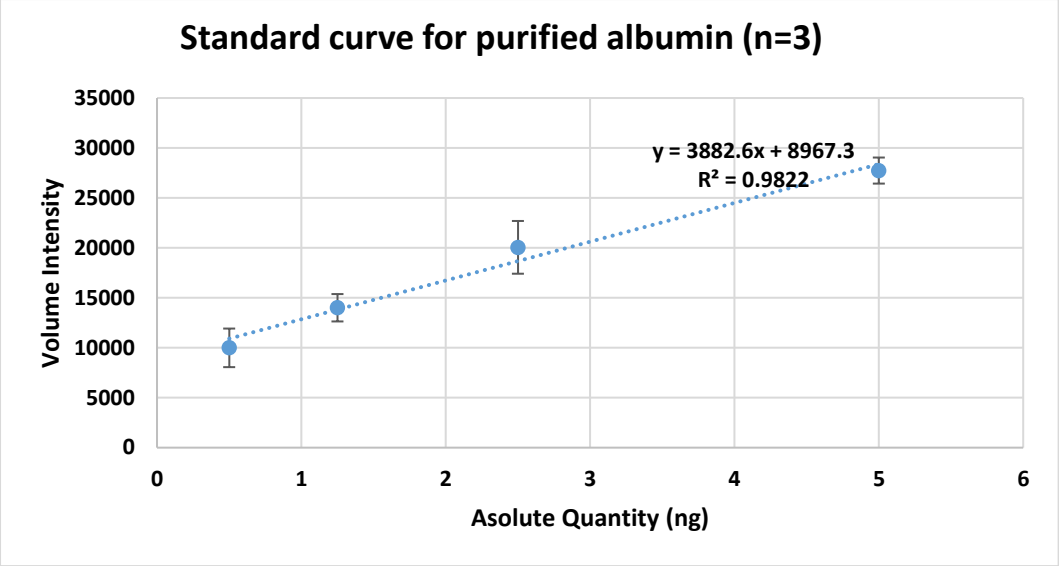


Figure 68 (b): *Standard curve of the purified albumin generated from volume intensities mean (SD) against the absolute quantity(ng) (n=3) and was used to quantify the absolute quantity of proteins in the in vitro AEP samples.*

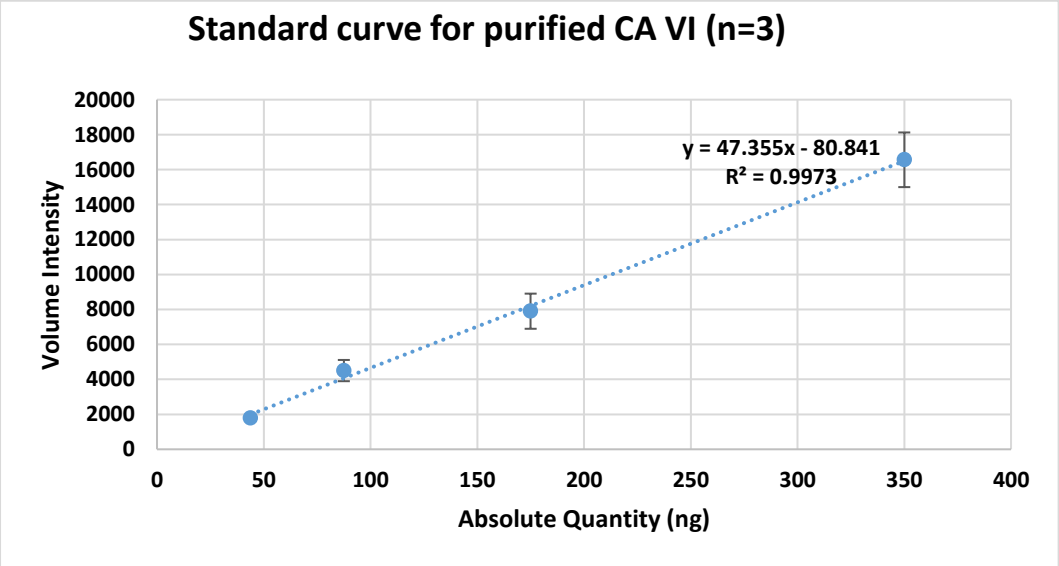


Figure 68 (c): *Standard curve of the purified CA VI generated from volume intensities (mean (SD) against the absolute quantity (ng) (n=3) and was used to quantify the absolute quantity of proteins in the in vitro AEP samples.*

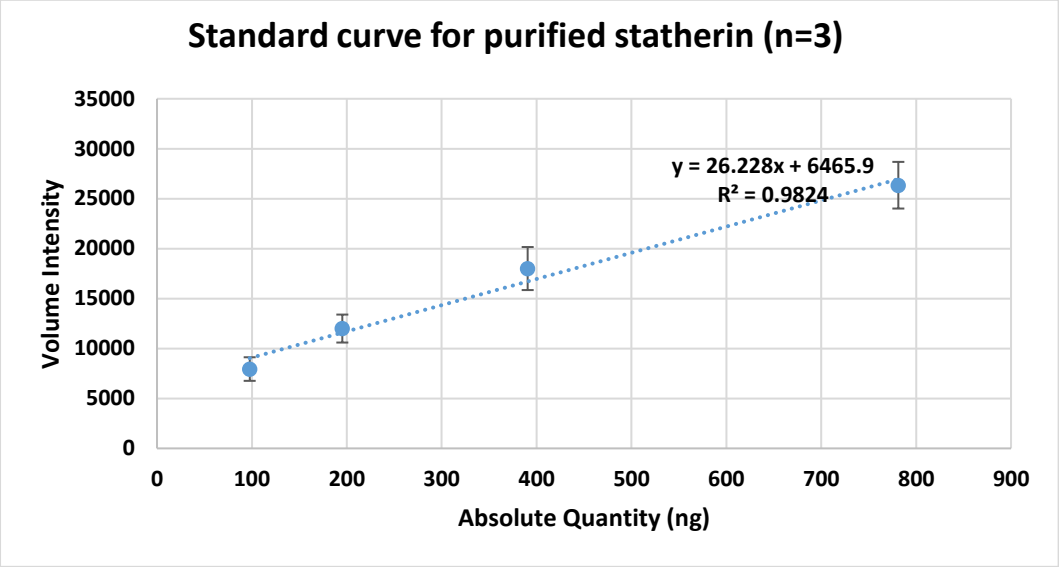


Figure 68 (d): *Standard curve of the purified statherin generated from volume intensities mean (SD) against the absolute quantity (ng) (n=3) and was used to quantify the absolute quantity of proteins in the in vitro AEP samples.*

Table 33 shows the mean (SD) and median (IQR) amount of the four specific proteins *in vitro* AEP from WMS and PS after 24 hours immersion in the corresponding solution [before erosion, control (C)] and after one erosion cycle (E1). Figure 69 shows the mean (SD) amount of mucin5b, CA VI and statherin after 24 hours immersion in the corresponding solution [before erosion, control (C)] and after one erosion cycle. As the amount of albumin (ng) before (control) and after one erosion cycle was small compared to the amount of the other three proteins, albumin was presented in a separate figure (Figure 70). In *in vitro* AEP from WMS at control and after one erosion cycle, the mean (SD) amount of mucin5b was [57.5 (33.3) ng and 238.9 (25.2) ng respectively], albumin was [1.4 (0.8) ng and 2.4 (0.5) ng respectively], CA VI was [6.3 (2.3) ng and 44.8 (9.6) ng respectively] and statherin was [19.4 (6.3) ng and 62.8

(14.4) ng respectively]. In *in vitro* AEP from PS at control and after one cycle erosion, the mean (SD) amount of albumin was [0.3 (0.2) ng and 0.2 (0.1) ng respectively], CA VI was [60.7 (22.5) ng and 92.3 (17.1) ng respectively] and statherin was [210.4 (25.8) ng and 415.8 (43.6) ng respectively]. Mucin5b was not detected in AEP from PS.

Before and after one erosion cycle, mucin5b and albumin were significantly more dominant in *in vitro* AEP from WMS than that from PS ($p < 0.0001$) whereas CA VI and statherin were significantly more dominant in AEP from PS ($p<0.0001$). The amount of mucin5b before erosion [57.5 (33.3) ng] significantly increased by more than four folds after one cycle erosion [238.9 (25.2) ng $P< 0.0001$].

Albumin in *in vitro* AEP from WMS before erosion [1.4 (0.74) ng] increased significantly by two folds after one erosion cycle [2.4 (0.54) ng $p<0.05$]. Albumin amount in AEP from PS experienced no significant differences ($p>0.05$).

The amount of CA VI in *in vitro* AEP from WMS before erosion [6.3 (2.3) ng] increased significantly by more than seven folds after one erosion cycle [44.7 (9.6) ng < 0.0001]. In *in vitro* AEP from PS, CA VI before erosion [60.7 (22.6) ng] also increased significantly by more than two folds after one erosion cycle [153.9 (23.2) ng $p<0.000$].

For statherin, its amount in *in vitro* AEP from WMS before erosion [19.4 (6.3 ng] increased by nearly three folds after one erosion cycle [62.8 (14.4) ng $P<0.0001$]. For statherin in *in vitro* AEP from PS, its amount before erosion [210.4 (25.9) ng] increased significantly ($P<0.0001$) by two folds after one erosion cycle [415.8 (43.6) ng].

Type of saliva and erosion condition	Mucin5b amount of protein(ng)		Albumin amount of protein(ng)		CA VI amount of protein(ng)		Statherin amount of protein(ng)	
	Mean (SD)	**Median (IQR)**	**Mean (SD)**	**Median (IQR)**	**Mean (SD)**	**Median (IQR)**	**Mean (SD)**	**Median (IQR)**
Whole mouth saliva Control (no acid exposure) **(WSC)**	57.5 (33.3)π	38.0 (46.0)	1.4 (0.8)∆∑	1.2 (0.1)	6.3 (2.3)#β	6.6 (3.7)	19.4 (6.3)ϕ	21.0 (9.0)
Whole mouth saliva One cycle erosion **(WSEl)**	238.9 (25.2)π	227.2 (14.7)	2.4 (0.5)∆α	2.6 (0.5)	44.8 (9.6)#€	45.7 (8.8)	62.8 (14.4)ϕ	66.3 (17.9)
Parotid saliva Control (no acid exposure) **(PSC)**			0.3 (0.2)∑	0.3 (0.3)	60.7 (22.5)¥β	61.2 (27.0)	210.4 (25.8)ϕ	205.2 (7.6)
Parotid saliva One cycle erosion **(PSE1)**			0.2 (0.1)α	0.2 (0.03)	154.0 (23.2)¥€	146.2 (28.3)	415.8 (43.6)ϕ	403.2 (13.02)

Table 33*: Mean (SD) and median (interquartile range) amount of proteins (nanogram) in vitro AEP formed on enamel specimens immersed in WMS (n=5) or PS (n=5) for 24 hour. The AEP was then eluted before(control) or after one cycle erosion using 0.5% SDS and quantified using ImageLab software. Same symbols in the table indicate significant differences (p<0.0001). (WMSC: whole mouth saliva at control; WMSE1: whole mouth saliva after one erosion cycle; PSC: parotid saliva at control; PSEV: parotid saliva after one erosion cycle).*

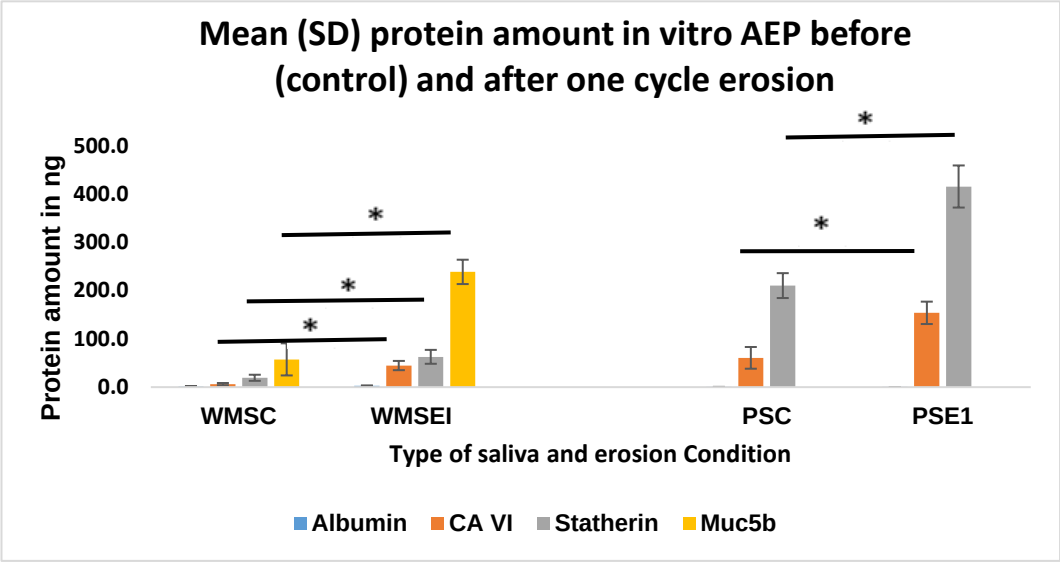


***Figure 69**: Mean (SD) amount of proteins (nanogram) in vitro AEP formed on enamel specimens immersed in WMS(n=5) or PS (n=5) for 24 hours. in vitro AEP were then eluted before(control) or after one cycle erosion using 0.5% SDS and quantified using ImageLab software. Asterisk shapes in the table indicate significant differences (p<0.0001). (WMSC: whole mouth saliva at control; WMSEI: whole mouth saliva after one erosion cycle; PSC: parotid saliva at control; PSEI: parotid saliva after one erosion cycle).*

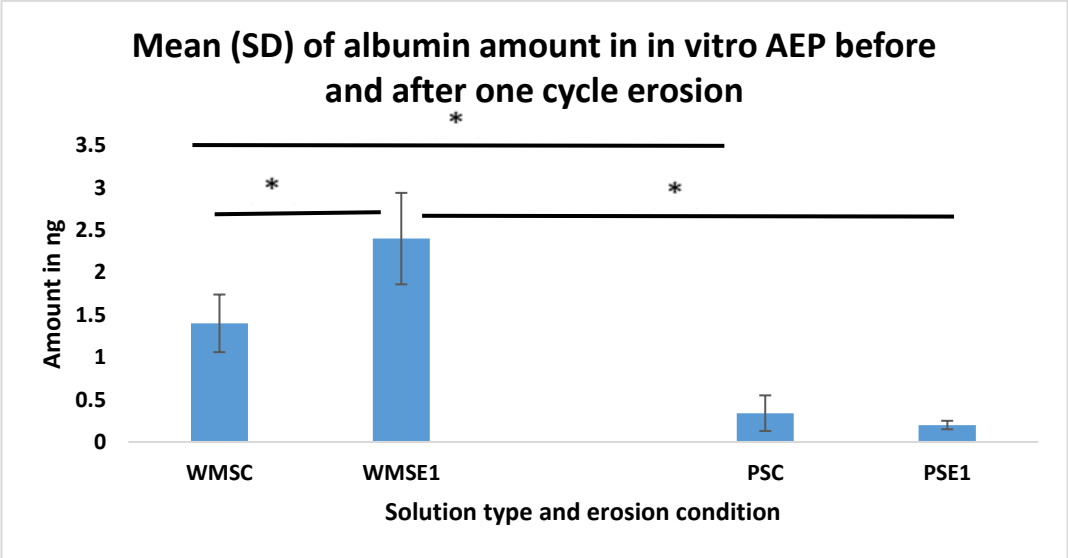


***Figure 70**: Mean (SD) amount of albumin (nanogram) in vitro AEP formed on enamel specimens immersed in WMS (n=5) or PS (n=5) for 24 hours. in vitro AEP samples were then eluted before (control) or after one erosion cycle using 0.5% SDS and quantified using ImageLab software. Asterisk shapes in the figure indicate significant differences (p<0.0001). (WSC: whole mouth saliva at control; WSEI: whole mouth saliva after one cycle erosion; PSC: parotid saliva at control; PSEI: parotid saliva after one cycle erosion).*

5.2.4.3 Calcium and phosphorus analysis

Table 34 and Figure 71 show the mean (SD) concentration of calcium and phosphorus (nM/mm^2) in the *in vitro* AEP samples from WMS and PS after 24 hours immersion in the corresponding solution [before erosion, control (C)] and after one erosion cycle (E1). In the *in vitro* AEP from WMS at control, the mean (SD) concentration of calcium and phosphorus was [0.06 (0.07) (nM/mm^2) and 0.14 (0.05) nM/mm^2 respectively] whereas after one erosion cycle was [0.19 (0.06) nM/mm^2 and 0.22 (0.09) nM/mm^2 respectively]. In the *in vitro* AEP from PS at control, the mean (SD) concentration of calcium and phosphorus was [0.03 (0.02) nM/mm^2 and 0.17 (0.10) nM/mm^2 respectively] whereas after one erosion cycle was [0.88 (0.13) nM/mm^2 and 1.20 (0.33) nM/mm^2 respectively]. When comparing the *in vitro* AEP from WMS and that from PS at control, there was neither significant difference in the concentration of calcium (P=0.17) nor in that of phosphorus (P=0.20). After one cycle of erosion, *in vitro* AEP from WMS the concentration of calcium and phosphorus was signifinatly lower than that from PS (p=0.0001).

When comparing within groups, the concentration of calcium in the *in vitro* AEP from WMS at control [0.06 (0.08) nM/mm^2] was significantly lower than its concentration after one cycle erosion [0.19 (0.06) nM/mm^2 p= 0.04]. For phosphorus, its concentration did not experience any significant differences in the *in vitro* AEP from WMS (P > 0.05). For AEP from PS, the concentration of calcium and phosphorus at control [0.03 (0.02) nM/mm^2 and 0.17 (0.10) nM/mm^2 respectively] was significantly lower than its concentration after one cycle erosion [0.88 (0.13) nM/mm^2 and 1.20 (0.33) nM/mm^2 respectively p<0.0001].

Group name and erosion condition	Calcium concentration (nM/mm^2)		Phosphorus concentration (nM/mm^2)	
	Mean (SD)	**Median (IQR)**	**Mean (SD)**	**Median (IQR)**
Whole mouth saliva Control (n=5) (no acid exposure) (WMSC)	0.06 (0.07)β	0.03 (0.08)	0.14 (0.05)	0.12 (0.07)
Whole mouth saliva One cycle erosion (n=5) (WMSEI)	0.19 (0.06)β	0.19 (0.06)	0.22 (0.09)	0.16 (0.16)
Parotid saliva Control (n=5) (no acid exposure) (PSC)	0.03(0.02)Δ	0.03 (0.02)	0.17 (0.10)*	0.13 (0.03)
Parotid saliva One cycle erosion (n=5) (PSE1)	0.88 (0.13)Δ	0.85 (0.11)	1.20 (0.33)*	1.20 (0.64)

Table 34 *: Mean (SD) and median (interquartile range) amount of calcium and phosphorus (nM/mm^2) in vitro AEP formed on enamel specimens immersed in either WMMS or PS for 24 hours. In vitro AEP was then eluted before or after one cycle erosion using 0.5% SDS and quantified using ICP-MS. Same symbols in the table indicate significant differences ($p<0.0001$). (WSC: whole mouth saliva at control; WMSEI: whole mouth saliva after one erosion cycle; PSC: parotid saliva at control; PSEI: parotid saliva after one erosion cycle).*

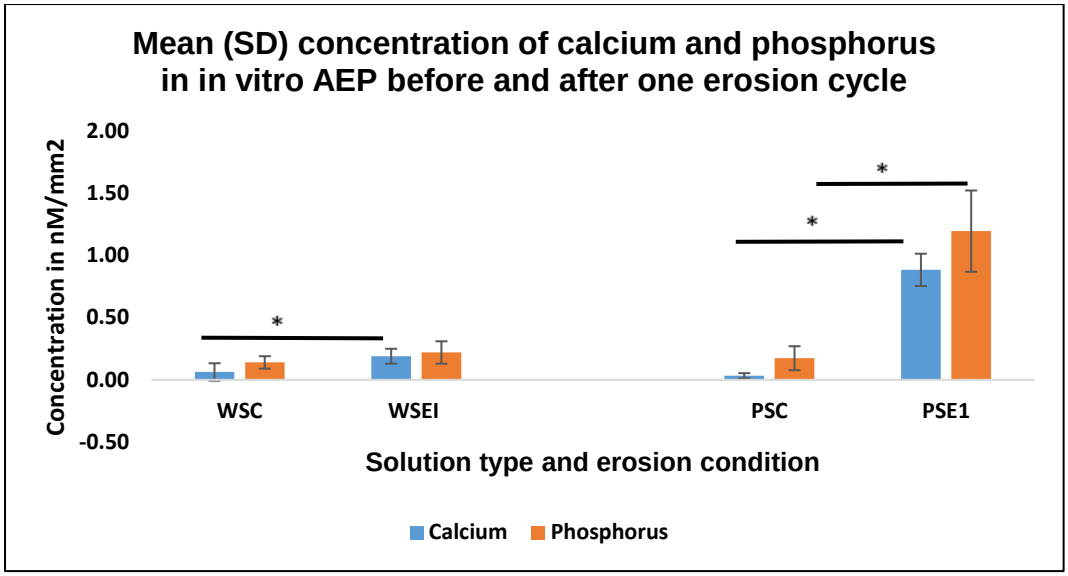
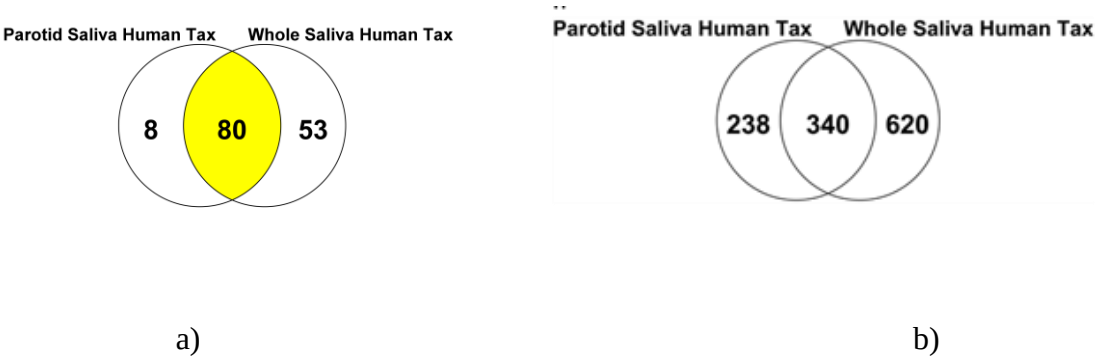


Figure 71 : *Mean (SD) amount of calcium and phosphorus (nM/mm^2) in in vitro AEP formed on enamel specimens immersed in either WMS or PS for 24 hours. In vitro AEP was then eluted before or after one erosion cycle using 0.5% SDS and quantified using ICP-MS. Asterisk shapes in the graph indicate significant differences (p<0.0001). (WSC: WSC= whole mouth saliva at control; WSEI: whole mouth saliva after one erosion cycle; PSC: parotid saliva at control; PSEI: parotid saliva after one erosion cycle).*

5.2.4.4 Proteomics results:

The protein identification from the individual database searches are visualised in Figure 72. LC/MS/MS sequencing successfully identified many proteins within each of the two stack gel samples. A much larger number of unique proteins were detected in the WMS sample (53) when compared to the PS sample. In total, 133 proteins were detected in the WMS sample. Eighty eight proteins were detected in the PS sample with only 8 proteins unique to this sample when compared to the WMS sample.

All protein identifications including those common to both samples and unique to the individual samples can be seen in the supplementary excel files (Appendix IX).



a) b)

Figure 72 *: Venn diagram representing the number of proteins in in vitro AEP from WMS versus in vitro AEP from PS a) number of proteins assigned for each individual database search; b) total unique peptides assigned for each individual database search using the uniprot database selecting Human Taxonomy (HT).*

Figure 73 classifies proteins in WMS sample into categories according to their function and gene ontology. Figure 74 shows the quantification of three individual proteins: mucin5b, albumin and CA VI. They were identified from the two gel bands of the AEP from WMS versus the AEP from PS following database searching against the human portion of the Uniprot database. Statherin was not identified in both gel bands which was due to the amount of statherin was below the limit of detection in this sample. The amount of statherin was very low compared to the rest of the sample which may have had a large dynamic range of concentrations across the total sample.

