Evaluation of the remineralisation of enamel by different formulations and concentrations of fluoride toothpastes *in vitro*.

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Submitted in accordance with the requirements for the degree of Doctor of Clinical Dentistry

The University of Leeds

Leeds Dental Institute

Division of Child Dental Health

September, 2017

The candidate confirms that the work submitted is his own and that appropriate credit has been given where reference has been made to the work of others.

Dedicated to my family

“Like branches on a tree, we may grow in different directions, yet our roots remain as one.”

ACKNOWLEDGEMENTS

I would like to express my sincere appreciation to my supervisors, Professor Jack Toumba, and Dr Marina Malinowski, for their invaluable advice during the study and the preparation of the dissertation.

I am grateful to Dr Simon Strafford for all his guidance and assistance in the laboratory.

I would like to express my gratitude to the staff of the Department of Child Dental Health, and to the many people whose advice and encouragement have helped me reach my goals.

I also need to give the warmest thanks to my wife Sarah, who stood by my side through the good times and the bad, giving me never ending support and inspiration, to my daughters Rayan and Jenan for filling my life with joy and laughter.

Most importantly and above all, I wish to thank my parents for allowing me to realise my own potential. All the support they have provided me over the years, was the greatest gift anyone has ever given me. Without them I would not have gotten to where I am today.

ABSTRACT

Aims:

To investigate the remineralising potential of toothpastes with different formulations of fluoride (F): amine fluoride (AmF), sodium monofluorophosphate (MFP), sodium fluoride (NaF) and stannous fluoride (SnF) on artificial subsurface caries lesions in vitro. A secondary aim was to investigate the remineralising potential of toothpastes containing sodium fluoride (NaF) formulation at different F concentrations (500, 1000, 1450, 2800 and 5000 ppm F) on artificial subsurface caries lesions in vitro.

Materials and methods:

Bovine enamel slabs were subjected to a pH cycling model after 2 weeks of immersion in a demineralisation buffer, to produce subsurface enamel lesions. The pH cycling regime ran for 28 days. Enamel subsurface lesion images were taken using a Quantitative Light-Induced Fluorescence (QLF) system under controlled conditions at baseline and endpoint of the experiment. All fluorescence images were examined with analysing software (QA2 version 1.16; Inspektor Research Systems).

Results:

For the different F compounds, significant ($p < 0.05$) remineralising potential was observed for the NaF, SnF and MFP groups in descending order. Lesion remineralisation for the AmF and F-free groups was not significant. As for the different fluoride concentrations, all fluoride concentrations showed significant ($p < 0.05$) remineralisation

potential when compared to the 0 ppm F control group, but no significance was found between groups.

Conclusions:

From the results of phase A of this *in vitro* study, it was concluded that: A statistically significant remineralisation of enamel subsurface lesions in comparison with the baseline was found in all groups except the **AmF** group. Furthermore, **NaF** toothpaste had the highest remineralising potential on artificial subsurface carious lesions in vitro, followed by **SnF** then **MFP**, while **AmF** was less than the **F-free** toothpaste.

The results of phase B of this *in vitro* study, concluded that: A statistically significant remineralisation of enamel subsurface lesions in comparison with the baseline was found in all groups. However, there was no difference in the effect of toothpastes with sodium fluoride (NaF) formulation and different concentrations (**500**, **1000**, **1450**, **2800**, and **5000 ppm F**) on remineralisation of artificial subsurface carious lesions *in vitro*, and no apparent dose response was present related to the concentration of fluoride.

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Chapter 1 REVIEW OF LITERATURE

1.1 Dental caries

Dental caries is defined as a transmissible disease process that causes localised destruction of susceptible dental hard tissues by acidic by-products from bacterial fermentation of dietary carbohydrates (Featherstone, 2008).

Dental caries is a chronic, reversible, preventable and multifactorial disease that occurs due to microbiological shifts in the plaque biofilm. Dental caries is affected by salivary flow and composition, fluoride exposure, frequency of dietary sugar consumption, and preventative behaviours such as tooth brushing (Selwitz et al., 2007).

1.1.1 Pathogenesis of dental caries

Interaction between acid producing bacteria (mainly *Streptococcus mutans*, *Streptococcus sobrinus* and *Lactobacillus* Spp) and fermentable substrate over a period of time, with the presence of host factors including teeth and saliva leads to an imbalance of the physiological equilibrium between tooth minerals and oral microbial biofilms, which in turn produces dental caries (Scheie and Petersen, 2004).

Acid produced as a by-product of fermentation causes local pH values to fall below the critical value resulting in diffusion of calcium, phosphate and carbonate out of the tooth causing demineralisation (Dawes, 2003, Featherstone, 2008). If pH continues to drop, the balance between demineralisation and remineralisation of tooth tissue tips towards demineralisation and if allowed to continue, cavitation that is clinically obvious will eventually occur (Featherstone, 2008).

1.1.2 Demineralisation and remineralisation

Under physiological conditions, the saliva in the oral cavity is supersaturated with hydroxyapatite and fluoroapatite, and therefore the enamel surface is in a state of dynamic equilibrium with its surrounding environment. The solubility of the enamel apatite on the tooth surface is directly related with the pH of the surrounding medium (saliva). Therefore a drop in salivary pH would result in an increase of apatite solubility (Kidd, 2016). In general, the solubility of apatite increases 10 times with a decrease of 1.0 pH unit. For hydroxyapatite, the critical pH is around 5.5, while it is approximately 4.5 for fluoroapatite (Buzalaf et al., 2011)

At the critical pH, equilibrium exists (no mineral loss or gain). When the pH is over the critical level, mineral precipitation occurs (Remineralisation). On the other hand, when pH is below the critical value, mineral dissolution occurs (Demineralisation) (Buzalaf et al., 2011).

The dynamic process of demineralisation and remineralisation occurs on multiple occasions throughout the day, but as long as there is no net irreversible loss of minerals, the damage to the tooth is reversible (Fejerskov and Kidd, 2008) .

For this reason demineralisation can be defined as “The chemical loss of calcified material from the structure of the tooth, which can be biofilm mediated (caries) or chemically mediated (erosion) from exogenous or endogenous sources of acid (diet, environment, or stomach)” (Longbottom et al., 2009).

On the other hand remineralisation can be defined as “The net gain of calcified material within the tooth structure, replacing that which was previously lost through demineralization” (Longbottom et al., 2009).

1.1.3 Enamel caries (white spot lesion) formation

Enamel is a translucent tissue and is the hardest calcified matrix in the body. Fully formed enamel consists of approximately 96% mineral and 4% organic material and water, and the inorganic content of enamel is a crystalline calcium phosphate (hydroxyapatite) (Nanci and Ten Cate, 2013).

Kidd (2016), explains how incipient enamel lesions develop and progress. Dental carious lesions are a result of an imbalance in physiological equilibrium between tooth mineral and biofilm fluid, and they present as a consequence of biofilm activity. As the pH is lowered in the oral fluids below the critical pH of hydroxyapatite, the hydroxyapatite in saliva is dissolved and drops from supersaturated to saturated. Fluorapatite has a lower critical pH than hydroxyapatite and therefore maintains its integrity, and the plaque saliva maintains fluorapatite at a supersaturated level. When the hydroxyapatite level drops, demineralisation of enamel occurs and a sub-surface enamel carious lesion begins to forms, while fluorapatite continues to be deposited at the surface of the lesion forming an intact surface zone. This surface zone exerts a protective effect, to prevent further dissolution of the of lesion body as long as the pH fluctuations are above the critical pH of fluorapatite.

If the pH drop is constant or maintained for a prolonged period, the dissolution of mineral continues along the naturally occurring enamel rods in a cone shape, with the base of the cone at the enamel surface and the apex pointing toward the dentin-enamel junction, and eventually cavitation will occur (Kidd, 2016).

Enamel caries can be described histopathologically in ground sections as having four distinct zones. The optical properties of each zone reflect different degrees of demineralisation (Gustafon, 1957, Kidd and Fejerskov, 2004, Soames and Southam, 2005):

1. The translucent zone: found at the advancing edge of the lesion, is more porous than normal enamel (1% volume of spaces compared to 0.1% pore volume respectively). Dissolution of minerals occurs mainly at the junctional areas between the prismatic and inter-prismatic enamel.
2. The dark zone: contains 2-4% by volume of pores , some pores are large but others are smaller than the translucent zone indicating that some mineralisation has occurred due to re-precipitation of mineral lost from the translucent zone, this leads to the theory that in rapidly advancing lesions the dark zone is narrower as less remineralisation is occurring (Shellis et al., 2002).
3. The Body of the lesion: has a pore volume of between 5 and 25% and contains apatite crystals larger than those found in normal enamel, suggesting re-precipitation of mineral dissolved from deeper zones. However with continuing pH challenge mineral continues to dissolute from both the periphery and the core. There is an increase in prominence of striae of Retzius in this zone. The explanation for this is unknown.
4. The Surface zone: is approximately 40 µm thick with minimal changes in early lesions. This is because of mineral re-precipitation from both the plaque and from the dissolved deeper zones of the lesion as ions diffuse outwards.

The incipient enamel lesion develops initially as a subsurface translucent zone, which then enlarges and develops a dark zone at it centre. As more mineral is lost, the lesion enlarges and the centre of the dark zone becomes the body of the lesion. At this stage the lesion will be clinically recognisable and will present as a white spot (Murray et al., 2003).

1.1.4 The role of saliva

Saliva is a mixed fluid in the oral cavity in contact with the teeth and oral mucosa. Saliva is composed of more than 99% water, and less than 1% solids (mostly electrolytes and proteins), and is produced by the salivary glands at a rate of 0.5-1.0 Litres per day (Humphrey and Williamson, 2001, Fejerskov and Kidd, 2008)

Saliva has a major role in protection against dental caries. It protects the dentition by clearing it from bacteria and debris, saliva also has a buffering action due its bicarbonate and phosphate ion constituents which help raise the pH after an acidic challenge. Some basic salivary proteins may also contribute to the buffering action of saliva. Saliva also maintains tooth integrity because of its supersaturation with calcium and phosphate and fluoride ions, which when present at the tooth surface increase surface hardness and resistance to demineralisation, and facilitate remineralisation of incipient lesions. Last but not least, saliva has antimicrobial properties due to the presence of immunoglobulin A (IgA) and lysosomes, which help decrease bacterial colonisation of oral tissues (Humphrey and Williamson, 2001, Dodds et al., 2005).

1.2 Fluoride's role in caries prevention and remineralisation

Dental caries is the most prevalent chronic disease, afflicting a significant proportion of the world population, including around 60% to 90% of school-aged children and the vast majority of adults (Marcenes et al., 2013).

Over 70 years ago, fluoride was introduced into dentistry, and it is now recognised as the main factor responsible for the dramatic decline in caries prevalence that has been observed worldwide (Featherstone, 1999, Petersen and Ogawa, 2016).

1.2.1 The Fluoride ion

The Fluoride ion is an inorganic anion of fluorine with the chemical formula F^-. Fluoride is the simplest anion of fluorine. Its salts and minerals are important chemical reagents and industrial chemicals, mainly used in the production of hydrogen fluoride for fluorocarbons. In terms of charge and size, the fluoride ion resembles the hydroxide ion. Fluoride is odourless and tasteless (Wells, 2008).

Fluoride occurs naturally in soil, water, foods, and several minerals, such as fluorapatite and fluorite. Fluoride concentration in seawater averages 1.3 ppm. In the ground water the concentration of fluoride depends on the nature of the rocks and the occurrence of fluoride-bearing minerals. Natural fluoride is seen in high concentrations in well water because fluoride is dissolved from rocks to groundwater (Fawell et al., 2006). Fluorides reach their highest concentration in siliceous rocks, alkaline rocks, geothermal waters, hot springs and volcanic gases (Axelsson, 2004)

It has been estimated that around 60-80% of human intake of fluoride occurs from drinking water and beverages, 6-8% from cereal products and grains, 5-7% from meat, fish and poultry, and 10-14% from all other foods (Axelsson, 2004).

1.2.2 Mechanism of action of fluoride

It has been suggested that fluoride has several caries protective modes of action, including both topical and systemic effects. During tooth development fluoride has a systemic effect that is exerted onto developing enamel that leads to replacement of hydroxyapatite crystals with the more stable and acid resistant fluorapatite crystals (Robinson, 2009). Furthermore fluoride has been found to have an effect on tooth morphology, as teeth that are formed in fluoridated environments tend to be smaller and have shallower pits and fissures than those formed in non-fluoridated environment. The

advantage of this is decreased plaque retention in the shallower pits and fissures. Unfortunately the evidence for this is poor (Lovius and Goose, 1969, Featherstone, 1999).

Research has shown that even though fluoride has a systemic action, its topical action is significantly more important. Inhibition of demineralisation and promotion of remineralisation of enamel are crucial to the caries protection process. When the pH of oral and plaque fluids drop below the critical pH of hydroxyapatite, Fluoride promotes remineralisation of demineralised enamel by substituting hydroxyapatite crystals with fluorapatite. Fluorapatite crystals are larger than hydroxyapatites, more stable and more resistant to acid dissolution as they have a lower critical pH (ten Cate, 1999, Buzalaf et al., 2011).

Although the main action of fluoride is on the prevention of demineralisation and the promotion of remineralisation of hard dental tissues, it has also been proposed that the fluoride ion can affect the physiology of the microbial cell. Fluoride exerts it effects on bacteria by direct inhibition of cellular enzymes, or by enhancing proton permeability of cell membranes in the form of hydrogen fluoride HF (ten Cate, 1999, Marquis et al., 2003, Koo, 2008, Fernandez et al., 2016).

1.2.3 Fluoride toxicity

As is true of virtually all substances to which humans are exposed, including water, oxygen and table salt, exposure to high amounts of fluoride can cause adverse effects. Fluoride toxicity can be either acute which is associated with ingestion of a large amount of fluoride over a short period of time, or it can be chronic which is associated with ingestion of smaller amounts of fluoride but over a prolonged period of time. In either case the signs and symptoms are dose dependant (Whitford, 2011).

1.2.3.1 Acute Fluoride Toxicity

Historically, there have been many cases and reports of accidental fluoride poisoning. From those reports, researchers have been able to determine the signs and symptoms of acute fluoride toxicity, and estimate the doses of fluoride ingestion that may cause serious toxicity (Lidbeck et al., 1943, Hodge and Smith, 1965, Eichler et al., 1982, McIvor et al., 1983).

Following ingestion of al large amount of fluoride, the first organ to be affected is the stomach. Symptoms of acute fluoride toxicity are nausea, bloody or normal vomiting, diarrhoea and fatigue. This will be followed by general collapse accompanied by pallor, weakness, shallow breathing, weak heart sounds, wet cold skin, cyanosis and equally dilated pupils. Death may occur within 2-4 hours, but if delayed for up to 20 hours, muscle paralysis, carpopedal spasm and spasms of extremities occur. This is associated with electrolyte imbalance, particularly severe hypocalcaemia and hyperkalaemia (Whitford, 2011).

Based on the reports of the mass poisoning of the Oregon state hospital, where about 10 gallons of scrambled eggs were mistakenly prepared with 17 pounds of sodium fluoride instead of powdered milk, causing 263 cases of acute poisoning of which 47 were fatal (Lidbeck et al., 1943), Hodge and Smith (1965) estimated that the certainly lethal dose was between 32 and 64 mg/kg sodium fluoride.

The potentially toxic dose, which is “The minimum dose that could cause serious life threatening systemic signs and symptoms and that should trigger immediate therapeutic intervention and hospitalisation”, was estimated to be 5.0 mg/kg (Dukes, 1977).

The immediate treatment of acute fluoride toxicity should be aimed at reducing the amount of fluoride available for absorption from the gastrointestinal tract. Vomiting

should be induced if the patient is conscious, and has a gag reflex. If the patient is unconscious avoid induction of vomiting to prevent aspiration into the lungs. Because of the strong affinity of calcium for fluoride absorption can be slowed by oral administration of 1% calcium chloride or calcium gluconate or, if these solutions are unavailable, as much milk as the patient can tolerate. The hospital emergency department should be informed, and the patient transported as soon as possible. A gastric lavage may be performed with a solution containing calcium or activated charcoal, and blood samples should be obtained to check for hypocalcaemia and hyperkalaemia (Whitford, 2011)

1.2.3.2 Chronic Fluoride Toxicity

Excess ingestion of fluoride over a prolonged period of time, can cause dental fluorosis, skeletal fluorosis and kidney damage. Fluoride is incorporated into the forming apatite crystals of both bone and teeth as they act as a reservoir for fluoride. The severity of dental fluorosis is related to the concentration of fluoride in the plasma, the stage of crown formation, and the duration of exposure to fluoride (Dean et al., 1950, Denbesten and Li, 2011).

1.2.4 Oral Fluoride Reservoirs

Teeth, saliva, oral mucosa and dental plaque fluids all act as reservoirs for the fluoride ion in the oral cavity. Of those previously mentioned, plaque fluids and saliva are the most important fluoride reservoirs due to their close association with the tooth surface. Oral fluoride reservoirs can be broadly classified into two broad types, both of which involve calcium (Ca) (Vogel, 2011):

1. The mineral deposits of fluoride, which include calcium fluoride (CaF_2) and fluorapatite (FAp)
2. The biologically or bacterially bound calcium fluoride deposits (Ca-F)

The importance of maintaining a caroiostatic concentration of fluoride in the oral fluids has been emphasized in current research to facilitate anti caries effects of fluoride. Fluoride present in solution at low levels amongst the enamel crystals can markedly inhibit dissolution of tooth mineral by acid (Featherstone, 1999). Salivary fluoride levels as low as 0.01-0.10 ppm have been shown to be effective in the prevention of the enamel dissolution (Hellwig and Lennon, 2004).

Multiple systematic reviews and studies have shown that the principle action of fluoride is through its topical rather than its systemic effect (Marinho et al., 2003, Twetman et al., 2003, Twetman, 2009). Steady presence of fluoride at low levels (sub-ppm) in the plaque enamel interface during acid insult will inhibit demineralisation, and when PH is restored traces of fluoride in solution will speed up the remineralisation process (Buzalaf et al., 2011). This indicates that the frequency of application and constant availability of fluoride is more crucial than the quantity of fluoride administered.

1.3 Toothpastes

Over the years, toothpaste has evolved, developed, taken many forms and contained multiple constituents. It started out as tooth powder containing crushed egg shells and ashes invented by the ancient Egyptians (3000-5000 BC), and continued to be modified by different cultures and civilisations until it reached its modern structure and appearance (Lippert, 2013).

Nowadays toothpastes are a mixture of abrasive suspended in an aqueous humectant phase by means of a hydrocolloid. In this matrix, surfactants, active (therapeutic) ingredients, preservatives colourings, sweeteners, flavour compounds and other ingredients are embedded (Lippert, 2013). Toothpastes have become accepted

worldwide as the dominant vehicle of topical fluoride delivery, for caries preventative means (Zero, 2006, Walsh et al., 2010).

1.3.1 Toothpaste excipients (Vranic et al., 2004, Davies et al., 2010, Yavnai, 2010, Lippert, 2013):

1.3.1.1 Abrasives:

Abrasives are substances that clean and polish the tooth surfaces. The most commonly used abrasives are calcium carbonate, alumina and dicalcium phosphate. Calcium phosphate and alumina are cheap ingredients but cannot be used with sodium fluoride as they will have unfavourable reaction with the free fluoride ions forming calcium fluoride. However, dicalcium phosphate can be formulated with either sodium phosphate or sodium monofluorophosphate.

Nowadays, most toothpastes contain silica, which is more expensive but can be combined with many fluoride salts. Its use has enabled adding sparkles and colouring agents to toothpastes. The concentration of silica varies between 10-20%.

1.3.1.2 Surfactants:

Surfactants are not only responsible for the foaming action of toothpastes, but they also aid in their intraoral dispersion. The most widely used agent is sodium lauryl sulphate, which is usually included at a concentration between 0.5 - 2.0%.

1.3.1.3 Viscosity and rheology modifiers:

Their primary function is to produce a gel phase containing a homogenous distribution of all ingredients, and to prevent components from separating during long periods of storage. Furthermore they are responsible for easy flow and clear break rather than stringy appearance when applied to a toothbrush. The most common are

carboxymethylcellulose, xanathan gum and cellulose gum at conc. ranging from 0.5-2.0%.

1.3.1.4 Humectants:

Used to avoid water separation and evaporation (drying of toothpaste), and to provide smooth glossy appearance. Glycerine and sorbitol are most commonly used due to their compatibility with other materials and their raw material cost.

1.3.1.5 Flavours and sweeteners:

Added primarily for palatable reasons, they mask the often unpleasant taste of surfactants, provide breath freshening and sensorial cues such as cooling, heating or tingling. Flavours are the most expensive and most volatile excipient and can be used at concentrations below 0.5%. All commonly used sweeteners are artificial, and the majority of toothpaste manufactures utilize either sodium saccharine or sucralose. Xylitol can also be considered a sweetener, although its main and still discussed purpose is caries prevention.

1.3.1.6 Fluoride (Active ingredient):

Of the many active ingredients found in different toothpastes Fluoride is by far the most important in caries prevention and enamel remineralisation. According to the EAPD policy document on the use of fluoride in children ‘’ The extensive use of fluoridated tooth pastes has probably been one of the major reasons for the dramatic reduction in dental caries recorded over the past 30 years.’’ (European Academy of Paediatric Dentistry, 2009).

1.3.2 Fluoride reservoirs in toothpastes

In toothpastes there are distinct fluoride reservoirs: ionic fluoride which is readily available in aqueous solutions; ionisable fluorides like MFP which will release ionic fluoride in the mouth upon action of oral phosphates; and insoluble fluoride formed by undesirable reaction of fluoride with calcium based abrasives, such as dicalcium phosphate dehydrate ($CaH_2PO_4.2H_2O$) and calcium carbonate ($CaCO_3$) (Tenuta and Cury, 2013). To avoid these unwanted reactions, silica can be used as an abrasive material instead of the calcium based abrasives, although this will render the product more expensive to manufacture. Another way to elude the reaction of fluoride with calcium is by using sodium MFP/$CaCO_3$ however it may hydrolyse with time, perhaps resulting in a less soluble fluoride (Tenuta and Cury, 2013).

A key factor in the cariostatic efficiency of fluoride toothpaste is the ability to form calcium fluoride-like globules when contacting the enamel or dentine surface (ten Cate, 1997, Featherstone, 1999). This desirable reaction is not to be confused with the undesirable reaction of fluoride with calcium based abrasives in the tube. During the time between fluoride exposures fluoride is slowly released from the compound into the oral fluids, maintaining a steady level of fluoride protection (Petzold, 2001). Petzold demonstrated the different calcium fluoride deposition rates between multiple fluoride formulations, (AmF, NaF, and MFP) showing that AmF had the highest and fasted accumulation rate, followed by NaF, then NaMFP. The result of this study was in agreement with previous investigations on, calcium fluoride deposition (Cruz et al., 1992)

1.3.3 Different Fluoride Formulations in toothpaste

Fluoride bioavailability in the oral fluids in the form of the $\mathbf{F^-}$ ion is essential for it to be effective in the demineralisation and remineralisation process. It can be delivered from toothpastes in different fluoride formulations. Different fluoride formulations differ in their

chemical structures, which in turn has implications on the mode of action for each formulation. According to Axelsson (2004) the three main categories are:

1. Inorganic compounds: including sodium fluoride (NaF) and stannous fluoride (SnF). These are readily soluble salts that provide free fluoride
2. Monofluorophosphate containing compounds: such as sodium monofluorophosphate (MFP). The Fluoride is covalently bound to phosphate ions, and requires hydrolysis to release the fluoride ions
3. Organic fluorides: such as amine fluoride (AmF). Fluoride is bound to organic compounds

Sodium fluoride (NaF):

Sodium fluoride is an inorganic compound, and is by far the most commonly used fluoride formulation in topical fluoride agents both for self-application and professional use. When in solution, NaF salt readily releases fluoride into saliva, dental plaque, pellicle and enamel crystallites. NaF is widely used in many fluoride vehicles including dentifrices, mouth rinses, chewing gums, solutions, gels, varnishes, prophylaxis pastes and slow release devices (Axelsson, 2004, Fejerskov and Kidd, 2008, Pessan et al., 2011).

Stannous fluoride (SnF):

SnF is an inorganic compound that releases both $\mathbf{F^-}$ which have both cariostatic and antibacterial properties, and $\mathbf{Sn^{+2}}$ ions which have antimicrobial properties into the oral environment. Tooth staining and instability are the main disadvantages of this formulation. SnF is used in dentifrices, mouth rinses, solutions, gels, and prophylaxis pastes (Axelsson, 2004, Fejerskov and Kidd, 2008, Pessan et al., 2011).

Sodium monofluorophosphate (Na_2FPO_3)(NaMFP):

NaMFP is a monofluorophosphate containing compound, that can be used in both neutral and acidic vehicles. Fluoride is covalently bound in Na_2FPO_3 and requires hydrolysis in order to release fluoride ions. The NaMFP containing products provide FPO_3 (MFP) ions together with some free F^- ions, both which can diffuse through the plaque and into enamel. This provides a source of the free F^- ion prior to hydrolysis. FPO_3 can be hydrolysed in plaque. Under acidic conditions, FPO_3 can also be hydrolysed at the surface of apatite crystals, providing phosphate and fluoride ions (Axelsson, 2004).One of the main advantages of NaMFP is its compatibility with calcium based abrasives as FPO_3 requires hydrolysis prior to release of free F^- therefore avoiding the production of insoluble fluoride formed by undesirable reaction of fluoride with calcium based abrasives, such as dicalcium phosphate dehydrate ($CaH_2PO_4.2H_2O$) and calcium carbonate ($CaCO_3$) (Tenuta and Cury, 2013). NaMFP can is used in dentifrices (at neutral pH) and gels (both acidic and neutral pH) (Axelsson, 2004, Fejerskov and Kidd, 2008, Pessan et al., 2011).

Amine fluoride (AmF):

AmF is an organic fluoride compound, that readily provides free fluoride. Its enhanced caries protective action has been attributed to the greater affinity of hydrophilic counter-ions to the enamel, which will reduce the surface energy and thereby the plaque adhesiveness of enamel . In addition, AmF provides a complexed store of fluoride ions and may enhance diffusion through carious enamel (Axelsson, 2004, Fejerskov and Kidd, 2008, Pessan et al., 2011)

In light of the multiple available fluoride formulations, a question comes to mind. What optimum fluoride formulation gives the highest extent of remineralisation of a

demineralised enamel lesion? The answer has been a matter of heated debate over the years. There have been a vast number of studies comparing the anti-caries effects between different fluoride compounds in dentifrices with the majority of them comparing only between two compounds, and only a handful comparing all the main fluoride compounds in dentifrices (Toda and Featherstone, 2008).

1.3.3.1 Studies reporting no difference between fluoride formulations

A Cochrane review by Marinho et al. (2003) to determine the effectiveness and safety of fluoride toothpastes in the prevention of caries in children, compared toothpastes containing MFP (22 trials), SnF (19 trials), NaF (10 trials) and AmF (5 trials) and did not find an link between the type of fluoride compound in the dentifrice and the magnitude of treatment effect. In spite of their findings the authors considered their results to be less reliable than evidence from head to head comparisons (Pessan et al., 2011).

A meta-analysis of clinical studies comparing the anti-caries protection of NaF against NaMFP had come to the same conclusion a few years before it (Proskin, 1993), these results are harmonious with other pieces of literature: (DePaola et al., 1993, Volpe et al., 1995, Saporito et al., 2000).

Furthermore some animal and in vitro studies have also concluded that both NaF and AmF have the same caries reducing (prevent demineralisation and promote remineralisation) abilities (Warrick et al., 1999, Holler et al., 2002, Toda and Featherstone, 2008).

1.3.3.2 Studies reporting NaF Superiority

Stookey et al. (1993) published a meta-analysis of clinical studies that gave inconsistent results with the studies mentioned previously. Results of the analysis demonstrated that NaF was significantly more effective than MFP in preventing caries by 5-20%. Based

upon their clinical findings the authors recommend that NaF be used as the active system in fluoridated dentifrices whenever practically feasible.

An in vitro pH cycling model demonstrated that NaF was statistically better at enamel remineralisation than MFP when lesions were assessed by cross sectional micro hardness (Toda and Featherstone, 2008). As with most reports claiming the superiority of NaF against MFP, their claims were built on the assumption that fluoride only exerts its effects on demineralisation and remineralisation as a free ion. Unlike NaF (which releases free F^-), fluoride in MFP formulation is covalently bound to phosphate and requires enzymatic hydrolysis to release free F^- (Pessan et al., 2011).

In a recent in vitro study evaluating remineralisation of carious lesions and fluoride uptake by enamel exposed to various fluoride dentifrices, it was found that enamel remineralisation and fluoride uptake was significantly greater when using NaF compared to MFP. It was also concluded that efficacy of the fluoride dentifrice was dependant on ionic fluoride levels (Hattab, 2013).

1.3.3.3 Studies reporting AmF superiority

Adding to the controversy, more inconsistent results have been published elsewhere in the literature. A recent in vitro study by Patil and Anegundi (2014), evaluated the remineralisation, and fluoride uptake by tooth enamel from four different fluoride dentifrices (Naf, MFP, SnF, and AmF). Results from the study indicated that enamel treated with amine fluoride had the highest fluoride uptake. These results were consistent with other in vitro studies (Arnold et al., 2006, Chan et al., 1991, Klimek, 1998, Cate, 2008, Altenburger et al., 2010), in situ studies (Buchalla et al., 2002), clinical studies (Cahen et al., 1982) and a review of Hungarian studies on AmF (Madlena, 2013).

In Madlena's review, it was concluded that the use of products containing, AmF and SnF_2 resulted in beneficial clinical effects on development of carious lesions. Amine fluoride is an organic fluoride unlike the other inorganic fluoride compounds used in the previous studies. The reason behind AmF's high uptake into enamel is the result of high distribution of organic material in enamel after its demineralisation, and that the cations in AmF have a hydrophilic and hydrophobic part (as in other surfactants). The hydrophobic part is aligned towards the oral cavity away from the tooth surface and the hydrophilic part, containing the fluoride ions towards the enamel or dentin surface. This causes the fluoride ions to accumulate close to the tooth surface facilitating the production of calcium fluoride as a labile reservoir or for immediate remineralisation. The surface layer of calcium fluoride is stabilised by the hydrophobic part of the molecule pointing towards the oral cavity, which reduces moistening by saliva and prevents it being washed out rapidly. Other fluoride compounds mentioned previously are inorganic fluorides which are lost by way of ionic exchange in saliva (Patil and Anegundi, 2014, Madlena, 2013).

The pH of AmF containing dentifrices also has a direct effect on its ability to remineralise enamel. Slightly acidified AmF containing dentifrices may have a positive effect on enamel remineralisation (Arnold et al., 2007). This phenomenon may be explained by the inverse relationship between calcium fluoride and pH, and also by increased plaque fluoride uptake in low pH environment when compared to neutral formulations (Pessan et al., 2011).

1.3.4 Different Fluoride Concentrations

The association between the concentration of fluoride in toothpaste and its clinical effectiveness has long been debated in the literature. Multiple studies have compared

high concentration fluoride versus low concentration fluoride toothpastes (Walsh et al., 2010) .

A randomised controlled trial conducted by Davies et al. (2002) assessed the impact of providing free fluoride toothpaste containing either 450ppm fluoride or 1450ppm fluoride on the level of caries in the deciduous dentition. The toothpaste was provided at 3 monthly intervals from the age of 12 months until 5-6 years. The results indicated a statistically significant difference of the dmft (16% reduction) between the 1450ppm and the control group. Though, the difference between the 440ppm and the control group was not statistically significant.

Biesbrock et al. (2003a) conducted a randomised double-blind study to assess the anti-caries effectiveness of placebo, 500ppm fluoride and 1450ppm fluoride dentifrices. The 657 subjects were randomly assigned to the 3 different groups for the first 9 months of the study. Subjects of the placebo group were then assigned to the 500ppm or 1450ppm fluoride groups for the rest of the study time, whilst all other subjects continued with their original treatment assignments.

The results of the study differed from those of Davies et al. (2002) in that both 500ppm and 1450ppm fluoride toothpastes delivered statistically significant lower dmft scores than the placebo toothpaste at 9 months and the same significant result was found for the 500ppm and the 1450ppm fluoride dentifrice when compared to placebo/500ppm and placebo/1450ppm fluoride at 21 months.

In October of the same year Biesbrock et al. (2003b) published another randomised double-blind study to assess anti-caries effectiveness of placebo, 1100 ppm sodium fluoride and 2800 ppm sodium fluoride dentifrices. The 644 subjects were randomly assigned into three groups as in the previous trial. Results were consistent with the previous study, as both 1100 and 2800 ppm fluoride showed a statistically significant

lower DMFS score when compared with placebo. Furthermore 2800 ppm group presented with a statistically significant lower DMFS score at 21 months indicating a dose response to fluoride.

Twetman et al. (2003) carried out a systematic review of the literature between the years 1966-2003 and found limited evidence for an anti-caries difference between low-fluoride (<1000ppm) and standard fluoride (1000-1100ppm) toothpastes in the young permanent dentition. Strong evidence was reported for the superior preventive effect of toothpastes with 1500ppm of fluoride compared with standard ones with 1000ppm fluoride when used daily during the young permanent dentition. An update of the systematic review (Twetman, 2009), produced results that reinforced the original findings.

A meta-analysis by Steiner et al. (2004) comparing the effect of 1000 ppm to the effect of 250 ppm fluoride toothpaste found a 13-14% reduction in caries increments for the group using 1000 ppm fluoride toothpaste. These results were in line with the systematic reviews published by (Twetman et al., 2003, Twetman, 2009).

A randomised controlled trial by Lima et al. (2008), evaluated the effect of low-fluoride dentifrice on children with different caries experience. One hundred and twenty 2 to 4 year old children, half with and half without active caries lesions were randomly divided into two groups which used either 500ppm or 1100ppm dentifrices. The results pointed out that the anti-caries effect of the 500ppm dentifrice was similar to the 1100ppm when used by caries inactive children. Though, 1100ppm toothpaste was more effective than 500ppm for caries active young patients. One of the major shortcomings of this clinical trial was that there was insufficient follow up time (one year) allowed to assess caries progression or arrestment.

A Cochrane review by Walsh et al. (2010) that included 75 studies, indicates that the caries preventive effect of fluoride toothpaste increases significantly with higher fluoride

concentrations. When compared to placebo. Concentrations of 440,500,550ppm fluoride and below show no statistically significant effect, but a statistically significant effect is evident for 1000,1055,1100,1250ppm fluoride concentrations (prevented fraction: 25%) and for highest fluoride concentrations (prevented fraction: 45%). For the active interventions, only the prevented fraction for comparisons of 250ppm with 2400,2500,2800ppm and 1000,1055,1100,1250ppm with 2400,2500,2800ppm attain statistical significance. The authors concluded that only fluoride toothpaste at a concentration of 1000 ppm fluoride and above is efficient at preventing dental caries.

1.4 Model systems used in coronal caries research

A well conducted randomised controlled clinical trial is considered the gold standard model used to study the caries process and progression in enamel (Scottish Intercollegiate Guideline Network, 2017). However conducting a randomised controlled trial is both costly and requires a prolonged period of time. Therefore different models that can mimic the oral environment have been developed and adopted.

1.4.1 *In vitro* model

Prior knowledge of the *in vivo* situation is required in order to set up an *in vitro* model. *In vitro* caries models in general have been adopted due to their ability to help us understand the complex process of caries development and prevention. They help us accurately predict a clinical outcome in a controlled and simplified way. Even though a model cannot capture all the details involved with caries formation, it can give us a means of performing reproducible experiments under controlled conditions (Buzalaf et al., 2010).

In vitro or laboratory models are the most commonly used systems in caries research. They have several advantages including (Xuedong, 2016):

1. Lower cost, and can be produced more rapidly.
2. The possibility of carrying out single variable experiments under highly controlled conditions, which are more sensitive and precise.
3. Best approach to screen a large number of agents to determine their modes of action.
4. The ability to bypass ethical issues that are associated with *in vivo* models (Salli and Ouwehand, 2015).

However in vitro models also have limitations which include:

1. Inability to simulate the complex biological processes associated with caries (Xuedong, 2016).
2. Inability to replicate and reproduce precisely the conditions of the oral environment (Higham et al., 2005).

1.4.1.1 pH cycling model

In vitro pH cycling models mimic the dynamics of mineral loss or gain involved in the caries process, and the have been used widely to evaluate the efficacy of fluoridated toothpastes on caries control (Buzalaf et al., 2010). The origin of the modern pH cycling models was produced by ten Cate and Duijsters (1982).

Stookey et al. (2011) published a study that looked at the robustness, and the ability to predict the anti-caries performance of fluoride containing products by using the *in vitro pH* cycling model. He compared data from three independent laboratories and concluded that the *in vitro* pH cycling model:

1. Was capable of measuring the dose response from 0-1100 ppm F.
2. The model was able to statistically separate positive from negative control.

3. Dentifrice formulations proven to be clinically effective against caries, performed in this model at a level that was not statistically less effective than the positive control.
4. This model was able to statistically differentiate between a product with attenuated fluoride activity (product formulated with the same level of fluoride as the positive control in addition to an ingredient known to compromise fluoride effectiveness) from the positive control.

For the reasons mentioned above, in vitro pH cycling continues to be an effective tool in evaluating the efficacy of fluoridated toothpastes on caries control.

1.4.1.2 Dental substrates that can be used in the pH cycling model

Human teeth can be regarded as the most appropriate source of dental substrate to be used in pH cycling models in terms of clinical relevance. However, their composition is variable, due to genetic influences, environmental conditions and age. These differences lead to large variations in their response under acidic challenges (Buzalaf et al., 2010). Furthermore, sources of human teeth are becoming more and more limited and there is a significant increase in difficulty of obtaining human teeth for research purposes (Stookey et al., 2011).

