The effect of post brushing mouthrinses on salivary fluoride retention

And

The effect of varying fluoride concentration on remineralisation of bovine enamel *in vitro*

Thesis submitted in partial fulfilment for the degree Doctor of Dental Sciences (Orthodontics)

Karen Elizabeth Shaw

February 2015

Acknowledgements

I would like to take this opportunity to thank the many people that have helped me in completing this project. To my supervisors Professor Sue Higham and Dr Norah Flannigan. To Lee Cooper , for all his advice and assistance in learning laboratory skills required for the study and also to Gleb Komarov for his assistance with consenting patients for the fluoride rinse study and also for his advice on QLF-D. To Elbert de Josselin de Jong for his help in identifying and understanding the problems encountered with TMR and in interpretation of the overall results of the *in vitro* study.

A special thanks also to Nichola Shaw for proof reading and to Girvan Burnside for his statistical advice.

Finally to all the people that kept me going through the tough times, you know who you are.

Table of Contents

Index of Tables

Index of Figures

Abstracts

The effect of post-brushing mouthwashes on salivary fluoride retention.

Objective: To assess the effect of post-brushing mouthwashes on salivary fluoride retention.

Methods: This was a three phase cross over study with thirty participants. Salivary F levels were measured before brushing with a 1450 ppm F toothpaste (Time 0) and after brushing, rinsing with water and then with one of either 0,225 or 500 ppm F mouthwash at time points 1,3,5,10,20,45,60 minutes using an ion-specific electrode.

Results: Significant differences in mean fluoride retention over the 60 minute period were found for all three pair wise groups using paired t tests ($p<0.001$). A 2660% increase in salivary fluoride retention over the 60 minutes was found with the 500 ppm F mouthwash when compared with the 0 ppm F group. With the 225 ppm F group a 120% increase was found.

Conclusion: The use of a fluoride mouthwash containing 225 ppm or 500 ppm produced a significant increase in salivary fluoride retention following brushing with 1450 ppm F toothpaste and rinsing with water. The use of the 500 ppm F mouthwash may be of particular benefit to those at high caries risk including orthodontic patients.

The effect of varying fluoride concentration on remineralisation of bovine enamel *in vitro.*

Objective: To assess the effect of varying fluoride concentration on the remineralisation of bovine enamel *in vitro* using the techniques of quantitative light induced fluorescence (QLF – D), multispectral imaging (MSI) and transverse microradiography (TMR).

Methods: artificially produced enamel carious lesions were produced following immersion in demineralisation solution (pH 4.5) for 72 hours. Baseline analysis was carried out with quantitative light induced fluorescence (QLF – D), multispectral imaging (MSI) and transverse microradiography (TMR). Samples were then randomised, using a computer generated randomisation sequence to one of 5 groups for remineralisation: 0 ppm, 225 ppm, 500 ppm, 1450 ppm or 2800 ppm Fluoride. The experimental phase lasted for eight weeks with weekly imaging with QLF-D and MSI. A post remineralisation phase of TMR was then carried out.

Results: Reliable results were obtained for QLF-D and MSI only due to problems with the preparation of the baseline TMR samples. The results from QLF-D and MSI indicated a significant change in mineral loss occurred over the eight week period. Groupwise differences were shown between the 2800 ppm group and the remaining groups only. However, this was in a negative direction indicating mineral loss not gain as would have been anticipated. It is likely that a true remineralisation process was not replicated in this study. However in comparison of QLF-D and MSI techniques moderate correlation between the results was seen. MSI found greater differences and at an earlier stage than QLF-D.

Conclusion: No remineralisation effect was seen in this study. Moderate correlation between the QLF-D and MSI results was demonstrated with greater differences detected with MSI.

Literature Review

Dental Caries

Dental caries is one of the most prevalent diseases in the world and is associated with significant morbidity and potential mortality if it develops into a dental abscess (Robertson and Smith 2009). In the UK the most common reason for children to be admitted to hospital for a general anaesthetic is for the extraction of teeth affected by dental caries (Moles 1997-2006).Data obtained from 1997 to 2006 revealed that an estimated 300000 children are admitted to hospital each year for tooth extractions, a number that has risen dramatically in recent years. (The number of emergency admissions for dental extractions has also increased(Moles and Ashely, 2009)). Dental caries is an area of great public health concern as it is, in itself, an entirely preventable disease (SIGN guideline 138, 2014).

Prevalence of Dental Caries

In the 2003 Child Dental Health Survey it was found that at the age of 8 years old 57% of children have caries experience of primary teeth. At age 15 years all permanent teeth are usually erupted but already at this stage of life 49% of children have caries experience of some permanent teeth (Lader, *et al* 2004, Pitts and Harker 2004). The permanent dentition should ideally last for life and caries experience this early could indicate a poor long term prognosis for the affected teeth and result in enforced extraction later in life (Gill,*et al* 2001).

Morbidity of Dental Caries

Significant morbidity can be associated with dental caries, most notably pain, but an adverse effect on quality of life in children has also been demonstrated (Gill, *et al*2001, Low, *et al*1999). Data obtained from questionnaires of 3342 parents in the 2003 survey also evaluated the prevalence of pain and other effects related to oral conditions in children.26% of 12 year olds and 20% of 15 year old children reported pain with 5% and 7% respectively reporting an effect of oral function. Dental caries can effect oral function such as the ability to chew foods when a tooth becomes tender to touch, also if a tooth becomes sensitive to temperature, avoidance of hot or cold foods and drinks is required to minimise pain. Symptoms of this nature are indicative of advanced dental caries.

If left untreated dental caries can affect the pulp of teeth and progress into an acute dental abscess and this is when the most acute symptoms of pain can arise. If this infection spreads into the surrounding facial tissues then it can be associated with facial swelling which can spread rapidly and if left untreated can cause severe sepsis (Robertson and Smith 2009).

Definition of Dental Caries

In 1962 the World Health Organisation defined dental caries as a localised, post-eruptive, pathological process of external origin involving the softening of the hard tooth tissue and proceeding to the formation of a cavity (WHO 1962).The process of destruction of the hard tissue components of tooth substance can affect any or all of enamel, dentine and cementum. This localised dissolution of tooth substance can occur on any tooth surface where there is biofilm coverage, also described as plaque. It is in the biofilm that the metabolic effects of bacteria occur, producing the

acidic environment that causes localised dissolution (or demineralisation)of the tooth surface, the first stages of dental caries (Fejerskov and Kidd 2008).

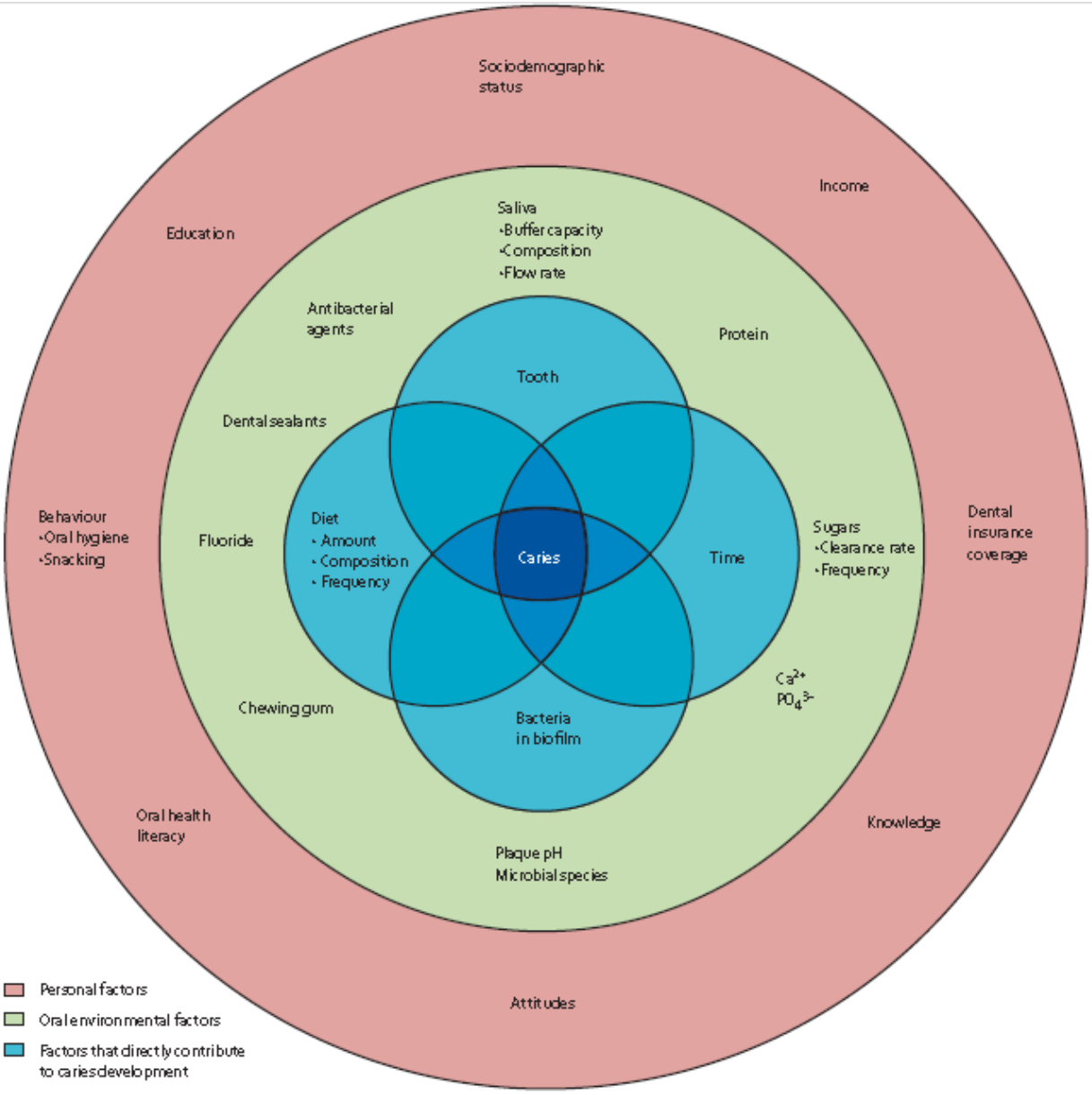
The progression of the carious lesion from initial demineralisation is complex and affected by several aetiological factors.

Aetiology of Dental Caries

There are four main factors required to produce dental caries - plaque or biofilm, a tooth surface, a substrate for bacteria to metabolise and time. The substrate is usually in the form of dietary sugars and the time required is for dietary sugars to be metabolised and for the production of the acidic environment. This then leads to tooth surface dissolution, enamel demineralisation, the first stage of dental caries. Without all four of these factors dental caries will not develop.(Kidd and Smith,1996)

Given these requirements it is easy to see that dental caries is a multifactorial disease with several additional factors affecting each of the four main requirements as seen in Figure 1.1

Figure 1. 1– Factors involved in dental caries development- Obtained from Selwitz *et al*, 2007.



The biofilm, plaque and the role of micro-organisms

The human body is comprised of an estimated 10^{14} cells of which only around 10% are mammalian (Fejerskov and Kidd 2008). The majority of cells are the organisms

that make up the resident microflora of the host. A new-born will have a sterile mouth, however, it will very quickly acquire microbes, usually via saliva from the mother that will establish the resident microflora. The oral microflora will change over time and more than 700 different species of microorganism have been identified in the mouth (Aas *et al* 2005).

Bacteria are essential for dental caries development as proven by experiments involving germ free animals (Soames and Southam 2005). The resident microflora acts as a barrier to colonisation by other transient organisms and is therefore part of the innate host defence. Resistance to potential pathogens occurs by saturation of attachment sites, creating unfavourable growth conditions for other organisms and by production of inhibitory factors such as bacteriocidins.

The dental biofilm is a community of microorganisms that are resident on the tooth surface. The development of dental biofilm can be separated into distinct stages:

Within seconds of cleaning an enamel surface glycoproteins present in saliva adsorb onto the enamel surface forming a pellicle.

Within a further 2 hours single bacterial cells, namely the coccal bacteria, are present adhering to and colonising this pellicle layer. These are most commonly the streptococci including *S.sanguis, S.oralis* and *S.mitis.* Also involved are other species such as *Actinomyces* and Gram-negative bacteria such as *Neisseria*. At this stage only around 2% of the coccal bacteria are *Streptococcus mutans* which is of importance as these are associated with caries lesion initiation.

From 4-24 hours there are many microcolonies formed following multiplication of the initial bacteria.

24 hours after surface cleaning streptococci bacteria account for 95% of the cultivable flora of plaque.

At this point there is increased microbial succession and the streptococcus dominated plaque changes with increased species diversity, the dominant species becoming *Actinomyces*. As the dental biofilms or plaque develop many of the bacteria produce polysaccharides, especially on metabolism of sucrose. As plaque becomes thicker, further microbial succession is driven by the change in conditions to a more anaerobic environment. Initially this is by facultative anaerobic species and after approximately 9 days obligatory anaerobic species (Fejerskov and Kidd 2008).

In 2 weeks plaque is considered mature, although there is significant site-to-site variation.

The biofilm formation and development is a dynamic process with attachment, growth, removal and reattachment of bacteria all occurring at the same time. Plaque therefore consists of bacteria within a matrix of salivary mucoids and bacterial polysaccharides or glucans.

The matrix consists mainly of salivary glycoproteins unless there is an excess of substrate in the environment in the form of fermentable carbohydrates or sugars. If this is the case then the plaque matrix principally consists of extracellular glucans that have been synthesised by plaque bacteria following breakdown of the excess sugars. This change in the matrix will produce local environmental changes favouring growth of more cariogenic species of bacteria. Frequent fermentable carbohydrate intake creates a repeatedly low pH environment which will favour colonisation and growth of more acidogenic bacteria such as *Streptococcus mutans* and lactobacilli. Further acid production by these species means more time below the critical pH for enamel demineralisation – around pH 5.5 (Marsh and Martin 1999). This will result in a tipping of the enamel demineralisation – remineralisation equilibrium to one favouring demineralisation and if persistent will enable the carious lesion to progress further.

Aside from an increased frequency of intake of fermentable carbohydrates other local environmental changes will also promote the colonisation of acidogenic bacteria. Placement of fixed orthodontic appliances is another example where plaque retention is increased and environmental change from a smooth surface to a more stagnant area is created.

The Role of Dietary Carbohydrates

Not all carbohydrates are of the same cariogenicity in that they may be fermented by bacteria at differing rates. Complex carbohydrates such as starch are not as harmful as they are not digested in the mouth whereas low molecular weight carbohydrates or sugars are more easily taken up into plaque and therefore have a more harmful effect.

Glucose, fructose and galactose are monosaccharides and sucrose, maltose and lactose are disaccharides. Of these lactose has been shown to be less cariogenic than the rest in animal studies (Rugg-Gunn,1993). Sucrose has been implicated more in the cariogenic process and is more cariogenic as it can support synthesis of extracellular glucans by mutans streptococci allowing it to accumulate more rapidly within plaque (Fejerskov and Kidd 2008). Fruits contain sugars namely fructose, sucrose and glucose that are present as intrinsic sugar that is integrated in the cellular structure of that fruit, and therefore less easily metabolised by oral bacteria. However, in fruit juices the sugars have been released in the juicing process and are therefore more cariogenic. In general, non-milk extrinsic sugars categorised as sugars present in a free form or added to foods are the most potent as they are the most easily metabolised (Kidd 2008).

These dietary sugars can diffuse through plaque and be rapidly metabolised by plaque bacteria resulting in production of acids, mainly lactic acid. As a result the pH

present in plaque can drop as much as 2 units 10 Minutes following ingestion of sugar (Soames and Southam 2005).

It takes 30 to 60minutes for the pH of plaque to return to normal. This occurs by diffusion of some of the acid out of the plaque matrix and diffusion of buffered saliva into the matrix, neutralising the acid. The pH of plaque is of importance as at a critical pH, normally in the region of pH5.5, there is dissolution of the hydroxyapatite crystals of the enamel surface as mineral ions diffuse into plaque (Marsh and Martin 1999).If there is continued diffusion of minerals such as calcium and phosphate out of the enamel surface then the first signs of demineralisation will become evident and eventually cavitation will occur (Selwitz, *et al*2007).

The frequency of intake of dietary carbohydrates has a key effect on the carious process. If there is a frequent intake then pH will remain lower over a longer period of time as there is not enough time for neutralisation between intakes.

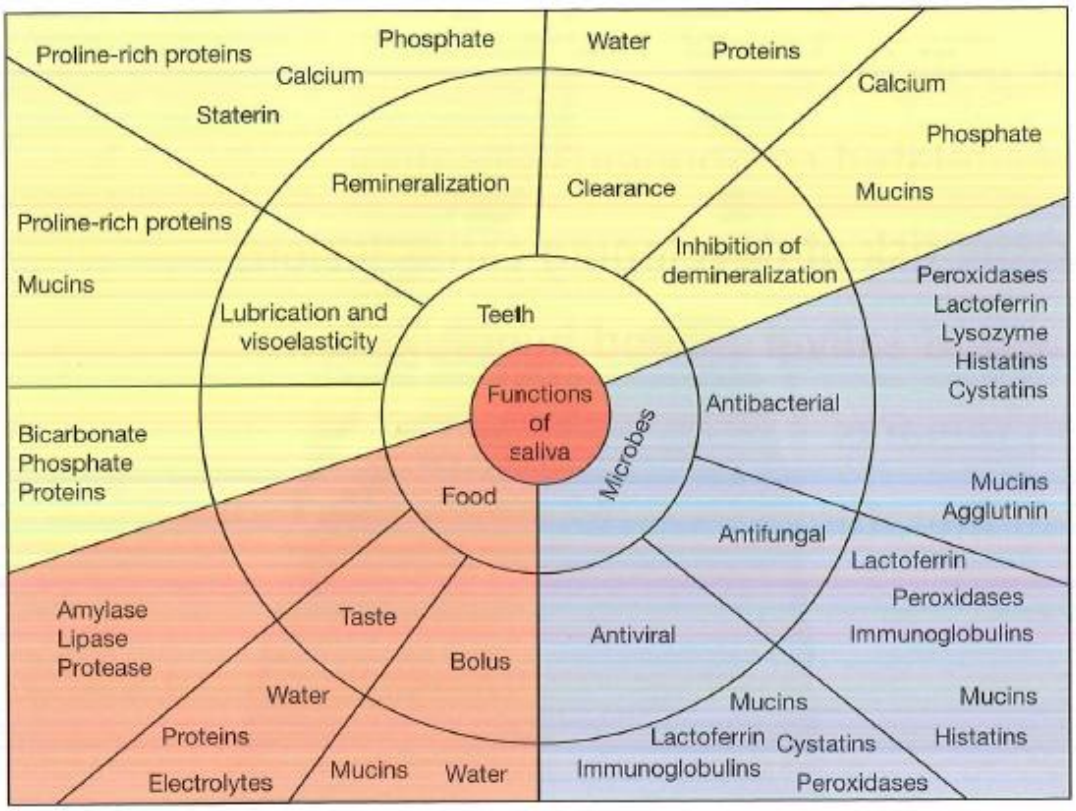
The role of saliva

Saliva is an important factor in the prevention of dental caries as all tissues in the oral cavity are coated with salivary mucous glycoproteins. The functional characteristics of saliva can be broadly categorised into 3 categories:

1. Coating the tissue surfaces such as enamel and epithelium;
2. Protection from the environment by bacterial agglutination and removal, inhibition of bacterial growth, metabolic effects on resident flora and specific immunity;
3. Enzymatic activities.(Kleinberg 1976)

These salivary functions and the interactions with teeth, food and microbes are further illustrated in Figure 1.2:

Figure 1. 2– Functions of saliva – from Fejerskov and Kidd 2008.



Saliva is composed of 99% water with just 1% of electrolytes and proteins. Normally 0.5-1 litre of saliva is produced per day which highlights the coating and the clearance effect of saliva. (Fejerskov and Kidd 2008)

Saliva and Dental Caries

Saliva has many functions – to aid swallowing and digestion, and to maintain the oral mucosa, teeth and tongue.

The protective role of saliva is mediated by its ability to clear food substances from the mouth and buffer organic acids formed by biofilm microorganisms. Additionally

saliva can reduce the demineralisation rate and enhance remineralisation by providing calcium, phosphate, and fluoride in the fluid phase of the biofilm whilst in close association with the tooth surface. (Hara and Zero, 2010)

Saliva also contains several antiviral and antibacterial properties including lysozymes which damage bacterial cell walls, lactoferrin which can restrict aggregation and lactoperoxidase which is antimicrobial, alongside specific antibodies in the form of secretary IgA.

The buffering effect of saliva or the ability to regulate pH of the oral cavity is due to the content of bicarbonate as well as phosphate and amphoteric proteins. These proteins have the potential, by creating an acquired pellicle of increased thickness, to restrict outward movement of calcium and phosphate from enamel.

Stimulated and unstimulated salivary flow rates also carry variable buffering capacity. Parotid saliva has a pH of 7.4 when flow rate is high but this reduces to a pH of 5.5 in unstimulated saliva. The submandibular gland again has a pH of 7.1 dropping to 6.4 for unstimulated flow (Kidd 2008).

The salivary flow reduces plaque accumulation on tooth surfaces and increases the rate of clearance of carbohydrates from the oral cavity. The buffering effect of saliva increases with increasing flow rate with a pH increase of 1.9 seen when the parotid salivary flow rate is high compared with unstimulated flow. This is due to an increase in the bicarbonate and sodium concentrations in saliva. Bicarbonate and phosphate buffer systems along with ammonia and urea present in saliva can neutralise the drop in pH that occurs when plaque bacteria metabolise dietary sugars.

Immunoglobulins specifically Immunoglobulin A (IgA) and non-immunological salivary components such as lactoferrin and lysozyme have an antibacterial action on plaque and can alter bacterial metabolism to become less acidogenic. (Kidd 2005).

In addition, calcium and phosphate are present at a supersaturated level in saliva, which is in direct contact with tooth surfaces and can therefore promote enamel remineralisation to demineralised enamel lesions.

Salivary flow and dental caries

Increased salivary flow increases the availability of further components that can favour remineralisation and neutralisation such as calcium phosphate and fluoride.

However, reduced flow can have an opposite and detrimental effect – shown to be associated with a higher caries rate due to the limited clearance and neutralising effects of saliva. In cases where salivary flow is significantly decreased, for example post radiation therapy, the effects can be associated with rampant caries (Brown,*et al* 1975).

The salivary clearance rate shows little change in an individual over time but there is great inter-individual variation.

Sites at highest risk of dental caries are sites that experience no disturbance by salivary flow as this is where plaque stagnation can occur more readily allowing cariogenic organisms to thrive. Approximal contact point and pits and fissures are most prone to this alongside local environmental changes such as restoration margins and orthodontic and prosthetic appliances whether fixed or removable. However, where there is good oral hygiene to these areas plaque stagnation will not happen so readily and therefore dental caries will not occur.

Development and progression of dental caries

The first clinically visible sign of a carious lesion is that of a white spot lesion. To be detected the tooth must be dried and plaque free. The white chalky appearance is due to subsurface mineral loss and the difference in refractory indices of enamel, water and air. Where there is subsurface mineral loss it is in-filled with a watery medium and has a refractive index of 1.33. Sound enamel has a refractive index of 1.62. Drying the surface with air from a 3 in 1 syringe allows air with a refractive index of 1.0 to replace the watery medium in the subsurface lesion. Since the difference in refractive index between 1.0 and 1.62 is much greater than the difference between 1.62 and 1.33 in the water filled lesion this allows a difference to be visually detected and hence the early lesion diagnosed (Kidd and Fejerskov 2004). Histological signs seen in polarised light are distinct, with white spot lesions showing 4 distinct regions:

1- Surface zone –relatively well mineralised (demineralisation accounts for only 1%) and intact zone approximately between 20-50µm thick.
2- Body of the lesion – deep to the surface zone the body of the lesion is more porous, when in water pores have a volume exceeding 5%. This is the area of greatest demineralisation.
3- Dark zone – seen when placed in quinolone not water, the dark zone outlines the body of the lesion and has a pore volume of between 2-4%.
4- Translucent zone –again visualised on placement in quinolone. This area may vary in width from 5-100µm and appears structure-less as dissolution of enamel initially occurs along the gap between enamel rod and interred enamel in this region. Quinolone penetrates more easily into the larger gaps and has the same refractive index as enamel at 1.62 and as such the result will appear structure-less. It is the first zone to show histological change. (Gorelick, *et al*1982)

Both the surface zone and the dark zone have potential for remineralisation and this is possible with improved oral hygiene, diet and with the aid of fluoride. However, if these changes are not implemented, then the lesion will progress from a white spot lesion until the surface zone is broken due to the loss of tooth structure and cavitation has occurred.

Orthodontic Treatment and Dental Caries

Placement of orthodontic appliances places an individual at higher risk of dental caries. This is mainly due to the plaque retentive factor of these appliances resulting in larger plaque accumulation and requiring a higher standard of oral hygiene to maintain dental health. White spot lesions are the most frequent iatrogenic effect associated with orthodontic treatment with incidences reported as high as 97% (Boersma, *et al*2005).On follow up it has also been noted that this increase in the prevalence of white spot lesions lasts for at least 5 years after treatment (Ogaard 1989).

Orthodontic appliances such as brackets and bands alter the local microbial environment and create stagnation sites, an environment that supports the proliferation of facultative bacteria which include *Streptococcus mutans*. This results in a lower pH within the plaque matrix and in the tipping of the remineralisation-demineralisation balance towards demineralisation of the enamel surface and increases the potential for white spot lesion creation. (Richter *et al*, 2011)

Detection of white spot lesions and dental caries

Methods for assessing enamel demineralisation

There are many clinical and imaging techniques useful in highlighting areas of enamel demineralisation. As previously mentioned drying and cleaning a tooth surface allows a white spot lesion, the first visible sign of enamel demineralisation, to be detected. Other techniques can be split into those available in the clinical environment such as laser light induced fluorescence, laser profilometery and electrical conductivity, and those of use in the *in vitro* setting such as transverse microradiography, surface microhardness, scanning electron microscopy and iodide permeability tests.

Assessment of mineral change

The ability to accurately assess mineral change in enamel is important to be able to diagnose the presence and extent of carious mineral loss. To be able to quantify this mineral loss is important, not only from the clinical aspect in the management of carious lesions, but also from an experimental aspect to be able to accurately assess any difference between agents being assessed for both the remineralisation and caries preventative properties.

The ideal technique in assessment of mineral change in enamel would be quick, easy and non-destructive providing accurate quantification of mineral loss or mineral gain. This would enable the longitudinal evaluation of carious lesions, providing valuable information to both clinician and patient.

Several mineral evaluation techniques are commonly used and can be broadly categorised into those that are destructive and therefore suitable only for *in situ* or *in*

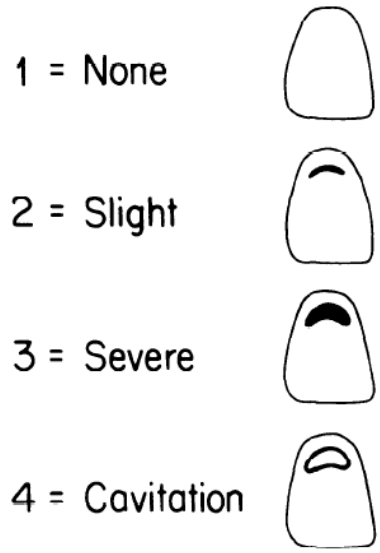
vitro studies or those that are non-destructive and suitable longitudinal evaluation of lesions, including those that can also be used in the clinical environment

Non-destructive methods of lesion analysis

Visual inspection:

The reported prevalence of white spot lesions varies with the technique used for detection. Many studies have used a white spot grading system developed by Gorelick *et al*(1982) which comprises a grading system of 1 to 4. 1 indicates no white spot lesion, 2 a slight lesion, 3 an excessive white spot lesion and 4 a white spot lesion with frank cavitation as represented in a schematic form (Figure 1.3)

Figure 1. 3- Schematic representation of white spot lesion grading system developed by Gorelick *et al* 1982.



This visual scale is useful for assessing presence or absence of lesions but does not highlight the area covered by a white spot lesion which is most likely to be of concern

to the patient. Another visual scale for assessing white spot lesions has been developed by Banks *et al* which assesses the site around a bracket and area covered by white spot lesion described as an enamel decalcification index (Banks *et al*2000). These differences in visual scales and often a lack of assessment for lesions prior to orthodontic treatment mean that valid conclusions as to prevalence of new lesions and best methods for prevention are difficult to produce, as concluded by a systematic review (Benson, *et al* 2005).

The local environment is crucial to the development of lesions and it is not therefore surprising that some teeth have a higher incidence of white spot lesion formation than others. Maxillary lateral incisor teeth have the highest incidence of white spot lesion formation noted to be three times the incidence of maxillary central incisors (Gorelick, *et al*1982, Banks and Richmond 1994).Factors influencing this may be tooth size and percentage area covered by the orthodontic bracket, salivary flow distribution and perhaps most crucially the available area between the bracket and gingival margin. 60% of white spot lesions have been noted to be in the gingival region (Banks and Richmond 1994).

Clinical photographs

Clinical photographs are useful aids to provide a permanent record of enamel characteristics that can then be monitored over time. Using a standardised technique it is possible to use clinical photographs to monitor lesion progression. Several techniques for this have been described, including those by Benson which projects photographic slides onto a 121-dot array grid with each dot being scored according to enamel condition (Benson *et al*,1998). A similar process using digital photographs and a computerised analysis has been described by the same group. However, they found it to be less reproducible than their morphographic grid technique. (Benson *et al*, 2000).

Regardless of the assessment tool used there are limitations in photographic assessment for enamel lesion quantification. One of the main difficulties is in the need for a standardised image capture technique. This should involve a set angulation for image capture and ideally also involve the drying of teeth to aid visual assessment. Changes in the angulation of image capture and the dryness of teeth along with differences in flash reflection or other obstructions such as cheek retractors have the potential to significantly affect enamel visualisation and assessment.

The analysis methods mentioned above also have a disadvantage in that they are largely subjective using a scoring system of 0 to 3 (0=no lesion, 1= diffuse lesion, 2= a white spot lesion with diffuse margins and 3= a pronounced white spot lesion. (Benson *et al*, 1998)

Quantitative Light-induced Fluorescence (QLF)

Quantitative light-induced fluorescence is based on the principle that blue light illuminates and excites tooth tissue and relies on the innate fluorescent properties of enamel. Fluorescence is the process of light absorption at a short wavelength which is then re-emitted at a different longer wavelength.

The concept was first described by Bjelkhagen *et al* in 1982 but it is only since the development of a clinical device based on Bjelkhagen's concept that the use of QLF has become more widely used within dental research.(de Josselin de Jong,1995)

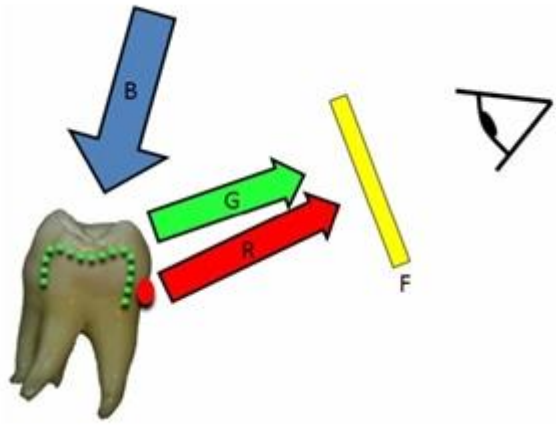
An intraoral charge coupled device (CCD) camera lens is used with a low cut-off filter of 520 nm in front of the camera lens to exclude the excitation beam coming from the image that will have been created by the camera. As such the filter ensures that no ambient light from the original light source is collected and that it is only fluorescent

light that is detected. Blue light with a peak intensity of 405 nm illuminates the tooth tissue.

The filter, set at 520 nm (yellow filter),removes the blues/pilot portion of the light spectrum leaving the green and red portions of the spectrum. (Figure 1.4)

Early enamel demineralisation (white spot lesions) can be detected by observing green fluorescence whilst red fluorescence can be used to indicate bacterial activity, for example on teeth or gingiva.(de Josselin de Jong,2009)

Figure 1. 4- QLF™ concept: a blue excitation light beam B with peak intensity at 405 nm illuminates a tooth; a yellow filter F transmits green G and red R fluoresced photons to the eye or camera. (from de Josselin de Jong ,2009)



Sound enamel fluoresces green light, demineralised enamel loses fluorescence, with the resultant areas seen to be darker on the QLF image. This occurs due to a decrease in the refractive index due to the increased porosity seen in the carious lesion of enamel. This increase in light scattering leads to reduced light absorption and a resultant decrease in autofluorescence. Localised mineral loss in enamel specimens has been studied for relative loss of fluorescence with a strong correlation found. (Hafstrom-Bjorkman *et al* 1992)

Quantitative light-induced fluorescence is designed to detect early carious changes in enamel, has the advantage of being a non-destructive technique and allows longitudinal assessment of lesions. Calculation of loss of fluorescence in these early lesions is done by subtracting the fluorescence of the lesion from the fluorescence of surrounding sound tissue. The area of the lesion can be quantified in millimetres along with percentage mean fluorescence loss and percentage maximum fluorescence loss(Fejerskov and Kidd 2008).The use of this technology can now be incorporated with a digital camera known as quantitative light-induced fluorescence digital (QLF-D).

The QLF technique has been validated by comparison with TMR with a high correlation of both techniques found. (Pretty, *et al*2004)

Multispectral imaging (MSI)

Multispectral imaging utilises wavelength technology to capture data across specific frequencies in the electromagnetic spectrum. Images taken at different wavelengths can be combined to produce a composite image of red, green or blue in a single image. The resultant composite images have colour patterns that can be used to analyse surface features of the object imaged, as different colour patterns have different characteristics with bright areas showing higher amounts of energy than darker areas.

Within dentistry multispectral imaging is used in the assessment of dental caries, enabled due to the change in fluorescence exhibited from sound or demineralised enamel and dentine. As with QLF an enamel sample can be excited with blue light (405nm) and fluorescence can be detected and captured again using a high pass colour light filter in the yellow spectrum.

The information obtained from MSI imaging is more detailed and has the potential to show significantly greater changes in mineral loss at an earlier stage than with QLF-

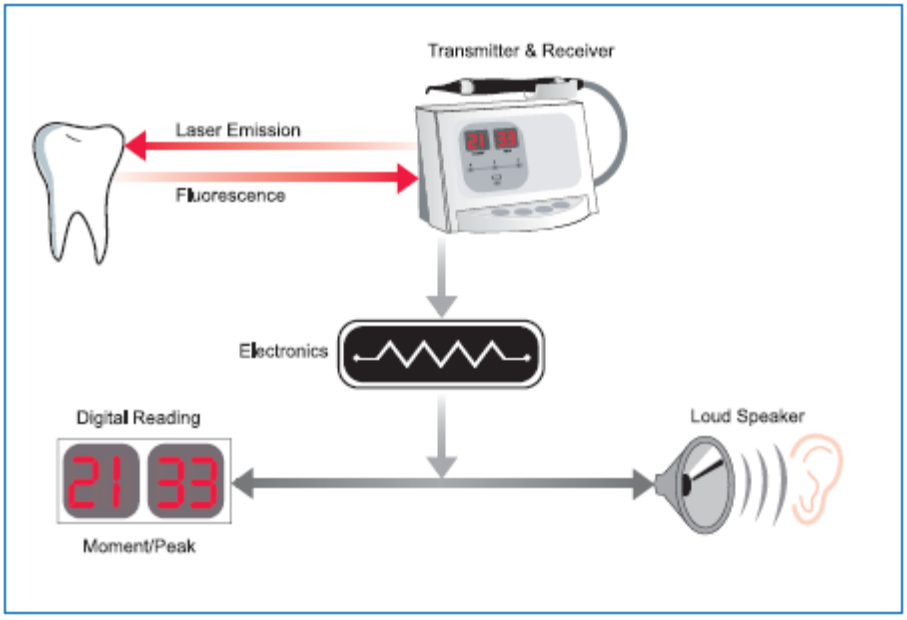
D(Desmons *et al* 2013).Multispectral imaging has been found to be a reliable technique in detecting enamel demineralisation(Adeyemi *et al* 2013) and a strong correlation between MSI and QLF-D has been found in the assessment of early enamel demineralisation(Desmons *et al* 2013).

However, a need for further research has been identified to compare it with currently used methods to fully evaluate its sensitivity and specificity. (Adeyemi *et al* 2013,Desmons *et al* 2013).

Laser fluorescence

DIAGNOdent (Kavo, Munich, Germany) is a laser fluorescence tool used for caries diagnosis. It operates at a wavelength of 655 nm which is in the red portion of the light spectrum. At this wavelength there is little fluorescence of sound enamel, resulting in a low reading. Where there is demineralisation present the lesion will start to fluoresce and the reading will increase with increasing severity of dental caries and increasing bacterial numbers.(Figure 1.5).

Figure 1. 5– DIAGNOdent process of laser fluorescence detection. (Obtained from www.Kavo.com)



It is advocated for use in clinical practice with the use of the DIANOdent pen as the laser transmitter for intraoral examination and diagnosis of carious lesions.

However, one of the limitations of the technique is in the high occurrence of false positives that occur, especially in the presence of staining. (Bader *et al*, 2004)

Electrical conductivity.

The conductivity of an electrical current varies depending on the material that is passing through. A change in the mineralisation of enamel results in a change in electrical conductivity, although this is also dependent on other factors such as fluid and electrolyte content.

The use of an electric signal as a caries detection tool was first developed in the 1950s with the first widely available device being the Vanguard electronic caries detector designed in the USA in the 1970s.(White *et al*, 1978)

The Vanguard electronic caries detector works by measuring the electrical conductivity of a probed surface and giving a result based on a numerical scale from 0 to 9. Evaluation of the validity of the Vanguard detector in the 1980s reported it had a sensitivity of 70% and a specificity of 85% at diagnosing dental caries.(Rock Kidd, 1988) This sounds impressive but it is a record only of the presence or absence of dental caries. For the diagnosis of early enamel demineralisation electrical conductive tests are of limited use and these tests are more suitable and reliable for extensive carious lesions.(Fejerskov and Kidd, 2008)

Despite this the use of electrical conductivity of caries as a diagnosis tool is still being explored with new devices such as the CarieScan Pro™ device being developed. There is still a current lack of evidence that these new devices are any more accurate in the diagnosis of enamel demineralisation than that of visual examination.(Teo *et al*, 2014)

Destructive methods of lesion analysis

Transverse microradiography (TMR)

Microradiography and particularly transverse microradiography originate from the work of Thewlis in 1940. Further development of the technique by Angmar *et al* in 1963 enabled it to be used quantitatively in the assessment of enamel mineralisation.