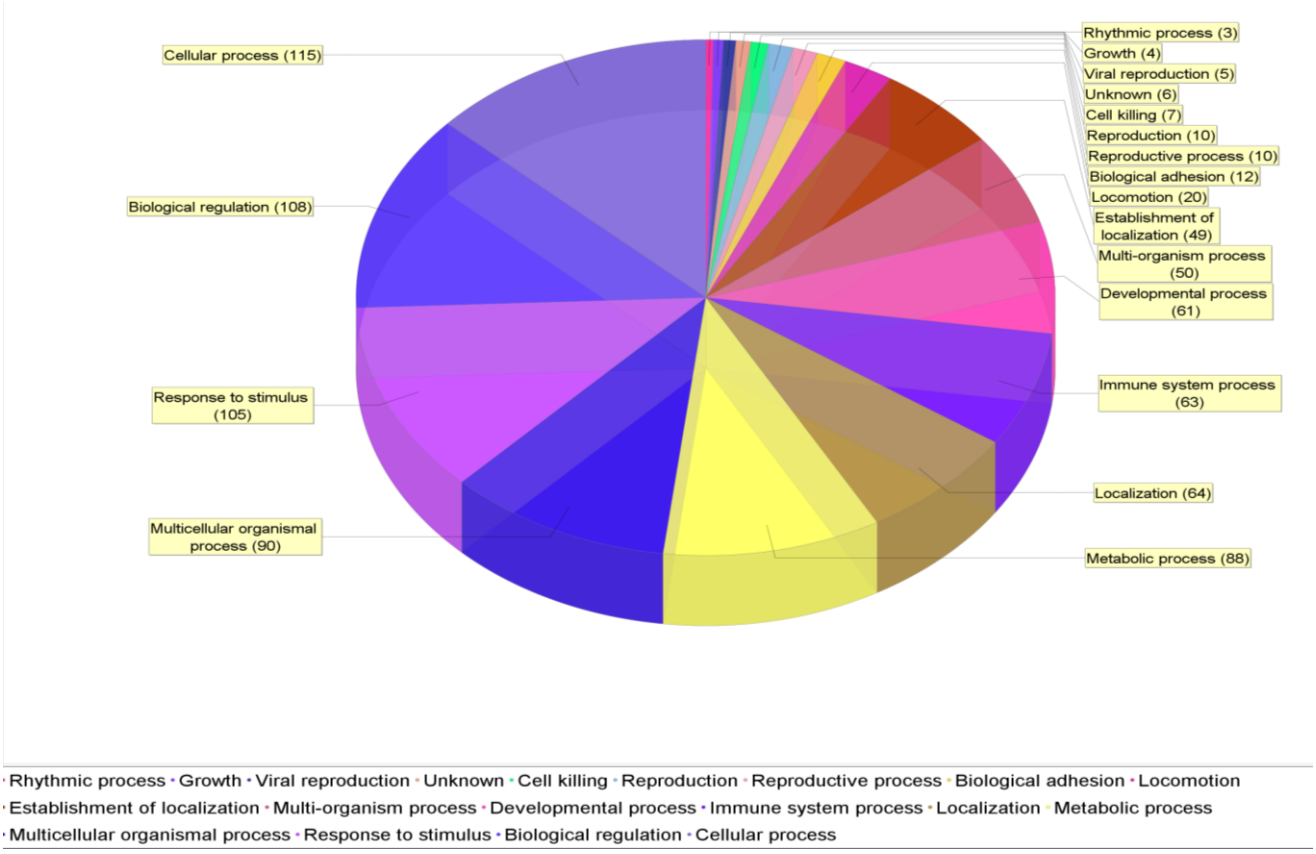


***Figure 73**: General distribution of identified proteins according to gene ontology and function involved*

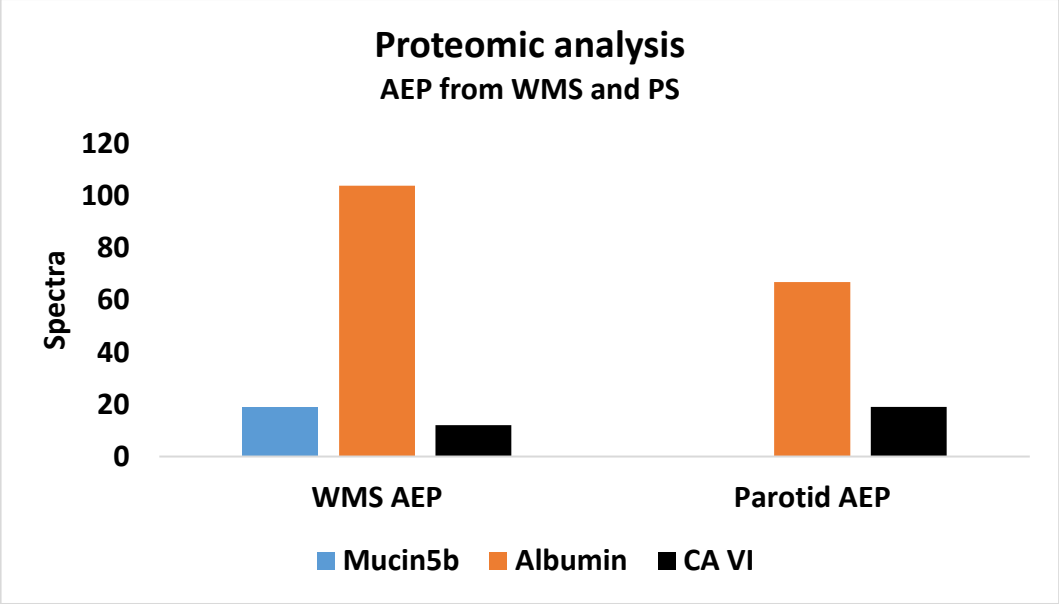


***Figure 74**: Fold change of mucin5b, albumin and CA VI in vitro formed AEP on enamel specimens after 24 h immersion in WMS or PS analysed using LC-MS/MS and searched using the Uniprot database selecting Human Taxonomy (HT).*

Figure 75 shows a wider overview of protein profile from the data file generated from the database which was uploaded into Scaffold 4 (v4.4.5) software (www.proteomesoftware.com). This shows a wider identification of other significant proteins found in AEP from WMS than that from PS. This represented protein identifications from the two gel bands following database searching against the human portion of the uniprot database (Appendix VIII). As can be seen from Figure 75, lactotransferrin, amylase, lysozyme, serotransferrin, IgM, cystatin SN,D,C and SA were dominant in AEP from WMS whereas PRPs were dominant in the AEP from PS.

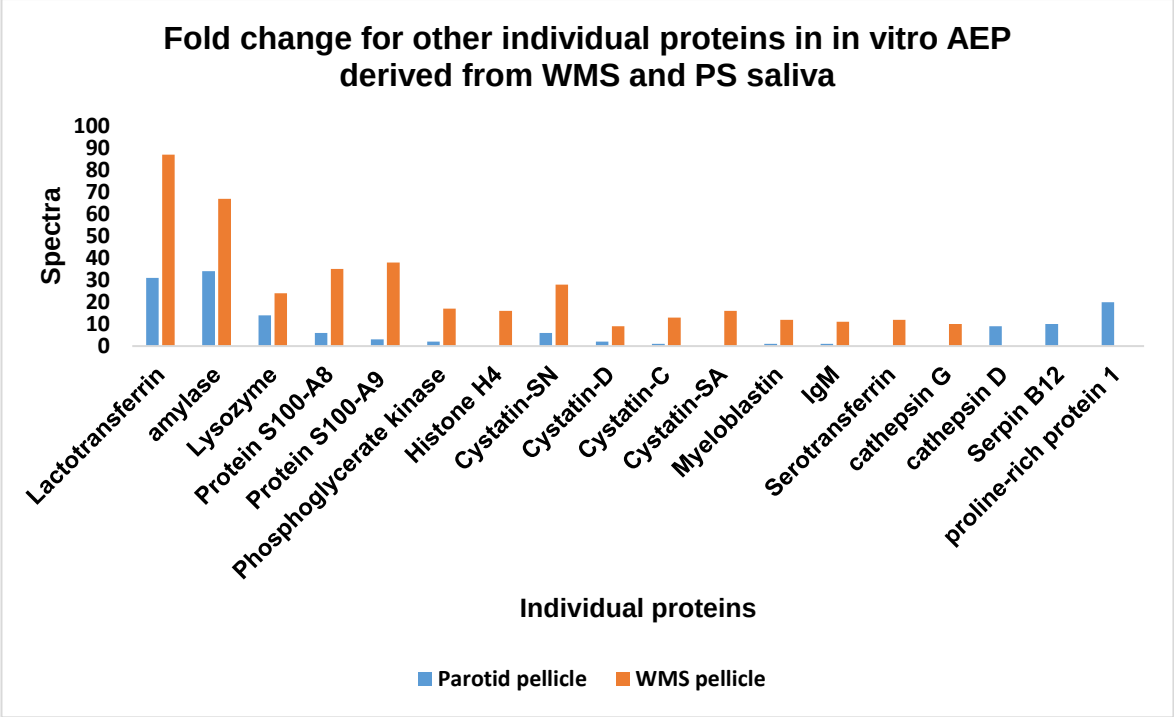


***Figure 75**: Wider identification of other salivary proteins found in in vitro formed AEP on enamel surfaces after 24 hours immersion in WMS versus PS saliva analysed using LC-MS/MS searched using the uniprot database selecting Human Taxonomy (HT).*

Section 5.3: Discussions of sections (5.1 and 5.2) (Early erosion)

In this laboratory study, WMS group showed significantly greater SMHC than PS and AS groups with no difference in SMHC between PS and AS groups.The results for SMHC were as follows: WMS [98.68 (8.5) KHN], PS [85.19 (6.07) KHN] and AS [63.97 (12.95) KHN]. WMS, PS and AS provided greater protection against erosion than DW as shown by a lower step height formation but greater microhardness change, leaving a softer layer in place. However,there were no significant differences between the step heights of WMS [1.13 (0.1)], PS [1.39 (0.3)] and AS [1.43 (0.3)]. This may be due to the fact that step height after one erosion cycle is less detectable by the white light SNCP. The step heights were measured using a white light profilometer with a spot size of 7 μm and resolution of 0.01 μm. This large spot size can not capture very early erosion and provides less detailed data that can not be compared to data obtained with a smaller spot size (Schlüter et al., 2011; Paepegaey et al. 2013). Looking at the SNCP and SMHC results, while the reduction in step height formation is a clear sign of enamel protection, it is difficult to interpret what the SMHC results mean in terms of protection against early erosion. Calcium (Ca^{2+}) concentration in the *in vitro* AEP from WMS increased significantly after one erosion cycle [0.19 (0.06) nM/mm^2] as compared to their concentration before erosion [0.06 (0.07) nM/mm^2]. Calcium (Ca^{2+}) and phosphorus (P) concentrations in the *in vitro* AEP from PS before erosion [0.03 (0.02) nM/mm^2 and 0.17 (0.10) nM/mm^2 respectively] significantly increased after one erosion cycle [0.88 (0.13) nM/mm^2 and 1.20 (0.33) respectively]. These ion concentrations in the AEP from PS after one

erosion cycle was more than five times greater than that from WMS. When combining the SMHC results with that of the calcium and phosphorus, it can be speculated that PS provided better protection in early erosive tooth wear in terms of a harder enamel surface compared to WMS.

The results of section 5.2 suggested that AEP from PS showed significantly greater amounts of statherin and CA VI compared to the AEP from WMS. The increase in the concentration of calcium and phosphorus in the AEP from PS also after one erosion cycle as compared to their concentration before erosion corresponded with the increased pattern of statherin in the AEP from PS after one cycle erosion. This is in agreement with Siqueira *et al.*, (2007b) who demonstrated, using mass spectrometry and proteomic approaches, that a significant part of the proteins present in the AEP are calcium- or phosphate-binding proteins (Siqueira *et al.*, 2007). It was also suggested that statherin plays a greater role in the selective and regulated biological process of AEP formation than other salivary proteins (Li *et al.*, 2004). The increase in the amount of statherin and ion contents in the AEP from PS after one cycle erosion may explain the harder enamel surface after one cycle erosion of enamel surfaces immersed in PS as compared to that immersed in WMS. The proteomic analysis also confirmed the results from SDS-PAGE and western blot that mucin5b and albumin were dominant in the AEP from WMS whereas the CA VI was more dominant in AEP from parotid saliva. Statherin was not detected using LC/MS/MS identification in neither of the AEP samples and mucin5b was also not detected in the AEP from PS.

Spectrophotometric analysis was used to estimate the total protein, whereas SDS-PAGE and western blot were used to quantify the specific protein in the

AEP samples. These methods were used as quantitative methods for protein separation. The use of another quantitative method with SDS-PAGE such as ELISA for protein analysis was not possible given the limited time frame of this PhD. SDS-PAGE and western blot are considered by many researchers as a practical alternative method for measuring proteins in biological samples (Taylor *et al*., 2013). They are also inexpensive, rapid, and more convenient when large numbers of samples to be analysed.

Proteomics analysis for all AEP samples (n=30) was not possible as it is expensive, time consuming, and an impracticable technique for analysing large numbers of samples, though more accurate. For the first time the proteomic analysis of 24 hour formed *in vitro* AEP was compared between WMS and PS providing larger scale analysis of the general protein profile in both types of AEP. The results of the proteomic analysis helped maximise our understanding of the possible cause of differences and changes in enamel protection. It was apparent that the different protection provided by the WMS and PS could at least partly, be attributed to the protein composition. The greater concentration of total protein on the WMS group may explain the softer enamel surface manifested by the greater microhardness change after exposure to WMS as compared to PS. This may be due to the formation of a more viscous and diffuse AEP from WMS as opposed to the more elastic and compact AEP from PS (Ash *et al*., 2014). This may also explain the presence or absence of some proteins in WMS which allowed the formation of a more viscous and diffuse AEP as opposed to other proteins in PS such as statherin, histatins and proline-rich proteins (Ash *et al*., 2014). Surface microhardness testing can however be influenced by many factors including the size and

thickness of formed AEP, operator load and time of indentation and type of indenter. Comparison between the effect of WMS and PS against early demineralisation has also previously been reported using different techniques from the ones used in this thesis. The significant difference in SMHC observed in this thesis, may be attributed to mucins which is the major difference between the protein profiles of AEP from WMS and PS. There are a number of rationales that may support the idea that parotid saliva may be more protective than WMS. First, a recent study demonstrated that proteins alone (WMS depleted of all ions), particularly calcium and phosphate-binding proteins, can provide better protection against 4 minutes erosive challenge than WMS or AS (Baumann *et al.*, 2016). The authors suggested that ions in saliva may compete for the binding sites on calcium and phosphate-binding proteins and that solutions having proteins without ions have more chance to bind into the enamel surface, improving its protection (Baumann *et al*., 2016). Furthermore, unique features of the oral environment such as enzymatic activities or mineral surface properties may also account for these differences between WMS and PS (Yao *et al.*, 2001). Some of the pure parotid derived proteins get degraded when they are in the WMS (Jensen *et al.*, 1992) which may alter their function. In addition, if WMS provided any protection against early erosion, this protection may be attributed to proteins derived from parotid saliva. Stimulated WMS, as that used in our studies, also contains approximately two thirds parotid-derived proteins and one third from submandibular and sublingual glands (Amerongen *et al.*, 1987). Finally, the results of section 5.2 of this thesis suggest that lactotransferrin, amylase, lysozyme and serotransferrin were dominant in the WMS AEP compared to

AEP from PS. This is in agreement with previous studies which demonstrated that proteolytic salivary enzymes such as the ones identified in this study (lysozyme, serotransferrin, lactotransferrin) were dominant in WMS (Helmerhorst *et al.*, 2006). These enzymes can degrade some salivary proteins in WMS such as histatins, statherin and PRPs undermining their function (Helmerhorst *et al.*, 2006; Siqueira *et al.*, 2010). *In vivo* studies are needed to measure salivary proteins in patients with erosion to compare to these *in vitro* results.

5.4 Conclusions:

- PS provided better protection against early erosion than WMS.
- CA VI and statherin were prevalent in AEP from PS after early erosion and resulted in a harder enamel surface whereas total protein, mucin5b and albumin were more prevalent in AEP from WMS.
- The concentration of calcium and phosphorus in *in vitro* AEP from PS after one erosion cycle was signifinatly greater than AEP from WMS.
- Considerable changes in the protein profile of 24-hour formed AEP derived from WMS compared to AEP from PS were also detected with more proteolytic enzymes in the AEP from WMS.
- Buffering and calcium homeostasis could be the main protective mechanisms in early erosion.

Overall discussion and summary of Chapter 4 and Chapter 5

Chapter 4 and 5 investigated two laboratory erosion models: advanced erosion (represented by five cycles of erosion) and early erosion (represented by one cycle of erosion) respectively. In this section, the results of profilometry (SNCP) and microhardness (SMHC) from Chapter 4 and 5 are discussed and summarised separately from the protein analysis. SNCP and microhardness were used to measure the surface loss and softening respectively after immersion of human enamel specimens in WMS, PS, AS and DW. This was to assess the role of ions and protein in preventing enamel erosion and to characterise the erosive process in an attempt to understand the exact component of saliva (proteins or ions or both) that are responsible to the protection against dental erosion. Chapter 4 and 5 also analysed the protein composition of the *in vitro* AEP that provide protection against advanced and early erosion. BCA assay, SDS-PAGE, ICP-MS and LC/MS/MS sequencing were used in order to identify and quantify the protein components of AEP that may have the potential to protect against enamel erosion.

SNCP and microhardness analysis

The findings of Chapter 4 and 5 on SNCP and microhardness testing indicate that the specimens immersed in the three types of saliva (WMS, PS and AS) had statistically lower step height in the five erosion cycle model [4.14 (0.9) µm, 6.42 (0.3) µm, 7.47 (1.0) µm respectively] and greater SMHC [224.11

(25.2) KHN, 208.16 (17.3) KHN, 194.0 (12.8) KHN] than DW group [10.89 (1.3) µm and 155.34 (18.4) KHN respectively].

The same pattern was observed in the one erosion cycle model where specimens treated in WMS,PS and AS had statistically lower step height [1.13 (0.1)µm, 1.39 (0.3) µm, 1.43 (0.3) µm and greater SMHC [98.68 (8.5) KHN, 85.19 (6.07) KHN, 63.97 (12.95) KHN respectively] than DW [2.29 (0.5) µm and 60.45 (11.3) KHN respectively]. While the reduction in step height formation is a clear sign of enamel protection, softness of enamel surface (i.e. greater microhardness change) may or may not be. This is expected since DW lacks minerals and proteins as opposed to saliva solutions. When comparing specimens immersed in WMS and PS to that immersed in AS, significant differences were observed in the step height and SMHC for the five cycles model whereas in the one cycle model, statistical differences were only observed in the SMHC between WMS and AS but not between PS and AS. The differences in protection exhibited between natural saliva (WMS and PS groups) as compared to AS group can be explained by the fact that WMS and PS contains both protein and ions as compared to ions only in the AS. It can also be explained by the incomplete role of ions alone as compared to ions plus proteins. As Martins *et al*. (2013) showed, ionic composition of saliva, independently of the type of saliva sample (WMS or PS), can further improve the reduction of enamel demineralisation as compared to protein without ions (dialyzed samples) (Martins *et al*., 2013).

When comparing specimens immersed in WMS to that immersed in PS, significant differences were observed only in the step height for the five cycles and only in SMHC for the one cycle erosion. This is may be explained by the suitability of the technique used in each model as well as the different protein composition between WMS and PS which allowed the formation of different quality of AEP (Ash *et al*., 2014).

Two different laboratory models were used in Chapter 4 and 5, five cycle and one cycle erosion respectively. These models were assessed by two different techniques, SNCP and SMH. To the author's knowledge, it is the first time that the difference in protection against dental erosion between WMS, PS, AS and DW has been assessed comparing two different laboratory models using SNCP and SMH. Five cycle erosion was used in this thesis, to represent advanced erosion in patients with an excessive intake of acidic drinks (Amaechi *et al.*, 1999). The repeated erosive challenges within the oral environment can be better represented by using multi-cycle laboratory models (Shellis *et al*. 2011, Young and Tenuta 2011).The five cycle model has also been used by our group as a well-established model to assess *in vitro* erosion (Austin *et al.,* 2011; Mistry, 2016; O'Toole *et al.*, 2015) so that useful comparison of results can be made. Furthermore, the five cycle erosion model was designed to generate measurable step height, to suit the specification of the white light used in the SNCP, and to accommodate the accuracy of our polishing protocol which was greater than 2 µm (Austin, 2011; Mistry, 2016). However, such extensive erosion may not always be the case in the *in vivo*

situation where minimal exposure to acid can occur. Therefore, the one cycle model was used to represent short exposure to acid which can lead to early erosion. Although it is still uncertain what early erosion means from a clinical perspective and laboratory modelling of *in vivo* early erosive tooth wear is difficult, the 10 minutes erosion (one cycle) model was selected in this thesis to represent early erosion. Previous studies have referred to *in vitro* early erosion, as an immersion period range between 5 seconds (Hannig *et al*., 2008) to 10 minutes (O'Toole *et al*., 2015) up to 2 hour (Mathews *et al*. 2012). In addition, 10 minutes has also been found to be the minimum time period for detecting statistical differences when developing the saliva protocol (section 2.1.6.1) and also useful comparison with the five cycle erosion model (5 X 10 minutes each) can be made.

This was referred to as early erosion within this thesis, as it is assumed that 10 minutes would cause initial softening of the surface and subsurface, following calcium and phosphate mineral loss rather than bulk tissue loss. However, the obstacle with this early erosion model was that the step height produced can be at the extreme of the white light laser resolution as indicated above. Therefore, the SMH technique was used to assess the surface and subsurface softening, caused by the early erosion model (one cycle).

From the above and as discussed in Chapter 4 and 5, it can be seen that SNCP provided meaningful results in the five cycle whereas microhardness testing was more useful in the one cycle model. The results from the SNCP can be easily interpreted as a clear protection against erosion whereas that

from microhardness requires further surface analytical techniques if more meaningful and reliable outcomes to be provided.

From the above, it can also be concluded that much more analysis of this enamel surface layer is required as there seems to be many saliva-related influential factors that greatly impact on enamel erosion. Therefore, further investigation of the of AEP from WMS and PS would provide more clear picture of the mechanism of AEP protection against enamel erosion as shown by the results from the proteins analysis.

Protein analysis

The findings of Chapter 4 and 5 demonstrated that total protein concentration in *in vitro* AEP from both WMS and PS was significantly lower after one and five erosion cycle compared to control (before erosion and after 24 hour immersion in either WMS or PS).

After one cycle of erosion, the concentration of calcium and phosphorus in *in vitro* AEP from WMS was signifinatly lower than that from PS whereas there was no significant difference in their amount after five erosion cycle. The concentration of calcium in the *in vitro* AEP from WMS at control was significantly lower than its concentration after one cycle of erosion. For phosphorus, its concentration did not experience any significant differences in the *in vitro* AEP from WMS. For AEP from PS, the concentration of calcium and phosphorus at control was significantly lower than its concentration after one cycle erosion. After five erosion cycles, no statistical difference was

observed in the concentration of calcium and phosphorus compared to that at control for both AEP from WMS and PS.

Before (at control) and after one and five erosion cycle, the amount of mucin5b and albumin were more significantly dominant in AEP from WMS whereas that of CA VI and statherin were dominant in AEP from PS ($p < 0.0001$). These results were also confirmed by the proteomics analysis which showed that the amount of mucin5b and albumin at control (before erosion and after 24 hour immersion in either WMS or PS) were dominant in AEP from WMS as opposed to CA VI which was dominant in the AEP from PS. Statherin was not detected in the proteomic analysis. After one erosion cycle (early erosion), the AEP from WMS experienced a significant increase in the amount of all four proteins compared to their amount at control whereas only CA VI and statherin significantly increased in the AEP from PS. After five erosion cycle (advanced erosion), in the AEP from WMS only mucin5b significantly increased whereas the other three proteins siginificantly decreased compared to their amount at control. In the AEP from PS after five cycle erosion, only CA VI significantly increased whereas statherin remained the same compared to control.

It can be concluded from the above that in early erosion mucin5b and albumin have the potential to protect against enamel erosion in the AEP from WMS whereas CA VI and statherin are the protective proteins in AEP from PS. This is because these proteins remain in the corresponding AEP at a statistical difference even after acidic challenges. For the same reason in adavnced erosion, the potential protein of protection was mucin5b in AEP from WMS

whereas that in the AEP from PS was CA VI. Therefore, it is clear that the proteins of the AEP behave differently depending on the severity of the acidic challenge and the results can be interpreted differently depending on the techniques used. There is no single method that is applicable for all stages of erosion but the selection of methods will depend on the laboratory model. Therefore, in order to obtain reliable interpretation of the result on erosion studies, it is important to study *in vitro* early erosion model separately from *in vitro* advanced erosion model with as many analytical techniques as possible should be combined.

Chapter 6: Comparison of AEP on eroded teeth to AEP on non-eroded teeth in the same subjects: An *in vivo* study.