Bovine teeth are easier to obtain, have a more uniform composition when compared to human teeth, and have been generally demonstrated to perform similarly to human teeth (Tanaka et al., 2008, Costa et al., 2015). For this reason, bovine enamel can offer a suitable alternative to human enamel for in vitro pH cycling models, and they provide a less variable response to both cariogenic challenge and anti-caries treatment such as fluoridated dentifrices (Mellberg, 1992, ten Cate and Mundorff-Shrestha, 1995). However, due to slight differences between bovine and human enamel in terms of mineral content and porosity (Edmunds et al., 1988), Stookey et al. (2011) has found that

slight adjustment and increase of demineralisation pH was necessary to achieve similar results. Although bovine enamel is more porous than human enamel, which leads to faster demineralisation and remineralisation, these differences result in quantitative and not qualitative differences in behavior (Buzalaf et al., 2010).

Artificial caries lesions produced from bovine teeth, have a mineral distribution and structure that resembles lesions produced from human teeth for both enamel and dentin (Featherstone and Mellberg, 1981, Mellberg, 1992).

1.4.1.3 Characteristics of the artificial caries lesion used in the pH cycling model

Different models for pH cycling have unique protocols for producing artificial caries lesions. Methods include immersion enamel substrates in buffered lactate or acetate gels, or the use of solutions under saturated in respect to apatite, with a pH ranging between 4.4 and 5.0, for a time ranging between 16 h and 28 days (Buzalaf et al., 2010). Lesions formed will depend on the protocol used and they include surface softened erosion like lesions or, subsurface caries like lesions. For caries research, subsurface enamel lesions are required to demonstrate the remineralisation potential of different treatments on the demineralised enamel (Buzalaf et al., 2010).

1.4.2 Animal caries models

Animal caries models are invaluable tools to simulate the natural progression of caries under biological conditions, and they have a long history of successful use in caries research. Controlled conditions can be created with the use of this model by manipulating oral microflora, and providing a specific diet. Furthermore, unlike *in vitro* or *in situ* models, which measure isolated components of the caries process, animal caries models truly measure caries (Stookey et al., 1995).

There are many similarities between carious lesions developed in rat models, and caries developed in human models which include; cariogenic microorganisms, the presence of fermentable carbohydrates in the diet, the demineralization pattern of the enamel, and the responsiveness to fluoride. However, the morphology of the teeth and the eating habits differ. Furthermore, the oral and plaque microflora are dissimilar and therefore this model has faced protest over the years (Stookey et al., 1995).

1.4.3 *In situ* model

In situ caries models involve the use of appliances or devices in the human mouth to simulate the natural process of dental caries. These models attempt to provide clinically relevant information in a relatively short period without causing irreversible tissue changes in the natural dentition. The advantages of in situ caries model systems compared with clinical trials include (Higham et al., 2005):

1. Fewer ethical and logistical problems.
2. Lower cost and results are obtained in a shorter time.
3. The experimental design can be more flexible, allowing the hypothesis to be tested.
4. The data is highly reproducible and recoverable from archives.

When comparing this model with the *in vitro* model, *In situ* caries model are also influenced by dietary eating habits, the presence of human saliva, plaque of varying composition and thickness, and a pellicle-coated tooth surface. All these factors make the test conditions more similar to the oral environment . However, The validation of these studies rely heavily on the compliance of the test subjects (Zero, 1995).

1.5 Methods used in Demineralisation and Remineralisation Evaluation

Multiple techniques are available for measuring mineral loss or gain during enamel demineralisation and remineralisation. Techniques available can either be destructive or non-destructive to enamel.

1.5.1 Quantitative Light-Induced Fluorescence (QLF)

The phenomenon of tooth fluorescence has long since been suggested as a useful tool for the detection of dental caries (Benedict, 1929).

Quantitative light induced fluorescence (QLF) is based on the principle that excitation of dentin with blue light (370 nm) causes it to fluoresce in the yellow-green region. By using a high pass filter ($\lambda \geq 540$ nm) to cut out the excitation light, this fluorescence can be observed (Neuhaus et al., 2009). When a subsurface enamel lesion that is occupied by water is present, an increase in light scattering can be observed relative to the surrounding enamel producing two important effects (De Josselin et al., 1995, Neuhaus et al., 2009):

1. Less excitation light reaches the dentin so that less fluorescence is produced underneath the lesion
2. Fluorescence that occurs is scattered through the lesion so that less light is observed.

De Josselin et al. (1995) developed a technique based on this optical phenomenon that was able to quantify the difference in fluorescence between intact and demineralised tooth structure. The currently marketed systems (Inspektor Research Systems BV, Amsterdam, The Netherlands) provide three quantitative metrics:

1. ΔF: Percentage fluorescence loss with respect to the fluorescence of sound tooth tissue; related to lesion depth (%),
2. ΔQ: The ΔF times the Area. Percentage fluorescence loss with respect to the fluorescence of sound tissue times the area. Related to lesion volume (% px2),
3. Area: The surface area of the lesion expressed in pixels2 (px2).

The QLF system has been tested in several *in vitro* studies. Hafstrom-Bjorkman et al. (1992) compared light induced fluorescence with longitudinal microradiography, and found results that indicated a lower discrimination threshold for laser fluorescence compared to longitudinal microradiography. His results were further reinforced by Emami et al. (1996), who found that there was a high correlation coefficient (r=0.73) light induced fluorescence and longitudinal microradiography, and concluded that light induced fluorescence is sensitive and valid method for quantification of mineral loss in enamel caries lesions. Al-Khateeb et al. (1997b) validated the QLF device against microradiographic and chemical analysis for the assessment of mineral changes in enamel.

Pretty et al. (2002b), looked at the intra- and inter-examiner reliability of QLF analysis. The authors concluded that the analysis stage of QLF is reliable between examiners and within multiple attempts by the same examiner, when analysing in vitro lesions.

The QLF method has also displayed similar results when used *in situ* studies. Al-Khateeb et al. (1997a) concluded that with the sensitive laser fluorescence method, it was possible to register the small changes in the enamel week by week during in situ remineralisation. he also found a highly significant linear dependence ($p < 0.001$) between the final measurements obtained with the laser fluorescence method and the data obtained from transverse microhardness, $r = 0.76$ which is considered to be the gold standard for the measurement of smooth surface enamel mineralisation.

Tranaeus et al. (2002) tested *In vivo* the repeatability and reproducibility of the QLF method with respect to three variables: lesion area, and average and maximum changes in lesion fluorescence. For the image-capturing stage, inter-examiner reliability showed an intra-class correlation coefficient (r) between 0.95 and 0.98. For the analytical stage, intra-examiner reliability for all three analysts showed a value of r between 0.93 and 0.99. Inter-examiner reliability showed a value of r between 0.95 and 0.99. The authors concluded that the in vivo repeatability and reproducibility of the QLF method were excellent.

One of the major limitations of the QLF method is that QLF readings can be affected with multiple factors, which include the presence of plaque, calculus and/or staining. The degree of dehydration of the tooth surface may also impact the readings obtained (Al-Khateeb et al., 2002). Therefore, In order to achieve reliable results, the application of compressed air for 15 seconds prior to the QLF imaging is suggested (Pretty et al., 2004).

Another limitation for QLF is that manufacturer recommendations include taking images in a dark environment. This may be suitable for *in vitro* uses but when used *in vivo* this would be impractical. Pretty et al. (2002a) found that a light level of 88 lux could be employed in areas where QLF is to be used without significantly affecting the reported values.

1.5.2 Indentation techniques

Indentation techniques include both micro-hardness (George et al., 2015) and nano-indentation techniques (Bertassoni et al., 2010). These methods have been used to measure the hardness of the tooth tissue using a diamond tip with known dimensions. The diamond tip is pressed against the tissue surface with a predetermined load (25-50 g) and duration, and a measurement of the resistance of tooth tissue to the diamond tip

is given. Data is then produced in arbitrary units, usually Knoop hardness number or Vickers hardness number (George et al., 2015).

The main criticism for this technique is that hardness increase is not identical to remineralisation, and that surface micro-harness measurements do not provide insight into mineral loss or gain nor do they detect redistribution of minerals within lesions following exposure to remineralising agents (Zero, 1995). However, surface micro-hardness has been found to be a highly sensitive and reproducible method for studying the very early stages of enamel demineralisation and remineralisation (Zero, 1995).

1.5.3 Transverse microradiography (TMR)

'Transverse microradiography (TMR) can be regarded as the gold standard for the evaluation of mineral distribution in cariology research.' (Buzalaf et al., 2010). TMR or contact microradiography can be used to measure the morphology of and the change in mineral content of dental hard tissue. Furthermore it provides a quantitative measurement of the amount of mineral, lesion depth and surface layer thickness (Clasen and Ogaard, 1999).

The limitation of this technique is that it is destructive to tooth tissue, and therefore studies looking at longitudinal mineral changes in the same lesions, cannot utilise this method (Nakata et al., 2012).

To prepare samples for TMR investigation, thin slices approximately 80 µm for enamel samples are cut perpendicular to the enamel surface. Radiographic exposure of the sample alongside a calibration aluminium wedge, using high resolution film produces a microradiographic image. The mineral can be automatically calculated from the grey levels of the image compared to the step wedge. ΔZ is the parameter of interest and it reflects the amount of mineral lost (White et al., 1992).

The main advantages of this technique are accuracy of determination of mineral loss or gain and the ability to detect mineral distribution in the lesion (Arends and ten Bosch, 1992).

1.5.4 Microcomputed tomography (Micro-CT)

A recently developed and promising method for assessment of demineralisation and remineralisation is microcomputed tomography (Micro-CT). Advantages of this technique are numerous and include Precise measurements and greater sensitivity to changes in mineral with time and position. More importantly, this method is non-destructive to tooth tissue and can be used to measure longitudinal changes in mineral content of dental tissues. Micro-CT also allows complementary analyses of fluoride, calcium and phosphorus present in the enamel (Buzalaf et al., 2010).

1.6 Research aims and hypotheses

1.6.1 Aims:

1. To investigate and compare the remineralising potential of toothpastes with different Fluoride (F) formulations: amine fluoride **(AmF)**, sodium monofluorophosphate **(MFP)**, sodium fluoride **(NaF)**, and stannous fluoride **(SnF)** on artificial subsurface caries lesions *in vitro*.
2. To investigate and compare the remineralising potential of toothpastes containing sodium fluoride (NaF) formulation at different concentrations of fluoride **(500, 1000, 1450, 2800 and 5000 ppm F)** on artificial subsurface caries lesions *in vitro*.

1.6.2 The null hypotheses:

1. There is no difference in the effect of toothpastes containing different fluoride formulations: **AmF, MFP, NaF** and **SnF** on remineralisation of artificial subsurface carious lesions *in vitro*.
2. There is no difference in the effect of toothpastes with sodium fluoride (NaF) formulation and different concentrations (500-5000 ppm F) on remineralisation of artificial subsurface carious lesions *in vitro*.

Chapter 2 MATERIALS AND METHODS:

This was a two phase *in vitro* study design to investigate the remineralisation of the enamel subsurface lesions under pH cycling conditions using different fluoride formulations (phase A), and different NaF concentrations (phase B). The methodology adopted in the present study including preparation of tissue samples and the pH cycling protocol as well as the materials and equipment used will be described in this section.

2.1 Power calculation:

Statistical advice was sought and the sample size was calculated by using data from a previous professional Doctorate thesis 'Comparison of the newer preventative therapies on remineralisation of enamel in vitro.' (Bataineh, 2014), A total of 23 enamel slabs per group was needed. This calculation was based on the assumption that the standard deviation of the response variable is 2.03, power 95%, 0.05 significance level. This is based on calculations by UCSF Biostatistics: Power and Sample Size Programs.

2.2 Experiment materials: Phase A

1. Fluoride free toothpaste (0 ppm F) - Boots Smile Non Fluoride.

Figure 1 Fluoride free toothpaste (0 ppm F) - Boots Smile Non Fluoride.

2. Sodium Fluoride **(NaF)** toothpaste (1450 ppm F) – Colgate Total Original Care.

Figure 2 Sodium Fluoride (NaF) toothpaste – Colgate Total Original Care.

3. Stannous Fluoride **(SnF)** toothpaste (1450 ppm F) (1100 ppm SnF + 350 ppm NaF) – Oral-B Pro-Expert.

Figure 3 Stannous Fluoride (SnF) toothpaste – Oral-B Pro-Expert.

4. Sodium Monofluorophosphate **(MFP)** toothpaste (1450 ppm F) – Colgate Sensitive Pro-Relief™.

Figure 4 Sodium Monofluorophosphate (MFP) toothpaste – Colgate Sensitive Pro-Relief™.

5. Amine Fluoride **(AmF)** toothpaste (1400 ppm F) – Elmex Kariesschutz.

Figure 5 Amine Fluoride (AmF) toothpaste – Elmex Kariesschutz.

2.3 Experiment materials: Phase B

1. Fluoride free toothpaste (0 ppm F) - Boots Smile Non Fluoride.

Figure 6 Fluoride free toothpaste (0 ppm F) - Boots Smile Non Fluoride.

2. **500 ppm F** Sodium Fluoride (NaF) toothpaste – Fluocaril Kids 2 to 6.

Figure 7 500 ppm F Sodium Fluoride (NaF) toothpaste – Fluocaril Kids 2 to 6.

3. **1000 ppm F** Sodium Fluoride (NaF) toothpaste – Aquafresh Milk Teeth 0-2.

Figure 8 1000 ppm F Sodium Fluoride (NaF) toothpaste – Aquafresh Milk Teeth 0-2.

4. **1450 ppm F** Sodium Fluoride (NaF) toothpaste – Colgate Total Original Care.

Figure 9 1450 ppm F Sodium Fluoride (NaF) toothpaste – Colgate Total Original Care.

5. **2800 ppm F** Sodium Fluoride (NaF) toothpaste – Colgate Duraphat 0.619%W/W.

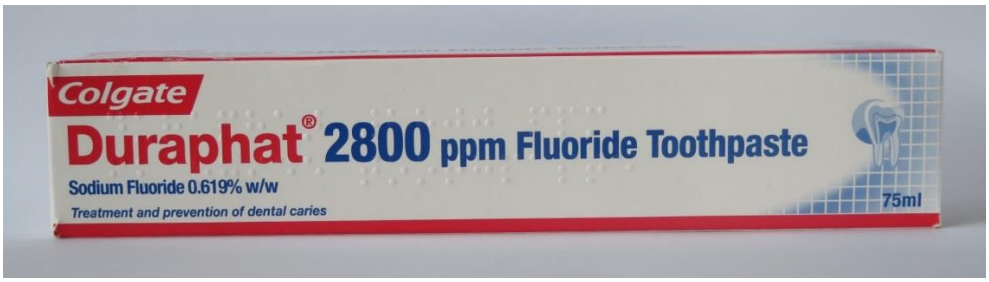


Figure 10 2800 ppm F Sodium Fluoride (NaF) toothpaste – Colgate Duraphat 0.619%W/W.

6. **5000 ppm F** Sodium Fluoride (NaF) toothpaste – Colgate Duraphat 1.1% W/W.

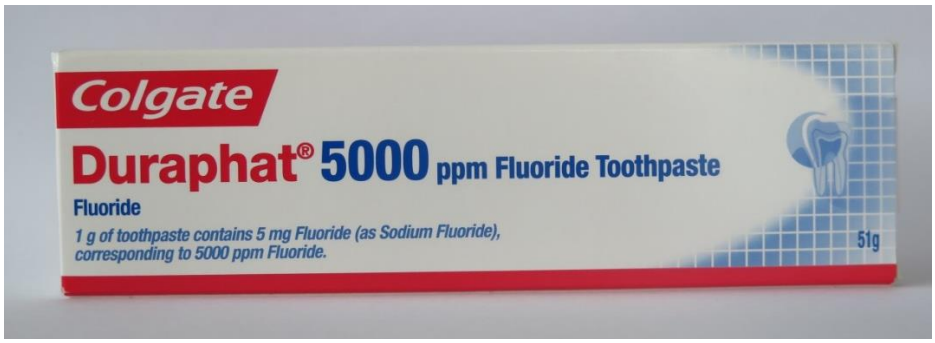


Figure 11 5000 ppm F Sodium Fluoride (NaF) toothpaste – Colgate Duraphat 1.1% W/W

2.4 Experimental and control groups

The enamel slabs were randomly assigned to two phases, each phase containing a number of groups:

2.4.1 Phase A: Different fluoride formulation toothpastes, with a fixed concentration, containing five groups:

1. Fluoride free toothpaste **(0 ppm F)** – 2 times/day (negative control).
2. Sodium fluoride **(NaF)** toothpaste (1450 ppm F) – 2 times/day.
3. Stannous Fluoride **(SnF)** toothpaste (1450 ppm F) (1100 ppm SnF + 350 ppm NaF) – 2 times/day.
4. Sodium Monofluorophosphate **(MFP)** toothpaste (1450 ppm F) – 2 times/day.
5. Amine Fluoride **(AmF)** toothpaste (1400 ppm F) – 2 times/day.

2.4.2 Phase B: Different sodium fluoride (NaF) concentration toothpastes containing six groups:

1. Fluoride free toothpaste **(0 ppm F)** – 2 times/day (negative control).
2. **500 ppm F** Sodium Fluoride (NaF) toothpaste – 2 times/day.
3. **1000 ppm F** Sodium Fluoride (NaF) toothpaste – 2 times/day.
4. **1450 ppm F** Sodium Fluoride (NaF) toothpaste – 2 times/day.
5. **2800 ppm F** Sodium Fluoride (NaF) toothpaste – 2 times/day.
6. **5000 ppm F** Sodium Fluoride (NaF) toothpaste – 2 times/day.

2.5 Enamel slab preparation

All enamel slabs used in the present study were obtained from bovine incisors. Approval for collection of bovine teeth was sought from the Food Standards Agency (Appendix 1) The teeth were obtained from an abattoir and stored immediately in distilled water and 0.1% thymol (Sigma Aldrich) at room temperature. Before sectioning, the teeth were cleaned using a spoon excavator and a toothbrush with pumice powder to remove any soft tissue remnants. To detect any defects, caries or cracks, all teeth were screened by trans-illumination and transmitted light using a low-power microscopy (Leitz, Wetzlar®, Germany). Suitable teeth were selected for the study.

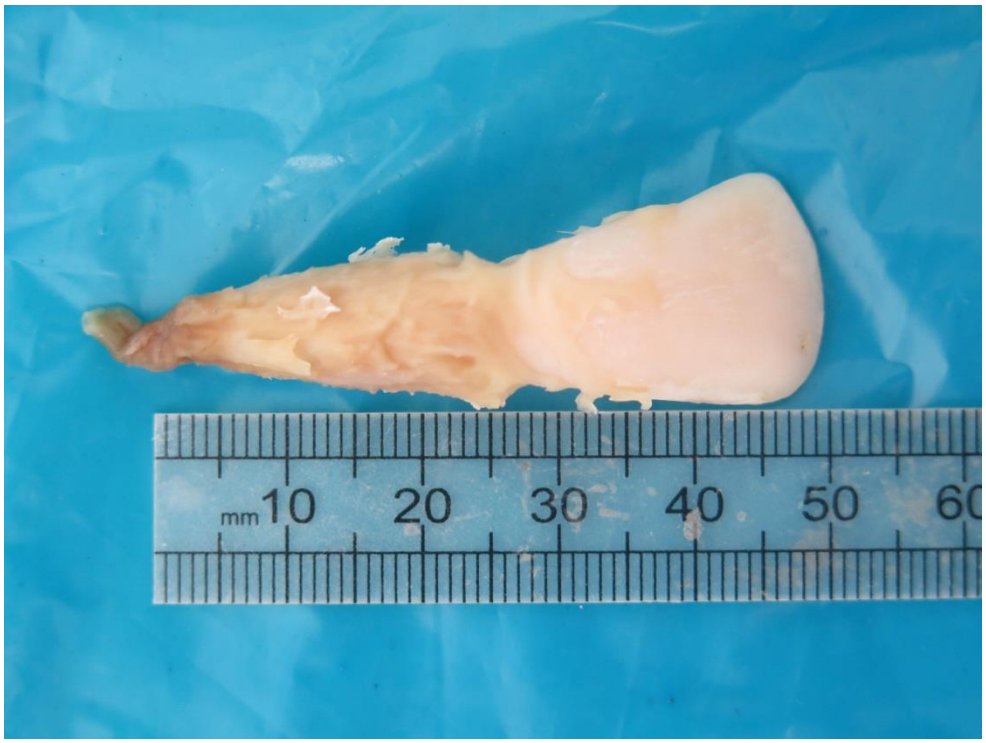


Figure 12 Extracted bovine incisor

Each tooth was mounted using ‘green stick’ impression compound (Kerr, UK) on plates. The crowns were sectioned using water cooled, diamond wire saw, cutting machine

(Well@Walter EBNER, CH-2400 Le Loche). The buccal and palatal surfaces of each crown were separated, and each buccal section was cut into three slabs that were approximately 6 x 5 x 3 mm in size.

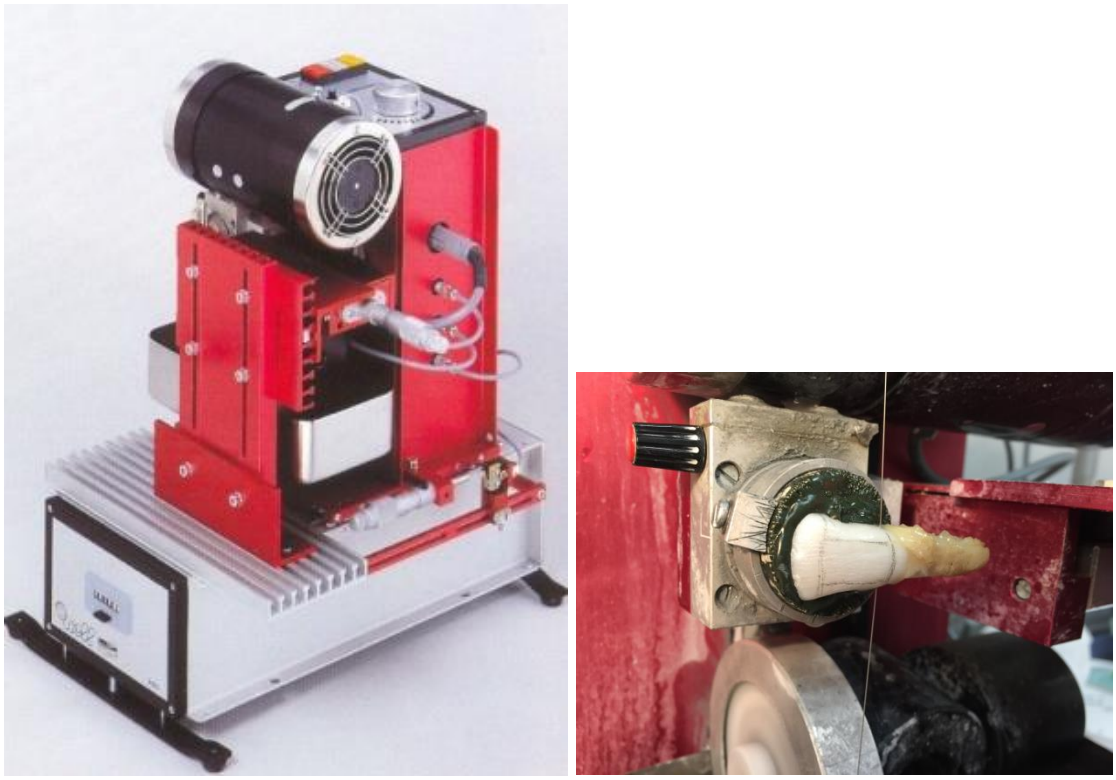


Figure 13 Diamond wire saw apparatus used for the teeth sectioning (Well® Walter EBNER, CH-2400 Le Loche).

Each enamel slab was mounted on a plastic rod using “sticky wax” to hold the slab in the demineralising gel. The rod was secured to the lid of a “Sterilin” type universal tube so that when the top was screwed onto the tube, the tooth was suspended in the centre of the tube free space. Two coats of an acid resistant, coloured nail varnish (Max Factor “Glossfinity”) were then applied on the enamel slabs, except for a small window of approximately 2 x 3 mm on the centre of each slab that was left exposed. An interval of 24 hours was left between the two applications to allow the nail varnish to dry completely.

Figure 14 Maxfactor Glossinfinity (Red Passion 110) nail varnish.

Once the enamel slabs were prepared, they were kept moist in plastic containers containing de-ionised water at room temperature to prevent dehydration.

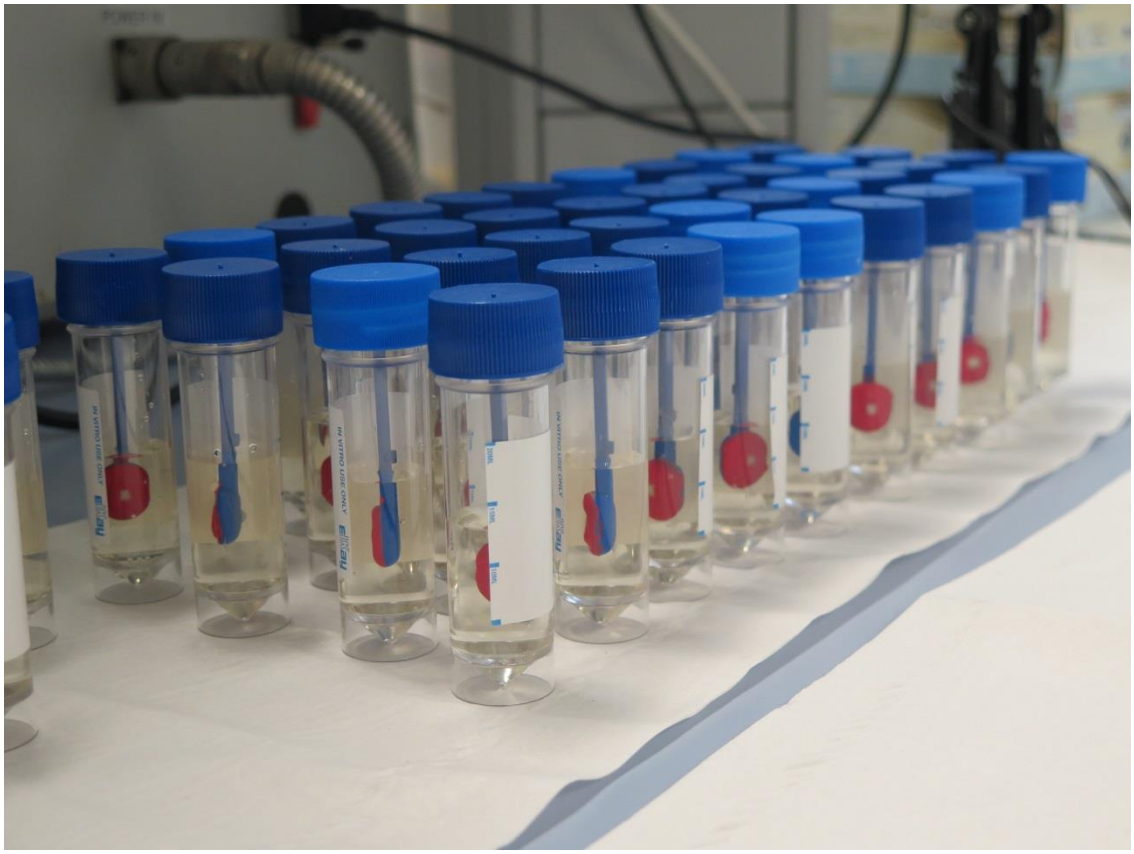


Figure 15 Enamel slabs suspended in sterilin tubes painted with nail varnish laving an exposed enamel window.

2.6 Preperation of the enamel sub-surface lesion:

In order to obtain a sub-surface caries-like lesion an acid demineralising gel was prepared. Preparation of the demineralising system (acidified hydroxyethyl cellulose gel) was performed by adding 0.1 M sodium hydroxide (BDH Analar Grade) to 0.1 M lactic acid (Sigma Aldrich D/L GPR 87% Lactic acid) to give a pH value of 4.5 and then 6% w/v hydroxyethyl cellulose (Sigma Aldrich) was added to the solution and stirred for one hour until a consistency similar to that of “wallpaper paste” was achieved. The mixture was left to settle for 24 hours. Once the demineralising gel was ready for use, it was poured into the universal tubes “Sterilin” into which the mounted teeth were then submerged (Figure 16). The enamel slabs were immersed in acid gel for 10 days to produce an artificial

enamel subsurface lesion. The enamel slabs were removed from the acid gel and washed with distilled water, the nail varnish was then removed using methanol to prepare the enamel slabs for the baseline QLF measurements.

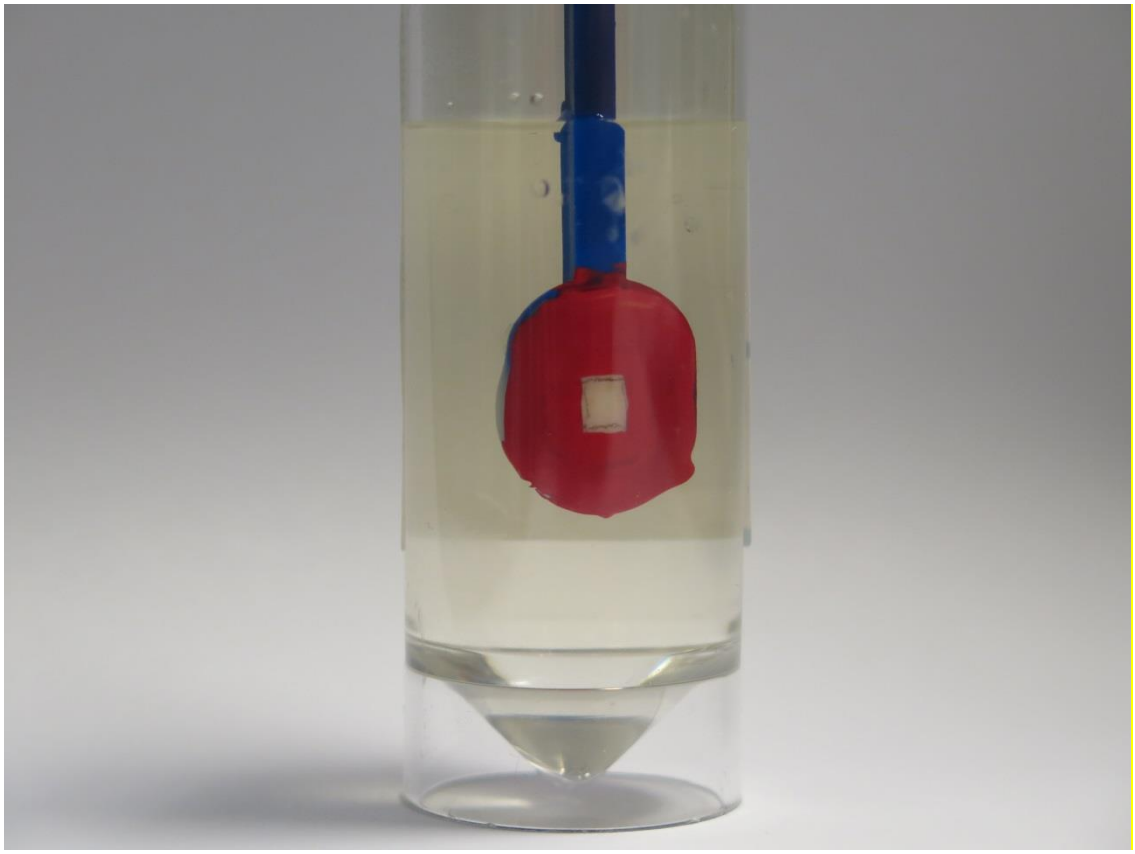


Figure 16 Enamel slab immersed in acidified hydroxyethyl cellulose gel.

2.7 Quantitative light-induced fluorescence (QLF) measurements

For each enamel slab, QLF measurements were taken after the creation of the enamel subsurface lesion and at the end of the 28 days experiment period using the QLF machine (QLF-D Biluminator™ 2) (Inspektor Research Systems BV, Amsterdam, The

Netherlands), Under controlled conditions. All the slabs were dried for 15 seconds with compressed air prior to imaging, and were then examined in a dark room.

QLF-D Biluminator™ 2 consists of a Biluminator™ mounted on a Single Lens Reflex (SLR) camera fitted with a 60 mm macro lens. The Biluminator™ provides the light sources and filters for making white-light and QLF™-images. Fluorescence images of all enamel specimens were captured with a 'Live View'-enabled digital full-sensor SLR camera (model 550D, Canon, Tokyo, Japan) at the following setting: shutter speed of 1/30 s, aperture value of 6.7, and ISO speed of 1600. All digital images were stored automatically on a personal computer with image-capturing software (C3 version 1.16; Inspektor Research Systems). All fluorescence images were examined with analysing software (QA2 version 1.16; Inspektor Research Systems). The analyses were performed by a single trained examiner.

To ensure that images of the enamel slab were always captured in the same camera positions and from the same angles, the camera was attached to a stand in the same position for all the images. The QLF camera was fixed at a position that provided optimum illumination of the enamel block surface. The camera specimen distance was standardised using the jig thereby controlling specimen stability light intensity and magnification.

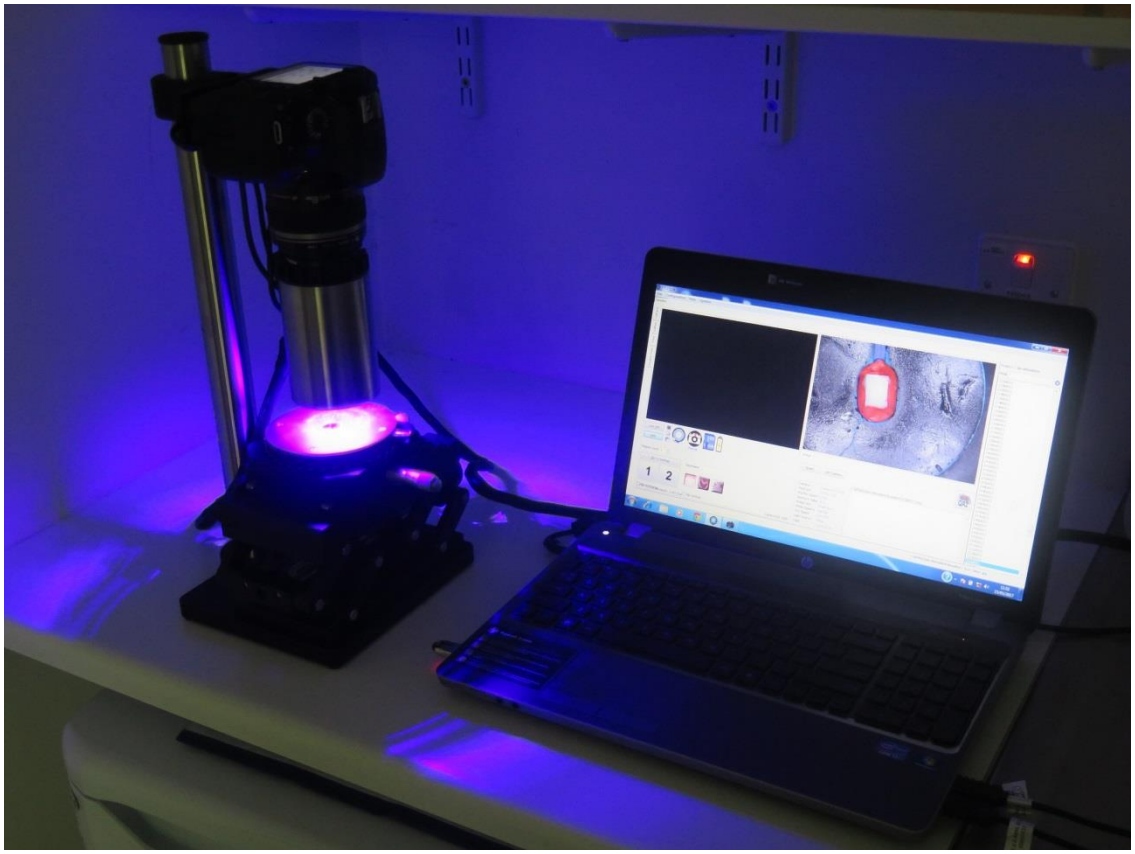


Figure 17 QLF machine, the SLR camera attached to the stand with standardised distance from the enamel slab.

A patch was drawn around the white spot lesion site by the study examiner with its borders on sound enamel (Figure 18). Inside this patch, the fluorescence levels of sound tissue were reconstructed by using the fluorescence radiance of the surrounding sound enamel. The percentage difference between the reconstructed and the original fluorescence levels was calculated. The same area of interest was used for the baseline and endpoint white spot lesion image identification.

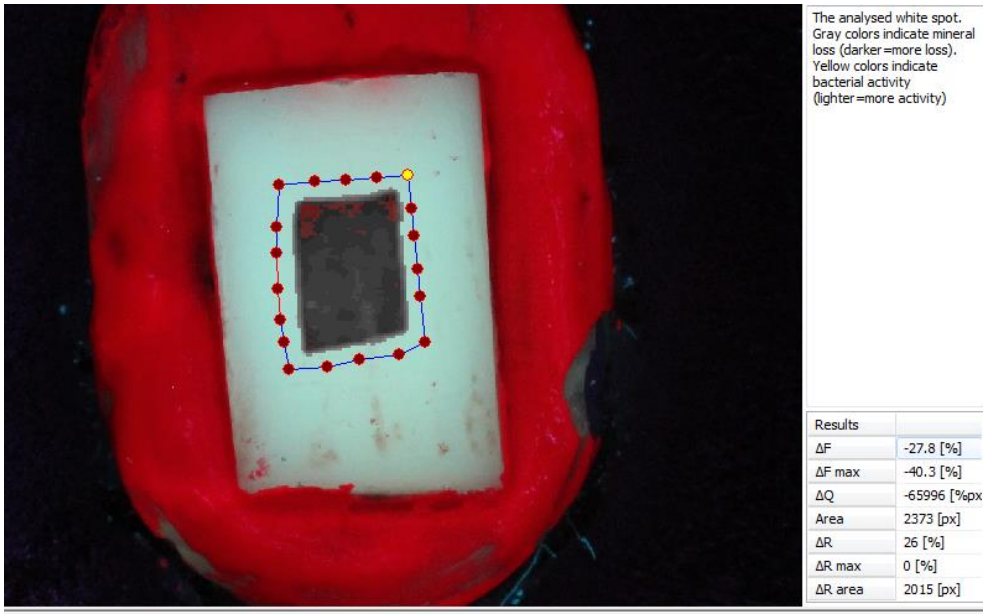


Figure 18 : QLF image taken with the blue light shows the demineralised lesion in the centre of the enamel slab as well as a patch drawn around the lesion with the border in sound enamel.