TMR is considered the gold standard for measuring mineral loss and gain in artificially created carious lesions (Damen, *et al* 1997). It is today still considered the most practical and widely accepted method in the assessment of demineralisation and remineralisation of enamel and dentine in *in vitro* and *in situ* studies.

The advantage of TMR is the ability to get accurate information of both mineral content and distribution within small sections of tissue. The main disadvantage is the destructive nature of the technique and that it therefore cannot be used longitudinally or in an *in vivo* setting. It also requires thin sections of tooth tissue which can be difficult to prepare. These sections also need to be flat to ensure the accuracy of the measurements.

The basis of TMR is comparison of the measurement of absorption of monochromatic x-rays of the tissue with that of a simultaneously exposed standard such as an aluminium step wedge. The x-ray beam is perpendicular to the direction of lesion progression allowing mineral loss to be determined in relation to the depth of the lesion as it is visualised cross sectionally.

Parameters for use have been evaluated and optimal parameters established for an improved TMR image analysis. These have been defined as a 40cm focus to specimen distance with exposure for 35min to a 20kV and 10mA x-ray source. Films should be in developing solution for 8 minutes and fixed for 8 minutes (Lovel 2008).

TMR software such as TMRW v1.22 (Inspektor Research System BV, Amsterdam, Netherlands) can then be used to automatically calculate lesion mineral content and characteristics.

Chemical analysis.

This technique uses a variety of methods to remove micro samples from an enamel lesion. The micro samples are dissolved in acid producing a solution which is then analysed for calcium and phosphate content. As such, this technique can only detect large changes in mineral content and can give no precise measurements of the lesion parameters such as mineral distribution or lesion depth (ten Bosch and Angmar-Mansson, 1991)

Polarised light microscopy.

Polarised light microscopy is based around the principle that the majority of crystals, with the main exception being cubic crystals are birefringent. This means that a ray of light is split into two rays at right angles to each other when entering the crystal. (ten Bosch and Angmar-Mansson, 1991) Hydroxyapatite crystals that make enamel are birefringent and react in this way.

The measured quantity of polarised light microscopy is the difference in optical path length of two light rays termed the retardation. Enamel sections are cut parallel to the long axis of tooth and prepared to 50 –100µm in thickness. Multiple measurements

of retardation are then taken along the length of the lesion and of sound enamel. This is carried out with a polarising microscope, a microscope fitted with two Nicol prisms or Polaroid plates. The section to be analysed is situated in between the two plates on a rotating stage. The technique only gives quantitative data on the mineral content if a compensator is used and adjusted until the image becomes dark (Arends and ten Bosch 1992).

Overall polarised light microscopy is very technique sensitive and time consuming. In addition, several factors can affect the birefringent of the section most notably the prism shape and orientation. It is a much more labour-intensive technique than TMR with results produced being less related to mineral content. (ten Bosch and Angmar-Mansson, 1991)

Microprobe analysis

A probe can be used to bombard a tooth surface with ions or electrons in order to stimulate the release of radiation or other particles from the tooth surface. This release of radiation or particles can be measured and analysed in relation to the energy of the initial bombarding particles. It is a very sensitive technique and its use in dental research is limited. The results are produced in ratio concentrations only, such as calcium to phosphate ratio, therefore it is not suitable for quantifying mineral content and distribution of dental hard tissues.(ten Bosch and Angmar-Mansson, 1991).

Prevention of Dental Caries – the Benefits of Fluoride

Changes in oral hygiene technique and reduced frequency of dietary carbohydrate intake will improve resistance to caries. By reducing substrate levels with dietary change and bacterial levels by improved mechanical plaque removal a patient's caries risk can be changed. In addition Fluoride is a key component in a regime to prevent dental caries.

The effect of fluoride on dental caries experience was first noted to be associated with mottled enamel, now described as dental fluorosis, and also a reduced prevalence of dental caries (McKay,1916). This finding led to research into levels of fluoride in water, which revealed a level of >2.0 parts per million of fluoride in drinking water was responsible for the levels of enamel mottling seen (Churchill 1931, Ainsworth 1933).The work by Dean *et al* in 1942 evaluated the caries reduction properties of fluoride further by analysing 12-14 year old children living in 20 different towns with varying amounts of fluoride in the drinking water. At a level of 1 part per million of fluoride they found that the reduction in the level of dental caries was seen without any of the signs of dental fluorosis. In addition, children in areas with no fluoride had twice the level of dental caries when compared to children drinking water with fluoride (Dean, *et al*1942).

From these early studies it was noted that to maintain the reduction of dental caries with fluoridated water that it must be maintained, the effect being lost if moving to a non-fluoridated area. The benefits post eruption of fluoride was also noted with people moving to fluoridated areas after their teeth had erupted, who also benefited from the reduced dental caries experience.

Being a porous structure, ions are able to diffuse in and out of enamel. Hydroxyapatite forms the lattice structure with a non apatite phase of carbonate or calcium phosphate and additional ions can be adsorbed onto the surface area of the

apatite crystals. Ions can be therefore be substituted for others of similar size and charge, for example phosphate can be exchanged for carbonate and crucially hydroxyl for fluoride. The crystal structure can be maintained even when part is demineralised and equally remineralisation can occur due to these features (Kidd 2008).

Fluoride can be incorporated into enamel at three different developmental stages. During formation low levels of fluoride can be incorporated into the apatite crystals from tissue fluids. Similarly more fluoride can be taken up in surface enamel after this calcification is complete but before eruption takes place. On eruption and continuing throughout life fluoride can be taken up into enamel from the environment around it.

The uptake of fluoride post eruption is greater in the newly erupted tooth than more mature enamel. Other factors such as enamel condition can affect uptake ability, especially if the surface is sound or carious with carious enamel having raised levels of fluoride. Permanent changes to the fluoride content of the mineralised enamel after eruption will only occur when there are fluctuations in pH over an extended period of time, for example when demineralisation and remineralisation is taking place (Fejerskov and Kidd 2008).

The caries preventative features of fluoride

The main benefit of fluoride in terms of caries prevention is its topical effect. Its uptake can reduce the rate of demineralisation and enhance continued mineral uptake slowing the progression of a carious lesion. It is for these reasons that exposure to fluoride must be ongoing for the effects to last.

Water Fluoridation

Fluoride occurs naturally and is present in many natural water supplies. This is how the caries preventive effect of fluoride was discovered by McKay, as mentioned earlier. Since this discovery fluoridation of all drinking water supplies has been advocated. However, the concentration of fluoride present in drinking water is low, with fluorosis noted where >2.0 parts per million of fluoride is present. The effect of fluoridated drinking water therefore, is more of a systemic rather than topical nature. Although water fluoridation is present in some areas of the country, it is not possible for it to be carried out in many regions due to political and also geographical reasons. Alternative methods of fluoride supplementation are therefore required.

Fluoride toothpaste

Fluoride toothpaste is the most common method of fluoride application and the primary agent for caries prevention. In the permanent teeth of children and adolescents its use is associated with a 24% reduction in caries (Marinho, *et al*2003). Most studies on this topic are relatively short at around 2-3 years and it is likely that over a lifetime the caries reduction benefit is much greater. Frequency of use, duration of brushing and rinsing behaviour all affect the outcome in terms of caries reduction alongside the fluoride concentration. It is recommended to brush twice daily for around two minutes, without water rinsing after brushing, with a fluoride toothpaste of 1450 ppm Fluoride to achieve maximum benefit.

A Cochrane systematic review has been carried out to assess the difference in caries reduction with varying fluoride concentration in toothpaste. 66 studies with a total of 74 trials were included in a meta-analysis to assess the effect of fluoride toothpaste on decayed, missing or filled surfaces in the mixed permanent dentition. They found that the caries preventative effect increased with increased fluoride concentration. For toothpaste with a concentration of 1000-1250 parts per million

(ppm) a 23% reduction in decayed, missing or filled surfaces (DMFS) was found increasing to 36% for toothpastes with a concentration of 2400-2800 ppm), but concentrations of 440-550 ppm and below showed no statistically significant effect when compared to placebo. The authors concluded that there was a statistically significant benefit in the use of fluoride toothpaste for caries prevention only when using a concentration of 1000 ppm or greater. (Walsh *et al* 2010)This supports the statement that fluoride concentration in toothpaste influences the effectiveness with an approximate reduction in caries of 6% for every 500 ppm of fluoride (Kidd 2008).

However, the benefits of increased fluoride concentration toothpaste have to be balanced against the risks of ingestion and of fluorosis in children, especially in those under the age of six that may not have developed the ability to spit out residual toothpaste. The Scottish Intercollegiate Guidelines Network guideline targeting prevention of caries in the permanent teeth of children aged 6 to 6 recommend twice daily brushing with a toothpaste containing at least 1000 ppm Fluoride (SIGN 47 2000).

Toothbrushing habits between individuals are highly variable. Duration of brushing is also highly varied. Although the recommended length of brushing time is around 2 minutes (SDCEP guideline, 2010, SIGN138) it has been established that it is generally less with reports in young adults of 33seconds (Macgregor, Rugg-Gunn 1985), 60seconds in the adolescent population (Macgregor and Rugg-Gunn 2009) and more recently in an older adult population 96seconds (Ganss, *et al*2009).

Rinsing with water after brushing, although not recommended, is common practice with 55% of 15-16 year old admitting to rinsing with water after brushing either always or often (Jensen, *et al*2012).

Fluoride Mouthrinses

Fluoride mouthrinses are available with concentrations from 0 to 900 ppm Fluoride (F) and are generally marketed for use post brushing. It has recently been demonstrated that a minimum concentration of 225 ppm F is required in a mouthrinse in order to maintain post brushing fluoride levels when using 1450 ppm or high concentration 5000 ppm F toothpaste (Mystikos,*et al* 2011). There is, however, a lack of evidence available especially with regards to the use of fluoride mouthrinses as a postbrushing rinse. Recommendations are usually to use a fluoride mouthrinse at a separate time from brushing (Kidd 2008).

Caries reduction with the use of fluoride mouthrinse has been reported as high as 44% and with an average reduction of 30%, although it must be noted this was using high concentration mouthrinse in school based interventions in a fluoride deficient area (Ripa 1991).

In the orthodontic patient population use of a fluoride mouthrinse has been associated with reduction in the prevalence of white spot lesions by 25%. Compliance with use of a daily mouthrinse in the same study was noted to be low at 12% (Geiger, *et al*1992).

Fluoride supplements

Fluoride supplements such as lozenges, tablets, drops or chewing gums were first introduced to gain the benefits of systemic fluoride in areas where fluoridated water was not available. However, their use has been questioned since topical fluorides are widely available.

In 2011 a Cochrane systematic review was published looking at the effects of fluoride supplements (Tubert-Jeannin *et al* 2011). The combined data of three studies found that fluoride supplements compared with no fluoride supplement had a 24% reduction in decayed, missing or filled surfaces. When comparing fluoride supplements with topical fluorides no difference in effect was seen. Due to an unclear risk of bias in several of the studies assessed the authors concluded that there was only weak evidence on the effect of using fluoride supplements. (Tubert-Jeannin *et al* 2011).

Fluoride gels and varnish

Fluoride gels are now rarely used due to the potential toxicity if swallowed in sufficient quantities.

The most commonly used ppm fluoride varnish is Colgate Duraphat Varnish which has a concentration of 22 600 ppm Fluoride. Similar to fluoride gels in its high fluoride concentration it has the added benefits of water tolerance and although best applied onto a clean dry tooth surface this enables it to be more easily applied and application can be targeted solely to at risk surfaces. Smaller quantities are used than fluoride gels and therefore the amount that may potentially be ingested is much less.

A systematic review to compare the effect of fluoride toothpaste, gels or varnish has been carried out with the conclusion being that there is no difference in caries reduction between these different topical agents (Marinho *et al*, 2003).

Fluoride varnish has the advantage in its ease of direct placement to high caries risk areas. However, fluoride toothpaste can be, and is, used more regularly with greater acceptability. The above mentioned systematic review could not provide any conclusions as to the potential adverse effects of fluoride gels or varnish due to lack of data reporting within the trials analysed (Marinho *et al*, 2003).

Fluoride in Saliva

Fluoride concentration in saliva is generally low and depends on fluoride present in the local environment. Salivary fluoride level is slightly higher in unstimulated saliva and is not affected by flow rate unlike most other salivary constituents.

Dietary sources of fluoride in saliva include fish and tea which are ingested. Also present are amounts of fluorides used for caries prevention, mainly toothpaste and mouthrinses. Small levels of fluoride from these sources are secreted in saliva but at 20-40% less than plasma levels. Crevicular fluid also excretes fluoride at a higher level, similar to plasma. Higher salivary concentrations of fluoride can be found closer to sources of fluoride, such as remnants of toothpaste. The main difficulty is in salivary fluoride retention as the clearance rate will be affected by salivary flow rate. Fluoride will diffuse into plaque from saliva and can increase fluoride plaque levels rapidly in a short time (Fejerskov and Kidd 2008).

Rinsing with low fluoride (<225 ppm) or no fluoride mouthrinses have also been shown to reduce the salivary fluoride concentration post brushing when compared with no rinsing (Jensen, *et al*2012). Higher concentration fluoride mouthrinses, however, have been shown to have an additional effect (Duckworth, *et al* 2009).Lower concentration mouthrinses cannot therefore be recommended for post brushing usage. Due to the fluoride clearance effect fluoride mouthrinses are generally recommended for use at a separate time from brushing with more research required to establish the benefits following usage postbrushing (Pitts, *et al* 2012).

Fluoride and Orthodontic treatment

The incidence of early enamel demineralisation or white spot lesions has been reported to be as high as 96% in orthodontic patients (Michell,1992).The plaque retentive features of orthodontic brackets and wires place orthodontic patients at high risk of dental caries. Orthodontic patients therefore need to have an increased level of oral hygiene with the British Orthodontic Society recommending orthodontic patients with fixed appliances brush for three minutes twice daily (BOS patient information leaflet – fixed appliances.)

Fluoride has been incorporated into many components of orthodontic appliances in an attempt to reduce the incidence of white spot lesions. Fluoride releasing elastomerics and fluoride realising bonding agents are examples of this.

A recent Cochrane review evaluated the effect of various fluoride delivery agents on the reduction in incidence of white spot lesions in orthodontic patients. Overall, they found the quality of evidence to be poor and recommended further research in this area (Benson *et al* 2013).

Fluoride varnish and orthodontic treatment

A single study identified in a Cochrane review showed significant benefit in the reduction of white spot lesions with 6 weekly application of a fluoride varnish. Comparison with the placebo group revealed a 70% reduction in the incidence of white spot lesions in the fluoride varnish group in the study (Stecksén-Blicks *et al* 2007).

A further *in vitro* study has been carried out examining the effects of two different fluoride varnishes on the prevention of enamel demineralisation adjacent to

orthodontic brackets(Nalbantgil*et al* 2013).Varnish was placed around orthodontic brackets bonded to extracted premolars prior to a period of immersion in demineralisation solution. Assessment with microhardness tests revealed no difference between the two fluoride varnishes but significant differences of both fluoride varnishes when compared to the control group. The authors concluded that there is a benefit in the use of a fluoride varnish in both prevention and inhibition of white spot lesions in orthodontic patients(Nalbantgil*et al* 2013).

Fluoride releasing elastomerics

Fluoride releasing elastomerics have been found to reduce post treatment demineralisation scores by 49% compared with standard elastomerics(Banks, *et al* 2000). This was assessed using the enamel decalcification index, a visual 5 point scale (Artun and Brobakken,1986).However, 63% of patients still exhibited white spot lesions at approximately 16% of sites and although significantly different to the control group, where 73% of patients had 26% sites affected, the incidence is still high (Banks, *et al* 2000).Their use also has disadvantages as they are less elastic and also swell over time which in itself may aid plaque retention.

In a separate randomised controlled trial Mattick *et al* employed a split mouth design randomised to have fluoride releasing modules to the right or left of the upper labial segment. Again using the enamel decalfication index, demineralisation was found on teeth ligated with fluoride releasing modules and conventional modules, but to a statistically significant lesser extent when using fluoride releasing modules (Mattick *et al* 2001).

The main problem encountered in both of the trials was the difference in the properties of fluoride releasing modules. They are less elastic, meaning that it is not possible to figure of eight tie an archwire into a bracket. Also, they were found to swell between visits becoming more plaque retentive. Patients were found to dislike

the fluoride modules since they were prone to staining and swelling and they are not available in a range of colours unlike conventional elastomeric modules (Banks *et al* 2000).

Fluoride releasing bonding agents

Glass ionomer is an alternative bonding agent to composite resins and has the benefit of fluoride leaching properties. There is only weak evidence as to its benefit in reducing the prevalence of white spot lesions (Benson, *et al* 2005).

An alternative fluoride and antibacterial releasing self-etch and primer Clearfil Protect Bond (Kuraray Medical, Okayama, Japan) has also been clinically evaluated. Unfortunately use of this self-etch primer was associated with an increased bracket failure rate with no differences in plaque accumulation or enamel demineralisation noted. It was acknowledged that patients involved in the trial were more motivated and maintained a higher level of oral hygiene than standard and that may contribute to bias (Pashos, *et al* 2009).

An *in vitro* study into fluoride release from orthodontic adhesives assessed a non-fluoride-releasing composite, a fluoride- releasing composite, a polyacid-modified composite (compomer), and two resin-modified glass-ionomer cements (RMGICs). Fluoride-containing adhesives initially showed higher rates of fluoride ion release, but significantly declined to lower levels with fluoride release found to last only 2 days (Sug-Joon, *et al*2010).

However, in an assessment of the potential of re-charging bonding materials with fluoride Lim *et al* found that with the use of RMGICs and periodic use of a high concentration fluoride mouthrinse it was possible to maintain the level of fluoride release. This was an *in vitro* study that used a 900 ppm fluoride mouthrinse, which is higher than would normally be recommended for orthodontic patients, but highlights the benefits of a high fluoride regime from the placement of orthodontic brackets and throughout treatment (Lim *et al* 2011).

There is currently a lack of good quality evidence as to the best regimen to prevent enamel demineralisation in orthodontic patients. A systematic review of the use of fluorides and demineralisation in orthodontic patients highlighted this lack of evidence with a current recommendation of daily use of a 0.05% sodium fluoride mouthrinse (Benson, *et al* 2005).

Fluoride, post brushing, will accumulate in reservoirs in saliva, oral tissues and plaque. It is from these local concentrations of fluoride that the caries prevention mechanism of fluoride is most dependent.

Alternatives to Fluorides

A milk derived product Casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) most commonly available as GC Tooth Mousse (GC International, Tokyo, Japan) has been developed following analysis of the anti-cariogenic properties of milk and other dairy products. Its action when incorporated into plaque is in increasing availability of calcium and phosphate at the tooth surface, thereby buffering free calcium and phosphate ions and preventing demineralisation and promoting remineralisation (Reynolds 1997). Comparing the effects of CPP-ACP to fluoride on enamel demineralisation *in vivo* and *in vitro* found no statistical differences between groups and therefore both can be recommended to protect against demineralisation (Uysal, *et al* 2010).

Summary

Enamel demineralisation associated with orthodontic components is a significant clinical problem. The primary goal would be to prevent demineralisation occurring and patient information and oral hygiene instruction is an important part of this. However, demineralisation will continue to occur and the development of techniques that can detect and analyse lesions as early as possible is an essential area of research.

Fluoride has been shown to be an important factor in the prevention of enamel demineralisation but also in remineralisation of early enamel lesions. Recommendations for patients with regards to fluoride regimes, especially whilst undergoing orthodontic treatment is lacking, with the use of mouthrinses in particular been highlighted as an area lacking in research. In addition, where demineralisation has occurred, the best formulations of fluoride to maximise remineralisation is also an area where further research is continuing.

Several studies have demonstrated the benefits of fluoride but few have been conducted using the concentrations of fluoride that are present in the majority of over-the-counter preparations available to patients. Further research is therefore required to in order to provide patients with evidence based recommendations of what regimen is best for them.

The aim of this study is to assess the effect of post-brushing mouthrinses of varying fluoride concentration on salivary fluoride retention, and to also assess the effect of varying fluoride concentration on the remineralisation of bovine enamel *in vitro.* The techniques of MSI, QLF-D and TMR will also be compared in the assessment of remineralisation.

Study 1 – The effect of post-brushing mouthrinse solutions on salivary fluoride retention.

Study 1 – The effect of post-brushing mouthrinse solutions on salivary fluoride retention.

Background

Fluoride (F) inhibits demineralisation and promotes remineralisation of enamel and dentine and is a key factor in the prevention of dental caries (Featherstone 2006). Toothpaste containing fluorides the most commonly used fluoride delivery system and is well established as one of the most effective means of caries prevention (Wong, *et al* 2011). The most commonly available toothpastes contain fluoride in the range of 500-1450 ppm F. In addition to toothpaste the use of a fluoride mouthrinse is on the increase (http://www.mintel.com).

Mouthrinses

Mouthrinses, of various concoctions, have been used for thousands of years. The use of mouthrinses date back as early as 3000B.C. where they were used for their curative properties or for religious reasons (McCormack, 1968). Table 2.1 illustrates the historical use and composition of mouthrinses

Table 2. 1– The history of mouthrinses – from McCormick 1968

Year	Composition	Purpose
3,000 B.C.	Powdered bones of small animals suspension	Calcium and phosphate abrasives
2,000 B.C.	Infants' urine	Plaque control?
1,000 B.C.	Code of Manu (religious edict) "All Indians must rinse after meals"	Mechanical cleaning "Swish and swallow"
1,000 B.C.	Jewish Traditional Laws: Marriage implies physical fitness . . . "If bad breath, marriage could be dissolved."	Importance of hygiene and health
400 B.C.	Hippocrates advocated a concoction of castorium and pepper	Hygiene and health
500 A.D.	Public cisterns for urine mouthwashes (Pliny)	Plaque control?
1752	Tolver recommended warm water	Mechanical cleaning
1869	Hayes recommended water, alcohol, sol-ammoniac, Peruvian bark, charcoal, burned bread and honey once a week. Cool wine and water after each meal. Eau de cologne every morning.	Antibacterial and deodorizing
1912	Acidic mouthwash (Wallace)	Stimulation of saliva
1921	Ammoniated mouthwashes (Grove)	Precipitation of saliva
1926	Mineralizing mouthwash (Andresen)	Mineralization of the enamel

Prior to the 19th century mouthrinses were used for halitosis, religious and hygiene reasons. Alongside the discovery of bacteria was the development of antiseptic mouthrinses and with the discovery of the caries preventative nature of fluoride was the development of fluoride mouthrinses. (McCormack 1968). Today these are still the two main categories of mouthrinses.

Antiseptic mouthrinses are useful before and after oral surgery altering the oral flora and plaque formation. This itself is useful in the prevention of dental caries and periodontal disease, especially when standard oral hygiene measures are more challenging or inappropriate post surgery.

Fluoride mouthrinses are used for the anti-cariogenic properties of fluoride. Table 2.2 illustrates the development of fluoride mouthrinses following the discovery of the effect of fluoride on enamel remineralisation in 1939 (McCormack 1968).

Table 2. 2 The history of fluoride mouthrinse – McCormack 1968.

Year	Mouthwash Solution	Principal Investigator	Demonstration
1939	NaF radioisotopes	Volker	1) Fluoride makes enamel less soluble. 2) Fluoride absorbed on the surface of enamel.
1940	NaF	Bibby	Fluoride inhibits acid formation by mouth bacteria.
1944	NaF 1,000 ppm	Epstine	Not toxic to gingiva after 6 months.
1944	NaF 4 ppm	Atkins	Decreased lactobacillus count.
1945	SnF_2 12–22 ppm	Van Huysen	Reduced enamel solubility.
1946	NaF 100 ppm	Bibby	No reduction in caries in adults.
1948	Acidulated NaF 100 ppm	Roberts	Increase in caries, 30%.
1959	1,000 ppm SnF_2	Konig	Decreased rat caries.
1960	NaF 1,000 ppm radioactive	Hellstrom	Retained 6% of dose, equivalent to water fluoridation.
1960	NaF 0.25%	Weisz	80–90% caries reduction.
1964	SnF_2 500 ppm	Shannon	"Safe and effective in protecting teeth from caries."
1964	SnF_2 5,000 ppm + NaCl	Shannon	Use as rinse instead of conventional topical.
1964	NaF, KF, FeF_2 (200 ppm–500 ppm)	Torell	2 × month or daily more effective than topicals or dentifrices.
1964	NaF 2,000 ppm	Lundstam	2 × month 36% reduction in 9 months, 2,400 children.
1965	NaF 3–40 ppm + Ca and PO_4	McCormick	Reduced interproximal caries 40%.
1965	NaF 10,300 ppm + acid	Brudevold	Reduced caries 70%.

Table 2.2 illustrates the different fluoride preparations and concentrations in mouthrinse which were researched in an attempt to identify the ideal caries preventative method. The demonstration indicated in the demonstration column is the effect of the fluoride mouthrinse against a control and not against an alternative method of fluoride delivery. At this time the use of fluoride in toothpaste was still relatively new with the first mass marketed fluoride toothpaste being introduced in the USA in 1956.

Previous research has demonstrated the importance of the oral soft tissues as the major site of fluoride retention in the mouth (Zero *et al* 1992) and it has also been shown that saliva, plaque and plaque fluid also act as significant fluoride reservoirs (Ten Cate 1990). For the oral soft tissues Zero *et al* demonstrated that the tongue and lower posterior vestibule retained the highest fluoride levels, followed by the upper posterior buccal vestibule and upper anterior labial vestibule, whilst the lowest fluoride levels were retained in the lower anterior vestibule and the floor of the mouth (Zero *et al* 1992).

Current recommendations on oral hygiene measures for dental caries prevention recommend the use of a fluoride toothpaste, brushing for two minutes and spitting and not rinsing with water.(SIGN138, SDCEP guideline 2010) Brushing with fluoride toothpaste is arguably the single most important factor in the prevention of dental caries. This is due to mechanical plaque removal achieved alongside the delivery of fluoride. Use of a fluoride mouthrinse alone therefore would not be recommended. However, the use of a fluoride mouthrinse as an adjunct to fluoride toothpaste is still a topic of debate.

In the UK, 31% of adults are reported to use a mouthrinse, a habit that spans all social groups(Chadwick 2009).An increasing trend in the use of mouthrinse has been seen with a 44% increase in the sales of mouthrinses in the UK reported between 2005 and 2010 (www.mintel.com).

A recent study on the use of mouthrinses carried out in Sweden reported 47% of adults to be using mouthrinse. Most reported the frequency of use of a mouthrinse to be daily with 87% reporting the use of the mouthrinse to be directly after brushing (Sarner *et al* 2012).

It is likely that post brushing mouthrinses have the capacity to enhance or diminish fluoride efficacy from toothpaste. Currently there is a lack of information regarding optimal levels of fluoride in mouthrinses used after brushing (Pitts *et al* 2012).

In 2011, Mystikos *et al* found that many mouthrinses available on the market contain little or no fluoride (Mystikos *et al,* 2011).Although these products can provide benefits such as reducing plaque and gingivitis they potentially have a washout effect if used directly after brushing with fluoride toothpaste. Duckworth *et al* recommended the use of a mouthrinse with at least 100 ppm (mg F/L) to maintain the fluoride levels achieved from toothpaste post rinsing and this might be particularly important for those at high caries risk (Duckworth *et al,* 2009). The use of higher fluoride levels in mouthrinses has also been recommended, especially when patients are at high caries risk (Marinho *et al*, 2003).

Mouthrinses are widely available with concentrations ranging from 0 to 990 ppm F and are generally marketed for use post brushing or at a time separate to when brushing takes place.

Aim

The aim of this study is to evaluate the effect of post-brushing mouthrinse solutions containing 0,225 and 500 ppm F on salivary fluoride retention when brushing with 1450 ppm fluoride toothpaste and rinsing with water post brushing.

Materials and Methods

The study was a single blind cross-over study with three cells and a minimum of 48 hours wash out period between each.

The study evaluated the effect of three treatments:

1) brushing with a 1450 ppm F (as NaF) toothpaste for 40 seconds, rinsing three times with ten ml of water, followed by rinsing with a 500 ppm mouthrinse solution for 60 seconds.

2) brushing with a 1450 ppm toothpaste for 40 seconds, rinsing three times with ten ml of water, followed by rinsing with a 225 ppm mouthrinse solution for 60 seconds.

3) brushing with a 1450 ppm toothpaste for 40 seconds, rinsing three times with ten ml of water, followed by rinsing with a 0 ppm mouthrinse solution for 60 seconds.

Subjects were blinded to the fluoride concentration in the mouthrinses with all examiners and administrators in the study also blind to the concentration. Mouthrinses were labelled as F,T or Z.

The study was approved (Project Ref 11227) by the University of Manchester Research Ethics Committee(Appendix VI).

Thirty one consented volunteers were recruited from staff and students of the University of Liverpool via telephone contact, email, poster, or word of mouth. They were aged between 18-65 years and fulfilled the following inclusion criteria with none of the exclusion criteria.

Inclusion criteria

1. Good general health with no medical conditions that the investigator considers may compromise the subjects' safety or the quality of the results.
2. Available for the duration of the study
3. Sign an informed Consent form and comply with the protocol (Appendix I)
4. A minimum of 24 teeth without extensive restorations, six in each quadrant
5. No sign of gingivitis or destructive periodontal disease or active caries lesions

Exclusion criteria

1. Presence of orthodontic bands
2. Presence of removable partial denture
3. Tumour of soft or hard tissue of the oral cavity
4. Any medical condition that the investigator considers may compromise the subjects' safety as well as quality of the study results
5. Pregnant women or women who are breastfeeding
6. Participation in any other dental study or participated in a dental study within the past one month
7. Allergies to Oral Care Products, Personal Care products or their ingredients.

All participants used the study designated toothpaste containing 1450 ppm F as Sodium Fluoride for one week prior to the first treatment and throughout the study period until the last treatment was completed. The toothpaste used in the study was Colgate® Triple Cool Stripe containing 1450 ppm F and the two mouthrinses were Colgate® Fluorigard Alcohol free mouthrinse containing 225 ppm F sodium fluoride (NaF) and Colgate® Duraphat Daily mouthrinse containing 500 ppm F as NaF. All the products, including the fluoride free mouthrinse which was formulated in the

same way as the other two mouthrinses, were supplied by Colgate-Palmolive (Europe).

Clinical examination of the oral hard and soft tissues was carried out at each visit. Each subjects' medical history was also checked with any changes in medical history noted at each visit (Appendix II). Subjects could be excluded if they had or developed any medical condition that compromised their safety or the quality of the study results. For example, taking medication that may have an effect on salivary flow. All participants were asked to refrain from having elective dental treatment during the course of the study.

For each of the study phases subjects were asked to brush their teeth for 40 seconds with 0.5g of the 1450 ppm toothpaste (without water) and then rinse with three 10ml water rinses. After this they had a single 10 ml rinse using a mouthrinse containing either 0, 225 or 500 ppm F according to a Latin square assigned randomisation. Subjects and examiners were blinded to the fluoride concentration of the mouthrinse with the mouthrinses labelled F,T or Z. Subjects were asked to make active lip and cheek movements whilst using the fluoride mouthrinse for one minute before then spitting out.

For saliva sample collections subjects were asked to pool saliva for 10 seconds before spitting out. Saliva samples were collected before brushing commenced (Time 0) and at various time points after the mouthrinse (1, 3, 5, 10, 20, 30, 45 and

60minutes). Subjects were not allowed to speak, eat or drink throughout this 60 minute period. Samples taken were of unstimulated saliva and eating, drinking or speaking about food would have had an effect on salivary flow.

Withdrawal of subject from the clinical trial

A genuine effort was made to determine the reason(s) why a subject failed to return for the necessary visit(s) or was withdrawn from the study. Subjects could be withdrawn from the study if any of the following occurs:

- The subject fails to substantially comply with the protocol requirements
- Subject fails to report for a scheduled examination
- Subject is treated with medication(s) during the course of the study that may interfere with the parameters under study
- Subject received emergency dental or medical treatment that may interfere with the parameters under study
- Subject develops a serious adverse reaction.
- Subject elects to terminate participation in the study

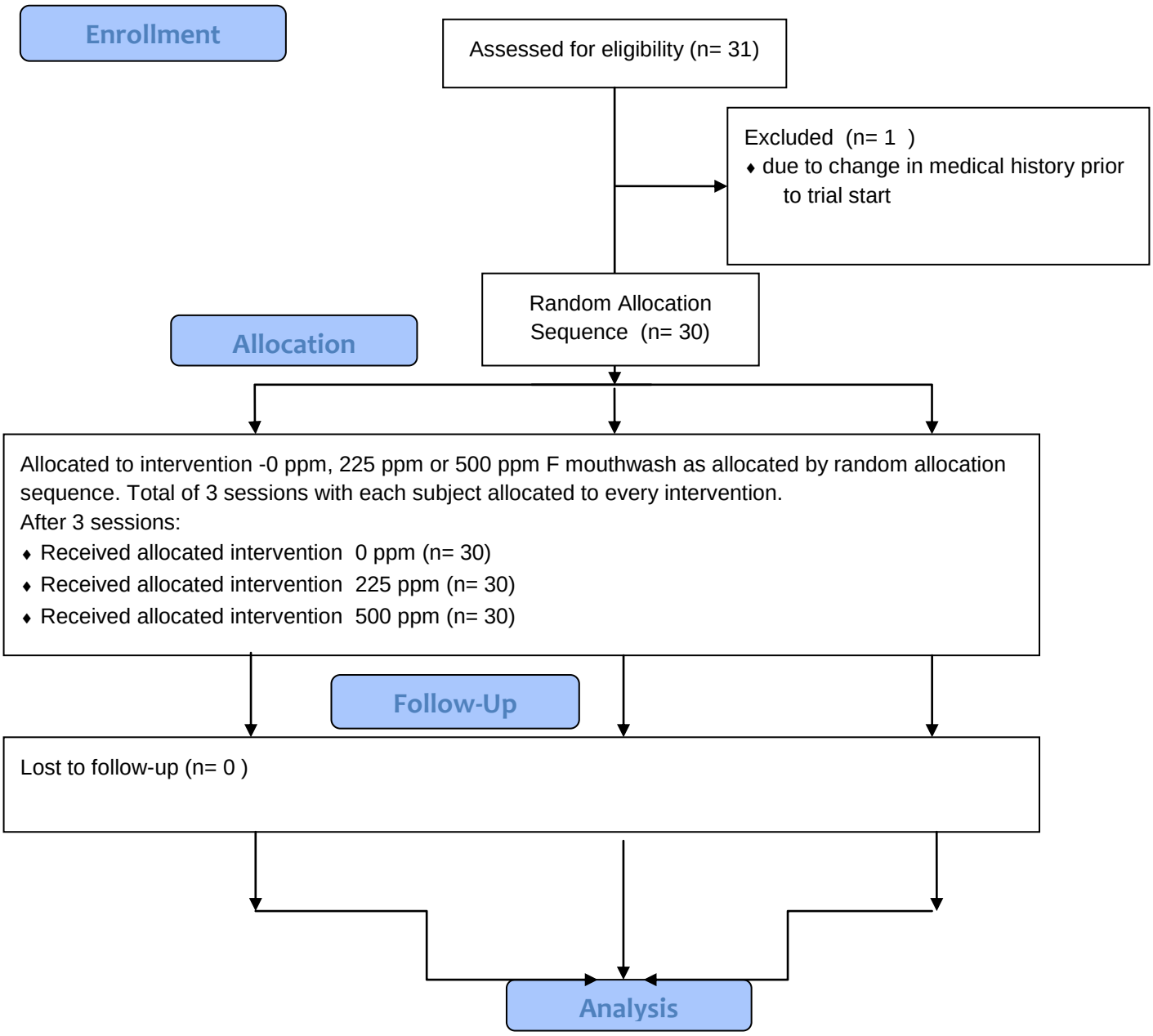
Subject remuneration

Subjects completing the three legs of the study received an out of pocket expense participation allowance of £60 (£20 per visit) in total.

Randomisation Procedures and Allocation of Treatments

The subjects were randomly assigned to one of the three treatment groups. Randomisation and product allocation were carried out based on a Latin square randomisation Table determining the order of the three mouthrinses over the three visits per subject. (Figure 2. 1)

Figure 2. 1- flow of subjects through the trial



The code break for the treatments was maintained in the Sponsor's study files in sealed envelopes. The Sponsor's code-break was broken at the end of the study when the statistical analysis has been completed. Staff involved in the clinical assessment of study subjects were unaware of the product assignment, the fluoride concentration of mouthrinse F,T or Z.

Concurrent treatments

The subjects were required to use their allocated 1450 ppm F washout toothpaste for the entire study. Subjects were not prevented from attending their dentist for emergency treatment but should have refrained from having elective dental treatment.

Fluoride analysis

All samples were analysed blind in terms of subjects and methods with mouthrinse treatments labelled as F, T or Z (Figure 2. 2).

Figure 2. 2– The 3 study mouthrinses labelled F, T and Z for blinding purposes.

100µm of saliva was first mixed with 10µm of TISAB III (Total Ionic Strength Adjustment Buffer, Thermo Electron Corp, Waltham, MA, USA). TISAB is commonly applied to enable fluoride ion analysis with an ion specific electrode. The main constituents of TISAB are CDTA (cyclohexylenedinitrilotetraacetate), sodium hydroxide, sodium chloride and acetic acid. Each constituent has a role in adjusting the activity of fluoride ions and also the pH of the solution. This enables fluoride ions to be exposed so that the concentration and activity of ions is equal to give an accurate representation in the reading obtained from the ion specific electrode. The electrode measures free fluoride ions that are present in a solution giving a millivolt reading.