6.1 Introduction:

In Chapters 4 and 5 of this thesis it was found that CA VI and statherin-rich AEP gave better protection than mucin5b and albumin-rich AEP in early erosive tooth wear, whereas the opposite was the case for advanced erosive tooth wear. It was speculated that calcium homeostasis and buffering could be the main protective mechanisms in early erosive tooth wear, whereas diffusion and lubrication could play a more major role in advanced erosive tooth wear. These findings were from *in vitro* studies and the role of these four proteins in *in vivo* AEP in preventing erosive tooth wear remains unknown. There are many differences between *in vitro* and *in vivo* AEP with the notable difference being the unique features of the oral environment compared to the *in vitro* models. Therefore, it would be of interest to assess the role of the total protein concentration and the four proteins: mucin5b, albumin, CA VI and statherin in an *in vivo* study. Previous *in vivo* studies have compared AEP from patients with erosive tooth wear to control subjects (Carpenter *et al*., 2014). However inter-subject variability of factors, such as salivary composition and the structure of enamel, could affect the results. Therefore, it would also be of interest to compare the protein composition of *in vivo* AEP between eroded and non-eroded surfaces within the same patients with erosive tooth wear. Thus this study aimed to measure the concentration of total protein and

amount of four specific salivary proteins *in vivo* salivary film and AEP between eroded and non-eroded teeth surfaces within patients exhibiting erosive tooth wear.

6.2 Aim, objectives and hypotheses

The aim of this study was to measure and compare total protein concentration and the amount of four specific salivary proteins in *in vivo* salivary film and AEP from eroded and non-eroded teeth surfaces in the same patients exhibiting erosive tooth wear.

The objectives were:

1. To compare the concentration of total protein in *in vivo* salivary film and AEP from eroded and non-eroded teeth in the same patients with erosive tooth wear.
2. To compare the amount of mucin5b, albumin, CA VI and statherin in *in vivo* film and AEP from eroded and non-eroded teeth surfaces in the same patients with erosive tooth wear.

The null hypotheses were that:

1. There is no difference in the concentration of total protein in *in vivo* salivary film and AEP between eroded and non-eroded teeth surfaces in the same patients exhibiting erosive tooth wear.
2. There is no difference in the amount of mucin5b, albumin, CA VI and statherin *in vivo* salivary film and AEP between eroded and non-eroded teeth surfaces in the same patients exhibiting erosive tooth wear.

6. 3 Materials and methods:

6.3.1 Human subjects:

As previous studies for comparing mean protein levels [Piangprach *et al.* (2009) and Carpenter *et al*. (2014)] showed a large effect size of 0.8, the power calculation in this *in vivo* study for comparing the mean protein levels was carried out based on paired t test with an effect size of 0.6 and 80% power which yielded a total sample of 24 to test the difference at 5% level using two tailed test. Therefore, thirty participants [17 females (58.6%)] and [12 males (41.4%)] ranging in age from 24 to 61 years [Mean (SD) = 37.7 (11.7) years] with erosive tooth wear were recruited for this study from the restorative clinics at King's College London Dental Institute, Guy's hospital London between December 2014 and February 2016. The baseline mean total BEWE score was 14.7 (SD =2.5). Only twenty nine of the collected samples were used as there were errors in collection for one subject. Ethical approval was obtained by the National Research Ethics Committee East Midlands (Nottingham 2, REC ref: 14/EM/1171). Patients who presented with moderate to severe erosive tooth wear were approached and were invited to take part in a screening examination to assess their eligibility to participate in the study. The full participant information sheet was explained to the patient thoroughly after which a consent and screening examination were then obtained. Details of the recruitment protocol, patient information sheet and consent form are shown in appendix X, XI and XII respectively.

The medical history was checked and a Basic Erosive tooth wear Examination (BEWE) was used to assess wear. The BEWE index was used to assess wear which used a 0-3 ordinal scale (0 = no wear, 1 = early surface loss, 2 = surface loss < 50% or specific defect, 3 = surface loss > 50%). The sextant BEWE score was allocated by recording the score on the most severely worn surface in each sextant. The total BEWE score was calculated by adding the sum of each sextant BEWE score which could range from 0-18. Each investigator performed the examination separately and was blinded to the scores of the other examiner. Teeth with restorations involving >50% of the tooth, traumatised or carious teeth were excluded. Examinations were carried out under normal dental surgery conditions with the patient in a reclined position and good lighting. The teeth were dried and cleaned with compressed air and the buccal, occlusal and palatal/lingual surfaces of each tooth excluding third molars were each examined without magnification. Diet was then assessed using a previously validated questionnaire (Bartlett *et al.*, 2013). Participants were questioned on the frequency and timing of dietary acid intake, the time spent consuming the acids and alternate drinking habits prior to swallowing. In addition, participants were questioned on the timing of their tooth brushing in relation to meals and dietary acid intake.

Detailed inclusion and exclusion criteria for participation in the study is shown in appendix X. Erosive wear patients included in this study had to have a minimum of 20 teeth (10 in each jaw) with a Basic Erosive tooth wear Examination (BEWE) cumulative score greater than or equal to 8 but with at

least one score of 3 on the occlusal surface of the lower molars or the buccal/palatal surface of the upper central incisor. In addition, this wear had to be as a result of a high acid diet which must include at least two daily incidences of acidic challenges. Patients were excluded if they showed any medical or dental problems such as severe dentine hypersensitivity, periodontitis or restoration of the occlusal or incisal surfaces of upper anterior teeth and first molars, as were those who had missing anterior teeth, anterior crowns/bridges or cavitated caries on more than one tooth. A history of eating disorders, gastro-oesophageal reflux, xerostomia, bruxism, prescribed xerostomic/heartburn medication, pregnancy, involvement in other research within the past 30 days or inability to speak or understand the English language also excluded the participant from this study. Those with medical histories likely to impact on compliance such as requiring antibiotic pre-medication prior to dental treatment or those preferring immediate restoration of their teeth were also excluded.

Participants found to be suitable based upon inclusion/exclusion criteria were invited to take part in the research and given a minimum of 24 hours to make an informed decision. Following agreement (n=29), a separate appointment was given and patients were given a unique identifier number from one to twenty nine based upon sequence of recruitment. Oral and written consents were obtained.

6.3.2 Sample collection

A single trained and calibrated investigator performed all wear and dietary assessments in addition to salivary film and AEP collection. Anything eaten or drank by the participants at least one hour prior to the study was documented. Detailed protocol of collection is shown in appendix XIII. Selected teeth were isolated with cotton wool rolls and filterpaper was applied to occlusal surfaces to collect salivary films and AEP samples from four surfaces. The eroded occlusal surface (E) of the lower first molars (n=58, 100%) and one non-eroded (N) adjacent posterior occlusal surface [premolars (n=21, 36%) and molars (n=37, 64%)] were selected from both the lower left (1) and right (2) sextants. This resulted in a total of eight samples per patient: salivary film samples from eroded surfaces (n=2) and non-eroded surfaces (n=2), AEP samples from eroded surfaces (n=2) and non-eroded surfaces (n=2). A total of two hundred and thirty two salivary films and AEP samples (n=116 each) were collected from twenty nine erosion patients. Samples were then allocated to four different groups: eroded film (EF, n=58), non-eroded film (NF, n=58), eroded AEP (EP, n=58), non-eroded AEP (NP, n=58). Detailed of sample collection patterns is shown in Figure 76.

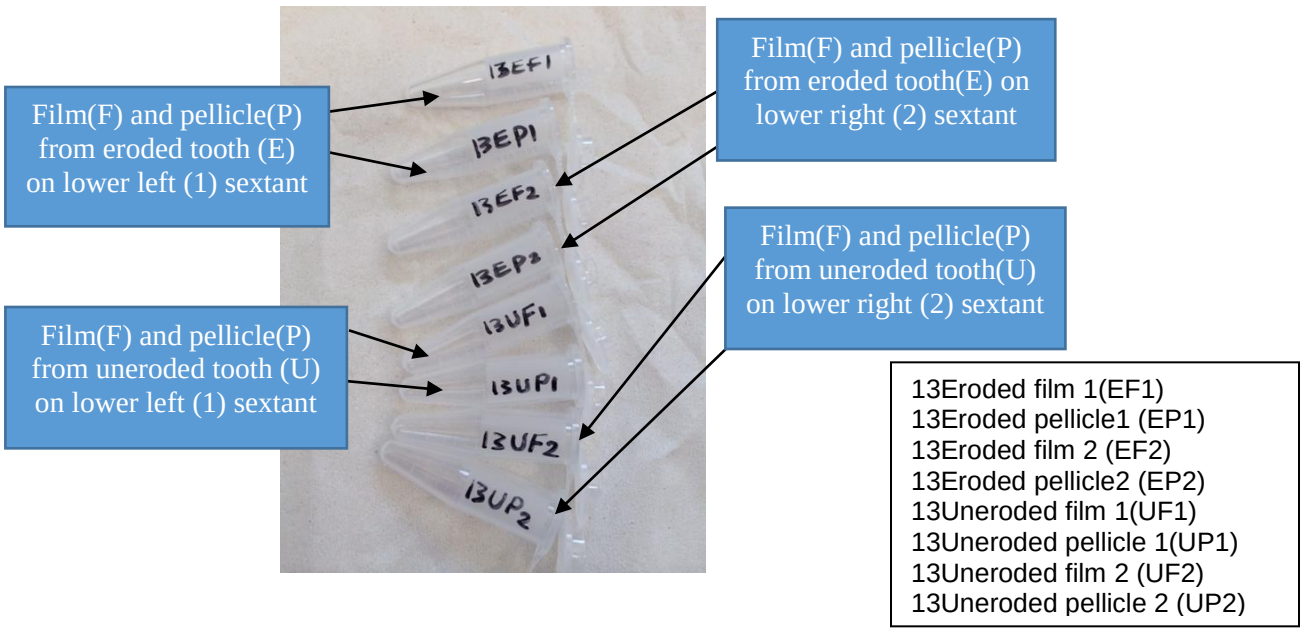


Figure 76: *An example of the labelling system for salivary film and pellicle during in vivo collection process from patient No. 13*

6.3.3 *In vivo* film and AEP harvest and recovery:

Firstly, the selected tooth was isolated using cotton wool rolls. Secondly, salivary films were collected by placing a dry, sterilised filterpaper against the surface for 5 seconds. This aimed to ensure that identified teeth were clear of salivary film before the subsequent AEP collection and also to analyse the protein contents of salivary films alongside AEP. The *in vivo* AEP was eluted from localised, dried enamel surface using sterilised filterpapers (VWR International Ltd, Leicestershire, England) of standardised size (21 mm length x 3 mm width). Approximately 5 mm length of the filterpaper was soaked in 3 µL SDS (0.5 % w/v) sodium dodecyl sulphate (SDS) sample buffer (Novex, Thermo Fisher Scientific Inc, UK) which was freshly made each morning. The soaked part of the filterpaper was then mechanically rubbed uniformly against a localised section of the surface (3 x 3 mm) for a timed period of 15 seconds

which was standardised for all AEP collections as per previously published protocols (Siqueira *et al.*, 2007; Svendsen *et al*., 2008; Carpenter *et al*., 2014). Each of the samples were collected in a universal tube before being placed immediately in ice and subsequently frozen at -20 º C until analysis. Analysis was performed by an investigator blinded to the erosion status of the surface the sample was collected from. Prior to laboratory analysis, the eight samples which were collected previously from each patient as detailed above were reduced to four samples. The two eroded films (n=2) from each patient were pooled to be analysed together producing a total of 29 eroded films (EF) instead of 58. Similarly, the two eroded AEP samples (n=2) from each patient were pooled to be analysed together producing a total of 29 eroded AEP (EP). The two non-eroded films (n=2) from each patient were also pooled to be analysed together producing a total of 29 non-eroded films (NF). The two non-eroded AEP (n=2) from each patient were pooled to be analysed together producing a total of 29 non-eroded AEP (NP). A total number of one hundred and sixteen samples from twenty nine patients were then subjected for analysis.

During laboratory analysis, two tubes (0.2 mL small tube and 1.5 mL Eppendorf tube) were used for the recovery of *in vivo* film and AEP from the filterpapers. Filterpapers carrying the *in vivo* film and AEP were then suspended in a small 0.2 mL-tube each, which in turn was placed in another 1.5 mL-tube as described in section 2.3.3 (Figure 12). The protein contents of

the *in vivo* film and AEP were then recovered from the filterpapers using the same procedure as described in section 2.3.3.

6.3.4 Testing

The concentration of total protein in the recovered salivary film and AEP samples was measured using the BCA (Pierce Chemical, Rockford, Ill., USA). All samples were also analysed for the amount of four specific salivary proteins by SDS-PAGE and immunoblotting against four antibodies: mucin5b, albumin, CA VI and statherin antibodies.

6.3.4.1 Total protein in films and AEP

Part (1 µL) of each *in vivo* recovered film (n=58) and AEP (n=58) samples were prepared for the analysis of total protein concentration. Each sample was diluted in DW at 1/100 to a final volume of 100 µL. Prepared *in vivo* films and AEP samples were placed into microtiter plates (96-wells, Fisher Scientific, Leicestershire). The total protein in the film and AEP samples were measured using the bicinchoninic acid assay (BCA) with bovine serum albumin (BSA) protein as a standard protein (2 mg/mL) (Pierce Chemical, Rockford, Ill., USA). A spectrophotometer (BioRad laboratories Ltd, Hemel Hempstead, UK) at wavelength of 562 nm was used to measure the absorbance of all samples as described in 2.4.4.

6.3.4.2 Specific protein analysis

Protein separation and transfer:

Prepared samples of films and AEP were run through precast gels and were separated consistently as explained in section 2.4.5.3. SDS–PAGE was used for the separation of protein fractions in their denatured state from the recovered film and AEP samples. Equal amount (15 µL) of each prepared film and AEP sample was loaded onto each lane on a 4–12 % Bis-Tris SDS-PAGE gel (Novex, Thermo Fisher Scientific Inc, UK). Electrophoresis was carried out in MES-SDS running buffer according to manufacturer's instructions. In each gel, the 15 lanes were occupied with protein samples and purified standards. 8 lanes were occupied by the salivary film and AEP samples and the other 4 lanes were occupied by a mixture of the four purified proteins for standards of known concentration (Figure 76). The purified standards used in the mixture were mucin5b (156 µg/mL), albumin (1 µg/mL), CA VI (140 µg /mL), statherin (382 µg/mL). Statherin was prepared by the author according to previous studies (Proctor *et al.*, 2005; Harvey *et al.*, 2011) as described in section 2.5.2.5.

The volume of purified proteins used in the mixture were mucin5b (10 µL), albumin (10 µL), CA VI (5 µL) and statherin (5 µL) to make a 30 µL mixture of purified standards. The volumes of purified standards loaded in the gels were 15 µL/lane1, 7.5 µL/lane2, 3.8 µL/lane3 and 1.5 µL/lane4 as shown in (Figure 77).

The loaded protein samples in the precast gels were then transferred onto a nitrocellulose membrane using western blot technique as described in 2.4.6.1.

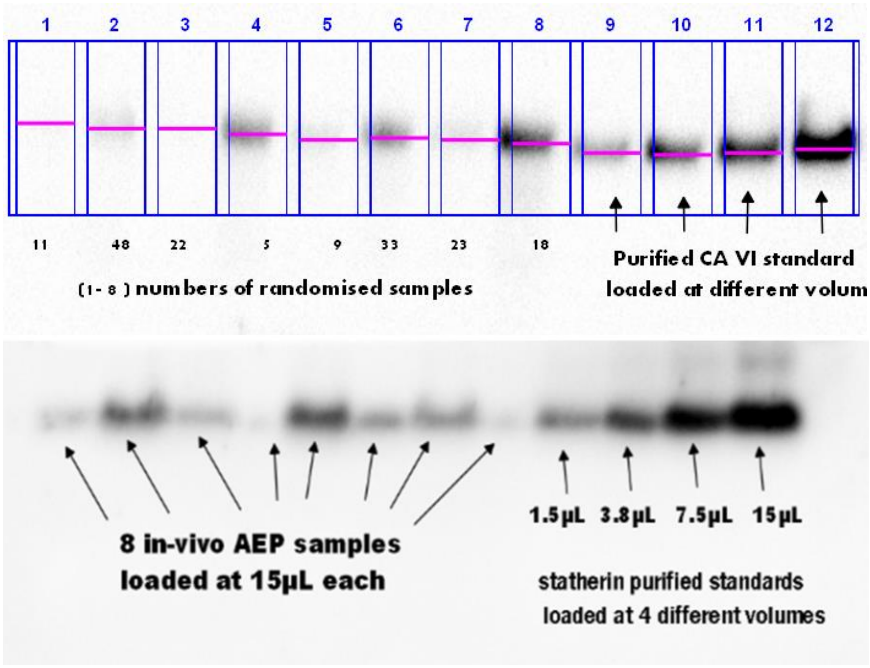


Figure 77: *Two examples of SDS-PAGE and western blotting of film and AEP samples and purified standards. Samples were immunoblotted against CA VI and statherin.*

Immunoblotting and immunodetection

Western blotting was completed according to the manufacturer's instructions and used to transfer proteins onto a nitrocellulose membrane as described in 2.4.6.1. Using a sterile razor, each nitrocellulose membrane was cut transversely into four sections corresponding to the four specific proteins of interest. At room temperature, the nitrocellulose membranes were then blocked in TTBS for 1 hour before membranes were probed with primary antibodies as described in section 2.4.6.3. The nitrocellulose membranes were then washed in TTBS for 15 minutes (5 minutes X 3 times) and then followed by incubation with the required secondary antibody. Details of the

primary and secondary antibodies used was given in section 2.5.2. A final 15-minutes wash in TTBS was completed before the membranes were developed with ECL substrate and were imaged as described in the next section.

Imaging analysis:

The amount of the four proteins of interest on the blotted and developed membranes were quantified as explained in section 2.4.6.4. ChemiDoc MP imaging analysis (Bio-Rad) was used to quantify the light intensity of the chemiluminescent reaction and exposure times optimised to prevent pixel saturation. The amounts of proteins on the blotted nitrocellulose membranes were quantified using tools of ImageLab software version 4.1 (Bio-Rad Laboratories Ltd., Hertfordshire, UK) to select and determine the background-subtracted density of the bands in all the gels (n=15) using purified protein of standards of known concentration. The standard curves of purified proteins were generated from the mean volume intensities (n=15) against the absolute quantities of the corresponding purified standard. This was used to generate a calibration curve using a linear formula. This formula was used to calculate the amount of each protein in the AEP samples. The bands of standard proteins on different SDS-PAGE gels (n=15) were used to assess reproducibility.

6.3.5 Statistical analysis

Statistical analysis was performed using SPSS version 23.0. Data were assessed for normality using histograms, boxplots and Shapiro-Wilks tests. Data for total protein, albumin and CA VI were observed to be normally

distributed and therefore descriptive statistics were presented as mean and standard deviation (SD) and data were analysed using paired t-test. As data for mucin5b and statherin did not follow normal distribution, therefore data were presented as median (min, max) and were analysed using Wilcoxon's matched-pair signed-rank test. The level of significance was set at a value of $p<0.05$.

6.4 Results:

Film (eroded: n=29; non-eroded: n=29) and AEP (eroded: n=29; non-eroded: n=29) samples were collected and analysed from twenty nine patients exhibiting erosive tooth wear.

6.4.1 Total protein

Table 35 and Figure 78 show the mean (SD) total protein concentration in the *in vivo* film (F) and AEP (P) samples from eroded (E) and non-eroded (N) surfaces of the same patient. The mean (SD) concentration of total proteins of EF was [1.27 (0.3) mg/mL], NF was [0.44 (0.04) mg/mL], EP was [0.41 (0.03) mg/mL] and NP was [0.61 (0.12) mg/mL].

For film samples, the concentration of total protein on non-eroded (NF) tooth surfaces [0.44 (0.04) mg/mL] was significantly lower than that on eroded (EF) surfaces [1.27 (0.3) mg/mL] ($p< 0.0001$).

For AEP, the total protein concentration on eroded surfaces [0.41 (0.03) mg/mL] was significantly lower protein concentration than non-eroded surfaces [0.61 (0.13) mg/mL] ($p<0.0001$).

Tooth surface	Total protein concentration (mg/mL) Mean (SD)
EP (n=29)	0.41 (0.03)
NP (n=29)	0.61 (0.12)
EF (n=29)	1.27 (0.3)
NF (n=29)	0.44 (0.04)

Table 35: *In vivo total protein concentration (mg/mL) in salivary films (F) and AEP (P) on eroded (E) and non-eroded (N) tooth surfaces in the same erosion patient using bicinchoninic acid (BCA) assay.*

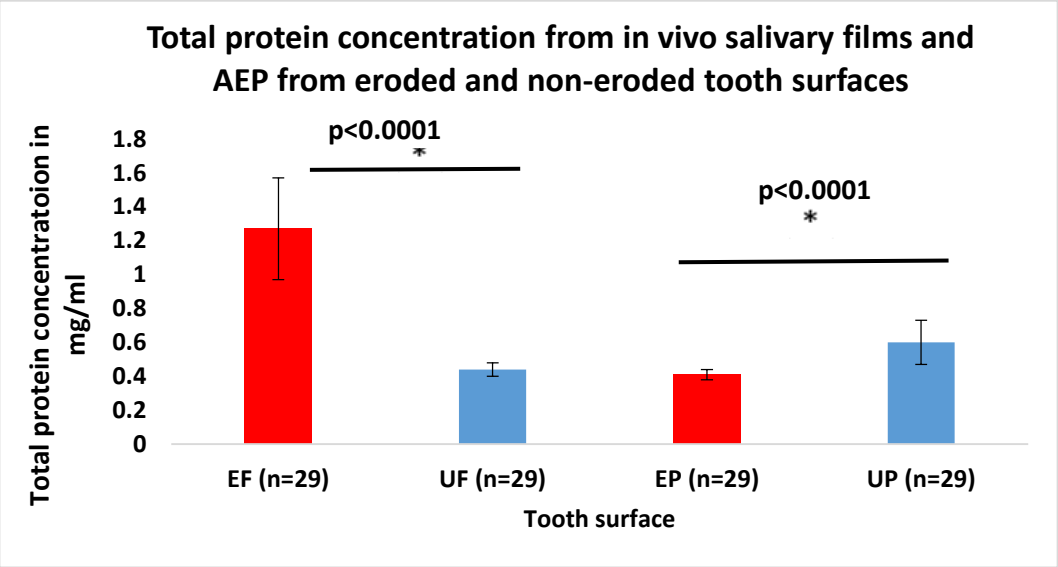


Figure 78*: Mean (SD) of in vivo total protein amount (mg/ml) in salivary films (F) AEP (P) from eroded (E) and non-eroded (U) tooth surfaces using bicinchoninic acid assay (BCA). Asterisks indicate significant differences.*

6.4.2 Specific protein amount

Figures 79 (a,b,c,d) show the standard curves of purified proteins for standards generated from the mean (SD) volume intensities against the absolute quantities of the corresponding purified standards in all gels (n=15). In this figure, it was demonstrated that the purified proteins used in this study were optimised in a way that the data points between a high volume of (15 µL) of purified proteins and low volume of (1 µL) provided a suitable curve range to calculate very little proteins in the *in vivo* AEP samples whilst producing a gradual change of intensities.