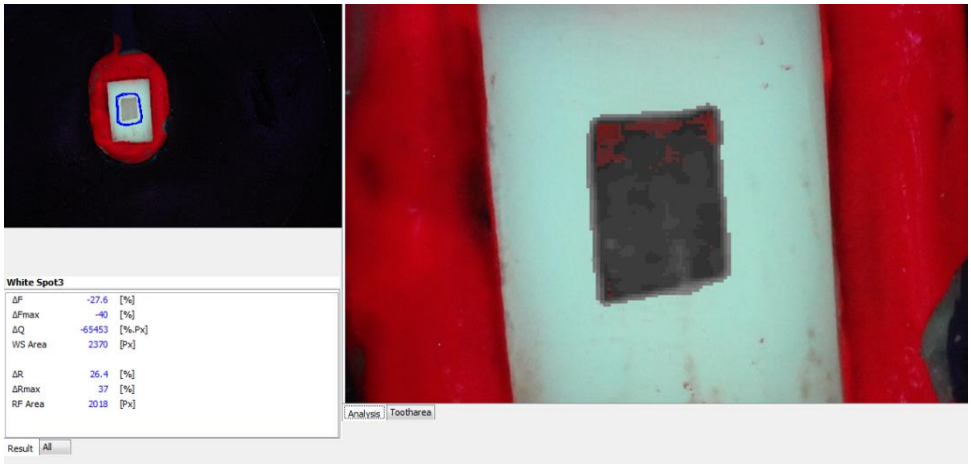
Demineralised areas appeared as dark spots. The fluorescent radiance of a white spot lesion viewed by QLF was lower than that of sound enamel. In order to enable calculation of loss of fluorescence in the white spot lesion, the fluorescent radiance of sound tissue at the lesion site was reconstructed by interpolation from the radiance of the sound tissue surrounding the lesion. Fluorescence radiance levels less than 95% of reconstructed sound fluorescence radiance levels were considered to be artificial early caries lesions and were displayed as shades of grey where darker grey corresponds to higher fluorescence loss. The difference between the measured values and the reconstructed values gave the resulting fluorescence loss in the lesion.

For each enamel lesion the following three metrics were obtained:

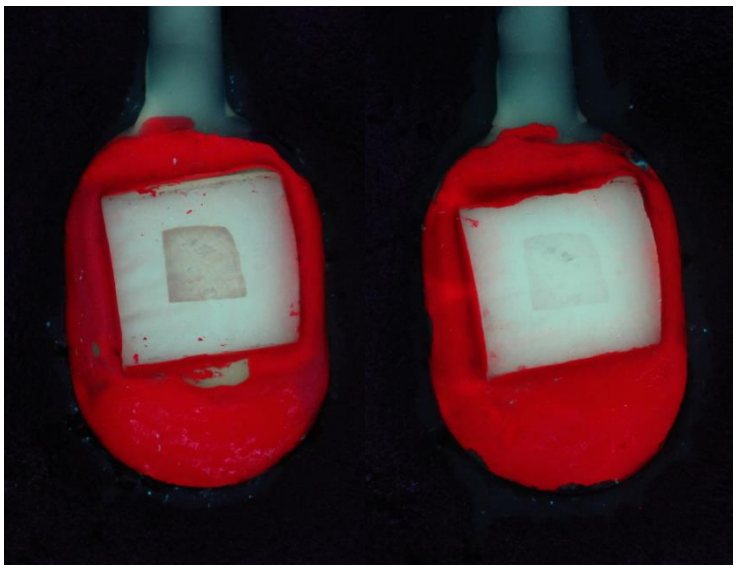
1. ΔF: Percentage fluorescence loss with respect to the fluorescence of sound tooth tissue; related to lesion depth (%).

2. ΔQ: The ΔF times the Area. Percentage fluorescence loss with respect to the fluorescence of sound tissue times the area. Related to lesion volume (% px2).
3. Area: The surface area of the lesion expressed in pixels2 (px2).

Example of the blue light image analysis results including ΔF, ΔQ and the lesion area values.



Example of the blue light image of demineralised enamel lesions before and after pH cycling.



Before lesion cycling **After lesion cycling**

2.8 The ΔF range of the artificial lesions

After performing the QLF baseline analysis for all enamel slabs, the range of ΔF values were found to vary between -5.97 and -32.53. The enamel slabs with the ΔF range (-8.57 to -20.83 with an average of -13.28) were selected to be involved in the experiment in order to pick up the differences in ΔF after treatment.

2.9 Randomisation and blindness

All enamel slabs were randomly assigned to five groups in phase A, and six groups in phase B using a random number generator. When the slabs were analysed with QLF, the investigator did not know to which group the enamel slab belongs, making the analysis blinded.

2.10 The pH cycling regime

Each enamel slab was attached to a plastic rod (holder). The enamel slabs were rinsed with distilled water for 1 minute then dipped in toothpaste slurry for 5 minutes. After that the enamel slabs were rinsed with distilled water for 1 minute and placed in day time artificial saliva for 60 minutes. The enamel slabs were then exposed to the first demineralisation challenge by dipping in acetic acid solution (pH 4.8) for 5 minutes, then rinsed with distilled water for 1 minute and placed in day time artificial saliva. This process was repeated until the enamel slabs were subjected to 5 demineralisation challenges. After the last cycle the enamel slabs were dipped in toothpaste slurry for 5 minutes.

Enamel slabs were then places in night time artificial saliva. The acetic acid was changed after each exposure. The day time saliva and the night time saliva were

changed every day. The enamel slabs were kept in the incubator at 37˚C at all times except during the dipping in the toothpaste slurry or the demineralisation solution (Figure 19).

Figure 19 Enamel slabs were kept in incubator at 37˚C at all times except during the dipping in the toothpaste slurry or the demineralisation solution

2.11 Toothpaste slurry

Toothpaste slurries were prepared by mixing the toothpaste with artificial day saliva in a volume ratio 1:4 (toothpaste: saliva) by weight, using a WhirliMixer® (Fisons) for 1 minute.

The toothpastes used were:

Phase A:

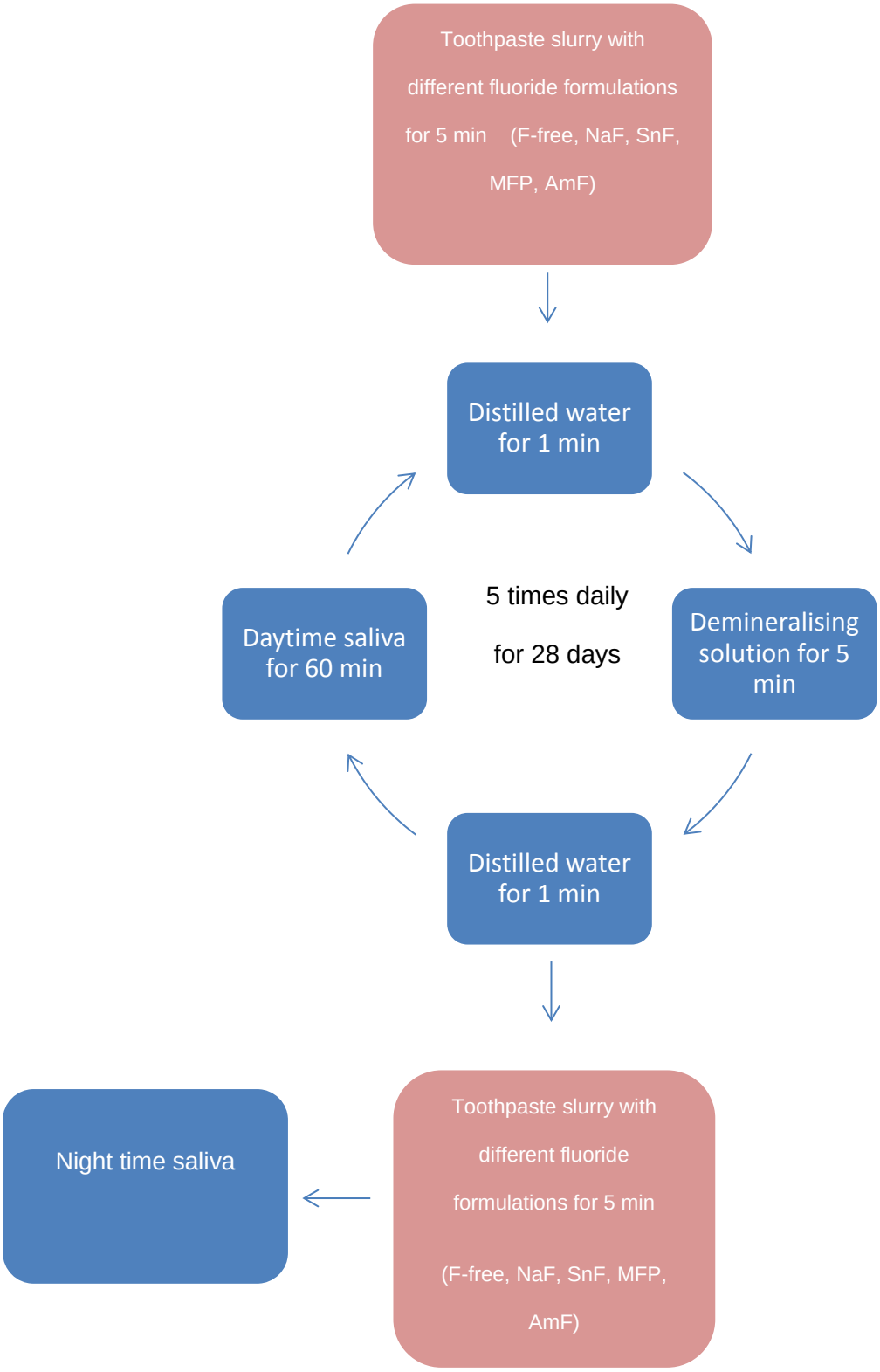
1. Fluoride free toothpaste **(0 ppm F)** – 2 times/day (negative control).
2. Sodium fluoride **(NaF)** toothpaste (1450 ppm F) – 2 times/day.
3. Stannous Fluoride **(SnF)** toothpaste (1450 ppm F) – 2 times/day.
4. Sodium Monofluorophosphate **(MFP)** toothpaste (1100 ppm SnF + 350 ppm NaF) – 2 times/day.
5. Amine Fluoride **(AmF)** toothpaste (1400 ppm F) – 2 times/day.

Phase B:

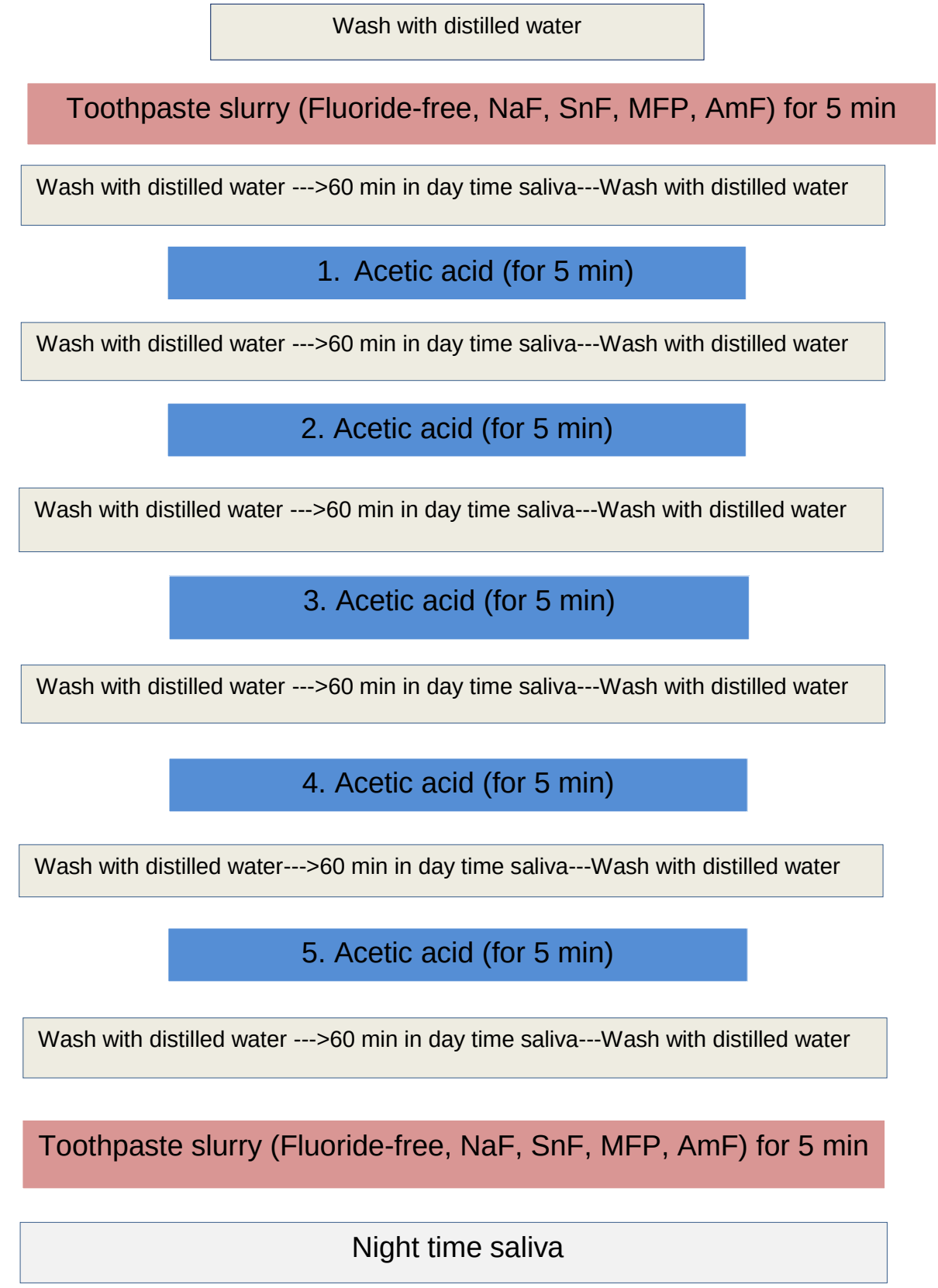
1. Fluoride free toothpaste **(0 ppm F)** – 2 times/day (negative control)
2. **500 ppm F** Sodium Fluoride (NaF) toothpaste – 2 times/day.
3. **1000 ppm F** Sodium Fluoride (NaF) toothpaste – 2 times/day.
4. **1450 ppm F** Sodium Fluoride (NaF) toothpaste – 2 times/day.
5. **2800 ppm F** Sodium Fluoride (NaF) toothpaste – 2 times/day.
6. **5000 ppm F** Sodium Fluoride (NaF) toothpaste – 2 times/day.

2.12 Flow charts

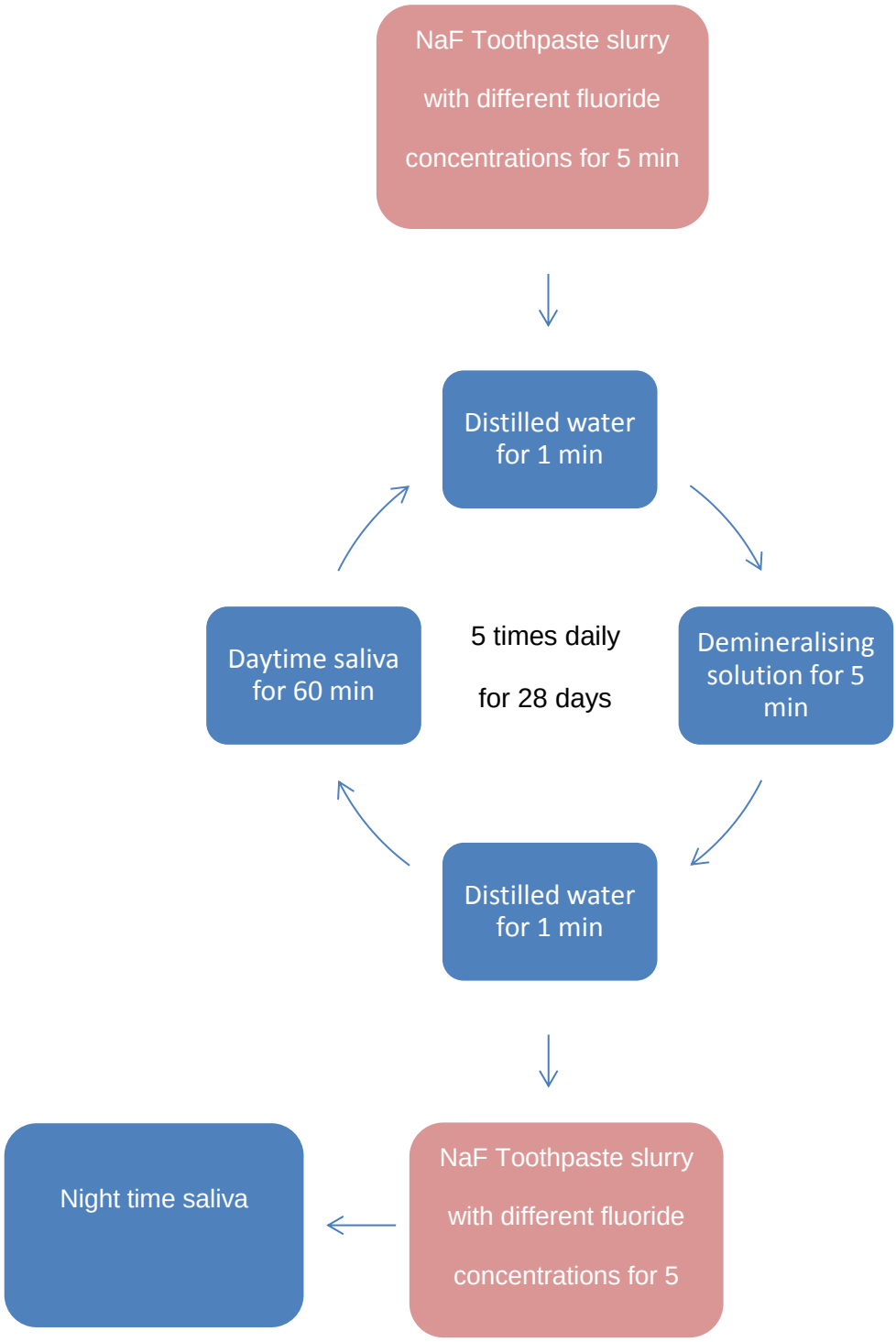
Flow chart for phase A-1: <u>Different fluoride formulation toothpaste</u> phase containing five groups (Fluoride-free, NaF, SnF, MFP, AmF):



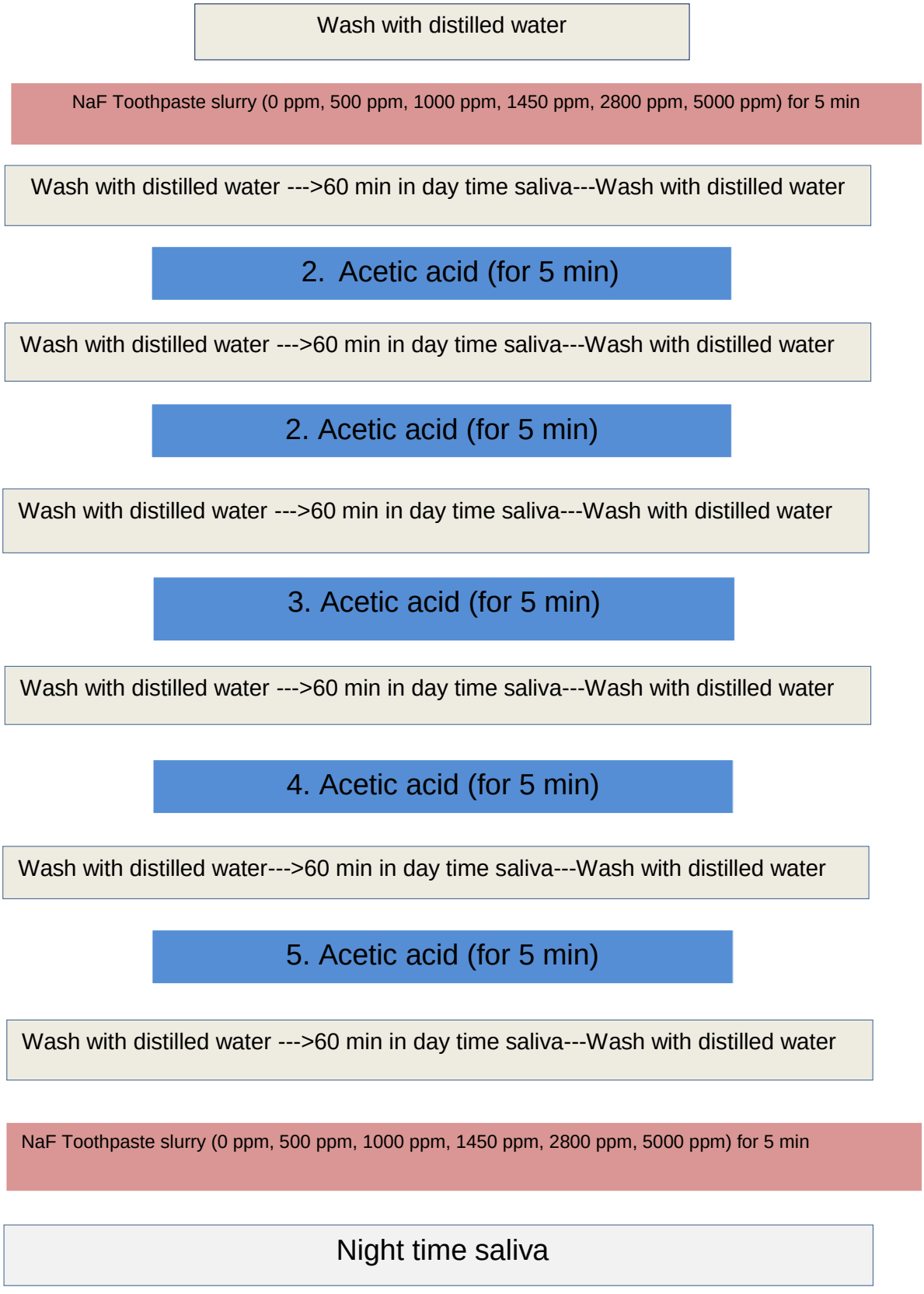
Flow chart for phase A-2: <u>Different fluoride formulation toothpaste</u> phase containing five groups (Fluoride-free, NaF, SnF, MFP, AmF):



Flow chart for phase B-1: <u>Different sodium fluoride (NaF) concentration toothpastes</u> containing six groups (0 ppm, 500 ppm, 1000 ppm, 1450 ppm, 2800 ppm, 5000 ppm):



Flow chart for phase B-2: <u>Different sodium fluoride (NaF) concentration toothpastes</u> containing six groups (0 ppm, 500 ppm, 1000 ppm, 1450 ppm, 2800 ppm, 5000 ppm):



2.13 Preparations of solutions used in the study

2.13.1 Artificial saliva

Two artificial saliva solutions were used in this study. The first solution was used for day time during the pH cycling, between the acid exposures. The second solution was used to store the slabs during the night. The day saliva was supersaturated solution that allowed remineralisation of enamel slabs, the night saliva was a saturated solution that maintained the enamel condition and did not provide any minerals exchange.

The artificial saliva composition was based on the electrolyte composition of natural saliva and it was advised to be used in order to eliminate any precipitation on the enamel surface (as provided by Dr R. P. Shellis, Department of Oral and Dental Science, University of Bristol, Bristol, UK).

2.13.1.1 The preparation of day time artificial saliva

The formulation of daytime saliva is shown in Table 1.

Table 1 Day time saliva formulation.

Salt	Concentration g/L
Calcium carbonate	0.07
Magnesium carbonate (hydrated basic)	0.019
Potassium di-hydrogen phosphate	0.554
HEPES buffer (acid form)	4.77
Potassium chloride	2.24

Using 900 mL distilled water 1.8 mL 1 mol/L HCL and above components are stirred using a shaker until it all dissolves. The pH will be adjusted to 6.8 by adding KOH solution that is made up to 1L with de-ionised water.

2.13.1.2 The preparation of night time artificial saliva

The formulation of the night time saliva is shown in Table 2.

Table 2 Night time saliva formulation.

Salt	Concentration g/L
Calcium carbonate	0.05
Magnesium carbonate (hydrated basic)	0.019
Potassium di-hydrogen phosphate	0.068
HEPES buffer (acid form)	4.77
Potassium chloride	2.24

Again using 900 mL distilled water 1.4 mL 1 mol/L HCL and above components are stirred using a shaker until it all dissolves. The pH will be adjusted to 6.8 by adding KOH solution that is made up to 1L with de-ionised water.

2.13.2 Acetic acid buffer

The preparation of acetic acid solution was done according to ten Cate et al. (2006), the constitution of acetic acid is shown in Table 3.

Table 3 Acetic acid formulation.

Contents	Concentration g/L
Calcium chloride	1.665 g
Potassium di-hydrogen phosphate	1.13 g
Acetic acid	28.73 ml

The above contents and 1 L distilled water were stirred using centrifuge until fully dissolved. The pH was adjusted using pH meter (ORION- model 920A) by adding KOH solution to reach pH 4.8.

2.14 Training and calibration

The study investigator had received training to use the QLF machine (QLF-D Biluminator™ 2) and was familiar with the QLF software before the study.

The training was provided by the manufacturer (Inspektor Research Systems BV, Amsterdam, The Netherlands), and included the image capturing as well as image analysis. The investigator assessment of sound, demineralised enamel and the border of the lesion during image analysis were calibrated.

2.15 Intra-examiner reproducibility

The study investigator randomly retested 15% of the enamel slabs with the QLF at the end of the experiment. Intra-examiner reproducibility was tested using Intra-class Correlation Coefficient (ICC).

2.16 Statistical analysis

The data were analysed using SPSS statistical software package for windows version 22.0. Descriptive statistics were used to calculate the mean, median, range, and standard deviation.

The normality of the data distribution was assessed using Shapiro-Wilk test and Kolmogorov-Smirnov test.

Paired sampled t-tests were performed to compare the changes in remineralisation at baseline and after treatment within the same group.

One way ANOVA was used to compare between the five groups when the data were normally distributed, and Kruskal-Wallis Test was used when the data were not normally distributed. Furthermore, Bonferroni test was used to assess if there was any significant difference between each of the groups. The test also calculated the 95% confidence interval. The significance level was set at $p < 0.05$.

Chapter 3 RESULTS

3.1 Quantitative Light-Induced Fluorescence (QLF) Results for phase A: Different Fluoride formulation toothpastes containing five groups (Fluoride-free, NaF, SnF, MFP, AmF)

Three main parameters for QLF were statistically analysed, these were:

- ΔF: Percentage fluorescence loss with respect to the fluorescence of sound tooth tissue. Related to lesion depth (%).
- ΔQ: ΔF times the Area. Percentage fluorescence loss with respect to the fluorescence of sound tissue times the area. This is related to lesion volume (%px2).
- Area: The surface area of the lesion expressed in pixels2 (px2).

3.1.1 The mean fluorescence loss ΔF:

The values of ΔF at baseline for all groups were checked to see if there was a difference between the groups. The normality tests (Shapiro-Wilk test and Kolmogorov-Smirnov test) were carried out to check the normality of the data (Appendix 2). The data were considered normally distributed if the p values from these tests were not statistically significant ($p > 0.05$). p values for all groups except for **MFP**, and **NaF** groups were not statistically significant therefore data was not considered to be normally distributed, and required a non-parametric test to assess baseline distribution of slabs.

The boxplot (Figure 20) for the distribution of the ΔF at the baseline, showing that all groups were similar in range with MFP having the least variation in ΔF compared to the other groups and containing a single outlier.

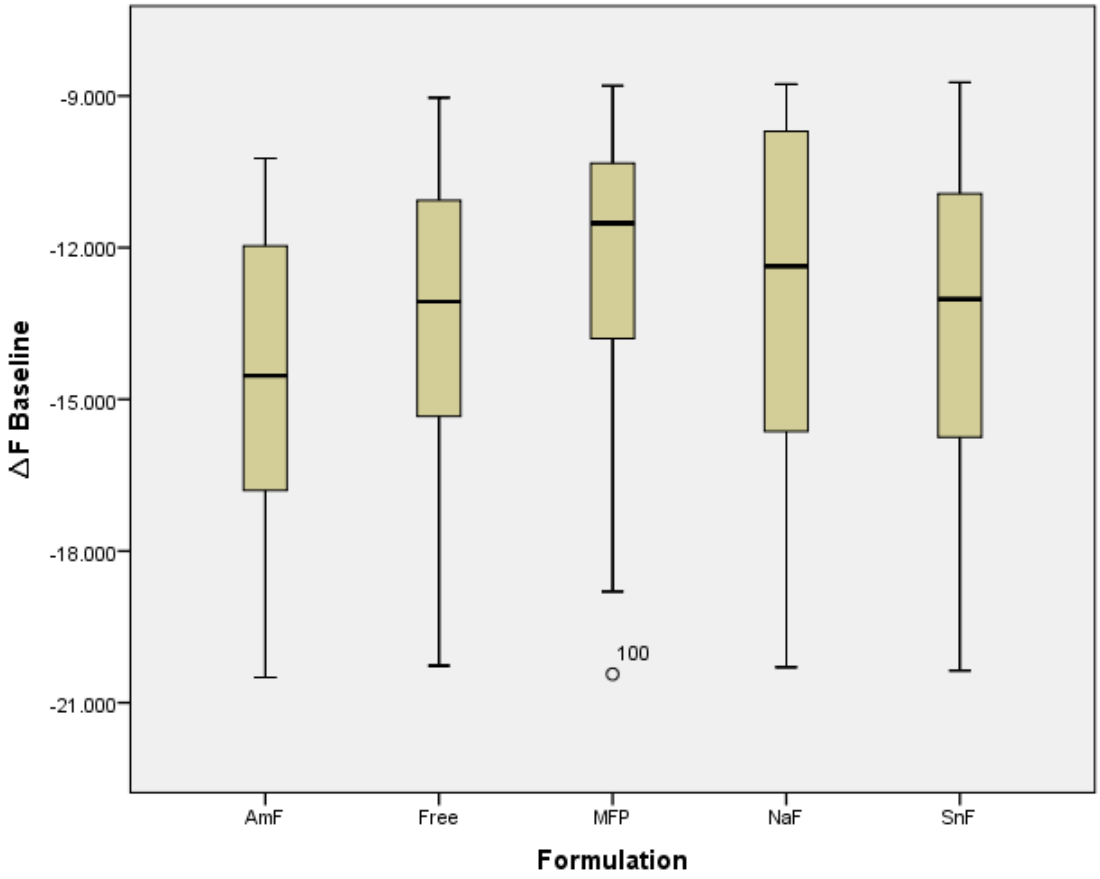


Figure 20 Boxplot for the distribution of the ΔF values at baseline for all groups. Error bars represent SD, the line in the box of Box-and-whisker plot is the median value of the data.

Kruskal-Wallis test (Table 4) was performed to assess if there was any statistically significant difference in ΔF values at the baseline between the lesions assigned to the five groups. No statistically significant difference was found.

Table 4 Kruskal-Wallis Test between groups for ΔF values at baseline

	ΔF at Baseline
Chi-Square	7.759
df	4
Asymptomatic Significance	0.101
a. Kruskal Wallis Test	
b. Grouping Variable: Formulation	

3.1.1.1 Difference in ΔF within each group

The ΔF mean values both at baseline and after treatment are shown in (Table 5). It can be seen that there was an improvement in ΔF values for all the groups in the study.

Table 5 Mean values of ΔF at baseline and after treatment for all groups.

Group	Mean ΔF at baseline ± SD	Mean ΔF after treatment ± SD	Mean Difference in ΔF at baseline and after treatment ± SD
F Free	-13.623 ± 3.39	-11.412 ± 2.54	2.211 ± 3.98
NaF	-13.375 ± 3.93	-7.417 ± 2.50	5.957 ± 4.11
SnF	-13.517 ± 3.50	-8.479 ± 2.30	5.038 ± 4.30
MFP	-12.249 ± 2.95	-9.224 ± 2.14	3.024 ± 3.48
AmF	-14.688 ± 3.14	-12.999 ± 5.49	1.689 ± 5.65

(Figure 21) shows the change in the mean of ΔF at baseline and after treatment with the standard deviation for all groups.

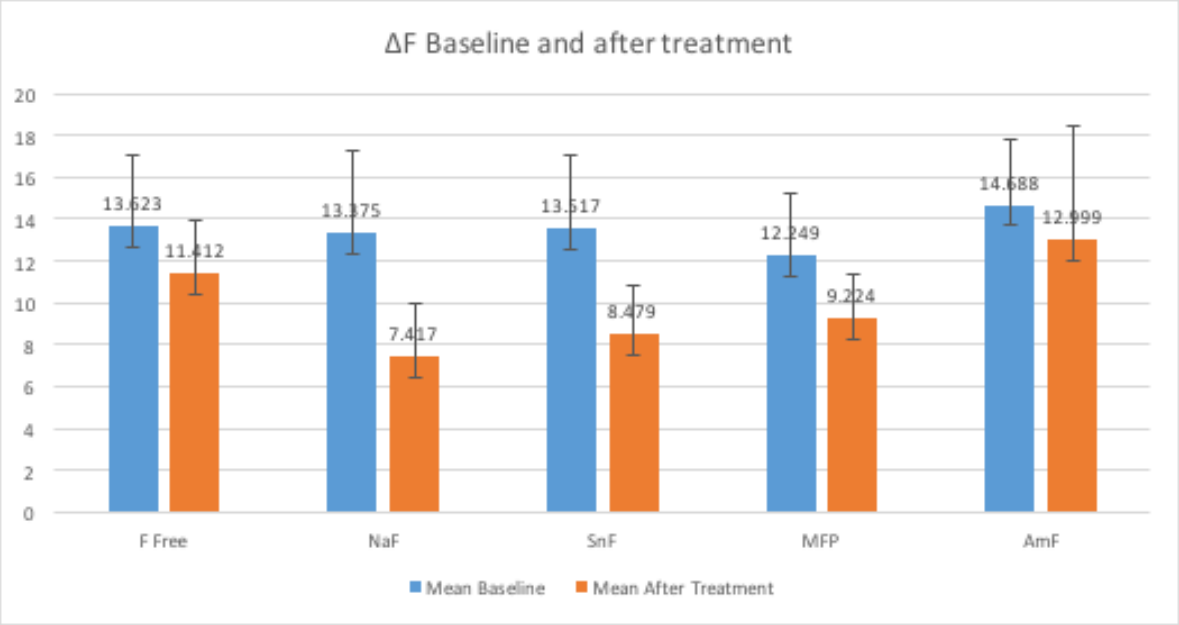


Figure 21 ΔF mean values at baseline and after treatment for all groups.

To assess whether the change in ΔF at baseline and after treatment was significantly different within the same group, paired T-Test was used. The results of the paired T-Test are shown in (Table 6).

It can be seen that there was a statistically significant improvement in the ΔF values after treatment compared with that at baseline in all groups except AmF ($p < 0.05$).

Table 6 Paired sampled T-Test results for ΔF values at baseline and after treatment.

		Paired Differences					Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference		
					Lower	Upper	
F Free	ΔF at baseline – ΔF after treatment	2.211	3.98	0.795	0.568	3.852	**0.010***
NaF		5.957	4.11	0.822	4.259	7.655	**0.000***
SnF		5.038	4.30	0.876	3.223	6.851	**0.000***
MFP		3.024	3.48	0.683	1.616	4.431	**0.000***
AmF		1.689	5.65	1.129	-0.641	4.020	**0.148**

3.1.1.2 Difference in ΔF between all groups:

The ΔF difference (change) was measured using the following formula:

Difference in ΔF = ΔF after treatment - ΔF at baseline

(Figure 22) shows the difference in ΔF in the five tested groups. In all groups the difference in ΔF was positive, meaning that there was decrease in ΔF (mean fluorescence loss) after treatment compared to that at baseline. The highest reduction in ΔF was seen in the **NaF** toothpaste group with a mean ΔF difference of (5.957 ± 4.11), closely followed by **SnF** Fluoride toothpaste group at a mean difference of (5.038 ± 4.30). The group with the least amount of reduction in mean ΔF, and highest standard

deviation was the **AmF** toothpaste at (1.689 ± 5.65). **MFP** and **F Free** toothpaste groups had a mean difference of (3.024 ± 3.48) and (2.211 ± 3.98) respectively (Table 7).

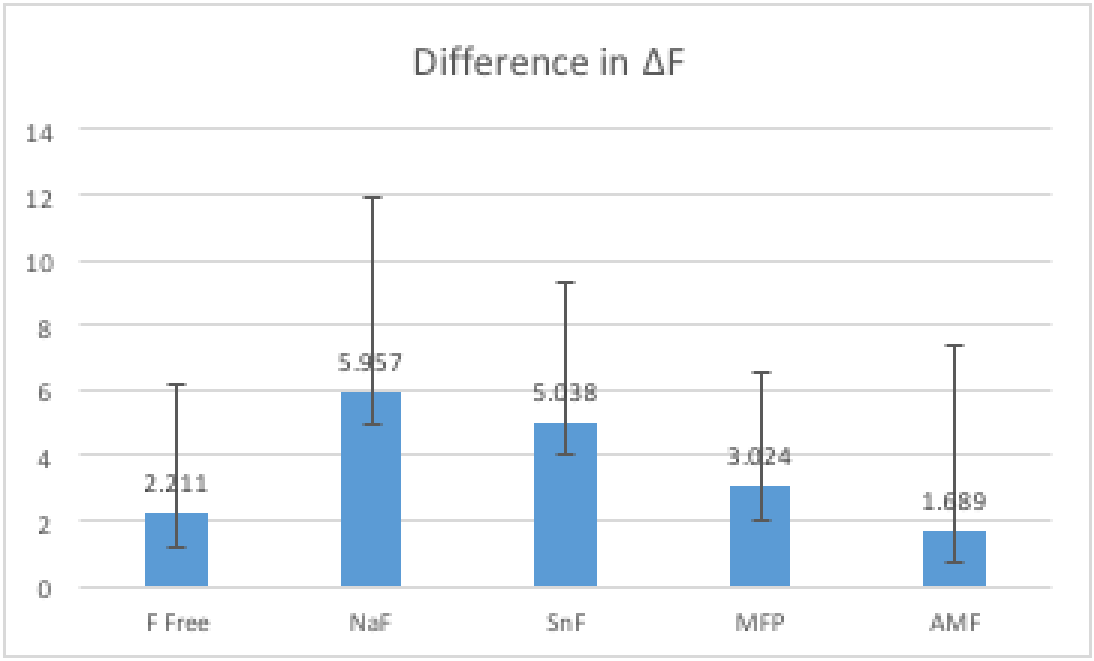


Figure 22 Means of the difference in ΔF at baseline and after treatment of all groups.