100µm of the solution was then placed in a 24 well tissue culture plate and millivolt readings were then obtained by lowering an ion specific electrode (model 96-09, Orion Research Inc. Fisher Scientific, Loughborough, UK) into the solution. Fluoride standards (1-1000µM) were prepared using a commercial standard (Orion 940906 0.1M Fluoride) to calibrate the electrode. Millivolt readings were then recorded for both standards and samples. (Figure 2.3)

Fluoride concentrations in ppm were calculated using 'F-calc' a bespoke Excel based software programme (Excel, Microsoft Inc, Redmond, California, USA) designed by L.Cooper. This software utilises a linear regression model to convert the millivolt fluoride readings of the saliva samples into ppm. (Figure 2.4)

Figure 2. 3– Ion specific electrode used to obtain millivolt readings

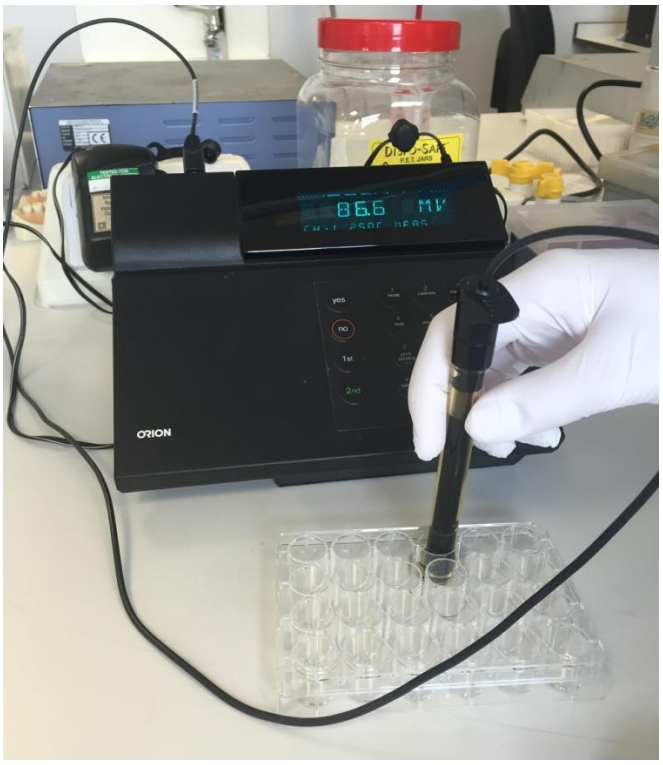
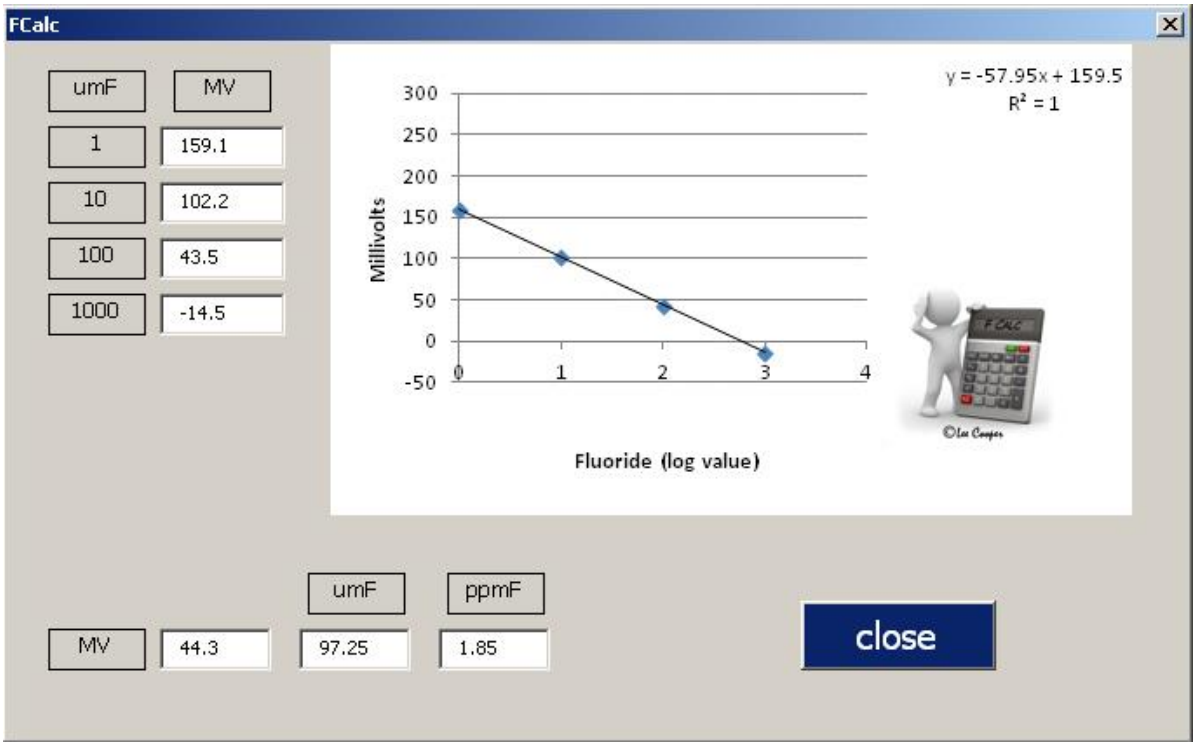


Figure 2. 4– Screenshot of 'F calc' software used to obtain ppm values from millivolt readings

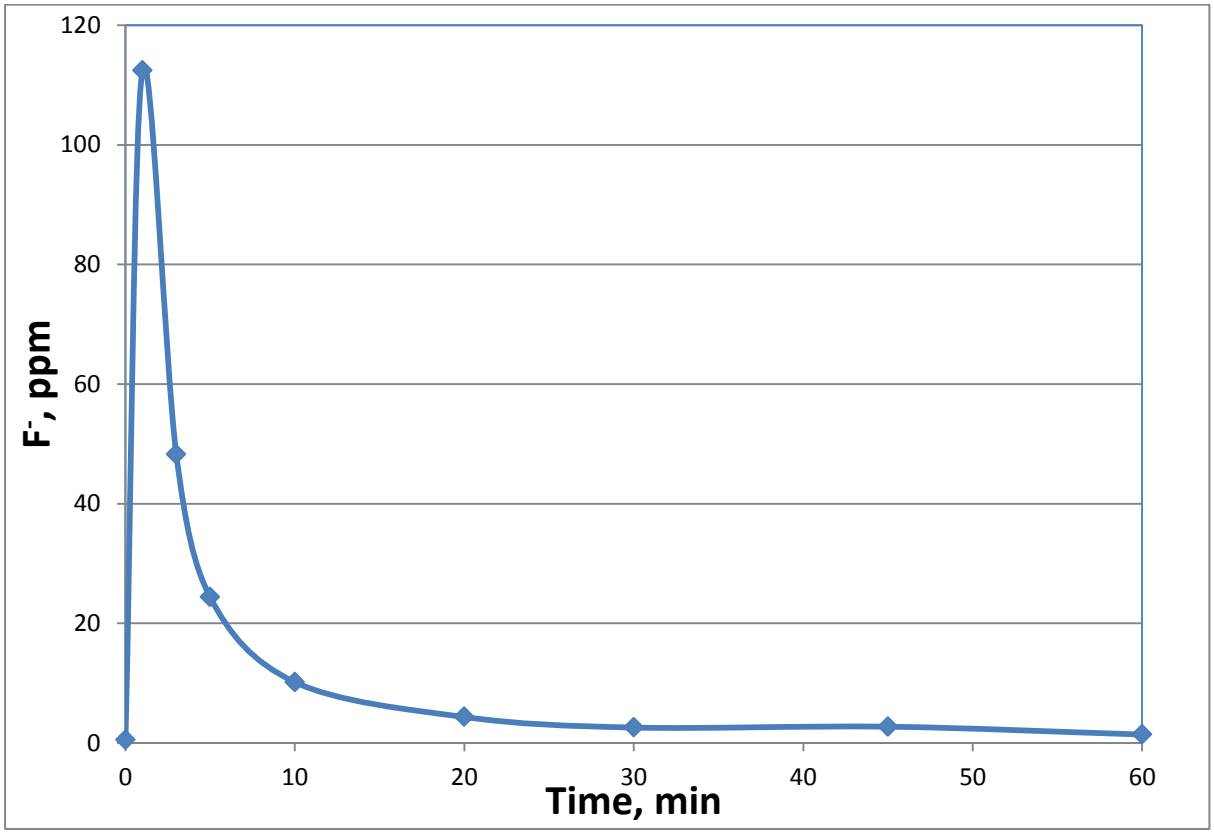


Data analysis

The Statistical Package for Social Sciences software (SPSS version 20.0,Chicago, Illinois, USA) was used for calculating descriptive statistics including mean and the standard deviations for each group.

Concentrations of fluoride (F) were plotted against time and the area under the curve over the 60 minutes of the study (AUC_{0-60}) calculated for each treatment for each subject. (Figure 2.5)

Figure 2. 5 -Concentration of fluoride curve for calculation of area under the curve values.



The $AUC_{0\text{-}60}$ values represent the total fluoride exposure over the 60 minute experimental period and are useful in giving an indication of salivary fluoride concentration over time. Additionally, in this study the $AUC_{0\text{-}60}$ values were useful in giving an indication of salivary fluoride clearance and the time wise effect of varying the fluoride concentration on salivary fluoride retention.

Comparison of the $AUC_{0\text{-}60}$ values was carried out using analysis of variance (ANOVA) and paired t-tests for the pair wise comparisons. The primary efficacy variable was the integrated area under the curve for fluoride (F) concentration as a function of time ($AUC_{0\text{-}60}$). A secondary analysis was performed after applying a $\log_{10}$ transformation to the fluoride (F) concentrations prior to calculating the $AUC_{0\text{-}60min}$.

Analysis of the difference between mouthrinses at each time point was carried out to assess the effect of time on the statistical significance of salivary fluoride levels between mouthrinses. This was done using analysis of covariance (ANCOVA) with the covariate being the baseline T0 salivary fluoride level with all other time points T1-T60 as dependant variables. A bonferroni multiple comparison correction was also applied due to the large number of pairwise comparisons. Again a secondary analysis was performed after applying a $\log_{10}$ transformation to the fluoride(F) concentrations as these were found to be more normally distributed and therefore more suitable for parametric testing.

The significance level (α level) for all tests used was set at a α level of 0.05.

Results

A total of 31 subjects (15 females, 16 males) were recruited into the study and 30 completed it. One male was excluded due to a change in medical history. The age range of the subjects was 18-47 years with a mean age of 25.3years. There were no adverse events reported associated with the use of any of the study products.

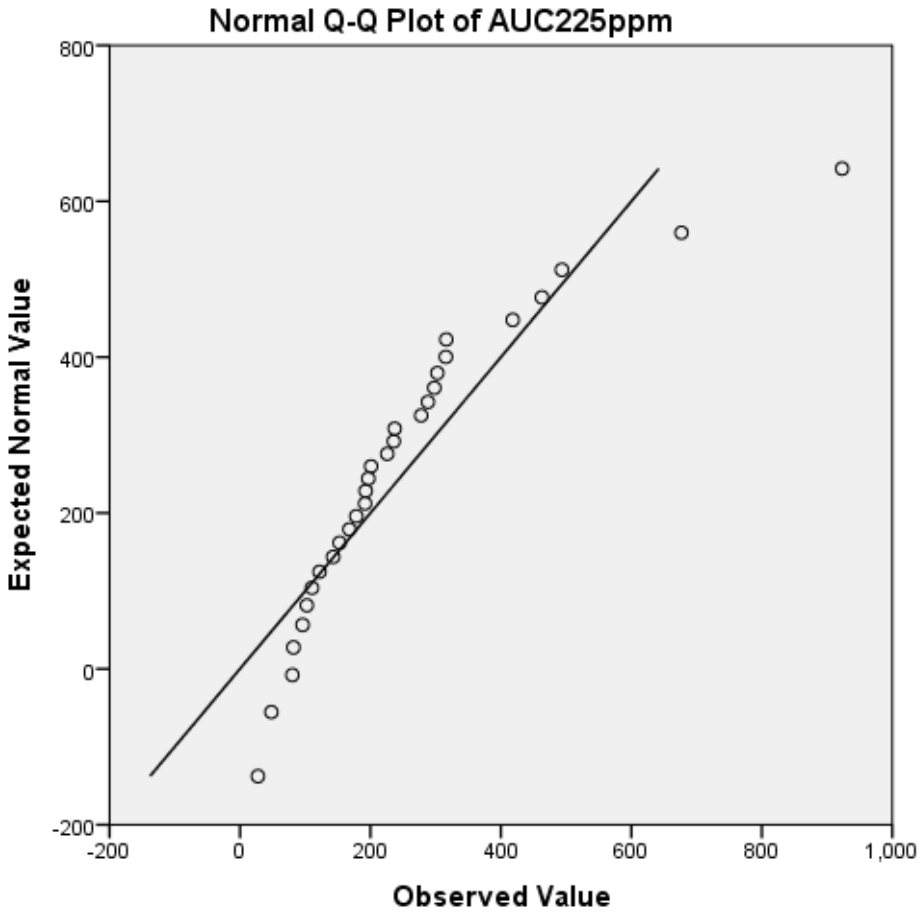
Saliva ppm fluoride concentrations for all time measurements in the three treatment groups are shown in Appendix VII Table 2. 10, 12 and 14 and AUC_{0-60} – in Appendix VII Table 2. 11, 13 and 15.

Normality testing

Normality testing of the AUC_{0-60} was assessed graphically with the use of Q-Q plots and Frequency histograms(Figures 2.6 and 2.7). Normality was tested in a group wise basis for the AUC values as a groupwise effect itself may skew normality of data spread when looking at a combined dataset.

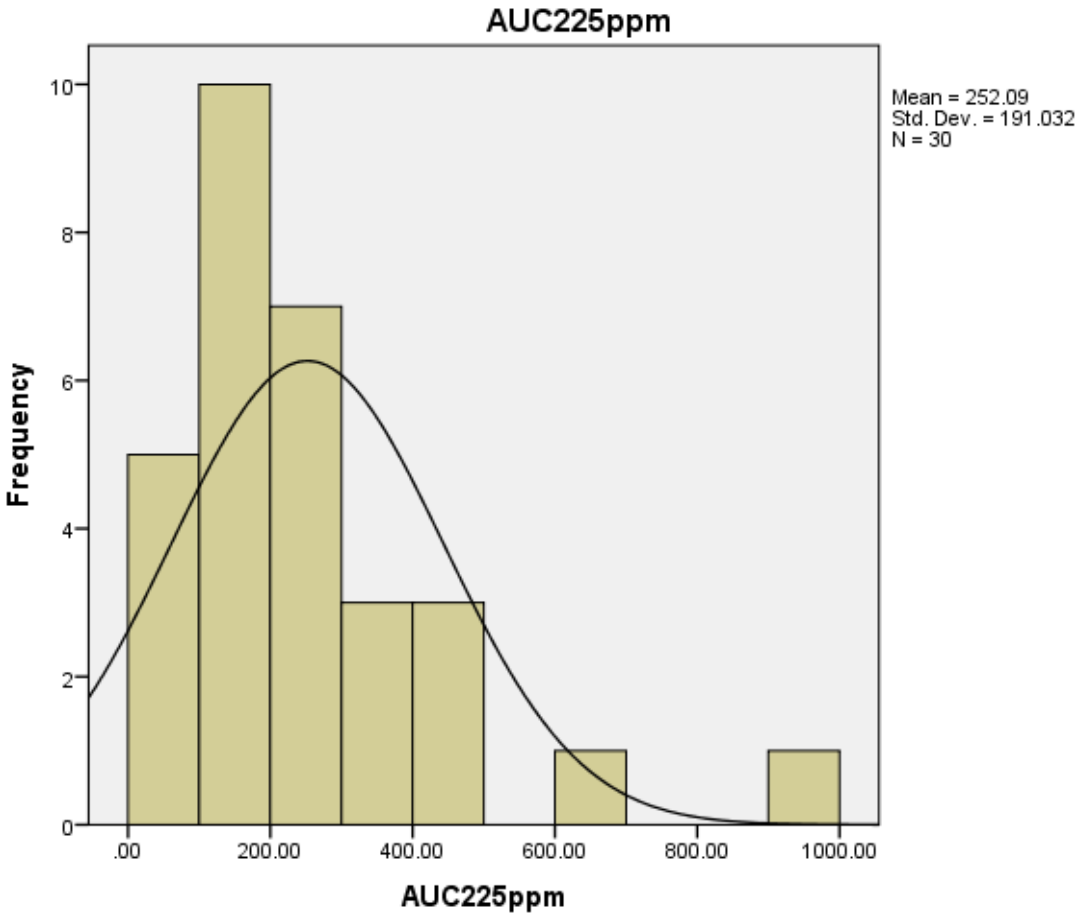
On a Q-Q plot normality of data spread is assessed in relation to the diagonal line. If all data is close to the line then it is normally distributed. However, if data strays away from the line in an obvious non-linear fashion then it is not normally distributed. The Q-Q plot based on the AUC data for the 225 ppm group is shown in Figure 2.6 which indicates that the data is not normally distributed.

Figure 2. 6Q-Q plot of AUC data for the 225 ppm group.



Frequency histograms are also graphical means of testing data normality. Normal distribution is indicated by a 'bell shaped' curve. The maximum height indicates the mean with the width of the curve indicative of the standard deviation. The frequency histogram for the AUC data for the 225 ppm group is shown in Figure 2. 7.

Figure 2. 7– Frequency histogram for AUC 225 ppm data.



This shows the data to be slightly skewed to the right as seen with several outliers on the frequency histogram (Figure 2. 7).

When the spread of data does not appear entirely normal, numerical normality testing can be used as a means of quantifying the normality of data spread. Shapiro-Wilk test was carried out. This is suitable for cases where the sample size is less than 50. The results are shown in Table 2. 3.

Table 2. 3-Tests of Normality

group		Shapiro-Wilk		
		Statistic	df	Sig.
ppm	0	.561	30	.000
	225	.829	30	.000
	500	.761	30	.000

This statistical significance is determined with a Shapiro-Wilk test which revealed that the data spread of the zero ppm, 225 ppm and 500 ppm groups were not normally distributed. Parametric testing on this dataset therefore, is not entirely suitable as normal data distribution is an assumption for the use of such test and would have to be interpreted with extreme caution.

When the data spread variable is not normally distributed data transformation can be carried out and if found to be normally distributed can be substituted in for analysis. The most common data transformations used are the logarithmic transformation, the square root transformation and the inverse transformation.

All three data transformations were carried out and assessed for normality to determine the best dataset for parametric testing. The Shapiro Wilk tests carried out on the transformed data are displayed in Table 2. 4.

Table 2. 4-Tests of Normality on Transformed data

group		Shapiro-Wilk		
		Statistic	df	Sig.
Logarithmic ppm	0	.878	30	.003
	225	.982	30	.876
	500	.973	30	.624
Inverse ppm	0	.946	30	.135
	225	.660	30	.000
	500	.938	30	.079
Square root ppm	0	.712	30	.000
	225	.958	30	.271
	500	.888	30	.004

The Shapiro Wilk test revealed that the logarithmic transformed data showed the best normality. The QQ plot and frequency histogram for logarithmic transformed treaty five ppm AUC data is shown in Figures 2.8 and 2.9. This shows the improvement in data normality seen with the transformed data when compared with Figure 2.5 and 2.6 for the untransformed data.

Figure 2. 8Q-Q plot for logarithmic transformed 225 ppm AUC data

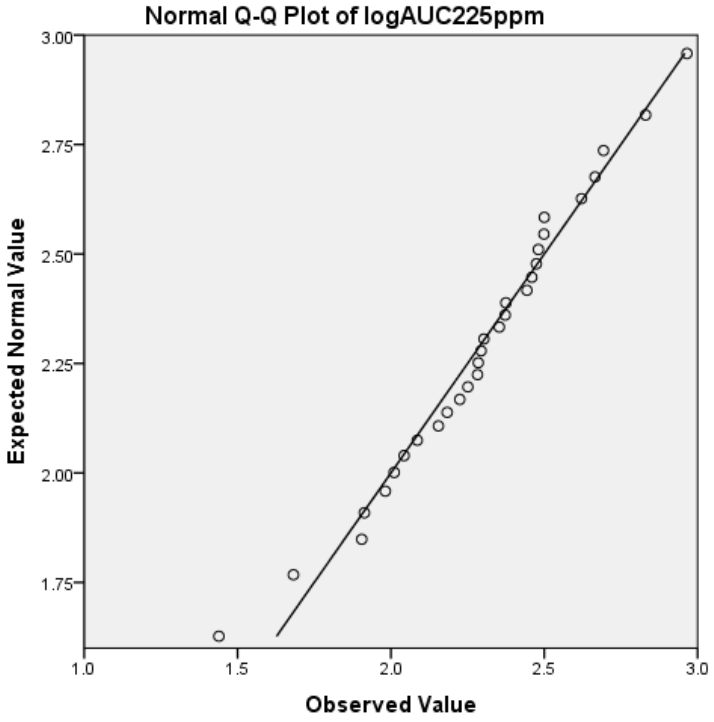
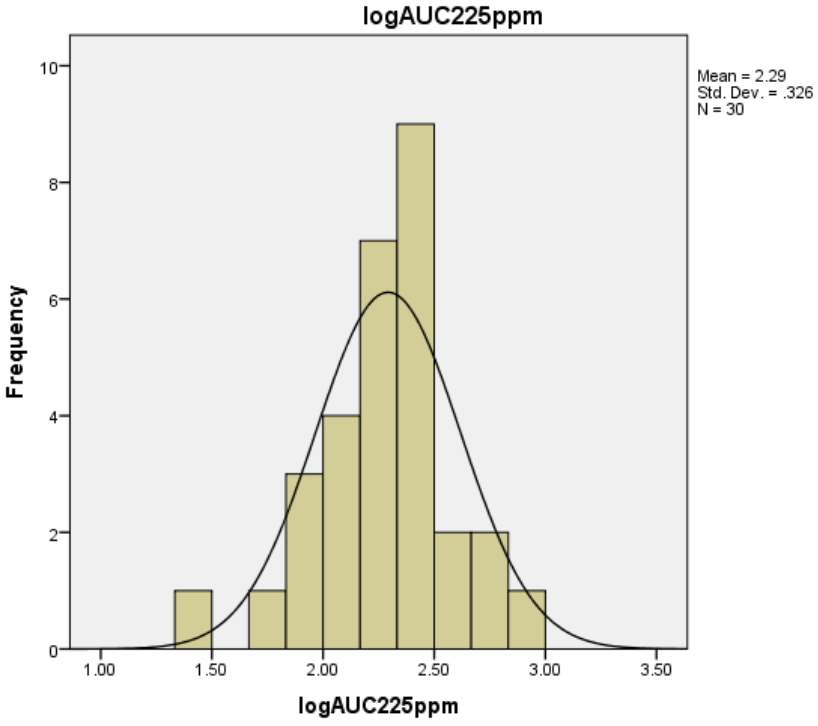


Figure 2. 9- frequency histogram for logarithmic transformed 225 ppm AUC data



This was weakest for the 0 ppm logarithmic transformed data. Therefore, a Q-Q plot and frequency histogram were carried out to visually assess the data spread. (Figure 2.s 10,11).

Figure 2. 10- frequency histogram of logarithmic transformed 0 ppm AUC data

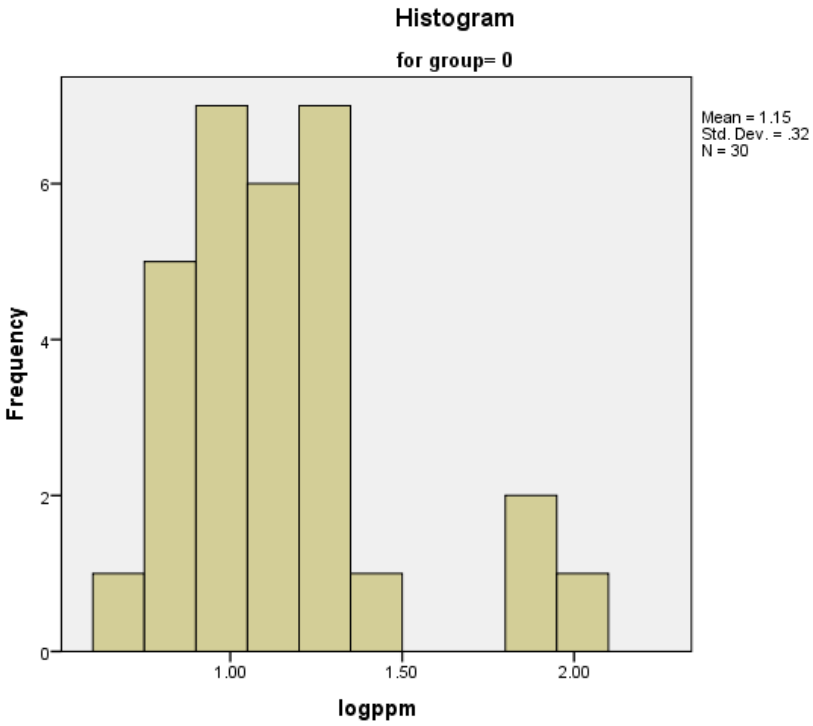
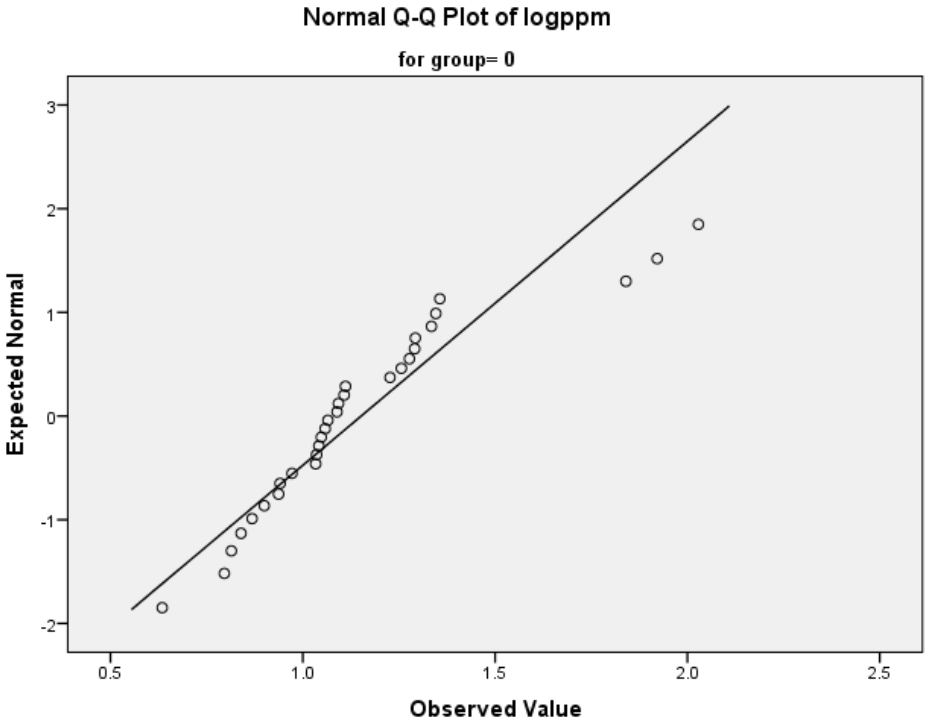


Figure 2. 11- Q-Q plot for logarithmic transformed 0 ppm AUC data.



The frequency histogram reveals an almost normalised distribution with the exception of slight outliers to the right. The Q-Q plot shows a variable distribution of data to the line but with an overall linear pattern. For this reason the data spread was concluded to be sufficiently normal to enable parametric testing.

To illustrate potential differences in the data sets data analysis was carried out on both the untransformed and the logarithmic transformed data.

Group wise comparisons

The AUC_{0-60} means were calculated and were 554, 252 and 20 for the 500, 225 and 0 ppm F mouthrinse groups respectively (Table 2. 5, Figure 2.s 12, 13).

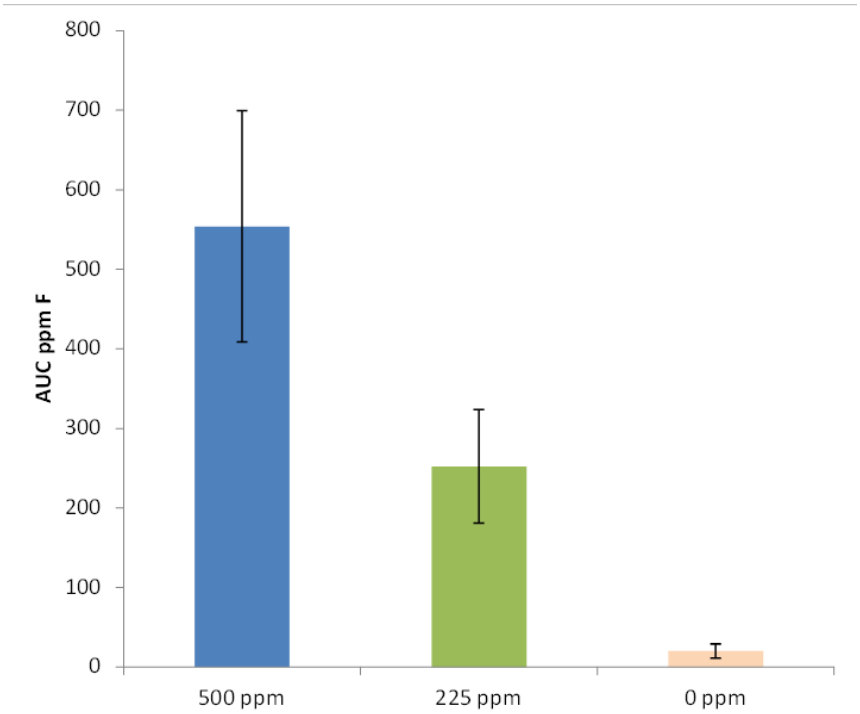
Table 2. 5- Mean and standard deviation of fluoride concentration integrated area under curve in human saliva 0-60 minutes post brushing with rinsing with 500, 225 and 0 ppm F.

Experiment regime	$AUC_{0-60min}$			
	Mean	SD	Log_{10} mean	SD
500 ppm F	554	389	25	19
225 ppm F	252	191	8	21
0 ppm F	20	24	-42	19

Table 2. 6-Statistical significance of pair-wise comparison of the three study treatments for $AUC_{0-60min}$.

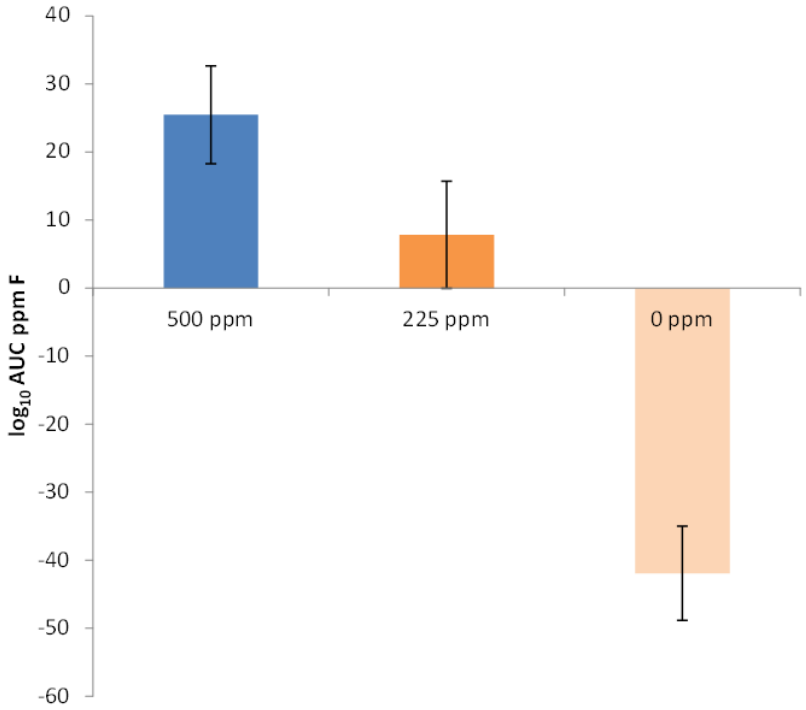
Treatment comparison	Difference for pair-wise comparison			
	$AUC_{0-60min}$	Significance	Log_{10} $AUC_{0-60min}$	significance
500 ppm vs. 225 ppm F	302	<0.0001	18	0.0002
500 ppm vs. 0 ppm F	534	<0.0001	67	<0.0001
225 ppm vs. 0 ppm F	232	<0.0001	50	<0.0001

Figure 2. 12 Mean $AUC_{0\text{-}60min}$ ppm saliva fluoride level during 60 minutes post brushing after rinsing with mouthrinses containing 500, 225 and 0 ppm F



The differences between the groups were statistically significant (ANOVA, $p<0.001$). The pair wise differences (Table 2. 6) between the three groups using a two-tailed paired t-test were also statistically significant ($p <0.001$). The use of the 500 ppm F mouthrinse resulted in a 2660% increase in total F salivary retention over 60 minutes when compared with the 0 ppm F group and a 120% overall increase when compared with the 225 ppm group.

Figure 2. 13-Mean log_{10} AUC ppm saliva fluoride level during 60 minutes post brushing after rinsing with mouthrinses containing 500, 225 and 0 ppm F



Similar results were found for the log_{10} AUC_{0-60} measurements with means of 25, 8 and -42 for the 500, 225 and 0 ppm F mouthrinse groups respectively. The pair wise differences between the 3 groups were also statistically significant ($p<0.05$) (Table 2.6)

The mean fluoride levels in saliva for each of the three mouthrinses for all time points measured are plotted in Figure 2.s14 and 2.15.

Figure 2. 14. Saliva fluoride level ppm during 60 minutes post brushing after rinsing with mouthrinses containing 500, 225 and 0 ppm F

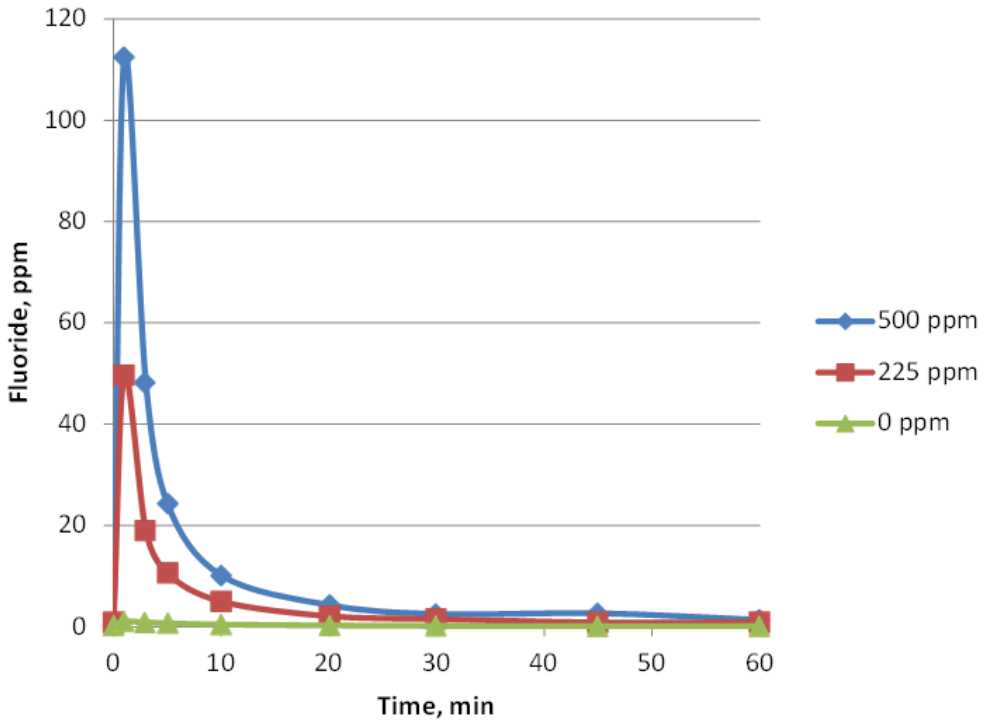
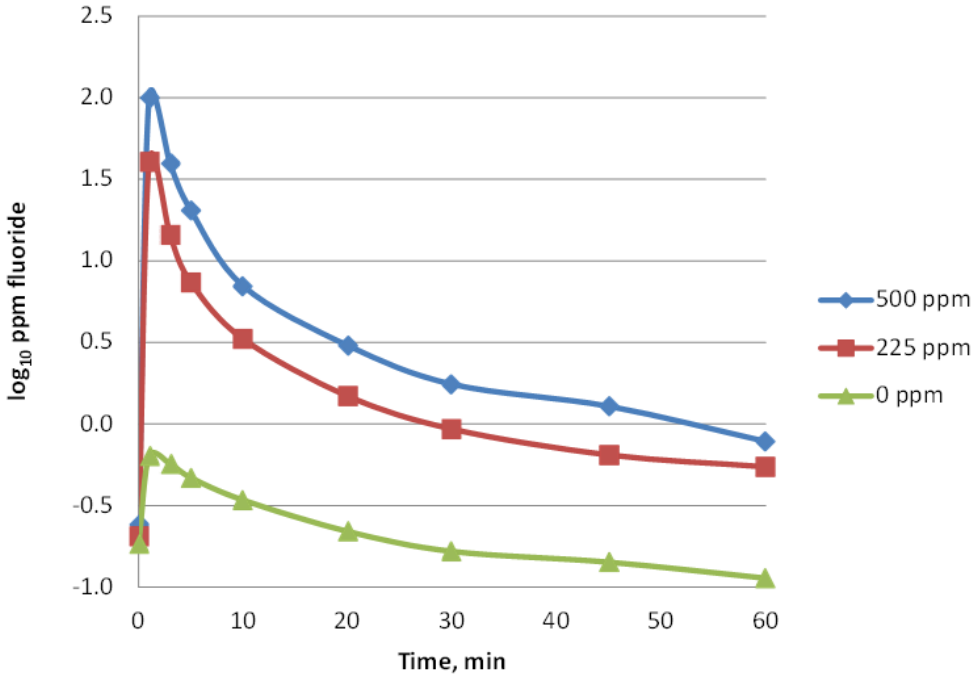


Figure 2. 15. Saliva fluoride level $\log_{10}$ ppm during 60 minutes post brushing after rinsing with mouthrinses containing 500, 225 and 0 ppm F



A sharp drop-off following the peak in salivary fluoride concentration is seen, which is due to the effect of salivary clearance. Following the statistically significant result in the comparison of all three mouthrinses based on the area under the curve values for the 60 minute period, further analysis of the data were carried out to determine the effect of time on the statistically significant differences between the mouthrinses.