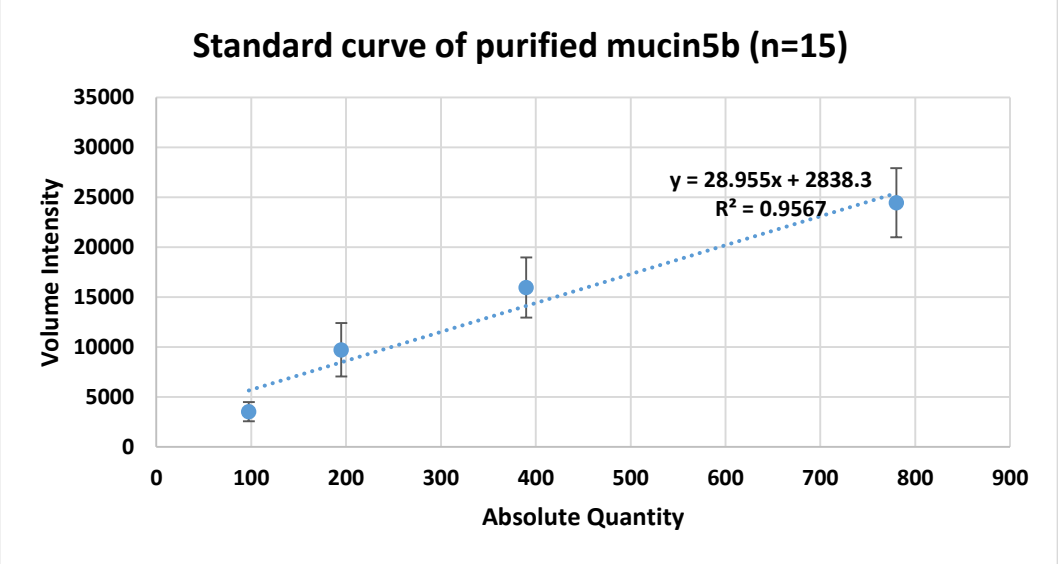


Figure 79 (a) : *Standard curve of the purified mucin5b generated from the mean (SD) volume intensities against the absolute quantity in nanogram (n=15).*

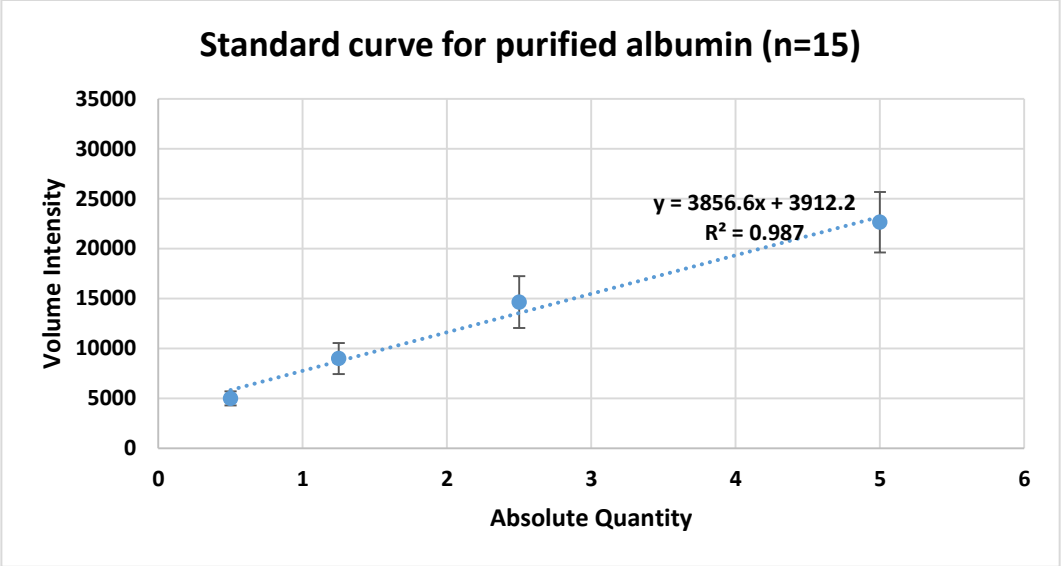


Figure 79 (b) : *Standard curve of the purified albumin generated from the mean (SD) volume intensities against the absolute quantity in nanogram (n=15).*

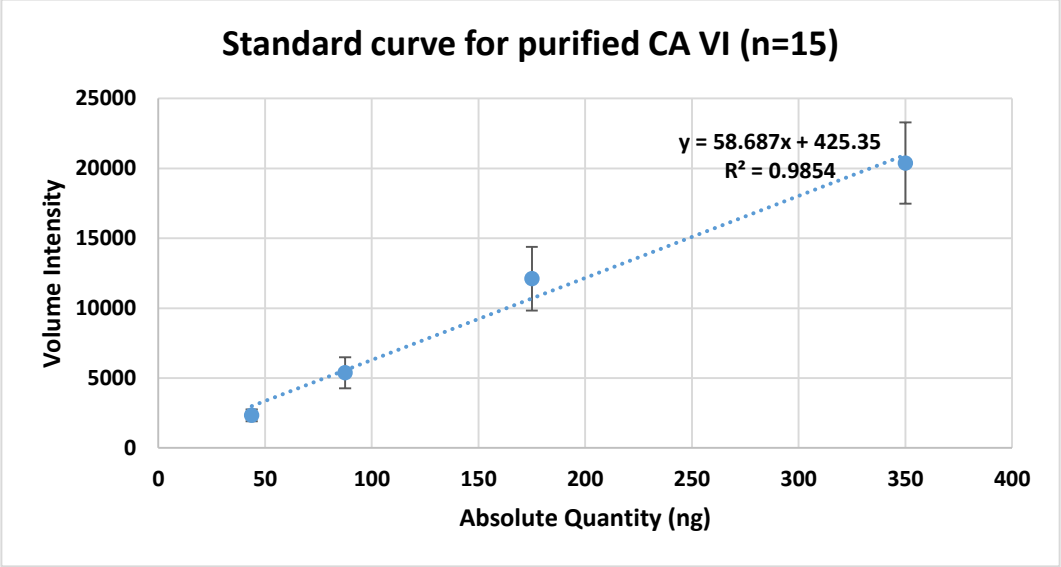


Figure 79 (c): *Standard curve of the purified CA VI generated from the mean (SD) volume intensities against the absolute quantity in nanogram (n=15).*

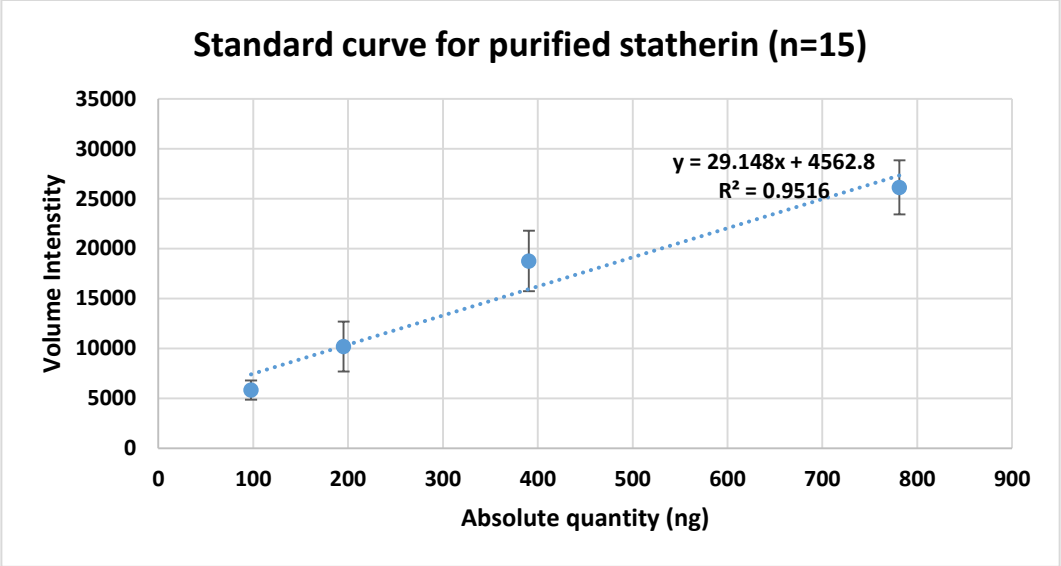


Figure 79 (d) : *Standard curve of the purified statherin generated from the mean (SD) volume intensities against the absolute quantity in nanogram (n=15).*

Recovered film and AEP samples were analysed for the amount of four specific salivary proteins. Table 36 shows the mean (SD) and median (min, max) amount of the four investigated salivary proteins in *in vivo* film on eroded (EF) and non-eroded (NF) tooth surfaces. Table 37 shows the mean (SD) and median (min, max) amount of the four investigated salivary proteins in *in vivo* AEP on eroded (EP) and non-eroded (NP) tooth surfaces.

In films, the median (min, max) amount of mucin5b on eroded and non-eroded tooth surface was [97.0 (81.1, 148.4) ng and 99.4 (56.2,888.6) ng respectively]. The median (min, max) amount of statherin on eroded and non-eroded tooth surface was [99.0 (62.1,477.1) ng and 98.0 (22.0, 720.8) ng respectively]. The mean (SD) amount of albumin on eroded and non-eroded tooth surface was [4.2 (1.9) ng and 3.9 (1.6) ng respectively]. The mean (SD)

amount of CA VI on eroded and non-eroded tooth surface was [113.3 (100.9) ng and 105.3 (81.0) ng]. There was no significant differences in the amount of mucin5b, albumin and CA VI or statherin between eroded and non-eroded occlusal surfaces.

In AEP, the median amount of mucin5b (min, max) collected from eroded surfaces was 96.0 (80.0, 328.2) ng and from non-eroded surfaces [96.0 (40.7, 574.5) ng]. This difference was not statistically significant (p=0.878). The median (min, max) amount of statherin collected on the eroded occlusal surfaces was 84.1 (20.0, 221.8) ng and on non-eroded surfaces in the same subjects was 97.1 (30.0, 755.6) ng. This difference was statistically significant (p=0.002). The mean amount of albumin (SD) collected from eroded teeth surfaces was 3.8 (1.9) ng and from non-eroded surfaces was 3.7 (1.7) ng. This difference was not statistically significant (p=0.702). The mean amount of CA VI (SD) collected from eroded teeth surfaces was 60.8 (49.6) ng and from non-eroded surfaces was 101.3 (72.3) ng. This again, was not statistically significant (p=0.059).

Protein investigated	Eroded surfaces film (EF)		Non-eroded surfaces film (NF)		Sig
	Mean (SD)	Median (min, max)	Mean (SD)	Median (min, max)	
Mucin5b (ng)	100.3 (16.3) (n=26)	97.0 (81.1, 148.4) (n=23)	137.6 (164.9) n=26	99.4 (56.2,888.6) n=23	P = 0.135
Albumin (ng)	4.2 (1.9) n=29	4.3 (1.3, 8.4) n=29	3.9 (1.6) n=29	3.5 (1.6,7.9) n=29	P = 0.284
Carbonic anhydrase VI (ng)	113.3 (100.9) (n=27	78.6 (43.0, 511.2) (n=23)	105.3 (81.0) n=26	81.7 (8.8, 305.7) n=27	P = 0.386
Statherin (ng)	134.5 (85.8) (n=27)	99.0 (62.1,477.1) (n=27)	173.8 (195.7) (n=27)	98.0 (22.0, 720.8) (n=27)	P = 0.946

Table 36: *Amount of four proteins (ng) in in vivo film (F) samples eluted from eroded (E) and non-eroded (N) tooth surfaces in the same patient using 0.5% SDS and quantified using ImageLab software using purified protein of standards.*

Protein investigated	Eroded surfaces AEP (EP)		Non-eroded surfaces AEP (NP)		Sig
	Mean (SD)	Median (min, max)	Mean (SD)	Median (min, max)	
Mucin5b (ng)	112.3 (65.2) (n=13)	96.0 (80.0, 328.2) (n=13)	119.9 (114.6) n=18	96.0 (40.7, 574.5) n=18	P = 0.878
Albumin (ng)	3.8 (1.9) n=29	3.8 (0.9, 9.3) n=29	3.7 (1.7) n=29	3.7 (1.1, 6.7) n=29	P = 0.702
Carbonic anhydrase VI (ng)	60.8 (49.6) (n=27	48.1 (0.2, 207.2) (n=27)	101.3 (72.3) n=26	69.5 (25.0, 262.5) n=27	P = 0.059
Statherin (ng)	90.1 (42.6) (n=23)	84.1 (20.0, 221.8) (n=23)	167.2 (186.3) (n=27)	97.1(30.0, 755.6) (n=27)	P = 0.002

Table 37: *Amount of four proteins (ng) in in vivo AEP (P) samples eluted from eroded (E) and non-eroded (N) tooth surfaces using 0.5% SDS and quantified using ImageLab software using purified protein standards.*

Figures 80 (a,b,c,d) shows the mean (SD) amount of each individual proteins: mucin5b, albumin, CA VI and statherin respectively in *in vivo* film and AEP samples.

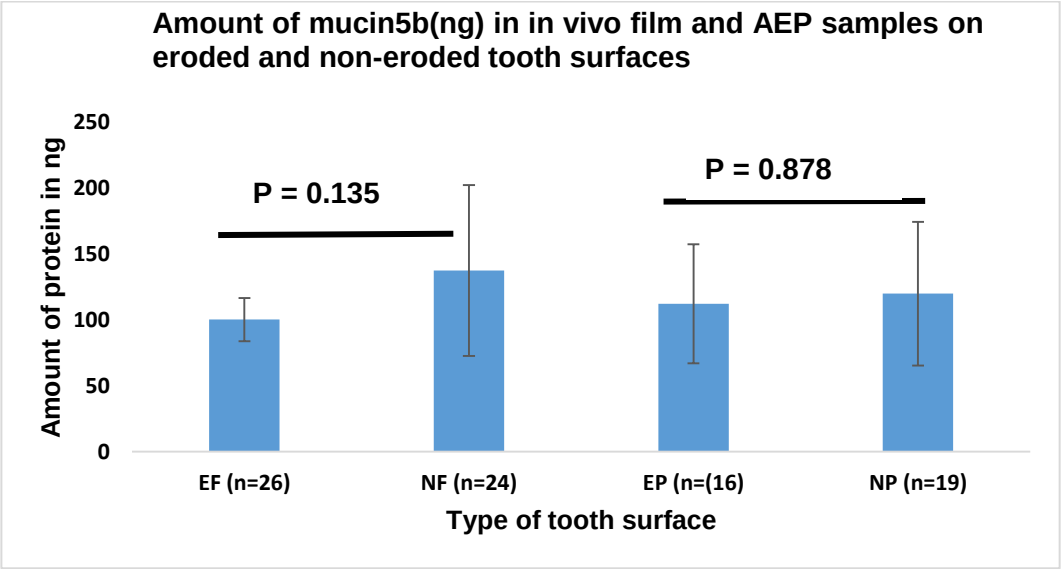


Figure 80 (a): *Mean (SD) amount (ng) of mucin5b in in vivo salivary film and AEP samples on eroded and non-eroded tooth surfaces in erosion patients .*

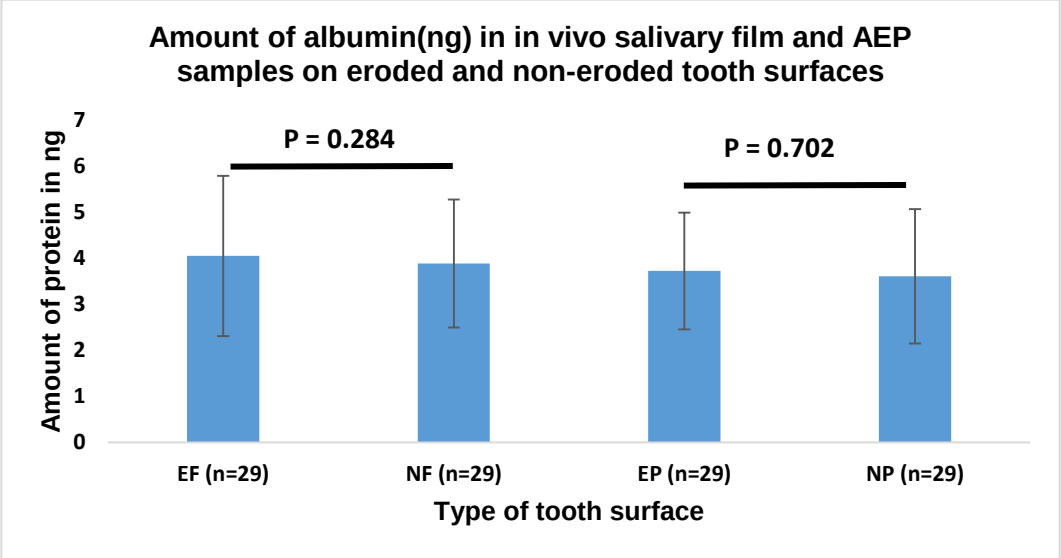


Figure 80 (b)*: Mean (SD) albumin amount (ng) in vivo salivary film and AEP samples on eroded and non-eroded teeth of erosion patients.*

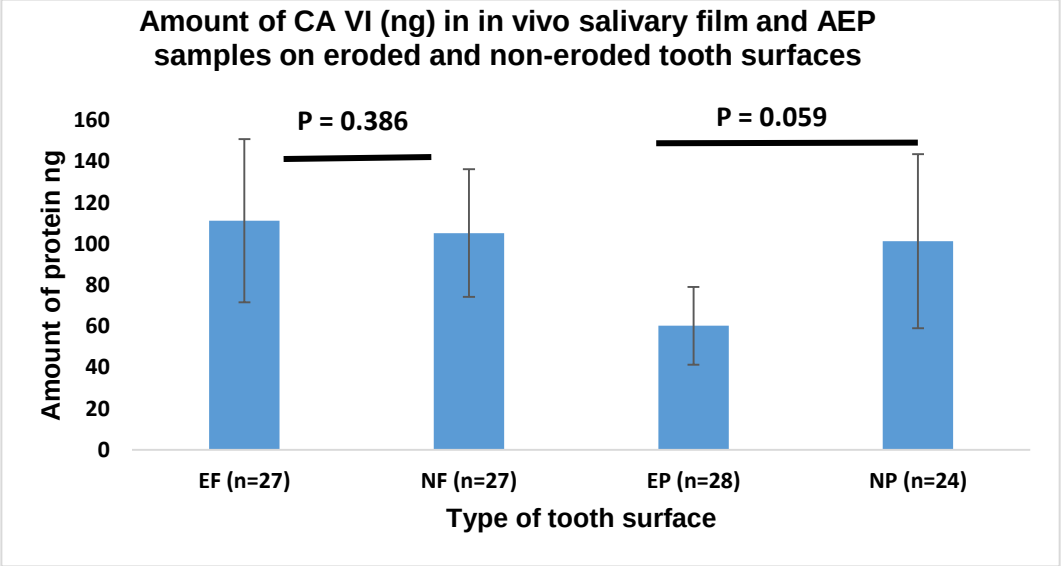


Figure80 (c): *Mean (SD) amount of CA VI (ng) in in vivo salivary film and AEP samples on eroded and non-eroded teeth of erosion patients.*

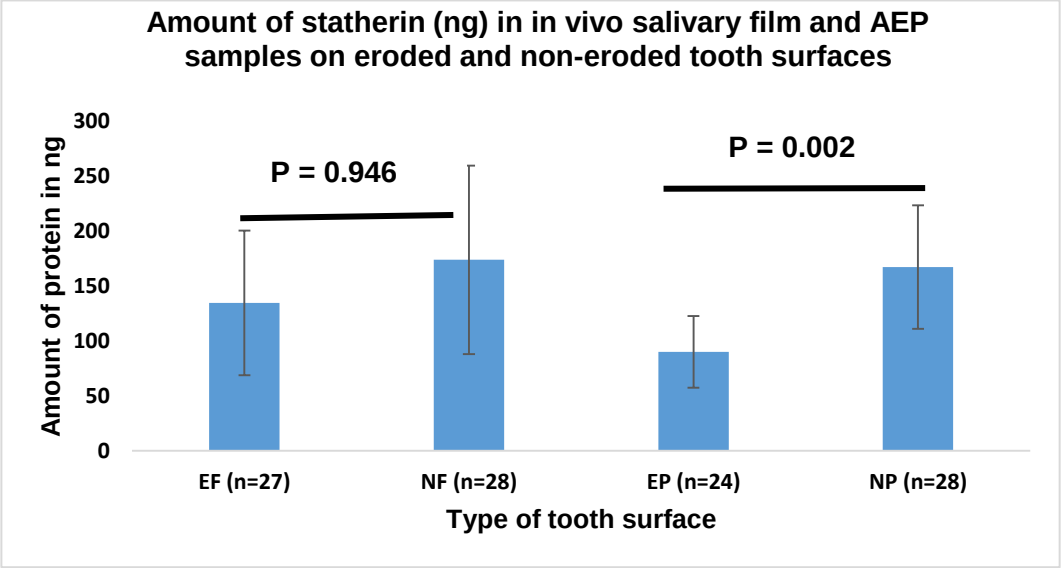


Figure 80 (d)*: Mean (SD) amount of statherin (ng) in vivo salivary film and AEP samples on eroded and non-eroded teeth of erosion patients.*

6.5 Discussion:

The total AEP protein concentration was observed to be significantly lower on eroded occlusal surfaces compared to non-eroded surfaces in the same sextant and within the same patient. The total protein concentration in salivary film was significantly greater on eroded tooth surfaces than that of non-eroded teeth. In addition, the amount of statherin was significantly lower in the AEP of eroded surfaces compared to statherin amounts observed in the AEP of non-eroded surfaces but no significant difference in the amount of statherin was observed in the salivary film. Mucin5b, albumin and carbonic anhydrase VI were detected in the film and AEP of eroded and non-eroded teeth surfaces of the same patient but their amounts did not differ significantly between the surfaces.

To the author's knowledge, no *in vivo* AEP studies have quantified mucin5b, albumin and CA VI. Only a few studies (Li *et al.*, 2004; Carpenter *et al.*, 2014) have directly quantified statherin in the AEP. Interestingly, part of *in vivo* AEP remains in place even after severe erosive challenges, indicating that some proteins remain in place and have the potential to prevent erosive tooth wear (Hannig *et al.*, 2005; Zimmerman *et al.*, 2013). In the present study, the selection of the four proteins was based on their hypothesised mechanisms of actions against erosive tooth wear. These included the physical permeable barrier and lubrication properties of mucin5b and albumin, buffering capacity of CA VI and calcium binding mechanism of statherin. In addition, albumin is also believed to bind to calcium ions in the enamel crystals and contribute

significantly to the formation of AEP (Hemingway *et al*., 2008; Jager *et al.*, 2011) but such affinity for hydroxyapatite was reported to be low (Carlen *et al.*, 1998).

The results of our study agree with a previous study carried out by Carpenter *et al.*, (2014) who compared the levels of total proteins and statherin in the AEP between thirty participants with and without erosive tooth wear (Carpenter *et al*., 2014). Both studies agree that total proteins concentration and amount of statherin in AEP were lower from patients with erosive tooth wear, or as is the case in the current study, from eroded surfaces than that from healthy subjects, or non-eroded surfaces. However, Carpenter *et al.*(2014) investigated the difference in the amount of mucin5b and CA VI in resting saliva but not in AEP from participants with and without erosive tooth wear (Carpenter *et al.*, 2014). They demonstrated that WMS from erosive wear patients had reduced amounts of mucin5b and CA VI compared to patients without erosive tooth wear. This is different from results observed in our study on the salivary film and AEP whereby the amounts of mucin5b, CA VI and albumin in AEP were not significantly different between eroded and non-eroded surfaces in the current study. This suggests that statherin is not adsorbing to the eroded tooth surface as well as the other proteins studied. Combining our results with that of Carpenter *et al*. (2014), the level of mucin5b and CA VI may suggest that the delivery of these two proteins from salivary film to the AEP as well as the flow and viscosity properties of the salivary film are important to the quality of AEP (Carpenter *et al.*, 2014). Albumin is

abundant in AEP and amongst the first proteins to adsorb to enamel (Siqueira *et al.*, 2012). Siqueira *et al*, (2012) suggested that albumin may be less likely to alter structurally or functionally in the mouth before it is incorporated into the AEP (Siqueira *et al.*, 2012). This agrees with the results in this study that albumin has the same affinity to eroded and non-eroded surfaces in both salivary film and AEP and that neither the saliva status nor the surface topography altered its adsorption.

The inherent protection against erosive tooth wear may be dependent on individual AEP proteins, in combination with the properties of enamel substrate including topography, tribology and surface roughness. This study may suggest that saliva may deliver proteins e.g statherin more effectively to non-eroded compared to eroded surfaces. Statherin was found to be amongst the first AEP proteins to be adhered competitively to hydroxyapatite (Siqueira *et al*., 2012) which supports the idea of favourable adsorption of statherin onto such tooth surfaces compared to other salivary proteins. The increased level of statherin, a calcium binding protein, on non-eroded surfaces indicates that calcium and phosphorus ions are possibly modulated around the enamel crystals and that statherin is potentially a major mediator against erosive tooth wear. Another possible explanation is that the adsorbed layer of statherin on the non-eroded surfaces may modify the adhesive and lubrication properties of the non-eroded surfaces (Harvey *et al.*, 2011). This could change its tribology influencing the wear and friction properties of the surface. Saliva/enamel interaction and AEP formation are influenced by the surface

roughness, surface free energy, surface chemical composition, wettability and many other interaction forces (Lindh, 2001; Svendsen and Lindh, 2009). In this regard, the competitive absorption of statherin onto non-eroded surfaces may also have influenced the adsorption of other proteins due to the competition and synergism among all *in vivo* AEP proteins during the dynamic event of AEP formation (Yin *et al*., 2006).

The salivary film was initially removed from the underlying AEP in order to assess the amount of the various proteins more accurately. A well-established method of collecting AEP from tooth surfaces was also applied in order to remove all organic materials from the enamel surfaces and avoid any contamination (Siqueira *et al*., 2007; Svendsen *et al*., 2008). The increased concentration of total protein in the salivary film on eroded tooth surfaces compared to non-eroded tooth surfaces may be as a result of the tooth cavitation induced by erosive wear. Although the data in the literature on surface texture is still contradictory, it is generally understood that erosive challenges increase enamel roughness to a certain degree before smoothing of the surface takes place (Las Casas *et al*., 2008). With regard to roughness, the greater intake of acidic food and drinks in erosion patients is likely to cause clinical signs of erosive tooth wear such as roughened or smooth surfaces which may change the surface binding affinity to certain proteins, including statherin. Further investigation with longitudinal erosion studies is necessary to establish the interaction between tribology and topography of the tooth surface.

The current study has some limitations. Calcium ions in the *in vivo* AEP samples were not analysed in this study due to the small amount of fluid collected. In addition, proteomic analysis of AEP at a larger scale was not feasible due to cost and time limitations. The relatively small sample size of 29 patients recruited for this study was however compensated for by the experimental design where comparisons were made between several samples obtained from the same participant. This reduces inter-subject variation that could cause bias such as diet and lifestyle, age, reflux, salivary properties, local topography of tooth surfaces. In this study, the lower total protein concentration and statherin levels observed in the *in vivo* AEP were collected solely from eroded occlusal surfaces of the lower first molars and compared to non-eroded surfaces in the same posterior sextant. However, this was not verified with other eroded surfaces which is a limitation of the study. Future work could compare protein concentrations on other eroded versus non-eroded surfaces in different sextants but within the same patient to attempt to replicate these findings.