Table 7 Descriptive statistics for the difference in ΔF at baseline and after treatment for all groups.

Group	N	Minimum	Maximum	Mean	Median	Std. Deviation
F Free	25	-6.033	9.067	2.210	1.366	3.977
NaF	25	-0.200	13.867	5.957	4.500	4.113
SnF	24	-1.333	15.400	5.037	4.000	4.296
MFP	26	-2.800	8.733	3.024	2.516	3.484
AmF	24	-8.567	12.567	1.689	0.966	5.647

Determination of the normality of the data for Difference in ΔF

In order to check if the ΔF differences between the baseline and after treatment were normally distributed, data normality test was carried out. This included Shapiro-Wilk test and Kolmogorov-Smirnov test. p value was not statistically significant therefore data was considered to be normally distributed (p >0.05).

The boxplot (Figure 23) of difference in ΔF at baseline and after treatment showed normal distribution of data in all groups, with **AmF** tooth paste group having a very large standard deviation.

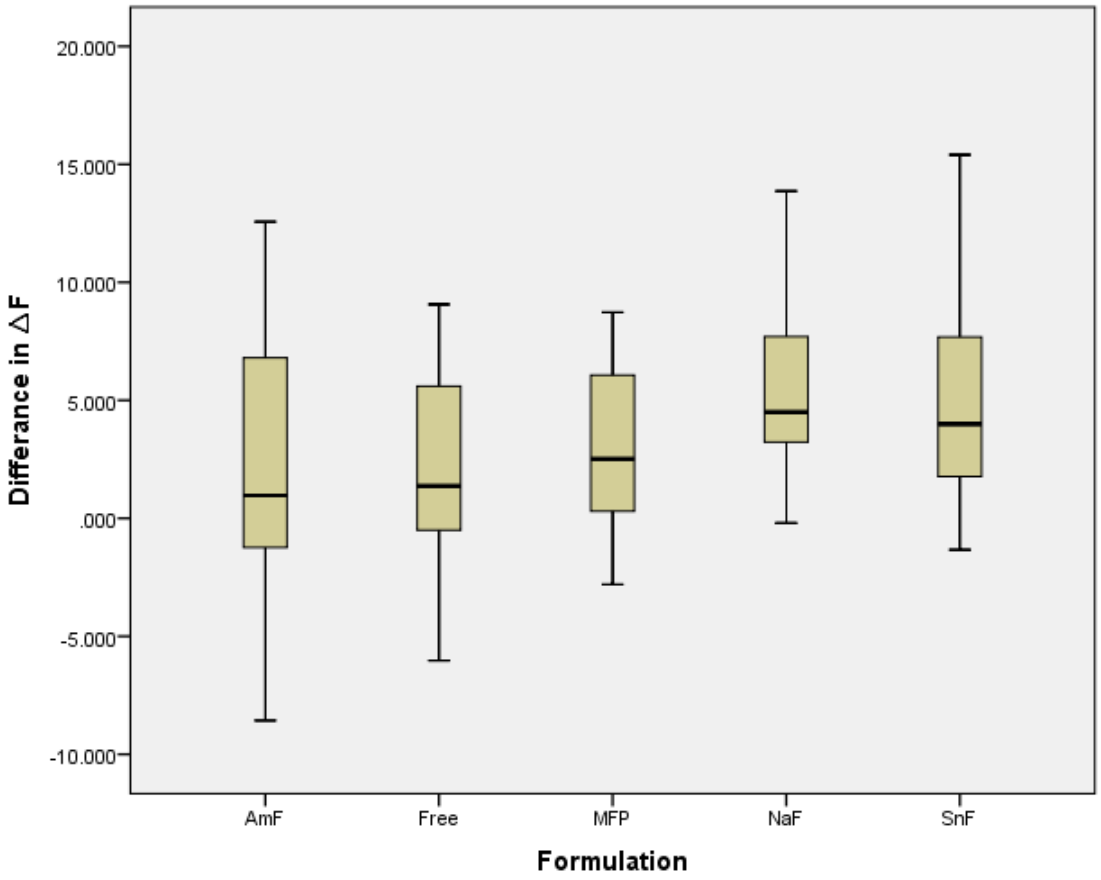


Figure 23 Boxplot for the difference in ΔF at baseline and after treatment for all groups.

One way ANOVA test (Table 8) was performed to assess if the difference in ΔF was statistically significant between the five groups. It showed that the mean difference in ΔF was statistically significant between the groups ($p < 0.05$).

Table 8 One way ANOVA between groups for the difference in ∆F at baseline and after treatment.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	333.527	4	84.132	4.429	**0.002***
Within Groups	2279.322	120	18.994		
Total	2615.849	124			

In order to determine which groups were statistically significant different, pairwise comparisons were conducted using a Bonferroni test. The Bonferroni tests corrects for multiple testing. The results of the Bonferroni tests are shown in (Table 9).

Table 9 Multiple comparisons of the difference in ΔF at baseline and after treatment between all test groups and control with Bonferroni correction.

(I) Group	(J) Group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Free	AmF	0.521	1.233	1.000	-3.004	4.047
	MFP	-0.814	1.221	1.000	-4.305	2.678
	NaF	-3.747	1.233	**0.029***	-7.272	-0.221
	SnF	-2.827	1.245	0.250	-6.389	0.735
NaF	AmF	4.268	1.233	**0.007***	0.743	7.793
	Free	3.747	1.233	**0.029***	0.221	7.272
	MFP	2.933	1.221	0.178	-0.558	6.424
	SnF	0.920	1.245	1.000	-2.642	4.482
SnF	AmF	3.348	1.245	0.082	-0.214	6.910
	Free	2.827	1.245	0.250	-0.735	6.389
	MFP	2.013	1.234	1.000	-1.515	5.541
	NaF	-0.920	1.245	1.000	-4.482	2.642
MFP	AmF	1.335	1.221	1.000	-2.156	4.826
	Free	0.814	1.221	1.000	-2.678	4.305
	NaF	-2.933	1.221	0.178	-6.424	0.558
	SnF	-2.013	1.234	1.000	-5.541	1.515
AmF	Free	-0.521	1.233	1.000	-4.047	3.004
	MFP	-1.335	1.221	1.000	-4.826	2.156
	NaF	-4.268	1.233	**0.007***	-7.793	-0.743
	SnF	-3.348	1.245	0.082	-6.910	0.214

*. The mean difference is significant at the 0.05 level.

It can be seen that the mean difference in ΔF of the **F Free** toothpaste group is significantly lower than the mean difference in ΔF of the **NaF** toothpaste group, but this is not the case when compared with **AmF**, **MFP**, and **SnF** as there is no significance.

As for the **NaF** group, its mean difference in ΔF is significantly higher than both **AmF** and **F Free** groups, but there is no significance when compared with **MFP**, and **SnF** groups.

3.1.1.3 The percentage change in ΔF at baseline and after treatment (%ΔF) was calculated using the following formula:

(Difference in ΔF at baseline and after treatment / ΔF at baseline) × 100

(Figure 24) shows the % change in ΔF values for all groups which was highest in the **NaF** toothpaste group at 41.4% and lowest for both **F Free** and **AmF** toothpaste groups 11.7% and 9% respectively. As for the **SnF** and **MFP** they were at 32.8% and 21.1% respectively.

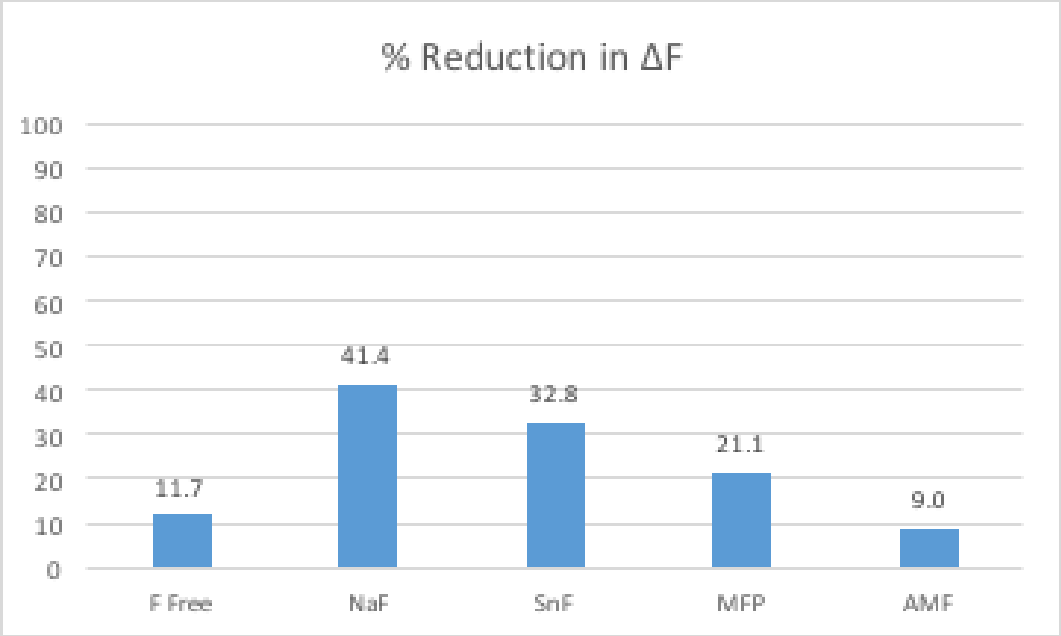


Figure 24 The % ΔF values for all groups.

Determination of the normality of data for Percentage reduction in ΔF:

In order to check if the percentage in reduction of ΔF was normally distributed, data normality test was carried out. This included Shapiro-Wilk test and Kolmogorov-Smirnov test. P value was statistically significant therefore data was not considered normally distributed ($p<0.05$).

The Boxplot (Figure 25) of the percentage of reduction in ΔF showed two outliers in the **NaF** group and a median indicating greater percentage reduction. All other groups were similar in medians, with similar standard deviations and no outliers.

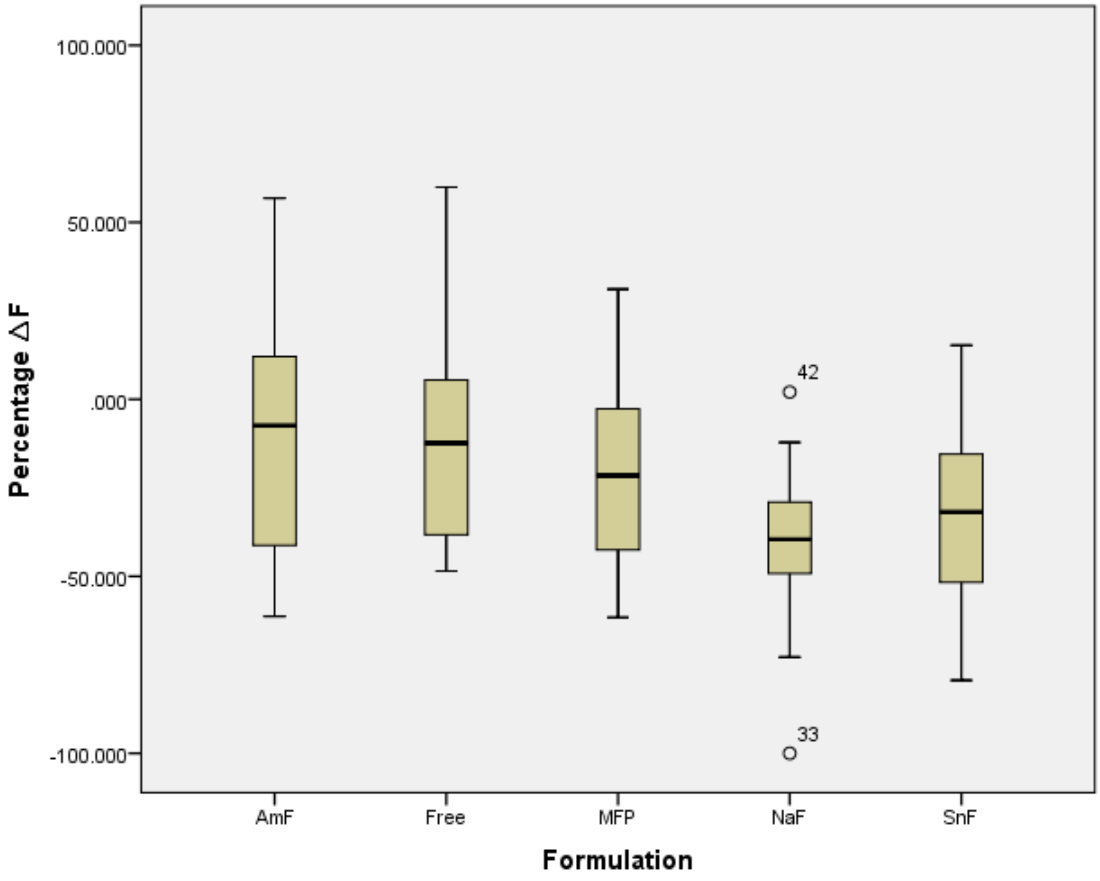


Figure 25 Boxplot for the Percentage of reduction in ΔF for all groups.

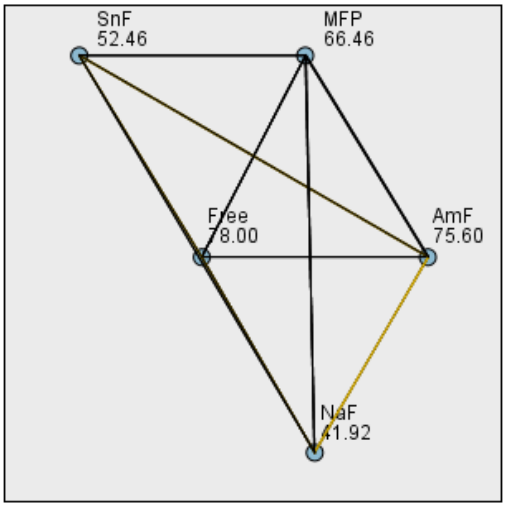
Non parametric Kruskal-Wallis test (Table 10) was performed to assess if the percentage reduction in ΔF was statistically significant between the five groups. It showed that the mean percentage reduction of ΔF was statistically significant between groups ($p<0.05$).

Table 10 Kruskal-Wallis Test results for the Percentage of reduction in ΔF.

	Percentage of reduction in ΔF
Chi-Square	18.043
df	4
Asymp. Sig.	**.001**
a. Kruskal Wallis Test	
b. Grouping Variable: Formulation	

In order to determine which groups were statistically significant different, pairwise comparison was performed (Figure 26). The test showed that **NaF** had a significantly higher percentage of reduction in ΔF when compared to **AmF** and **F Free** groups ($p<0.05$). No other significant differences were found.

Pairwise Comparisons of Formulation



Each node shows the sample average rank of Formulation.

Sample1-Sample2	Test Statistic	Std. Error	Std. Test Statistic	Sig.	Adj.Sig.
NaF-SnF	-10.538	10.353	-1.018	.309	1.000
NaF-MFP	24.542	10.148	2.418	.016	.234
NaF-AmF	33.680	10.247	3.287	.001	.015
NaF-Free	36.080	10.247	3.521	.000	.006
SnF-MFP	14.003	10.255	1.365	.172	1.000
SnF-AmF	23.142	10.353	2.235	.025	.381
SnF-Free	25.542	10.353	2.467	.014	.204
MFP-AmF	9.138	10.148	.901	.368	1.000
MFP-Free	11.538	10.148	1.137	.256	1.000
AmF-Free	-2.400	10.247	-.234	.815	1.000

Each row tests the null hypothesis that the Sample 1 and Sample 2 distributions are the same.
Asymptotic significances (2-sided tests) are displayed. The significance level is .05.

Figure 26 Pairwise comparison between percentages of reduction in ΔF between all formulations

3.1.1.4 Intra-examiner reproducibility for ΔF

The intra-examiner reproducibility was tested using intra-class correlation coefficient. 19 enamel slabs (15%) were randomly selected and re-analysed. The Intra-class correlation coefficient (Table 11) was found to be (0.99) which represents excellent reproducibility.

Table 11 Intra-class Correlation Coefficient for ΔF measurements.

	Intra-class Correlation	95% Confidence Interval		F Test with True Value 0			
		Lower Bound	Upper Bound	Value	df1	df2	Sig
Single Measures	.989	.978	.994	176.103	19	19	.000
Average Measures	.994	.989	.997	176.103	19	19	.000

3.1.2 ΔQ: ΔF times the Area. Percentage fluorescence loss with respect to the fluorescence of sound tissue times the area. Lesion volume

Normality tests (Shapiro-Wilk test and Kolmogorov-Smirnov test) were done for the ΔQ values at baseline and showed that the data were normally distributed for all groups (Appendix 3).

The boxplot (Figure 27) for the distribution of the ΔQ at the baseline showed similar distribution of medians between the groups. **AmF** and **F Free** toothpaste groups had a larger standard deviation than the rest of the groups.

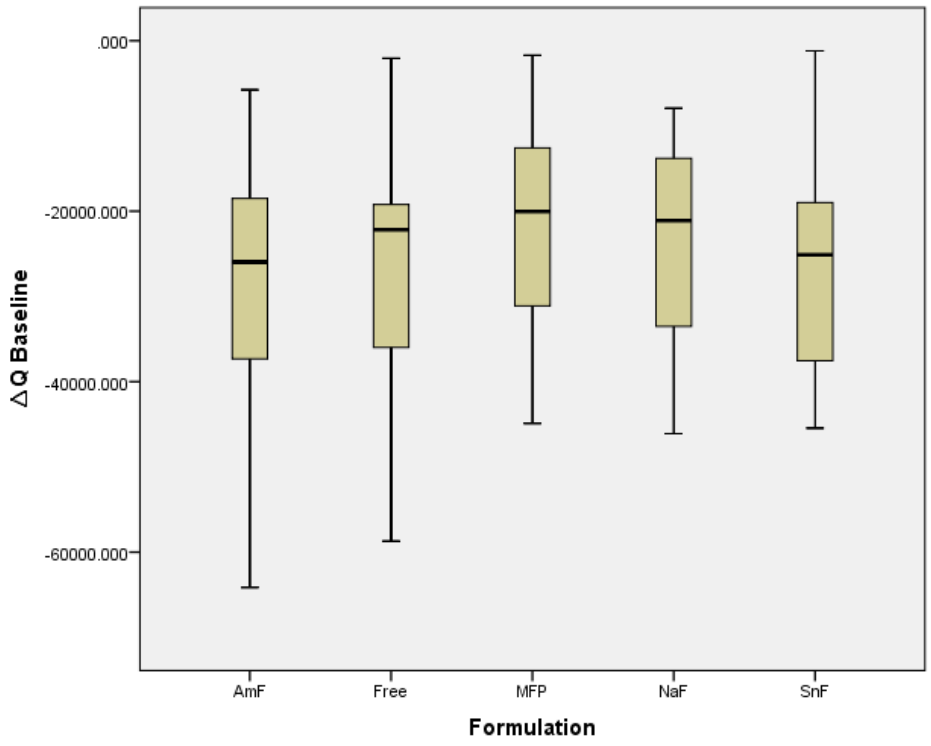


Figure 27 Boxplot for the distribution of the ΔQ values at baseline for all groups.

One way ANOVA test (Table 12) was performed to assess if there was any statistically significant difference in ΔQ at the baseline between the lesions assigned to the five groups. No statistically significant difference was found.

Table 12 One way ANOVA results for ΔQ values at baseline.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	6.53E+8	4	1.63E+8	1.057	0.381
Within Groups	1.9E+10	120	1.54E+8		
Total	1.9E+10				

3.1.2.1 Difference in ΔQ within each group

The ΔQ mean values both at baseline and after treatment are shown in (Table 13). It can be seen that there was an improvement in ΔQ values for all the groups in the study.

Table 13 The mean values of ΔQ at baseline and after treatment for all groups.

Group	Mean ΔQ at baseline ± SD	Mean ΔQ after treatment ± SD	Mean Difference in ΔQ at baseline and after treatment ± SD
F Free	-26468.027 ± 14321.27	-19435.373 ± 8249.20	7032.653 ± 13554.28
NaF	-23800.893 ± 11342.64	-852.733 ± 957.35	22948.160 ± 10979.76
SnF	-26468.292 ± 12189.41	-8936.972 ± 7830.86	17531.319 ± 12108.89
MFP	-21548.813 ± 11257.30	-7290.731 ± 7129.00	14258.082 ± 12823.84
AmF	-27872.920 ± 12803.14	-22636.520 ± 13429.10	5236.400 ± 18679.27

(Figure 28) shows the change in the mean of ΔQ at baseline and after treatment with the standard deviation for all groups.

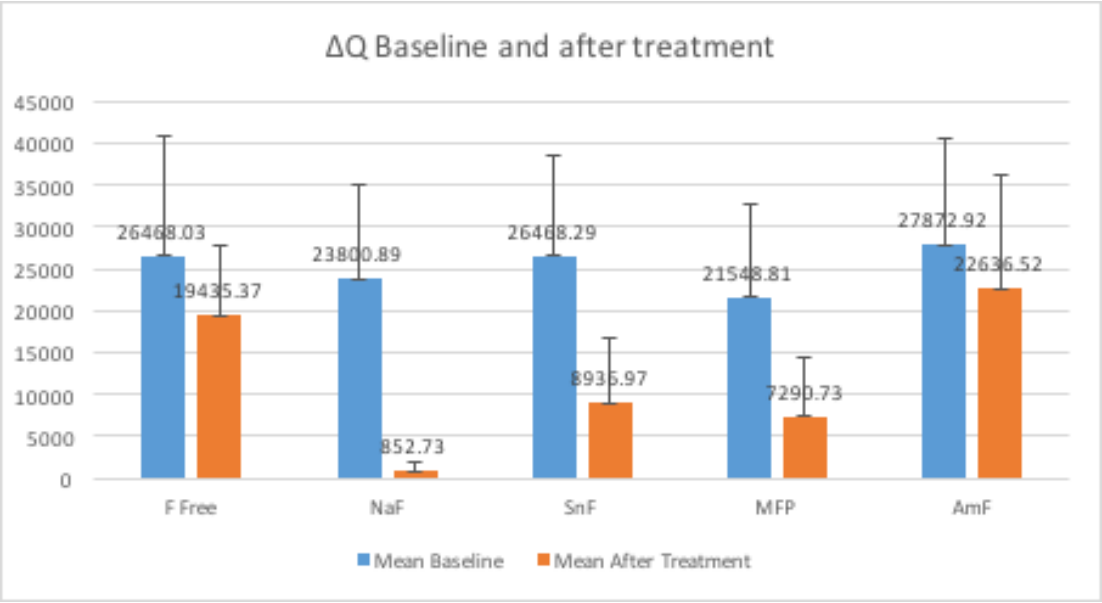


Figure 28 ΔQ mean values at baseline and after treatment for all groups.

To assess whether the change in ΔQ at baseline and after treatment was significantly different within the same group, paired T-Test was carried out and the results (Table 14) showed that there was a statistically significant improvement in the ΔQ values after treatment compared with that at baseline in **Naf**, **SnF** and **MFP** toothpaste groups ($p < 0.001$), but there was no such significance in the **AmF** and **F Free** groups.

Table 14 Paired sampled T-Test results for ΔQ values at baseline and after treatment.

		Paired Differences					Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference		
					Lower	Upper	
F Free	ΔQ at baseline – ΔQ after treatment	7032.653	13554.28	2710.856	1437.721	12627.59	**0.16**
NaF		22948.16	10979.76	2195.953	18415.94	27480.38	**0.000***
SnF		17531.32	12108.89	2471.717	12418.18	22644.45	**0.000***
MFP		14258.08	12823.84	2514.961	9078.422	19437.74	**0.000***
AmF		5236.400	18679.27	3735.854	-2474.02	12946.82	**0.174**

3.1.2.2 Difference in ΔQ between groups

The ΔQ difference (change) was measured using the following formula:

Difference in ΔQ = ΔQ after treatment - ΔQ at baseline

(Figure 29) shows the difference in ΔQ in the five tested groups. In all groups the difference in ΔQ was positive, meaning that there was decrease in ΔQ after treatment compared to that at baseline. The highest reduction in ΔQ was in the **NaF** toothpaste group with a mean difference of 22948.160 ± 10979.76, while the lowest reduction was in the **AmF** group with only 5236.400 ± 18679.27 mean difference.

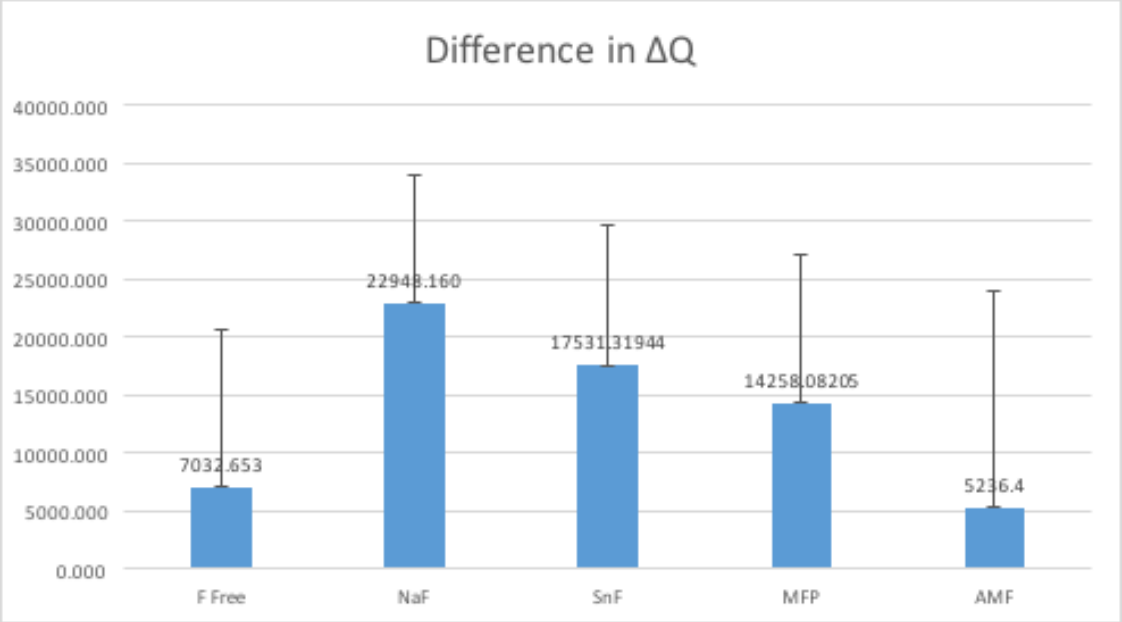


Figure 29 Means of the difference in ΔQ at baseline and after treatment of all groups.

Table 15 Descriptive statistics for the difference in ΔQ at baseline and after treatment for all groups.

Group	N	Minimum	Maximum	Mean	Median	Std. Deviation
F Free	25	-10204.333	37578.667	7032.65333	4418.0000	13554.280763
NaF	25	7813.667	44060.667	22948.16000	20896.33333	10979.763462
SnF	24	-1785.333	41874.667	17531.31944	15983.33333	12108.888777
MFP	26	-10580.000	32893.000	14258.08205	16264.66667	12823.837482
AmF	24	-26172.333	38388.333	5236.40000	6833.00000	18679.267525

Determination of the normality of the data

Shapiro-Wilk test and Kolmogorov-Smirnov test was carried out to check if the difference in ΔQ at baseline and after treatment was normally distributed. The data was considered normally distributed, as there was no statistical significance.

The boxplot (Figure 30) of difference in ΔQ at baseline and after treatment shows similar Medians of **AmF** and **F Free** groups, both at a lower level than **MFP**, **NaF** and **SnF** which also have similar median level. The standard deviation for the **AmF** group is also very wide.

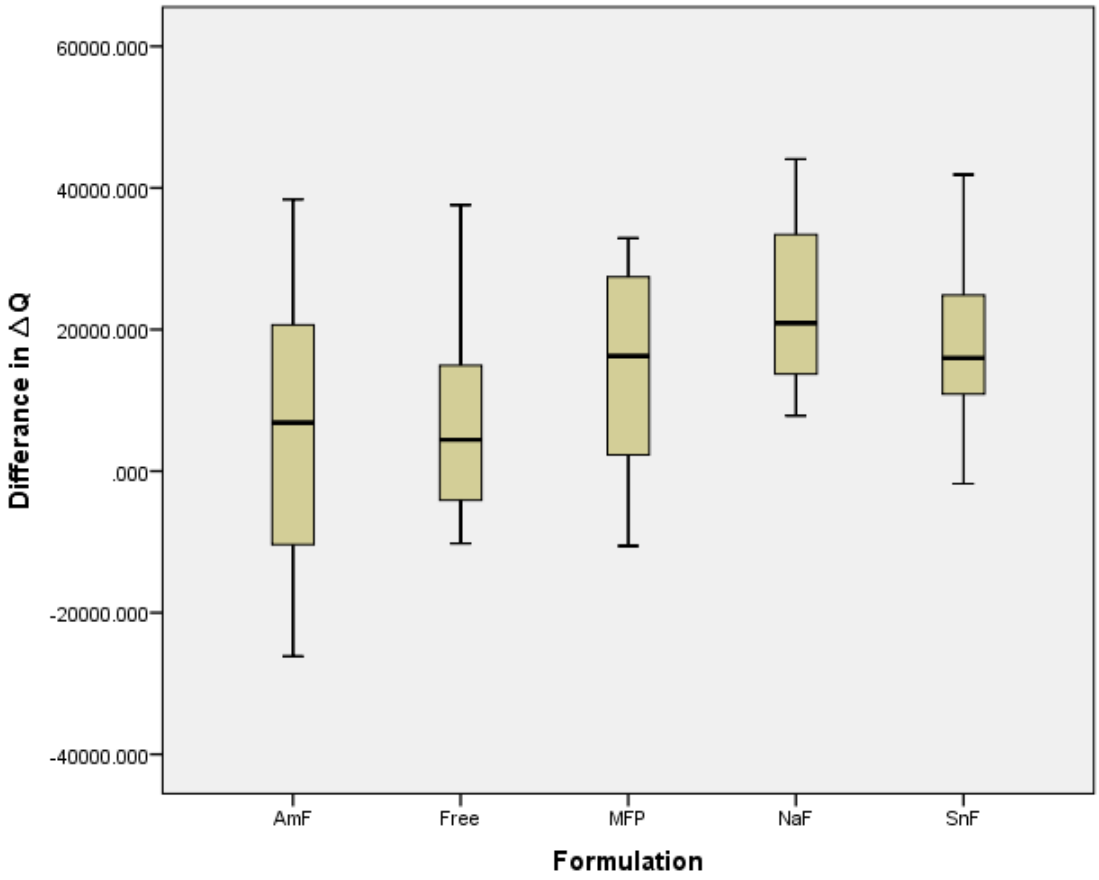


Figure 30 Boxplot for the difference in ΔQ at baseline and after treatment for all groups.

One way ANOVA test (Table 16) was performed to assess if the difference in ΔQ was statistically significant between the five groups. It showed that the mean difference in ΔQ was statistically significant between the groups ($p < 0.001$).

Table 16 One way ANOVA between groups for the difference in ∆Q at baseline and after treatment.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	5387564560.720	4	1346891140.180	6.979	**.000***
Within Groups	23160179936.438	120	193001499.470		
Total	28547744497.158	124			

In order to determine which groups were statistically significant different, pairwise comparisons were conducted using Bonferroni test. The Bonferroni tests corrects for multiple testing. The results of the Bonferroni tests are shown in (Table 17). It can be seen that the mean difference in ΔQ of the **F Free** toothpaste group is significantly lower than the mean difference in ΔQ of the **NaF** toothpaste group but this is not the case when compared with **AmF**, **MFP**, and **SnF** as there is no significance.

As for the **NaF** group, its mean difference in ΔQ is significantly higher than **AmF**, and **F Free** groups only.

SnF group had statistically higher mean difference in ΔQ than **AmF** group only.

Mean difference in ΔQ for the **MFP** group was not statistically significant when compared to all other groups.

Table 17 Multiple comparisons of the difference in ΔQ at baseline and after treatment between all test groups and control.

(I) Group	(J) Group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Free	AmF	1796.253333	3929.391805	1.000	-9441.27617	13033.78283
	MFP	-7225.428718	3891.425776	.658	-18354.38051	3903.52307
	NaF	-15915.506667*	3929.391805	**.001***	-27153.03617	-4677.97717
	SnF	-10498.666111	3970.111979	.093	-21852.64980	855.31758
NaF	AmF	17711.760000*	3929.391805	**.000***	6474.23050	28949.28950
	Free	15915.506667*	3929.391805	**.001***	4677.97717	27153.03617
	MFP	8690.077949	3891.425776	.274	-2438.87384	19819.02974
	SaF	5416.840556	3970.111979	1.000	-5937.14314	16770.82425
SnF	AmF	12294.919444*	3970.111979	**.024***	940.93575	23648.90314
	Free	10498.666111	3970.111979	.093	-855.31758	21852.64980
	MFP	3273.237393	3932.539096	1.000	-7973.29293	14519.76772
	NaF	-5416.840556	3970.111979	1.000	-16770.82425	5937.14314
MFP	AmF	9021.682051	3891.425776	.221	-2107.26974	20150.63384
	Free	7225.428718	3891.425776	.658	-3903.52307	18354.38051
	NaF	-8690.077949	3891.425776	.274	-19819.02974	2438.87384
	SnF	-3273.237393	3932.539096	1.000	-14519.76772	7973.29293
AmF	Free	-1796.253333	3929.391805	1.000	-13033.78283	9441.27617
	MFP	-9021.682051	3891.425776	.221	-20150.63384	2107.26974
	NaF	-17711.760000*	3929.391805	**.000***	-28949.28950	-940.93575
	SnF	-12294.919444*	3970.111979	**.024***	-23648.90314	-940.93575

***. The mean difference is significant at the 0.05 level.**

3.1.2.3 The percentage of the changes in ΔQ at baseline and after treatment (%ΔQ) was calculated using the following formula:

(Difference in ΔQ at baseline and after treatment / ΔQ at baseline) × 100

(Figure 31) shows the % ΔQ change values for all groups with **NaF** group having near 100% reduction in ΔQ, while **AmF** shows a negative reduction percentage -1%. **AmF** group was also showing a very low percentage reduction at 3.1%. **SnF** and MFP were at 53.8% and 31.8% respectively.

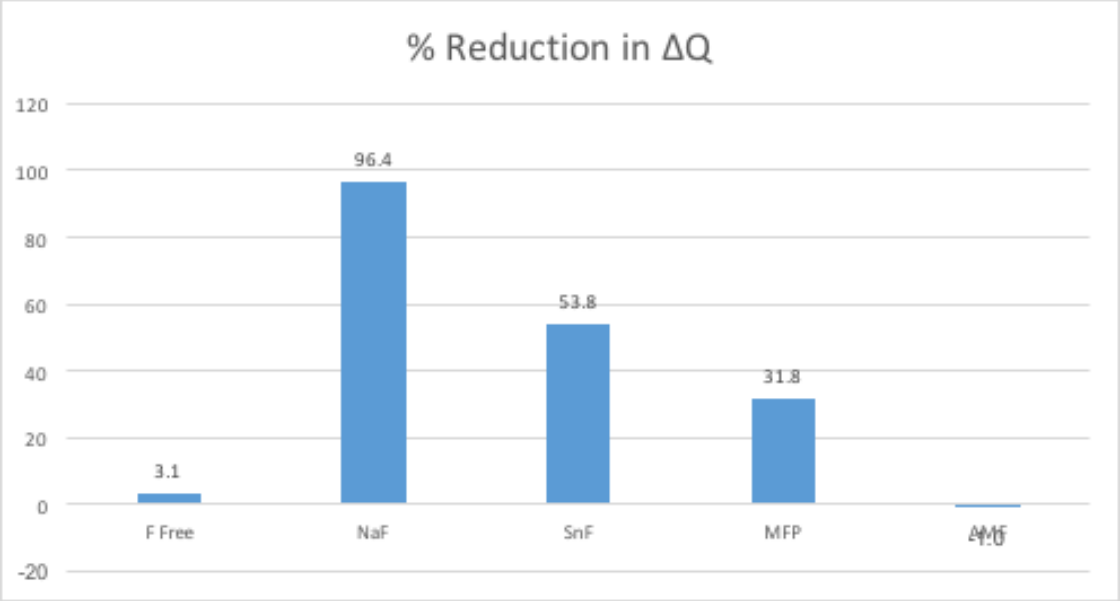


Figure 31 The %ΔQ values for all groups.

Determination of the normality of data for Percentage reduction in ΔQ:

In order to check if the percentage in reduction of ΔQ was normally distributed, data normality test was carried out. This included Shapiro-Wilk test and Kolmogorov-Smirnov test. P value was statistically significant therefore data was not considered normally distributed ($p<0.001$).

The Boxplot (Figure 32) of the percentage of reduction in ΔQ for all groups shows that the data is not normally distributed, and that there are several outliers in all groups except **AmF** group. Also noticeable, was the very narrow distribution of data in the **NaF** group (most of the data was clustered around the median), which was showing a very high percentage of reduction in ΔQ.