Pairwise effect between groups over time

Prior to assessment of group wise effect over the time points the normality of the data were again examined. As with the area under the curve values, data normality was tested per group at each time point as the data might not otherwise appear normal should a strong group wise effect exist. Frequency histogram and Q-Q plots showed a lack of normal data distribution, confirmed with a Shapiro-Wilk test. Therefore, as with the area under the curve values, the data were transformed and values assessed for normality (Table 2.7).

Table 2. 7–Test of normality – Shapiro-Wilk

Group		ppm			log ppm			square root ppm			inverse ppm		
		Statistic	df	Sig.	Statistic	df	Sig.	Statistic	df	Sig.	Statistic	df	Sig.
T0	0	.361	30	.000	.960	30	.316	.926	30	.039	.722	30	.000
	225	.361	30	.000	.938	30	.082	.564	30	.000	.545	30	.000
	500	.487	30	.000	.933	30	.058	.700	30	.000	.736	30	.000
T1	0	.967	30	.457	.868	30	.002	.643	30	.000	.952	30	.195
	225	.967	30	.457	.801	30	.000	.951	30	.178	.430	30	.000
	500	.921	30	.028	.969	30	.517	.958	30	.276	.920	30	.027
T3	0	.916	30	.021	.856	30	.001	.649	30	.000	.923	30	.033
	225	.916	30	.021	.937	30	.078	.985	30	.938	.591	30	.000
	500	.759	30	.000	.975	30	.677	.901	30	.009	.930	30	.049
T5	0	.723	30	.000	.861	30	.001	.662	30	.000	.941	30	.096
	225	.723	30	.000	.989	30	.982	.921	30	.029	.666	30	.000
	500	.831	30	.000	.981	30	.840	.926	30	.039	.940	30	.090
T10	0	.725	30	.000	.886	30	.004	.725	30	.000	.968	30	.476
	225	.725	30	.000	.982	30	.875	.887	30	.004	.792	30	.000
	500	.657	30	.000	.962	30	.340	.839	30	.000	.924	30	.033
T20	0	.701	30	.000	.873	30	.002	.699	30	.000	.950	30	.166
	225	.701	30	.000	.972	30	.604	.863	30	.001	.881	30	.003
	500	.583	30	.000	.953	30	.205	.800	30	.000	.891	30	.005
T30	0	.595	30	.000	.923	30	.031	.773	30	.000	.947	30	.144
	225	.595	30	.000	.948	30	.152	.787	30	.000	.911	30	.015
	500	.582	30	.000	.957	30	.258	.790	30	.000	.882	30	.003
T45	0	.785	30	.000	.961	30	.330	.873	30	.002	.927	30	.041
	225	.785	30	.000	.957	30	.253	.899	30	.008	.886	30	.004
	500	.413	30	.000	.927	30	.040	.663	30	.000	.842	30	.000
T60	0	.557	30	.000	.943	30	.112	.841	30	.000	.889	30	.005
	225	.557	30	.000	.984	30	.927	.824	30	.000	.800	30	.000
	500	.592	30	.000	.888	30	.004	.731	30	.000	.938	30	.081

Non-significant differences indicating normal data distribution are highlighted in light blue. This clearly indicates logarithmic transformed data is, again, the most appropriate for parametric testing acknowledging the variation in distribution that occurs between groups at different time points. Therefore in addition to analysis of the untransformed data (Table 2. 8) analysis was also carried out on the logarithmic transformed data.(Table 2. 9)

The group wise effect over time was assessed using an analysis of covariance (ANCOVA) repeated measures test. The T0 values were used as the covariate with the T1-T60 values entered as dependent variables. A Bonferroni multiple comparisons correction was also applied due to the higher degree of type I error, that of a false positive result, associated with multiple comparisons. The use of the covariate as the fluoride level at baseline (T0) is important as the dependant variables T1-T60, will be affected by this baseline value. By placing it into the ANCOVA model the change assessed will be in relation to the baseline values and therefore attributable to the effect of the mouthrinses.

Table 2. 8-ppm–Pairwise Comparisons

Dependent Variable			Mean Difference (I-J)	Std. Error	Sig.[b]	95% Confidence Interval for Difference[b]	
						Lower Bound	Upper Bound
T1	0	225	-44.242*	8.950	.000	-66.097	-22.388
		500	-110.153*	8.833	.000	-131.721	-88.584
	225	0	44.242*	8.950	.000	22.388	66.097
		500	-65.910*	8.891	.000	-87.619	-44.201
	500	0	110.153*	8.833	.000	88.584	131.721
		225	65.910*	8.891	.000	44.201	87.619
T3	0	225	-15.212*	5.566	.023	-28.802	-1.621
		500	-46.555*	5.493	.000	-59.968	-33.142
	225	0	15.212*	5.566	.023	1.621	28.802
		500	-31.343*	5.529	.000	-44.843	-17.843
	500	0	46.555*	5.493	.000	33.142	59.968
		225	31.343*	5.529	.000	17.843	44.843
T5	0	225	-7.355*	2.478	.012	-13.405	-1.306
		500	-22.922*	2.445	.000	-28.892	-16.951
	225	0	7.355*	2.478	.012	1.306	13.405
		500	-15.567*	2.461	.000	-21.576	-9.557
	500	0	22.922*	2.445	.000	16.951	28.892
		225	15.567*	2.461	.000	9.557	21.576
T10	0	225	-3.129	1.668	.192	-7.201	.943
		500	-9.276*	1.646	.000	-13.295	-5.257
	225	0	3.129	1.668	.192	-.943	7.201
		500	-6.147*	1.657	.001	-10.192	-2.102
	500	0	9.276*	1.646	.000	5.257	13.295
		225	6.147*	1.657	.001	2.102	10.192

T20	0	225	-1.184	.735	.332	-2.978	.610
		500	-3.832*	.725	.000	-5.602	-2.061
	225	0	1.184	.735	.332	-.610	2.978
		500	-2.647*	.730	.001	-4.429	-.865
	500	0	3.832*	.725	.000	2.061	5.602
		225	2.647*	.730	.001	.865	4.429
T30	0	225	-.950	.522	.217	-2.224	.324
		500	-2.227*	.515	.000	-3.484	-.969
	225	0	.950	.522	.217	-.324	2.224
		500	-1.277*	.518	.047	-2.543	-.011
	500	0	2.227*	.515	.000	.969	3.484
		225	1.277*	.518	.047	.011	2.543
T45	0	225	-.559	.855	1.000	-2.648	1.529
		500	-2.470*	.844	.013	-4.531	-.408
	225	0	.559	.855	1.000	-1.529	2.648
		500	-1.910	.850	.081	-3.985	.165
	500	0	2.470*	.844	.013	.408	4.531
		225	1.910	.850	.081	-.165	3.985
T60	0	225	-.705	.366	.173	-1.599	.189
		500	-1.221*	.361	.003	-2.104	-.339
	225	0	.705	.366	.173	-.189	1.599
		500	-.516	.364	.479	-1.405	.372
	500	0	1.221*	.361	.003	.339	2.104
		225	.516	.364	.479	-.372	1.405

Based on estimated marginal means

*. The mean difference is significant at the .05 level.

b. Adjustment for multiple comparisons: Bonferroni.

The ANCOVA results of the ppm values (Table 2. 8) show statistically significant differences between all three mouthrinses seen for the first five minutes. At 10 minutes the difference in the 0 ppm and the 225 ppm groups becomes non-significant. A statistically significant difference between the 225 ppm and 500 ppm groups is seen until the 45 minute point. However, at 60 minutes a statistically significant difference is still noted between the 500 ppm and 0 ppm groups.

Table 2. 9- log ppm–Pairwise Comparisons

Dependent Variable			Mean Difference (I-J)	Std. Error	Sig.[b]	95% Confidence Interval for Difference[b]	
						Lower Bound	Upper Bound
T1	0	225	-1.794*	.079	.000	-1.987	-1.601
		500	-2.173*	.079	.000	-2.367	-1.980
	225	0	1.794*	.079	.000	1.601	1.987
		500	-.379*	.079	.000	-.572	-.186
	500	0	2.173*	.079	.000	1.980	2.367
		225	.379*	.079	.000	.186	.572
T3	0	225	-1.393*	.081	.000	-1.590	-1.196
		500	-1.813*	.081	.000	-2.011	-1.615
	225	0	1.393*	.081	.000	1.196	1.590
		500	-.420*	.081	.000	-.618	-.223
	500	0	1.813*	.081	.000	1.615	2.011
		225	.420*	.081	.000	.223	.618
T5	0	225	-1.184*	.079	.000	-1.377	-.990
		500	-1.605*	.080	.000	-1.799	-1.410
	225	0	1.184*	.079	.000	.990	1.377
		500	-.421*	.079	.000	-.615	-.227
	500	0	1.605*	.080	.000	1.410	1.799
		225	.421*	.079	.000	.227	.615
T10	0	225	-.973*	.083	.000	-1.177	-.769
		500	-1.273*	.084	.000	-1.478	-1.069
	225	0	.973*	.083	.000	.769	1.177
		500	-.300*	.084	.002	-.504	-.096
	500	0	1.273*	.084	.000	1.069	1.478
		225	.300*	.084	.002	.096	.504
T20	0	225	-.812*	.074	.000	-.993	-.631
		500	-1.094*	.074	.000	-1.276	-.913

	225	0	.812*	.074	.000	.631	.993
		500	-.282*	.074	.001	-.463	-.101
	500	0	1.094*	.074	.000	.913	1.276
		225	.282*	.074	.001	.101	.463
T30	0	225	-.731*	.079	.000	-.925	-.538
		500	-.978*	.079	.000	-1.171	-.784
	225	0	.731*	.079	.000	.538	.925
		500	-.246*	.079	.008	-.439	-.053
	500	0	.978*	.079	.000	.784	1.171
		225	.246*	.079	.008	.053	.439
T45	0	225	-.638*	.085	.000	-.847	-.429
		500	-.907*	.086	.000	-1.117	-.698
	225	0	.638*	.085	.000	.429	.847
		500	-.269*	.086	.007	-.478	-.060
	500	0	.907*	.086	.000	.698	1.117
		225	.269*	.086	.007	.060	.478
T60	0	225	-.664*	.090	.000	-.884	-.444
		500	-.791*	.090	.000	-1.011	-.570
	225	0	.664*	.090	.000	.444	.884
		500	-.127	.090	.488	-.347	.093
	500	0	.791*	.090	.000	.570	1.011
		225	.127	.090	.488	-.093	.347

The ANCOVA results of the Log ppm(Table 2. 9) values show statistically significant differences between all three mouthrinses at all time points up to and including 45 minutes. At 60 minutes a difference is still seen between the 0 ppm in comparison with the 225 ppm or 500 ppm groups. However, there is no significant difference between the 225 ppm and 500 ppm groups at 60 minutes.

The results from the untransformed data (ppm), (Table 2. 8) and the transformed data (logarithmic ppm) (Table 2. 9) show differences in the significance of pairwise comparisons over time. It is an assumption that data are normally distributed when using parametric tests, such as an ANCOVA test. Since this is not necessarily the case in the untransformed datasets, the results from this may be questionable and therefore the results using the transformed data will be accepted. This demonstrates the difference that can occur where a normal data distribution is assumed and not assessed prior to parametric testing.

Summary of results

Statistically significant differences in salivary fluoride retention were observed between the 0 ppm, 225 ppm, and 500 ppm group, based on the area under the curve and the logarithmic area under the curve data. The ANCOVA results based on the logarithmic salivary fluoride ppm data found that significant differences were found between all three groups at all time points, with the only exception being between the 225 ppm and 500 ppm groups where significance was lost at the 60 minute time point.

Discussion

Tooth brushing regimens and the amount of toothpaste used by individuals can be highly variable. In this project 0.5g of 1450 ppm F toothpaste was used which represents a pea sized amount or covering approximately half the head of a small toothbrush. This is the recommended amount in the SIGN guideline – dental interventions to prevent caries in children. (SIGN guideline 138, 2014) Studies have shown that this is also in line with the amounts used by children (Wiener *et al* 2009, Bentley *et al* 2008).

The duration of brushing is also highly variable between individuals. Although the recommended length of brushing time is around 2 minutes (SDCEP guideline,2010) it has been established that it is generally less in practice and has been reported in young adults to be as little as 33seconds (Macgregor and Rugg-Gunn 1985), 60seconds in an adolescent population (Macgregor and Rugg-Gunn 2009) and, more recently, 96 seconds in an older adult population (Ganss *et al* 2009). In this study 40 seconds was chosen since it was considered to reflect the likely brushing time of a caries risk population. Salivary fluoride retention can be influenced by both the amount of toothpaste used and the duration of tooth brushing with an increase in both showing an increased fluoride retention (Zero *et al* 2010). It is known that, in general, people use less toothpaste and brush for shorter times than is recommended (Wiener *et al* 2009, Bentley*et al*2008, Macgregor and Rugg-Gunn 1985, Macgregor and Rugg-Gunn 2009, Ganss*et al*2009). These factors might be particularly important for caries risk patients who could benefit from the increased fluoride retention and improved caries prevention.

A third factor that influences fluoride retention is the use of water to rinse toothpaste slurry in the mouth. Rinsing with water is not normally recommended after brushing as it can reduce the fluoride retention (Mystikos*et al*2011, Duckworth *et al* 2009). However, most people dip their toothbrush under water prior to brushing and rinsing with water after tooth brushing is common with 55% of 15-16 year old describing rinsing with water as often or always post brushing (Jensen*et al*2012). In this study, participants rinsed 3 times with 10ml of water after brushing, but no water was used on the toothbrushes prior to this. It was anticipated that this represented a realistic scenario of salivary fluoride post brushing whilst offering a more controlled method of tooth brushing.

Unstimulated saliva samples were collected which differs in composition and flow rate from stimulated saliva. To ensure the comparability of saliva samples over the study subjects were asked to refrain from eating, drinking or speaking for the duration of each study period.

A late and second increase in salivary ppm was observed in a few isolated subjects which was likely due to residual toothpaste, most likely in the buccal sulcus becoming disrupted on salivary pooling prior to the sample being taken. The baseline values at T0 varied and it is plausible that some subjects brushed their teeth before the trial although they were advised not to do so. Drinking tea before the trial period may also affect the fluoride levels seen at baseline since this contains fluoride. As far as possible these factors were controlled and the timewise analysis utilised the baseline T0 values as a covariate to adjust for the potential effects of additional fluorides. The overall analysis was conducted on area under the curve values and this also would account for baseline rises ensuring that as far as possible these confounding factors were accounted for.

In this study the use of the 500 ppm mouthrinse produced a significant increase in fluoride salivary retention when compared with the 225 ppm mouthrinse ($p<0.001$) which in turn achieved significantly more fluoride retention than the non fluoride mouthrinse. Statistically significant differences were seen between the higher and lower fluoride mouthrinses up to the 45-60 minutes post brushing time interval ($p<0.05$) and both the 225 ppm and 500 ppm fluoride mouthrinses showed significantly elevated salivary fluoride levels compared to the non fluoride mouthrinse throughout the entire 60 minute post brushing period. This gives an indication of the lasting impact of fluoride retention following the use of post brushing fluoride containing mouthrinses.

A recent study also found that a 225 ppm fluoride mouthrinse significantly increased salivary fluoride retention when compared with no rinsing and that a 500 ppm mouthrinse provided significant increases in retention of fluoride when compared with the 225 ppm mouthrinse (Sköld *et al* 2012). These results support the findings of the current study and others highlighting the benefits of mouthrinses containing 225 ppm or 500 ppm fluoride post brushing in achieving a significant increase in salivary fluoride retention.

Although the populations considered in the current study and that described above are different, it is interesting to compare the fluoride retention outcome for the different post brushing rinse regimes. In the current study subjects brushed with 1450 ppm fluoride toothpaste and then rinsed three times with 10ml of water before using the mouthrinses. In the other study participants brushed with the same toothpaste but did not rinse out with water prior to rinsing with either a 500 ppm or 225 ppm fluoride mouthrinse or not rinsing at all after brushing. When post brushing rinsing with water was employed in the current study, prior to use of the mouthrinses, the $AUC_{0\text{-}60}$ means were 554, 252 and 20 for the 500, 225 and 0 ppm fluoride mouthrinse groups respectively. For the other study when no water rinsing was performed, for the 500 ppm F, 225 ppm F and no rinse treatments the mean $AUC_{0\text{-}60}$ were 626, 380 and 237 ppm F (Sköld *et al* 2012). This perhaps highlights the amount of fluoride washout that can occur when rinsing with water. In the current study rinsing with water was carried out after brushing since water wasn't used when brushing as a means of standardising the amount of fluoride toothpaste used and preventing over dilution.

High caries risk groups are likely to conduct suboptimal brushing regimes even when brushing occurs. In particular, brushing for the recommended time of 2 minutes (SDCEP guideline,2010) should not be underestimated in caries prevention. However, the results of this study suggest that even when brushing is suboptimal, particularly with regard to post brushing rinsing with water, the adjunctive use of fluoride mouthrinses of at least 225 ppm can increase salivary fluoride retention significantly. This is particularly important when considering the high number of the population, especially adolescents, who report regular post brushing rinsing with water and who could benefit significantly from using a post brushing fluoride mouthrinse.

It is interesting to note that in order to achieve post brushing salivary fluoride retention of periods of up to 60 minutes it is not necessary to rinse with solutions

containing very high levels of fluoride. The results of this study suggest that using a post brushing mouthrinse with a fluoride concentration of at least 225 ppm will increase salivary fluoride retention. It is speculated that if toothbrushing with post brushing rinsing occurs just before bedtime, then the anti-caries benefits of dentifrices may be enhanced further. This is due to the decrease in salivary flow during sleep which would result in an increased fluoride retention.

Conclusion

Use of a fluoride mouthrinse containing 225 ppm or 500 ppm produced a significant increase in salivary fluoride retention following brushing with 1450 ppm F toothpaste and rinsing with water. The use of the 500 ppm F mouthrinse may be of particular benefit to those at high caries risk.

Study 2 – The effect of varying fluoride concentration on remineralisation of bovine enamel *in vitro.*

Study 2 – The effect of varying fluoride concentration on remineralisation of bovine enamel *in vitro.*

Background

See also literature review Section 1.

The *in vitro* approach in cariology research

Due to the multifactorial nature of the carious process caries research based on *in vitro* creation and assessment of carious lesions is an accepted model. The advantage of an *in vitro* approach is that it enables the experimental conditions to be controlled to allow for the assessment of set variables. These factors can include variables such as fluoride level, pH and solution composition. In addition a large number of confounding factors that are encountered with *in vivo* and *in situ* studies are removed.

However, as an artificial setting the results from *in vitro* studies and the applicability of these results to the oral environment may be questionable. The acquired pellicle and dental plaque biofilms are difficult to create and are often absent in *in vitro* studies such as in this study. Further developments in this area are ongoing in order to improve the general applicability of *in vitro* results.

Use of Bovine incisors

Sound human teeth would be the ideal choice for experimentation, however, these are not readily available. It is only in a few situations that sound human teeth would be extracted. Examples would be for orthodontic or periodontal reasons, or where an impacted tooth is removed. For this reason the use of bovine teeth in cariology research is widely accepted.

The main difference between human and bovine enamel is that bovine enamel is softer and more porous being more alike to that of deciduous tooth enamel than permanent (Arends *et al*, 1989).Being more porous bovine enamel

demineralises more readily when creating artificial caries-like lesions. Research in this area has found that consistent caries-like lesions can be produced when bovine enamel is immersed in a partially saturated acidic buffer solution within 72 hours (Amaechi *et al*, 1998).

Laboratory techniques for the assessment of *in vitro* carious lesions.

As previously mentioned transverse microradiography is considered the gold standard technique. Examination of the caries lesion is by microradiography of cross sections of the lesion. This gives a true representation of mineral loss and depth. The main disadvantage of the technique is in its destructive nature and therefore its use can only be confined to *in vitro* or *in situ* studies. It is also technique sensitive and time consuming, which may be the reason why several other techniques are often favoured, although TMR is still the only technique that examines the carious lesion cross sectionally.

Quantitiative light fluorescence bases its assessment on the innate fluorescent properties of enamel to detect and also quantify areas of enamel demineralisation. It is not a destructive technique and therefore has the advantage of being able to be used in *in vitro* and *in situ* studies but can also be used *in vivo*. It can also be used as a longitudinal evaluation technique of caries lesions with the ability to quantify enamel demineralisation.

Multi spectral imaging also has the advantage of being a non destructive technique that can be used longitudinally. It uses wavelength technology to build a series of images produced from a series of wavelength analysis, creating a ‘cube’ of data for each exposure. This technology has been shown to detect changes in enamel mineralisation earlier and with greater margins when compared with QLF-D (Desmons *et al* 2013).

Fluoride concentrations

Fluoride is available in many different preparations and from various sources, from water to toothpaste. The most commonly used toothpaste concentration of fluoride is 1450 ppm F, with 2800 ppm being recommended for those at particularly high risk of dental caries. The concentration most commonly found in a fluoride mouthrinse is 225 ppm or 0.5%F, with higher fluoride mouthrinses now available at a concentration of 500 ppm F. 5000 ppmF toothpaste and 900 ppmF mouthrinse are produced but are not commercially available to the general public. With this in mind the preparations used in this study were a control group of 0 ppm with experimental groups of 225 ppm,500 ppm, 1450 ppm and 2800 ppm fluoride.

Aim

To investigate the effect of varying fluoride concentration on the remineralisation of bovine enamel *in vitro* using the techniques of quantitative light induced fluorescence (QLF – D), multispectral imaging (MSI) and transverse microradiography (TMR).

Objectives

Subsurface caries-like lesions were created and assessed with TMR, QLF-D and Multi Spectral Imaging. Enamel sections were then immersed in artificial saliva with 0, 225, 500, 1450 or 2800 ppm fluoride for 8 weeks. Percentage mineral gain or loss was assessed with weekly QLF-D and Multi Spectral Imaging for 8 weeks followed by TMR.

Method

Bovine incisor selection and preparation

Bovine incisors were extracted at a local abattoir from freshly culled animals and were stored in 10% (w/v) thymol solution (Sigma-Aldrich.Co, Ltd,UK) to prevent bacterial growth and desiccation.

Teeth were examined and those with enamel irregularities such as cracks, hypoplasia or any enamel malformations were discarded.

Sound teeth with no irregularities were debrided of remaining soft tissue using a scalpel.

The buccal surface was then polished with wet sandpaper of varying coarseness– p120, p240, p1000, p1500 (English Abrasives and Chemicals, UK) until the outermost ridged enamel and surface pellicle was removed leaving a polished surface. They were then rinsed with water to remove remnants of the abrasive and allowed to ‘bench dry’ on blue roll (Lotus professional, Hydrotec) for several minutes.

QLF-D was used to assess the prepared teeth. Teeth with any enamel malformations that were previously undetected were excluded or further polishing was carried out until imaging was clear of irregularities and there was a clear polished surface.

The crowns were then sectioned using a rotary diamond disk to give ideally 2 samples of buccal enamel per tooth as marked in Figure 3. 1. Samples varied in size due to the size difference between individual teeth and due to differences in incisor morphology.

Figure 3. 1- prepared enamel surface marked with pencil to give two buccal enamel samples.

The sections of the teeth were then coated in transparent acid resistant nail varnish (Max Factor Nailfinity, clear, Proctor and Gamble, Weybridge UK). The buccal enamel was bordered by 2-3mm of nail varnish leaving an exposed window of the most homogenous enamel as highlighted by QLF-D examination and left overnight to dry.

Sections were then mounted onto glass rods using greenstick impression compound (Kerr Inc,Orange, California, USA) leaving the windows of enamel exposed, and placed in 50ml containers and refrigerated prior to the demineralisation phase.

Demineralisation

Partially saturated acidic buffer solution was then prepared using the following method:

For 1 litre:

299mg Potassium dihydrogen orthophosphate (KH_2PO_4)

Place in a 1L beaker with a magnetic stirrer and add 900ml deionised distilled water.

Then add:

2.2ml of 1M $CaCl_2$ AVS grade

2.85ml of glacial Acetic acid (HAC) AR grade

Adjust pH to 4.5 with conc KOH solution

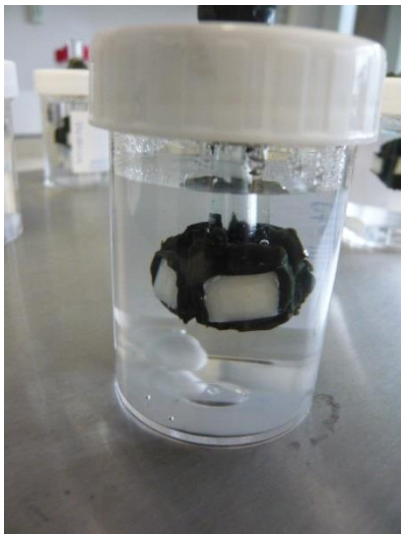
Add 0.5ml (500µl) of NaF

Make up to 1L with additional distilled deionised water.

Creation of artificial subsurface carious lesions

The samples were then immersed in a demineralising solution. A small magnetic flea was placed in each pot and set on a Stuart SB301 Stirrer set at 150rpm for 72 hours (Amaechi *et al*, 1998) (Figure 3. 2).

Figure 3. 2- samples mounted and in demineralisation solution



Samples were then rinsed in distilled water, removed from the greenstick rods and dried, being left to bench dry for at least 30 minutes prior to imaging with QLF-D to assess lesion creation.

Baseline assessment with QLF-D

All samples were baseline imaged with QLF-D. The QLF-D images were captured using C3 V1.23 QLF-D software (Inspektor Research System, BV Amsterdam, The Netherlands). The camera was held in a fixed position with white light and blue light images collected. All images were taken in a darkened room with the sample surface orientated to be at 90° to the light source. All images were labelled allocating a sample number to each sample and stored on the computer's hard drive.

Samples with non-uniform demineralisation were excluded. Samples with insufficient demineralisation, where the lesions were presumed to be shallow by little change in fluorescence between the lesion and surrounding sound enamel, were then remounted and labelled and placed in demineralisation solution for a further 24 hours prior to drying and re-imaging with QLF-D (Figure 3. 3 A and 3. 4).

Figure 3. 3 – QLF-D assessment -Sufficient uniform demineralisation

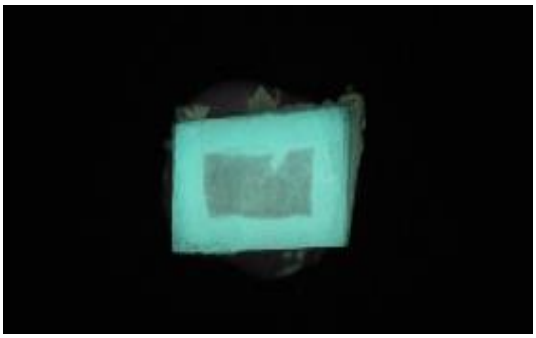
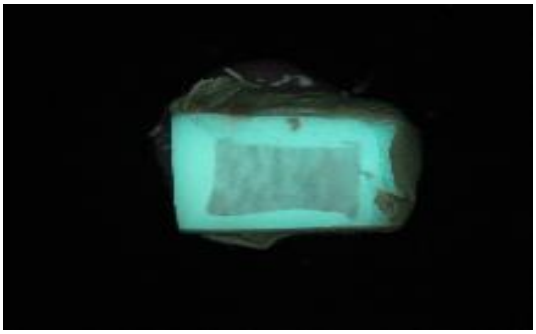


Figure 3. 4- non-uniform demineralisation

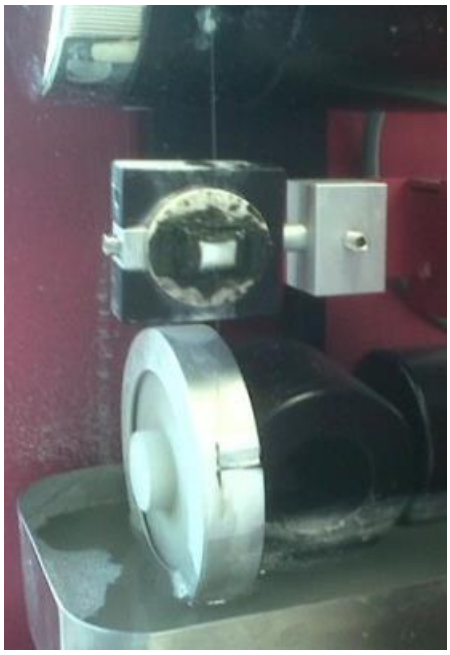


The samples with sufficient and uniform demineralisation as assessed with QLF-D imaging were then considered suitable for inclusion in the study and were prepared for baseline analysis with transverse micro radiography.

TMR preparation

Samples suitable for TMR were mounted with green stick and sectioned with a 0.17mm diameter wire on a water-cooled diamond wire saw (Well, Walter Evber, Le Locle, Switzerland)(Figure 3.5). 3 sections approximately 110µm in thickness were taken from each section and stored in distilled water prior to mounting on brass anvils.

Figure 3. 5– water – cooled diamond wire saw for sectioning samples.



Sections were mounted on brass anvils with nail varnish (Mac Factor Nailfinity) (Figure 3.6). Sections were polished on a diamond disc (Figure 3.7) then to ensure the sections were planoparallel, they were soaked in acetone to remove them from the brass anvils and remounted. The second side was polished to give a section thickness of 80+/- 10µm. Sections were then again removed by soaking in acetone and stored in distilled water in labelled 2ml containers prior to mounting on templates.

Figure 3. 6– Sample sections mounted on brass anvils

Figure 3. 7- Diamond disk to hand polish the samples mounted on brass anvils

Sample sections were mounted on templates, leaving the lesion visible in an exposed window (Figure 3. 8). To identify sections on microradiograph a template outline was drawn labelling each section (Figure 3. 9). The mounted sections were then placed in a microradiographic plate holder which housed an aluminium stepwedge with ten layers of 25µm steps. Microradiographs were taken at a 35 minutes exposure on Kodak high-resolution plates (type1A). Phillips x-ray set (Philips B.V, Eindhoven, The Netherlands) was used for x-ray generation utilising a copper anode with nickel filter operating at 25Kv and 10mA with a focus to the specimen distance of 40 cm. The plates were then developed and fixed using Kodak HRP chemistry (Figure 3. 10).

Figure 3. 8– Sample sections mounted on a template with lesions exposed in a prepared window.

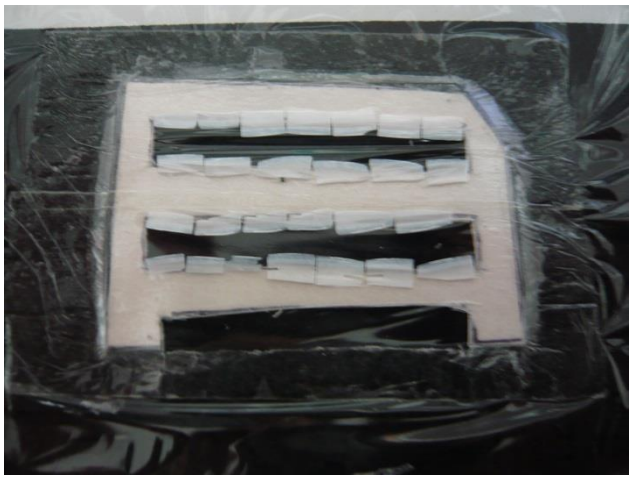


Figure 3. 9– Paper template outline to identify sections.

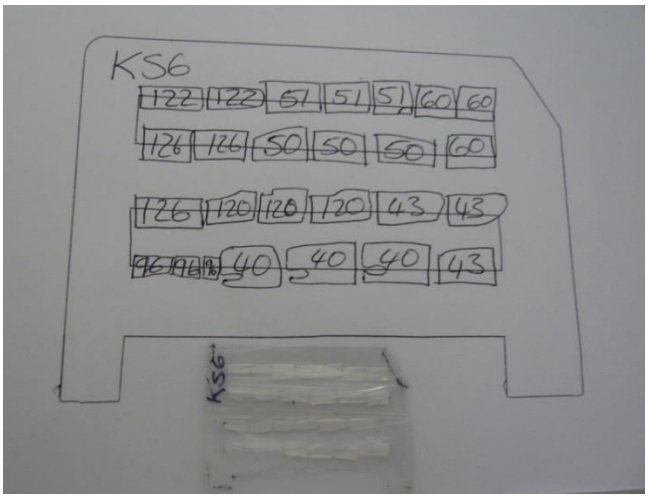
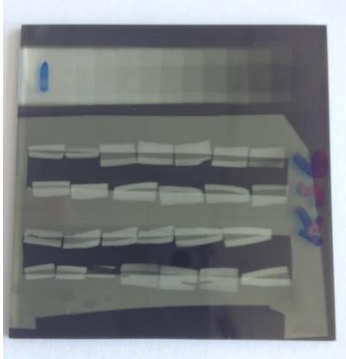


Figure 3. 10– Complete TMR plate

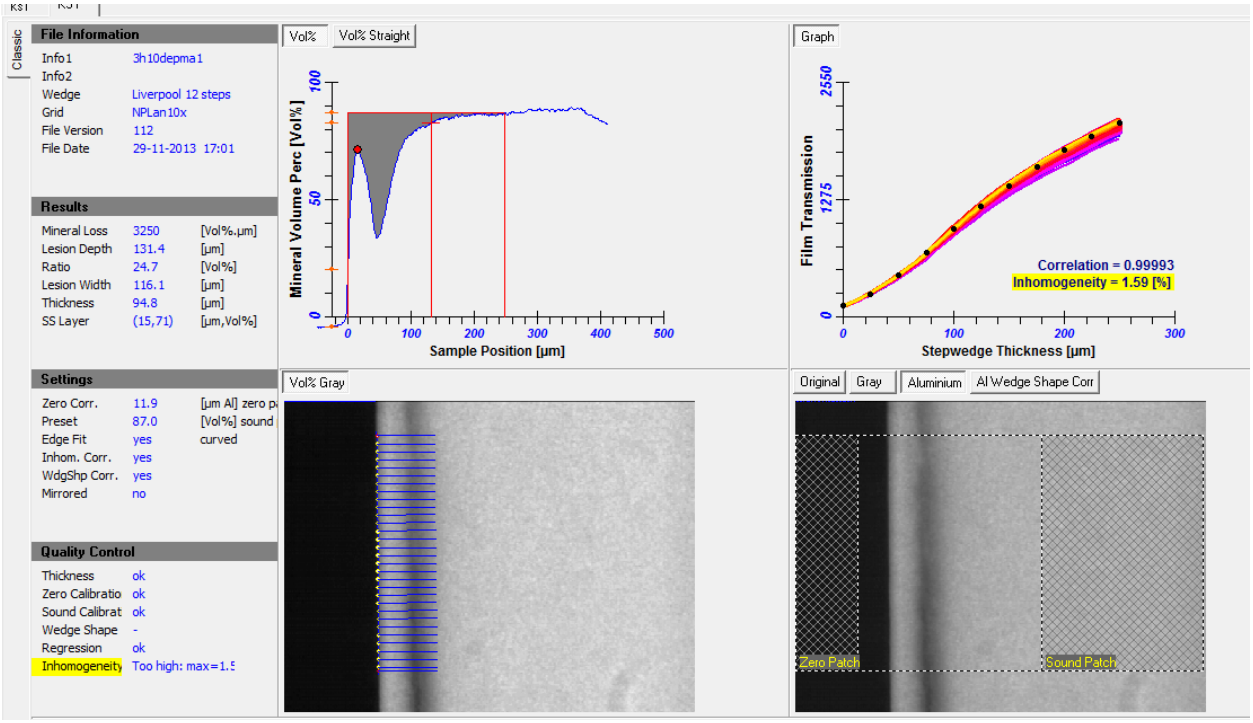


A Leica Leitz optical microscope (Leiza, Wetzlar, Germany) was used to view the microradiographs. The aluminium stepwedge was calibrated before images

were captured of the samples along the length of the samples lesion. This was achieved using a CCD video camera module (Sony XC75CE, Sony Corporation, Tokyo, Japan) linked to a computer (Viglen PC, London, UK).

TMR image analysis was carried out using TMR 2006 3.0.0.15 software(Inspektor Research System BV, Amsterdam, The Netherlands) with results for mineral loss (Vol%.µm), lesion depth (µm), and lesion width (µm) taken. Sound areas and zero areas were identified with profiles across the lesion created automatically. These were then modified to avoid any artefacts on the image or damaged areas of the lesion (Figure 3. 11).

Figure 3. 11– Screenshot of TMR analysis obtained showing lesion profile graphs and correlation to the stepwedge.



Once baseline values were obtained, samples with successful QLF-D and TMR results were randomised to create 5 groups for remineralisation. Randomisation was done using a computer generated random number sequence run in Excel (Excel, Microsoft Inc, Redmond, California, USA). The remineralisation phase

involved immersion in artificial saliva with concentration of either 0, 225, 500, 1450 or 2800 ppm F .

Groups were checked for equivalence before clear nail varnish was again applied ensuring that the sectioned ends of the samples were covered. Samples were then mounted on green stick impression compound, labelled with a cotton thread and colour coded label indicating the group, and labelled with the sample number . Samples were then immersed in artificial saliva with 0, 225, 500, 1450 or 2800 ppm F again on a Stuart stirrer with a magnetic flea (Figure 3. 12).

Figure 3. 12– samples suspended in solutions per group with magnetic flea in each pot and placed on a stuart stirrer.

Artificial saliva with the varying fluoride concentrations was produced with the following constituents:

- 2.0 g Methylhydroxybenxoate
- 10.0 g Sodiumcarboxymethylcellulose
- 0.625 g Potassium chloride
- 0.059 g Magnesium chloride hexahydrate
- 0.804 g Postassium hydrogen orthophosphate
- 0.326 g Potassium dihydrogen orthophosphate
- 0.166 Calcium chloride diyhydrate
- Sodium fluoride
    - 0 g in 0 ppm group
    - 0.497 g in 225 ppm group
    - 1.105 g in 500 ppm group
    - 3.205 g in 1450 ppm group
    - 6.189 g in 2800 ppm group

The saliva was made up with distilled water and the pH adjusted to 7.2 using concentrated Potassium hydroxide.

Experimental period

Baseline and then weekly QLF-D and Multi Spectral Imaging images were taken with the samples being removed from the solutions and allowed to air dry for at least 30 minutes prior to imaging. Solutions were changed weekly before the samples were re-immersed. This was repeated for the duration of the study

that lasted for 8 weeks. TMR was then carried out to assess overall mineral change.