There is potential that a reduced amount of statherin on a tooth surface may serve as a biomarker for risk of erosive tooth wear progression, although further studies with a larger number of participants are needed to confirm these preliminary results. Other similar studies are required to investigate other salivary proteins which may also play the role in the protection against erosive tooth wear, potentially improving oral diagnostic, therapeutic and

preventive measures. Further research could also focus on the adsorption behaviour of other individual proteins within AEP to different enamel surfaces. Looking at the differences in *in vivo* AEP noted in chapter 6, there seem to be two underlying theories. First, erosive tooth wear has already occurred on the surface which affects the tribology of the surface leading to preferential binding of the proteins to other surfaces. The adsorbed layer of statherin on the non-eroded surfaces may modify the adhesive and lubrication properties of the non-eroded surfaces (Harvey *et al.*, 2011) changing its tribology which in turn could influenced the wear and friction properties of such tooth surfaces. Second, there is something about the topography of occlusal surfaces of first molars which increases total protein concentration in salivary film but reduces the total protein and the statherin concentration in the AEP. This impairs the ability of the AEP to maintain calcium homeostasis, inducing the occurrence of erosive tooth wear. Occlusal surfaces of the lower first molars were used in the *in vivo* study within this thesis. These teeth surfaces have been reported to be the most commonly affected surface by erosive tooth wear alongside the buccal surfaces of the maxillary incisors (Jaeggi and Lussi, 2014). However it is unknown why these are the most commonly affected surfaces (Jaeggi and Lussi, 2014). Those investigating wear in children have assumed it is due to the early eruption of these teeth and hence the length of time exposed to the oral environment (Wiegand *et al.*, 2006; Murakami *et al.*, 2016). It is interesting to note that the *in vivo* study in adults within this thesis observed the occlusal surfaces of the lower first molars to have a lower total protein concentration

and statherin amount in AEP compared to non-eroded surfaces in the same posterior sextant. This may help to explain the results of longitudinal studies who observed presence of wear at baseline was a predictor of wear progression (Knight *et al.*, 1997; Ganss *et al.*, 2001). Ganss *et al,* (2001) found this association to be particularly strong for the occlusal surfaces of the lower first molars, even when behavioural risk factors were fully controlled for (Ganss *et al.*, 2001). The authors suggested a salivary/physiological predisposition to erosive tooth wear was possible (Ganss *et al.*, 2001). The small number of participants in the *in vivo* study within this thesis could be a limitation. Another limitation for the *in vivo* study within this thesis was that although it provided evidence for a physiological predisposition to increased wear in posterior sextants, this was not verified with other eroded surfaces and is another limitation of the study.

6.6 Conclusions

The null hypotheses were rejected since the total protein and statherin in the *in vivo* film and AEP were different between eroded and non-eroded tooth surfaces of the same patient.

Total protein concentration in AEP was reduced on eroded teeth compared to non-eroded teeth in the same subjects. This highlights the importance of tribology and topography in erosive tooth wear process which warrant further investigation. The calcium-binding statherin was also correlated best, out of the four proteins studied, to erosive tooth wear. Calcium homeostasis could be the main protective mechanisms in *in vivo* erosive tooth wear.

Chapter 7: General discussion and summary

The aim of this thesis was to investigate the role of salivary AEP and proteins/ions on erosion. This thesis identified that although salivary ions offer some protection against erosion, combination of ions and proteins provide better protection. There were several novel findings within the thesis. Comparing WMS and PS resulted in interesting findings regarding specific proteins that may contribute to this protection. In a laboratory model representing advanced erosion (five erosion cycles), mucin5b and albumin played a more major role in protection against tissue loss as measured by SNCP. In contrast, statherin and CA VI were the more prevalent proteins in a model representing early erosion (one erosion cycle) and resulted in a harder enamel surface as measured by SMHC. This would suggest that the protective mechanisms in early erosion could be through buffering and calcium homeostasis and this was further confirmed by an increase in the amount of calcium and phosphate in the *in vitro* AEP after the one erosion cycle. The mechanisms in more advanced erosion seem to be through provision of a physical and a diffusion barrier as well as lubrication. Total protein concentration was also a determinant in the level of protection, increasing concentrations resulting in less tissue loss. In Chapter 3, the importance of long term build up of salivary AEP against erosion shown from results obtained by SNCP and SMH teachniques was further confirmed by AFM. AFM is becoming increasingly commonly used in the study of erosion due to the potential to combine high resolution imaging with surface profilometry. Consequently, images can be obtained at a comparable resolution to scanning

electron microscopy from which roughness measurements can be easily obtained. The most commonly reported roughness parameters in the literature, are either Ra (number average roughness) or Rq (root mean square roughness) (Field *et al.*, 2010) measured from 2D profiles. In this study, we measured Ra, Rq and Rt to give an overview of how the actual surface deviates from an ideal and perfectly flat surface even though Rt in the case of the roughness data in Chapter 3 did not add any information other than that provided by Sa or Sq.

SNCP, SMH techniques, SDS-PAGE and western blot were used as the main methods in this thesis. SNCP is the a gold standard method to measure the amount of enamel tissue loss but it has some disadvantages. These include the requirement for having a very flat enamel surface in order to obtain accurate measurments of the step height using the white laser light of the SNCP. Also, in this thesis SNCP used white laser light with a spot size of 7 microns. A laser light of this size is not good at capturing very detailed structures and requires a gross tissue loss in order for a step hight to be captured. This problem was clear in Chapter 5 where using SNCP to detect the enamel surface changes after early erosive wear. SMH was also used in this thesis to provide information about the enamel softening after saliva and acid treatment. However, SMH had many challenges including the inability to detect advanced erosion and the difficulty to visualise the microhardness indentor on eroded enamel surfaces, making SMH a subjective measurmement. This can be evident by the variable results produced with

high standard deviations even when the indentor was calibrated and enamel surfaces were standarised. SMH measurements could also be influenced by AEP adherent to the enamel surfaces - which could be different in composition before and after acid treatment. Another challenge for both SNCP and SMH methods was that they did not provide information on the AEP layers adherent to the enamel surface following saliva treatment and whether any AEP are still present after erosive challenege. Much more analysis of this surface layer was required to provide a more reliable interpretation of the SNCP and SMH results. SDS-PAGE and western blot were used to analyse the composition of AEP before and after acid treatment. These methods are current, appropriate, reliable and well documented in life science for seprating, identifying and quantifying proteins in protein mixtures. A major challenege of these methods was the great deal of practice required to master the technique and the many steps involved in protein analysis as well as the reproducibility of different blots. Reproducibility of the western blots within this thesis was assessed using standards bands on different SDS-PAGE gels and was improved using same scanner setting (intensity) for every blot for which the exposure times were optimised to prevent pixel saturation.

A clinically relevant *in vitro* model using natural saliva was developed and used for all studies within this thesis in an attempt to provide results that are more representative of the clinical situation. This developed model is important as it can be used in future *in vitro* studies for investigating dental products and develop anti-erosive formulations and preventive strategies. It is however

important to note that this *in vitro* model may not accurately represent the clinical situation and future modifications may be needed as our understanding of the erosion processes in the complexity of the oral cavity improves. For the saliva *in vitro* model, pooled natural saliva (WMS and PS) was used to reducevarability and allowed sufficient saliva for all *in vitro* studies. Although natural saliva (WMS and PS) used within this thesis was collected from healthy volunteers according to a well-designed protocol, the calcium and fluoride concentration in the collected saliva were not measured which was a limitation of this *in vitro* studies.

Buccal and lingual surfaces from extracted human molar teeth were also used for the preparation of enamel specimens as described in section 2.1.1. Before any experiment was conducted, initial surface microhardness values of all prepared specimens were taken to ensure that their hardness values fall within the accepted range. The SMH values of sound enamel surfaces have been reported to range between 270 KHN and 440 KHN (Meredith *et al.*, 1996; Lussi *et al.*, 2011; Austin *et al.*, 2011). In addition, the selected mean SMH values of the enamel specimens were statistically analysed for any differences and the results indicated that there were no statistical differences between their mean values. Previous studies have also investigated the effects of tooth surfaces on *in vitro* erosive wear using SNCP (Ganss *et al.*, 2000) and Knoop SMH (Carvalho and Lussi, 2015). These studies have reported no significant differences between the buccal and lingual surfaces of molars in relation to erosive tooth wear.

The results of the labortary study within this thesis led to an interest to undertake an *in vivo* study to further assess the differences in total protein and the four specific proteins which were assessed in the laboratory studies. Total protein and the amount of statherin were the main two differences between surfaces with and without erosive wear in the *in vivo* study of erosive tooth wear patients. This shows the possible important role of proteins in protection against erosive wear and in particular, the role of calcium homeostatis in this process clinically. The *in vivo* results of this thesis suggest that there seems to be two underlying theories in the role of AEP against erosive tooth wear. First, erosive tooth wear has already occurred on the surface which affects the tribology of the surface leading to preferential binding of the proteins to other surfaces. The adsorbed layer of statherin on the non-eroded surfaces may modify the adhesive and lubrication properties of the non-eroded surfaces (Harvey *et al.*, 2011) changing its tribology which in turn could influence the wear and friction properties of such tooth surfaces. A second possible theory may be that there is something about the topography of occlusal surfaces of first molars which increases total protein concentration in salivary film but reduces the total protein and the statherin concentration in the AEP. This impairs the ability of the AEP to maintain calcium homeostasis, inducing the

occurrence of erosive tooth wear. *In vivo* data are of great interest as most laboratory models do not replicate the clinical dynamics accurately.

Overall findings in this thesis imply that proteins in AEP play a major role in protection against erosion. The most important factors in early erosion in this context and in the clinical environment are the total protein concentration and statherin, which is a calcium binding protein, pointing to calcium hemeostasis as one of the more important protective mechanisms.

Chapter 8: Clinical implications and suggestions for future work

8.1 Clinical implications

The results for the early erosion model (one erosion cycle) were more similar to the clinical situation than the advanced erosion model (five cycles erosion). The results can not be directly translated into clinical practice, but provide interesting information regarding protection against early erosive wear, which can be further evaluated in future *in-situ* and *in vivo* studies. The aim would be to have a targeted approach in enhancing the natural protective abilities of saliva and AEP, as well as possibly developing methods to detect individual proteins as biomarkers of erosive tooth wear. These measures could enhance individualised preventive care plans in patients at risk of erosive wear.

8.2 Future recommendations

There are several aspects of this thesis that warrant further investigation:

The findings suggested that WMS reacted with the enamel surface even before the acid challenge resulted in SMH reduction as compared to AS and DW. This effect has possibly been overlooked in previous *in vitro* erosion studies. There is therefore a greater need for this to be considered in future work on *in vitro* models of erosion using surface SMH if more reliable interpretation of the results is to be provided. This highlights the importance of considering natural saliva as the immersion medium in *in vitro* erosion models as it appears to significantly affect the results compared with artificial saliva.

Measuring calcium and fluoride ions in the collected natural saliva is an important recommendation for future studies in order to equilibrate the calcium and fluoride concentration of the natural and artificial saliva. Measuring calcium in saliva can also possibly help correct the calcium loss measured from enamel surface for that already present in the saliva and AEP. In addition, as the *in vitro* model has assessed solutions containing proteins and ions (NS) and ions only (AS) compared to that containing neither proteins nor ions (DW), it would be interesting to compare the results to a protein only solution. It is also recommended for future studies that either buccal or lingual enamel surfaces used to avoid the variability in mineral contents.

It would be interesting to assess the *in vitro* protocols in an *in-situ* model, to compare the effect of *in vivo* formed AEP to the *in vitro*. Adding abrasion/attrition elements to the developed erosion model would also be of interest to mimic the action of the teeth and soft tissues.

Morover, it would be interesting to compare the results for patients with and without erosion. This will provide further insight into the individual proteins playing a role in protection and increase our understanding of the processes involved. It will also be of interest to identify other proteins from the proteomic results and assess them in similar models developed in this thesis.

Further studies with a larger number of participants are needed to confirm the preliminary results of the *in vivo* study. Future work could compare amounts of protein on an increased number of eroded versus non-eroded surfaces in

different sextants but within the same patient, to attempt to replicate these findings.

Our results in chapter 6 of this thesis also indicate that the delivery system of proteins from the salivary film to tooth surfaces may play an important role in protection against erosive tooth wear. In addition, it would be of interest if *the in vivo* study in Chatpter 6 of this thesis is repeated but on patients with erosive tooth wear as a results of GORD or other intrinsic factors as the protein profile is different.

Protein-protein interaction of salivary proteins may act as vehicles to deliver other proteins to their site of action. This needs to be investigated further in both *in vitro* and *in vivo* studies in order to better understand the novel molecules or fragments formed as a result of the proteolytic and post-secretion processing of WMS which may protect oral soft and hard tissues.

Appendices

Appendix I: Patient information sheet for teeth collection

Guy's, King's and St Thomas' Dental Institute Department of Fixed and Removable Prosthodontics

25th Floor, Guy's Tower
Guy's Hospital
London Bridge
London
SE1 9RT
Tel 0207 188 5390
Fax 0207 188 7486

Volunteer information sheet (Version 2) 15/07/2015

Title of project: Protection of erosive tooth wear (donation of extracted tooth)

You will be given a copy of the information sheet and a signed consent form to keep.

Part 1
Invitation paragraph

You are being invited to donate your tooth for a research study. Before you decide it is important for you to understand why the research is being done and what it will involve:

Part 1 tells you the purpose of the studies and what will happen if you decide to participate.
Part 2 gives you more detailed information about the conduct of the studies.

Please take time to read the following information carefully. Ask us if there is anything that is not clear. Talk to others about the research if you wish and the following organization could give you independent advice:
Guy's and St Thomas' Hospital NHS Foundation Trust Patient Advice and Liaison Service Telephone 020 7188 8801 or 020 7188 8803 email: pals@gstt.nhs.uk
Post: Patient information team, Knowledge and information centre, St Thomas' Hospital London, Westminster Bridge Road, SE1 7EH

What is the purpose of the study?
Tooth wear is a condition where the teeth wear away faster than normal and is caused by acid erosion (from acidic foods and drinks and stomach acid), tooth grinding and over brushing. Tooth wear is a common condition that can affect anyone and it appears to be

happening more and more nowadays. Severe tooth wear can cause teeth to become very sensitive, as well as causing cosmetic and chewing problems due to shortened teeth and even in severe cases can cause tooth loss. Certain toothpastes and mouth rinses have the potential to prevent and treat tooth wear. However the scientific evidence for this is lacking and the studies we plan to carry out may provide important information regarding the disease process, progression of the disease and possible prevention of the disease.

Why have I been chosen?

You are suitable for this study because you are a healthy individual who needs a tooth removed.

Do I have to take part?

It is up to you to decide whether or not to take part. If you do, you will be given this information sheet to keep and be asked to sign a consent form. You are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive.

What will happen to me if I decide to take part?

At your first visit, when you are consulted about the tooth extraction, you will be invited to join the study by a clinician. At your second visit we will confirm that you still want to donate your tooth and then you will have your tooth removed in the normal way. After your tooth is extracted it will be transferred to the Biomaterials laboratory at King's College Hospital Dental Institute (Department of Biomaterials, 17th Floor, Guy's Tower, Guy's Hospital, London Bridge SE1 9RT). Once the tooth is extracted your participation in the study is over.

What do I have to do?

You will just have to attend your set appointments as normal.

What is the drug, device or procedure being tested?

Various methods of studying the surface changes of the extracted teeth and the effects of dietary acids, fluorides and other protective agents are being investigated in this study on the extracted teeth.

What are the alternatives for diagnosis or treatment?

The research does not involve any volunteer treatment and you will receive your routine standard treatment as usual.

What are the side effects of any treatment received when taking part?

There are no risks associated with this study, other than the usual risks of a tooth extraction which will be explained to you by the clinical team who are carrying out the treatment.

What are the other possible disadvantages or risks of taking part?

There are no risks associated with this study, other than the usual risks of a tooth extraction which will be explained to you by the clinical team who are carrying out the treatment.

What are the possible benefits of taking part?

We do not expect that you will receive any benefit from taking part in this study.

What happens when the research study stops?
We aim to publish the results in medical journals.

What if there is a problem? And contact details:
No problems can be foreseen however the contact number for complaints or concerns is for:
Professor David Bartlett 0207 188 5390 or email david.bartlett@kcl.ac.uk

Will my taking part in the study be kept confidential?
We will not be collecting any information about you and your confidentiality is safeguarded during and after the study. Our procedures for handling, processing, storage and destruction of your data are compliant with the Data Protection Act 1998.

Contact for further information:
Professor David Bartlett 0207 188 5390 or email david.bartlett@kcl.ac.uk

This completes Part 1 of the Information Sheet. If the information sheet in Part 1 has interested you and you are considering participation, please continue to read the additional information in Part 2 before making any decision.

Part 2

What if relevant new information becomes available?
We are a leading establishment in this area of research and if any new information relevant to this study becomes available the researchers will discuss this with you. You are free to withdraw from the study at any time.

What will happen if I don't want to carry on with the study?
You can withdraw from study. Just advise the clinician treating you that you do not want to donate your tooth and your tooth will be disposed of once extracted, or you can keep it to take home.

What if there is a problem?
If you have any concern about any aspect of this study, you should ask to speak with the researchers who will do their best to answer their questions.
Professor David Bartlett 0207 188 5390 or email david.bartlett@kcl.ac.uk

If you remain unhappy and wish to complain formally, you can do this through the NHS complaints procedure. If you are harmed by taking part in this research project there are no special compensation arrangements. If you are harmed due to someone's negligence, then you may have grounds for a legal action but you may have to pay privately for it. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way that you have been approached or treated during the course of this study, the normal NHS complaints mechanisms should be available to you.

Details of how to complain can be obtained from the Volunteer Advice and Liaison Service (PALS)

Guy's and St Thomas' Hospital NHS Foundation Trust Patient Advice and Liaison Service

Telephone 020 7188 8801 or 020 7188 8803 email: pals@gstt.nhs.uk

Post: Patient information team, Knowledge and information centre, St Thomas' Hospital London, Westminster Bridge Road, SE1 7EH

Will my taking part in this study be kept confidential?

We will not be collecting any information about you and your confidentiality is safeguarded during and after the study. Our procedures for handling, processing, storage and destruction of your data are compliant with the Data Protection Act 1998.

What will happen to any samples that I give?

After your tooth has been removed, it will be anonymised (i.e. there will be no way of linking the tooth to your personal data or medical records) and then transported to the Biomaterials laboratory at King's College Hospital Dental Institute (Department of Biomaterials, 17th Floor, Guy's Tower, Guy's Hospital, London Bridge SE1 9RT). The tooth will be used in a laboratory study or a clinical study investigating erosive tooth wear. The study may be laboratory experiment which involves simulating erosive tooth wear on the enamel blocks from the donated teeth in the laboratory, as well as exposure to topical protection or it may be a clinical study where participants wear mouth guards (like sports guards) containing sterilised blocks containing the enamel from the donated teeth. In both cases, measurements of the amount of wear on the tooth surface are taken.

What will happen to the results of the research study?

The results of the study will be published in medical journals. Participants will not be identified in any report or publication.

Who has reviewed the study?

This study was given a favourable ethical opinion REC ref: 12/LO/1836

Will any genetic tests be done?

No.

Thank you for considering taking part and for taking time to read this sheet – please ask any questions if you need to.

Appendix II: Consent form for teeth collection

Guy's, King's and St Thomas' Dental Institute Department of Fixed and Removable Prosthodontics

25th Floor, Guy's Tower
Guy's Hospital
London Bridge
London
SE1 9RT
Tel 0207 188 5390
Fax 0207 188 7486

Consent Form (Version 2: 22/07/2015)

Title of project: Protection of erosive tooth wear (donation of extracted tooth)

REC ref: REC ref: 12/LO/1836

Please complete this form after you have read the Information Sheet and/or listened to an explanation about the research

Patient Identification: Date

Thank you for considering taking part in this research. The person organising the research and/or a member of the clinical team who is trained for this purpose must explain the project before you agree to take part.
If you have any questions arising from the Information Sheet or explanation given to you, please ask the researcher before you decide whether or not to join in. You will be given a copy of this Consent Form to keep and refer to at any time.

I confirm that I have read and understand the information sheet version 2, 15/07/2015) for the above study.
I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily.
I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected.
I agree to take part in the above study.

Name of Patient.....
Signature.....Date.....

Name of Person taking consent.....
Signature.....Date.....

Appendix III: Volunteer information sheet for saliva collection

Participant Information Sheet

Healthy Volunteers Group

Study Title: Role of Saliva/pellicle in dental erosion and dental caries

REC ref: Northampton REC, 14/EM/0183

Investigator: Dr Rebecca Moazzez

Invitation paragraph

You are being invited to donate saliva for a research study. You should only participate if you want to. Choosing not to take part will not disadvantage you in any way. It is up to you to decide whether to take part or not. If you decide to take part you are still free to withdraw from the study at any time and without giving a reason. You can withdraw your data at any point up until the conclusion of your final clinic visit. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. Before you decide it is important for you to understand why the research is being done and what it will involve.

Part 1 tells you the purpose of the study and what will happen if you decide to participate.

Part 2 gives you more detailed information about the conduct of the study.

Part 1: Purpose of the study and what will happen

What is the purpose of the study?

The goal of this study is to collect saliva from healthy individuals, individual with dental erosion (wear of teeth by acids) and individual with dental caries (tooth decay).

Dental erosion is a condition where the teeth wear away faster than normal and is caused by acids (from acidic foods and drinks and stomach acid). Dental erosion is a common condition that can affect anyone and it appears to be happening more and more nowadays. Severe dental erosion can cause teeth to become very sensitive, as well as causing cosmetic and chewing problems due to shortened teeth and even in severe cases can cause tooth loss.

Dental caries (tooth decay) results when foods and drinks high in sugary carbohydrates, bacteria in plaque (a sticky film that forms on the teeth when they are not brushed) use these carbohydrates to produce acid. Acid in plaque begins to break down the tooth's surface and result in decay. Left untreated it can result in pain and death of the nerve inside the tooth and tooth loss.

A number of research studies have shown a relationship between the properties of saliva and salivary pellicle (a thin film formed from saliva on the tooth surface immediately after brushing) and dental erosion and dental decay. Some proteins in saliva and pellicle may offer a protective role against these two conditions developing. However the scientific

evidence is lacking about the role of these proteins in the hardening and loss of enamel and dentin through these conditions. This study will help us in our understanding of the role of saliva and pellicle in preventing dental erosion and decay.

Why have I been chosen?

You are suitable for this study because you do not have any signs of dental caries (tooth decay) or dental erosion (Abnormal wear of teeth by acids).

Do I have to take part?

You do not have to take part. It is up to you decide whether or not to take part. If you do, you will be given this information sheet to keep and be asked to sign a consent form. You are still free to withdraw at any time and without giving a reason. This will not affect the standard of care you receive.

What will happen to me if I decide to take part?

At your first visit, you will be invited to join the study by a clinician and given this patient information sheet. At your second visit we will confirm that you still want to donate saliva. After your saliva is collected it will be anonymised and transferred to the Biomaterials laboratory at King's College Hospital Dental Institute and used in a Laboratory study. After the completion of the study the sample will be discarded.

What do I have to do?

You will just have to attend your set appointments as normal.

Once your consent is taken, you will be given a general oral exam and we will ask you some questions regarding your medical history to ensure that you meet our study criteria. You will then be asked to provide a sample of unstimulated saliva by dribbling any saliva

collected in your mouth into a tube. Following this you will be asked to chew on a tasteless piece of paraffin wax for 5 minutes and dribble any saliva collected in your mouth into another tube (Stimulated saliva).

Next, a saliva sample will be collected from the sides of your cheeks inside your mouth from one of the salivary glands (parotid gland). This will be collected by placing a sterile suction cup on the inside of your mouth on the surface of your cheeks. The whole process will take up to 30 minutes. The saliva secretion will be stimulated by placing 2 drops of citric acid 2% solution on the back of your tongue every 30 seconds.

What are the side effects of any treatment received when taking part?

There is no treatment and no side effects.

What are the other possible disadvantages or risks of taking part?

There are no risks associated with this study.

What are the possible benefits of taking part?

We do not expect that you will receive any benefit from taking part in this study.

Will any genetic tests be carried out?

No

What happens when the research study stops?

We aim to publish the results in medical journals. Our procedures for handling, processing, storage and destruction of your data are compliant with the Data Protection Act 1998. Any samples collected for the study will be discarded.

What will happen if I don't want to carry on with the study?

You can withdraw from participation at any time. Just advise the clinical researcher or the chief investigator that you do not want to continue taking part and any collected saliva, if any, will be discarded.