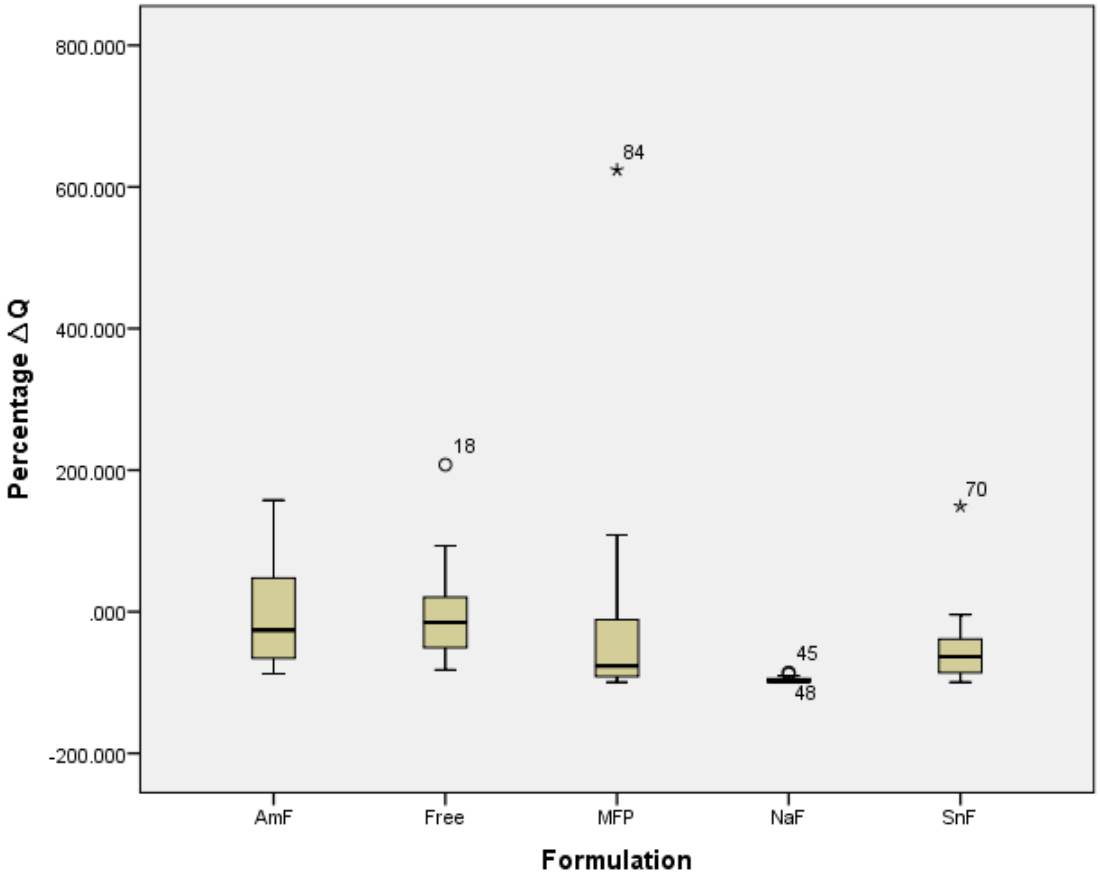


Figure 32 Boxplot for the Percentage of reduction in ΔQ for all groups.

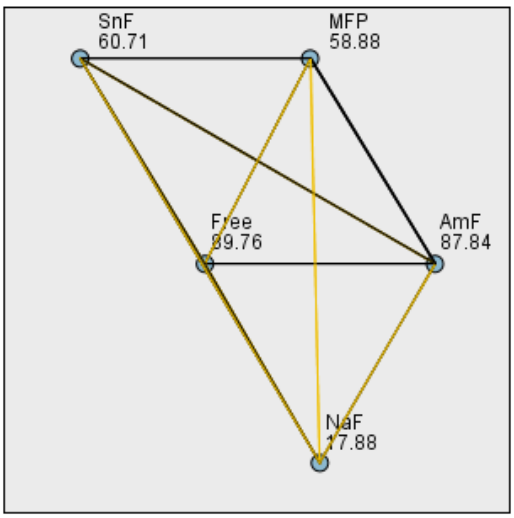
Non parametric Kruskal-Wallis test (Table 18) was performed to assess if the percentage reduction in ΔQ was statistically significant between the five groups. It showed that the mean percentage reduction of ΔQ was statistically significant between groups ($p<0.001$).

Table 18 Kruskal-Wallis Test results for the Percentage of reduction in ΔQ.

	Percentage of reduction in ΔQ
Chi-Square	64.602
df	4
Asymp. Sig.	**0.000**
a. Kruskal Wallis Test	
b. Grouping Variable: Formulation	

In order to determine which groups were statistically significant different, pairwise comparison was performed (). **NaF** group had a statistically higher mean percentage of reduction in ΔQ when compared to all other groups ($p<0.05$). **MFP** group also had a statistically higher mean percentage of reduction in ΔQ when compared to **F Free** group only ($p<0.05$). There was no further statistical significance in any other group.

Pairwise Comparisons of Formulation



Each node shows the sample average rank of Formulation.

Sample1-Sample2	Test Statistic	Std. Error	Std. Test Statistic	Sig.	Adj.Sig.
NaF-MFP	41.005	10.148	4.041	.000	.001
NaF-SnF	-42.828	10.353	-4.137	.000	.001
NaF-AmF	69.960	10.247	6.827	.000	.000
NaF-Free	71.880	10.247	7.015	.000	.000
MFP-SnF	-1.824	10.255	-.178	.859	1.000
MFP-AmF	28.955	10.148	2.853	.004	.065
MFP-Free	30.875	10.148	3.043	.002	.035
SnF-AmF	27.132	10.353	2.621	.009	.132
SnF-Free	29.052	10.353	2.806	.005	.075
AmF-Free	-1.920	10.247	-.187	.851	1.000

Each row tests the null hypothesis that the Sample 1 and Sample 2 distributions are the same.
Asymptotic significances (2-sided tests) are displayed. The significance level is .05.

Figure 33 Pairwise comparison between percentages of reduction in ΔQ between all formulations

3.1.2.4 Intra-examiner reproducibility for ΔQ

The intra-examiner reproducibility was tested using intra-class correlation coefficient. 19 enamel slabs (15%) were randomly selected and re-analysed. The Intra-class Correlation Coefficient (Table 19) was found to be (0.99) which represents excellent reproducibility.

Table 19 Intra-class Correlation Coefficient for ΔQ measurements.

	Intra-class Correlation	95% Confidence Interval		F Test with True Value 0			
		Lower Bound	Upper Bound	Value	df1	df2	Sig
Single Measures	.989	.978	.994	174.246	19	19	.000
Average Measures	.994	.989	.997	174.246	19	19	.000

3.1.3 Area of the white spot lesion

The values of the white spot lesion area at baseline for all groups were checked to see if there was a difference between the groups. The normality tests (Shapiro-Wilk test and Kolmogorov-Smirnov test) showed that the data were normally distributed (Appendix 4).

The boxplot (Figure 34) for the distribution of the area of white spot lesion at the baseline showed very close median values for all groups with one outlier in the **SnF** group. The Standard deviation range for **AmF**, **F Free** and **MFP** groups was also similar. **NaF** Group had the narrowest standard deviation of all groups.

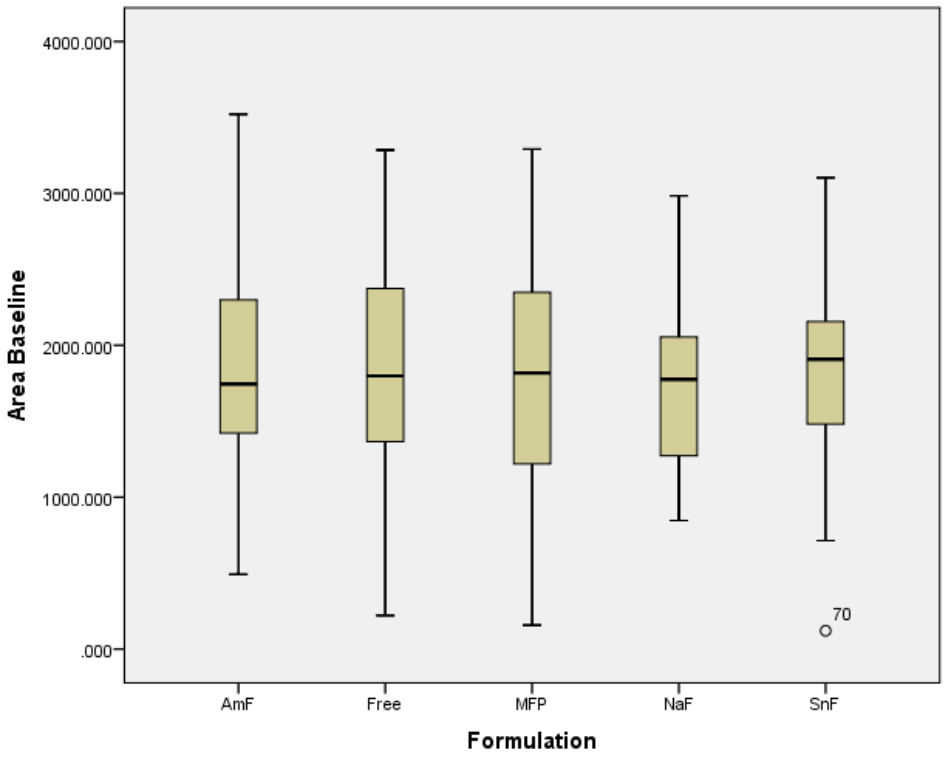


Figure 34 Boxplot for the distribution of the Area values at baseline for all groups.

One way ANOVA test (Table 20) was performed to assess if there was any statistically significant difference in the area values at the baseline between the lesions assigned to the five groups. No statistically significant difference was found.

Table 20 One way ANOVA results for Area values at baseline.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	285761.4	4	71440.34	0.150	0.963
Within Groups	5.71E+7	120	475586.5		
Total	5.74E+7	124			

3.1.3.1 Difference in lesion area within each group

The lesion area mean values both at baseline and after treatment are shown in (Table 21). It can be seen that there was a decrease in the lesion area for all groups in the study.

Table 21 Mean values of Area at baseline and after treatment for all groups.

Group	Mean Area at baseline ± SD	Mean Area after treatment ± SD	Mean Difference in Area at baseline and after treatment ± SD
F Free	1869.253 ± 714.61	1683.200 ± 576.10	-186.053 ± 502.14
NaF	1778.693 ± 575.41	98.987 ± 104.45	-1679.707 ± 548.76
SnF	1842.736 ± 668.68	879.333 ± 628.24	-963.403 ± 879.51
MFP	1743.936 ± 760.12	710.051 ± 617.68	-1033.885 ± 991.53
AmF	1849.400 ± 711.20	1639.707 ± 544.75	-209.693 ± 788.68

(Figure 35) shows the change in the mean of area at baseline and after treatment with the standard deviation for all groups.

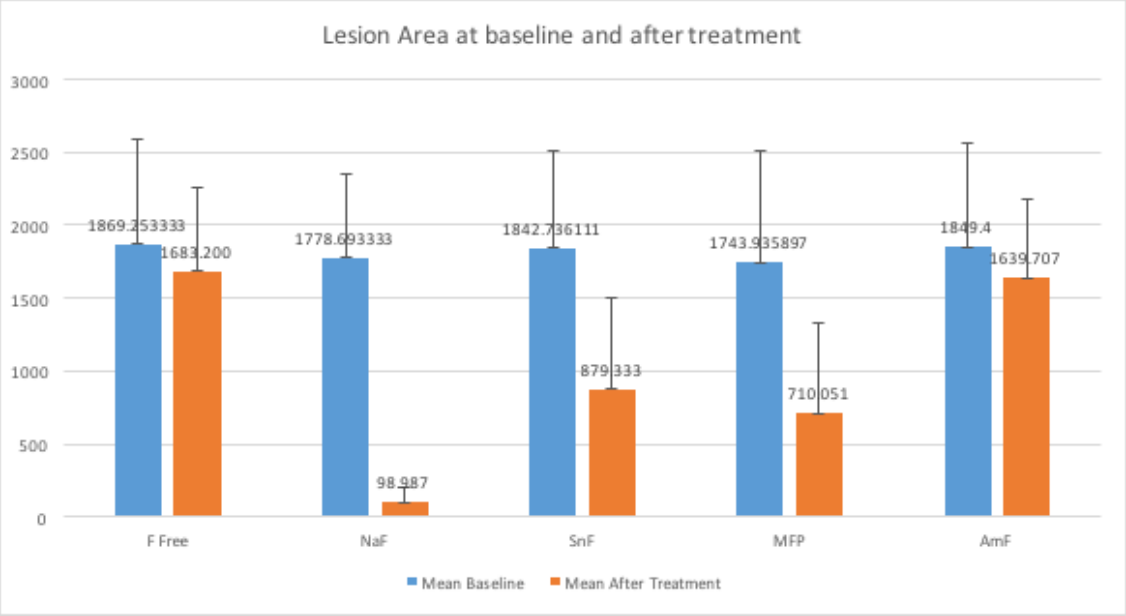


Figure 35 Lesion area at baseline and after treatment for all groups.

To assess whether the change in the area at baseline and after treatment was significantly different within the same group, paired T-Test was used.

The paired T-Test results shown in (Table 22) showed that there was a statistically significant improvement in the lesion area values after treatment compared with that at baseline in **NaF**, **SnF** and **MFP** toothpaste test groups ($p < 0.001$), while there was no significant difference between baseline and after treatment in the **AmF** test group and **F Free** control group.

Table 22 Paired sampled T test results for the lesion area values at baseline and after treatment for all groups.

		Paired Differences					Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference		
					Lower	Upper	
F Free	Area at baseline – Area after treatment	-186.053	502.1382	100.4276	-393.326	21.21913	**0.76**
NaF		-1679.71	548.7572	109.7514	-1906.22	-1453.19	**0.000***
SnF		-963.403	879.5119	179.5296	-1334.79	-592.017	**0.000***
MFP		-1033.88	991.5311	194.4553	-1434.37	-633.397	**0.000***
AmF		-209.693	788.6843	157.7369	-535.246	115.8595	**0.196**

3.1.3.2 Difference in lesion area between groups

The lesion area difference (change) was measured using the following formula:

Difference in lesion area= lesion area after treatment – lesion area at baseline

(Figure 36) shows the difference in the lesion area for the five tested groups. In all test groups, the mean difference was negative indicating a decrease in area of the lesion after treatment compared to that at baseline.

The highest reduction in area size was found in the **NaF** toothpaste group -1679.707 ± 548.76. **SnF** and **MFP** showed similar reduction in lesion size -963.403 ± 879.51 and -

1033.885 ± 991.53 respectively. The lowest reduction in lesion size was in the **F Free** group -186.053 ± 502.14 followed by the **AmF** group at a mean difference of -209.693 ± 788.68.

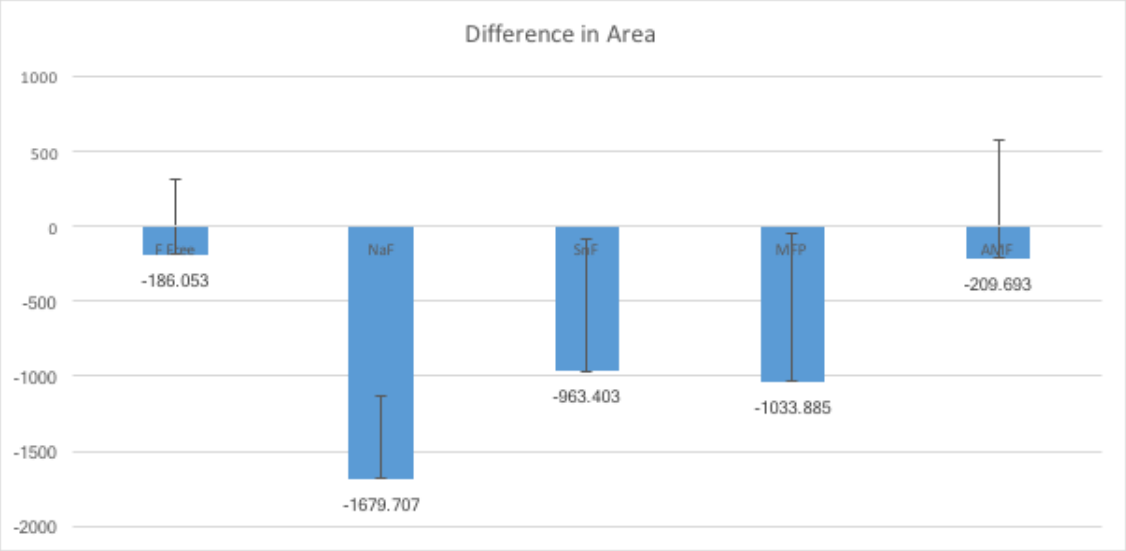


Figure 36 Means of the difference in the lesion area at baseline and after treatment of all tested groups.

Table 23 Descriptive statistics for the difference in Lesion Area at baseline and after treatment for all groups.

Group	N	Minimum	Maximum	Mean	Median	Std. Deviation
F Free	25	-1312.667	600.667	-186.05333	-130.33333	502.138212
NaF	25	-2804.333	269.667	-1679.70667	-1751.66667	548.757210
SnF	24	-2804.333	269.667	-963.40278	-657.00000	879.511947
MFP	26	-3249.000	1189.667	-1033.88462	-1192.33333	991.531134
AmF	25	-1791.667	979.667	-209.69333	-223.66667	788.684282

Determination of the normality of lesion area difference at baseline and after treatment data:

In order to check if the differences in the lesion area at baseline and after treatment were normally distributed, data normality test was carried out. This included Shapiro-Wilk test and Kolmogorov-Smirnov test. The data was considered normally distributed as there was no significance.

The boxplot (Figure 37) of difference in the lesion area at baseline and after treatment shows similar range of median values for all groups with the **MFP** group having the widest standard deviation.

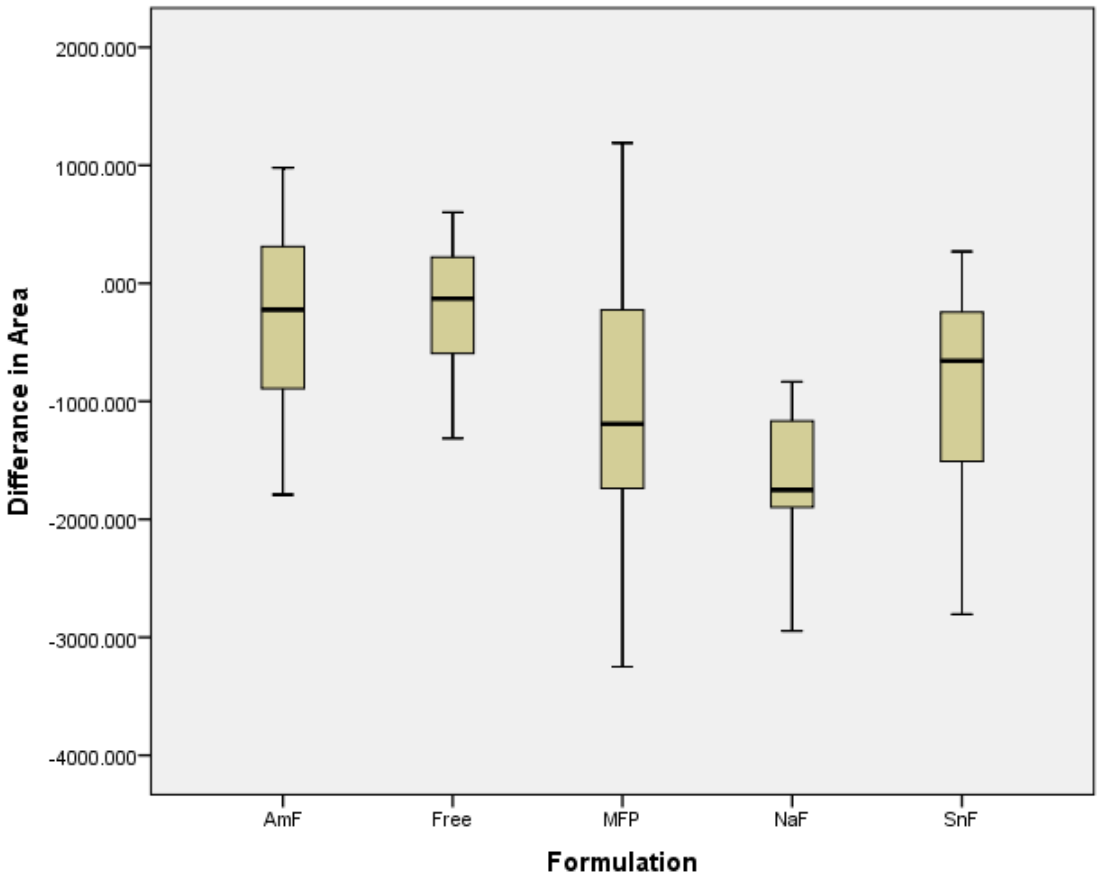


Figure 37 Boxplot for the difference in the lesion Area at baseline and after treatment for all groups

One way ANOVA test (Table 24) was performed to assess if the difference in area was statistically significant between the five groups. It showed that the mean difference in area was statistically significant between the groups ($p < 0.001$).

Table 24 One way ANOVA between groups for the difference in Area at baseline and after treatment.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	39516424.245	4	9879106.061	16.797	**.000***
Within Groups	70577002.631	120	588141.689		
Total	110093426.876	124			

In order to determine which groups were statistically significant different, pairwise comparisons were conducted using Bonferroni test. The Bonferroni tests corrects for multiple testing. The results of the Bonferroni tests are shown in (Table 25). The mean reduction in Area of the lesion was significantly higher in the **NaF** group when compared to all other groups.

The mean reduction in area for the **F Free** group was statistically lower than all other groups except for the **AmF** group.

There was no significance when comparing **SnF** and **MFP** groups.

Table 25 Multiple comparisons of the difference in Area at baseline and after treatment between all test groups and control.

(I) Group	(J) Group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Free	AmF	23.640000	216.913197	1.000	-596.70243	643.98243
	MFP	847.831282*	214.817368	**.001***	233.48264	1462.17992
	NaF	1493.653333*	216.913197	**.000***	873.31091	2113.99576
	SnF	777.349444*	219.161062	**.006***	150.57843	1404.12046
NaF	AmF	-1470.013333*	216.913197	**.000***	-2090.35576	-849.67091
	Free	-1493.653333*	216.913197	**.000***	-2113.99576	-873.31091
	MFP	-645.822051*	214.817368	**.032***	1260.17069	-31.47341
	SnF	-716.303889*	219.161062	**.014***	-1343.07491	-89.53287
SnF	AmF	-753.709444*	219.161062	**.008***	-1380.48046	-126.93843
	Free	-777.349444*	219.161062	**.006***	--1404.12046	-150.57843
	MFP	70.481838	217.086936	1.000	-550.35746	691.32114
	NaF	716.303889*	219.161062	**.014***	89.53287	1343.07491
MFP	AmF	-824.191282*	214.817368	**.002***	-1438.53992	-209.84264
	Free	-847.831282*	214.817368	**.001***	-1462.17992	-233.48264
	NaF	645.822051*	214.817368	**.032***	31.47341	1260.17069
	SnF	-70.481838	217.086936	1.000	-691.32114	550.35746
AmF	Free	-23.640000	216.913197	1.000	-643.98243	596.70243
	MFP	824.191282*	214.817368	**.002***	209.84264	1438.53992
	NaF	1470.013333*	216.913197	**.000***	849.67091	2090.35576
	SnF	753.709444*	219.161062	**.008***	126.93843	1380.48046

***. The mean difference is significant at the 0.05 level.**

3.1.3.3 The percentage change in lesion area at baseline and after treatment (% Area) was calculated using the following formula:

(Difference in area at baseline and after treatment / area at baseline) × 100

(Figure 38) shows the % Area values for all groups, with **NaF** group having the highest % reduction in area of lesion 94.4%, followed by **SnF** and **MFP** Groups at 37.6% and 24.5% respectively. The lowest % reduction in area was located in the **F Free** Group at 0.1% followed closely by **AmF** group at 1.4%.

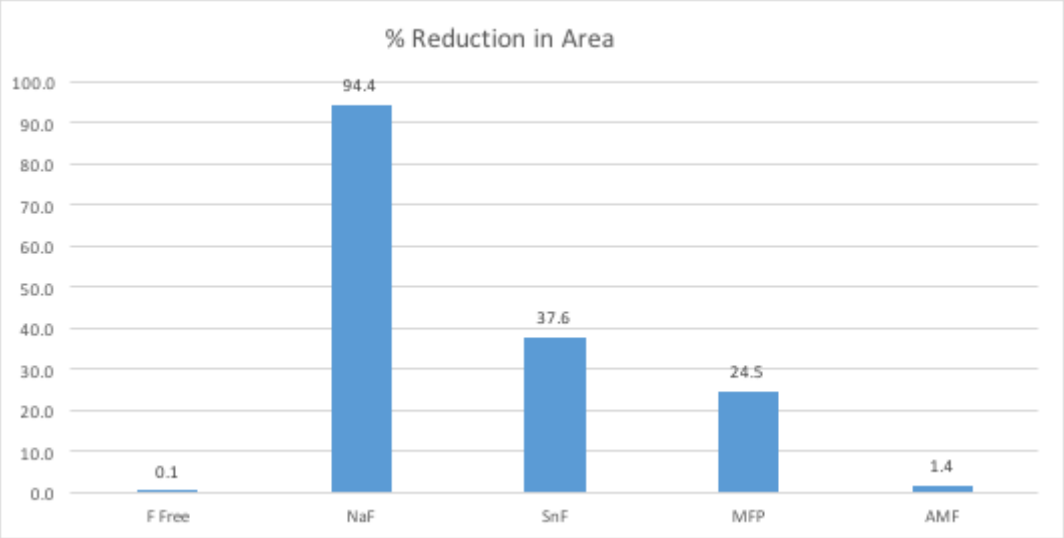


Figure 38 The % Area values for all groups.

Determination of the normality of data for Area:

In order to check if the percentage in reduction of area was normally distributed, data normality test was carried out. This included Shapiro-Wilk test and Kolmogorov-Smirnov test. P value was statistically significant therefore data was not considered normally distributed ($p<0.001$).

The Boxplot (Figure 39) of the percentage of reduction in Area for all groups shows that the data is not normally distributed, and that there are several outliers in all groups. Also noticeable, was the very narrow distribution of data in the **NaF** group (most of the data was clustered around the median), which was showing a very high percentage of reduction in Area.

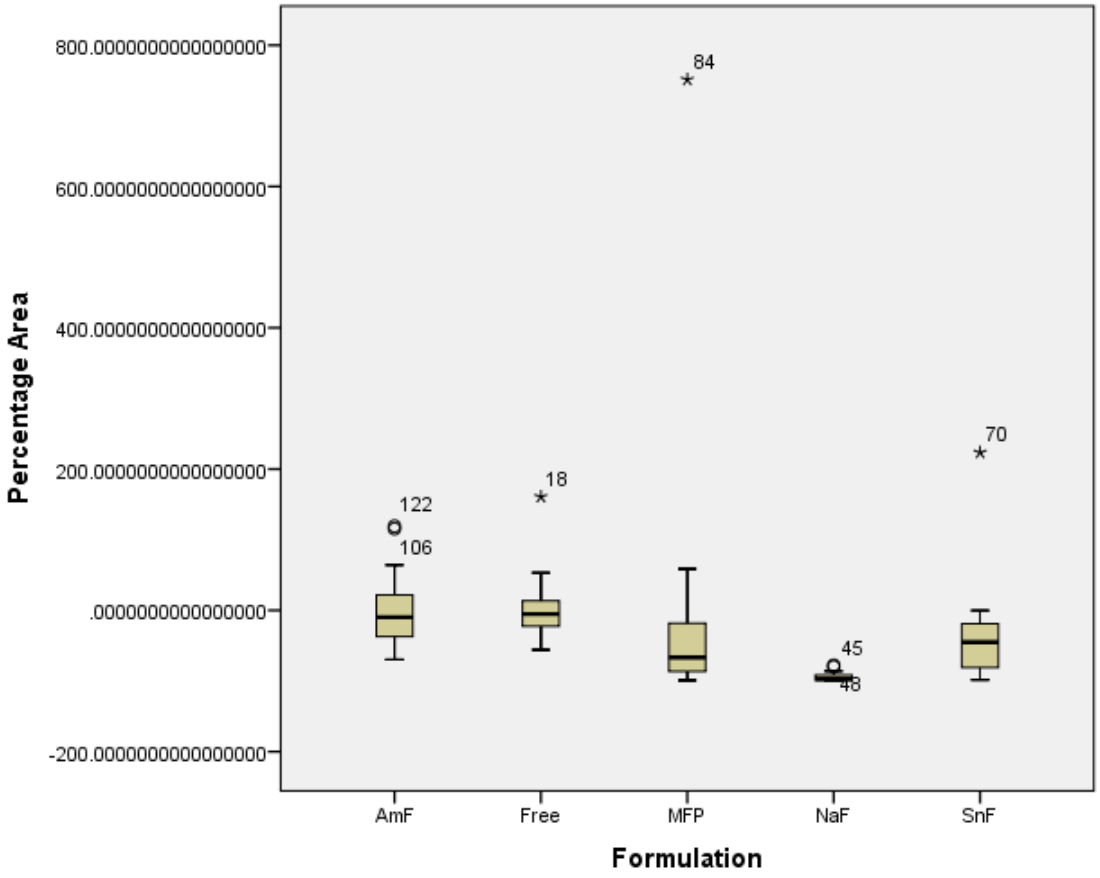


Figure 39 Boxplot for the Percentage of reduction in Area for all groups.

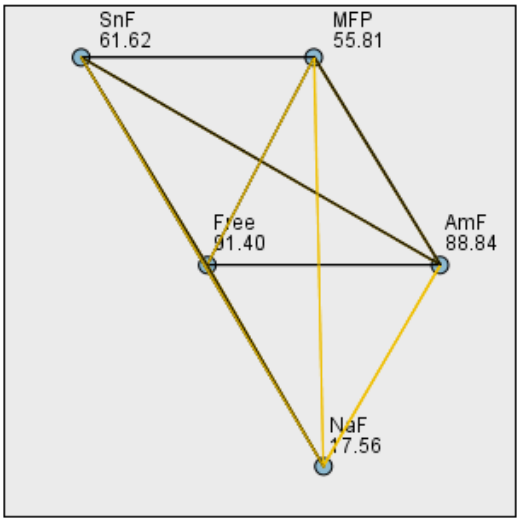
Non parametric Kruskal-Wallis test (Table 26) was performed to assess if the percentage reduction in area was statistically significant between the five groups. It showed that the mean percentage reduction of area was statistically significant between groups ($p<0.001$).

Table 26 Kruskal-Wallis Test results for the Percentage of reduction in Area.

	Percentage of reduction in Area
Chi-Square	68.470
df	4
Asymp. Sig.	**0.000**
a. Kruskal Wallis Test	
b. Grouping Variable: Formulation	

In order to determine which groups were statistically significant different, pairwise comparison was performed (Figure 40). **NaF** group had a statistically higher mean percentage of reduction in Area when compared to all other groups ($p<0.05$). **MFP** group also had a statistically higher mean percentage of reduction in Area when compared to **AmF**, and **F Free** groups ($p<0.05$). There was no further statistical significance in any other group.

Pairwise Comparisons of Formulation



Each node shows the sample average rank of Formulation.

Sample1-Sample2	Test Statistic	Std. Error	Std. Test Statistic	Sig.	Adj.Sig.
NaF-MFP	38.248	10.148	3.769	.000	.002
NaF-SnF	-44.065	10.353	-4.256	.000	.000
NaF-AmF	71.280	10.247	6.956	.000	.000
NaF-Free	73.840	10.247	7.206	.000	.000
MFP-SnF	-5.817	10.255	-.567	.571	1.000
MFP-AmF	33.032	10.148	3.255	.001	.017
MFP-Free	35.592	10.148	3.507	.000	.007
SnF-AmF	27.215	10.353	2.629	.009	.129
SnF-Free	29.775	10.353	2.876	.004	.060
AmF-Free	-2.560	10.247	-.250	.803	1.000

Each row tests the null hypothesis that the Sample 1 and Sample 2 distributions are the same.
Asymptotic significances (2-sided tests) are displayed. The significance level is .05.

Figure 40 Pairwise comparison between percentages of reduction in Area between all formulations.

3.1.3.4 Intra-examiner reproducibility for Area of the white spot lesion

The intra-examiner reproducibility was tested using intra-class correlation coefficient. 19 enamel slabs (15%) were randomly selected and re-analysed. The Intra-class Correlation Coefficient (Table 27) was found to be (0.99) which represents excellent reproducibility.

Table 27 Intra-class Correlation Coefficient for Area measurements.

	Intra-class Correlation	95% Confidence Interval		F Test with True Value 0			
		Lower Bound	Upper Bound	Value	df1	df2	Sig
Single Measures	.992	.983	.996	230.060	19	19	.000
Average Measures	.996	.992	.998	230.060	19	19	.000

3.1.4 Summary of the results for all three parameters for phase A:

The ΔF results: the results showed that there was statistically significant improvement in the ΔF values between baseline and after treatment for all groups except **AmF** group. The greatest improvement was seen in the **NaF** toothpaste group, closely followed by **SnF** toothpaste group, then **MFP** and finally **fluoride free**.

When comparing the groups against each other, the difference in ΔF at baseline and after treatment in NaF group was statistically significantly higher than F Free and AmF groups. No other statistical significance was found.

The ΔQ results: A statistically significant improvement in the ΔQ values between baseline and after treatment was found in **NaF**, **SnF** and **MFP** toothpaste groups, but there was no such significance in the **AmF** and **F Free** groups. The highest improvement was achieved by the **NaF** group followed by the **SnF** and finally the **MFP** group.

When comparing the groups against each other, the difference in ΔQ at baseline and after treatment was statistically significantly higher in the **NaF** group when compared to **AmF** and **F Free** toothpaste groups only. **SnF** group was significantly higher than **AmF** group only. No other statistical significance could be found.

For the lesion area results: A significant reduction in the area of the white spot lesion was seen in the **NaF**, **MFP** and **SnF** groups in descending order. Reduction in area of lesion for the **AmF** and **F Free** groups was not significant.

When comparing the groups against each other, the difference in the lesion area at baseline and after treatment was statistically significant in **NaF** group when compared to all other groups. All groups showed statistical significance when compared to **F Free** group except **AmF** group. There was no statistical significance between **SnF** and **MFP** groups.

3.2 Quantitative Light-Induced Fluorescence (QLF) Results for phase B: Different Sodium fluoride (NaF) concentration toothpastes containing six groups (0 ppm, 500 ppm, 1000 ppm, 1450 ppm, 2800 ppm, 5000 ppm)

Three main parameters for QLF were statistically analysed, these were:

- ΔF: Percentage fluorescence loss with respect to the fluorescence of sound tooth tissue. Related to lesion depth (%).
- ΔQ: ΔF times the Area. Percentage fluorescence loss with respect to the fluorescence of sound tissue times the area. This is related to lesion volume (%px2).
- Area: The surface area of the lesion expressed in pixels2 (px2).

3.2.1 The mean fluorescence loss ΔF:

The values of ΔF at baseline for all groups were checked to see if there was a difference between the groups. The normality tests (Shapiro-Wilk test and Kolmogorov-Smirnov test) were carried out to check the normality of the data (Appendix 5). The data were considered normally distributed if the p values from these tests were not statistically significant ($p > 0.05$). p values for **500 ppm, 1450 ppm,** and **5000 ppm** groups were statistically significant, therefore data was not considered to be normally distributed, and required a non-parametric test to assess baseline distribution of slabs.

The boxplot (Figure 41) for the distribution of ΔF at the baseline showing that the **5000 ppm** group was heavily skewed, while all the other groups were somewhat similar in distribution.

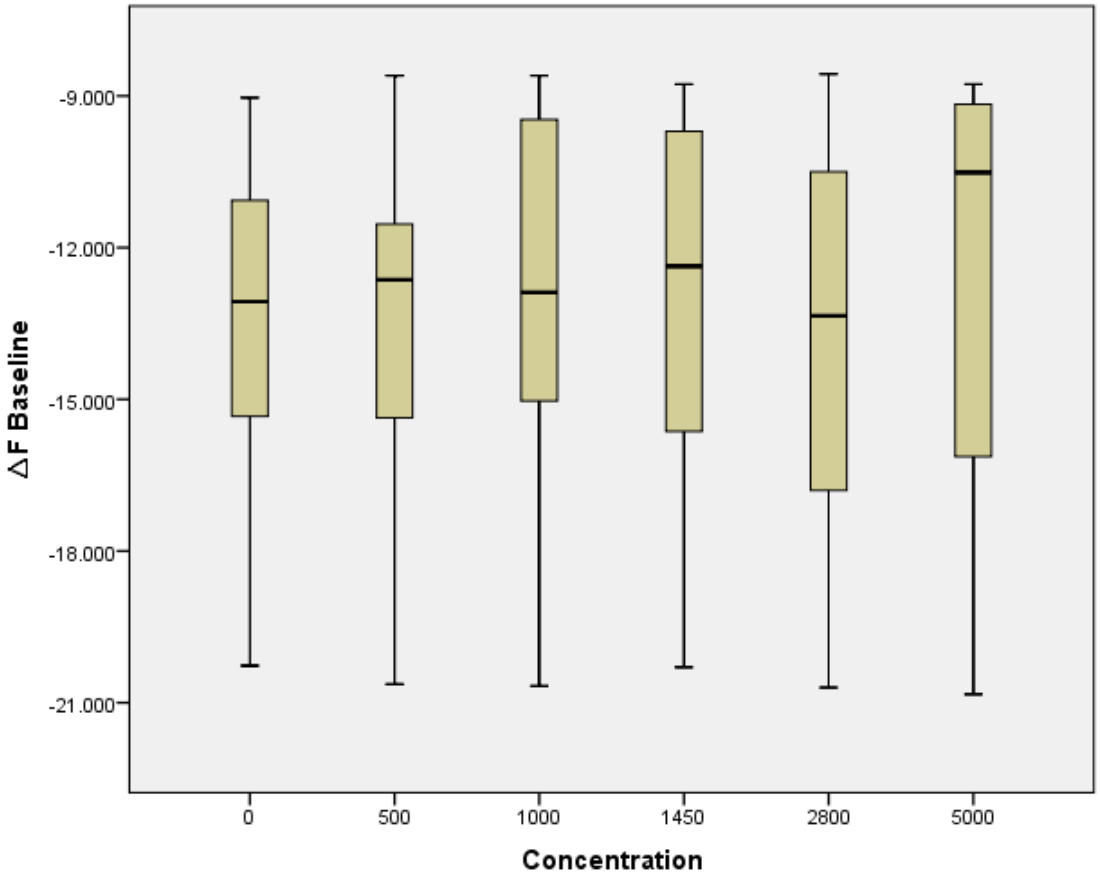


Figure 41 Boxplot for the distribution of the ΔF values at baseline for all groups. Error bars represent SD, the line in the box of Box-and-whisker plot is the median value of the data.