Multi Spectral Imaging

Multi Spectral Images (MSI) were captured using Nuance 3.0 Nuance multispectral imaging system (CRi, Woburn, USA) Samples were illuminated with a 405 nm exciting light source. Fluorescence and white light images were captured with Nuance TM fitted with a 460 nm highpass colour filter. The camera was held in a fixed position with samples orientated at 90° to the camera with all images taken in a darkened room. Images were collated for 420nm to 720nm at 10nm increments. This produced a composite image called the data cube. The cubes of data were labelled by week and sample number and saved on the computer's hard drive for later analysis.

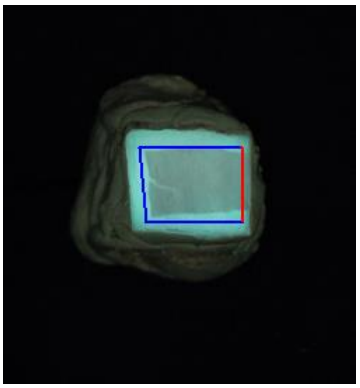
Software for total analysis of MSI images is not yet available therefore the image cubes produced from MSI were split, isolating the 520nm section for each image set as this has been shown to be the most sensitive for demineralisation analysis (Desmons *et al* 2013). The 520 nm sections were saved as Tiff files to maintain image quality before being converted into bitmap files, compatible with the analysis software.

QLF-D and MSI image analysis

All image analysis of QLF-D and the 520nm MSI sections were analysed using QA2 v1.23 software (Inspektor Research System, BV Amsterdam, The Netherlands). All images were analysed by a single examiner following a set of predetermined rules (Pretty *et al* 2002) for box placement around the lesion (Figure 3. 13). The box places the blue region on sound areas with a red area

required where this crosses over the demineralised lesion, which is required in these samples since the sound end was removed to obtain sections for the baseline TMR. Values were obtained for fluorescence loss - ΔF at the 5% threshold level.

Figure 3. 13– box placement around the lesion to mark area for analysis.



Statistical Analysis

The Statistical Package for Social Sciences software (SPSS version 20.0,Chicago, Illinois, USA) was used for calculating descriptive statistics including mean and the standard deviations for each group.

Reliability of the QLF-D and MSI

data were tested with the repeated analysis of 10% of the data at least 4 weeks after the initial analysis. The variability was assessed in relation to the standard deviation of the mean, and plotted on Bland and Altman plots(Bland and Altman, 1986). The normality of the data for the QLF, MSI and TMR analyses was tested with frequency histograms and Q-Q plots and confirmed with Shapiro-Wilk tests. The data were accepted as being normally distributed for the QLF-D and MSI. For the TMR data not all datasets were normally distributed and therefore a secondary analysis was carried out on logarithmic transformed data. With the normality assessed and accepted parametric tests were suitable for data analysis. The data were then analysed using a two-way

analysis of co-variance (ANCOVA). For the QLF-D and MSI data the repeated measures test was used with week 0 as the covariate and week 1 to week 8 as dependent variables. The significance level (α level) for all tests used was set at an α level of 0.05. A bonferroni correction for multiple comparisons was applied as there are 10 sets of pairwise comparisons between groups which would otherwise be at risk of type 1 error. Due to the variation in difference between groups over time an additional ANCOVA test was applied to the week 0 and week 8 data only, using a univariate model.

Correlations between QLF-D, MSI and TMR were also carried out using the Pearsons correlation test to compare the agreement of the data sets.

Results

A total of 80 bovine incisors were prepared and demineralised giving a total of 160 samples. Only 85 showed sufficiently uniform demineralised lesions to proceed to baseline TMR. Due to the error with the diamond plate and anvils, several sample sections were extensively damaged and extremely thin with the result that values from the TMR were not possible. An extra slice was taken from each sample without sufficient data from the first baseline round of TMR in an effort to improve the baseline data for each sample and increase the number of samples with sufficient data to proceed with the study. Again many sections were damaged in the process but following this 66 samples were available with baseline data and the sample with the least data available from TMR was excluded, giving 65 samples and 13 in each of the experimental groups.

The post remineralisation phase of TMR was not affected by the above mentioned grinding problems and data were successfully obtained for 61 of the 65 samples. 2 samples disintegrated on sectioning with the wire saw, 1 sample lesion was separated from the rest of the sample during grinding and removing

the sample from the anvil in acetone, and the final sample was unfortunately broken due to human error on removing sections from the 2ml storage containers. All samples affected were from different groups resulting in a minimum of 12 samples per group for TMR analysis.

Repeatability testing of QLF-D and MSI measurements

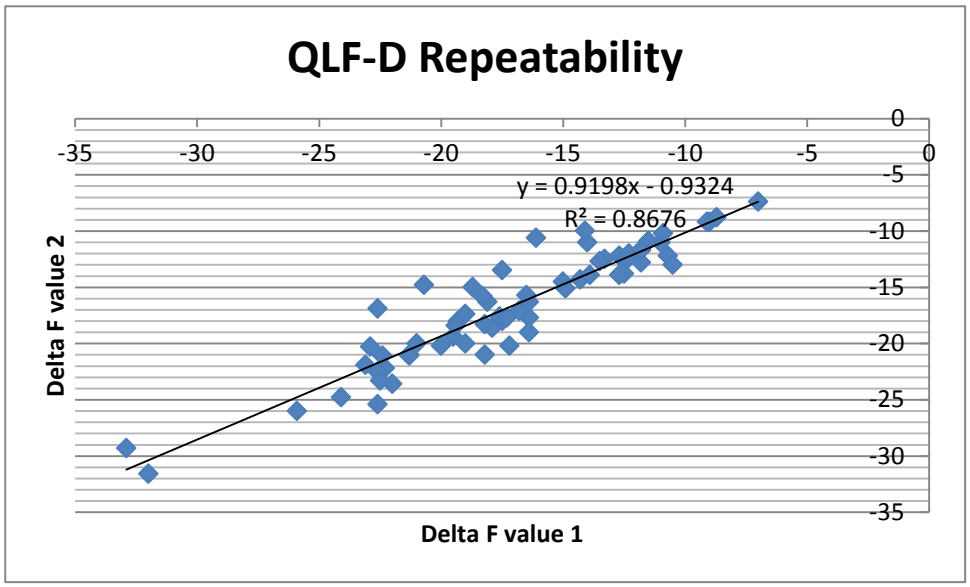
To assess the reliability of the values obtained from QLF-D and MSI image analysis, repeated measurements were taken. 65 QLF-D images and 65 MSI images were re-analysed representing 11% of the total. These were re-analysed at least 4 weeks after initial analyses with the same operator, under the same conditions, blind to the original readings. The two data sets were compared.

QLF-D repeatability:

The values of measurements with differences in ΔF and in standard deviation of the mean are in Appendix VIII Table 3. 15.

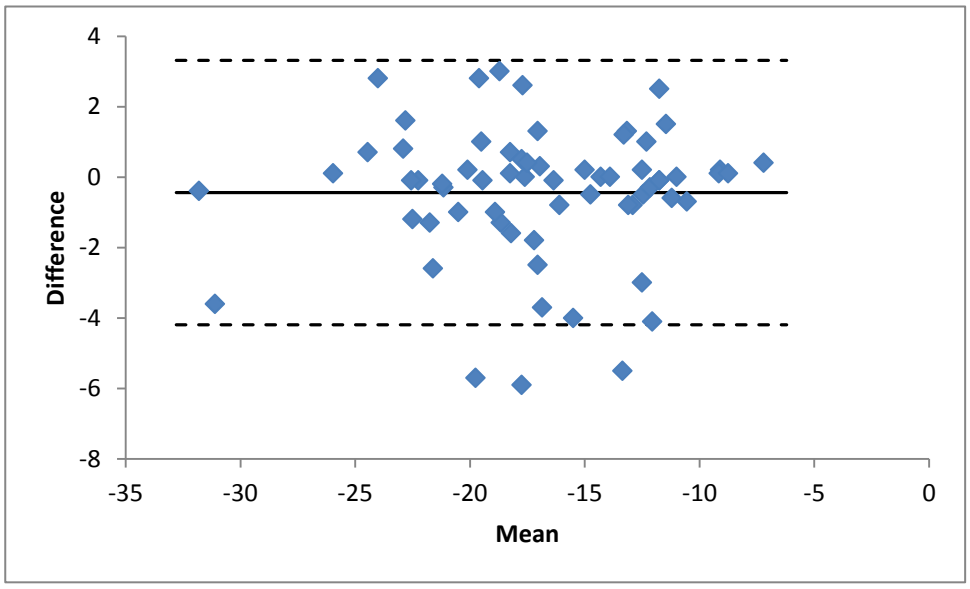
A strong correlation between the data sets is seen below where the value sets are plotted showing a linear agreement (Figure 3. 14).

Figure 3. 14– Correlation of QLF-D repeated values.



To better assess the reliability of the measurements the pairs of data were plotted ona Bland-Altman plot. This plots the mean of the two measurements on the x-axis and the difference between them on the y-axis. The mid horizontal line indicates the mean difference with the upper and lower lines highlighting the limits of agreement. 95% of repeated measures would be expected to lie within the limits of agreement(Altman 1991),(Figure 3. 15).

Figure 3. 15– Bland-Altman plot for QLF-D



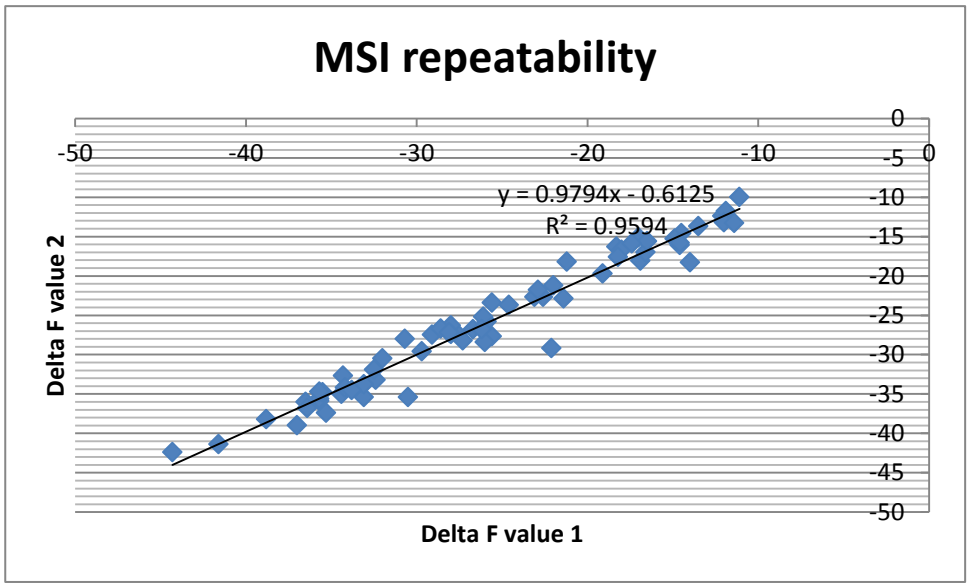
The mean difference between the pairs of data were -0.44, within the limits of agreement of -4.19 and 3.31. Three pairs of data did not lie within the limits of agreement. However, since these represent less than 5% the QLF-D analysis was considered to be sufficiently reliable.

MSI Repeatability testing

Values of both data sets for the 65 images with differences and differences to standard deviation are reported (Appendix VIII Table 3. 16).

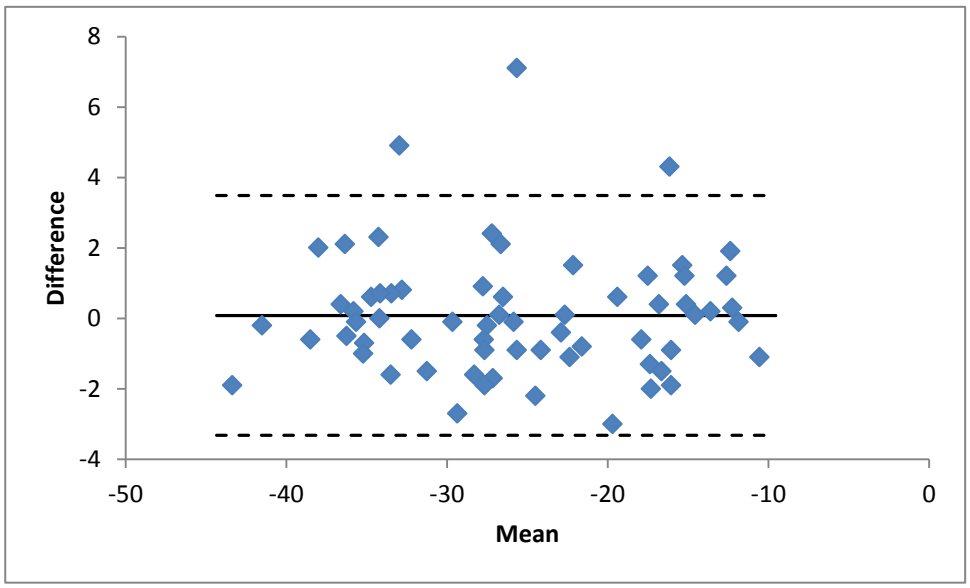
Again the paired data are plotted below showing a strong linear agreement (Figure 3. 16).

Figure 3. 16– correlation of MSI repeated values.



The differencesbetween the pairs of data for MSI were smaller than for the pairs of QLF-D data indicating a greater agreement. This is quantified above with a correlation coefficient of 0.9594. A Bland-Altman plot was again carried out in order to better assess the reliability of the measurements within the 95% limits of agreement(Figure 3. 17), (Altman 1991).

Figure 3. 17–Bland-Altman plot for MSI



The mean difference between the pairs was found to be 0.09, with 95% limits of agreement of -3.32 and 3.49. The MSI analysis can therefore be concluded to be of good reliability.

QLF-D Assessment of mineral change – ∆F

All samples were considered to be suitable to be included in the study at baseline and although samples would be showing baseline ∆F values that would be greater or lesser than other samples the computerised generated randomisation sequence allocating the groups would have been predicted to create equal groups. Means and standard deviations of all groups are shown below (Table 3. 1). This also shows the minimum and maximum baseline ∆F value for the samples within those groups. It is on the basis of the spread of high and low ∆F baseline values that groups can be considered equal.

Table 3. 1- Descriptive Statistics QLF-D

	N	Minimum∆F	Maximum∆F	Mean∆F	Std. Deviation∆F
Group	13	0	0	.00	.000
ppm0	13	-27.90	-10.50	-20.2769	5.04615
ppm225	13	-32.00	-7.60	-17.2385	8.11445
ppm500	13	-24.70	-8.10	-16.0769	5.80175
ppm1450	13	-31.30	-9.20	-20.3769	6.68732
ppm2800	13	-28.70	-9.40	-18.0231	5.40018
Valid N (listwise)	13				

Normality testing

Prior to data analysis the distribution of the data per group at each time point was checked and assessed for normality. Graphically normality was assessed with the use of Q-Q plots and frequency histograms. Based on these graphs there was some doubt as to the normality in distributing the data at certain time

points, therefore, numerical normality testing was also carried out in the form of the Shapiro-Wilk test.

This confirmed that the data in each group at each time point was normally distributed. Parametric tests were therefore suitable to be used.

ANCOVA testing of QLF-D Data

An analysis of co-variance (ANCOVA) was used to test for difference between group effects. Using ANCOVA allows for the differences between groups to be assessed whilst also controlling for an additional variable described as the covariate. It is important to statistically control a known variable that may have an effect on the dependant variable. In this study the covariate is the baseline mineral loss -ΔF.

The variation in dependant variables that is due to the covariate is corrected by regression procedures before a standard analysis of variance is carried out. This enables the analysis to be carried out only on the ΔF values that have been corrected for by the baseline ΔF value. This process increases the power of the test meaning that should a true difference between the groups exist the likelihood of this being detected is greater.

Testing of a groupwise effect

The model used was a 2-way ANCOVA with the categorical independent variable being the group and covariate as previously mentioned being baseline ΔF. The dependant variables, therefore, were the week 1 to week 8 ΔF values.

Table 3. 2 - Tests of Between-Subjects Effects

Source	Type III Sum of Squares	Df	Mean Square	F	Sig.
Intercept	679.133	1	679.133	11.703	.001
week0	12390.618	1	12390.618	213.522	.000
group	1888.529	4	472.132	8.136	.000
Error	3423.746	59	58.030		

A statistically significant groupwise effect is seen with a significant level of $p < 0.05$ (Table 3. 2).

Pairwise comparisons of between group effects

The above ANCOVA test confirms an overall groupwise effect as indicated with a significance level of $p<0.05$.

Pairwise comparison over the 8 week experimental period

Testing of between group effects was therefore carried out using the same ANCOVA model to highlight whether individual pairwise groups were significant(Table 3. 3).With 5 groups and therefore 10 pairwise comparisons a bonferroni correction was applied to adjust for the effect of multiple comparisons.

Table 3. 3 - Pairwise comparisons of all groups

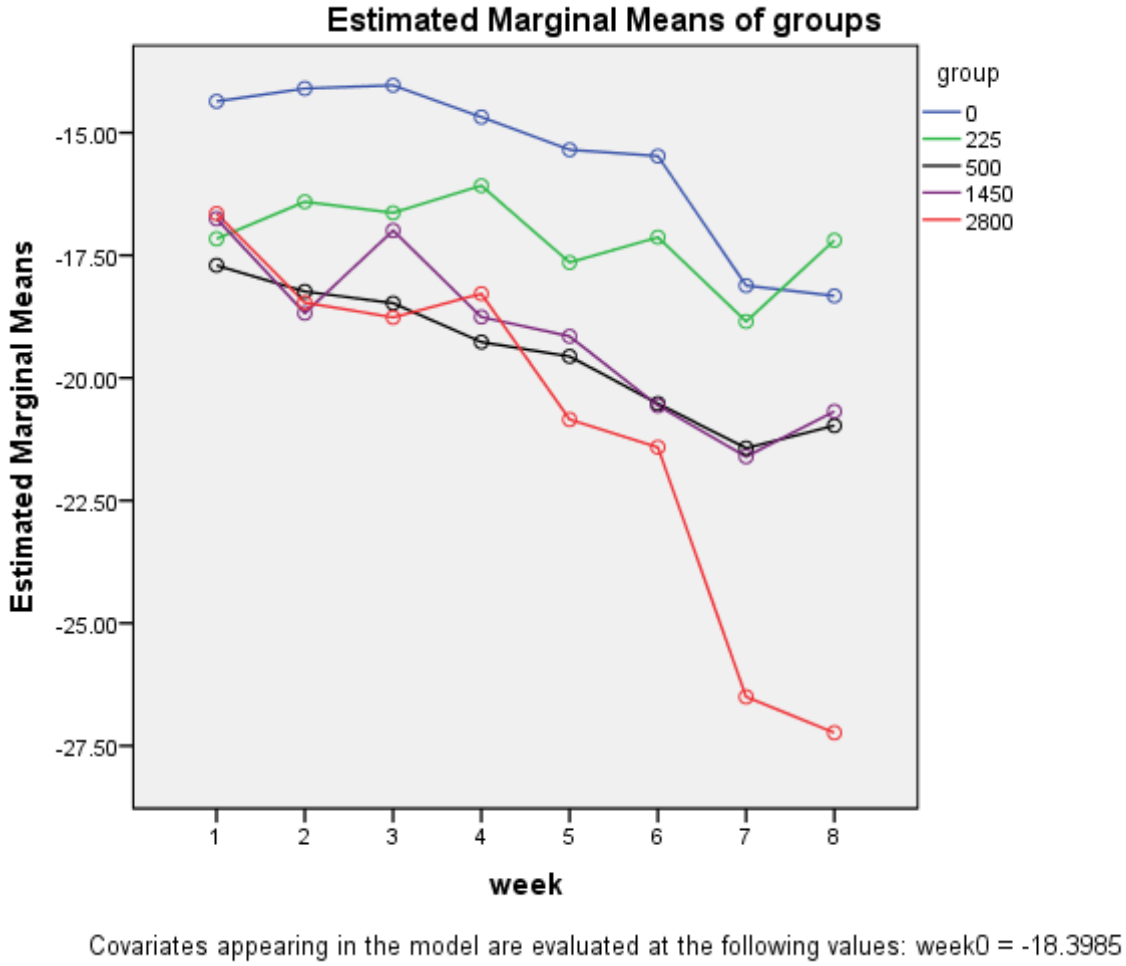
(I) group		Mean Difference (I-J)	Std. Error	Sig.[b]	95% Confidence Interval for Difference[b]	
					Lower Bound	Upper Bound
0	225	1.583	1.070	1.000	-1.537	4.702
	500	**3.966***	**1.081**	**.005**	**.812**	**7.120**
	1450	**3.593***	**1.056**	**.012**	**.513**	**6.674**
	2800	**5.465***	**1.064**	**.000**	**2.363**	**8.567**
225	0	-1.583	1.070	1.000	-4.702	1.537
	500	2.384	1.058	.280	-.703	5.470
	1450	2.011	1.070	.653	-1.111	5.133
	2800	**3.882***	**1.057**	**.005**	**.799**	**6.966**
500	**0**	**-3.966***	**1.081**	**.005**	**-7.120**	**-.812**
	225	-2.384	1.058	.280	-5.470	.703
	1450	-.373	1.083	1.000	-3.530	2.785
	2800	1.498	1.062	1.000	-1.598	4.595
1450	**0**	**-3.593***	**1.056**	**.012**	**-6.674**	**-.513**
	225	-2.011	1.070	.653	-5.133	1.111
	500	.373	1.083	1.000	-2.785	3.530
	2800	1.871	1.064	.839	-1.233	4.975
2800	**0**	**-5.465***	**1.064**	**.000**	**-8.567**	**-2.363**
	225	**-3.882***	**1.057**	**.005**	**-6.966**	**-.799**
	500	-1.498	1.062	1.000	-4.595	1.598
	1450	-1.871	1.064	.839	-4.975	1.233

Based on estimated marginal means

*. The mean difference is significant at the .05 level.

b. Adjustment for multiple comparisons: Bonferroni.

Figure 3. 18– plotted estimated marginal mean per group over the 8 week experimental period.



Statistically significant differences are highlighted in Table 3.3with the plotted estimated marginal means of each group at each week seen in Figure 3. 18 above.

The difference between the individual groups each week varied without any apparent linear groupwise effect over time. Due to this variation across the experimental period, a second analysis was carried out based on the final data at week eight compared to the baseline data at week 0 only (Table 3. 4, Table 3. 5).

Between group effects at week 8

Again a groupwise effect was seen, (Table 3. 4) with significant difference also noted to the baseline data indicating the overall experimental effect. Pairwise comparisons based on the baseline and final data were therefore carried out to assess for groupwise differences. The same ANCOVA model was used.(Table 3. 5)

Table 3. 4– Tests of between group effects

Dependent Variable: week8 ΔF

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	2564.308[a]	5	512.862	37.763	.000
Intercept	175.852	1	175.852	12.948	.001
week0	1720.590	1	1720.590	126.689	.000
Group	786.105	4	196.526	14.470	.000
Error	801.290	59	13.581		
Total	31708.110	65			
Corrected Total	3365.598	64			

a. R Squared = .762 (Adjusted R Squared = .742)

Pairwise comparison of between group effects at week 8

Table 3. 5– Pairwise group comparison of week 0 baseline and week 8 final data

(I) group		Mean Difference (I-J)	Std. Error	Sig.[b]	95% Confidence Interval for Difference[b]	
					Lower Bound	Upper Bound
0	225	-1.135	1.464	1.000	-5.404	3.133
	500	2.643	1.480	.792	-1.672	6.959
	1450	2.354	1.446	1.000	-1.862	6.569
	2800	**8.906***	**1.455**	**.000**	**4.661**	**13.151**
225	0	1.135	1.464	1.000	-3.133	5.404
	500	3.779	1.448	.115	-.445	8.002
	1450	3.489	1.465	.205	-.783	7.761
	2800	**10.041***	**1.447**	**.000**	**5.822**	**14.261**
500	0	-2.643	1.480	.792	-6.959	1.672
	225	-3.779	1.448	.115	-8.002	.445
	1450	-.290	1.481	1.000	-4.610	4.031
	2800	**6.263***	**1.453**	**.001**	**2.025**	**10.500**
1450	0	-2.354	1.446	1.000	-6.569	1.862
	225	-3.489	1.465	.205	-7.761	.783
	500	.290	1.481	1.000	-4.031	4.610
	2800	**6.553***	**1.456**	**.000**	**2.305**	**10.800**
2800	**0**	**-8.906***	**1.455**	**.000**	**-13.151**	**-4.661**
	225	**-10.041***	**1.447**	**.000**	**-14.261**	**-5.822**
	500	**-6.263***	**1.453**	**.001**	**-10.500**	**-2.025**
	1450	**-6.553***	**1.456**	**.000**	**-10.800**	**-2.305**

Based on estimated marginal means

*. The mean difference is significant at the .05 level.

This reveals statistical significances between groups where $p<0.05$ and where the 95% confidence interval does not span 0. The significant differences are highlighted and this indicates differences between the 2800 ppm fluoride group and all other groups. Difference between the 0 ppm, 225 ppm, 500 ppm and 1450 ppm groups were not significant. Interestingly, the differences between the groups is in a negative direction indicating increasing mineral loss and not mineral gain that would have been predicted.

MSI Assessment of mineral change – ΔF

As with the QLF-D data, all samples were considered to be suitable to be included in the study. Means and standard deviations of all groups are shown below (Table 3.6).

Table 3. 6-Descriptive Statistics - MSI

	N	Minimum (ΔF)	Maximum (ΔF)	Mean (ΔF)	Std. Deviation (ΔF)
group	13	0	0	.00	.000
ppm0	13	-37.40	-11.80	-28.4538	7.62677
ppm225	13	-41.40	-10.00	-23.2308	10.09648
ppm500	13	-38.20	-13.30	-22.8462	8.00413
ppm1450	13	-42.40	-12.40	-29.7154	8.78321
ppm2800	13	-36.00	-15.10	-24.2231	6.85531
Valid N (listwise)	13				

The descriptive statistics in Table 3.9 above show the minimum and maximum ΔF values in each group. It also demonstrates the mean ΔF values and standard deviations of those groups. As with the QLF-D data it would appear that a mix of lower and higher ΔF values are included in each group.

As for the QLF-D values, normality of the data distribution was examined and confirmed numerically with the Shapiro-Wilk test. Only two datasets were considered not to be of normal distribution as determined with the Shapiro-Wilk test. These were weak 4 for the 500 ppm group and week 6 for the 0 ppm group. The frequency histograms and QQ plots for these two datasets were examined to assess the deviation from normal (Figure 3.s 19-22). The frequency histogram of week 4 of the 500 ppm group showed that the data were skewed to the left (Figure 3. 19). The Q-Q plot also showed some

deviation from the line, but was of an overall linear appearance (Figure 3. 20). The week 6 0 ppm data again highlighted a slight skew, this time to the right (Figure 3. 21). The Q-Q plot indicated some outliers, but was again of an overall linear appearance (Figure 3. 22). They were both therefore included in data analysis as the distribution of data were not sufficiently lacking from normal to warrant exclusion. Parametric tests were used as the data were considered to be normally distributed.

Figure 3. 19– Frequency histogram for week 4 of the 500 ppm data, highlighted as being not normally distributed as indicated by the left skewed data.

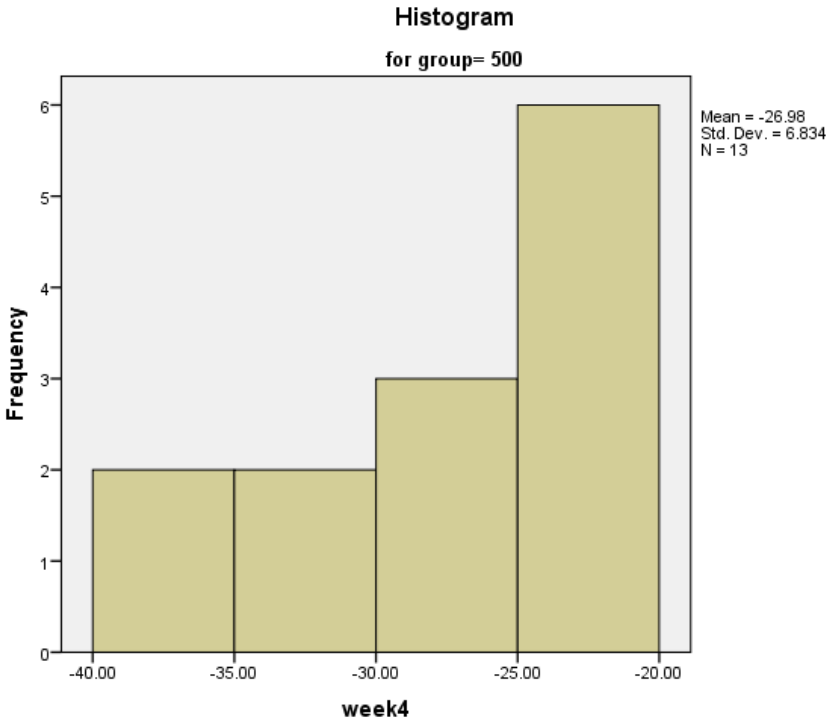


Figure 3. 20– Q-Q plot of week 4 for the 500 ppm data showing some deviation from the line indicating the data distribution is not entirely normal.

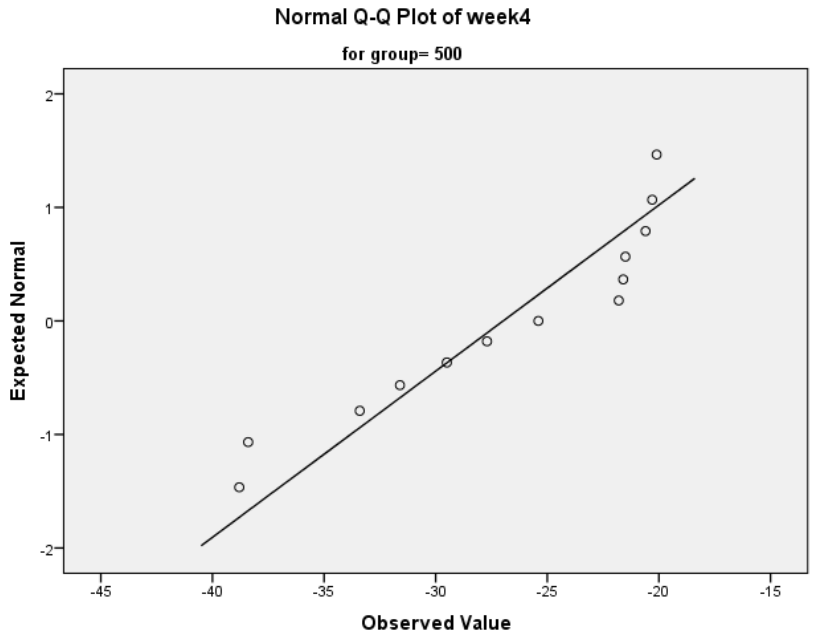


Figure 3. 21– Frequency histogram of week 6 for the 0 ppm data indicating a slight skew to the right.

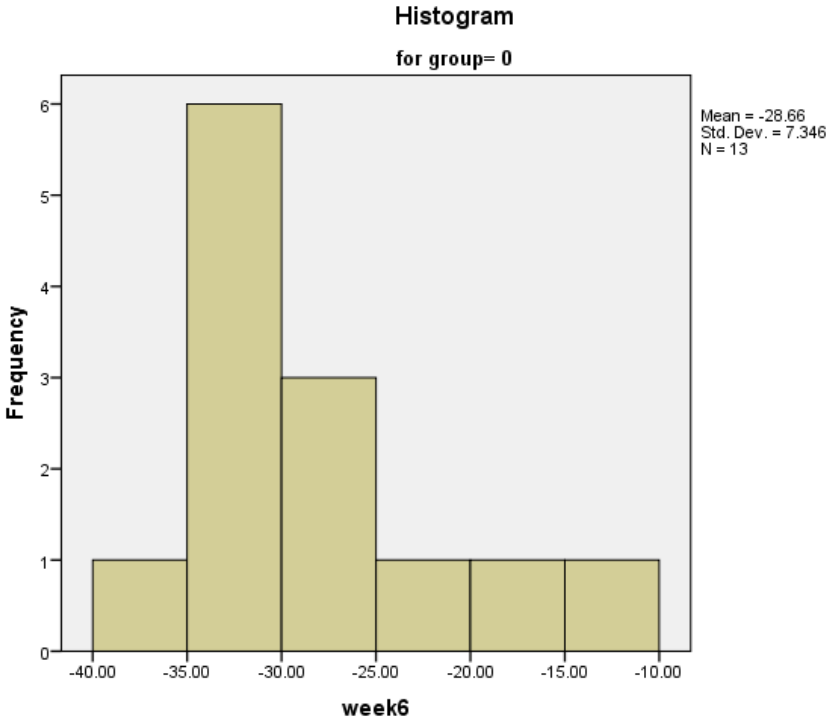
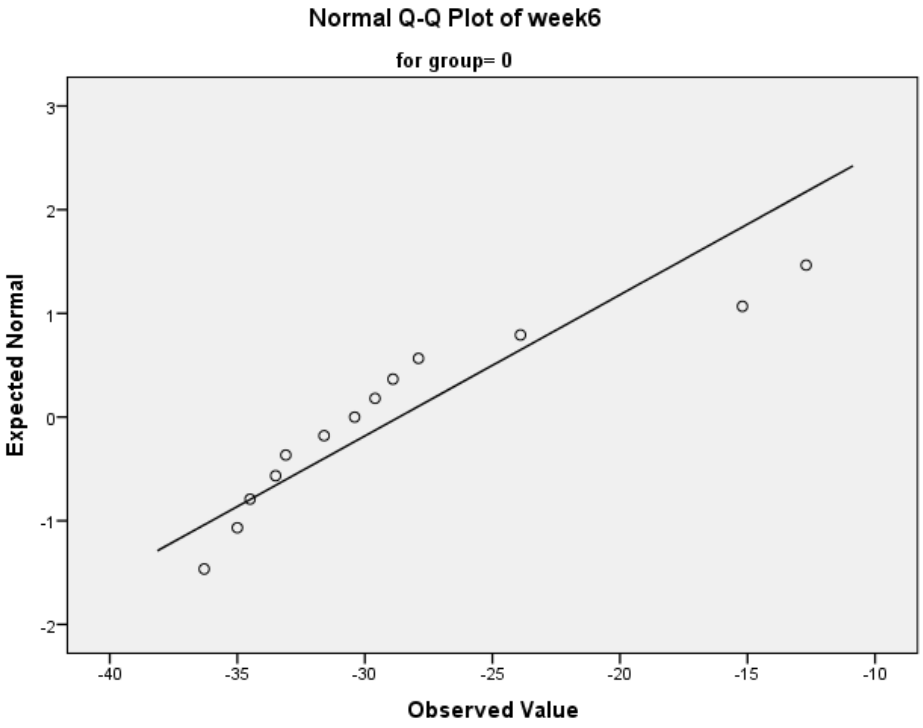


Figure 3. 22–Q-Q plot of week 6 for the 0 ppm data highlighting some outliers may account for the lack of complete normal data distribution.



Test of groupwise effect

An ANCOVA test with the same parameters as for the QLF-D data, with week 0 as a covariate was used to assess for a groupwise difference.(Table 3.7)

This confirms an overall groupwise effect was seen with a statistical significance of $p<0.05$. A statistical difference is also noted with the week 8 and weeks 0 data indicating an experimental effect.

Pairwise comparisons of between group effects

As for the QLF-D data an ANCOVA model was again used with week 0 as a covariate and with the bonferroni correction for multiple comparisons applied, was carried out to assess for pairwise differences between the groups.

Table 3. 7 - Tests of Between-Subjects Effects

Dependent Variable: Week8

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	4493.064[a]	5	898.613	50.762	.000
Intercept	290.158	1	290.158	16.391	.000
Week0	2966.290	1	2966.290	167.563	.000
group	1499.174	4	374.793	21.172	.000
Error	1044.447	59	17.702		
Total	59215.210	65			
Corrected Total	5537.511	64			

a. R Squared = .811 (Adjusted R Squared = .795)

Table 3. 8-Pairwise Comparisons

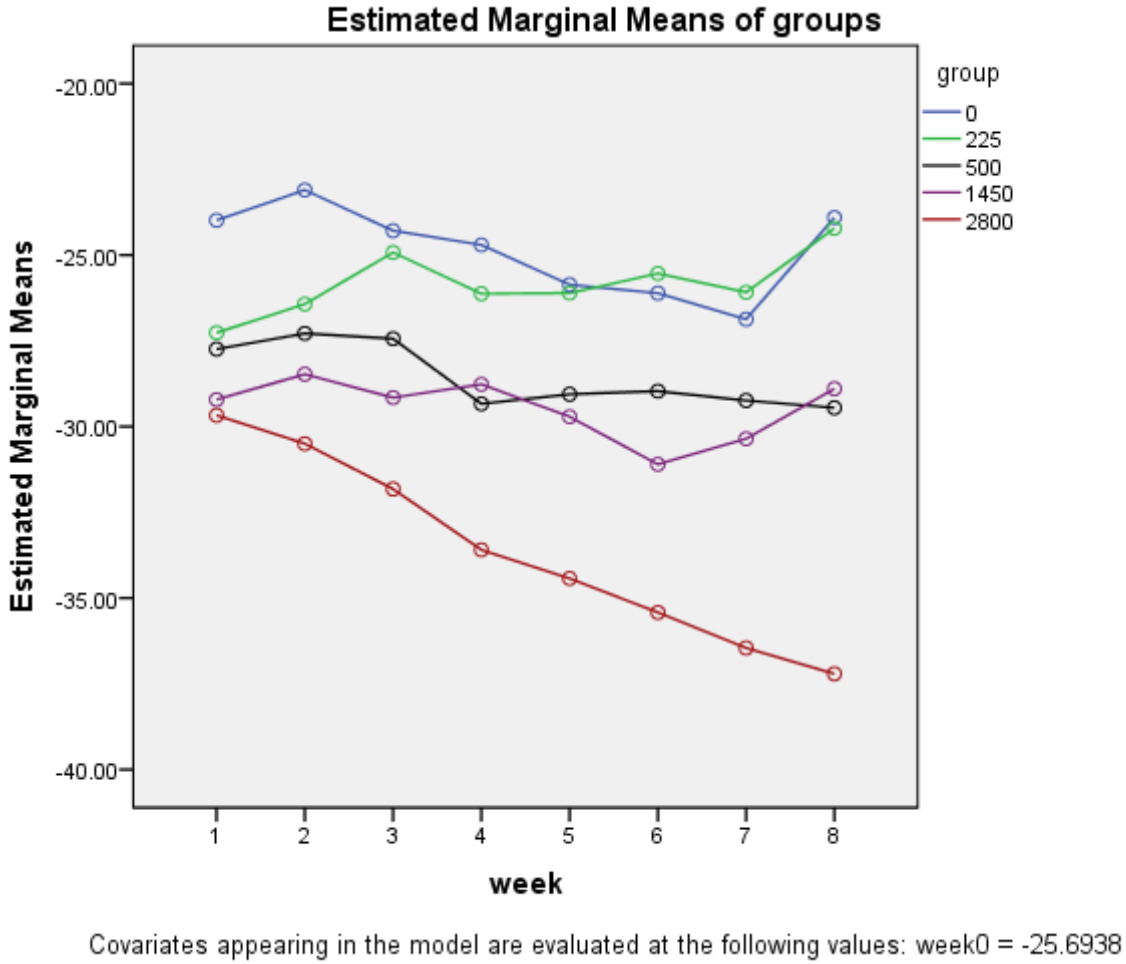
(I) group		Mean Difference (I-J)	Std. Error	Sig.[b]	95% Confidence Interval for Difference[b]	
					Lower Bound	Upper Bound
0	225	.982	1.197	1.000	-2.508	4.472
	500	**3.710***	**1.200**	**.030**	**.210**	**7.211**
	1450	**4.605***	**1.173**	**.002**	**1.182**	**8.027**
	2800	**8.783***	**1.188**	**.000**	**5.318**	**12.249**
225	0	-.982	1.197	1.000	-4.472	2.508
	500	2.728	1.172	.234	-.690	6.147
	1450	**3.622***	**1.210**	**.040**	**.094**	**7.150**
	2800	**7.801***	**1.173**	**.000**	**4.381**	**11.222**
500	**0**	**-3.710***	**1.200**	**.030**	**-7.211**	**-.210**
	225	-2.728	1.172	.234	-6.147	.690
	1450	.894	1.214	1.000	-2.647	4.435
	2800	**5.073***	**1.174**	**.001**	**1.650**	**8.496**
1450	**0**	**-4.605***	**1.173**	**.002**	**-8.027**	**-1.182**
	225	**-3.622***	**1.210**	**.040**	**-7.150**	**-.094**
	500	-.894	1.214	1.000	-4.435	2.647
	2800	**4.179***	**1.199**	**.009**	**.681**	**7.676**
2800	**0**	**-8.783***	**1.188**	**.000**	**-12.249**	**-5.318**
	225	**-7.801***	**1.173**	**.000**	**-11.222**	**-4.381**
	500	**-5.073***	**1.174**	**.001**	**-8.496**	**-1.650**
	1450	**-4.179***	**1.199**	**.009**	**-7.676**	**-.681**

Based on estimated marginal means

*. The mean difference is significant at the .05 level.

b. Adjustment for multiple comparisons: Bonferroni.