This completes Part 1 of the Information Sheet. If the information sheet in Part 1 has interested you and you are considering participation, please continue to read the additional information in Part 2 before making any decision.

Part 2: Study Conduct

What if relevant new information becomes available?

We are one of the leading establishments in this area of research and if any new information relevant to this study becomes available the researchers will discuss this with you. You are free to withdraw from the study at any time. If you decide to withdraw your research doctor will make arrangements for your care to continue. If you decide to continue in the study you will be asked to sign an updated consent form.

Also, on receiving new information the researchers might consider it to be in your best interests to withdraw you from the study. They will explain the reasons and arrange for your care to continue. At the end of the study the results will be presented to the scientific community.

Will my taking part in the study be kept confidential?

Once you have agreed to take part in this study, you will be allocated a study number which will be used at all times during your subsequent visits. This means that all information which is collected about you during the course of the research will be kept strictly confidential. Any information about you which leaves the hospital will be anonymised and have your personal details removed so that you cannot be recognised from it.

What if there is a problem?

If you have a concern about any aspect of this study, you should ask to speak to the researchers who will do their best to answer your questions.

Please contact:

Dr Rebecca Moazzez

Rebecca.v.moazzez@kcl.ac.uk

0207 188 1856

If you have a complaint, you should talk to your research doctor who will do their best to answer your questions. If you remain unhappy, you may be able to make a formal complaint through the NHS complaints procedure. Details can be obtained through the Guy's and St Thomas' Patient Advisory Liaison Service (PALS) on 0207 1887188, address: PALS, KIC, Ground floor, north wing, St Thomas' Hospital, Westminster Bridge Road, London, SE1 7EH .

This trial is co-sponsored by King's College London and Guy's and St Thomas' NHS Foundation Trust. The sponsors will at all times maintain adequate insurance in relation to the study independently. Kings College London, through its own professional indemnity (Clinical Trials) and no fault compensation and the Trust having a duty of care to patients via NHS indemnity cover, in respect of any claims arising as a result of clinical negligence by its employees, brought by or on behalf of a study patient.

What will happen to the results of the research study?

The results of the study will be published in medical journals. Participants will not be identified in any report or publication.

Who has reviewed the study?

This study has been reviewed by an internal reviewer at King's College London and was given a favourable ethical opinion by Northampton REC, 14/EM/0183.

Contact for Further Information

Dr Rebecca Moazzez, Room 365, Floor 25, Tower Wing, Guy's Hospital, London Bridge.
0207 188 1856, rebecca.v.moazzez@kcl.ac.uk

Thank you for considering taking part and for taking time to read this sheet – please ask any questions if you need to.

You will be given a copy of the information sheet and a signed consent form to keep.

Guy's and St Thomas' NHS
NHS Foundation Trust

KING'S College LONDON

Appendix IV: Consent form for saliva collection

Informed Consent Form

Study title: Role of Saliva/pellicle in dental erosion and dental caries

Principal Investigator: Dr Rebecca Moazzez

	Please Initial box
I confirm that I have read and understood the information sheet (dated 27/07/2014, Version no.4) for the above study. I have had an opportunity to consider the information, ask questions and have these answered satisfactorily.	
I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected.	
I understand that data collected during the study, may be looked at by responsible individuals from King's College clinical staff, regulatory authorities or from the NHS Trust, where it is relevant to my taking part in this research. I give my permission for these individual to have access to my records.	
I understand that if the study is published, none of my personal details will be identifiable.	
I agree to take part in this study.	

Participant's Legal Name	Date	Signature
Name of person taking consent (if different from researcher)	Date	Signature
Researcher	Date	Signature

Appendix V: Saliva collection protocol

Informed consent was obtained. The participants were asked to abstain from eating and drinking for at least one hour before saliva sample collection. Two types of saliva were collected. Firstly, stimulated saliva were collected by asking the participants to chew on a piece of paraffin wax. Paraffin-stimulated saliva samples from the volunteers will be collected over 5 minutes. The saliva was collected into 20-ml sterile polypropylene universal tubes Saliva. Secondly, saliva sample from parotid glands was collected. This was collected through a number of steps. The orifice of the parotid gland was located then its area was dried with gauze for better vision. The parotid collector, a lashley cup, was placed on the mucosa so that the inner ring surrounds the duct orifice. The collector was held on the mucosa by suction from the outer ring by pulling back on the syringe and allowing the pressure to come to equilibrium. The syringe can then be rested on the patient's shoulder. A medium binder clip will then be attached to the tygon tubing going from the collector to the syringe to lock in air in the tubing. The suction created should be sufficient so that the cup is in place without occluding the inner chamber of the parotid collector with tissue.Saliva from the parotid gland will then flow passively into the inner ring and through the attached tubing. The subject should avoid unnecessary movement of their head or jaw to prevent dislodging this cup. The flowing saliva was collected into an ice-cooled pre-weighed and pre-labeled container.

The parotid saliva secretion was then stimulated using 2 drops of citric acid 2% solution every 30 second applied to the posterior lateral surface of the tongue bilaterally.

Flow might not begin for a minute or two after stimulation has been applied. A maximum of 5 minutes was allowed for saliva to appear in the clear portion of the tubing. Once saliva flow is observed, an additional 10 minutes will be allowed for the saliva to reach the end of the tubing. When the saliva began to exit the tygon tube, a 10-minute collection period was started. The collection tubes of all types of saliva will be re-weighed again after saliva collection. Time of collection and the general oral condition of volunteers were recorded. The pH of the collected saliva was immediately be measured using a pH meter. The buffering capacity of the saliva was also immediately measured using a specified saliva kit provided by GC Company (GC America INC, 3737 W. 127th Street Alsip, IL 60803).

The tubes are pre- and re-weighed, and the saliva was collected over a fixed period of time (5 min) in order to determine salivary flow rate using the following formula:

$$\text{Salivay Flow rate (ml/min)} = \frac{\text{Weight of tube with saliva} - \text{Weight of pre-weighed tube with no}}{\text{Time of collection (mins)}}$$

Although saliva will be collected from different individuals, the flow rate and buffering capacity will be determined by calculating the mean of individual flow rates.

All collected saliva samples will be anonymised and stored in a HTA freezer at- 80 °C. Once the saliva has been used and the study completed, saliva samples will be discarded.

Appendix VI: Surface microhardness testing after 24 hour immersion in solutions

Testing:

Surface microhardness (SMH) measurements after 24 hour immersion in solutions:

Referring to chapter 3 section 3.3.3.2, only for the 24 hour groups (3), the surface microhardness values (SMH) before 24 hour immersion in the corresponding solutions (WMS,AS,DW) and prior to the first erosion cycle were also calculated, in order to assess the effect of the solution alone on the SMH values. SMH values after 24 hour immersion in solution were selected as the baseline (KHNb) for calculating the surface microhardness change (SMHC) after five cycles immersion in acid.

Results:

Surface microhardness measurements (SMH) after 24 hour immersion in solutions

Figure 89 shows the results of SMH values before and after 24 hour immersion in the pertaining solutions prior to the erosion cycle. The mean (SD) SMH values of enamel samples before 24 hour immersion in WMS and AS [343.63 (12.21) KHN and 354.09 (15.71) KHN respectively] exhibited significant reduction after 24 h immersion in WMS and AS [315.72 (11.74) $p<0.0001$ and 321.79 (10.49) $p<0.001$ respectively]. The mean (SD) SMH value at baseline for DW group [345.32 (15.29)] was not significantly different than that after 24 hour immersion in water [329.00 (19.31)] $p<0.05$].

Therefore, the SMH values after 24 hour immersion in solutions were selected as the baseline (SMHb) for calculating the surface microhardness change (SMHC) after five cycles erosion (section 3.3.3.2). The selected baseline values for the three groups were: WMS3 group [315.72 (11.74)], AS group [321.79 (10.49)] and DW group [329.00 (19.31)].

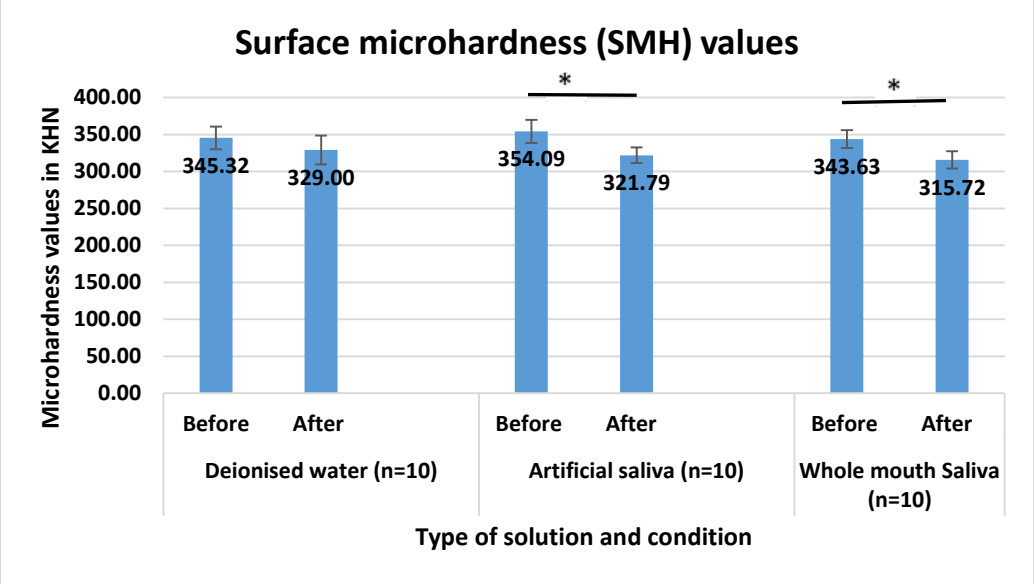


Figure 80: *Mean (SD) knoop surface microhardness values (SMH) for three groups according to the solution before and after 24 hour immersion in the pertaining solution (WMS,AS,DW). Asterisks indicates significant differences.*

Appendix VII: Western blot protocol

1) Make running buffer:

   475ml UHQ H_2O
   25ml 20x Nupage MES SDS Running Buffer (NP0002)
2) Assemble gel in ring and make sure upper chamber does not leak

   4-12% 15 well Nupage Bis-Tris gel
3) Prepare and heat samples on 100 °C heating block for 5 min
4) Add samples to the wells of the gel accordingly
5) Run gels at 200V constant, 125mA, for 32 minutes
6) Make transfer buffer: 425ml UHQ H_2O
   +50ml methanol (10% final)
   +25ml of 20x Nupage Transfer Buffer
7) Soak sponges and filter paper (cut to size) in transfer buffer
8) Disassemble ring and discard buffer, make transfer sandwich from bottom up:
   Cathode plate on bench
   3 x sponges
   1x filter paper
   GEL
   Nitrocellulose membrane (cut to size) smooth out any bubbles with roller/blunt syringe
   1x filler paper
   3x sponges
9) Fill inner chamber with transfer buffer to top of sponges, not all the way to the top
10) Transfer at 30V constant, 150amps for 1 hour
11) Place membrane contact side UP in clean plastic tray
12) Treat membrane w/FITC stain for 20 minutes
    10mg FITC in 1ml DMSO up to 100ml in carbonate buffer
13) Rinse off FITC in tap H_2O
14) Photograph on ChemiDot to check for protein transfer
15) Rinse off remaining FITC in tap H_2O
16) Store dry on bench in clean plastic tray overnight
17) Block membrane at R.T. for 1 hour in TBST (TTBS)

    TBST: 2.42g Tris base
    +9.0g NaCl
    +up to 1000ml in UHQ dH_2O, pH to 7.6 with HCl, mix,

    +1ml Tween 20, mix
20) Wash membrane in TBST (3 x 5 minutes)

21) Place membrane in secondary antibody for 1 hour at R.T. on rocker

Goat anti-Mouse HRP (Dako #p0447, Lot #00071312)
At 1:2000 in TBST (5ul in 10ml)

22) Wash membranes in TBST (3 x 5 minutes)

23) Develop with chemiluminescent reagent western C kit

Appendix VIII: detailed protocol of the proteomic analysis

Proteomics Facility – Denmark Hill

In-gel Digestion Protocol for Proteins Separated by PAGE for Analysis by Mass Spectrometry

Measures to take to avoid contamination with keratin

- All procedures including band/spot excision to be carried out in a dust-free area or laminar flow hood.
- Wear colourless, powder-free, latex gloves rinsed with water and changed often.
- Prior to a new digestion prepare fresh ammonium bicarbonate (Ambic) in glass durans rinsed with fresh ultra-pure 18MΩ water and decant fresh acetonitrile into a rinsed universal.
- Clean glassware/scalpel blades with ethanol.
- Use fresh eppendorf tube from sealed bag. Open eppendorf lids carefully, avoid flicking open.
- Replace lids on all solutions straight after use to prevent contamination with keratin.
- Care when preparing new trypsin stock solutions as keratin contamination here will affect all subsequent digests

Materials

Ammonium Bicarbonate – Sigma; A6141
Dithiothreitol (DTT) – Sigma; D5545-5G
Iodoacetamide (IAA) – Sigma; I1149-5G
Acetonitrile (ACN) – Fischer; A0627PB17
Trypsin – Roche; bovine sequencing grade Cat No. 1418 475 (4 vials/box; 25µg/vial)
Eppendorfs
TFA – MERCK; 1.08262.0100

Solutions

- 100mM Ambic – Weigh out 0.79g into a 200ml Duran, make up to 100ml mark with water, dissolve by swirling gently.
- 50mM Ambic – Using a 100 ml Duran make up to the 40ml mark with 100mM Ambic, dilute with water to 80ml mark.
- 10mM DTT – Make 100mM stock; weigh out 15mg of DTT into an eppendorf and add 1ml of 100mM Ambic. Dilute 10 fold for working solution (100µl 10mM DTT and 900µl 100mM Ambic)
- 55mM IAA – Weigh out 10mg of IAA into an eppendorf and add 1ml 100mM Ambic.

- Trypsin - Make 0.1% TFA fresh in rinsed universal (5ml water [5x 1ml pipette], 5ul TFA]. Add 250µl of 0.1% TFA, vortex gently with lid on to ensure solubilisation. Divide into 8x 30µl aliquots in Eppendorfs and freeze at -20^{o}C for future use.

Tryptic digestion

1. Excise bands of interest cutting as close to the edge of the band as possible. Chop into ~2 mm^2 pieces and transfer into an eppendorf. NB. Store gel pieces at 4^{o}C in water until required.
2. Wash the gel cubes with 100mM Ambic for 5 mins (decant off water first if gel pieces have been stored) and decant. Volumes of Ambic and ACN (steps 2 to 8) are added in excess.
3. Add acetonitrile, decant after one round and add same volume again to fully and quickly dehydrate the gel pieces, decant and dry in speed vac for 5 mins. Whilst samples are drying prepare DTT solution.
4. Rehydrate the gel with 10mM DTT and heat at 56^{o}C for 30 mins.
5. Decant DTT, add ACN (2x slugs; see step 3), dehydrate and dry in speed vac (5mins). Whilst samples are drying prepare 55mM IAA solution.
6. Add 55mM IAA; incubate at ambient temperature for 20 mins in the dark.
7. Discard the supernatant wash briefly with 100mM Ambic buffer then replace and wash for a further 5 mins and discard the buffer (go to step 9 if de-staining not required).
8. If not completely de-stained at this stage (colloidal Coomassie) wash with 1:1 solution of 100mM Ambic:ACN in excess. Incubate at 37^{o}C shaking for 30 mins (repeat this step until de-stained).
9. Decant the liquid, dehydrate once again with acetonitrile as in step 3 and dry off in a speed vac for 5 mins
10. Take trypsin aliquot/s (30µl) and add 200µl of 50mM Ambic to give a final trypsin concentration of 13ng/µl. Rehydrate the gel pieces in a minimal volume of trypsin solution (i.e. enough to cover and rehydrate the gel pieces) at 4^{o}C for 20 mins. Remove unabsorbed trypsin and add a minimal volume of 50mM Ambic (10-20µl) to cover the gel pieces and keep them wet during enzyme cleavage. Incubate at 37^{o}C for 2 hours then overnight at room temperature.

Peptide extraction

1. Decant supernatant from gel pieces and collect into a new eppendorf tube.
2. Wash gel pieces with 50mM Ambic for 5 mins at 37^{o}C, decant and pool into tube from step 1. Use a minimal volume that will immerse the gel pieces.
3. Dehydrate gel pieces with ACN for 10 mins at 37^{o}C, decant and pool supernatant into tube from step 1.
4. Repeat steps 2 and 3.
5. Freeze the peptide extract and dry down the pooled supernatants to completion; avoid over drying. Store at -80^{o}C until required.

Appendix IX: Supplementary Excel files for the protein identifications using the Uniprot database selecting Human Taxonomy

Bio View:Identified Proteins (141/142) Including 0 Decoys	Accession Number	Molecular Weight	Fold Change by Sample	PS	WMS
Serum albumin OS=Homo sapiens GN=ALB PE=1 SV=2	P02768	69 kDa	1.6	67	104
Lactotransferrin OS=Homo sapiens GN=LTF PE=1 SV=6	P02788	78 kDa	2.8	31	87
Alpha-amylase 1 OS=Homo sapiens GN=AMY1A PE=1 SV=2	P04745	58 kDa	2	34	67
Desmoplakin OS=Homo sapiens GN=DSP PE=1 SV=3	P15924	332 kDa	0.05	80	4
Zymogen granule protein 16 homolog B OS=Homo sapiens GN=ZG16B PE=1 SV=3	Q96DA0	23 kDa	1.7	24	41
Actin, cytoplasmic 2 OS=Homo sapiens GN=ACTG1 PE=1 SV=1	P63261	42 kDa	3.4	14	48
BPI fold-containing family A member 2 OS=Homo sapiens GN=BPIFA2 PE=1 SV=2	Q96DR5	27 kDa	1	26	26
Glyceraldehyde-3-phosphate dehydrogenase OS=Homo sapiens GN=GAPDH PE=1 SV=3	P04406	36 kDa	2.1	15	32

Polymeric immunoglobulin receptor OS=Homo sapiens GN=PIGR PE=1 SV=4	P01833	83 kDa	3.8	9	34
Protein S100-A8 OS=Homo sapiens GN=S100A8 PE=1 SV=1	P05109	11 kDa	5.8	6	35
Protein S100-A9 OS=Homo sapiens GN=S100A9 PE=1 SV=1	P06702	13 kDa	13	3	38
Lysozyme C OS=Homo sapiens GN=LYZ PE=1 SV=1	P61626	17 kDa	1.7	14	24
Desmoglein-1 OS=Homo sapiens GN=DSG1 PE=1 SV=2	Q02413	114 kDa	0.3	28	9
Ig gamma-1 chain C region OS=Homo sapiens GN=IGHG1 PE=1 SV=1	P01857	36 kDa	2.3	11	25
Annexin A1 OS=Homo sapiens GN=ANXA1 PE=1 SV=2	P04083	39 kDa	2	12	24
Ig alpha-1 chain C region OS=Homo sapiens GN=IGHA1 PE=1 SV=2	P01876	38 kDa	1.5	14	21
Myeloperoxidase OS=Homo sapiens GN=MPO PE=1 SV=1	P05164	84 kDa	17	2	33
Cystatin-SN OS=Homo sapiens GN=CST1 PE=1 SV=3	P01037	16 kDa	4.7	6	28
Ig kappa chain C region OS=Homo sapiens GN=IGKC PE=1 SV=1	P01834	12 kDa	2.8	9	25
Annexin A2 OS=Homo sapiens GN=ANXA2 PE=1 SV=2	P07355	39 kDa	0.4	23	9

Carbonic anhydrase 6 OS=Homo sapiens GN=CA6 PE=1 SV=3	P23280	35 kDa	0.6	19	12
Zinc-alpha-2-glycoprotein OS=Homo sapiens GN=AZGP1 PE=1 SV=2	P25311	34 kDa	0.9	16	15
Pyruvate kinase isozymes M1/M2 OS=Homo sapiens GN=PKM PE=1 SV=4	P14618	58 kDa	4.4	5	22
Junction plakoglobin OS=Homo sapiens GN=JUP PE=1 SV=3	P14923	82 kDa	0.1	24	3
Protein-glutamine gamma-glutamyltransferase E OS=Homo sapiens GN=TGM3 PE=1 SV=4	Q08188	77 kDa	1.2	12	14
Lactoperoxidase OS=Homo sapiens GN=LPO PE=1 SV=2	P22079	80 kDa	1.2	11	13
Prolactin-inducible protein OS=Homo sapiens GN=PIP PE=1 SV=1	P12273	17 kDa	1.3	10	13
Mucin-5B OS=Homo sapiens GN=MUC5B PE=1 SV=3	Q9HC84	596 kDa	19		19
BPI fold-containing family B member 1 OS=Homo sapiens GN=BPIFB1 PE=2 SV=1	Q8TDL5	52 kDa	2.3	6	14
Deleted in malignant brain tumors 1 protein OS=Homo sapiens GN=DMBT1 PE=1 SV=2	Q9UGM3	261 kDa	9	2	18
Basic salivary proline-rich protein 1 OS=Homo sapiens GN=PRB1 PE=1 SV=2	P04280	39 kDa	0	20	

Ig lambda-2 chain C regions OS=Homo sapiens GN=IGLC2 PE=1 SV=1	P0CG05 (+1)	11 kDa	2.8	5	14
Phosphoglycerate kinase 1 OS=Homo sapiens GN=PGK1 PE=1 SV=3	P00558	45 kDa	8.5	2	17
Alpha-enolase OS=Homo sapiens GN=ENO1 PE=1 SV=2	P06733	47 kDa	1.6	7	11
Serpin B3 OS=Homo sapiens GN=SERPINB3 PE=1 SV=2	P29508	45 kDa	0.3	13	4
Arginase-1 OS=Homo sapiens GN=ARG1 PE=1 SV=2	P05089	35 kDa	0.2	13	3
Hornerin OS=Homo sapiens GN=HRNR PE=1 SV=2	Q86YZ3	282 kDa	0.07	15	1
Fructose-bisphosphate aldolase A OS=Homo sapiens GN=ALDOA PE=1 SV=2	P04075	39 kDa	INF		16
6-phosphogluconate dehydrogenase, decarboxylating OS=Homo sapiens GN=PGD PE=1 SV=3	P52209	53 kDa	INF		16
Histone H4 OS=Homo sapiens GN=HIST1H4A PE=1 SV=2	P62805	11 kDa	INF		16
Antileukoproteinase OS=Homo sapiens GN=SLPI PE=1 SV=2	P03973	14 kDa	1.5	6	9
Cystatin-A OS=Homo sapiens GN=CSTA PE=1 SV=1	P01040	11 kDa	1	7	7
Dermcidin OS=Homo sapiens GN=DCD PE=1 SV=2	P81605	11 kDa	0.4	10	4

Cystatin-C OS=Homo sapiens GN=CST3 PE=1 SV=1	P01034	16 kDa	13	1	13
Apolipoprotein A-I OS=Homo sapiens GN=APOA1 PE=1 SV=1	P02647	31 kDa	INF		14
Peptidyl-prolyl cis-trans isomerase B OS=Homo sapiens GN=PPIB PE=1 SV=2	P23284	24 kDa	12	1	12
Myeloblastin OS=Homo sapiens GN=PRTN3 PE=1 SV=3	P24158	28 kDa	12	1	12
Desmocollin-1 OS=Homo sapiens GN=DSC1 PE=1 SV=2	Q08554	100 kDa	0.3	9	3
Ig gamma-3 chain C region OS=Homo sapiens GN=IGHG3 PE=1 SV=2	P01860	41 kDa	2.8	6	17
Ig mu chain C region OS=Homo sapiens GN=IGHM PE=1 SV=3	P01871	49 kDa	11	1	11
Ig alpha-2 chain C region OS=Homo sapiens GN=IGHA2 PE=1 SV=3	P01877	37 kDa	2.5	11	28
Histone H2B type 1-K OS=Homo sapiens GN=HIST1H2BK PE=1 SV=3	O60814 (+8)	14 kDa	INF		12
Serotransferrin OS=Homo sapiens GN=TF PE=1 SV=3	P02787	77 kDa	INF		12
Profilin-1 OS=Homo sapiens GN=PFN1 PE=1 SV=2	P07737	15 kDa	INF		12
Leukocyte elastase inhibitor OS=Homo sapiens GN=SERPINB1 PE=1 SV=1	P30740	43 kDa	INF		12
Myeloid cell nuclear differentiation antigen	P41218	46 kDa	INF		12