Kruskal-Wallis test (Table 28) was performed to assess if there was any statistically significant difference in ΔF values at the baseline between the lesions assigned to the six groups. No statistically significant difference was found.

Table 28 Kruskal-Wallis Test between groups for ΔF values at baseline

	ΔF at Baseline
Chi-Square	5.803
df	5
Asymptomatic Significance	0.326
a. Kruskal Wallis Test	
b. Grouping Variable: Formulation	

3.2.1.1 Difference in ΔF within each group

The ΔF mean values both at baseline and after treatment are shown in (Table 29). It can be seen that there was an improvement in ΔF values for all the groups in the study.

Table 29 The mean values of ΔF at baseline and after treatment for all groups.

Group	Mean ΔF at baseline ± SD	Mean ΔF after treatment ± SD	Mean Difference in ΔF at baseline and after treatment ± SD
0 ppm	-13.623 ± 3.39	-11.412 ± 2.54	2.211 ± 3.98
500 ppm	-13.504 ± 3.17	-7.553 ± 2.51	5.951 ± 3.81
1000 ppm	-12.641 ± 3.22	-7.899 ± 2.24	4.742 ± 3.44
1450 ppm	-13.375 ± 3.93	-7.417 ± 2.50	5.957 ± 4.11
2800 ppm	-13.740 ± 3.63	-7.879 ± 1.95	5.860 ± 3.84
5000 ppm	-12.160 ± 3.63	-6.700 ± 1.95	5.460 ± 3.78

(Figure 42) shows the change in the mean of ΔF at baseline and after treatment with the standard deviation for all groups.

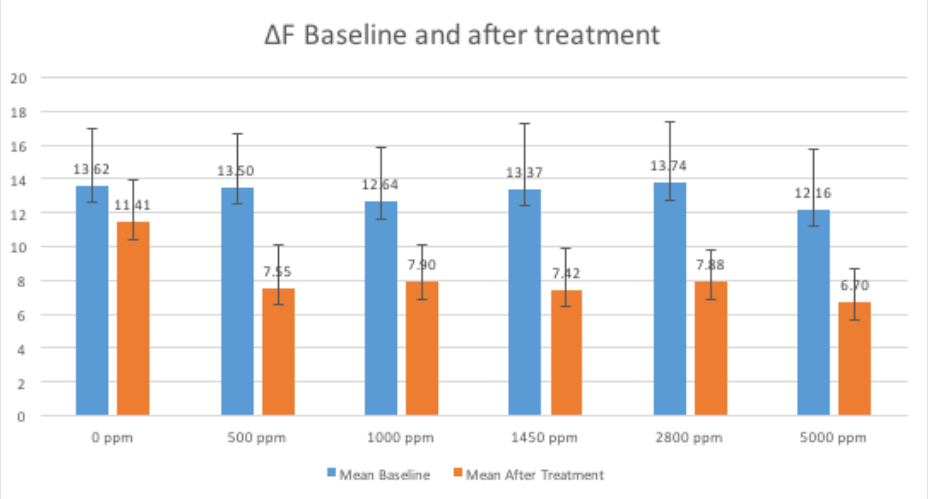


Figure 42 ΔF mean values at baseline and after treatment for all groups.

To assess whether the change in ΔF at baseline and after treatment was significantly different within the same group, paired T-Test was used. The results of the paired T-Test are shown in (Table 30).

It can be seen that there was a statistically significant improvement in the ΔF values after treatment compared with that at baseline in all groups ($p < 0.001$).

Table 30 Paired sampled T-Test results for ΔF values at baseline and after treatment.

		Paired Differences					Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference		
					Lower	Upper	
0 ppm	ΔF at baseline – ΔF after treatment	2.210	3.977	0.795	0.568	3.852	**0.010***
500 ppm		5.951	3.809	0.747	4.412	7.489	**0.000***
1000 ppm		4.742	3.442	0.675	3.351	6.132	**0.000***
1450 ppm		5.957	4.113	0.822	4.259	7.655	**0.000***
2800 ppm		5.860	3.837	0.752	4.310	7.410	**0.000***
5000 ppm		5.460	3.777	0.740	3.934	6.986	**0.000***

3.2.1.2 Difference in ΔF between all groups:

The ΔF difference (change) was measured using the following formula:

Difference in ΔF = ΔF after treatment - ΔF at baseline

(Figure 43) shows the difference in ΔF in the six tested groups. In all groups the difference in ΔF was positive, meaning that there was decrease in ΔF (mean fluorescence loss) after treatment compared to that at baseline. The reduction in ΔF was almost identical in the **500, 1450**, and **2800 ppm** groups (5.951 ± 3.81) (5.957 ± 4.11) (5.860 ± 3.84) respectively. As for the **1000**, and **5000 ppm** groups, they were slightly lower and more similar with the former being at (4.742 ± 3.44) and the latter at (5.460 ± 3.78). The lowest reduction in ΔF was found in the **0 ppm** Group showing only (2.211 ± 3.98) mean reduction (Table 31).

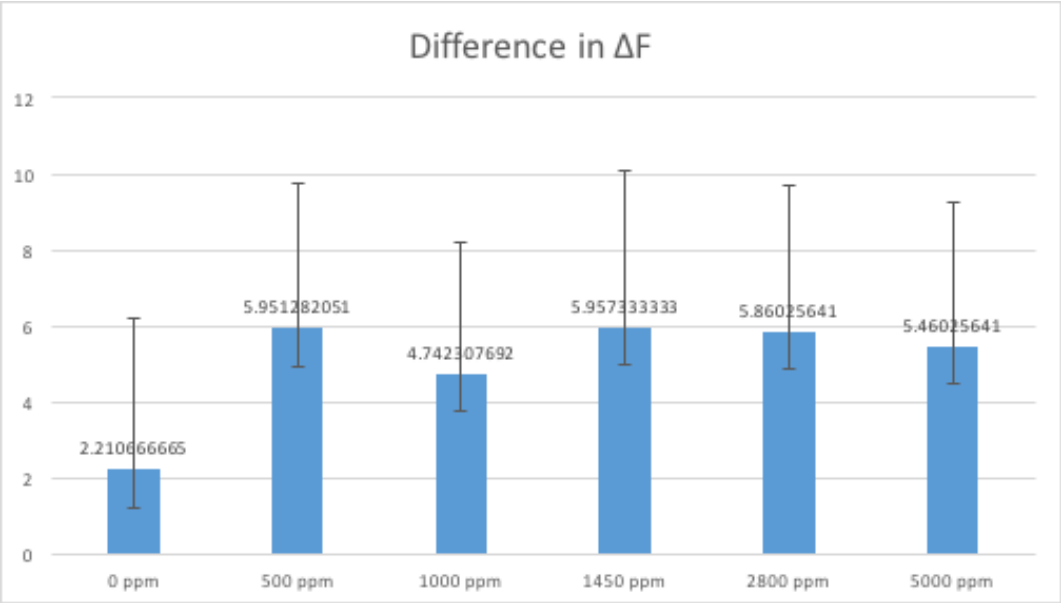


Figure 43 Means of the difference in ΔF at baseline and after treatment of all groups.

Table 31 Descriptive statistics for the difference in ΔF at baseline and after treatment for all groups

Group	N	Minimum	Maximum	Mean	Median	Std. Deviation
0 ppm	25	-6.033	9.067	2.210	1.366	1.366
500 ppm	26	-1.867	13.467	1.36667	1.36667	3.809290
1000 ppm	26	-2.867	11.833	4.74231	4.43333	3.442771
1450 ppm	25	-0.200	13.867	5.95733	4.50000	4.113151
2800 ppm	26	-0.567	12.733	5.86026	4.88333	3.837036
5000 ppm	26	-0.500	13.200	5.46026	4.68333	3.777666

Determination of the normality of the data for Difference in ΔF:

In order to check if the ΔF differences between the baseline and after treatment were normally distributed, data normality test was carried out. This included Shapiro-Wilk test and Kolmogorov-Smirnov test. p value was not statistically significant therefore data was considered to be normally distributed ($p > 0.05$).

The boxplot (Figure 44) of difference in ΔF at baseline and after treatment showed normal distribution of data in all groups. The boxplot also shows 0 ppm group having the lowest median value when compared to all other test groups.

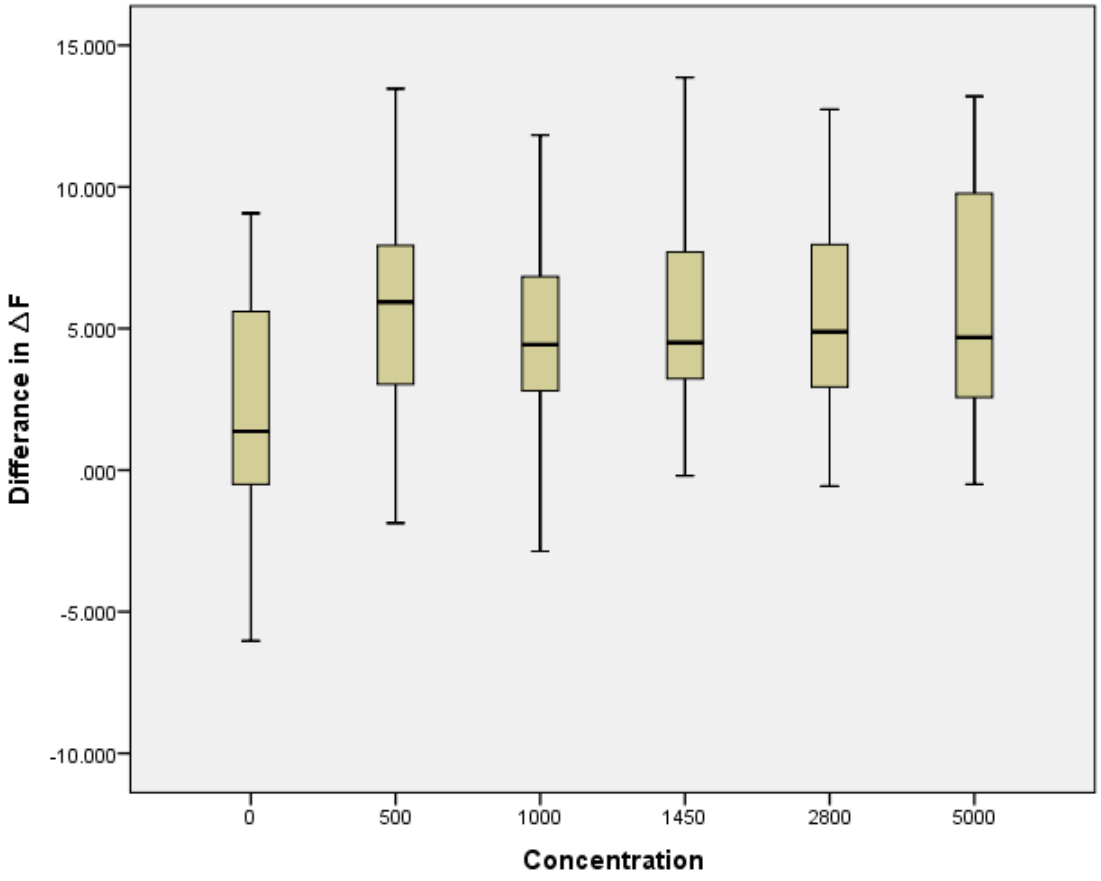


Figure 44 Boxplot for the difference in ΔF at baseline and after treatment for all groups.

One way ANOVA test (Table 32) was performed to assess if the difference in ΔF was statistically significant between the six groups. It showed that the mean difference in ΔF was statistically significant between the groups ($p < 0.05$).

Table 32 One way ANOVA between groups for the difference in ΔF at baseline and after treatment.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	267.145	5	53.429	3.645	0.004*
Within Groups	2169.647	148	14.660		
Total	2436.792	153			

In order to determine which groups were statistically significant different, pairwise comparisons were conducted using a Bonferroni test. The Bonferroni tests corrects for multiple testing. The results of the Bonferroni tests are shown in (Table 33)

Table 33 Multiple comparisons of the difference in ΔF at baseline and after treatment between all test groups and control with Bonferroni correction.

(I) Group	(J) Group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
0 ppm	500	-3.740615*	1.072487	**.010***	-6.94048	-.54075
	1000	-2.531641	1.072487	.293	-5.73151	.66823
	1450	-3.746667*	1.082951	**.011***	-6.97775	-.51558
	2800	-3.649590*	1.072487	**.013***	-6.84946	-.44972
	5000	-3.249590*	1.072487	**.043***	-6.44946	-.04972
500 ppm	0	3.740615*	1.072487	**.010***	.54075	6.94048
	1000	1.208974	1.061920	1.000	-1.95937	4.37732
	1450	-.006051	1.072487	1.000	-3.20592	3.19382
	2800	.091026	1.061920	1.000	-3.07732	3.25937
	5000	.491026	1.061920	1.000	-2.67732	3.65937
1000 ppm	0	2.531641	1.072487	.293	-.66823	5.73151
	500	-1.208974	1.061920	1.000	-4.37732	1.95937
	1450	-1.215026	1.072487	1.000	-4.41489	1.98484
	2800	-1.117949	1.061920	1.000	-4.28629	2.05039
	5000	-.717949	1.061920	1.000	-3.88629	2.45039
1450 ppm	0	3.746667*	1.082951	**.011***	.51558	6.97775
	500	.006051	1.072487	1.000	-3.19382	3.20592
	1000	1.215026	1.072487	1.000	-1.98484	4.41489
	2800	.097077	1.072487	1.000	-3.10279	3.29695

	5000	.497077	1.072487	1.000	-2.70279	3.69695
2800 ppm	0	3.649590*	1.072487	**.013***	.44972	6.84946
	500	-.091026	1.061920	1.000	-3.25937	3.07732
	1000	1.117949	1.061920	1.000	-2.05039	4.28629
	1450	-.097077	1.072487	1.000	-3.29695	3.10279
	5000	.400000	1.061920	1.000	-2.76834	3.56834
5000 ppm	0	3.249590*	1.072487	**.043***	.04972	6.44946
	500	-.491026	1.061920	1.000	-3.65937	2.67732
	1000	.717949	1.061920	1.000	-2.45039	3.88629
	1450	-.497077	1.072487	1.000	-3.69695	2.70279
	2800	-.400000	1.061920	1.000	-3.56834	2.76834
***. The mean difference is significant at the 0.05 level.**						

Table 33 (continued).

It can be seen that the mean difference in ΔF of the **0 ppm (Control)** toothpaste group is significantly lower than the mean difference in ΔF of all other test groups except for **1000 ppm** toothpaste. As for the significance between different concentrations of NaF fluoride toothpaste, there was no significance in any of the groups.

3.2.1.3 The percentage change in ΔF at baseline and after treatment (%ΔF) was calculated using the following formula:

(Difference in ΔF at baseline and after treatment / ΔF at baseline) × 100

(Figure 45) shows the % change in ΔF values for all groups which was identical at 41.7% in both the **500 ppm** and **5000 ppm** groups. The lowest % change in ΔF values was found in the control **0 ppm** group at 11.7%.

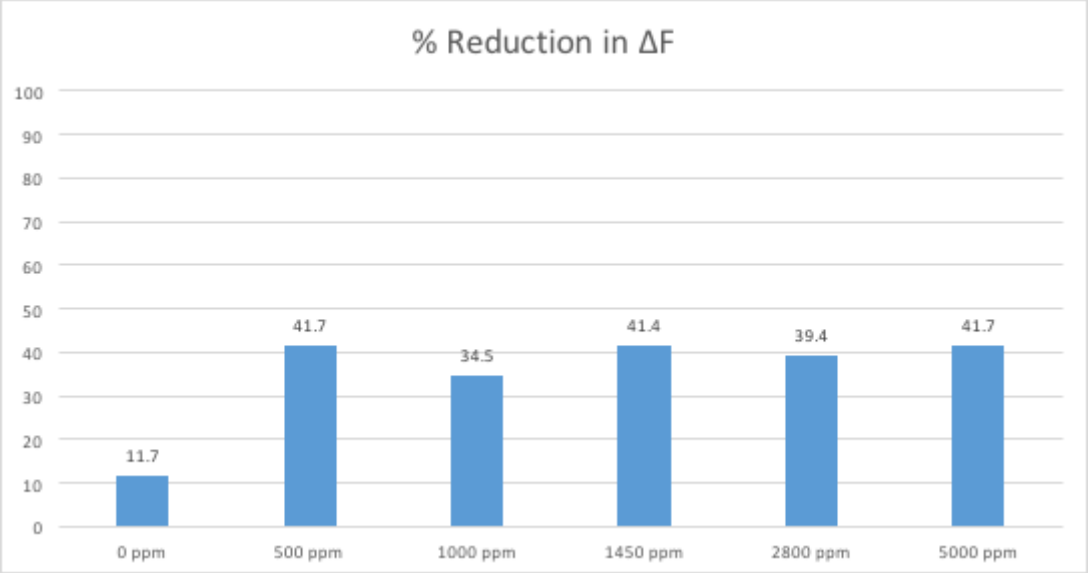


Figure 45 The % ΔF values for all groups.

Determination of the normality of data for Percentage reduction in ΔF:

In order to check if the percentage in reduction of ΔF was normally distributed, data normality test was carried out. This included Shapiro-Wilk test and Kolmogorov-Smirnov test. p value was statistically significant therefore data was not considered normally distributed ($p<0.05$).

The Boxplot (Figure 46) of the percentage of reduction in ΔF showed one outlier in the **500 ppm** group and 2 outliers in the 1450 ppm group. All groups showed similar medians except for 0 ppm group which had a lower percentage reduction in ΔF.

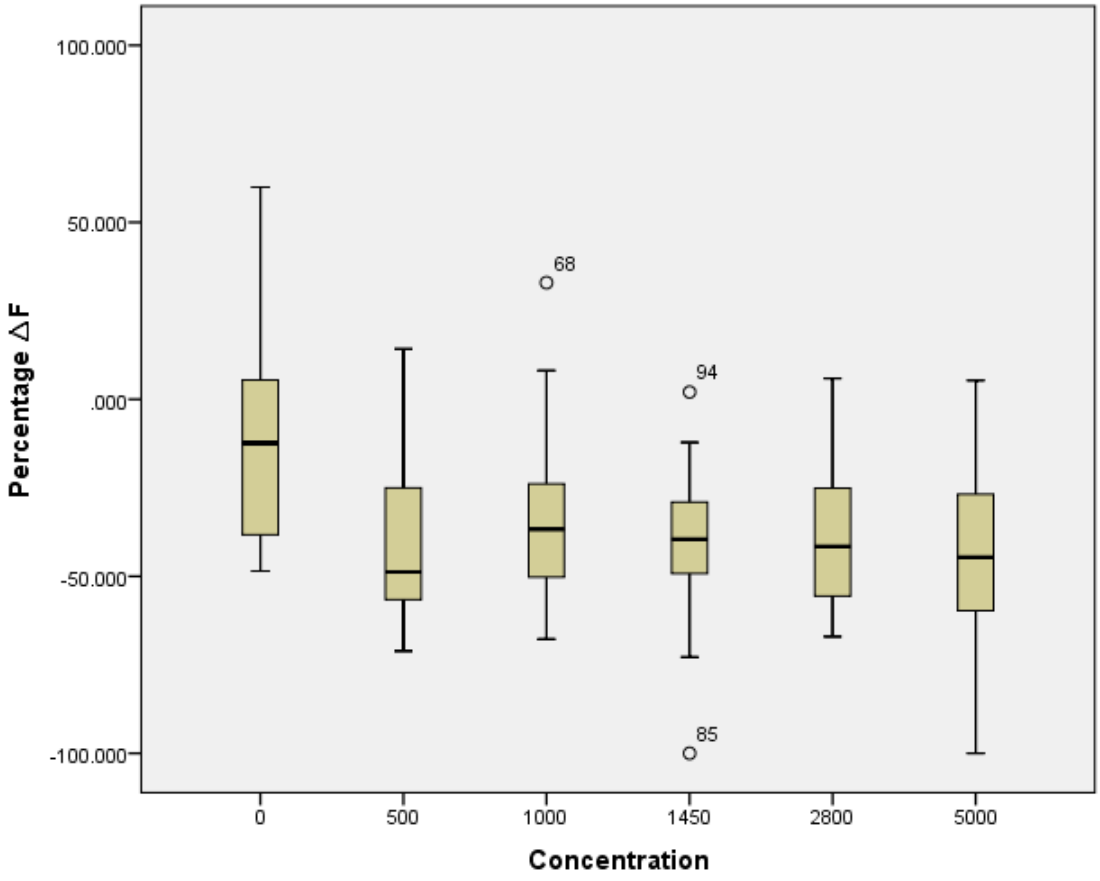


Figure 46 Boxplot for the Percentage of reduction in ΔF for all groups.

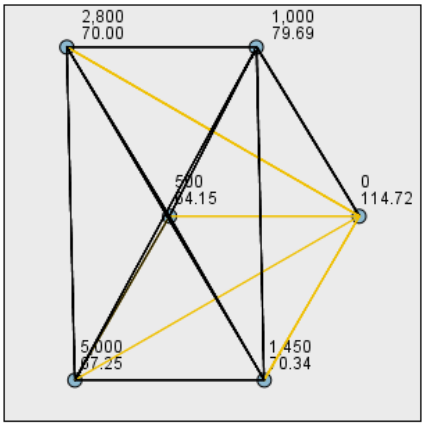
Non parametric Kruskal-Wallis test (Table 34) was performed to assess if the percentage reduction in ΔF was statistically significant between the five groups. It showed that the mean percentage reduction of ΔF was statistically significant between groups ($p<0.05$).

Table 34 Kruskal-Wallis Test results for the Percentage of reduction in ΔF.

	Percentage of reduction in ΔF
Chi-Square	13.421
df	5
Asymp. Sig.	**.020**
a. Kruskal Wallis Test	
b. Grouping Variable: Formulation	

In order to determine which groups were statistically significant different, pairwise comparison was performed (Figure 47). The test showed significant % reduction in ΔF for all test groups when compared to **0 ppm** control group except for the **1000 ppm** group which showed no significant difference. No other significant differences were found.

Pairwise Comparisons of Concentration



Each node shows the sample average rank of Concentration.

Sample1-Sample2	Test Statistic	Std. Error	Std. Test Statistic	Sig.	Adj.Sig.
500-5,000	-3.096	12.370	-.250	.802	1.000
500-2,800	-5.846	12.370	-.473	.636	1.000
500-1,450	-6.186	12.493	-.495	.620	1.000
500-1,000	-15.538	12.370	-1.256	.209	1.000
500-0	50.566	12.493	4.048	.000	.001
5,000-2,800	2.750	12.370	.222	.824	1.000
5,000-1,450	3.090	12.493	.247	.805	1.000
5,000-1,000	12.442	12.370	1.006	.314	1.000
5,000-0	47.470	12.493	3.800	.000	.002
2,800-1,450	.340	12.493	.027	.978	1.000
2,800-1,000	9.692	12.370	.784	.433	1.000
2,800-0	44.720	12.493	3.580	.000	.005
1,450-1,000	9.352	12.493	.749	.454	1.000
1,450-0	44.380	12.615	3.518	.000	.007
1,000-0	35.028	12.493	2.804	.005	.076

Each row tests the null hypothesis that the Sample 1 and Sample 2 distributions are the same.
Asymptotic significances (2-sided tests) are displayed. The significance level is .05.

Figure 47 Pairwise comparison between percentages of reduction in ΔF between all Concentrations

3.2.1.4 Intra-examiner reproducibility for ΔF

The intra-examiner reproducibility was tested using intra-class correlation coefficient. 24 enamel slabs (15%) were randomly selected and re-analysed. The Intra-class Correlation Coefficient (Table 35) was found to be (0.99) which represents excellent reproducibility.

Table 35 Intra-class Correlation Coefficient for ΔF measurements.

	Intra-class Correlation	95% Confidence Interval		F Test with True Value 0			
		Lower Bound	Upper Bound	Value	df1	df2	Sig
Single Measures	.989	.978	.994	176.103	24	24	.000
Average Measures	.994	.989	.997	176.103	24	24	.000

3.2.2 ΔQ: ΔF times the Area. Percentage fluorescence loss with respect to the fluorescence of sound tissue times the area. Lesion volume

Normality tests (Shapiro-Wilk test and Kolmogorov-Smirnov test) were done for the ΔQ values at baseline and showed that the data were normally distributed for all groups (Appendix 6).

The boxplot (Figure 48) for the distribution of the ΔQ at the baseline showed similar distribution of medians between the groups. **5000 ppm** group had one outlier.

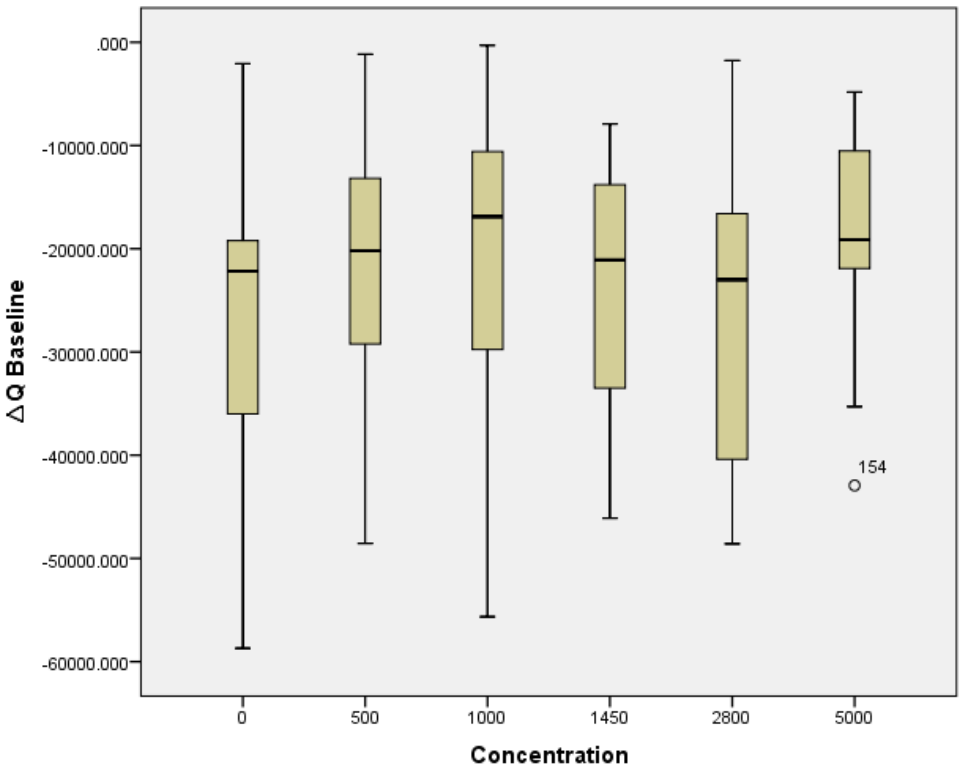


Figure 48 Boxplot for the distribution of the ΔQ values at baseline for all groups.

One way ANOVA test (Table 36) was performed to assess if there was any statistically significant difference in ΔQ at the baseline between the lesions assigned to the six groups. No statistically significant difference was found.

Table 36 One way ANOVA results for ΔQ values at baseline.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1.38E+9	5	2.76E+8	1.740	0.129
Within Groups	2.3E+10	148	1.58E+8		
Total	2.5+10	153			

3.2.2.1 Difference in ΔQ within each group:

The ΔQ mean values both at baseline and after treatment are shown in (Table 37). It can be seen that there was an improvement in ΔQ values for all the groups in the study.

Table 37 The mean values of ΔQ at baseline and after treatment for all groups.

Group	Mean ΔQ at baseline ± SD	Mean ΔQ after treatment ± SD	Mean Difference in ΔQ at baseline and after treatment ± SD
0 ppm	26468.03 ± 14321.27	19435.37 ± 8249.20	7032.65 ± 13554.28
500 ppm	20878.17 ± 12330.29	2515.14 ± 4794.04	18363.03 ± 13133.36
1000 ppm	20155.91 ± 13009.99	2411.55 ± 3877.49	17744.36 ± 14014.81
1450 ppm	23800.89 ± 11342.64	852.73 ± 957.35	22948.16 ± 10979.76
2800 ppm	26162.40 ± 13975.89	3848.77 ± 4683.02	22313.63 ± 13585.87
5000 ppm	18643.92 ± 10059.03	2278.05 ± 3205.92	16365.87 ± 9117.05

(Figure 49) shows the change in the mean of ΔQ at baseline and after treatment with the standard deviation for all groups.

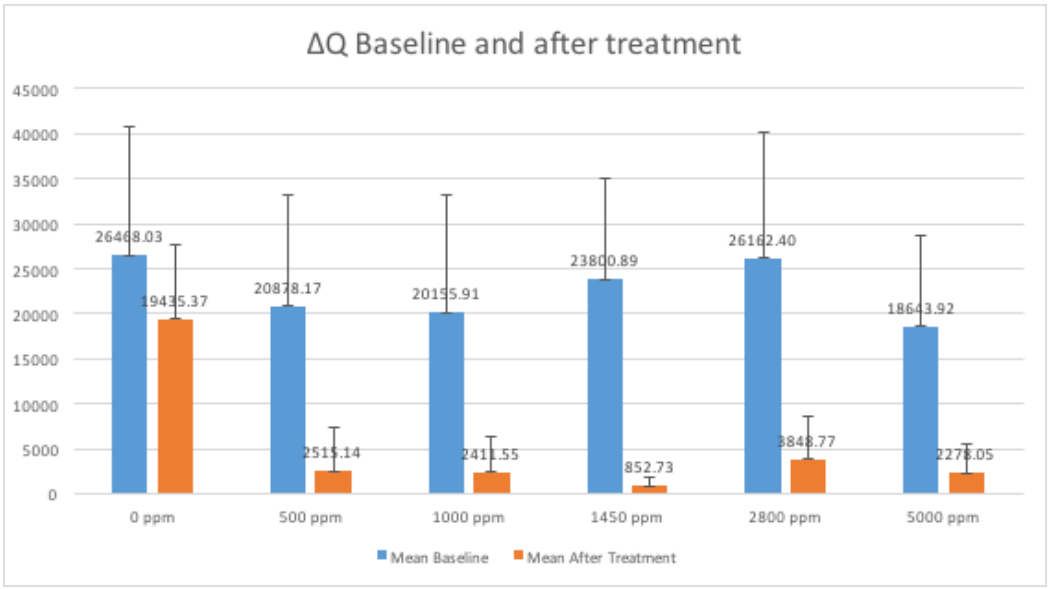


Figure 49 ΔQ mean values at baseline and after treatment for all groups.

To assess whether the change in ΔQ at baseline and after treatment was significantly different within the same group, paired T-Test was carried out and the results (Table 38) showed that there was a statistically significant improvement in the ΔQ values after treatment compared with that at baseline in all treatment groups ($p<0.05$).

Table 38 Paired sampled T-Test results for ΔQ values at baseline and after treatment.

		Paired Differences					Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference		
					Lower	Upper	
0 ppm	ΔQ at baseline – ΔQ after treatment	7032.653	13554.28	2710.856	0.568	3.852	**0.016***
500 ppm		18363.03	13133.36	2575.664	13058.35	23667.70	**0.000***
1000 ppm		17744.36	14014.81	2748.530	3.351	6.132	**0.000***
1450 ppm		22948.16	10979.76	2195.953	18415.94	27480.38	**0.000***
2800 ppm		22313.63	13585.87	2664.408	16826.18	27801.08	**0.000***
5000 ppm		16365.87	9117.048	1788	12683.42	20048.33	**0.000***

3.2.2.2 Difference in ΔQ between groups

The ΔQ difference (change) was measured using the following formula:

Difference in ΔQ = ΔQ after treatment - ΔQ at baseline

(Figure 50) shows the difference in ΔQ in the six tested groups. In all groups the difference in ΔQ was positive, meaning that there was decrease in ΔQ after treatment compared to that at baseline. The highest reduction in ΔQ was in the **1450 ppm F** toothpaste group with a mean difference of (22948.16 ± 10979.76) , while the lowest reduction was in the **0 ppm F** group with only (7032.65 ± 13554.28) mean difference. The rest of the groups were roughly similar in range.

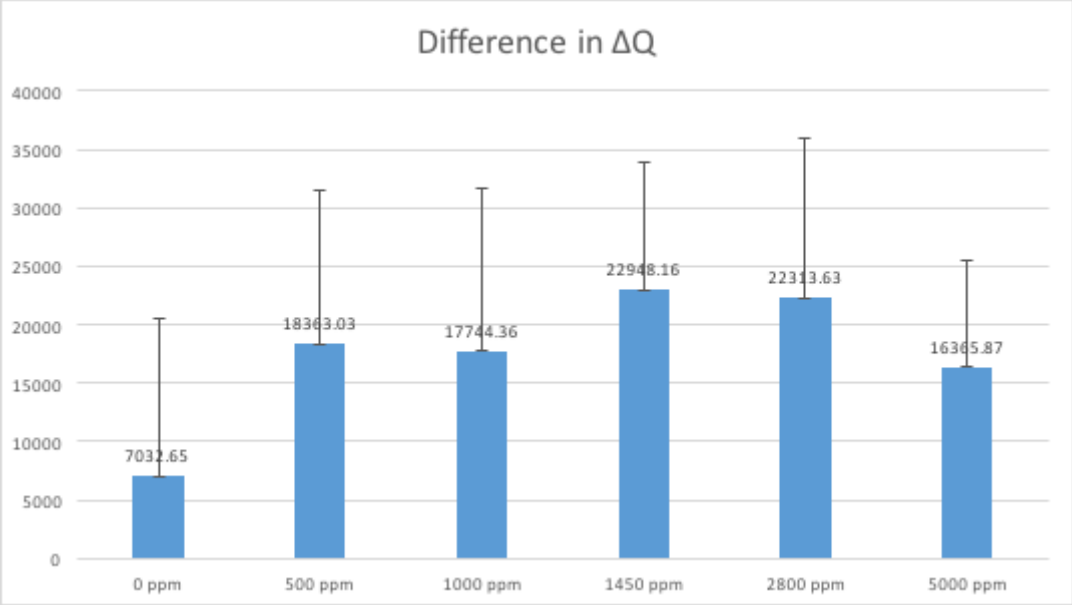


Figure 50 Means of the difference in ΔQ at baseline and after treatment of all groups.

Table 39 Descriptive statistics for the difference in ΔQ at baseline and after treatment for all groups.

Group	N	Minimum	Maximum	Mean	Median	Std. Deviation
0 ppm	25	-10204.333	37578.667	7032.65333	4418.00000	13554.280763
500 ppm	26	.000	48433.333	18363.02564	18866.33333	13133.360146
1000 ppm	26	-10443.667	52795.667	17744.35897	14795.00000	14014.806388
1450 ppm	25	7813.667	44060.667	22948.16000	20896.33333	10979.763462
2800 ppm	26	1127.667	47357.000	22313.62821	21675.83333	13585.866188
5000 ppm	26	4816.333	38843.000	16365.87179	14379.16667	9117.048450

Determination of the normality of the data

Shapiro-Wilk test and Kolmogorov-Smirnov test was carried out to check if the difference in ΔQ at baseline and after treatment was normally distributed. The data was considered normally distributed, as there was no statistical significance.

The boxplot (Figure 51) of difference in ΔQ at baseline and after treatment shows 0 ppm F group having the lowest median value with all other groups having a similar range.