Figure 3. 23- plotted estimated marginal mean per group over the 8 week experimental period.



Statistically significant differences in the pairwise group comparisons are highlighted in Table 3.8. The 2800 ppm group was significantly different to all other groups in a negative direction indicating demineralisation. The 0 ppm group showed the least progressive mineral loss. The MSI data indicates greater differences between groups than shown with the QLF-D data however this does not appear to be a linear fluoride concentration related effect.

Figure 3.23 illustrates the plotted estimated marginal means of each group over time from week one to week eight. As was indicated by the QLF–D data, the plotted difference between groups varies at each week, as demonstrated with intersecting lines. The 2800 ppm fluoride group is the only group that exhibited a near linear trend, however, this is downwards in a direction indicating an increasing level of demineralisation and not of remineralisation that would have been predicted. All other groups appear relatively linear indicating little change in mineral loss.

Further analysis was therefore carried out based on the week 0 and week 8 data only.

Pairwise group effect week 0 to week 8

Table 3. 9 -Pairwise Comparisons

(I) group		Mean Difference (I-J)	Std. Error	Sig.[b]	95% Confidence Interval for Difference[b]	
					Lower Bound	Upper Bound
0	225	.315	1.685	1.000	-4.599	5.229
	500	**5.547***	**1.690**	**.017**	**.618**	**10.476**
	1450	**4.991***	**1.652**	**.037**	**.172**	**9.810**
	2800	**13.303***	**1.673**	**.000**	**8.423**	**18.182**
225	0	-.315	1.685	1.000	-5.229	4.599
	500	**5.232***	**1.650**	**.024**	**.418**	**10.045**
	1450	4.676	1.703	.080	-.292	9.644
	2800	**12.987***	**1.652**	**.000**	**8.170**	**17.804**
500	**0**	**-5.547***	**1.690**	**.017**	**-10.476**	**-.618**
	225	**-5.232***	**1.650**	**.024**	**-10.045**	**-.418**
	1450	-.556	1.710	1.000	-5.542	4.430
	2800	**7.755***	**1.653**	**.000**	**2.935**	**12.575**
1450	**0**	**-4.991***	**1.652**	**.037**	**-9.810**	**-.172**
	225	-4.676	1.703	.080	-9.644	.292
	500	.556	1.710	1.000	-4.430	5.542
	2800	**8.311***	**1.689**	**.000**	**3.387**	**13.236**
2800	**0**	**-13.303***	**1.673**	**.000**	**-18.182**	**-8.423**
	225	**-12.987***	**1.652**	**.000**	**-17.804**	**-8.170**
	500	**-7.755***	**1.653**	**.000**	**-12.575**	**-2.935**
	1450	**-8.311***	**1.689**	**.000**	**-13.236**	**-3.387**

Based on estimated marginal means

*. The mean difference is significant at the .05 level.

b. Adjustment for multiple comparisons: Bonferroni.

Pairwise analysis revealed statistical significances between groups where $p<0.05$ and where the 95% confidence interval does not span 0. The significant differences are highlighted and indicates differences between:

- The 2800 ppm fluoride group and all other groups.
- 1450 ppm and 0 ppm and 2800 ppm groups
- 500 ppm and 0 ppm, 225 ppm and 2800 ppm groups
- 225 ppm and 500 ppm and 2800 ppm groups
- 0 ppm and 500 ppm, 1450 ppm and 2800 ppm

Again, the difference between groups is in a negative direction indicating increasing mineral loss and not the mineral gain with the increase in fluoride concentration that would have been expected. The changes between the groups do not indicate a linear dose dependant change highlighted, for example, with no significant difference between the 1450 ppm group and 225 ppm or 500 ppm fluoride groups.

TMR analysis of mineral change

The descriptive statistics of the TMR mineral loss (ΔZ) baseline data is shown in the Table 3.10 below.

Table 3. 10– TMR Descriptive Statistics at baseline

	N	Minimum (ΔZ)	Maximum (ΔZ)	Mean (ΔZ)	Std. Deviation (ΔZ)
ppm0	13	2590.00	9180.00	4592.3498	1811.18177
ppm225	13	1660.00	8913.33	4407.8016	1893.32028
ppm500	13	2455.00	7740.00	4499.5907	1517.28586
ppm1450	13	2294.00	7730.00	4491.5534	1508.62786
ppm2800	13	2235.00	7605.71	4470.1158	1502.97194
Valid N (listwise)	13				

As shown the mean mineral loss values and standard deviations were similar across all groups at baseline.

However, there were difficulties in obtaining the baseline values. Due to an unforeseen error in alignment between the diamond grinding plates and anvils used for baseline TMR preparation, sample sections were thin and damaged at the end of the preparation prior to being radiographed. This affects the reliability of the TMR values and the assessment of the demineralised lesions at baseline.

The data obtained for these samples was calibrated on the aluminium step wedge. The difference in thickness of the baseline and the final sections for TMR analysis are illustrated in Figures 3. 24 and 3. 25. The 25 µm increments seen in the Figures give an indication of the number of steps in the step wedge to which each sample would be calibrated to. An increase in steps gives an

increased reliability of the values obtained. The ideal thickness for TMR analysis is between 80-100μm. The baseline data can therefore be considered unreliable.

Overall, to be interpreted with caution due to the baseline data reliability, the TMR data indicated that remineralisation was present in all groups, but to a non-statistically significant level between the groups. This is in contrast to both the QLF-D and MSI results. (Table 3. 11)

Figure 3. 24- Thickness of sections used for baseline TMR analysis

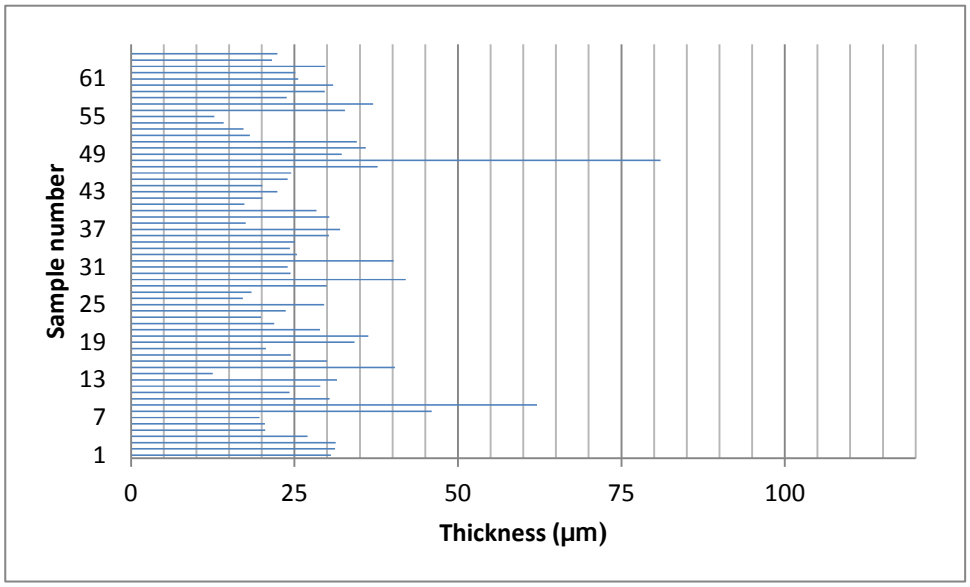


Figure 3. 25- Thickness of sections used for final TMR analysis

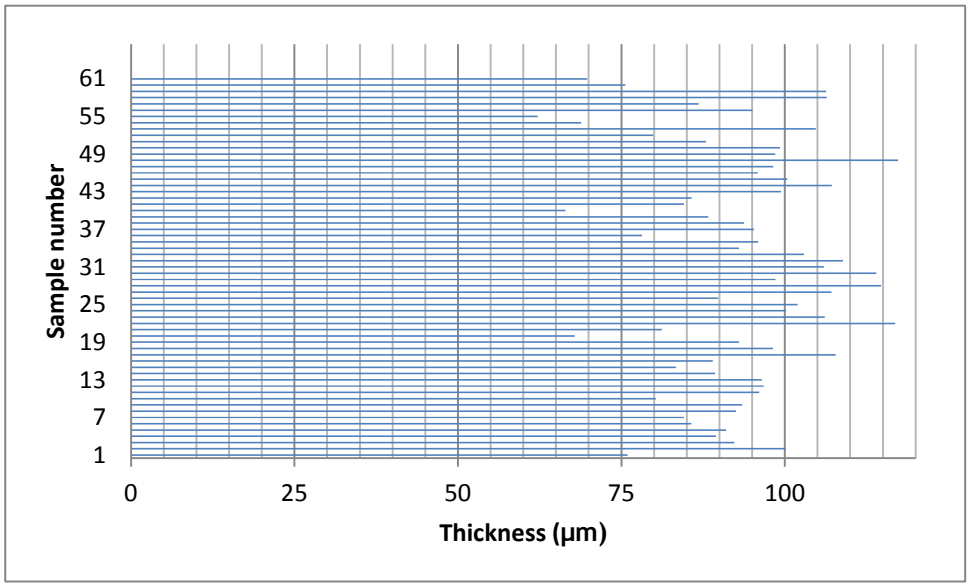


Table 3. 11– TMR Pairwise Comparisons

(I) group		Mean Difference (I-J)	Std. Error	Sig.[a]	95% Confidence Interval for Difference[a]	
					Lower Bound	Upper Bound
0	225	358.567	269.612	1.000	-429.966	1147.100
	500	-252.578	270.147	1.000	-1042.677	537.521
	1450	-300.723	270.939	1.000	-1093.139	491.692
	2800	46.234	264.420	1.000	-727.116	819.585
225	0	-358.567	269.612	1.000	-1147.100	429.966
	500	-611.145	270.102	.276	-1401.112	178.822
	1450	-659.290	270.868	.182	-1451.497	132.917
	2800	-312.333	264.408	1.000	-1085.647	460.981
500	0	252.578	270.147	1.000	-537.521	1042.677
	225	611.145	270.102	.276	-178.822	1401.112
	1450	-48.146	269.788	1.000	-837.195	740.904
	2800	298.812	264.652	1.000	-475.217	1072.841
1450	0	300.723	270.939	1.000	-491.692	1093.139
	225	659.290	270.868	.182	-132.917	1451.497
	500	48.146	269.788	1.000	-740.904	837.195
	2800	346.958	265.280	1.000	-428.907	1122.822
2800	0	-46.234	264.420	1.000	-819.585	727.116
	225	312.333	264.408	1.000	-460.981	1085.647
	500	-298.812	264.652	1.000	-1072.841	475.217
	1450	-346.958	265.280	1.000	-1122.822	428.907
Based on estimated marginal means						
a. Adjustment for multiple comparisons: Bonferroni.						

Correlation testing

Correlation testing is carried out in order to describe the strength and direction of a linear relationship between two datasets. Normally distributed continuous data (i.e. parametric data) were obtained from TMR, QLF-D and MSI techniques. The Pearson correlation coefficient is designed to test correlation of two parametric datasets. This test gives a range of values from -1 to +1 to indicate a positive and negative correlation. Total agreement between two datasets would be indicated by a correlation coefficient of +1.

The data above appears to show some agreement between the QLF-D and MSI results, which is in contrast to the results of the TMR analysis. Pairwise correlation coefficients were therefore carried out to indicate the strength and direction of correlation between the datasets between the three different techniques.

Correlation of Baseline Values:

Table 3. 12– Correlation of baseline values.

Techniques compared	Correlation
QLF-D Vs TMR	-0.34
MSI Vs TMR	-0.35
QLF-D Vs MSI	0.9

The above baseline correlation values indicate a strong correlation between the QLF- D and MSI values with a correlation coefficient of 0.9. There is only a poor correlation in a negative direction between the TMR results and those of the QLF-D and MSI, as indicated by the correlation coefficients of -0.34 and -0.35

respectively. This indicates not only correlation, but also that there is conflicting data with the QLF-D and MSI showing mineral loss, but with the TMR results showing mineral gain (Table 3.12).

Comparison of Week 8 final Data

Table 3. 13- Correlation of week 8 data

Techniques compared	Correlation
QLF-D Vs TMR	-0.098
MSI Vs TMR	-0.3
QLF-D Vs MSI	0.73

Correlation based on the final data values again indicates a strong correlation between the QLF-D and MSI datasets with a correlation coefficient of 0.73. There is no correlation demonstrated between the QLF-D and TMR datasets, and only a poor correlation between the MSI and TMR datasets. Again this highlights the conflicting results of mineral loss and gain, with the correlation coefficient of -0.3 (Table 3.13).

Correlation of change detected from baseline (week 0) to Final data (week 8)

Table 3. 14– Correlation of change in values from week 0 baseline to week 8 final data.

Techniques compared	Correlation
QLF-D Vs TMR	-0.075
MSI Vs TMR	0.008
QLF-D Vs MSI	0.64

The main parameter of interest in this study is the change in mineral loss from the baseline at week 0 to the end of the data collection at week 8. For this reason correlation coefficients were also carried out based on the change in values of mineral loss from weeks 0 to week 8 (Table 3. 14).The correlations are also demonstrated in scatter plots (Figure 3.s 26-28).

Figure 3. 26– Scatter plot of mineral loss values for QLF-D (Delta F Y-axis) and TMR data (Delta Z X-axis) indicating a lack of correlation.

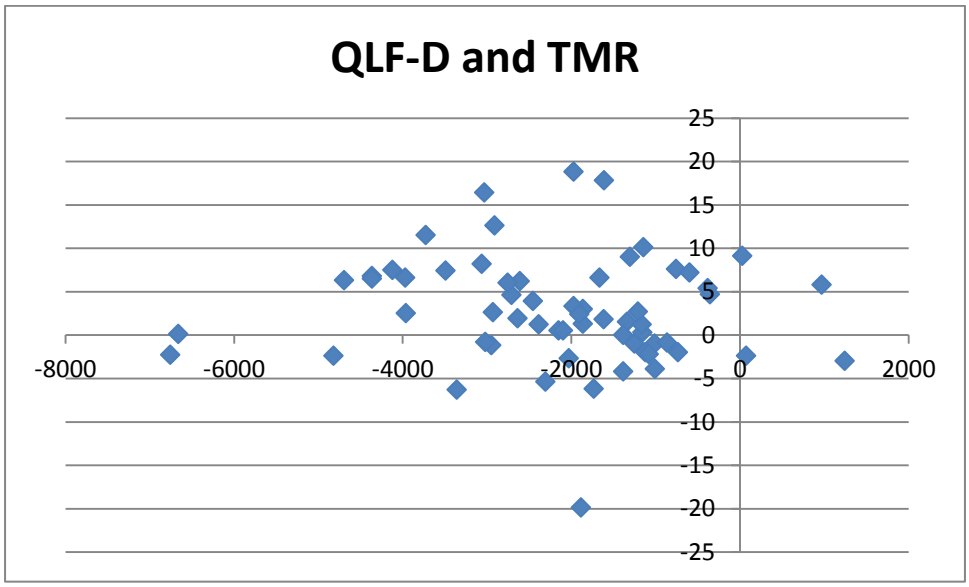


Figure 3. 27– Scatter plot ofmineral loss values for TMR (Delta Z X-axis) and MSI (Delta F Y-axis) data indicating a lack of correlation.

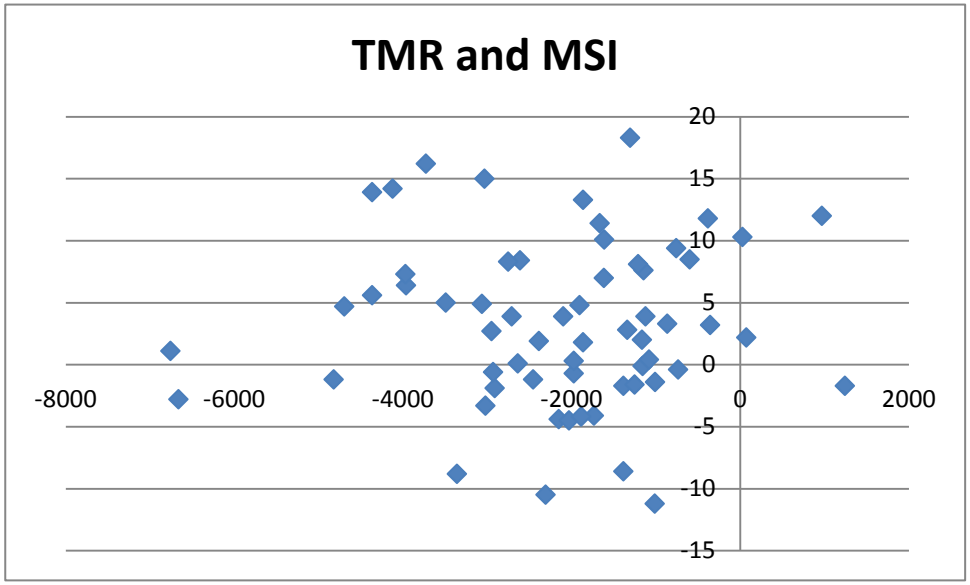
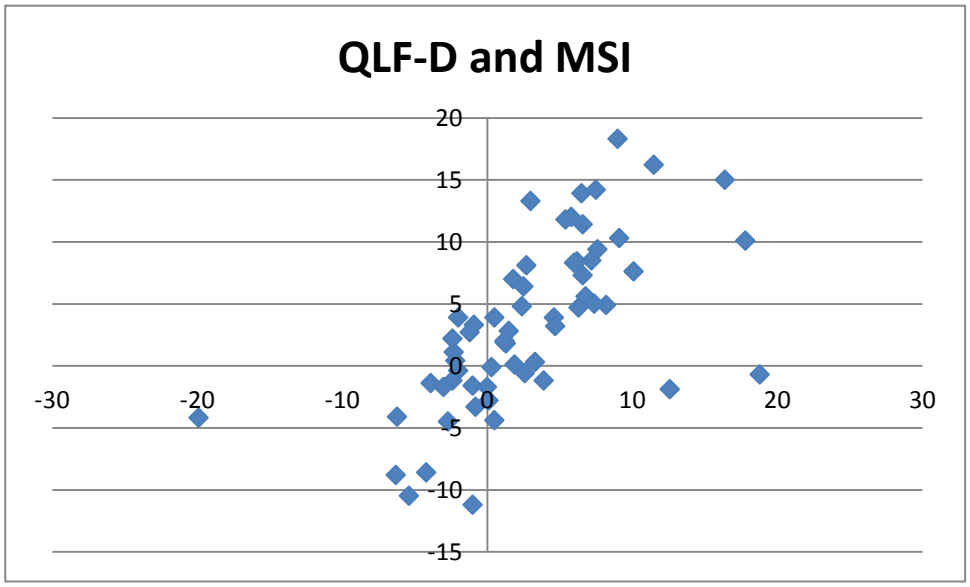


Figure 3. 28- Scatter plot of mineral loss values for QLF-D (Delta F Y-axis) and MSI (Delta F X-axis) data highlighting a moderate correlation.



This revealed a moderate correlation between the QLF-D and MSI values as demonstrated with a correlation coefficient of 0.64. No correlation was found between the TMR data and that of the QLF-D or MSI.

Summary of results

Overall, a fluoride concentration based effect was not seen. No difference was found between the groups with TMR. QLF-D highlighted a difference between the 2800 ppm group and remaining groups only, and this was in a negative direction indicating further mineral loss not gain. MSI found greater differences between group effects, but again little overall change between groups was shown, with the exception of the 2800 ppm group.

The TMR results did not correlate with the QLF-D or MSI results. However, moderate correlation was shown between the results from QLF-D and MSI.

Results of this study show no difference on the amount of remineralisation seen with varying fluoride concentration on the remineralisation of bovine enamel based on the TMR results. There is a lack of correlation between the TMR and the QLF-D or MSI results, but moderate to strong correlation between the QLF-D and MSI results. However, these results show an increasing level of demineralisation and not remineralisation with varying fluoride concentration. There was also no linear effect seen with the increase in fluoride concentration between the groups.

Discussion

Lesion remineralisation was assessed by the change in mineral content as measured with QLF-D (ΔF), MSI(ΔF), or TMR (ΔZ) values. The baseline lesions varied in the severity of demineralisation. Samples were randomly

allocated to groups using a computer generated random number sequence producing five groups of approximately similar mean values for mineral loss. The main statistical model used for analysis was an ANCOVA due to the influencing effect of the baseline mineral loss values on the final mineral loss values.

Fluoride has well known and demonstrated effects on the remineralisation of carious lesions. The results of this study show remineralisation based only on the TMR results. QLF-D and MSI results indicated further mineral loss, increasing demineralisation. No fluoride concentration based effect on remineralisation was demonstrated. However, several factors may have had an effect on the overall results of this study and these will be discussed below.

Baseline lesions

The mean depth of the subsurface lesions was moderate as defined by Benson in 2009. Moderate lesions are those with a subsurface mineral loss value of between 1800-2500 (ΔZ). More advanced carious lesions are more delicate and more prone to damage in the preparation for TMR.

Samples were randomised to the fluoride concentration groups by computer generated randomisation. The mean mineral loss in each group was approximately equal. It was necessary to have a spread of mineral loss values within each group as larger lesions have been found to remineralise faster (Strang *et al*, 1987)

As previously mentioned, during preparation of the samples for baseline TMR analysis there was an issue with the diamond disk used to grind sections. The contact section of the diamond disk to the anvil had become misaligned, resulting in uneven grinding and over polishing. This resulted in sections that were too thin and many were extensively damaged. Unfortunately the precise

nature of the problem was not identified until the majority of the sections had been prepared for TMR. Baseline data were available for sufficient number of samples to proceed with the study. However, since the resulting sections prepared were much thinner than the ideal this reduced the number of steps in stepwedge that is used to correlate the data and may have had the resultant effect that the values produced from the TMR analysis may be less reliable than those of adequate thickness. Radiographs of thinner sections produce a much darker image. This is illustrated in Appendix VIII Figure 3.29 with a thickness of 23 µm and Appendix VIII Figure 3.30 showing greater contrast with adequate thickness of 98 µm. The ideal thickness is between 80 µm and 100 µm.

This was the basis of undertaking a second analysis including only those with baseline sections of greater than 25 µm,although this analysis did not show any difference from the total data. The resultant small sample size would be likely too small to have the power to detect a difference.

Lesion Erosion

Purcell *et* al 2006 showed erosive changes can occur on artificial creation of demineralised lesion of bovine incisors (Purcell *et al,* 2006). Two effects can be produced on bovine enamel when placed in demineralisation solution, that of demineralisation and also that of erosion. The damage to the lesions made full visualisation of the lesion impossible as they were not intact with several sections having multiple fragments of the lesion missing. Since this study focuses on remineralisation the ideal scenario would have been to include only those samples that showed demineralisation and not erosion in response to the solution.

Erosion is detected by assessment at the end of the lesion where it contacts sound enamel. Where only demineralisation has occurred the lesion would be

flushed to th3.e sound area of enamel. Where an element of erosion has occurred there would be a dip from the sound area of enamel into the lesion. (Appendix VIII Figure 3. 31, Appendix VIII Figure 3. 32)

It is impossible to detect for erosion if the end of the lesion where it contacts the sound portion of enamel is broken or damaged (Appendix VIII Figure 3. 33). The additional difficulty in detecting for erosion where damage has occurred during grinding is that the end of lesions are particularly prone to damage and may produce the appearance of an eroded lesion. Distinguishing between damage and erosion is therefore incredibly difficult (Appendix VIII Figure 3. 34).

The vast majority of sections showed no signs of erosion. The sections that did had sufficient damage to question a diagnosis of erosion. Therefore the study proceeded on the basis that erosion was not present. Should erosion have been present and continued throughout the experimental period it may have had the effect of masking any remineralisation that occurred resulting in a non effect. Since this is what the results of this study showed based on the TMR data, it is possible that the enamel was affected by an erosive process in addition to demineralisation had occurred and was present at baseline.

Preparation of sections for TMR analysis after the experimental period was not affected by the grinding problems that occurred in preparation for the baseline analysis. Therefore, all post –remineralisation sections were assessed for signs of erosion, as described above.

Five samples were found with indications of an erosive process after the remineralisation phase. However, the remaining samples showed no signs of erosion. Due to the damage to the baseline sections it was not possible to assess if the five samples with erosion following the experimental period, had erosion present at baseline. It is possible that erosion could have occurred during the eight week experimental period which would indicate that the remineralisation solutions used were too acidic over the eight week period.

Remineralisation solutions

All solutions were created to a pH 7.2 which is within a neutral range. A stock of solutions was made of sufficient volume to last throughout the study. It is possible that the solution properties changed over time with the potential to become more acidic since 10 weeks lapsed from the creation of the solutions to the end of the experimental phase. However, all solutions were pH tested at the end of the experimental phase and showed little change in pH.

The samples were immersed in solution for eight weeks only being removed for drying prior to imaging once each week. This is not representative of the oral environment but was designed to highlight what would be considered an accelerated effect of remineralisation and highlight the effect of varying fluoride concentration on the extent of remineralisation.

Topical application of high concentration fluorides have been shown to create a hyper mineral surface layer with blocking of surface layer pores. (ten Cate *et al* 1981). Where this occurs, it is possible for the subsurface lesion to progress undermining the hyper mineralised surface layer and ultimately leading to cavitation. It is possible that in this study the concentrations of fluoride used were too high for continual immersion as in this design. Hyper mineralised surface areas may have occurred blocking the remineralisation of the deeper layers. This in combination with variable areas of baseline erosion that would have opened pores, therefore becoming more susceptible to further erosion or demineralisation may account for some of the results shown in the study, in particular with reference to the 2800 ppm group.

Mineral change detected with QLF-D and MSI

The results of the QLF-D and MSI data indicated increasing mineral loss over time, which was most significant in the 2800 ppm group. This was an unexpected finding, but was confirmed by not only by the data in the reliability

study, but by the increased darkness that can be seen on images that would be an indication of further fluorescence loss, likely due to demineralisation. Appendix VIII Figure 3. 35 shows MSI images from a sample in the 2800 ppm group at the start, week 0, and Appendix VIII Figure 3. 36 the final week 8 MSI images from a sample in the 2800 ppm group.

An uneven pattern is also seen in Appendix VIII Figure 3. 36 with greater contrast and a mixture of much lighter and darker areas than seen in week 0. Two potential reasons for this increase in darkness are that there had been ongoing erosion in parts of the sample only, or uneven demineralisation occurring. The light patches may be areas unaffected by further erosion or demineralisation. Alternatively, these may be areas of remineralisation. The TMR results would support the latter.

Variation in bovine enamel

Dowker *et al*, in 2003 carried out a 3-D analysis on the development of artificial subsurface enamel lesions *in vitro* (Dowker *et al*, 2003). They found that the distribution of mineral across the surface of the lesion was varied and was not uniform. Most advanced areas of the lesion were, in general, associated with the areas of lowest mineralisation at the surface of the lesion. The authors describe a lack of uniformity of the initial subsurface lesions created. They found that on exposure to demineralisation solution the initial mineral loss at the surface increases the porosity of the enamel which then allows access of the demineralisation solution to the deeper layers. Localised variations in the solubility within the enamel were found leading to differential degrees of demineralisation.

It is due to this differential degree in solubility of enamel that a range of demineralisation in the baseline samples was found. As such the samples will have reacted differently to the demineralisation solution, but also to the remineralisation solutions that they were placed into during the experimental

phase. This individual variation within the samples may account for some of the unexpected response to remineralisation. In addition the artificial creation of erosion lesions in enamel is associated with increased porosity of the enamel. If baseline erosion had been present this increased porosity may have left areas of the lesions affected more susceptible to further demineralisation and less likely to significantly remineralise.

Storage of extracted bovine incisors

Another complicating factor that may have influenced the response of enamel to both demineralisation and remineralisation phases became apparent during the experimental phase. The same stock of bovine incisors used for this study, showed unexpected changes whilst still in storage in thymol solution. Extracted incisors are stored in a solution of thymol that is normally clear, in this case the solution itself turned purple with the uptake of purple stain also evident in the root of these incisors.

The cause of this change has not been found and it may simply be due to residual chemical in the storage container that reacted to the thymol solution. The problem has not occurred since. However, since the bovine incisors used for this study were stored in the same container it is possible that a changing solution may have had an effect on the enamel.

Correlation of techniques

The results from TMR did not correlate to that from MSI or QLF-D in this study. TMR is the gold standard technique since the lesion is visualised cross sectionally. Correlations between TMR and QLF have been studied showing good correlation. (Pretty *et al* 2004, Van der Veen *et al, 2007*). Increased correlation between TMR and QLF is found using human enamel, r= 0.64, when compared with bovine enamel r=0.84. (Al-Khateeb *et al,* 1997). However

the correlation between TMR and QLF does vary and inverse correlation has also been reported. (Lovel,2008)

These studies report correlation with QLF and not QLF-D. However it is the same technology behind both techniques with the main difference being in the incorporation with a digital camera in QLF-D. Both techniques have been validated with good compatibility between QLF and QLF-D. (Inaba *et al*, 2010)

The information obtained from MSI imaging was more detailed as indicated with higher mean mineral loss volumes. MSI has been shown to highlight significantly greater changes in mineral loss at an earlier stage than with QLF-D (Desmons *et al* 2013).Multispectral imaging has been found to be a reliable technique in detecting enamel demineralisation(Adeyemi *et al* 2013) and a strong correlation between MSI and QLF-D has been found in the assessment of early enamel demineralisation (Desmons *et al* 2013). However as it is still a relatively new technique further research is required.

The potential effects of ongoing erosion may have influenced the greater fluorescence loss identified with MSI and QLF-D and the apparent increased mineral loss. This is especially likely where access to the deeper layers would be blocked where hypermineralisation of the surface layers had occurred.

Limitations of this study

Several factors that have been identified both subsequently and during the experimental period may have had an effect on the outcome. Unfortunately, therefore, the results of this study in terms of assessment of remineralisation with varying fluoride concentration, cannot be considered reliable.

In terms of study design, the *in vitro* design, although this does allow for examination of specific factors such as fluoride by controlling all other factors, it is not necessarily generalisable to the oral environment. The main factors in

this are the lack of bacteria and therefore the acquired pellicle that is crucial in the development and remineralisation of carious lesions.

Artificial creation of demineralised areas is also not representative of the oral environment and the enamel response *in vitro* to demineralisation solutions, as we have seen in this study can be variable.

Preparation of bovine incisors for *in vitro* studies involves smoothing and polishing ridged areas of enamel to create a smooth surface to replicate that more of human incisors. However, this involves removing the outer layers of enamel which may differ structurally from more inner layers of enamel and therefore the effects seen again might not be entirely representative of responses that would be seen clinically.

Samples were immersed for 8 weeks with only the time taken for drying and imaging each week out of solution. This is not representative of the oral environment where fluoride would rapidly be eliminated through salivary clearance.

In addition to the factors already mentioned the lack of plaque biofilms and of normal pH cycling reduce the generalisability of results. However, the *in vitro* model used here has been successfully used in other studies assessing remineralisation. (Preston *et al*, 2007)

Conclusion

The results of this study did not show an effect attributable to the fluoride concentration following the 8 week remineralisation period of demineralised bovine lesions. Further research in this area is still required.

Overall Discussion

The two studies presented here illustrate the difficulties and complexities in conducting research looking at the efficacy of fluoride. Study one looking at the effect of post-brushing mouth rinses and salivary fluoride retention found significant benefit in the use of fluoride mouthwashes after brushing. This may be of particular benefit to those at high risk of dental caries, including orthodontic patients. However, the benefit to those that don't rinse out after brushing is unknown as is the potential preventative effects against enamel demineralisation associated with orthodontic treatment where fluoride mouthwashes are routinely used. It would be interesting to know how significant the effect would be using a post brushing fluoride mouthwash after a brush time of two minutes or three minutes for orthodontic patients. Orthodontic appliances will potentially alter salivary retention, which may be increased around brackets and wires as these are not naturally cleansable. Again it would be interesting to know the effect of post-brushing rinses specifically in orthodontic population and in comparison with a non-orthodontic population. This would allow for fluoride regimes that are better tailored to the individual patient.

Study two was aimed at a better understanding the effect of fluoride concentration on remineralisation of enamel lesions. Unfortunately, this study was not successful in assessment of a fluoride concentration effect on remineralisation. The study with setup using the most common over-the-counter concentrations available to patients in terms of the concentration of fluoride mouthwash and also in toothpaste. The *in vitro* study design has been discussed earlier, but has the benefit of removing other factors and allow for the assessment of the effects of fluoride. It would be interesting to know the differences in remineralisation in detail with regards to high versus standard concentration fluoride toothpaste in particular. This would again allow better recommendations to patients on the most suitable fluoride regimes for them, and especially on any alterations to this where enamel demineralisation especially associated with orthodontic treatment is noted.

Two main questions with regards to fluoride and orthodontic treatment still exists, One: what is the best fluoride regime in the prevention of enamel

demineralisation associated with orthodontic treatment, and Two: what is the best fluoride regime to advise where enamel demineralisation associated with orthodontic treatment is seen, in order to promote remineralisation, and finally, do these differ?

These are all areas where further research is still required.

References

Aas JA, Paster BJ, Stokes LN, Olsen I, Dewhirst F, (2005) Defining the normal bacterial flora of the oral cavity. Journal clinical microbiology 43:5721-32

Adeyemi AA , Desmons S, Miles E, Burnside G, Valappil SP, Lynch R, de Josselin de Jong E, Higham SM. (2013) The Visual Assessment of Early *in vitro* Chemical Demineralisation Using a Multispectral Imaging Method Caries Research 47:433–531 Abstracts: 60th ORCA Congress

Ainsworth NJ.1933 Mottled teeth. British Dental Journal. 55, 233-250.

Al-Khateeb S, ten Cate JM, Anger-Mansson B, de Josselin de Jong E, Sundstrom G, Exterkate RAM, Oliveby A, (1997) Quantification of formation and remineralisation of artificial enamel lesions with a new portable fluorescence device. Advances in Dental Research, 11(4): 502-506

Altman DG. (1991) Practical Statistics for Medical Research. Chapman and Hall.London

Amaechi BT,Higham SM, Edgar WM (1998) Factors effecting the development of carious lesions in bovine teeth *in vitro*. Archives of Oral Biology. 43:619-628

American Dental Association. Oral Health Topics A-Z. Cleaning Your Teeth and Gums (Oral Hygiene). http://www.ada.org/public/topics/cleaning.asp Accessed 06/08/2012

Angmar B, Carlström D, Glas J-E. (1963) Studies on the ultrastructure of dental enamel.IV. The mineralisation of normal human enamel. Journal Ultrastructural Reseach;8:12-33.

Arends J.(1989) Remineralisation of bovine dentine *in vitro* – caries research 23:309314

Arends J, ten Bosch JJ. (1992) Demineralisation and remineralisation evaluation techniques. Journal Dental Research ;7(Spec iss):924-928.

Artun J, Brobakken BO.(1986) Prevalence of carious white spots after orthodontic treatment with multibonded appliances. European Journal Orthodontics. Nov;8(4):229-34.

Artun,J and Brobakken,B.O. (1986) Prevalence of white spots after orthodontic treatment with multi-bonded appliances, European Journal of Orthodontics, 8,229-234

Bader JD, Shugars DA.(2004) A systematic review of the performance of a laser fluorescence device for detecting caries. Journal American Dental

Association 135; 1413-1426.

Banks PA, Chadwick SM, Asher-McDade C, Wright JL.(2000) Fluoride-releasing elastomerics–a prospective controlled clinical trial. European Journal of Orthodontics; 22: 401–7.

Banks PA, Richmond S (1994) Enamel sealants:a clinical evaluation of their value during fixed appliance therapy. European Journal of Orthodontics 16: 19-25.