OS=Homo sapiens GN=MNDA PE=1 SV=1					
Cystatin-D OS=Homo sapiens GN=CST5 PE=1 SV=1	P28325	16 kDa	4.5	2	9
14-3-3 protein zeta/delta OS=Homo sapiens GN=YWHAZ PE=1 SV=1	P63104	28 kDa	10	1	10
Gasdermin-A OS=Homo sapiens GN=GSDMA PE=2 SV=4	Q96QA5	49 kDa	0.2	9	2
Complement C3 OS=Homo sapiens GN=C3 PE=1 SV=2	P01024	187 kDa	INF		11
Fibrinogen beta chain OS=Homo sapiens GN=FGB PE=1 SV=2	P02675	56 kDa	INF		11
Plastin-2 OS=Homo sapiens GN=LCP1 PE=1 SV=6	P13796	70 kDa	INF		11
Matrix metalloproteinase-9 OS=Homo sapiens GN=MMP9 PE=1 SV=3	P14780	78 kDa	INF		11
Caspase-14 OS=Homo sapiens GN=CASP14 PE=1 SV=2	P31944	28 kDa	0.2	8	2
Cystatin-S OS=Homo sapiens GN=CST4 PE=1 SV=3	P01036	16 kDa	6.2	4	25
Transketolase OS=Homo sapiens GN=TKT PE=1 SV=3	P29401	68 kDa	9	1	9
Cathepsin G OS=Homo sapiens GN=CTSG PE=1 SV=2	P08311	29 kDa	INF		10
Moesin OS=Homo sapiens GN=MSN PE=1 SV=3	P26038	68 kDa	INF		10

Serpin B12 OS=Homo sapiens GN=SERPINB12 PE=1 SV=1	Q96P63	46 kDa	0	10	
Peroxiredoxin-1 OS=Homo sapiens GN=PRDX1 PE=1 SV=1	Q06830	22 kDa	2	3	6
Neutrophil defensin 1 OS=Homo sapiens GN=DEFA1 PE=1 SV=1	P59665 (+1)	10 kDa	3.5	2	7
Ig gamma-2 chain C region OS=Homo sapiens GN=IGHG2 PE=1 SV=2	P01859	36 kDa	2.6	7	18
Cystatin-B OS=Homo sapiens GN=CSTB PE=1 SV=2	P04080	11 kDa	8	1	8
Hemoglobin subunit beta OS=Homo sapiens GN=HBB PE=1 SV=2	P68871	16 kDa	8	1	8
Secreted frizzled-related protein 1 OS=Homo sapiens GN=SFRP1 PE=1 SV=1	Q8N474	35 kDa	0.1	8	1
Cathepsin D OS=Homo sapiens GN=CTSD PE=1 SV=1	P07339	45 kDa	0	9	
Heat shock protein beta-1 OS=Homo sapiens GN=HSPB1 PE=1 SV=2	P04792	23 kDa	1.7	3	5
Immunoglobulin J chain OS=Homo sapiens GN=IGJ PE=1 SV=4	P01591	18 kDa	7	1	7
Filaggrin-2 OS=Homo sapiens GN=FLG2 PE=1 SV=1	Q5D862	248 kDa	0.1	7	1
Myosin-9 OS=Homo sapiens GN=MYH9 PE=1 SV=4	P35579	227 kDa	7	1	7

Eosinophil cationic protein OS=Homo sapiens GN=RNASE3 PE=1 SV=2	P12724	18 kDa	INF		7
Alpha-1-antitrypsin OS=Homo sapiens GN=SERPINA1 PE=1 SV=3	P01009	47 kDa	INF		8
Gelsolin OS=Homo sapiens GN=GSN PE=1 SV=1	P06396	86 kDa	INF		8
Fatty acid-binding protein, epidermal OS=Homo sapiens GN=FABP5 PE=1 SV=3	Q01469	15 kDa	0.8	4	3
Clusterin OS=Homo sapiens GN=CLU PE=1 SV=1	P10909	52 kDa	0.4	5	2
Catalase OS=Homo sapiens GN=CAT PE=1 SV=3	P04040	60 kDa	0.8	4	3
Cathelicidin antimicrobial peptide OS=Homo sapiens GN=CAMP PE=1 SV=1	P49913	19 kDa	INF		6
High mobility group protein B2 OS=Homo sapiens GN=HMGB2 PE=1 SV=2	P26583	24 kDa	INF		7
Coronin-1A OS=Homo sapiens GN=CORO1A PE=1 SV=4	P31146	51 kDa	INF		7
Cysteine-rich secretory protein 3 OS=Homo sapiens GN=CRISP3 PE=1 SV=1	P54108	28 kDa	INF		7
Glutathione S-transferase P OS=Homo sapiens GN=GSTP1 PE=1 SV=2	P09211	23 kDa	1	3	3
Lipocalin-1 OS=Homo sapiens GN=LCN1 PE=1 SV=1	P31025	19 kDa	2	2	4
BPI fold-containing family B member 2 OS=Homo sapiens GN=BPIFB2 PE=1 SV=2	Q8N4F0	49 kDa	5	1	5

Galectin-7 OS=Homo sapiens GN=LGALS7 PE=1 SV=2	P47929	15 kDa	0.2	5	1
Heat shock 70 kDa protein 1A/1B OS=Homo sapiens GN=HSPA1A PE=1 SV=5	P08107	70 kDa	5	1	5
Alpha-2-macroglobulin OS=Homo sapiens GN=A2M PE=1 SV=3	P01023	163 kDa	2	2	4
Hemoglobin subunit alpha OS=Homo sapiens GN=HBA1 PE=1 SV=2	P69905	15 kDa	5	1	5
Actin-related protein 2/3 complex subunit 3 OS=Homo sapiens GN=ARPC3 PE=1 SV=3	O15145	21 kDa	INF		6
Kallikrein-10 OS=Homo sapiens GN=KLK10 PE=1 SV=3	O43240	30 kDa	INF		6
Cystatin-SA OS=Homo sapiens GN=CST2 PE=1 SV=1	P09228	16 kDa	INF		16
Histone H2A type 1 OS=Homo sapiens GN=HIST1H2AG PE=1 SV=2	P0C0S8 (+6)	14 kDa	INF		6
Bactericidal permeability-increasing protein OS=Homo sapiens GN=BPI PE=1 SV=4	P17213	54 kDa	INF		6
Azurocidin OS=Homo sapiens GN=AZU1 PE=1 SV=3	P20160	27 kDa	INF		6
Peroxiredoxin-6 OS=Homo sapiens GN=PRDX6 PE=1 SV=3	P30041	25 kDa	INF		6

Protein-arginine deiminase type-4 OS=Homo sapiens GN=PADI4 PE=1 SV=2	Q9UM07	74 kDa	INF		6
14-3-3 protein sigma OS=Homo sapiens GN=SFN PE=1 SV=1	P31947	28 kDa	INF		9
BPI fold-containing family A member 1 OS=Homo sapiens GN=BPIFA1 PE=1 SV=1	Q9NP55	27 kDa	1.5	2	3
Polyubiquitin-B OS=Homo sapiens GN=UBB PE=1 SV=1	P0CG47 (+3)	26 kDa	1.5	2	3
Thioredoxin OS=Homo sapiens GN=TXN PE=1 SV=3	P10599	12 kDa	0.7	3	2
Peroxiredoxin-2 OS=Homo sapiens GN=PRDX2 PE=1 SV=5	P32119	22 kDa	0.2	4	1
Sulfhydryl oxidase 1 OS=Homo sapiens GN=QSOX1 PE=1 SV=3	O00391	83 kDa	INF		5
L-lactate dehydrogenase A chain OS=Homo sapiens GN=LDHA PE=1 SV=2	P00338	37 kDa	INF		5
Fibrinogen gamma chain OS=Homo sapiens GN=FGG PE=1 SV=3	P02679	52 kDa	INF		5
Peroxiredoxin-5, mitochondrial OS=Homo sapiens GN=PRDX5 PE=1 SV=4	P30044	22 kDa	INF		5
Actin-related protein 3 OS=Homo sapiens GN=ACTR3 PE=1 SV=3	P61158	47 kDa	INF		5

Small proline-rich protein 3 OS=Homo sapiens GN=SPRR3 PE=1 SV=2	Q9UBC9	18 kDa	INF		5
Annexin A3 OS=Homo sapiens GN=ANXA3 PE=1 SV=3	P12429	36 kDa	INF		5
Protein S100-A7 OS=Homo sapiens GN=S100A7 PE=1 SV=4	P31151	11 kDa	3	1	3
Cofilin-1 OS=Homo sapiens GN=CFL1 PE=1 SV=3	P23528	19 kDa	3	1	3
Alpha-1-antichymotrypsin OS=Homo sapiens GN=SERPINA3 PE=1 SV=2	P01011	48 kDa	3	1	3
Synaptic vesicle membrane protein VAT-1 homolog OS=Homo sapiens GN=VAT1 PE=1 SV=2	Q99536	42 kDa	3	1	3
Proteasome subunit beta type-1 OS=Homo sapiens GN=PSMB1 PE=1 SV=2	P20618	26 kDa	INF		4
Heat shock protein HSP 90-alpha OS=Homo sapiens GN=HSP90AA1 PE=1 SV=5	P07900	85 kDa	INF		4
Peptidyl-prolyl cis-trans isomerase A OS=Homo sapiens GN=PPIA PE=1 SV=2	P62937	18 kDa	INF		4
Neutrophil elastase OS=Homo sapiens GN=ELANE PE=1 SV=1	P08246	29 kDa	INF		4
Protein S100-A12 OS=Homo sapiens GN=S100A12 PE=1 SV=2	P80511	11 kDa	INF		4
Ras-related C3 botulinum toxin substrate 2 OS=Homo	P15153 (+1)	21 kDa	INF		4

sapiens GN=RAC2 PE=1 SV=1					
Histone H3.1 OS=Homo sapiens GN=HIST1H3A PE=1 SV=2	P68431 (+3)	15 kDa	INF		4
Metalloproteinase inhibitor 1 OS=Homo sapiens GN=TIMP1 PE=1 SV=1	P01033	23 kDa	INF		3
Semenogelin-1 OS=Homo sapiens GN=SEMG1 PE=1 SV=2	P04279	52 kDa	0	3	
IgGFc-binding protein OS=Homo sapiens GN=FCGBP PE=1 SV=3	Q9Y6R7	572 kDa	INF		3
Adenylyl cyclase-associated protein 1 OS=Homo sapiens GN=CAP1 PE=1 SV=5	Q01518	52 kDa	INF		3
Alpha-1-acid glycoprotein 1 OS=Homo sapiens GN=ORM1 PE=1 SV=1	P02763	24 kDa	INF		3
DnaJ homolog subfamily C member 3 OS=Homo sapiens GN=DNAJC3 PE=1 SV=1	Q13217	58 kDa	0	3	
Haptoglobin OS=Homo sapiens GN=HP PE=1 SV=1	P00738	45 kDa	INF		3
Neutrophil gelatinase-associated lipocalin OS=Homo sapiens GN=LCN2 PE=1 SV=2	P80188	23 kDa	INF		3
UPF0762 protein C6orf58 OS=Homo sapiens GN=C6orf58 PE=1 SV=2	Q6P5S2	38 kDa	INF		3
Serpin A12 OS=Homo sapiens GN=SERPINA12 PE=2 SV=1	Q8IW75	47 kDa	0	3	

Ezrin OS=Homo sapiens GN=EZR PE=1 SV=4	P15311	69 kDa	INF		9
Serum amyloid A-1 protein OS=Homo sapiens GN=SAA1 PE=1 SV=1	P0DJI8	14 kDa	0	3	
Striated muscle preferentially expressed protein kinase OS=Homo sapiens GN=SPEG PE=1 SV=4	Q15772	354 kDa	0	3	

Appendix X: Protocol of recruiting erosion patients

PROTOCOL TITLE:
Impact of dietary advice on the progression of tooth wear

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CONTENTS

Study Synopsis

Title of clinical trial	Impact of dietary advice on the progression of tooth wear
Protocol Short Title/Acronym	RCT on diet and tooth wear
Study Phase if not mentioned in title	n/a
Sponsor name	Kings College London Guy's and St Thomas's Trust
Chief Investigator	David Bartlett
REC number	To be notified
Medical condition or disease under investigation	Tooth wear
Purpose of clinical trial	RCT on the effect of dietary intervention and progression of tooth wear
Primary objective	Assess the impact of diet advice intervention in achieving a dietary change
Secondary objective (s)	Assess the change in the rate of tooth wear following diet advice
Trial Design	Randomised clinical trial
Endpoints	tbn
Sample Size	60
Summary of eligibility criteria	Tooth wear
Version and date of final protocol	
Version and date of protocol amendments	

1**. Background & Rationale**

The prevalence and severity of tooth wear in the UK is increasing [Adult Dental Health Survey 2009]. The most common cause of tooth wear is derived from acids, which damage the enamel surface through a process known as dental erosion [Larsen, 1990]. The acids may be derived from either dietary or gastric sources, the former being probably the most common cause and the latter being the most severe. Tooth wear is known to pass through active and inactive phases (Rodriguez *et al.*, 2012) however, the origins of the acid are believed not to be mutually exclusive and can behave together or independently. Previous work has shown that patients presenting with reflux or gastric acid causing tooth wear have more active and more destructive outcome (Bartlett *et al.*, 1997) (Rodriguez *et al.*, 2012).

Severe erosive tooth wear reduces the lifespan of affected teeth and can reduce the oral health related quality of life of affected individuals [Al Omiri et al 2006]. Despite this, there is a knowledge deficit as to how best to prevent or slow the disease [DoH Evidence Based Toolkit for Prevention 2009]. To date there have been no studies investigating dietary intervention to prevent dental erosion. A recent literature review revealed that dietary advice is rarely provided by practitioners and called for an investigation into dietary advice specific to the prevention of acid erosion (Franki, Hayes, & Taylor, 2014). A Cochrane review (Harris, Gamboa, Dailey, & Ashcroft, 2012) investigated dietary interventions to change dental behaviour and found that there is evidence suggesting that one-to-one dietary interventions in the dental setting can change behaviour but more rigorous trials need to be carried out.

Rodriguez et al (2012) measured progression by super-imposing data sets of all teeth and compared step heights against internal mathematically calculated reference points over time. The occlusal surface of each tooth was scanned sequentially every 6 months. The topographical data set for each tooth was then compared against each other to determine the presence of any change. Any change in step height from the same tooth indicated progression of wear. In this study we will assess the progression of tooth wear on upper first molars and upper central incisors, which are key teeth for tooth wear progression.

2 Trial Objectives, Design and Statistics

2.1. Trial Objectives

The aim of this research is to investigate the impact of dietary advice on the progression of tooth wear over 6 months using a randomised clinical trial design. The null hypothesis is that dietary advice does not impact the progression of tooth wear.

The primary objective is to measure a change in diet following specific dietary advice.
The second objective is to assess the change in the rate of tooth wear.
The third objective is to assess if the change in diet will affect the saliva of the patient.

2.2 Trial Design & Flowchart

This is a randomised clinical trial assessing the impact of dietary information on patients presenting with signs of tooth wear.

Based on previous work, a sample size of 60 patients will be recruited separately from the epidemiology study following informed consent. Patients presenting with moderate to severe tooth wear will be recruited. Those with a Basic Erosive tooth wear Examination (BEWE) cumulative score greater than or equal to 8 but with at least one score of 3 on the occlusal surfaces of the lower molars or the incisal edge of the upper central incisor. After randomisation, an impression will be taken of the upper and lower teeth using a silicone material. One group will receive one-to-one dietary advice (with dietary information sheets) as the intervention and the other group will not receive any intervention. The silicone impressions will be repeated 6 months later. Impressions will be cast in stone and the occlusal surfaces of the upper or lower molars and the buccal surface of the upper central and lateral incisors will be scanned to monitor tooth wear progression.

Measurement of tooth wear

Silicone putty/wash impressions (Aquasil, DENTSPLY Caulk, Milford, Del., USA) will be made of participants mouths to measure tooth wear of index teeth. The silicone impressions will be converted to moonstone type IV dental stone (Bracon Ltd., Etchingham, UK) which will then be scanned using a non-contacting laser profilometer (Xyris 2000TL, TaiCaan, Southampton, UK) accurate to 1.3 microns and repeatable to 1.6 microns. Tooth wear will be measured by superimposition of sequential scans using Geomagic Qualify 11 surface matching software (Geomagic Inc., Morrisville, N.C., USA). Scans will be cleaned, transformed into a computer-aided design format and superimposed using a best fit alignment algorithm. The software randomly selects and aligns 300 data points to find regions of best fit. After this rough alignment, fine alignment using 1,000 additional data points will be automatically performed until differences in the Z axis are minimised. The two scans are then superimposed allowing three-dimensional comparisons between surfaces, using individual data points that do not alter in the Z axis as reference points. To measure tooth wear, a digital mesh of measuring points, separated 1 mm on the X, Y and Z axes, are laid over the superimposed surfaces. Each digital point measures a circle around it of 1 mm in diameter. Thus the point mesh covers the majority of the superimposed surfaces. Tooth wear in microns will then be measured by calculating the mean of all points.

Measurement of saliva

The patient will be asked to chew a piece of flavourless chewing gum. They will then be asked to expectorate into a vial for 5 minutes. A filter paper shall be placed against two of their teeth to measure the thickness of their saliva. Salivary content will be analysed.

	Screen Visit	Day 1	6 months
1. Patient informed and information given (10 minutes)	X		
2. Consent (10 minutes)		x	
3. Physical examination (10 minutes)		x	x
4. Dietary Questionnaire (10 minutes)		x	x
5. Dietary intervention for half the group (10 minutes)		x	x
6. Impression of teeth (10 minutes)		x	x
7. Saliva collection (5 minutes)		x	x
8. Filter paper placed on two teeth (5 minutes)		x	x

Dietary intervention
Computer-generated numbers will randomise the 60 patients into two groups based on the dietary intervention. All patients will be asked a dietary assessment questionnaire to assess current acid intake and this will be repeated at the end of the study and the results compared. One group of patients will receive non-individualised dietary advice which is the current standard of care. The other group will receive a detailed chair-side dietary analysis and an individualised plan to target their most destructive behaviour. They will get this to take home with them as well as a dietary information leaflet.
All patients will be encouraged to consume a healthy diet.
At the end of the study those participants not receiving the dietary intervention will be given the same dietary advice, specific plan and information leaflet.

2.4 Trial Statistics
Impressions of teeth, taken at two time points, at start and six months later will be cast. The occlusal surface of the lower molar and the incisal/ buccal surface of the upper central incisor will be scanned by an operator, who is blinded to the clinical condition using non-contacting surface profilometers. The gold standard for the measurement of tooth wear using this scanner is step height. Data points from surface maps will be superimposed and compared to the baseline map and then step height changes and surface roughness outputs described as mean and volume step height per tooth. Summary data compared between individuals will give progression of tooth wear for the cohort.

2.4.1 Sample Size

Previous research investigated tooth wear progression in 60 patients and more recent research investigated tooth surface roughness in 30 patients [Rodriguez, 2012]. Based on the data from this research, this study aims to recruit up to 60 subjects with tooth wear. The subjects will be divided into two groups (one group with a dietary intervention plan and one group without a dietary intervention plan). The total time needed for the study will be 24 months to collect the data.
Sample size calculation for this study was carried out to find the effect size based on the selected sample of 60 using independent samples t test for comparing control and dietary advice groups for the difference in step height (tooth wear) at baseline and post treatment (after 6 months of dietary advice) time points. With this total sample size of 60 (30 control and 30 dietary advice group), the study with 80% power at 5% level of significance will be able to detect the difference between the two groups with an effect size of 0.74 using independent samples t test. The power calculation for this study was carried out using Gpower 3.1.

2.4.2 Randomisation Procedures
A list of 60 patients will be selected based on inclusion/exclusion criteria. All of these patients will be given unique identifier numbers from one to sixty. 30 patients will receive the dietary advice intervention and the remaining 30 will receive the current standard of care. Patients will be allocated to these groups based on simple random sampling procedure (SRS). Random numbers will be generated using Excel software and patients will be allocated accordingly to these two groups. The clinical investigator will be responsible for the randomisation procedure, allocating patients to the groups and documenting this within an enrolment log.

2.4.3 Analysis
Basic data from the surface profiles will be uploaded into geo-magic software to analyse, using superimposition software the vertical step height data over the 6 month period.

Descriptive statistics will be used to define the sample characteristics and the step height for the two groups. The change in step height between the two groups will be compared using independent samples t test. Linear models may be used to find out the significant predictors of change in step height. Other relevant analyses will be carried out if necessary. All the analyses will be carried out using SPSS version 21.

3. Selection and Withdrawal of Subjects

3.1 Inclusion Criteria

Severe tooth wear with a BEWE score of 3 on the occlusal surface of the first lower molars or incisal/buccal surface of the upper central incisor.
This wear will be as a result of a high acid diet i.e. as at least two dietary acidic challenges a day.
Adult 25-70 years old.
Minimum of at least 10 occluding tooth pairs (i.e. at least 10 upper teeth which bite against 10 lower teeth) – including the opposing upper molars and lower incisors
No anterior crowns/ bridges or implants opposing the lower molars or upper incisors
Written consent to the study

3.2 Exclusion Criteria

Pregnancy or breast feeding
Medical history likely to impact on attendance or mobility
Presence of periodontal disease or caries on more than one tooth. BPE score of 2 or above.
Unable to speak or understand English
Saliva diagnoses (xerostomia- dry mouth)
Orthodontic appliances
Severe dentine hypersensitivity
Restoration of the occlusal or incisal surfaces of upper anterior teeth and first molars.
Have factors which could contraindicate their participation, such as any condition requiring the need for antibiotic premedication prior to a dental treatment, a condition requiring the need for long-term antibiotic use, blood thinning medications that prohibit the safe conduct of a dental cleaning or previous use of the weight loss medications.
Participation in other research within 30 days
Preferring restoration of their teeth rather than dietary intervention

3.3 Withdrawal of Subjects

Data from subjects who fail to attend for the 2nd visit will be discarded as the data will be incomplete.

4 Assessment of Efficacy

The primary outcome of the study will be to assess whether a dietary change occurs as a result of enhanced dietary advice. The diet questionnaires taken at the start and end of the 6 month trial will be compared to establish what changes to the diet occurred. The second outcome is to compare the wear/change on the teeth from the intervention group compared to the controls. The median/mean change in wear over the time will be compared with teeth and by subject to assess the impact of dietary change.

4.1 Efficacy Parameters

Step height measurement on tooth surfaces

8. Ethics & Regulatory Approvals

Ethical journey

Potential patients seen on specialized tooth wear clinic held at Guy's Dental Hospital will be issued with a patient information sheet at that time. Of those who elect not to have their teeth restored will be informed of the study. Not all patients elect or prefer to have restorations to cover their teeth from future wear. Their clinical decision to participate in the research will

not be affected by the decision to participate in the study. Consent will be taken from participants agreeing to the study after at least 24 hours at a separate appointment. After consent a silicone impression will be taken with a silicone material (low viscosity, Aquasil, Dentsply UK).

From an ethical perspective there is some concern about the delivery of individualised dietary advice to one group and not the other. The RCT is designed to test the hypothesis that patients with tooth wear will alter their diet when given individualised specific advice. There is no evidence in the literature to suggest that dietary intervention will prevent progression of tooth wear and this has not been tested on patients. This is the first study to utilise a dietary intervention. Based on previous work progression of tooth wear can range from 15-100um over one year and a sample size of [Rodriguez, 2012 #1798]. Even at the highest levels of progression this will not significantly risk the longevity of the teeth. Even for those patients with 0.1mm of progression in one year the time needed to remove 2mm of tooth will be 40 years. Therefore an additional exclusion criterion will be that for those patients in this study the lowest age limit will be 35years old. This is likely to slightly influence recruitment but from an ethical perspective it is safer. If any patient develops tooth wear quicker than that then they will be withdrawn from the study but in 20 years of clinical experience I have never seen tooth wear progress so fast that it is clinically detectable by the naked eye. Multiple other authors have reached the same conclusion (Pintado, Anderson et al, 1997, Lambrechts, Braem et al, 1989) There is no way of assessing what dietary advice has occurred prior to the recruitment. In theory the referring dentist may have given advice or the patient may have found information on the web. However, this investigation tests a planned dietary intervention in the form of an individual plan by a trained professional and dietary advice information sheets.

9. Quality Assurance, Data Handling, Publication Policy and Finance

The impressions will be categorised and stored and scanned within 24 hours.

10. Signatures

To be signed by Chief Investigator minimum and statistician if applicable.

____ _____

_____

Chief Investigator Date 14/7/14

Print name BARTLETT

_____

_____

Statistician Date

Print name

Appendix XI: Patient Information Sheet for recruiting erosion patients

Patient information sheet (Version 2 Dated 24th September 2014)

Title of project: Impact of dietary advice on the progression of tooth wear

REC ref 14/EM/1171
Investigator: Professor David Bartlett

Part 1

Invitation paragraph

You are being invited to participate in this research study to assess how dietary advice impacts on the progression of your tooth wear. Before you decide it is important for you to understand why the research is being done and what it will involve:
Please take time to read the following information carefully. Ask us if there is anything that is not clear. Talk to others about the research if you wish and if you would like to ask the research team any further questions please contact:
Dr Saoirse O'Toole or Professor David Bartlett 02071885390 emails Saoirse.otoole@kcl.ac.uk or david.bartlett@kcl.ac.uk

What is the purpose of the study?