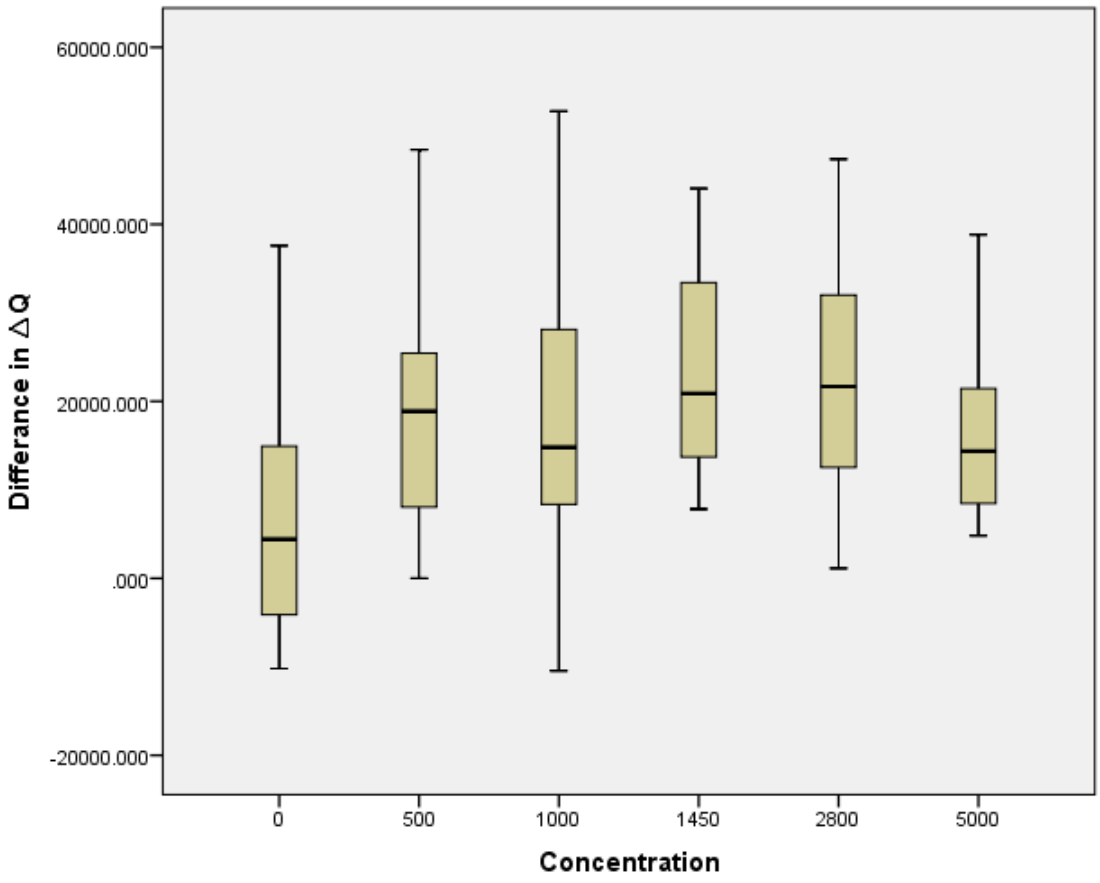


Figure 51 Boxplot for the difference in ΔQ at baseline and after treatment for all groups.

One way ANOVA test (Table 40) was performed to assess if the difference in ΔQ was statistically significant between the five groups. It showed that the mean difference in ΔQ was statistically significant between the groups ($p < 0.001$).

Table 40 One way ANOVA between groups for the difference in ∆Q at baseline and after treatment.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	4137994560.022	5	827598912.004	5.276	0.000*
Within Groups	23217476567.744	148	156874841.674		
Total	27355471127.766	153			

In order to determine which groups were statistically significant different, pairwise comparisons were conducted using Bonferroni test. The Bonferroni tests corrects for multiple testing. The results of the Bonferroni tests are shown in (Table 41).

The results show that there was a statistically significant difference in ΔQ of all groups except **5000 ppm F** when compared to **0 ppm F** (Control) group. No other statistical significance was found between groups.

Table 41 Multiple comparisons of the difference in ΔQ at baseline and after treatment between all test groups and control.

(I) Group	(J) Group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
0 ppm	500	-11330.372308*	3508.367344	.023*	-21797.92485	-862.81977
	1000	-10711.705641*	3508.367344	.040*	-21179.25818	-244.15310
	1450	-15915.506667*	3542.596129	.000*	-26485.18409	-5345.82925
	2800	-15280.974872*	3508.367344	.000*	-25748.52741	-4813.42233
	5000	-9333.218462	3508.367344	.130	-19800.77100	1134.33408
500 ppm	0	11330.372308*	3508.367344	.023*	862.81977	21797.92485
	1000	618.666667	3473.801306	1.000	-9745.75476	10983.08809
	1450	-4585.134359	3508.367344	1.000	-15052.68690	5882.41818
	2800	-3950.602564	3473.801306	1.000	-14315.02399	6413.81886
	5000	1997.153846	3473.801306	1.000	-8367.26758	12361.57527
1000 ppm	0	10711.705641*	3508.367344	.040*	244.15310	21179.25818
	500	-618.666667	3473.801306	1.000	-10983.08809	9745.75476
	1450	-5203.801026	3508.367344	1.000	-15671.35356	5263.75151
	2800	-4569.269231	3473.801306	1.000	-14933.69066	5795.15220
	5000	1378.487179	3473.801306	1.000	-8985.93425	11742.90861
1450 ppm	0	15915.506667*	3542.596129	.000*	5345.82925	26485.18409
	500	4585.134359	3508.367344	1.000	-5882.41818	15052.68690
	1000	5203.801026	3508.367344	1.000	-5263.75151	15671.35356

	2800	634.531795	3508.367344	1.000	-9833.02074	11102.08433
	5000	6582.288205	3508.367344	.939	-3885.26433	17049.84074
2800 ppm	0	15280.974872*	3508.367344	**.000***	4813.42233	25748.52741
	500	3950.602564	3473.801306	1.000	-6413.81886	14315.02399
	1000	4569.269231	3473.801306	1.000	-5795.15220	14933.69066
	1450	-634.531795	3508.367344	1.000	-11102.08433	9833.02074
	5000	5947.756410	3473.801306	1.000	-4416.66502	16312.17784
5000 ppm	0	9333.218462	3508.367344	.130	-1134.33408	19800.77100
	500	-1997.153846	3473.801306	1.000	-12361.57527	8367.26758
	1000	-1378.487179	3473.801306	1.000	-11742.90861	8985.93425
	1450	-6582.288205	3508.367344	.939	-17049.84074	3885.26433
	2800	-5947.756410	3473.801306	1.000	-16312.17784	4416.66502
***. The mean difference is significant at the 0.05 level.**						

Table 41 (continued).

3.2.2.3 The percentage of the changes in ΔQ at baseline and after treatment (%ΔQ) was calculated using the following formula:

(Difference in ΔQ at baseline and after treatment / ΔQ at baseline) × 100

(Figure 52) shows the % ΔQ change values for all groups with **1450 ppm F** group having 96.4% reduction in ΔQ, while **0 ppm F** group had on 3.1% reduction. All other groups were similar in percentage range.

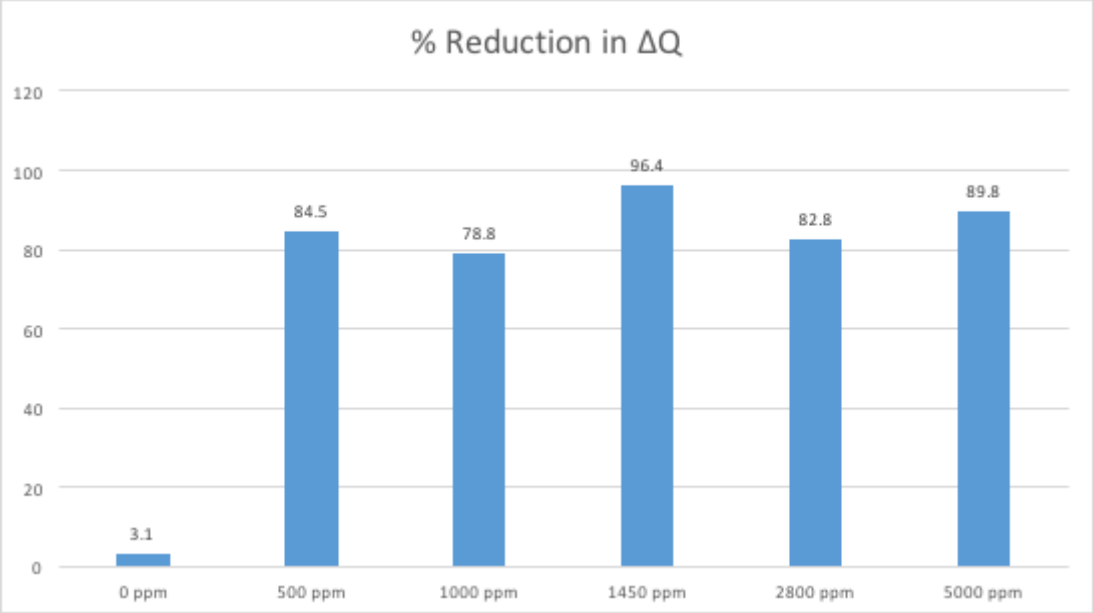


Figure 52 The %ΔQ values for all groups.

Determination of the normality of data for Percentage reduction in ΔQ:

In order to check if the percentage in reduction of ΔQ was normally distributed, data normality test was carried out. This included Shapiro-Wilk test and Kolmogorov-Smirnov test. p value was statistically significant therefore data was not considered normally distributed ($p<0.001$).

The Boxplot (Figure 53) of the percentage of reduction in ΔQ for all groups shows that the data is not normally distributed, and that there are several outliers in all groups, most of these outliers being in the **500**, and **1000 ppm F** groups. The median decrease in percentage value is lowest in the **0 ppm** group while all other groups have similar median values.

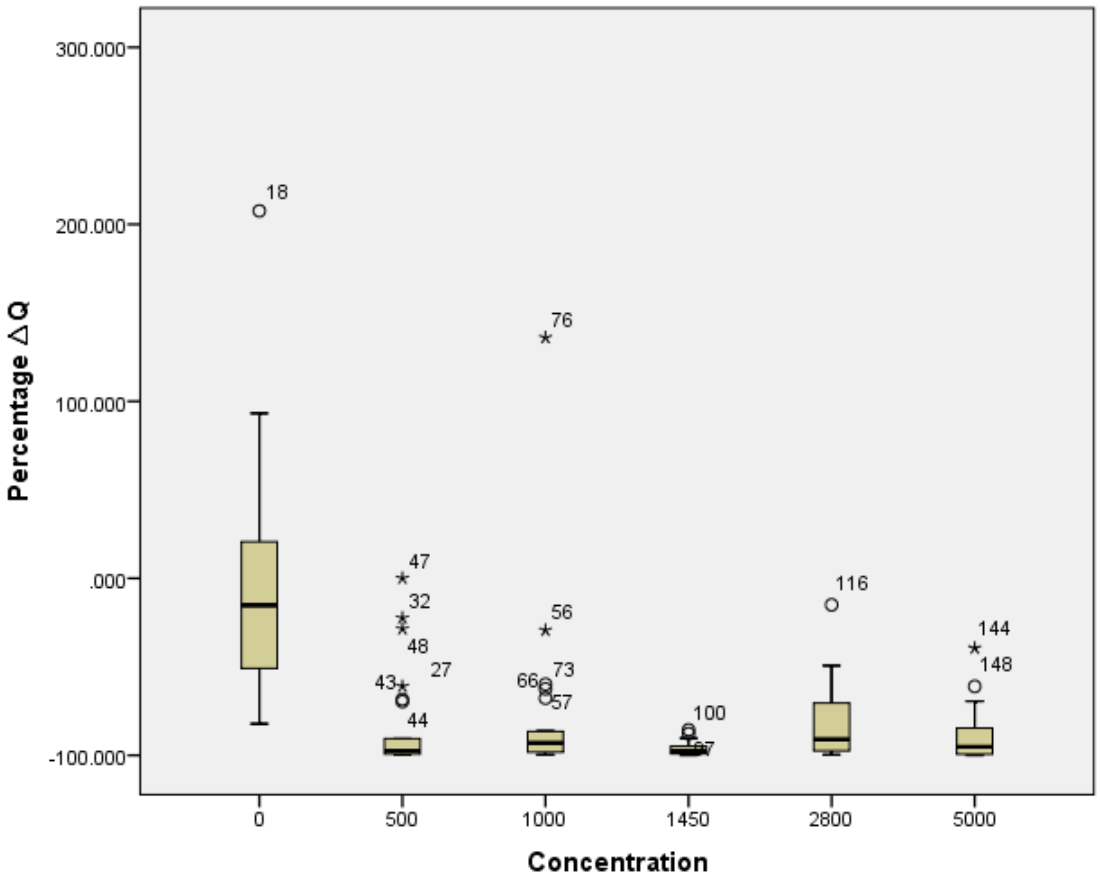


Figure 53 Boxplot for the Percentage of reduction in ΔQ for all groups.

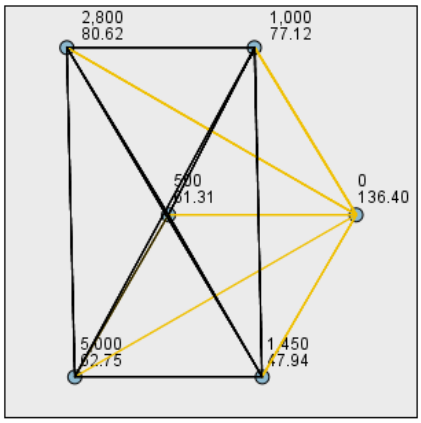
Non parametric Kruskal-Wallis test (Table 42) was performed to assess if the percentage reduction in ΔQ was statistically significant between the six groups. It showed that the mean percentage reduction of ΔQ was statistically significant between groups ($p<0.001$).

Table 42 Kruskal-Wallis Test results for the Percentage of reduction in ΔQ.

	Percentage of reduction in ΔQ
Chi-Square	60.983
df	5
Asymp. Sig.	**0.000**
a. Kruskal Wallis Test	
b. Grouping Variable: Formulation	

In order to determine which groups were statistically significant different, pairwise comparison was performed (Figure 54). The test shows that there was significant difference in percentage reduction of ΔQ found in all groups when compared to **0 ppm** F toothpaste (Control) group. There was no statistical significance between test groups.

Pairwise Comparisons of Concentration



Each node shows the sample average rank of Concentration.

Sample1-Sample2	Test Statistic	Std. Error	Std. Test Statistic	Sig.	Adj.Sig.
1,450-500	13.368	12.493	1.070	.285	1.000
1,450-5,000	-14.810	12.493	-1.185	.236	1.000
1,450-1,000	29.175	12.493	2.335	.020	.293
1,450-2,800	-32.675	12.493	-2.616	.009	.134
1,450-0	88.460	12.615	7.012	.000	.000
500-5,000	-1.442	12.370	-.117	.907	1.000
500-1,000	-15.808	12.370	-1.278	.201	1.000
500-2,800	-19.308	12.370	-1.561	.119	1.000
500-0	75.092	12.493	6.011	.000	.000
5,000-1,000	14.365	12.370	1.161	.246	1.000
5,000-2,800	17.865	12.370	1.444	.149	1.000
5,000-0	73.650	12.493	5.895	.000	.000
1,000-2,800	-3.500	12.370	-.283	.777	1.000
1,000-0	59.285	12.493	4.745	.000	.000
2,800-0	55.785	12.493	4.465	.000	.000

Each row tests the null hypothesis that the Sample 1 and Sample 2 distributions are the same.
Asymptotic significances (2-sided tests) are displayed. The significance level is .05.

Figure 54 Pairwise comparison between percentages of reduction in ΔQ between all concentrations.

3.2.2.4 Intra-examiner reproducibility for ΔQ

The intra-examiner reproducibility was tested using intra-class correlation coefficient. 24 enamel slabs (15%) were randomly selected and re-analysed. The Intra-class Correlation Coefficient (Table 43) was found to be (0.99) which represents excellent reproducibility.

Table 43 Intra-class Correlation Coefficient for ΔQ measurements.

	Intra-class Correlation	95% Confidence Interval		F Test with True Value 0			
		Lower Bound	Upper Bound	Value	df1	df2	Sig
Single Measures	.989	.978	.994	174.246	24	24	.000
Average Measures	.994	.989	.997	174.246	24	24	.000

3.2.3 Area of the white spot lesion:

The values of area of white spot lesion at baseline for all groups were checked to see if there was a difference between the groups. The normality tests (Shapiro-Wilk test and Kolmogorov-Smirnov test) were carried out to check the normality of the data. The data were considered normally distributed if the p values from these tests were not statistically significant ($p > 0.05$). p values for all groups except for **5000 ppm F** group were not statistically significant therefore data was considered to be normally distributed (Appendix 7).

The boxplot (Figure 55) for the distribution of the area of white spot lesion at the baseline showed variation of median values between groups with one outlier in the **2800 ppm F** group.

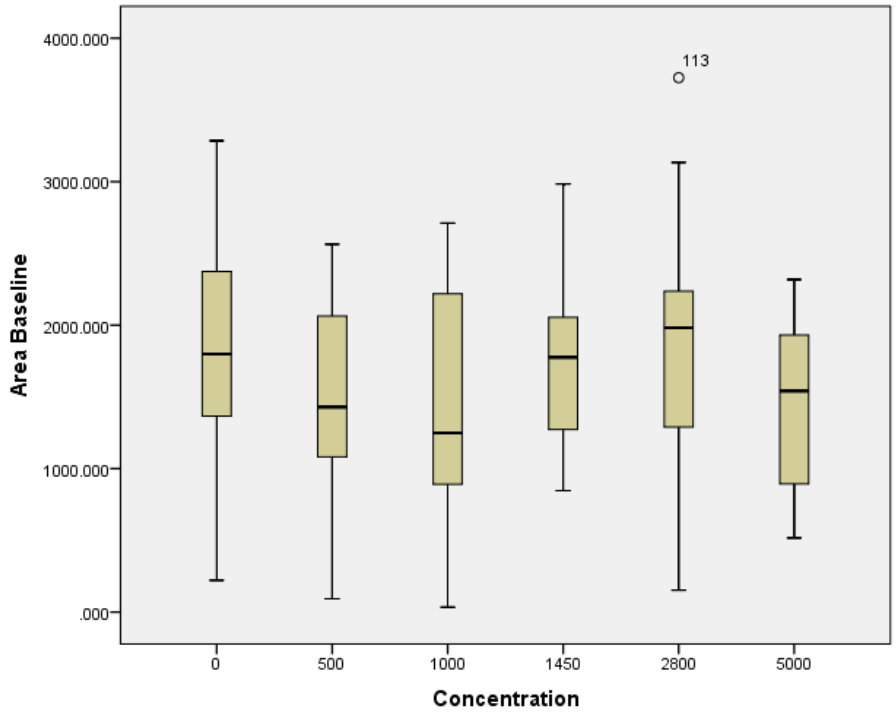


Figure 55 Boxplot for the distribution of the Area values at baseline for all groups.

One way ANOVA test (Table 44) was performed to assess if there was any statistically significant difference in the area values at the baseline between the lesions assigned to the six groups. No statistically significant difference was found.

Table 44 One way ANOVA results for Area values at baseline.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	5074366	5	1014873	2.036	0.077
Within Groups	7.38E+7	148	498485.4		
Total	7.89E+7	153			

3.2.3.1 Difference in lesion area within each group

The lesion area mean values both at baseline and after treatment are shown in (Table 45). It can be seen that there was a decrease in the lesion area for all groups in the study.

Table 45 The mean values of area at baseline and after treatment for all groups.

Group	Mean area at baseline ± SD	Mean area after treatment ± SD	Mean Difference in area at baseline and after treatment ± SD
0 ppm	1869.25 ± 714.61	1683.2 ± 576.10	-186.05 ± 502.13
500 ppm	1515.21 ± 757.09	208.27 ± 341.80	-1314.94 ± 796.84
1000 ppm	1487.82 ± 772.72	270.09 ± 392.63	-1217.73 ± 907.03
1450 ppm	1778.69 ± 575.41	98.99 ± 104.45	-1679.70 ± 548.75
2800 ppm	1869.40 ± 792.07	437.12 ± 493.30	-1432.28 ± 874.73
5000 ppm	1447.72 ± 587.61	257.86 ± 344.89	-1189.85 ± 500.73

(Figure 56) shows the change in the mean of area at baseline and after treatment with the standard deviation for all groups.

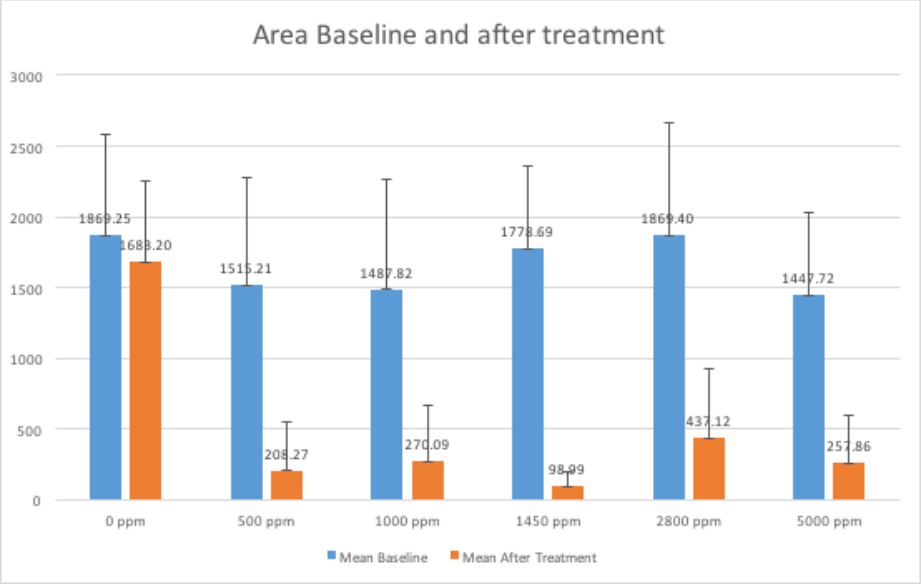


Figure 56 Lesion area at baseline and after treatment for all groups.

To assess whether the change in the area at baseline and after treatment was significantly different within the same group, paired T-Test was used.

The paired T-Test results shown in (Table 46) showed that there was a statistically significant improvement in the lesion area values after treatment compared with that at baseline, for all toothpaste test groups ($p < 0.001$) except the **0 ppm F** (Control) toothpaste ($p > 0.05$).

Table 46 Paired sampled T test results for the lesion area values at baseline and after treatment for all groups.

		Paired Differences					Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference		
					Lower	Upper	
0 ppm	Area at baseline – Area after treatment	-186.053	502.1382	100.42	-393.326	21.219	**0.076**
500 ppm		-1314.95	796.84	156.27	-1636.80	-993.09	**0.000***
1000 ppm		-1217.73	907.04	177.88	-1584.09	-851.36	**0.000***
1450 ppm		-1679.71	548.75	109.75	-1906.22	-1453.19	**0.000***
2800 ppm		-1432.28	874.73	171.55	-1785.60	-1078.97	**0.000***
5000 ppm		-1189.86	500.73	98.20	-1392.11	-987.606	**0.000***

3.2.3.2 Difference in lesion area between groups

The lesion area difference (change) was measured using the following formula:

Difference in lesion area= lesion area after treatment – lesion area at baseline

(Figure 57) shows the difference in the lesion area for the six tested groups. In all test groups, the mean difference was negative indicating a decrease in area of the lesion after treatment compared to that at baseline.

The highest reduction in area size was found in the **1450 ppm F** toothpaste group -1679.707 ± 548.76. While The lowest reduction in lesion size was in the **0 ppm F** (control) group -186.053 ± 502.14. All other test groups were similar in range of difference in area.

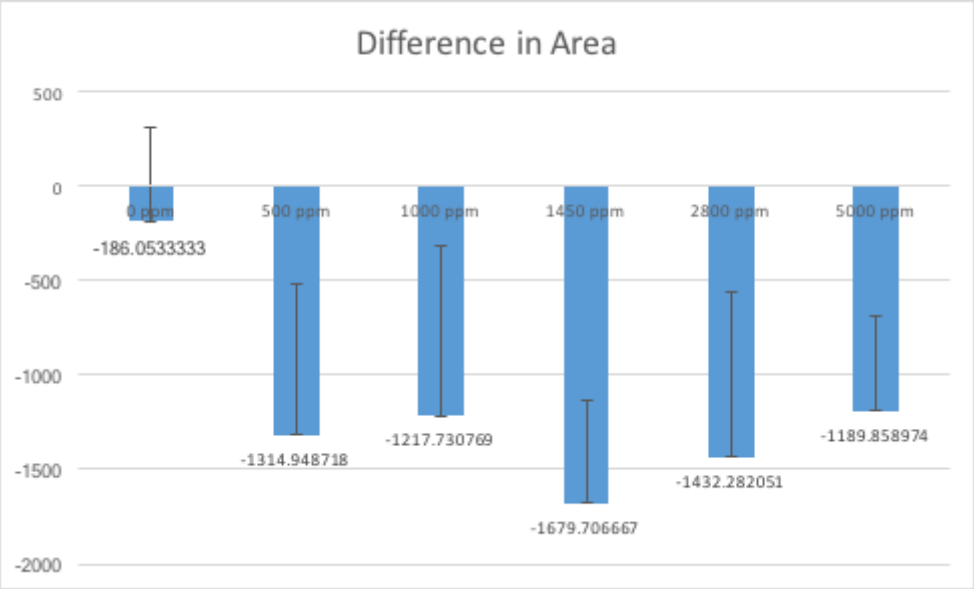


Figure 57 Means of the difference in the lesion area at baseline and after treatment of all tested groups.

Table 47 Descriptive statistics for the difference in Lesion Area at baseline and after treatment for all groups.

Group	N	Minimum	Maximum	Mean	Median	Std. Deviation
0 ppm	25	-1312.667	600.667	-186.05333	-130.33333	502.138212
500 ppm	26	-2544.333	13.333	-1314.94872	-1344.33333	796.849935
1000 ppm	26	-2688.000	1051.333	-1217.73077	-1140.00000	907.039982
1450 ppm	25	-2804.333	269.667	-1679.70667	-1751.66667	548.757210
2800 ppm	26	-3366.667	202.333	-1432.28205	-1220.50000	874.737912
5000 ppm	26	-2297.000	-419.667	-1189.85897	-1155.50000	500.738088

Determination of the normality of lesion area difference at baseline and after treatment data:

In order to check if the differences in the lesion area at baseline and after treatment were normally distributed, data normality test was carried out. This included Shapiro-Wilk test and Kolmogorov-Smirnov test. The data was considered normally distributed as there was no significance.

The boxplot (Figure 58) of difference in the lesion area at baseline and after treatment shows similar range of median values for all test groups except **0 ppm** F Control group, which had a much lower median value. The data appeared normally distributed and no outliers were detected.

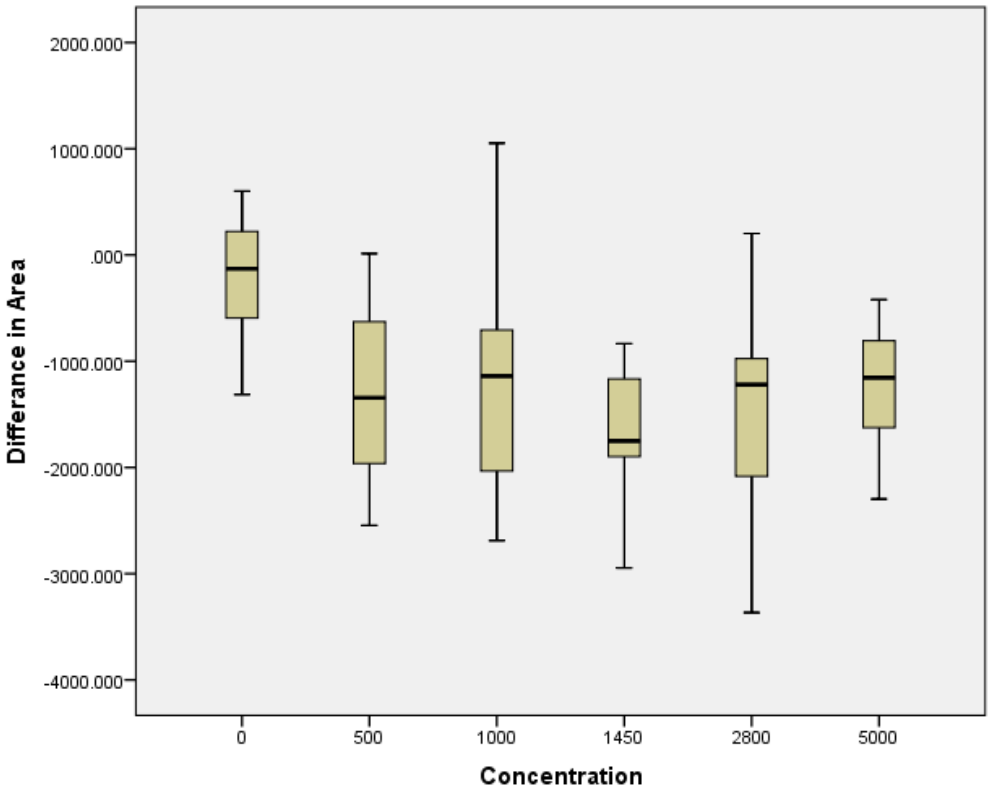


Figure 58 Boxplot for the difference in the lesion Area at baseline and after treatment for all groups.

One way ANOVA test (Table 48) was performed to assess if the difference in area was statistically significant between the five groups. It showed that the mean difference in area was statistically significant between the groups ($p < 0.001$).

Table 48 One way ANOVA between groups for the difference in Area at baseline and after treatment.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	33101583.243	5	6620316.649	13.043	.000
Within Groups	75118564.128	148	507557.866		
Total	108220147.371	153			

In order to determine which groups were statistically significant different, pairwise comparisons were conducted using Bonferroni test. The Bonferroni tests corrects for multiple testing. The results of the Bonferroni tests are shown in (Table 49). The results revealed that there was significant difference in area of white spot lesions of all test groups when compared to **0 ppm F** (control) group. No other significant differences were found between the test groups.

Table 49 Multiple comparisons of the difference in Area at baseline and after treatment between all test groups and control.

(I) Group	(J) Group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
0 ppm	500	1128.895385*	199.558941	**.000***	533.49203	1724.29874
	1000	1031.677436*	199.558941	**.000***	436.27408	1627.08079
	1450	1493.653333*	201.505904	**.000***	892.44102	2094.86564
	2800	1246.228718*	199.558941	**.000***	650.82536	1841.63208
	5000	1003.805641*	199.558941	**.000***	408.40228	1599.20900
500 ppm	0	-1128.895385*	199.558941	**.000***	-1724.29874	-533.49203
	1000	-97.217949	197.592795	1.000	-686.75512	492.31922
	1450	364.757949	199.558941	1.000	-230.64541	960.16131
	2800	117.333333	197.592795	1.000	-472.20384	706.87051
	5000	-125.089744	197.592795	1.000	-714.62692	464.44743
1000 ppm	0	-1031.677436*	199.558941	**.000***	-1627.08079	-436.27408
	500	97.217949	197.592795	1.000	-492.31922	686.75512

	1450	461.975897	199.558941	.330	-133.42746	1057.37926
	2800	214.551282	197.592795	1.000	-374.98589	804.08845
	5000	-27.871795	197.592795	1.000	-617.40897	561.66538
1450 ppm	0	-1493.653333*	201.505904	**.000***	-2094.86564	-892.44102
	500	-364.757949	199.558941	1.000	-960.16131	230.64541
	1000	-461.975897	199.558941	.330	-1057.37926	133.42746
	2800	-247.424615	199.558941	1.000	-842.82797	347.97874
	5000	-489.847692	199.558941	.229	-1085.25105	105.55567
2800 ppm	0	-1246.228718*	199.558941	**.000***	-1841.63208	-650.82536
	500	-117.333333	197.592795	1.000	-706.87051	472.20384
	1000	-214.551282	197.592795	1.000	-804.08845	374.98589
	1450	247.424615	199.558941	1.000	-347.97874	842.82797
	5000	-242.423077	197.592795	1.000	-831.96025	347.11410
5000 ppm	0	-1003.805641*	199.558941	**.000***	-1599.20900	-408.40228
	500	125.089744	197.592795	1.000	-464.44743	714.62692
	1000	27.871795	197.592795	1.000	-561.66538	617.40897
	1450	489.847692	199.558941	.229	-105.55567	1085.25105
	2800	242.423077	197.592795	1.000	-347.11410	831.96025
***. The mean difference is significant at the 0.05 level.**						

Table 49 (continued).

3.2.3.3 The percentage change in lesion area at baseline and after treatment (% Area) was calculated using the following formula:

(Difference in area at baseline and after treatment / area at baseline) × 100

(Figure 59) shows the % Area values for all groups, with **1000 ppm F** group having the highest % reduction in area of lesion 94.4%, followed by **5000**, and **500 ppm F** Groups at 84.5% and 82.5% respectively. The lowest % reduction in area was located in the **0 ppm F** (control) Group at 0.1%.

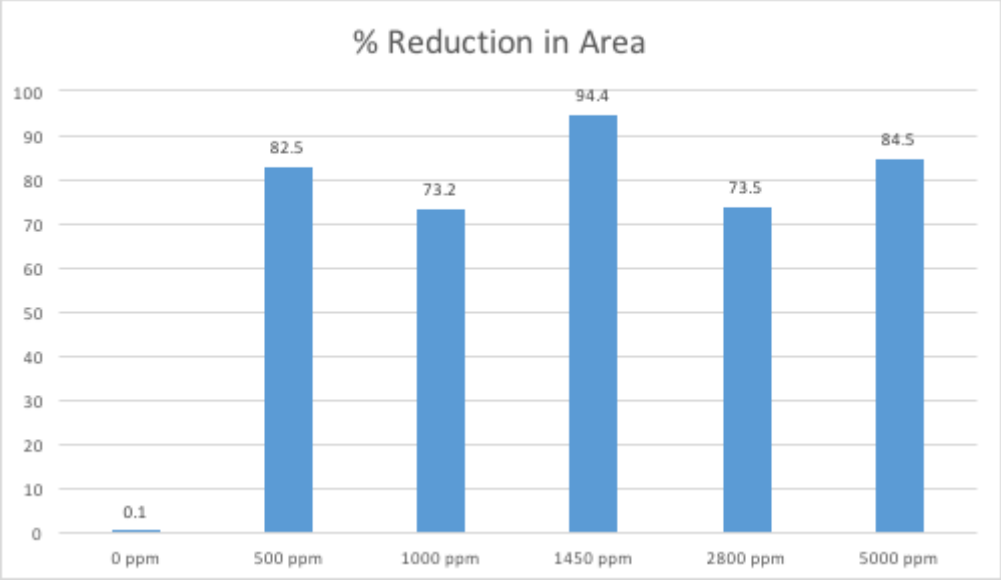


Figure 59 The % Area values for all groups.

Determination of the normality of data for Area:

In order to check if the percentage in reduction of area was normally distributed, data normality test was carried out. This included Shapiro-Wilk test and Kolmogorov-Smirnov test. P value was statistically significant therefore data was not considered normally distributed ($p<0.001$).

The Boxplot (Figure 60) of the percentage of reduction in Area for all groups shows that the data is not normally distributed, and that there are several outliers in all groups.

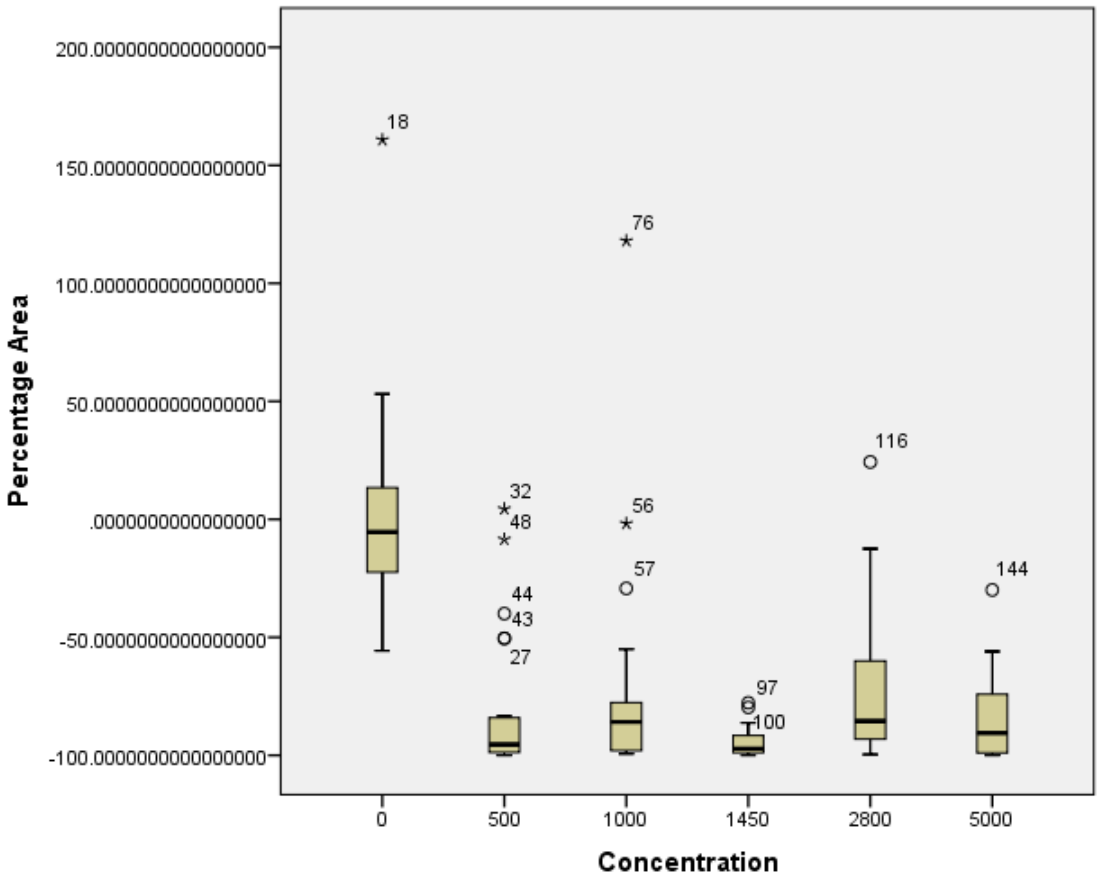


Figure 60 Boxplot for the Percentage of reduction in Area for all groups.

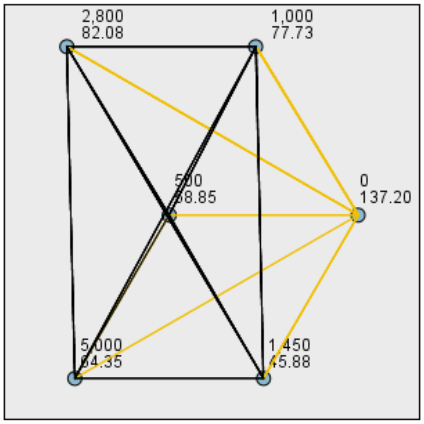
Non parametric Kruskal-Wallis test (Table 50) was performed to assess if the percentage reduction in area was statistically significant between the six groups. It showed that the mean percentage reduction of area was statistically significant between groups ($p<0.001$).

Table 50 Kruskal-Wallis Test results for the Percentage of reduction in Area.