Benson PE, Parkin N, Dyer F, Millett DT, Furness S, Germain P. (2013) Fluorides for the prevention of early tooth decay (demineralised white lesions) during fixed brace treatment. Cochrane database of systematic reviews.

Benson PE, Pender N, Higham SM, (1998). Morphometric assessment of enamel demineralisation from photographs. Journal of Dentistry 26:669-677

Benson PE, Pender N, Higham SM, (2000). Enamel demineralisation assessed by computerised image analysis of clinical photographs. Journal of Dentistry 28: 319-326

Benson PE, Shah AA, Millett DT, Dyer F, Parkin N, Vine RS. (2005) Fluorides, orthodontics and demineralization: a systematic review. Journal

of Orthodontics. Jun;32(2):102-14.

Benson R, (2009). *In vitro* remineralisation of caries-like lesions ranging in severity with casein phosphopeptide. Master of Dental Science dissertation, University of Liverpool.

Bentley EM, Ellwood RP, Davies RM.(2008) Fluoride ingestion from toothpaste by young children. Oral Health and Preventative Dentistry. 6: 61-5

Bjelkhagen H , Sundström F , Angmar-Månsson B , Ryden H (1982) Early detection of enamel caries by the luminescence excited by visible laser light . Swedish Dental Journal 6 : 1 – 7

Bland M, Altman DG. (1986) Statistical Methods for Assessing the Difference Between Two Methods of Measurement. Lancet; 1:307-310.

Boersma JG, Vander Veen MH, Lagerweij MD, Bokhout B. (2005) Caries prevalence measured with QLF after treatment with fixed orthodontic appliances: influencing factors. Caries Research;39:41-7.

British Orthodontic Society Patient Information Leaflet (2012)Fixed appliances.

Brown LR, Dreizen S, Handler S, (1975) Effect of radiation-induced xerostomia on human oral microflora. Journal Dental Research;54(4):740–

50.

Buchalla W. (2005) Comparative fluorescence spectroscopy shows differences in noncavitated enamel lesions. Caries Research;39:150-156.

Chadwick B,White D,Lader D,Pitts N. (2011) Preventive behaviour and risks to oral health - a report from the Adult Dental Health Survey 2009 The Health and Social Care Information Centre.

Churchill HV (1931) The occurrence of fluorides in some waters of the United States. Journal of Dental Research 12 141-148

Damen JJM, Eterkate RAM, Ten Cate JJ. (1997) Reproducibility of TMR for the determination of longitudinal mineral changes in dental hard tissues. Advances in Dental Research 11 (4) 415-419.

De Josselin de Jong E , Sundström F , Westerling H , Tranaeus S , ten Bosch JJ , Angmar-Mansson B (1995) A new method for *in vivo* quantification of changes in initial enamel caries with laser fluorescence . Caries Research 29 : 2 – 7

De Josselin de Jong E,Higham SM,Smith PW,van Daelen CJ, and van der Veen MH (2009) Quantified light-induced fluorescence, review of a diagnostic tool in prevention of oral disease. Journal of Applied Physics Vol. 105 Issue 10, p102031-102038

Dean HT, Arnold FA, and Evolve E. (1942) Domestic water and dental caries ,V Additional studies of the relation of fluoride domestic water to dental caries experience in 425 white children aged 12-14, of 13 cities in 4 states. Public Health Report 57, 1155-1179

Desmons S , Adeyemi AA, Miles E, Burnside G, de Josselin de Jong E, Valappil SP, Lynch R, Higham SM. (2013) Comparing Two Methods in the Early Detection of *in vitro* Chemical Demineralisation: A Pilot Study. Caries Research;47:433–531 Abstracts: 60th ORCA Congress

Dowker SEP, Elliot JC, Davies GR, Wassif HS, (2003). Longitudinal study of the three-dimensional development of subsurface enamel lesions during *in vitro* demineralisation. Caries Research, 37:237-245

Duckworth A. Maguire, N. Omid, I.N. Steen, G.I. McCracken, F.V. Zohoori, (2009) Effect of Rinsing with Mouthwashes after Brushing with a Fluoridated Toothpaste on Salivary Fluoride Concentration. Caries Research;43:391–39

Featherstone JD. (2006) Caries prevention and reversal based on the caries balance. Paediatric dentistry ; 28:128-32

Fejerskov O, Kidd E, (2008) Dental Caries: The disease and its clinical management, 2nd edition. Blackwell Munsgaard.Chapters 8,10 and 11

Ganss C. & Schlueter N. & Preiss S. & Klimek J. (2009) Tooth brushing habits in uninstructed adults—frequency, technique, duration and force. Clinical Oral Investigations. Jun;13(2):203-8. Epub 2008 Oct 14.

Geiger AM, Gorelick L, Gwinett AJ, Benson BJ (1992) Reducing white spot lesions in orthodontic populations with fluoride rinsing. American Journal of Orthodontics and Dentofacial Orthopaedics 101 vol5 403-407.

Gill D.S., Lee R.T. and Tredwin C.J. (2001) Treatment Planning for the Loss of First Permanent Molars. Dental Update; 28: 304-308

Gorelick L, Geiger AM, Gwinnett AJ.(1982) Incidence of white spot formation after bonding and banding. American Journal Orthodontics;81:93-8.

Hafström-Björkman U, Sundström F, de Josselin de Jong E, Oliveby A, Angmar-Månsson B. (1992) Comparison of laser fluorescence and longitudinal microradiography for quantitative assessment of *in vitro* enamel caries. Caries Research.;26(4):241-7.

Hara AT; Zero DT. (2010) The Caries Environment: Saliva, Pellicle, Diet, and Hard Tissue Ultrastructure - Current Concepts in Cariology, Dental Clinics of North America.54(3):455-467

Inaba D, de Josselin de Jong , E van der Veen M H. (2010) Red Fluorescence Detection from the Occlusal Aspect of Extracted Molars Related to Fluorescence. Caries Research. vol. 44, p.226,

Jensen O, Gabre P, Skold UM, Birkhed D. (2012) Is the use of fluoride toothpaste optimal? Knowledge, attitudes and behaviour concerning fluoride toothpaste and toothbrushing in different age groups in Sweden. Community Dentistry and Oral Epidemiology; 40: 175-84

Kidd E A M, Smith B G N. (1996) Pickard's Manual of Operative Dentistry. 7th Edition pp 58–59 Oxford: Oxford University Press; Chapter 1

Kidd E.A.M. and Fejerskov O. (2004) What Constitutes Dental Caries? Histopathology of Carious Enamel and Dentin Related to the Action of Cariogenic Biofilms. Journal of Dental Research 83: C35

Kidd, (2005) Essentials of Dental Caries,Third Edition, Oxford University Press. Chapters 1,2 and 5

Kleinberg, Iv, J. A. Kanapka, and D. Craw. (1976). Effect of saliva and salivary factors on the metabolism of the mixed oral flora, p. 433-464. In H. M. Stiles, W.J. Loesche, and T. C. O'Brien (ed.), Proceedings: Microbial Aspects of Dental Caries (a special supplement to Microbiology Abstracts), vol. 2. Information Retrieval Inc., Washington, D.C.

Lader D, Chadwick B, Chestnutt I, et al.(2004) Children’s Dental Health in

the United Kingdom, 2003 – Summary Report. London: Office of National Statistics,

Lim BS, Lee SJ, Lim YJ, Ahn SJ. (2011) Effects of periodic fluoride treatment on fluoride ion release from fresh orthodontic adhesives. Journal of Dentistry. Nov;39(11):788-94.

Lovel S. *In vitro* remineralisation of carious lesions by casein phosphopeptide.(2008)Master of Dental Science Dissertation, University of Liverpool.

Low W, Tan S, Schwartz S.(1999) The effect of severe caries on the quality of life in young children. Pediatric Dentistry; 21: 325–26.

MacGregor IDM. and Rugg-Gunn A. J. (2009) Survey of toothbrushing duration in 85 uninstructed English schoolchildren Clinical Oral Investigations 13:203–208

Macgregor IDM, Rugg-Gunn AJ (1985) Toothbrushing duration in 60 uninstructed young adults. Community Dentistry and Oral Epidemiology; 13: 121-2.

Marinho VC, Higgins JP, Logan S, Sheiham A. (2003) Fluoride mouthrinses for preventing dental caries in children and adolescents. Cochrane Database Systematic Reviews.; CD002284.

Marinho VC, Sheilham A, Logan S and Higgins JP. (2003) Fluoride toothpastes for preventing dental caries in children and adolescents. Cochrane Database Systematic Reviews

Marsh P, Martin MV (1999) Oral Microbiology, Fourth Edition, Reed Educational and Professional Publishing limited, Oxford Chapter 6 pp82-103.

Mattick CR, Mitchell L, Chadwick SM, Wright J. (2001) Fluoride-releasing elastomeric modules reduce decalcification: a randomized controlled trial. Journal of Orthodontics Sep;28(3):217-9.

McCormack J. (1968). A Critical Review of the Literature on Mouthwashes. Annals New York Academy of Sciences p374-385

McKay FS (1916) An investigation into mottled teeth. Dental Cosmos, 58 477-484

Mitchell L. (1992) Decalcification during orthodontic treatment with fixed appliances – an overview. British Journal of Orthodontics 19: 199–205.

Moles D. R., Ashley P. (2009) Hospital admissions for dental care in children: England 1997-2006 British Dental Journal 206, Online article E14 doi:10.1038/sj.bdj.2009.254

Mystikos C, Yoshino T, Ramberg P, Birkhed D. (2011) Effect of post-brushing mouthrinse solutions on salivary fluoride retention. Swedish Dental Journal;35(1):17-24.

Nalbantgil D, Oztoprak MO, Cakan DG, Bozkurt K, and Arun T (2013).Prevention of demineralization around orthodontic brackets using two different fluoride varnishes European Journal of Dentistry. Jan; 7(1): 41–47.

Ogaard B. (1989) Prevalence of white spot lesions in 19-year-old: a study on untreated and orthodontically treated persons 5 years after treatment. American Journal of Orthodontics and Dentofacial Orthopaedics;96:423-7.

Pashos E, Kuronchkina N, Huth KS, Hanson CS, Rudzki-Janson (2009) Failure rate of brackets bonded with antimicrobial and fluoride-releasing, self-etching primer and the effect on prevention of enamel demineralisation. American Journal of Orthodontics and Dentofacial Orthopaedics 135 (5):613-620.

Pitts N, Duckworth M, Marsh P, Mutti B,Parnell C and Zero D, (2012) Post-brushing rinsing for the control of dental caries: exploration of the available evidence to establish what advice we should give our patients. British Dental Journal; Vol. 212 Issue 7, p315-320

Pitts, N., Harker, R. (2004) Obvious Decay Experience Children's Dental Health in the United Kingdom 2003. Office for National Statistics October .

Preston K P, Higham S M and Smith P W (2007) The efficacy of techniques for the disinfection of artificial sub-surface dentinal caries lesions and their effect on demineralization and remineralization *in vitro*. Journal of Dentistry volume 35 issue 6 pp 490-5

Pretty IA, Edgar WM, Higham SM, (2004) The validation of quantitative light-induced fluorescence (QLF) to quantify acid erosion of human enamel. Archives of Oral Biology 49: 285-294.

Pretty IA, Hall AF, Smith PW, Edgar WM, Higham SM, (2002) The intra- and inter- examiner reliability of quantitative light-induced fluorescence (QLF) analyses. British Dental Journal 193(2): 105-109

Purcell V, Pender N, Higham SM, (2006) Effect of Tooth Mousse on demineralised enamel - a comparative study using quantitative light-induced fluorescence. Master of Dental Science dissertation, University of Liverpool

Reynolds EC.(1997) Remineralization of enamel subsurface lesions by casein phosphopeptide-stabilized calcium phosphate solutions. Journal of Dental Research;76:1587–1595.

Richter AE, Arruda AO, Peters MC, Sohn W. (2011) Incidence of caries lesions among patients treated with comprehensive orthodontics. American Journal of Orthodontics and Dentofacial Orthopaedics May;139(5):657-64.

Ripa LW (1991) A critique of topical fluoride methods (dentifrices, mouthrinses, operator and self applied gels) in an area of decreased caries and increase fluorosis prevalence. Journal of Public Health Dentistry vol51 p23-41

Robertson D. Smith A.J. (2009) The microbiology of the acute dental abscess. Journal of Medical Microbiology volume 58 no. 2 155-162

Rock, W.P. & Kidd, E.A. (1988) The electronic detection of demineralisation in occlusal fissures. British Dental Journal 164, 243-7.

Rugg-Gunn,AJ (1993) Nutrition diet and oral health. Oxford university press.

Sarner B, Sundin E, Abdulrahman S, Birkhed D:, Lindstrom P (2012) Use of different mouthrinses in an adult Swedish population. Swedish Dental Journal 6:53-60

Scottish Dental Clinical Effectiveness Programme. (2010) Prevention and management of dental caries in children. Dundee; Available from url: http://www.sdcep.org.uk/index.aspx?o=2332

Selwitz RH, Ismail AI, Pitts NB, (2007) Dental Caries, Lancet; 369:51-59

SIGN 138: (2014) Dental interventions to prevent caries in children, A National Clinical Guideline, Scottish Intercollegiate Guidelines Network, March 2014

SIGN 47: (2000) Preventing Dental Caries in Children at High Caries Risk Targeted prevention of dental caries in the permanent teeth of 6-16 year olds presenting for dental care, A National Clinical Guideline, Scottish Intercollegiate Guidelines Network, December 2000

Sköld UM, Birkhed D, Ellwood RP, (2012) Effect on salivary fluoride retention post-brushing after rinsing with mouthwashes containing 225 or 500 ppm F compared to not rinsing. Journal Clinical Dentistry;23(3):97-100.

Soames, Southam,(2005) Oral Pathology, 3rd Edition, Oxford University Press

Stecksén-Blicks C, Renfors G, Oscarson ND, Bergstrand F, Twetman S. (2007) Caries-preventive effectiveness of fluoride varnish: a randomized controlled trial in adolescents with fixed orthodontic appliances. Caries Research;41(6):455-9.

Strang R, Damato FA, Creanor SL, Stephen KW, (1987) The effect of baseline lesion mineral loss on *in situ* remineralisation. Journal of Dental Research 66(11): 1644-1646

Sug-Joon A; Shin-Jae L; Dong-Yul L; Bum-Soon L. (2010) Effects of different fluoride recharging protocols on fluoride ion release from various orthodontic adhesives. Journal of Dentistry. 39(3):196-201

Ten Bosch JJ. Angmar-Mansson, (1991) A review of quantitative methods for studies of mineral content of intra-oral incipient caries lesions. Journal of Dental Research 70(1): 2-14

Ten Cate JM, Jongebloed WL, Arends J. (1981) Remineralization of artificial enamel lesions *in vitro*. IV. Influence of fluorides and diphosphonates on short- and long-term remineralisation. Caries Research;15:60–69.

Ten Cate JM. (1990) *In vitro* studies on the effects of fluoride on de- and remineralization. Journal of Dental Research.; 69 Special edition No:614-9

Ten Cate, J. M., Jongebloed, W. L., and Arends, J.(1981) Remineralization of artificial enamel lesions *in vitro*. IV. The influence of fluorides and diphosphonates on short and long term remineralization, Caries Research, 15, 60,

Teo, TKY; Ashley, PF; Louca, C. (2014) An *in vivo* and *in vitro* investigation of the use of ICDAS, DIAGNOdent pen and CarieScan PRO for the detection and assessment of occlusal caries in primary molar teeth.Clincal Oral Investigations; 3; p737-p744,

Thewlis J. (1940). The structure of teeth as shown by x-ray examination. Medical Research Council Report Series No 238.

Tubert-Jeannin S, Auclair C, Amsallem E,Tramini P, Gerbaud L, Ruffieux C, Schulte A, Koch M , Rège-Walther M, Ismail A. (2011) Fluoride supplements (tablets, drops, lozenges or chewing gums) for preventing dental caries in children. Cochrane Database of Systematic Reviews[Cochrane Database Syst Rev],ISSN: 1469-493X, 2011 Dec 07; (12)

Uysal T, Amasyali M, Ozcan S, Koyuturk AE, Akyol M, Sagdic D. (2010) *In vivo* effects of amorphous calcium phosphate-containing orthodontic composite on enamel demineralization around orthodontic brackets Australian Dental Journal. Sep;55(3):285-91.

Uysal, T; Amasyali, M; Koyuturk, AE; Ozcan, S (2010) Effects of different topical agents on enamel demineralization around orthodontic brackets: an *in vivo* and *in vitro* study. Australian Dental Journal; 55; 3; p268-p274

Van der Veen M, Mattousch T, Boersma JG, (2007) Longitudinal development of carious lesions after orthodontic treatment evaluated by quantitative light fluorescence. American Journal of Orthodontics and Dentofacial Orthopaedics, 131(2):223-228

Walsh,T,Worthington HV, Glenny A, Appelbe P, Marinho VCC,Shi X. (2010) Fluoride toothpastes of different concentrations for preventing dental caries in children and adolescents. Cochrane Database Systematic

Reviews[Cochrane Database Syst Rev], ISSN: 1469-493X, 2010 Jan 20; (1)k

White, G.E., Tsamtsouris, A. & Williams, D.L. (1978) Early detection of occlusal caries by measuring the electrical resistance of the tooth. Journal of Dental Research 57,195-200.

Wiener RC, Crout RJ, Wiener MA. (2009) Toothpaste Use By Children, Oral Hygiene, and Nutritional Education: An assessment of parental performance. Journal of Dental Hygiene 83: 141-5

Wong MC, Clarkson J, Glenny AM, Lo EC, Marinho VC, Tsang BW, Walsh T, Worthington HV. (2011) Cochrane reviews on the benefits/risk of fluoride toothpastes. Journal of Dental Research.; 90:573-9

World Health Organization technical report series (1962) No.2 Standardization of reporting of dental diseases and conditions. World Health Organization. Geneva

www.kavo.com/Products/Dental-Instruments/Prophylaxis/Preventative-instruments/DIAGNOdent-pen-2190.aspx?sstr=1accessed 04/08/2014

http://www.mintell.com accessed 07/2014

Zero DT, Creeth JE, Bosma ML, Butler A, Guibert RG, Karwal R, Lynch

RJ, Martinez-Miler EA, Gonzalez-Cabezas C, Kelly SA. (2010) The Effect of Brushing Time and Dentifrice Quantity on Fluoride Delivery *in vivo* and Enamel Surface Microhardness *in situ.* Caries Research;44:90-100

Zero DT, Raubertas RF, Pedersen AM, Fu J, Hayes AL, Featherstone JD. (1992) Studies of fluoride retention by oral soft tissues after the application of home-use topical fluorides. Journal of Dental Research;71:1546-52.

Appendix I Patient Information and Informed Consent Form (Page 1 of 5)

Subject ID No: ____ Subject Initials: _____ Date of Birth: _____

Study title: Effect of post-brushing mouthrinse solutions on salivary fluoride retention

What is the purpose of the study?

You are being invited to take part in a study in which the amount of fluoride you retain in your saliva will be assessed after brushing and rinsing with different fluoride mouthrinses.

It is important that you read this leaflet carefully. If you have any additional questions about the study please feel free to ask the investigator or other clinical staff who will be happy to help you. If you do agree to take part in this study you will be asked to sign the informed consent form, which constitutes part of this leaflet.

Will I get any benefit from taking part?

You may not gain any direct benefit from taking part. The information we obtain from this study may help us use fluoride mouthrinses more effectively. All products supplied for use in the study will be provided free of charge.

How many subjects are involved?

The study will involve approximately 30 subjects. The subjects will be males and females, aged 18-65 years old.

How long is the study?

The study duration is approximately 3 weeks. There are 3 visits after recruitment; each visit will last approximately 70 minutes. If selected you will be invited to participate in this study and you will be asked to use a standard fluoride toothpaste at home.

Subject Initials:_____ Date:_____

How will I be selected?

In order to take part you must be in good general health, have no sign of significant gum disease and no tooth decay, you must have a minimum of 24 teeth without extensive restorations, six in each quadrant of the mouth. You must also not be wearing any orthodontics appliance or dentures. You must not be taking part (or have taken part in the last month) any other dental research study. You must not be pregnant or breast feeding. You must be available for the duration of the study (Approximately 1 week) and also be willing to use only the toothpaste and toothbrush we will provide you with for the duration of the study period. You must not be allergic to oral care products, personal care products or their ingredients. A dentist will check your mouth to ensure that your mouth, teeth and gums are healthy and are suitable for the study.

What do I have to do?

During the course of the 3 week study and for one week before it commences you will be required to brush with a standard fluoride toothpaste (1450 ppm F from Sodium Fluoride) and toothbrush that we will provide. At each of the study visits you will be required to brush your teeth with the same toothpaste and brush and then rinse with one of three mouthrinses containing different levels of fluoride (225/500/0 ppm F from Sodium Fluoride). After this procedure an unstimulated saliva sample (≈0.3 ml) will be collected and at various time-points after brushing-rinsing (0, 1, 3, 5, 10, 20, 30, 45 and 60 min). You will not be able speak, eat or drink during these 60-min test period. You will have a minimum 48 hours washout phase between the study visits where you will use the designated commercially available toothpaste.

What do I do between visits?

You must use only the products provided. You are requested to inform the examining dentist immediately, if you receive emergency dental treatment or if you become pregnant or start breast feeding.

Whom do I contact in case of emergency?

In case of emergency, or if you should notice any abnormal conditions other than your existing condition, you should notify the clinical staff at the study site immediately, Investigator Professor Susan Higham (phone 07970 247633).

Subject Initials: _____ Date:_____

Patient Information and Informed Consent Form (Page 3 of 5)

What else will happen during the study?

At the end of the study you will return all study products (including any empty tubes of toothpaste).

Will I be paid for taking part in this study?

At the end of the study you will receive a study participation fee of £60 for your time and inconvenience. If for any reason you do not complete the study you may receive a pro rated amount based on the number of visits you attend.

Will I experience any unpleasant side effects?

The materials in the study products have been used in currently marketed products, without any significant safety problems. Most people will have no side effects with the products that we are using in this study but as with any product some side effects may occur in certain people. They are most commonly mouth or gum irritation. They are mild in nature and generally resolve once the product usage stops. In the unlikely event you do experience any unusual effects, please contact Professor Susan Higham (phone 07970 247633). Should you experience any side effect as a direct result of using the investigational products in the study, the Sponsor will bear the cost of any reasonable expense incurred during the medical treatment of the side effect.

Confidentiality?

All information will be treated with confidence to comply with the Data Protection laws. Your identification will only be in the form of a number and your initials. They will be made accessible to the sponsor or sponsor's representatives including the ethics committee and the regulator authorities. In the event that the results of this study are published, your identity will remain confidential. You have the right to refuse to provide or allow to be recorded any information collected as a result of participating in this study. You may have access to any of your information recorded as a result of participation in the study.

Subject Initials: _____ Date:_____

Patient Information and Informed Consent Form (Page 4 of 5)

What happens if I decide not to take part?

Your participation in this study is entirely voluntary. If you do not wish to take part you may hand back the forms, you do not have to give a reason. Once the study has started you are free to stop taking part at any time without giving a reason and without loss of benefits to which you are otherwise entitled. However, you must contact the clinic staff and inform them of your decision to withdraw. The investigator, or the sponsor of the study, or the study site may discontinue you from the study for safety reasons, or if you fail to follow the instructions of this study. Any new important information which discovered during the course of the study and which may influence your willingness to continue participation in the study will be made available to you by the investigator or clinic staff.

Who is organising and funding the research?

This study is being funded by Colgate Palmolive Ltd.

Who has reviewed the study?

This study has been reviewed by the University Manchester Research Ethics Committee, Manchester University, United Kingdom

Contact for Further Information

Further information about this study can be obtained by calling Professor Susan Higham (phone 07970 247633).

Subject Initials:_____ **Date:**_____

PATIENT INFORMATION AND INFORMED CONSENT FORM (page 5 of 5)

Study:Effect of post-brushing mouthrinse solutions on salivary fluoride retention

Principle Investigator: Professor Susan Higham

Subject Identification Number for this trial: ☐

CONSENT FORM

Please initial box

1. I confirm that I have read and understand the information sheet (**ProtocolNumber**DHU-Rinse-06/11-Liverpool***)*** for the above study and have had the opportunity to ask questions. ☐

2. 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. ☐

3. I understand that sections of any of my medical notes may be looked ☐

at by responsible individuals from the Colgate Palmolive Dental Health Unit or from regulatory authorities where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records.

☐

4. I agree to take part in the above study.

_____	_____	_____
Name of Subject	Date	Signature

_____	_____	_____
Name of Person taking consent (If different from Examining Dentist)	Date	Signature

_____	_____	_____
Examining Dentist	Date	Signature

Appendix II: Confidential Medical History

Subject ID No:……Subject Initials…….Date of Birth……..

Before beginning any study we need you to complete and return this form. Please tick the appropriate response

A yes answer does not necessarily mean you will not be able to do the study.

1. Are you attending or receiving treatment from a doctor? YES___NO___

2. Are you taking or using any medicines, pills, tablets, ointments, injections or any other drug, either from your doctor or on your own accord? YES___NO___

3. Are you allergic to or have you ever had any unfavourable reaction to any medicine, food or any other substance ? YES___NO___

4. Have you had any serious illnesses as a child or adult? YES___NO___

5. Have you ever been a hospital in-patient or ill at home for a long period? YES___NO___

6. Do you have or have you ever had any heart or blood pressure problems? YES___NO___

7. Have you ever had rheumatic fever or chorea (St. Vitus` dance)? YES___NO___

8. Do you have a heart murmur? YES___NO___

9. Do you have a heart pacemaker? YES___NO___

10. Have you ever had any heart surgery? YES___NO___

11. Do you have any chest or breathing problems? YES___NO___

12. Do you suffer from eczema, asthma or any form of allergy? YES___NO___

13. Do you suffer from fainting attacks, fits or seisures? YES___NO___

14. Have you ever suffered from hepatitis, jaundice, liver or kidney disease? YES___NO___

15. Are you diabetic? YES___NO___

16. Have you had any problems arising from a blood sample, a blood donation or transfusion? YES___NO___

17. Do you carry a warning card from your doctor or specialist? YES___NO___

18. Is there anything else concerning your health, such as a joint replacement,

you think we should know about? YES___NO___

19. Following extraction, surgery or injury have you or any other member of your

family bled for such a time as to cause you to be worried? YES___NO___

20. Do you suffer from a dry mouth when eating food i.e. do you

have to drink liquids to swallow easily ? YES___NO___

Please inform us immediately if there is any change in this information

To the best of my knowledge this information is correct. I understand this information may be inspected by authorised personnel and will be treated in strict confidence

PLEASE Initial HERE …… Date …..……..

Checked by.....……. Date …….....

Confidential Medical History Continued

Please write information on questions overleaf in the box below:

Question Number	Medication	Description	Subject Initials	Date

Medical History Review Record

Visit	Date	Any change in MH- details	Staff to initial and date	Subject to initial and date	Study Dentist to sign and date *
2					
3					
4					

***Only if there has been a change in medical history**

Appendix III: Oral Soft and Hard Tissue Assessment Form

Subject ID No: ____Subject Initials _____Date of Birth (DD/MM/YYYY)**: _____**

Male ☐ **Female** ☐

Tick the appropriate box

	AREA	NORMAL	ABNORMAL
1.	Perioral area/lips	☐	☐
2.	Buccal mucosa	☐	☐
3.	Labial mucosa	☐	☐
4.	Sublingual mucosa	☐	☐
5.	Gingiva free/attached	☐	☐
6.	Tongue	☐	☐
7.	Palate hard/soft	☐	☐
8.	Uvula	☐	☐
9.	Oropharynx	☐	☐
10.	All other soft/hard tissues	☐	☐

DESCRIBE ANY IRREGULARITIES:

_____

_____ _____

Signature of Examining Dentist **Date**

Appendix IV: Screening Form

Subject ID No: _____ Subject Initials_____Date_____Date of Birth_____

1. Is the age of the subject between 18 and 65 (both inclusive)? YES ___NO ___

2. Is subject available for the duration of the study? YES ___NO ___

3. Is subject in good health with no medical conditions that the investigator considers may compromise the subject's safety or the quality of the results? YES ___NO ___

4. Has the subject signed an Informed Consent Form and ready to comply with protocol? YES ___NO ___

5. Does the subject have 24 natural teeth free of extensive restorations and tooth decay. There should b least 6 teeth in each Quadrant YES ___NO ___

If answer to any of questions 1-5 is No, subject is ineligible for study. Subject should be dismissed and question 14 completed. If subject is eligible, continue to questions 6-14

6. Does subject have an orthodontic appliance? YES ___NO ___

7. Does subject use a removable partial denture? YES ___NO ___

8. Does subject have a soft or hard tissue tumour of the oral cavity? YES ___NO ___

9. Does subject have active caries lesion, gingivitis or advanced periodontal disease? YES ___NO ___

10. Is the subject participating in any other dental study or participated in a dental study within the past one month? YES ___NO ___

11. Is subject pregnant or breast feeding? YES ___NO ___

12. Does subject have any medical condition that the investigator considers may compromise the subject's safety as well as the quality of the study results? YES ___NO ___

13. Does the subjects have a history of Allergy to oral care products, Personal care Products or their ingredients YES ___NO ___

If answer to any of questions 6-13 is YES, subject is ineligible for the next visit. Subject should be dismissed and Q 14 completed.

14. IS SUBJECT ELIGIBLE TO ATTEND THE NEXT VISIT YES ___NO __

_____ _____

Signature of Examining Dentist **Date**

Appendix V: Adverse Event Forms

Non-Serious Adverse Event Form

Instructions: Do not leave any field blank. Please indicate if information is unknown, not provided or not available (refused). Date format: 01/Apr/2010. Please complete form electronically as this form is expandable.

Date of Awareness (dd/mmm/yyyy):

Date of Report (dd/mmm/yyyy):

Protocol #:

Protocol Title:

Indication/Objective of Protocol (if applicable):

Investigator:

Study Originator/Manager:

Type of study:

☐Clinical	☐Consumer	☐Panel

Product Category:

☐Fabric Care	☐Household Surface Care	☐Oral Care
☐Personal Care	☐Other:	

Phase of study the earliest event (s) occurred during:

☐After consent	☐Wash-out	☐Pre-randomisation
☐Randomisation: no product exposure	☐Randomisation: product exposure	☐Other:

Subject/Patient information:

ID	Initials	Sex	Age or DOB (dd/mmm/yyyy)	Weight	Ethnic group

Product information:

Product name (PIM#):

Start date (dd/mmm/yyyy):	Stop Date or Duration (dd/mmm/yyyy):
Dosage:	Frequency:
Randomisation group:	

Reaction/Event information: Subject ID:

Onset date (dd/mmm/yyyy)	Stop date or duration (dd/mmm/yyyy):	Severity (Mild, Moderate, Severe)	Relationship to product (Possibly related, Related, Unrelated, Unknown)

Describe event(s)/reactions(s) in detail:

Outcome:

☐Resolved, Date (dd/mmm/yyyy):	☐Resolving	☐Unknown/Lost to F/U
☐Not resolved	☐Resolved with sequelae	☐Other:

Action taken with the product:

☐ Continued	☐ Reduced, Specify:
☐ Discontinued	☐ Unknown
☐ Temporarily discontinued	☐ Other:

Did the event(s) abate after product was stopped or dose reduced (Yes/No)?

Did the event(s) reappear after product was reintroduced (Yes/No)?

Protocol Status of Subject:

☐ Protocol Continued ☐ Protocol Discontinued

Treatment for the serious AE(s)/AR(s):

Relevant Medical History Data: ☐ Yes (List below) ☐ None ☐ Not provided ☐ Unknown

(Medical history with onset dates if known)

Relevant Concomitant Medications: ☐ Yes (List below) ☐ None ☐ Not provided ☐ Unknown

(Medication name, dose, frequency, start/stop dates [dd/mmm/yyyy] or duration of therapy if known)

Relevant Lab data: ☐ Yes (List below) ☐ None ☐ Not provided ☐ Unknown

(Lab test, results, dates [dd/mmm/yyyy] if known)

Subject ID:

Investigator/Designee Signature	**Date (dd/mmm/yyyy):**

Serious Adverse Event Report Form

Instructions: Please complete all fields of this form electronically. Please indicate if 'Not provided,' 'Not Available' or 'Unknown.' (Date eg: dd/mmm/yyyy 01/Dec/2009)

Case-ID-No:

A) Study site details	
Study No.:	Centre Name:
Study type : Clinical /Consumer test /Panel test	Investigator:
If clinical study, please define protocol type:	Address:
Study product(s) in protocol :	Country of occurrence:

B) Reporter Information	
Sender / Reporter	Health professional: ☐ YES ☐ NO
Name:	Profession (Speciality):
	Date of Awareness (dd/mmm/yyyy):
Address:	
Tel.:	Date of Report (dd/mmm/yyyy):
Fax:	
E-mail:	

C) Subject information	
Subject's ID-number:	
If randomised, please provide randomisation number:	
Sex: ☐ Male ☐ Female	Year of birth (dd/mmm/yyyy) or Age:
In case of intoxication: Weight (kg):	
If Female, Pregnancy: ☐ NO ☐ YES, how many months: ☐ Unknown	
D) Product information	
Which product is involved in event/reaction? ☐ Study/Test product: ☐ Other: ☐ Blinded ☐ Placebo ☐ Product Name: ☐ Controlled/Comparator product: Duration of product use:	

E) Clinical study products
Please indicate if Test product, Comparator (control product) or Placebo

Product/Lot No.	Dose [unit]	Route	Frequency	Indication	Start Date (dd/mmm/yyyy)	Stop Date (dd/mmm/yyyy)

F) Serious AE/AR Information			
Reason for report:			
☐ Death		☐Disability/Incapacity	
☐ Hospitalisation/Prolonged Hospitalisation		☐ Congenital Anomaly	
☐Life Threatening		☐ Other, Specify:	
☐Suspected transmission via a medicinal product of an infectious agent			
If hospitalisation, provide dates (dd/mmm/yyyy)		From:	To:
☐Hospitalisation ongoing			
Specify when the Serious AE/AR occurred during the study?			
☐After consent, before wash-out		☐ Wash-out	
☐ Pre-randomisation		☐ Randomised: with no product initiated	
☐ Randomised: with product initiated		☐ Unknown	
☐Other: Specify:			
Diagnosis / Symptoms			
Please, indicate diagnosis or main symptom (s) and list serious most significant AE/AR first: 1. 2. 3. 4.			
Date of primary symptom (dd/mmm/yyyy)**:**			
Severity:	☐Mild	☐ Moderate	☐Severe
Outcome of serious adverse event :			
☐ Not resolved	☐ Resolving		☐ Resolved with sequelae
☐ Resolved, date:	☐ Unknown/ lost to follow up		☐ *Death, date:

Cause of death if known:

Autopsy: ☐ YES ☐ NO ☐ Unknown ☐ Outcome:

Action taken with involved product:

☐ Continued

☐ Discontinued

☐ Temporarily discontinued

☐ Dose reduced, new dose:

☐ Unknown

☐ Other:

Protocol status of subject:

☐ Subject continued on protocol

☐ Subject discontinued from protocol

Was the subject treated for the event(s)/reaction(s) (Medical Intervention)? ☐ YES ☐ NO

If yes, please specify or describe:

Event/Reaction abated after drug stopped or dose reduced?

☐ YES ☐ NO ☐ N.A.

Event/Reaction reappeared after drug reintroduction?

☐ YES ☐ NO ☐ N.A.

Causality

☐ Related ☐ Possibly related ☐ Unrelated ☐ Unknown

*If unrelated, please provide an alternative causality:

Code broken (unblinded)

☐ YES ☐ NO

G) Case narrative

Please provide full details of the serious AE/AR, dechallenge/rechallenge information and vital signs. Attach any relevant reports from the source document or hospitalisation file. In case of death, report cause and attach a copy of the autopsy report, if performed.

Information enclosed: ☐ NO ☐ YES, Specify:

H) Relevant medical history

Description	Onset Date (dd/mmm/yyyy)	Resolved Date (dd/mmm/yyyy)

I) Concomitant medication

Please report the medication taken in the last 4 weeks prior to the serious AE(s)/AR(s)

Drug	Dose [unit]	Route	Frequency	Indication	Start Date (dd/mmm/yyyy)	Stop Date (dd/mmm/yyyy)

J) Laboratory data & other test procedure(s) relevant to the serious AE(s)/AR(s)

Test	Date (dd/mmm/yyyy)	Result (normal, abnormal, clinically significant)

Investigator/Designee Signature	**Date** (dd/mmm/yyyy)**:**

This report has to be sent within **1 Calendar day (No later than 1 business day)**:

- By the Investigator to the Study Manager:
  - Address
  - Phone
  - E-mail
- By the Study Manager to **Central (Pharmaco)vigilance at:**
  - The GVC mailbox via: Email: Global_PCV@colpal.com

Or Fax:+732.878.7844

- Additionally, copy the CRA at SCRR@colpal.com

Appendix VI: Ethical Approval

Secretary to Research Ethics Committee 5
Faculty Office - Devonshire House

Tel: 0161 275 0288

Email: jared.ruff@manchester.ac.uk

Professor Iain Pretty
School of Dentistry

16th November 2011

Dear Professor Pretty

Research Ethics Committee 5 (Flagged Humanities) - Project Ref 11227

I am writing to thank you for submitting your research project application to the University Ethics Committee which met on 10th October 2011 and providing follow up material to address the issues that I raised with you in my email of 17th October 2011. I can now confirm that by way of chair's action your project has now been formally approved by the University Ethics Committee 5 (flagged Humanities).

This approval is effective for a period of five years and if the project continues beyond that period it must be submitted for review. It is the Committee's practice to warn investigators that they should not depart from the agreed protocol without seeking the approval of the Committee, as any significant deviation could invalidate the insurance arrangements and constitute research misconduct. We also ask that any information sheet should carry a University logo or other indication of where it came from, and that, in accordance with University policy, any data carrying personal identifiers must be encrypted when not held on a university computer or kept as a hard copy in a location which is accessible only to those involved with the research.

Finally, I would be grateful if you could complete and return the attached form at the end of the project or by September 2012.

I hope the research goes well.