Dental erosion is a condition that erodes or dissolves teeth. Acids present in the diet can result in gradual softening and destruction of teeth. The condition is relatively common with upwards of 30% of European Adults showing some signs. The aim of this study is to assess the effectiveness of a dietary intervention and to measure the rate of tooth wear/dental erosion over a 6 month period.
We plan to recruit up to 60 participants to take part in a randomised controlled trial. This means you will be randomly placed into one of two groups, of which 30 will receive basic dietary advice and the other 30 will receive special dietary advice. The choice of which group you will be asked to join will be randomly decided by a computer. Therefore you might be asked to join the group given special dietary information or not.
You will be asked to participate in the study if the level of your tooth wear is not severe enough to justify re-building your teeth or that you choose not to re-build your teeth. If at the end of the study you choose to consider re-building your teeth your standard of care will be unaffected. It is entirely up to you to choose whether or not to participate in the study.

Why have I been chosen?

You have been asked to consider this study if you have signs of erosive tooth wear/dental erosion on your teeth. You will be consuming more than 2 intakes of acidic foods each day. The level of tooth wear will not be severe but there will be clinical signs visible to a Dentist. You will have more than 20 teeth in total and be between 35-70 years old. You will be prepared to have two dental impressions taken of your teeth and come and visit the clinic twice over a 6 month period.

Do I have to take part?

No, it is up to you decide whether or not to take part. If you do, you will be given this information sheet to keep and be asked to sign a consent form. You are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive.

What will happen to me if I decide to take part?

If you agree to participate in this study, you will be asked to read and sign this Research Participant Information Sheet and Consent Form before any study procedures begin. You will be given a copy of this Research Participant Information Sheet and Consent Form to keep.
To complete this study you will need to attend the study site on 3 occasions, including your screening visit and two more visits spread over 6 months.

1. Screening visit: (10 minutes) You will be asked to participate in the study. Information will be given to you about the study. You will be given time to ask any questions and decide if you choose to take part.

2. Second visit: (approximately 50 minutes). Following an explanation of the study we will ask you give signed consent to take part in this study. Your gender, date of birth and race will be recorded and a suitably qualified member of staff will take a medical history from you. The dentist will examine your mouth and then ask you a set of standard questions to make sure you can take part in the study. You will be asked for a sample of your saliva over 5 minutes. Very small filter papers will be lightly pressed against two of your teeth to further examine your saliva. Afterwards, they will take an impression (rubber mould) of your teeth so we can measure how much your teeth will wear over a 6 month period. All participants will be asked a questionnaire at the beginning of the study and at the end to establish your current dietary habits. This will take around 10 minutes. For half of the participants (randomly selected) you will be given dietary advice which is the current standard of care. The other half will be given a dietary advice plan. This will be in the form of a written and verbal plan to take home, as well as a dietary leaflet. This should not take more than 10 minutes and you will not receive any further information following this session

3. After a period of 6 months you will be asked to return. At that visit we will check to see that you remain dentally healthy, take the final impression of your teeth and repeat the salivary tests and the questionnaire. This will take around 35 minutes. The dietary advice plan and information leaflets will then be given to the group which did not receive them at the start of the study (10 minutes).

Expenses and payment

After the follow-up examination, at the end of this study you will receive £150 for your participation and to cover any out-of-pocket expenses. The money obtained from this study must be declared for tax and benefit purposes. If for any reason you do not complete the study, the sum you receive will be in proportion to the time you have committed to the study.

Is there anything I should or should not do?

You will be given as much time to think about this taking part in the study as needed. Normally this involves at least 24 hours to consider this project before it is started.
Once you have agreed to participate in the study, you will continue your lifestyle without any further interventions. If you become pregnant or become seriously ill during the investigation you may continue if you so choose but there is no obligation.

Are there any side effects?

We do not know how quickly teeth wear and this study will help us understand the process better. Over 6 months it is highly unlikely you will notice any difference in the appearance of your teeth. In similar studies we have calculated that the amount of wear likely to occur is around the thickness of a hair follicle. Our instruments can detect this level of wear but provided it remains around this level there will be no long term impact on your teeth.

Having dental impressions is a routine part of dentistry and many of you will have had them before. There are no known side effects but it can be a little uncomfortable keeping your mouth open whilst the material sets.

Are there any benefits in taking part?

There is no direct, immediate benefit to you from taking part in this research study. However, you will have helped the dental profession gain a better understanding of how teeth wear over time.

What happens when the research study ends?

When the study has finished your participation in the study ends and you return to your Dentist for continuing care.

What if there is a problem?

Any complaint about the way you have been dealt with during the study or any possible harm you might suffer will be addressed.

What if relevant new information becomes available?

Sometimes during the course of a research project, new information becomes available about the treatment that is being studied. If this happens, your research dentist will tell you about it and discuss whether you want to or should continue the study. If you decide to continue in the study you will be asked to sign an updated consent form. Also, on receiving new information your research dentist might consider it to be in your best interests to withdraw you from the study and he/she will explain the reasons why. If the study is stopped for any other reason, you will be informed why.

What if there is a problem?

If you have any concern about any aspect of this study, you should ask to speak with the researchers who will do their best to answer their questions. Please contact Prof. David Bartlett: email david.bartlett@kcl.ac.uk and phone 0207 188 5390

If you have a complaint, you should talk to your research doctor who will do their best to answer your questions. If you remain unhappy, you may be able to make a formal complaint through the NHS complaints procedure. Details can be obtained through the Guy's and St Thomas' Patient Advisory Liaison Service (PALS) on 0207 1887188, address: PALS, KIC, Ground floor, north wing, St Thomas' Hospital, Westminster Bridge Road, London, SE1 7EH .

What happens if something goes wrong?

If you are harmed due to someone's negligence, then you may have grounds for a legal action but you may have to pay for it. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way you have been approached or treated during the course of this study, the normal National Health Service complaints mechanisms should be available to you.

Will my taking part in this study be kept confidential?

All information will be kept strictly confidential. Your name and address and contact details will be kept in a secure room and only available to those listed in this information sheet.

What will happen to the results of the research study?

It is possible that the results of the study will be published in an internationally refereed scientific journal. Should this be the case any information about your data will be anonymised as detailed in 'Confidentiality' above. The protocol summary may be posted on a publicly available protocol register and that a summary of the study results will be posted on a publicly available results register.

Who has reviewed the study?

This study has been reviewed and given favourable ethical approval by a local Research Ethics Committee.

Intellectual property statement:

The information and any materials or items that you are given about or during the study (such as information regarding the study drug(s) or the type of study being performed) should be considered the confidential business information of the study sponsor. You are of course, free to discuss with your friends and family while considering whether to participate in this study or at any time when discussing your present or future healthcare.

Thank you for your help. If you have any further questions, please do not hesitate to ask.

REC Ref: 14/EM/1171
Patient Identification Number for this trial:

Appendix XII: Consent form for recruiting erosion patients

Title of project: Impact of dietary advice on the progression of tooth wear:
Version 2 dated 24th September 2014

Sponsored by Kings College London
Investigator: Professor David Bartlett

Name of Researcher: Professor D Bartlett

Please initial box

1. I have read and understand the information sheet (Version 1 dated 24th September 2014) for the above study and have had the opportunity to ask questions. ☐

2. I have had the opportunity to ask questions and all my questions have been answered to my satisfaction ☐

3. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. ☐

4. I understand that sections of any of my medical notes may be looked at by responsible individuals from regulatory authorities where it is relevant to my taking part in research. I give permission for these individuals to have access to my records. ☐

5. I agree to take part in the research and complete the study. ☐

_____ _____
Name of Patient Date Signature

_____ _____
Name of Person taking consent Date Signature

1 for patient; 1 for researcher; 1 to be kept with notes

Appendix XIII: Detailed protocol for in vivo salivary film and AEP collection.

The following steps are the steps will be followed when colleting the in vivo salivary film and AEP:
Note what and when the patient last ate and drank.
Identify two eroded teeth (E1 and E2) and two non-eroded teeth (U1 and U2), ideally 6 (E) and 7 (U) of each quadrant of the same arch.
From an eroded tooth – one surface (occlusal) will be recorded.
From An non-eroded tooth- one surface (occlusal) will be recorded.
Isolate the tooth
Collect the salivary film by placing a dry filter paper against the surface for 5 second.
Collect the salivary pellicle by placing a 0.5% SDS filter paper against the tooth surface for 15 seconds.
When collecting the pellicle, filter papers soaked in SDS (0.5% w/v) will be rubbed against the tooth surface using a blunt end of an instrument or the operator's finger for 15 seconds.
The pattern of collection of the samples will be as follows:
Eroded film 1(EF1) (ie Eroded tooth, Film, first surface)
Eroded pellicle1 (EP1) (ie Eroded tooth, Pellicle, first surface)
Eroded film2 (EF2) (ie Eroded tooth, Film, second surface)
Eroded pellicle2 (EP2) (ie Eroded tooth, Pellicle, second surface)

Non-eroded film 1(UF1) (ie Non-eroded tooth, Film, first surface)
Non-eroded pellicle 1(UP1) (ie Non-eroded tooth, Pellicle, first surface)
Non-eroded film 2 (UF2) (ie Ueroded tooth, Film, second surface)
Non-eroded pellicle 2 (UP2) (ie Ueroded tooth, Pellicle, second surface)

For example: for the 12th patient, we would have the following labelling system and order:

12Eroded film 1(EF1)
12Eroded pellicle1 (EP1)
12Eroded film2 (EF2)
12Eroded pellicle2 (EP2)

12Non-eroded film 1(UF1)
12Non-eroded pellicle 1(UP1)
12Non-eroded film 2 (UF2)
12Non-eroded pellicle 2 (UP2)

When collecting the samples, the clinician collecting the samples will say the type of tooth (i.e. eroded or non-eroded), the number (i.e. 1 or 2) and type of sample (i.e. film or pellicle) out loud and cross check with the sample collector to avoid any potential errors.
Each of the above samples will be collected into a universal tube, which in turn, will be placed immediately in the ice. All collected samples will then be transferred to the laboratory for processing.

An example of the labelling system for salivary film and pellicle collection from patient No. 13

Appendix XIV: List of publications in international peer-reviewed journals:

- **Mutahar, M.**, Carpenter, G., Bartlett, D., Mathew, M., and Moazzez, R., 2017. The presence of acquired enamel pellicle changes acid-induced erosion from dissolution to a softening process. *Scientific Reports* 7, 10920.

- **Mutahar, M.,** O'Toole, S., Carpenter, G., Bartlett, D., Andiappan, M. and Moazzez, R., 2017. Reduced statherin in acquired enamel pellicle on eroded teeth compared to healthy teeth in the same subjects: An in-vivo study. PloS one, 12(8), p.e0183660.

- O'toole S., Mistry M., **Mutahar M**., Moazzez R., Bartlett D. (2015) ''Sequence of stannous and sodium fluoride solutions to prevent enamel erosion''. J Dent, 43(12):1498-503.

Appendix XV: List of abstracts submitted to national and international conferences

Mutahar M., Carpenter G., Bartlett D., Moazzez R (2016). Mucin5b and albumin mediate greater protection against dental erosion than statherin (Oral/Poster presentation), ORCA meeting, July, Athens, Greece.

Introduction and Aim: Our previous data demonstrated that whole saliva (WS) provided better protection against erosion than parotid saliva (PS) after five erosion cycles. Using a profilometer, WS produced significantly less step height (4.16±0.57μm) than PS (6.41±0.71μm) ($p<0.0001$). The responsible proteins for protection are unknown. This study aimed to measure four key proteins in an in-vitro erosion model comparing WS and PS.

Methods: 30 human enamel samples were prepared and assigned to 2 groups: (WMS: n=15) and (PS: n=15); three subgroups each: control (n=5), one cycle (n=5), five cycle erosion (n=5). Samples were immersed in the corresponding saliva for 24h (control) followed by a further 30min prior to exposure to a 10min citric acid (pH 3.2) followed by 2min water rinse (one cycle). This cycle was repeated five times. Enamel pellicle was eluted using filter papers for all groups. Proteins were immunoblotted for: mucin5b, albumin, carbonic anhydrase VI (CA VI) and statherin. Antibody binding was quantified using ImageLab software using purified protein standards of known concentration (n=3) to assess quantity and reproducibility. Data were log transformed to attain normality and linear models and post hoc tests were used for the statistical analysis.

Results:

Albumin and mucin5b were more dominant in WMS pellicles than PS ($p<0.0001$) whereas CA VI and statherin were dominant in PS pellicles ($p<0.0001$). Mucin5b in WS pellicles, but absent in PS, at control [(57.5±33.3ng) significantly increased after five cycles (121.5±19.9ng) $p<0.0001$]. Statherin in PS pellicles increased after one cycle (415.8 ng ± 43.6ng) compared to control [210.4 ±25.9ng] ($P<0.0001$) but returned to control levels after five cycles (180.6 ±23.5ng).

Conclusion:

The greater resistance of WMS pellicles to enamel erosion compared to PS pellicles relates to protein composition. Thus Mucin5b and albumin-rich pellicles gave better protection than carbonic anhydrase and statherin-rich pellicles.

Mucin5b and Albumin Mediate Greater Protection Against Dental Erosion Than Statherin

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Introduction

Pellicle protects against dental erosion (1-3). Our previous data demonstrated that whole mouth saliva (WMS) provided better protection against erosion than parotid saliva (PS) in both early and advanced erosion. After one cycle erosion, Knoop microhardness showed that WMS had significantly greater microhardness change (98.68±17.5KHN) than PS (85.19±22.07KHN (p=0.002). Using a profilometer after five cycles, WMS produced significantly less step height (4.16±0.57µm) than PS (6.41±0.71µm) (p<0.0001). The responsible proteins for such protection are unknown.

Objective

This study aimed to measure four key proteins in an in-vitro erosion model comparing WMS and PS.

Materials and Methods

Figure 1 Photograph of polished, taped enamel sample

- Created window of exposed enamel
- Reference area for scanning

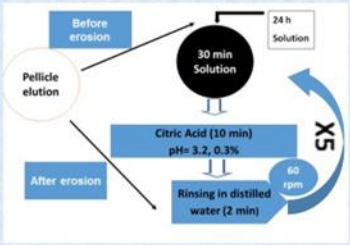

Figure 2 A flowchart representation of the one and five cycles erosion

Analytical and Measurement Techniques

- Gel electrophoresis (SDS-PAGE)
- Western blot
- ImageLab software using purified protein standards of known concentration (n=3).
- Liquid chromatography/mass spectrometry (LC/MS/MS).
- Proteome Discoverer

Stat:

- Linear models and post hoc tests

Results

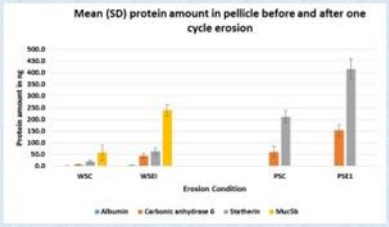

Figure 3: Amount of proteins (nanogram) in-vitro pellicle derived from PS and WMS before erosion [control] and after one cycle (EI).

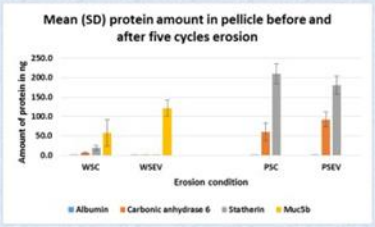

Figure 4: Amount of proteins (nanogram) in-vitro pellicle derived from PS and WMS before erosion [control (C)] and after five cycles erosion (EV).

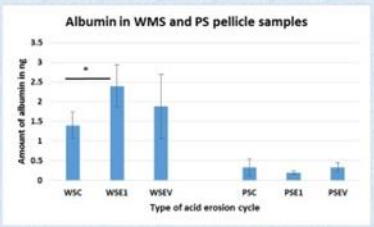

Figure 5: Amount of albumin (nanogram) in-vitro salivary pellicle derived from WMS and PS at control(C), after one (EI) and five cycles erosion(EV).

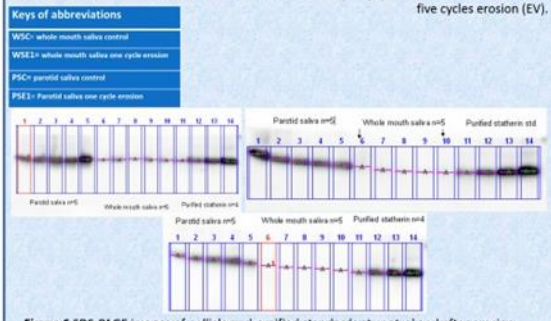

Figure 6 SDS-PAGE images of pellicle and purified standards at control and after erosion

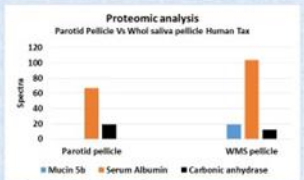

Figure 7: Fold change of three salivary proteins in WMS pellicle versus PS pellicle (n=1).

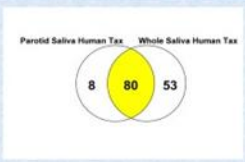

Figure 8: Venn diagram representing the number of proteins in WMS pellicle versus PS pellicle (n=1) .

Conclusion

The greater resistance of WMS pellicle to enamel erosion compared to PS pellicle relates to protein composition. Therefore, the likely mechanisms contributing to protection in both early and late erosion seem to be diffusion and buffering.

Mutahar M., Carpenter G., Bartlett D., Moazzez R (2015). Salivary proteins mediate greatest protection against dental erosion (Oral Presentation). BSODR meeting, September, Cardiff, UK.Salivary proteins mediate greatest protection against dental erosion

Abstract

Objectives: The aim is to investigate the effect of salivary ions and proteins on eroded enamel in a laboratory investigation.

Methods: 40 polished enamel specimens were prepared from extracted human teeth (Research ethics approval, Northampton REC, 14/EM/0183) and randomly assigned to 4 subgroups. 10 enamel samples per group were allocated to parotid, whole mouth, artificial saliva and water and immersed in the corresponding solution for 24 hours followed by a further 30 minutes prior to exposure to a 10-min erosion cycle in 80 ml of 0.3% pH 3.2, citric acid, agitated at room temperature, followed by 2-min water rinse. The 30 min immersion in the corresponding solution followed by the acid was repeated 5 times for all samples. Mean step height change from 5 randomly assigned points was measured using a non-contacting profilometer and Knoop microhardness measured at baseline (KHNb) and on the eroded surface of each sample (KHNe) and SMH change = (KHNb – KHNe) was calculated. Linear Regression model and Stata12.0 were used for the statistical analysis.

Results

Whole and parotid saliva produced significantly less step height (4.16±0.57 µm, 6.41±0.71 µm respectively) than artificial saliva (7.47±0.98µm) and these differences were statistically significant compared to water (10.89±0.98µm and $p < 0.0001$). Microhardness change, for whole mouth (224.11 ±29.29 KHN $p<0.0001$), parotid (208.16 ±50.20 KHN $p<0.0001$) and artificial saliva (194.0±19.75KHN $p<0.002$ was significantly greater than water (155.34±18.4 KHN). Whole mouth saliva had significantly greater microhardness change than artificial ($p<0.012$).

Conclusion: Saliva, containing proteins, appears to offer greater protection against dental erosion than artificial salvia and water. Whole mouth saliva provided less step height and greater hardness change than parotid saliva.

W*ord count: 268*

Mutahar M., Bartlett D., Mistry M., Moazzez R. (2014). Effect of Saliva on Dental Erosion (Poster presentation). IADR Pan European Regional (PER) Congress, September, Dubrovnik, Croatia.

Objectives: To assess, *in vitro*, the effect of immersion of human enamel samples in natural saliva, artificial saliva and distilled water for various time periods on dental erosion.
Methods: 90 specimens were prepared from extracted human teeth and were randomly assigned to 3 experimental groups, 30 samples per group: natural saliva (NS), artificial saliva (AS) and distilled water (DW). Within each group samples were then randomly allocated to 3 subgroups: 30 minutes immersion in solution (1), 60 min immersion (2) and 24 hours immersion followed by a further 30 minutes (3) prior to exposure to a 10-min erosion cycle. The erosion cycle consisted of 80 ml 0.3% citric acid, pH=3.2, at 22°C±1, followed by 2-min water rinse which was repeated 5 times. Step height change was measured using a non-contacting profilometer. Knoop microhardness was measured at baseline (KHNb) and for the eroded surface of each sample (KHNe) and % SMH change = (KHNb – KHNe) calculated. Two-ways ANOVA and Bonferroni tests were used for the statistical analysis.
Results: The NS and AS groups had significantly less enamel loss for all three immersion times (NS1: 6.33 µm; NS2: 5.91 µm; NS3: 3.80 µm) (AS1: 6.02 µm; AS2: 6.72 µm; AS3: 6.34 µm) compared with DW groups (DW1: 8.61 µm; DW2: 8.24 µm; DW3: 8.80 µm) ($P<0.0001$). When comparing NS with AS, there was only a significant difference between groups AS3 and NS3 ($p < 0.0001$).
A significantly greater % SMH change was observed for group NS3 (249.4±29.6KHN) compared with AS3 (181.3±31.0) and DW3 (167.1±30.3) $p<0.0001$). Within subgroups, only NS3 showed significantly less enamel loss and greater % SMH change than NS1 and NS2 ($P<0.0001$).
Conclusion: Natural saliva provided better protection against enamel loss compared with artificial saliva and water leaving a softened layer in place.

Key words: Saliva, erosion, profilometer, Knoop surface hardness.

Effect of Saliva on Dental Erosion

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Introduction

Dental erosion is caused by extrinsic or intrinsic sources. Salivary pellicle may play an important protective role against the progression of dental erosion. Some studies relate such protective effect to the mineral content whereas others have referred it to the protein components of the salivary pellicle

Objectives

To assess, in vitro, the effect of immersion of human enamel samples in natural saliva, artificial saliva and distilled water for various time periods on dental erosion.

Materials and Methods

- Microhardness tester
- Surface non-contacting profilometer

Figure 1 Photograph of polished, taped enamel sample

- Created window of exposed enamel
- Reference area for scanning

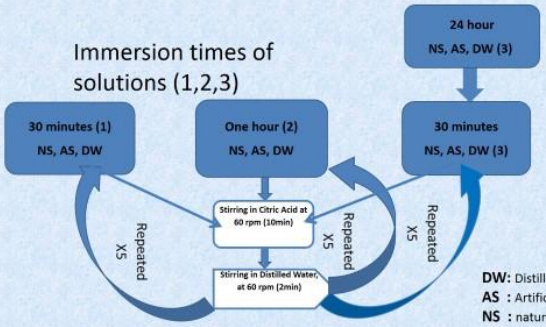

Figure 2 A flowchart representation of the erosion cycle and salivary pellicle formation protocol

DW: Distilled water
AS : Artificial saliva
NS : natural saliva
1 : 30 minutes
2 : 60 minutes
3 : 24hr followed by 30 min

Results

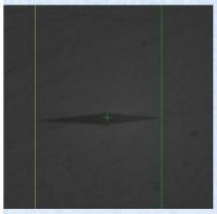

Figure 3 Image capture X 40 lens of Knoop microhardness indentations.

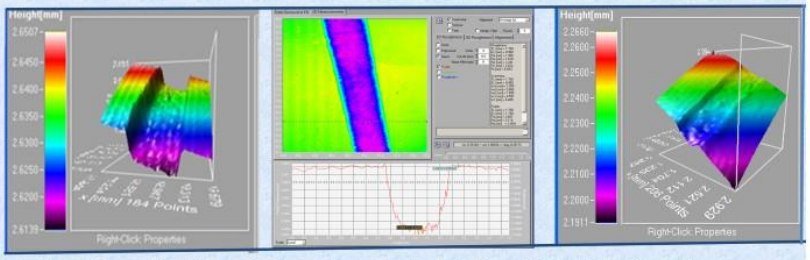

Figure 4 Representative 3D images of various treated enamel analysed using Taicaan XYRIS (Boddies) surface analysis software.

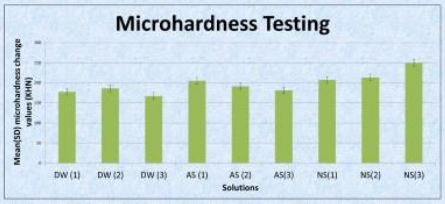

Figure 5 Mean (SD) % SMH change in Knoop microhardness units (KHN).

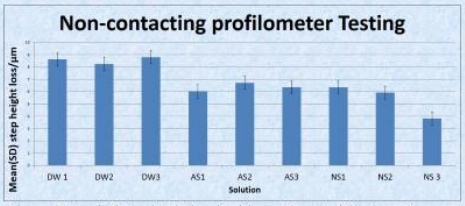

Figure 6 Mean (SD) step height loss (µm) (manual reading) for nine subgroups: three solutions (DW, AS & NS) and three immersion times (1, 2 & 3).

Conclusion

- Using a 0.3% citric acid cycling method, enamel surfaces had significantly greater microhardness change and less step height when exposed to natural saliva than to artificial salvia and distilled water.
- Enamel loss was significantly less after immersion in natural saliva for 24h , leaving an intact softened layer in place.

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