	Percentage of reduction in Area
Chi-Square	64.444
df	5
Asymp. Sig.	**0.000**
a. Kruskal Wallis Test	
b. Grouping Variable: Formulation	

In order to determine which groups were statistically significant different, pairwise comparison was performed (Figure 61). The results revealed a significant difference in mean percentage reduction of white spot lesion area of all test groups when compared to 0 ppm F (control) group. No other statistical significance was evident.

Pairwise Comparisons of Concentration



Each node shows the sample average rank of Concentration.

Sample1-Sample2	Test Statistic	Std. Error	Std. Test Statistic	Sig.	Adj.Sig.
1,450-500	12.966	12.493	1.038	.299	1.000
1,450-5,000	-18.466	12.493	-1.478	.139	1.000
1,450-1,000	31.851	12.493	2.550	.011	.162
1,450-2,800	-36.197	12.493	-2.897	.004	.056
1,450-0	91.320	12.615	7.239	.000	.000
500-5,000	-5.500	12.370	-.445	.657	1.000
500-1,000	-18.885	12.370	-1.527	.127	1.000
500-2,800	-23.231	12.370	-1.878	.060	.906
500-0	78.354	12.493	6.272	.000	.000
5,000-1,000	13.385	12.370	1.082	.279	1.000
5,000-2,800	17.731	12.370	1.433	.152	1.000
5,000-0	72.854	12.493	5.832	.000	.000
1,000-2,800	-4.346	12.370	-.351	.725	1.000
1,000-0	59.469	12.493	4.760	.000	.000
2,800-0	55.123	12.493	4.412	.000	.000

Each row tests the null hypothesis that the Sample 1 and Sample 2 distributions are the same.
Asymptotic significances (2-sided tests) are displayed. The significance level is .05.

Figure 61 Pairwise comparison between percentages of reduction in Area between all formulations.

3.2.3.4 Intra-examiner reproducibility for Area of the white spot lesion

The intra-examiner reproducibility was tested using intra-class correlation coefficient. 24 enamel slabs (15%) were randomly selected and re-analysed. The Intra-class Correlation Coefficient (Table 51) was found to be (0.99) which represents excellent reproducibility.

Table 51 Intra-class Correlation Coefficient for Area measurements.

	Intra-class Correlation	95% Confidence Interval		F Test with True Value 0			
		Lower Bound	Upper Bound	Value	df1	df2	Sig
Single Measures	.992	.983	.996	230.060	24	24	.000
Average Measures	.996	.992	.998	230.060	24	24	.000

3.2.4 Summary of the results for all three parameters for phase B:

The ΔF results: the results showed that there were statistically significant improvement in the ΔF values between the baseline and after treatment for all groups. The reduction in ΔF was highest, and almost identical in the **500, 1450, and 2800 ppm F** groups, followed by **5000 ppm, 1000 ppm** and the lowest improvement was seen in the **0 ppm F** group.

When comparing the groups against each other, it can be seen that the mean difference in ΔF of the **0 ppm F** (Control) toothpaste group is significantly lower than the mean difference in ΔF of all other test groups except for **1000 ppm F** toothpaste. As for the significance between different concentrations of NaF fluoride toothpaste, there was no significance in any of the groups.

The ΔQ results: A statistically significant improvement in the ΔQ values between the baseline and after treatment was found in all groups. The highest improvement was achieved by the **1450 ppm F** group followed by **2800 ppm F** and the least improvement was seen in the **0 ppm F** group (control).

When comparing the groups against each other, the difference in ΔQ at baseline and after treatment in all test groups was statistically significant higher than that for the **0 ppm F** group except for the **5000 ppm F** group. No other statistical significance was found between groups.

For the lesion area results: A significant reduction in the area of the white spot lesion was seen in all groups except for the **0 ppm F** group. The highest reduction was found in the **1450 ppm F** group followed by the **2800, 500, 1000, 5000 ppm F** groups In descending order.

When comparing the groups against each other, the difference in the lesion area at baseline and after treatment was statistically significant for the test groups compared with the **0 ppm F** group. No significant difference was found between the test groups.

Chapter 4 DISCUSSION

Multiple *in vitro*, *in situ*, and *in vivo* studies investigating the effects of different fluoride formulations or fluoride concentrations on enamel subsurface lesion remineralisation are available. However the vast majority of these studies only compare one or two formulations/ concentrations against fluoride-free or placebo toothpaste.

Furthermore, pH cycling *in vitro* studies published in the literature are usually of a limited time period and usually extend for no longer than 14 days, during which enamel is left in demineralising/ remineralising solutions of high concentrations for prolonged periods of time and at high concentrations unlike the conditions of the oral environment.

Therefore the current *in vitro* study aimed to investigate the remineralising potential of toothpastes with different Fluoride (F) formulations: amine fluoride **(AmF)**, sodium monofluorophosphate **(MFP)**, sodium fluoride **(NaF)**, and stannous fluoride **(SnF)** on artificial subsurface caries lesions.

Another aim was to investigate the remineralising potential of toothpastes containing sodium fluoride (NaF) formulation at different concentrations of fluoride **(500, 1000, 1450, 2800 and 5000 ppm F)** on artificial subsurface caries lesions *in vitro*.

4.1 In vitro model

This study used an in vitro model to study enamel remineralisation using different fluoride formulations and concentrations. In *in vitro* studies, the clinical efficacy of

fluoride toothpastes are estimated using different models that attempt to simulate the conditions of the oral cavity, and the process of caries formation.

The caries process is a continuum of demineralisation and remineralisation which has been referred to as the ionic seesaw. Therefore pH cycling models appear to be the most suitable for investigating the effect of anti-caries fluoride toothpastes (Tenuta and Cury, 2013). In vitro pH cycling models mimic the dynamics of mineral loss or gain involved in the caries process, and the have been used widely to evaluate the efficacy of fluoridated toothpastes on caries control (Buzalaf et al., 2010).

Stookey et al. (2011) published a study that looked at the robustness, and the ability to predict the anti-caries performance of fluoride containing products by using the *in vitro pH* cycling model. He compared data from three independent laboratories and concluded that the *in vitro* pH cycling model:

1. Was capable of measuring the dose response from 0-1100 ppm F.
2. The model was able to statistically separate positive from negative control.
3. Dentifrice formulations proven to be clinically effective against caries, performed in this model at a level that was not statistically less effective than the positive control.
4. This model was able to statistically differentiate between a product with attenuated fluoride activity (product formulated with the same level of fluoride as the positive control in addition to an ingredient known to compromise fluoride effectiveness) from the positive control.

For the reasons mentioned above, in vitro pH cycling continues to be an effective tool in evaluating the efficacy of fluoridated toothpastes on caries control.

In-spite of all the advantages in vitro studies provide, the main limitation continues to be the inability to simulate the complex biological processes associated with caries (Xuedong, 2016), and the inability to replicate and reproduce precisely the conditions of the oral environment (Higham et al., 2005).

4.2 Study design

This was a two phase *in vitro* study design to investigate the remineralisation of the enamel subsurface lesions under pH cycling conditions using different fluoride formulations (phase A), and different NaF concentrations (phase B).

Five fluoride toothpaste formulations were investigated in phase A including the negative control (**F-Free, NaF, SnF, MFP, and AmF**).

Six NaF toothpaste concentrations were investigated in phase B including the negative control (**0 ppm, 500 ppm, 1000 ppm, 1450 ppm, 2800 ppm, 5000 ppm F**).

A randomised, single-blinded design was used to prevent the introduction of bias in the study.

4.3 Bovine teeth

Human teeth can be regarded as the most appropriate source of dental substrate to be used in pH cycling models in terms of clinical relevance. However, their composition is variable, due to genetic influences, environmental conditions and age. These differences lead to large variations in their response under acidic challenges (Buzalaf et al., 2010). Furthermore, sources of human teeth are becoming more and more limited and there is a significant increase in difficulty of obtaining human teeth for research purposes (Stookey et al., 2011).

Bovine teeth are easier to obtain, have a more uniform composition when compared to human teeth, and have been generally demonstrated to perform similarly to human teeth (Tanaka et al., 2008, Costa et al., 2015). For this reason, bovine enamel can offer a suitable alternative to human enamel for in vitro pH cycling models, and they provide a less variable response to both cariogenic challenge and anti-caries treatment such as fluoridated dentifrices (Mellberg, 1992, ten Cate and Mundorff-Shrestha, 1995). However, due to slight differences between bovine and human enamel in terms of mineral content and porosity (Edmunds et al., 1988), Stookey et al. (2011) found that slight adjustment and increase of demineralisation pH was necessary to achieve similar results. Although bovine enamel is more porous than human enamel, which leads to faster demineralization and remineralization, these differences result in quantitative and not qualitative differences in behaviour (Buzalaf et al., 2010).

Artificial caries lesions produced from bovine teeth, have a mineral distribution and structure that resembles lesions produced from human teeth for both enamel and dentine (Featherstone and Mellberg, 1981, Mellberg, 1992).

Furthermore, the use of bovine teeth overcomes many disadvantages and obstacles laid down by the use of human teeth including (Yassen et al., 2011):

- Difficulties in obtaining a large quantity of human teeth with good quality, as most extracted teeth are severely carious or broken down.
- Difficulty in controlling the source and age of human teeth which may lead to large variations in outcome measures.
- Relatively small curved surface area of human teeth may also be a limitation to specific tests requiring flat surfaces of uniform thickness.
- Awareness of infection hazards and ethical issues has increased rendering it even more difficult to obtain a sufficient number of sound extracted human teeth.

4.4 Enamel slabs preparation and storage

The buccal section of the bovine teeth was used in the present study to allow a more uniform thickness of enamel as well as more flat surfaces. The enamel slabs were stored in distilled water and 0.1% thymol (Sigma Aldrich) with the aim of inhibition of the bacterial growth and prevention of enamel slabs dehydration. The antimicrobial properties of thymol were proven through its ability to perforate cell membranes, and subsequently destroy the pathogens that may be present on the teeth (Shapiro and Guggenheim, 1995). At the same time thymol has no detrimental effect on enamel but a few studies showed that it can affect dentine permeability (Preston et al., 2007).

The enamel and dentine thickness were found to influence the fluorescence, however; this confounding factor could not be absolutely standardised in the present study. Since only profound differences in the total thickness of the dental tissue was found to influence the caries assessment with the QLF (Ando et al., 2003), the impact of this factor is believed to be minimal on the results of the current study.

4.5 Artificial caries lesions

In the current study acidified hydroxyethyl cellulose gel was used to create a sub-surface caries-like lesion. The enamel slabs were immersed in the acidified gel for 10 days in order to produce enamel lesion with ΔF of similar range.

Acidified hydroxyethyl cellulose gel, has been shown to be easy to use and creates lesions with consistent depths of demineralisation. Lesions created with this gel have also demonstrated more rigidity when compared with lesions produced by acetic acid buffer (Issa, 2004).

Lesions produced by acid buffer solutions have been shown to produce larger and deeper lesions than acidified gels (including Acidified hydroxyethyl cellulose gel). This can be explained by the rapid diffusion rate of acid buffer solutions which does not allow re-precipitation of minerals and therefore does not allow formation of an intact surface layer over the lesion (produces an erosion-like lesion). Acidified gels on the other hand create more controlled demineralisation process, and allow re-precipitation of dissolved mineral ions to create an intact surface layer of the lesion that mimics the caries process (Amaechi et al., 1998).

4.6 pH cycling

The origin of the modern pH cycling models was produced by (ten Cate and Duijsters, 1982). In vitro pH cycling models mimic the dynamics of mineral loss or gain involved in the caries process, and the have been used widely to evaluate the efficacy of fluoridated toothpastes on caries control (Buzalaf et al., 2010). pH cycling *in vitro* studies published in the literature are usually of a limited time period and usually extend for no longer than 14 days, during which enamel is left in demineralising/ remineralising solutions of high concentrations for prolonged periods of time and at high concentrations unlike the conditions of the oral environment. However in the current study, a 28 day period of pH cycling was implemented in order to allow sufficient time to produce changes in the pre-demineralised enamel slabs.

In the current study, enamel slabs were exposed to 5 acidic challenges per day in a demineralising solution. The enamel slabs were initially dipped in toothpaste slurry for 5 minutes, then rinsed with distilled water for 1 minute and placed in day time artificial saliva. The enamel slabs were exposed to the first demineralisation challenge by dipping in acetic acid solution (pH 4.8) for 5 minutes, then rinsed with distilled water for 1 minute

and placed in day time artificial saliva. This process was repeated until the enamel slabs were subjected to 5 demineralisation challenges, which represent the acid in the cariogenic challenge. After the last cycle the enamel slabs were dipped in toothpaste slurry for 5 minutes. Enamel slabs were then placed in night time artificial saliva.

The day time saliva was supersaturated with calcium and phosphate in order to allow remineralisation of enamel slabs during the day and it was used in between the demineralisation challenges for 60 minutes. On the other hand, the night time saliva was a saturated solution and it was used overnight to maintain the enamel condition without providing any mineral exchanges.

The pH cycling protocol used for the current study was developed at the University of Leeds and has been used in previous caries studies at the Leeds Dental Institute-Paediatric Dentistry Department.

4.7 Quantitative Light-Induced Fluorescence (QLF)

QLF is a system based on the measurement of fluorescence loss following enamel demineralisation. This method has been employed in pH-cycling experiments and has shown to be efficient at measuring the remineralisation of enamel subsurface lesions (Gomez et al., 2014).

In the current study, QLF has been used to measure enamel demineralisation at baseline (after acid gel demineralisation for 10 days), and enamel lesion remineralisation following treatment with experimental toothpastes. QLF readings in the present study showed excellent reproducibility as intra-class correlation coefficients (ICCs) for inter-examiner reliability for the image analysis was found to be (0.99). These results are in line with previous studies (Hafstrom-Bjorkman et al., 1992, Emami et al., 1996, Al-Khateeb et al., 1997b, Tranaeus et al., 2002).

Pretty et al. (2002b), looked at the intra- (0.93) and inter-examiner reliability (0.96) of QLF analysis. The authors concluded that the analysis stage of QLF is reliable between examiners and within multiple attempts by the same examiner, when analysing in vitro lesions.

Image capture technique was standardised, and the environment controlled for all enamel slabs in order to reduce the chances of bias. To ensure that images of the enamel slabs were always captured in the same camera positions and from the same angles, the camera was attached to a stand in the same position for all the images. The QLF camera was fixed at a position that provided optimum illumination of the enamel block surface. The camera specimen distance was standardised thereby controlling specimen stability, light intensity, and magnification.

To further reduce the risk of bias, the investigator was trained by the manufacturer and was familiar with the QLF software prior to study commencement.

4.8 Quantitative Light-Induced Fluorescence (QLF) parameters

As mentioned before, QLF produces three parameters that include; the ΔF which represents the percentage fluorescence loss and related to lesion depth, the surface area of the lesion as well as ΔQ which is the ΔF times the area and represent the lesion volume. All these values were calculated in this study; however, the ΔQ value was considered as the main indicator for the mineral loss and the lesion progression or regression in the present study. Since it was indicated that the lesion area may increases or decreases while the ΔF value maintained the same or alternatively the lesion area may be maintained despite the increase or decrease in ΔF value (Ando et al., 2004). For this reason the independent evaluation for these two values may not give a good evaluation of the lesions progression or regression.

4.9 Remineralising potential of toothpastes with different Fluoride (F) formulations on artificial subsurface caries lesions

Phase A of the current study investigated the remineralising potential of toothpastes with different Fluoride (F) formulations: amine fluoride **(AmF)**, sodium monofluorophosphate **(MFP)**, sodium fluoride **(NaF)**, and stannous fluoride **(SnF)** on artificial subsurface caries lesions *in vitro*. Non-fluoride toothpaste was used as negative control.

The study results demonstrated that in all QLF parameters (ΔF, ΔQ and lesion area) statistically significant remineralisation between baseline and after treatment was found in **Naf**, **SnF** and **MFP** toothpaste groups, but there was no such significance in the **AmF** and **F Free** groups. The highest remineralisation was achieved by the **NaF** group followed by the **SnF** and finally the **MFP** group.

When comparing the groups against each other, the difference in remineralisation at baseline and after treatment was statistically significantly higher in the **NaF** group when compared to **AmF** and **F Free** toothpaste groups only. Remineralisation in **SnF** group was significantly higher than **AmF** group only. No other statistical significance could be found.

The reason behind the poor performance of AmF could possibly be due to the fact that pH of the environment has an effect on the effectiveness of AmF toothpastes. (Arnold et al., 2007) ran a pH cycling *in vitro* study to examine the effect of pH of amine fluoride containing toothpastes on enamel remineralisation. Results of their study demonstrated that lowering the pH of the environment during amine fluoride exposure had positive effects on enamel remineralisation *in vitro*. Furthermore the presence of fluoride at a pH between 4.5 and 5.1 caused the released mineral ions to be re-precipitated as mixed

fluor-hydroxyapatite enhancing remineralisation of the body of the lesion and the enamel surface layer.

The current study also conflicts the results published by (Arnold et al., 2006) who compared the effect of four different toothpastes with differing fluoride compounds **(AmF, NaF, MFP and F-Free)** on enamel remineralisation. In their study the authors concluded that **AmF** produced the highest amount of remineralisation followed by **MFP** and lastly **NaF**. However, the results of their study are less than reliable due to the fact that the enamel slabs were immersed in the demineralisation solution for 50 days. This was then followed by incubation of the enamel slabs in toothpaste slurries for 48 hours, which according to the authors, simulates 2 years of tooth brushing. Unlike the current study, their methods did not represent the natural conditions of the oral environment, which include cycles of demineralisation and remineralisation.

(Patil and Anegundi, 2014) conducted a study with a methodology identical to (Arnold et al., 2006), and found supporting results. Again the issue with their study was that conditions did not match the oral environment.

On the other hand, results of another *in vitro* pH cycling study (Casals et al., 2007) aiming to investigate remineralisation of human enamel after the use of commercially available toothpastes containing different fluoride compounds (**NaF, SnF, MFP and AmF**), concluded that **NaF** and **SnF** have superior remineralising potential on artificial subsurface carious lesions when compared to other fluoride compounds. The authors also concluded that **NaF** uptake into demineralised enamel *in vitro* was double the amount of **AmF** uptake at the same fluoride concentration. Their study included 3 daily dippings of enamel slabs into a demineralising solution followed by 3 daily dippings into toothpaste slurries. This cycle was repeated for five days and is somewhat

representative of the continuous rise and fall of pH found in the oral environment. These results are harmonious with the results found in the current study.

An in vitro pH cycling model demonstrated that **NaF** was statistically better at enamel remineralisation than **MFP** when lesions were assessed by cross sectional micro hardness (Toda and Featherstone, 2008). Also in line with results found in this current study, a recent in vitro experiment evaluating remineralisation of carious lesions and fluoride uptake by enamel exposed to various fluoride dentifrices, found that enamel remineralisation and fluoride uptake was significantly greater when using **NaF** compared to **MFP**. It was also concluded that efficacy of the fluoride dentifrice was dependant on ionic fluoride levels (Hattab, 2013). In both of these studies and as with most reports claiming the superiority of **NaF** against **MFP**, their claims were built on the assumption that fluoride only exerts its effects on demineralisation and remineralisation as a free ion. Unlike **NaF** (which releases free F^-), fluoride in **MFP** formulation is covalently bound to phosphate and requires enzymatic hydrolysis to release free F^- (Pessan et al., 2011). This unfortunately is one of the limitations of *in vitro* pH cycling models due to the lack of enzymes in artificial saliva.

When looking at clinical trials and *in vivo* experiments, a Cochrane review by (Marinho et al., 2003) to determine the effectiveness and safety of fluoride toothpastes in the prevention of caries in children, compared toothpastes containing **MFP** (22 trials), **SnF** (19 trials), **NaF** (10 trials) and **AmF** (5 trials) and did not find an link between the type of fluoride compound in the dentifrice and the magnitude of treatment effect. In spite of their findings the authors considered their results to be less reliable than evidence from head to head comparisons (Pessan et al., 2011).

4.10 Remineralising potential of toothpastes containing sodium fluoride (NaF) formulation at different concentrations of fluoride on artificial subsurface caries lesions in vitro.

Phase B of the current study investigated the remineralising potential of toothpastes with different Fluoride (F) concentrations: **500**, **1000**, **1450**, **2800**, **5000 ppm F** on artificial subsurface caries lesions *in vitro.* Non-fluoride toothpaste was used as a negative control.

The study results demonstrated slight differences according to the parameter used:

ΔF results: showed that there was statistically significant improvement in remineralisation between the baseline and after treatment for all groups. Remineralisation of the artificial subsurface carious lesions was highest, and almost identical in the **500, 1450, and 2800 ppm F** groups. Followed by **5000 ppm, 1000 ppm** and the lowest improvement was seen in the **0 ppm F** group.

When comparing the groups against each other, it can be seen that remineralisation of the **0 ppm F** (Control) toothpaste group is significantly lower than remineralisation of all other test groups except for **1000 ppm F** toothpaste. As for the remineralisation significance between different concentrations of NaF fluoride toothpaste, there was no significance when comparing any of the groups.

ΔQ results: A statistically significant improvement in remineralisation between the baseline and after treatment was found in all groups. The highest improvement was achieved by the **1450 ppm F** group followed by **2800 ppm F** and the least improvement was seen in the **0 ppm F** group (control).

When comparing the groups against each other, the remineralisation at baseline and after treatment in all test groups was statistically significantly higher than that for the **0 ppm F** group except for the **5000 ppm F** group. No other statistical significance was found between groups.

For the **lesion area results**: A significant reduction in the area of the white spot lesion was seen in all groups except for the **0 ppm F** group. The highest reduction was found in the **1450 ppm F** group followed by the **2800, 500, 1000, 5000 ppm F** groups In descending order.

When comparing the groups against each other, the difference in the lesion area at baseline and after treatment was statistically significant for the test groups compared with the **0 ppm F** group. No significant difference was found between the test groups.

As mentioned before, the ΔQ value was considered as the main indicator for the mineral loss and the lesion progression or regression in the present study. And therefore it seems as though in this current *in vitro* study design, there was no difference in the effect of toothpastes with sodium fluoride (NaF) formulation and different concentrations (**500-5000 ppm F**) on remineralisation of artificial subsurface carious lesions. Furthermore, no apparent dose response was present related to the concentration of fluoride.

These results were unexpected and surprising as they contradict the results of a Cochrane review by (Walsh et al., 2010) that included 75 studies, indicating that the caries preventive effect of fluoride toothpaste increases significantly with higher fluoride concentrations. When compared to placebo. Concentrations of **450-550ppm F** and below show no statistically significant effect, but a statistically significant effect was evident for **1000-1500 ppm F** concentrations (prevented fraction: 25%) and for highest fluoride concentrations **2400-2800 ppm F** (prevented fraction: 45%). However, the issue

with this review was, the conclusion that the efficacy of dentifrices containing **450-550 ppm F** is not significantly different from placebo was based only on two trials, while the number of studies comparing placebo with conventional concentrations **1000-1500 ppm F** was significantly higher (58 trials). Furthermore, no conclusion could be taken when comparing the clinical efficacy of low fluoride and conventional toothpastes, as only one trial met the inclusion criteria of the systematic review. This clearly indicates that further research is needed prior to making the assumption that higher fluoride concentrations should always be preferred (Pessan et al., 2011)

Furthermore, Biesbrock et al. (2003a) conducted a randomised double-blind study to assess the anti-caries effectiveness of placebo, **500 ppm F** and **1450ppm F** dentifrices, and found a dose response depending on the concentration of fluoride used. The higher the dose used the lower DMF score at 9 and 21 months.

Not all the evidence published in the literature supports the theory of a dose response to fluoride concentration toothpastes. A randomised controlled trial by Lima et al. (2008), evaluated the effect of low-fluoride dentifrice on children with different caries experience. One hundred and twenty 2 to 4 year old children, half with and half without active caries lesions were randomly divided into two groups which used either **500 ppm F** or **1100 ppm F** dentifrices. The authors results pointed out that the anti-caries effect of the **500 ppm F** dentifrice was similar to the **1100 ppm F** when used by caries inactive children. This supports the results found in the current study. However, a shortcoming of the before mentioned trial was its short follow-up time (one year).

Damato et al. (1990) carried out an in vitro pH-cycling experiment to investigate the effect of fluoride concentration on enamel demineralisation and remineralisation. Artificial carious lesions were exposed to **0, 1, 250, 500, 1000, 1750, and 2500 ppm F** toothpaste solutions in a pH cycling model. The authors determined that there was a cut-off point of

500 ppm F where any concentration below would not remineralise the artificially demineralised enamel lesions. Furthermore the authors also concluded that higher fluoride concentrations did not produce any further significant increase in remineralisation when compared to the **500 ppm F** group. This is in line with results of the current study.

The exact cause of why a dose response to different concentrations of NaF was not evident in the present study is difficult to explain, although speculations can be made. A possible explanation could be that an exceedingly high fluoride concentration toothpaste slurry produced a surface mineral rich layer which in turn compromised the remineralisation of the body of the lesion. In the literature there is evidence that the use of frequent applications of a low concentration of fluoride is preferable to the use of high fluoride concentration as the latter will cause rapid formation of an insoluble calcium fluoride precipitate on the surface layer of an incipient caries lesion. This will block the pores on the surface layer and prevent mineral re-precipitation into the subsurface body of the lesion arresting the process of remineralisation (ten Cate et al., 1981).

Another possible speculation for the lack of a dose response in this current study could be that, in the in vitro situation, (unlike the in vivo environment) the concentration of fluoride in toothpaste has no effect on the amount of remineralisation of demineralised enamel, and that a minimal amount of free fluoride ions are necessary to produce sufficient remineralisation.

4.11 Suggestions for future research

More research into the remineralising potential of different fluoride formulations and concentrations is needed. In the current *in vitro* study, artificial day and night time saliva was used as an alternative to natural human saliva. Future research may incorporate the use of human saliva to assess whether or not it has an effect on the action of the fluoride toothpastes, as human saliva contains enzymes that may aid in hydrolysis of some organic fluoride compounds including **MFP**.

The average ΔF (percentage fluorescence loss with respect to the fluorescence of sound tooth tissue - Related to lesion depth) reading at baseline for all the enamel slabs was -13.26 %. It would be interesting in the future, to see whether or not deeper baseline depths of the enamel lesions have an effect on the dose response to different fluoride concentrations. This poses the question, do deeper lesions require higher concentrations of fluoride to remineralise?

The pH cycling model used in this current study had a duration of 28 days. It would be interesting to see the same study repeated, but with a longer period of cycling e.g. 60, 90 and 120 days. Alternatively the sample size could be increased and this may show a difference between groups.

The pH of the acetic acid used for the demineralisatiion cycle for this study was 4.8. Looking at the results, it seems that all groups showed remineralisation of the artificial enamel lesion to a similar level. Future *in vitro* designs could use acetic acid at a pH of 4.5 as this would create a harsher environment for the enamel lesions. In this harsher environment, different concentrations/ formulations of fluoride may perform at significantly different levels.

Another possible modification to this current study would be the use of transverse micro-radiography for assessment of artificial caries lesions at baseline and after treatment with the fluoride toothpastes.

Furthermore, due to the limitations of *in vitro* experiments, future *in situ* and *in vivo* studies investigating the remineralisation of enamel by different formulations and concentrations of fluoride toothpaste may provide information that is directly associated with the clinical use of toothpastes.

4.12 Null hypotheses outcome

1. The null hypothesis “There is no difference in the effect of toothpastes containing different fluoride formulations: **AmF, MFP, NaF** and **SnF** on remineralisation of artificial subsurface carious lesions *in vitro*.” can be rejected as significant differences were found in the enamel remineralisation between the test groups.

2. The null hypothesis “There is no difference in the effect of toothpastes with sodium fluoride (NaF) formulation and different concentrations (**500-5000 ppm F**) on remineralisation of artificial subsurface carious lesions *in vitro*.” Can be accepted as no significant differences were found in the enamel remineralisation between the test groups.

CONCLUSIONS

From the results of phase A of this *in vitro* study, it can be concluded that:

1. A statistically significant remineralisation of enamel subsurface lesions in comparison with the baseline was found in all groups except the **AmF** group.
2. **NaF** toothpaste has the highest remineralising potential on artificial subsurface carious lesions in vitro, followed by **SnF** then **MFP**.
3. **AmF** remineralising potential on artificial subsurface carious lesions in vitro is less than fluoride-free toothpaste.

From the results of phase B of this *in vitro* study, it can be concluded that:

1. A statistically significant remineralisation of enamel subsurface lesions in comparison with the baseline was found in all groups.
2. There is no difference in the effect of toothpastes with sodium fluoride (NaF) formulation and different concentrations (**500**, **1000**, **1450**, **2800**, and **5000 ppm F**) on remineralisation of artificial subsurface carious lesions *in vitro*.
3. No apparent dose response was present related to the concentration of fluoride.

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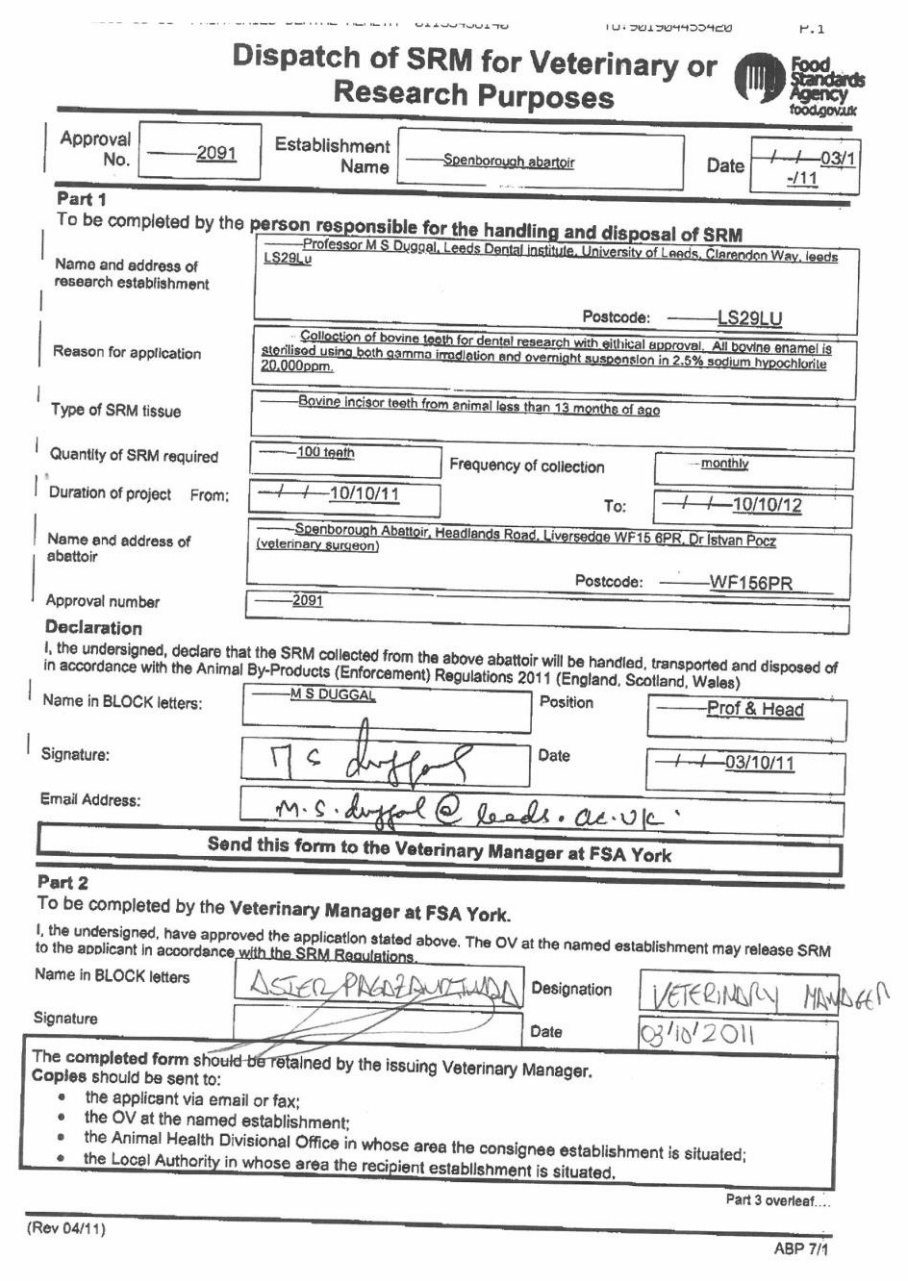
APPENDICES

Appendix 1: Approval for collection of bovine teeth.



Dispatch of SRM for Veterinary or Research Purposes

Food Standards Agency food.gov.uk

Approval No.	2091	Establishment Name	Spenborough abartoir	Date	03/11

Part 1

To be completed by the **person responsible for the handling and disposal of SRM**

Name and address of research establishment	Professor M S Duggal, Leeds Dental institute, University of Leeds, Clarendon Way, leeds LS29LU Postcode: LS29LU
Reason for application	Collection of bovine teeth for dental research with ethical approval. All bovine enamel is sterilised using both gamma irradiation and overnight suspension in 2.5% sodium hypochlorite 20,000ppm.
Type of SRM tissue	Bovine incisor teeth from animal less than 13 months of age
Quantity of SRM required	100 teeth — Frequency of collection: monthly
Duration of project From:	10/10/11 — To: 10/10/12
Name and address of abattoir	Spenborough Abattoir, Headlands Road, Liversedge WF15 6PR, Dr Istvan Pocz (veterinary surgeon) Postcode: WF156PR
Approval number	2091

Declaration

I, the undersigned, declare that the SRM collected from the above abattoir will be handled, transported and disposed of in accordance with the Animal By-Products (Enforcement) Regulations 2011 (England, Scotland, Wales)

Name in BLOCK letters:	M S DUGGAL	Position	Prof & Head
Signature:	M S duggal	Date	03/10/11
Email Address:	m.s.duggal@leeds.ac.uk		

Send this form to the Veterinary Manager at FSA York

Part 2

To be completed by the **Veterinary Manager at FSA York.**

I, the undersigned, have approved the application stated above. The OV at the named establishment may release SRM to the applicant in accordance with the SRM Regulations.

Name in BLOCK letters	[illegible]	Designation	VETERINARY MANAGER
Signature		Date	03/10/2011

The **completed form** should be retained by the issuing Veterinary Manager.
Copies should be sent to:

- the applicant via email or fax;
- the OV at the named establishment;
- the Animal Health Divisional Office in whose area the consignee establishment is situated;
- the Local Authority in whose area the recipient establishment is situated.

Part 3 overleaf....

(Rev 04/11) ABP 7/1

Appendix 2: Normality tests for ΔF at baseline phase A

Shapiro-Wilk and Kolmogorov-Smirnov Tests of Normality for ΔF at Baseline.

	Group	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
ΔF at baseline	F Free	.116	25	.200*	.940	25	.146
	NaF	.138	25	.200*	.893	25	.013
	SnF	.193	24	.200*	.928	24	.088
	MFP	.228	26	.001	.865	26	.003
	AmF	.137	25	.200*	.948	25	.225

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction

Appendix 3: Normality tests for ΔQ at baseline phase A

Shapiro-Wilk and Kolmogorov-Smirnov Tests of Normality for ΔQ at Baseline.

	Group	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
ΔQ at baseline	F Free	0.138	25	0.200*	0.957	25	0.286
	NaF	0.114	25	0.200*	0.943	25	0.177
	SnF	0.134	24	0.200*	0.957	24	0.385
	MFP	0.117	26	0.200*	0.977	26	0.806
	AmF	0.111	25	0.200*	0.953	25	0.286
*. This is a lower bound of the true significance.							
a. Lilliefors Significance Correction							

Appendix 4: Normality tests for area of the white spot lesion at baseline phase A

Shapiro-Wilk and Kolmogorov-Smirnov Tests of Normality for Area at Baseline.

	Group	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
Area at baseline	F Free	0.077	25	0.200*	0.987	25	0.982
	NaF	0.103	25	0.200*	0.952	25	0.278
	SnF	0.106	24	0.200*	0.972	24	0.722
	MFP	0.078	26	0.200*	0.991	26	0.997
	AmF	0.138	25	0.200*	0.956	25	0.339
*. This is a lower bound of the true significance.							
a. Lilliefors Significance Correction							

Appendix 5: Normality tests for ΔF at baseline phase B

Shapiro-Wilk and Kolmogorov-Smirnov Tests of Normality for ΔF at Baseline.

	Group	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
ΔF at baseline	0 ppm	0.116	25	0.200*	0.940	25	0.146
	500 ppm	0.214	26	0.004	0.909	26	0.025*
	1000 ppm	0.153	26	0.120	0.928	26	0.071
	1450 ppm	0.138	25	0.200*	0.893	25	0.013*
	2800 ppm	0.129	26	0.200*	0.941	26	0.144
	5000 ppm	0.216	26	0.003	0.823	26	0.000*
*. This is a lower bound of the true significance.							
a. Lilliefors Significance Correction							

Appendix 6: Normality tests for ΔQ at baseline phase B

Shapiro-Wilk and Kolmogorov-Smirnov Tests of Normality for ΔQ at Baseline.

	Group	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
ΔQ at baseline	0 ppm	.138	25	.200*	.957	25	.365
	500 ppm	.087	26	.200*	.972	26	.683
	1000 ppm	.119	26	.200*	.953	26	.267
	1450 ppm	.114	25	.200*	.943	25	.177
	2800 ppm	.159	26	.089	.938	26	.122
	5000 ppm	.142	26	.190	.941	26	.143

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction

Appendix 7:Normality tests for area of the white spot lesion at baseline phase B

Shapiro-Wilk and Kolmogorov-Smirnov Tests of Normality for Area at Baseline.

	Group	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
Area at baseline	0 ppm	.077	25	.200*	.987	25	.982
	500 ppm	.134	26	.200*	.939	26	.128
	1000 ppm	.153	26	.119	.932	26	.087
	1450 ppm	.103	25	.200*	.952	25	.278
	2800 ppm	.110	26	.200*	.984	26	.946
	5000 ppm	.161	26	.080	.917	26	.038
*. This is a lower bound of the true significance.							
a. Lilliefors Significance Correction							