Yours sincerely

Jared Ruff
Senior Research Manager
Faculty of Humanities and Secretary to URC 5 (Flagged Humanities)
0161 275 0288
Jared.ruff@manchester.ac.uk

UNIVERSITY OF MANCHESTER

COMMITTEE ON THE ETHICS OF RESEARCH ON HUMAN BEINGS

Progress or Completion Report Form on an Approved Project

The Committee's procedures require those responsible for projects which have been approved by the Committee to report on any of the following:

* Any incident, accident or untoward event associated with the project (*Please note that if the incident constitutes an accident or dangerous occurrence, the usual Health and Safety reporting mechanism must still be used*)
* Any variation in the methods or procedures in the approved protocol
* A termination or abandonment of the project (with reasons)
* A report on completion of the project or a progress report 12 months after approval has been given.

The report should be sent to the Secretary to the Committee, Dr T P C Stibbs, Room 2.004 John Owens Building, University of Manchester, Oxford Road, Manchester M13 9PL (*tel:* 0161-275-2046/2206).

Project:

UNIVERSITY OF MANCHESTER

COMMITTEE ON THE ETHICS OF RESEARCH ON HUMAN BEINGS

Progress or Completion Report Form on an Approved Project

The Committee's procedures require those responsible for projects which have been approved by the Committee to report on any of the following:

* Any incident, accident or untoward event associated with the project (*Please note that if the incident constitutes an accident or dangerous occurrence, the usual Health and Safety reporting mechanism must still be used*)

* Any variation in the methods or procedures in the approved protocol

* A termination or abandonment of the project (with reasons)

* A report on completion of the project or a progress report 12 months after approval has been given.

The report should be sent to the Secretary to the Committee, Dr T P C Stibbs, Room 2.004 John Owens Building, University of Manchester, Oxford Road, Manchester M13 9PL (*tel:* 0161-275-2046/2206).

Project:

Appendix VII

Table 2. 10. Fluoride concentration (ppm) in human saliva 0-60 minutes post brushing with rinsing with 500 ppm F.

Subj/time	0	1	3	5	10	20	30	45	60
1	0.43	176.87	59.71	25.54	11.90	4.56	1.91	1.04	0.72
2	0.19	55.20	20.69	8.72	2.86	1.06	1.21	0.58	0.45
3	0.23	159.25	81.66	46.86	3.20	1.93	1.12	0.58	0.34
4	0.69	83.49	24.72	11.02	3.67	1.57	2.46	1.78	1.47
5	0.10	142.03	61.47	28.13	12.78	5.09	2.78	1.62	0.94
6	0.31	44.78	22.71	16.49	6.51	2.98	1.25	0.73	0.37
7	3.54	65.05	39.53	21.96	12.40	5.87	2.82	1.50	0.89
8	0.08	89.59	23.26	17.08	4.58	1.88	0.73	0.45	0.36
9	1.38	266.18	193.58	62.97	36.88	14.96	11.01	3.21	2.70
10	0.09	63.29	18.27	13.70	4.80	1.71	0.96	3.89	0.24
11	0.31	101.78	39.45	19.75	10.34	5.15	3.39	1.70	0.83
12	0.20	160.67	69.78	37.33	3.19	1.90	1.36	0.93	0.64
13	0.33	135.22	70.62	33.36	2.95	1.50	1.22	0.87	0.54
14	0.17	56.79	30.26	17.90	8.16	4.62	2.39	1.42	0.84
15	0.06	136.86	63.63	39.48	23.35	3.10	2.30	1.07	0.72
16	0.07	46.60	19.94	10.44	7.15	3.57	0.72	8.00	0.42
17	3.19	211.31	114.99	77.58	55.63	27.02	15.50	8.79	7.62
18	0.03	141.89	51.03	20.05	1.89	0.86	0.47	0.26	0.38
19	0.15	145.33	66.62	43.68	16.44	8.37	4.76	3.94	7.15
20	0.22	213.28	70.17	28.77	16.50	5.65	4.12	1.37	1.01
21	0.35	104.95	36.82	20.62	6.42	4.15	2.27	0.99	0.56
22	0.21	141.61	46.43	18.51	8.72	4.56	2.83	1.28	0.95
23	0.30	56.32	13.40	10.67	3.13	1.48	0.80	0.53	0.21
24	0.07	79.33	26.95	11.65	4.07	1.47	0.92	0.25	0.30
25	0.32	118.39	45.68	23.63	11.78	3.86	1.53	30.41	5.31
26	0.37	78.87	30.02	13.30	4.86	2.96	1.67	1.04	0.54
27	0.23	80.29	31.00	14.75	6.27	2.50	1.40	0.75	4.17
28	0.10	42.63	14.68	7.17	2.15	0.85	0.43	0.35	0.24
29	0.38	85.80	38.18	21.26	8.73	3.42	2.10	1.50	0.94
30	0.31	89.80	22.60	8.60	3.70	1.52	0.84	0.68	0.39
mean	**0.48**	**112.45**	**48.26**	**24.37**	**10.17**	**4.34**	**2.58**	**2.72**	**1.41**
SD	**0.82**	**56.03**	**36.25**	**16.41**	**11.30**	**5.12**	**3.14**	**5.61**	**1.99**

Table 2. 11. Area under the curve values for fluoride concentration (ppm) in human saliva 0-60 minutes post brushing with rinsing with 500 ppm F.

Subj/time	0-1	1-3	3-5	5-10	10-20	20-30	30-45	45-60	0-60
1	88.65	236.58	85.25	93.60	82.30	32.35	22.13	13.20	**654.06**
2	27.70	75.89	29.41	28.95	19.60	11.35	13.43	7.73	**214.05**
3	79.74	240.91	128.52	125.15	25.65	15.25	12.75	6.90	**634.87**
4	42.09	108.21	35.74	36.73	26.20	20.15	31.80	24.38	**325.29**
5	71.07	203.50	89.60	102.28	89.35	39.35	33.00	19.20	**647.34**
6	22.55	67.49	39.20	57.50	47.45	21.15	14.85	8.25	**278.44**
7	34.30	104.58	61.49	85.90	91.35	43.45	32.40	17.93	**471.39**
8	44.84	112.85	40.34	54.15	32.30	13.05	8.85	6.08	**312.45**
9	133.78	459.76	256.55	249.63	259.20	129.85	106.65	44.33	**1639.74**
10	31.69	81.56	31.97	46.25	32.55	13.35	36.38	30.98	**304.72**
11	51.05	141.23	59.20	75.23	77.45	42.70	38.18	18.98	**504.00**
12	80.44	230.45	107.11	101.30	25.45	16.30	17.18	11.78	**590.00**
13	67.78	205.84	103.98	90.78	22.25	13.60	15.68	10.58	**530.47**
14	28.48	87.05	48.16	65.15	63.90	35.05	28.58	16.95	**373.32**
15	68.46	200.49	103.11	157.08	132.25	27.00	25.28	13.43	**727.09**
16	23.34	66.54	30.38	43.98	53.60	21.45	65.40	63.15	**367.83**
17	107.25	326.30	192.57	333.03	413.25	212.60	182.18	123.08	**1890.25**
18	70.96	192.92	71.08	54.85	13.75	6.65	5.48	4.80	**420.49**
19	72.74	211.95	110.30	150.30	124.05	65.65	65.25	83.18	**883.42**
20	106.75	283.45	98.94	113.18	110.75	48.85	41.18	17.85	**820.94**
21	52.65	141.77	57.44	67.60	52.85	32.10	24.45	11.63	**440.49**
22	70.91	188.04	64.94	68.08	66.40	36.95	30.83	16.73	**542.87**
23	28.31	69.72	24.07	34.50	23.05	11.40	9.98	5.55	**206.58**
24	39.70	106.28	38.60	39.30	27.70	11.95	8.78	4.13	**276.43**
25	59.36	164.07	69.31	88.53	78.20	26.95	239.55	267.90	**993.86**
26	39.62	108.89	43.32	45.40	39.10	23.15	20.33	11.85	**331.66**
27	40.26	111.29	45.75	52.55	43.85	19.50	16.13	36.90	**366.23**
28	21.37	57.31	21.85	23.30	15.00	6.40	5.85	4.43	**155.50**
29	43.09	123.98	59.44	74.98	60.75	27.60	27.00	18.30	**435.14**
30	45.06	112.40	31.20	30.75	26.10	11.80	11.40	8.03	**276.73**
mean	**56.46**	**160.71**	**72.63**	**86.33**	**72.52**	**34.57**	**39.70**	**30.94**	**553.85**
SD	**28.12**	**90.01**	**51.39**	**66.33**	**81.25**	**41.08**	**51.62**	**51.70**	**389.46**

Table 2. 12. Fluoride concentration (ppm) in human saliva 0-60 minutes post brushing with rinsing with 225 ppm F.

Subj/time	0	1	3	5	10	20	30	45	60
1	0.39	80.30	32.48	13.51	5.47	1.72	0.85	0.68	0.48
2	0.12	3.23	1.46	0.84	0.50	0.32	0.32	0.30	0.17
3	0.24	87.28	42.37	22.50	13.96	5.53	1.53	0.97	0.71
4	1.41	34.20	12.18	4.66	2.19	1.81	1.49	1.36	1.60
5	0.05	53.30	2.97	1.60	0.87	0.43	0.30	0.21	0.15
6	0.25	12.65	5.99	3.94	1.31	0.92	0.65	0.34	0.64
7	0.37	60.98	26.60	14.75	4.12	2.00	1.03	0.97	0.39
8	0.33	65.34	25.57	15.31	5.99	2.39	1.05	0.85	0.66
9	12.10	108.00	60.67	54.59	24.72	8.99	7.34	3.98	2.11
10	0.23	35.38	12.63	7.74	3.60	1.29	0.66	2.72	0.30
11	0.16	39.80	2.87	1.95	1.08	0.58	0.40	0.23	0.16
12	0.10	44.56	16.71	8.30	3.94	1.46	0.62	0.53	0.46
13	0.27	55.18	24.51	13.45	5.86	2.60	1.86	1.28	0.87
14	0.09	37.33	16.65	8.37	4.23	2.27	1.38	0.57	0.28
15	0.07	44.38	16.11	9.99	9.25	3.91	2.65	2.18	1.08
16	0.05	42.23	10.50	4.19	1.53	0.53	0.24	0.17	0.08
17	9.50	105.26	37.27	30.38	15.31	9.81	5.06	2.78	2.05
18	0.03	70.76	29.10	13.34	5.18	1.19	10.52	0.26	2.79
19	0.05	44.66	19.44	10.80	4.15	2.09	1.31	0.70	0.41
20	0.15	59.01	24.39	10.21	3.91	1.40	0.65	0.58	0.51
21	0.66	77.22	31.16	19.51	13.92	4.64	2.47	1.40	1.79
22	0.12	65.59	15.25	5.64	2.12	0.84	0.45	0.67	0.40
23	0.08	21.89	6.12	2.98	1.46	0.54	0.35	0.21	0.12
24	0.30	59.51	24.61	8.68	2.68	0.60	0.41	1.95	7.81
25	0.07	36.39	14.71	4.67	2.50	0.82	0.40	0.23	0.94
26	0.69	25.08	9.73	4.02	1.29	0.65	0.61	0.32	0.32
27	0.01	35.35	13.75	7.00	2.58	1.13	0.76	0.36	0.27
28	0.04	4.06	5.11	2.24	1.00	1.00	0.22	0.19	0.11
29	0.73	39.72	16.83	9.65	5.03	2.29	1.94	1.02	1.09
30	0.42	42.98	12.52	5.20	2.19	1.84	0.79	0.62	0.77
mean	**0.97**	**49.72**	**19.01**	**10.67**	**5.06**	**2.19**	**1.61**	**0.95**	**0.98**
SD	**2.71**	**25.76**	**13.08**	**10.63**	**5.39**	**2.32**	**2.26**	**0.92**	**1.46**

Table 2. 13. Area under the curve values for fluoride concentration (ppm) in human saliva 0-60 minutes post brushing with rinsing with 225 ppm F.

Subj/time	0-1	1-3	3-5	5-10	10-20	20-30	30-45	45-60	0-60
1	40.35	112.78	45.99	47.45	35.95	12.85	11.48	8.70	**315.54**
2	1.68	4.69	2.30	3.35	4.10	3.20	4.65	3.53	**27.49**
3	43.76	129.65	64.87	91.15	97.45	35.30	18.75	12.60	**493.53**
4	17.81	46.38	16.84	17.13	20.00	16.50	21.38	22.20	**178.23**
5	26.68	56.27	4.57	6.18	6.50	3.65	3.83	2.70	**110.37**
6	6.45	18.64	9.93	13.13	11.15	7.85	7.43	7.35	**81.92**
7	30.68	87.58	41.35	47.18	30.60	15.15	15.00	10.20	**277.73**
8	32.84	90.91	40.88	53.25	41.90	17.20	14.25	11.33	**302.55**
9	60.05	168.67	115.26	198.28	168.55	81.65	84.90	45.68	**923.03**
10	17.81	48.01	20.37	28.35	24.45	9.75	25.35	22.65	**196.74**
11	19.98	42.67	4.82	7.58	8.30	4.90	4.73	2.93	**95.90**
12	22.33	61.27	25.01	30.60	27.00	10.40	8.63	7.43	**192.66**
13	27.73	79.69	37.96	48.28	42.30	22.30	23.55	16.13	**297.93**
14	18.71	53.98	25.02	31.50	32.50	18.25	14.63	6.38	**200.96**
15	22.23	60.49	26.10	48.10	65.80	32.80	36.23	24.45	**316.19**
16	21.14	52.73	14.69	14.30	10.30	3.85	3.08	1.88	**121.96**
17	57.38	142.53	67.65	114.23	125.60	74.35	58.80	36.23	**676.76**
18	35.40	99.86	42.44	46.30	31.85	58.55	80.85	22.88	**418.12**
19	22.36	64.10	30.24	37.38	31.20	17.00	15.08	8.33	**225.67**
20	29.58	83.40	34.60	35.30	26.55	10.25	9.23	8.18	**237.08**
21	38.94	108.38	50.67	83.58	92.80	35.55	29.03	23.93	**462.87**
22	32.86	80.84	20.89	19.40	14.80	6.45	8.40	8.03	**191.66**
23	10.99	28.01	9.10	11.10	10.00	4.45	4.20	2.48	**80.32**
24	29.91	84.12	33.29	28.40	16.40	5.05	17.70	73.20	**288.07**
25	18.23	51.10	19.38	17.93	16.60	6.10	4.73	8.78	**142.84**
26	12.89	34.81	13.75	13.28	9.70	6.30	6.98	4.80	**102.50**
27	17.68	49.10	20.75	23.95	18.55	9.45	8.40	4.73	**152.61**
28	2.05	9.17	7.35	8.10	10.00	6.10	3.08	2.25	**48.10**
29	20.23	56.55	26.48	36.70	36.60	21.15	22.20	15.83	**235.73**
30	21.70	55.50	17.72	18.48	20.15	13.15	10.58	10.43	**167.70**
mean	**25.35**	**68.73**	**29.68**	**39.33**	**36.26**	**18.98**	**19.24**	**14.54**	**252.09**
SD	**13.73**	**37.86**	**23.31**	**39.67**	**38.03**	**20.19**	**20.93**	**15.22**	**191.03**

Table 2. 14. Fluoride concentration (ppm) in human saliva 0-60 minutes post brushing with rinsing with 0 ppm F.

Subj/time	0	1	3	5	10	20	30	45	60
1	0.18	0.27	0.29	0.32	0.29	0.21	0.19	0.09	0.08
2	0.07	0.56	0.49	0.30	0.22	0.14	0.09	0.07	0.08
3	0.19	1.43	1.28	0.83	0.61	0.38	0.26	0.16	0.13
4	0.34	0.35	0.29	0.38	0.35	0.29	0.36	0.33	0.18
5	0.07	0.63	0.34	0.28	0.20	0.16	0.11	0.11	0.08
6	0.66	0.54	0.63	0.58	0.41	0.28	0.26	0.16	0.12
7	0.41	0.37	0.35	0.25	0.18	0.17	0.18	0.15	0.13
8	0.21	0.97	0.52	0.68	0.42	0.29	0.23	0.25	0.25
9	0.24	4.41	5.14	4.46	2.30	1.61	0.52	0.56	0.34
10	0.09	0.75	0.54	0.44	0.27	0.21	0.14	0.10	0.06
11	0.36	0.86	0.69	0.50	0.33	0.17	0.12	0.10	0.08
12	0.32	9.85	6.47	4.41	2.56	1.51	1.19	0.69	0.53
13	0.59	0.76	0.78	0.87	0.47	0.33	0.21	0.38	0.13
14	0.06	0.56	0.47	0.36	0.26	0.13	0.09	0.06	0.04
15	0.05	0.46	0.45	0.37	0.38	0.20	0.11	0.09	0.07
16	0.05	0.18	0.15	0.14	0.12	0.08	0.05	0.04	0.04
17	0.89	3.43	2.39	2.11	1.69	1.16	1.02	0.66	0.60
18	0.02	0.33	0.42	0.32	0.18	0.07	0.07	0.06	0.03
19	0.06	0.26	0.28	0.19	0.14	0.10	0.09	0.06	0.07
20	0.17	0.74	0.76	0.84	0.75	0.12	0.27	0.19	0.12
21	0.86	0.53	0.44	0.29	0.25	0.18	0.17	0.11	0.08
22	0.11	0.48	0.35	0.26	0.18	0.15	0.09	0.07	0.07
23	0.39	0.49	0.56	0.44	0.22	0.17	0.12	0.20	0.11
24	0.07	0.86	0.81	0.49	0.36	0.11	0.08	0.18	0.10
25	0.35	0.38	0.43	0.42	0.25	0.18	0.17	0.12	0.10
26	1.19	0.90	0.56	0.48	0.55	0.38	0.27	0.24	0.26
27	0.10	0.25	0.22	0.16	0.14	0.21	0.08	0.09	0.06
28	0.05	0.29	0.35	0.25	0.15	0.11	0.08	0.05	0.12
29	0.39	0.71	0.85	0.48	0.42	0.23	0.12	0.24	0.38
30	0.35	0.34	0.31	0.33	0.29	0.24	0.21	0.15	0.12
mean	**0.30**	**1.10**	**0.92**	**0.74**	**0.50**	**0.32**	**0.23**	**0.19**	**0.15**
SD	**0.29**	**1.88**	**1.40**	**1.07**	**0.60**	**0.39**	**0.26**	**0.17**	**0.14**

Table 2. 15. Area under the curve values for fluoride concentration (ppm) in human saliva 0-60 minutes post brushing with rinsing with 0 ppm F.

Subj/time	0-1	1-3	3-5	5-10	10-20	20-30	30-45	45-60	0-60
1	0.23	0.56	0.61	1.53	2.50	2.00	2.10	1.28	**10.80**
2	0.32	1.05	0.79	1.30	1.80	1.15	1.20	1.13	**8.73**
3	0.81	2.71	2.11	3.60	4.95	3.20	3.15	2.18	**22.71**
4	0.35	0.64	0.67	1.83	3.20	3.25	5.18	3.83	**18.93**
5	0.35	0.97	0.62	1.20	1.80	1.35	1.65	1.43	**9.37**
6	0.60	1.17	1.21	2.48	3.45	2.70	3.15	2.10	**16.86**
7	0.39	0.72	0.60	1.08	1.75	1.75	2.48	2.10	**10.86**
8	0.59	1.49	1.20	2.75	3.55	2.60	3.60	3.75	**19.53**
9	2.33	9.55	9.60	16.90	19.55	10.65	8.10	6.75	**83.43**
10	0.42	1.29	0.98	1.78	2.40	1.75	1.80	1.20	**11.62**
11	0.61	1.55	1.19	2.08	2.50	1.45	1.65	1.35	**12.38**
12	5.09	16.32	10.88	17.43	20.35	13.50	14.10	9.15	**106.81**
13	0.68	1.54	1.65	3.35	4.00	2.70	4.43	3.83	**22.17**
14	0.31	1.03	0.83	1.55	1.95	1.10	1.13	0.75	**8.65**
15	0.26	0.91	0.82	1.88	2.90	1.55	1.50	1.20	**11.01**
16	0.12	0.33	0.29	0.65	1.00	0.65	0.68	0.60	**4.31**
17	2.16	5.82	4.50	9.50	14.25	10.90	12.60	9.45	**69.18**
18	0.18	0.75	0.74	1.25	1.25	0.70	0.98	0.68	**6.52**
19	0.16	0.54	0.47	0.83	1.20	0.95	1.13	0.98	**6.25**
20	0.46	1.50	1.60	3.98	4.35	1.95	3.45	2.33	**19.61**
21	0.70	0.97	0.73	1.35	2.15	1.75	2.10	1.43	**11.17**
22	0.30	0.83	0.61	1.10	1.65	1.20	1.20	1.05	**7.94**
23	0.44	1.05	1.00	1.65	1.95	1.45	2.40	2.33	**12.27**
24	0.47	1.67	1.30	2.13	2.35	0.95	1.95	2.10	**12.91**
25	0.37	0.81	0.85	1.68	2.15	1.75	2.18	1.65	**11.43**
26	1.05	1.46	1.04	2.58	4.65	3.25	3.83	3.75	**21.60**
27	0.18	0.47	0.38	0.75	1.75	1.45	1.28	1.13	**7.38**
28	0.17	0.64	0.60	1.00	1.30	0.95	0.98	1.28	**6.91**
29	0.55	1.56	1.33	2.25	3.25	1.75	2.70	4.65	**18.04**
30	0.35	0.65	0.64	1.55	2.65	2.25	2.70	2.03	**12.81**
mean	**0.70**	**2.02**	**1.66**	**3.10**	**4.09**	**2.75**	**3.18**	**2.58**	**20.07**
SD	**0.97**	**3.25**	**2.46**	**4.15**	**4.92**	**3.14**	**3.16**	**2.29**	**23.61**

Appendix VIII

Table 3. 15– Repeated QLF-D ΔF values with difference in relation to the standard deviation of the mean.

Measurement Pair	Value 2 ΔF	Value 1 ΔF	Difference	Difference in Standard Deviations of the mean
1	-10.7	-12.2	1.5	0.49333
2	-11.8	-11.7	0.1	-0.03289
3	-16.1	-10.6	5.5	-1.80888
4	-14	-11	3	-0.98666
5	-13.3	-12.5	0.8	-0.26311
6	-22.9	-20.3	2.6	-0.85511
7	-9	-9.2	0.2	0.065777
8	-18.2	-21	2.8	0.920883
9	-24.1	-24.8	0.7	0.230221
10	-12.7	-12.2	0.5	-0.16444
11	-17.9	-18.6	0.7	0.230221
12	-16.4	-17.7	1.3	0.427553
13	-17.5	-13.5	4	-1.31555
14	-20	-20.2	0.2	0.065777
15	-11	-11	0	0
16	-22.3	-22.2	0.1	-0.03289
17	-10.5	-13	2.5	0.822217
18	-17.5	-18	0.5	0.164443
19	-16.8	-17.1	0.3	0.098666
20	-18.1	-16.3	1.8	-0.592
21	-20.7	-14.8	5.9	-1.94043
22	-18.3	-15.8	2.5	-0.82222
23	-14.1	-10	4.1	-1.34844
24	-19.3	-18	1.3	-0.42755
25	-13.5	-12.7	0.8	-0.26311
26	-32.9	-29.3	3.6	-1.18399
27	-10.9	-10.2	0.7	-0.23022
28	-19.5	-19.4	0.1	-0.03289
29	-21	-20	1	-0.32889

30	-16.4	-19	2.6	0.855106
31	-9.1	-9.2	0.1	0.032889
32	-11.5	-10.9	0.6	-0.19733
33	-8.7	-8.8	0.1	0.032889
34	-12.3	-12	0.3	-0.09867
35	-7	-7.4	0.4	0.131555
36	-11.8	-12.8	1	0.328887
37	-25.9	-26	0.1	0.032889
38	-16.4	-16.3	0.1	-0.03289
39	-22.6	-22.5	0.1	-0.03289
40	-15	-14.5	0.5	-0.16444
41	-17.6	-17.6	0	0
42	-14.9	-15.1	0.2	0.065777
43	-23.1	-21.9	1.2	-0.39466
44	-22.6	-16.9	5.7	-1.87466
45	-22	-23.6	1.6	0.526219
46	-19	-17.4	1.6	-0.52622
47	-14.3	-14.3	0	0
48	-32	-31.6	0.4	-0.13155
49	-21.3	-21	0.3	-0.09867
50	-16.5	-15.7	0.8	-0.26311
51	-12.4	-12.6	0.2	0.065777
52	-18.2	-18.3	0.1	0.032889
53	-13.9	-13.9	0	0
54	-21.3	-21.1	0.2	-0.06578
55	-22.4	-21.1	1.3	-0.42755
56	-22.5	-23.3	0.8	0.26311
57	-12.5	-13.8	1.3	0.427553
58	-19	-20	1	0.328887
59	-19.4	-18.4	1	-0.32889
60	-17.3	-17.7	0.4	0.131555
61	-11.8	-11.7	0.1	-0.03289
62	-22.6	-25.4	2.8	0.920883
63	-12.7	-13.9	1.2	0.394664
64	-17.2	-20.2	3	0.986661
65	-18.7	-15	3.7	-1.21688

Table 3. 16– MSI repeated ΔF values with the difference in relation to the standard deviation of the mean.

Measurement Pair	Value 2 ΔF	Value 1 ΔF	Difference	Difference in standard deviations of the mean
1	-16.9	-18.1	1.2	-0.140549619
2	-22.6	-22.7	0.1	-0.011712468
3	-18.3	-16.3	2	0.234249365
4	-13.5	-13.7	0.2	-0.023424937
5	-17	-15.1	1.9	0.222536897
6	-35.3	-37.4	2.1	-0.245961834
7	-12.1	-12.4	0.3	-0.035137405
8	-32	-30.5	1.5	0.175687024
9	-35.7	-35.9	0.2	-0.023424937
10	-18	-16.7	1.3	0.152262088
11	-22.1	-29.2	7.1	-0.831585247
12	-27.6	-27.4	0.2	0.023424937
13	-28	-26.3	1.7	0.199111961
14	-27.3	-28.2	0.9	-0.105412214
15	-21.4	-22.9	1.5	-0.175687024
16	-34.3	-32.7	1.6	0.187399492
17	-29.1	-27.5	1.6	0.187399492
18	-25.9	-25.8	0.1	0.011712468
19	-32.4	-33.2	0.8	-0.093699746
20	-26.7	-26.8	0.1	-0.011712468
21	-35.7	-35.6	0.1	0.011712468
22	-24.6	-23.7	0.9	0.105412214
23	-25.6	-27.7	2.1	-0.245961834
24	-16.6	-17	0.4	-0.046849873
25	-19.1	-19.7	0.6	-0.07027481
26	-37	-39	2	-0.234249365
27	-11.9	-11.8	0.1	0.011712468
28	-22	-21.2	0.8	0.093699746
29	-36.5	-36	0.5	0.058562341
30	-26.1	-25.2	0.9	0.105412214
31	-14.5	-14.6	0.1	-0.011712468

32	-12	-13.2	1.2	-0.140549619
33	-14.9	-15.3	0.4	-0.046849873
34	-14.6	-16.1	1.5	-0.175687024
35	-11.1	-10	1.1	0.128837151
36	-18.2	-17.6	0.6	0.07027481
37	-33.1	-35.4	2.3	-0.26938677
38	-23.1	-22.7	0.4	0.046849873
39	-35.5	-34.8	0.7	0.081987278
40	-21.2	-18.2	3	0.351374048
41	-30.7	-28	2.7	0.316236643
42	-25.6	-23.4	2.2	0.257674302
43	-34.4	-35	0.6	-0.07027481
44	-35.7	-34.7	1	0.117124683
45	-33.1	-33.8	0.7	-0.081987278
46	-38.8	-38.2	0.6	0.07027481
47	-28.6	-26.7	1.9	0.222536897
48	-41.6	-41.4	0.2	0.023424937
49	-29.7	-29.6	0.1	0.011712468
50	-32.5	-31.9	0.6	0.07027481
51	-16.5	-15.6	0.9	0.105412214
52	-34.2	-34.2	0	0
53	-14.6	-15.8	1.2	-0.140549619
54	-33.8	-34.5	0.7	-0.081987278
55	-26	-28.4	2.4	-0.281099239
56	-44.3	-42.4	1.9	0.222536897
57	-14	-18.3	4.3	-0.503636136
58	-28	-27.4	0.6	0.07027481
59	-26.2	-26.8	0.6	-0.07027481
60	-22.9	-21.8	1.1	0.128837151
61	-11.4	-13.3	1.9	-0.222536897
62	-36.4	-36.8	0.4	-0.046849873
63	-17.4	-15.9	1.5	0.175687024
64	-28.1	-27.2	0.9	0.105412214
65	-30.5	-35.4	4.9	-0.573910945

Figure 3. 29– TMR image of a 23 µm sample showing a lack of contrast

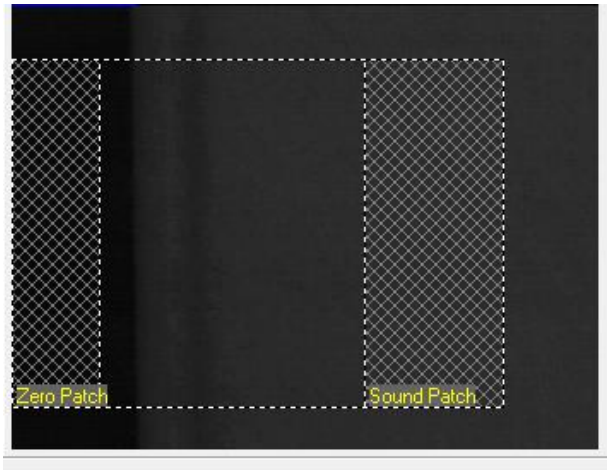


Figure 3. 30– TMR image of a 98 µm sample showing greater contrast

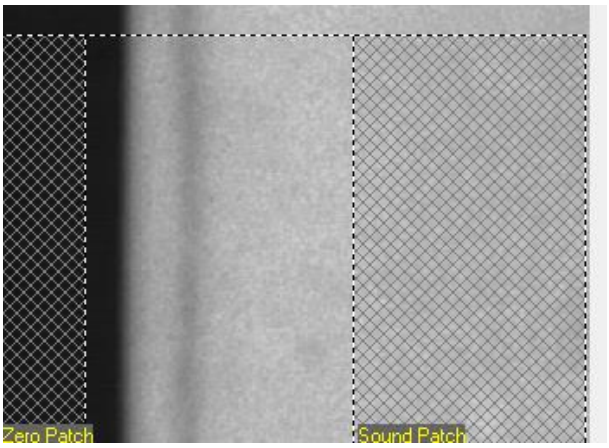


Figure 3. 31-TMR screenshot showing erosion of the lesion

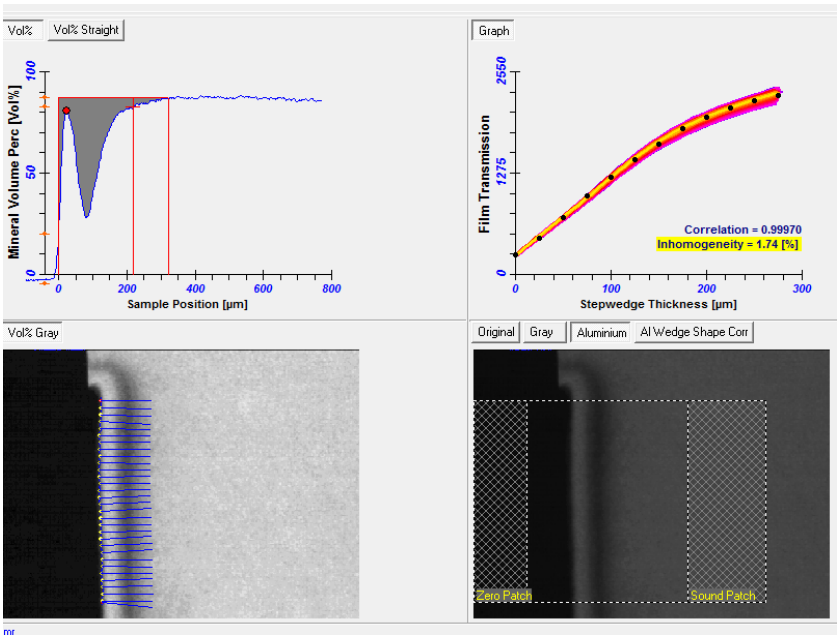


Figure 3. 32- TMR screenshot showing no sign of erosion with a smooth transition from the lesion to sound enamel.

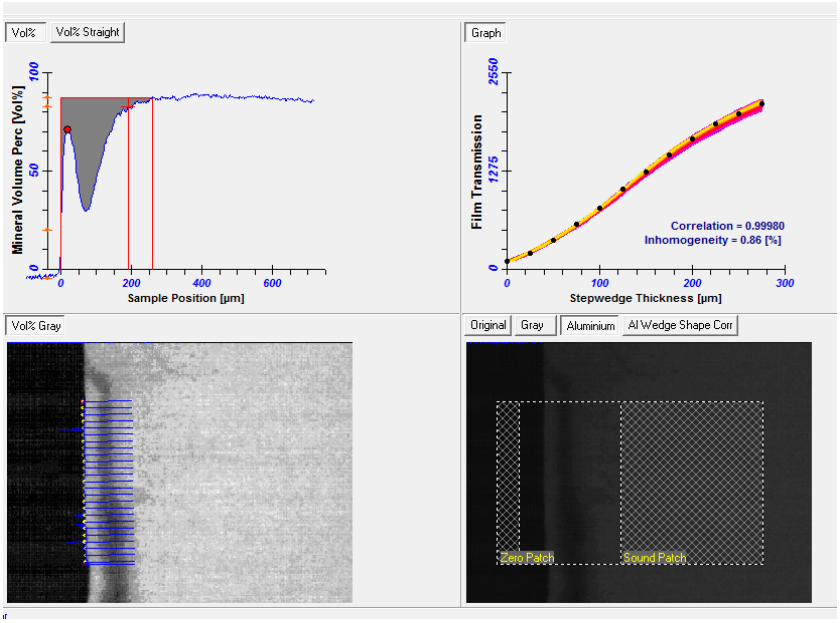


Figure 3. 33– TMR of damage section with only fragments of the remaining

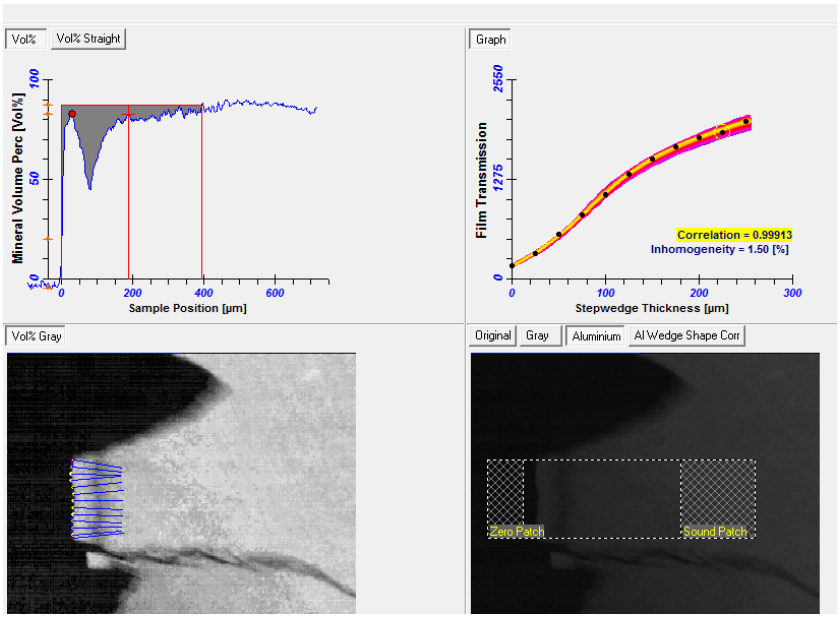


Figure 3. 34– TMR with a break in continuity from the lesion to sound enamel that may be due to erosion or the damage.

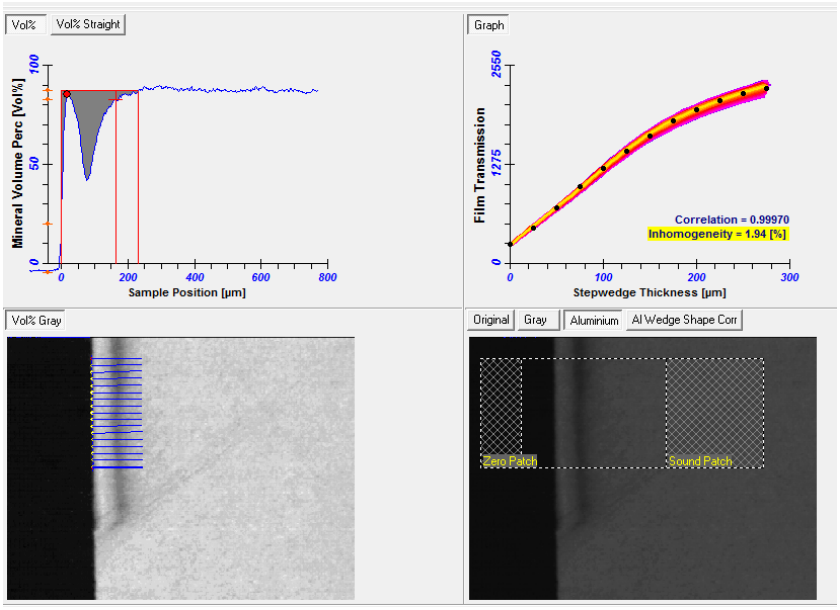


Figure 3. 35– MSI image at week 0 baseline.

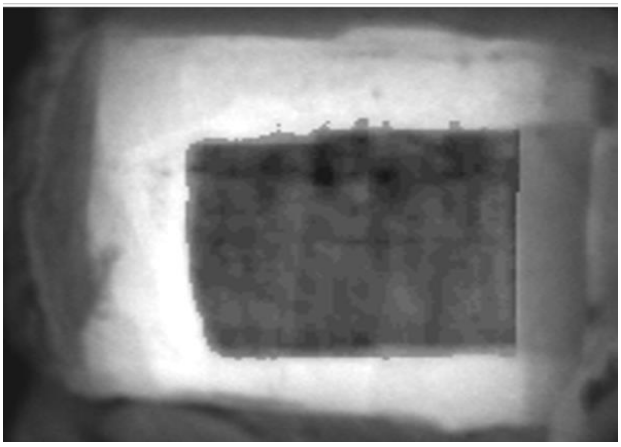


Figure 3. 36- MSI image at week 8 showing increased darkened areas.